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Investigation of Forssman Antigen Expression in Erythrocytes among Palestinian Population

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Declaration:

I certify that this thesis submitted for the degree of Master is the result of my own research, except where otherwise acknowledged, and that this study (or any part of the same) has not be submitted for a higher degree to any other university or institute.

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Date: 11-06-2016

Dedication

I dedicate my dissertation work to my family especially to my husband "Fuad" for his tolerance to take care of my duties for a long time, and for reinforcing me to be the best. A special feeling of gratitude to my parents, whose words of encouragement and push for tenacity ring in my ears. I also dedicate this dissertation to my teachers, supervisors and friends at Al-Quds University and Gothenburg University, who have supported me throughout the master thesis work. I will always appreciate all they have done.

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Abstract

Background: Expression of Forssman antigen (FORS1) has been recently described on human erythrocytes and consequently the FORS blood group system was recognized as the *31*st system.

Objective: To investigate the expression of FORS1 antigen in human erythrocytes and characterize anti-Forssman antibody among Palestinian population and compare it to a group of European donors.

Methods and Materials: In this study, 211 adult and 73 newborn donors from Palestine and 65 adult donors from Europe were included. The RBCs from the Palestinian donors were typed for FORS1 antigen using monoclonal anti-Fs antibodies and plasma were tested for anti-Forssman antibody using sheep RBCs. The molecular biology of FORS1 antigen negativity was investigated by DNA sequencing of exon 7 of GBGT1 gene that encodes the Fs synthetase, the enzyme that catalyzes the terminal step of FORS1 antigen biosynthesis.

Results: All study samples tested were negative for FORS1 antigen by serologic typing. Anti-Fs antibodies (IgG and IgM) were present in 96.2 % of adult Palestinian samples. The absence of anti-Fs was more common among males compared to females, and stronger reactions (agglutinations) were observed also in females. Stronger agglutination reactions (grades +3 or +4) for IgG and IgM anti-Fs were observed in A and O blood groups, 50% and 50.6%, respectively, compared to other blood groups. DNA sequencing of exon 7 of GBGT1 gene revealed that all samples have the 887A>G [p.Gln296Arg] and 688G>A [p.Gly230Ser] mutations which were previously reported to be responsible for the FORS1 antigen negativity. Additionally, four SNPs were detected with an allele frequency ranging from 0.2% to 3.6%. One SNP, namely R243C is a novel mutation detected in a Palestinian donor and has a damaging effect on the Fs synthetase as predicted by PolyPhen-2. There was no significant difference between presence/absence of anti-Forssman IgM (P= 0.408) and IgG (P= 0.433) antibodies and GBGT1 gene polymorphisms.

Conclusion: This is the first study to investigate FORS blood group system in Palestine. Our results confirmed that the FORS is a low prevalence blood group system - at least

among our studied populations- with naturally occurring anti-Forssman in the majority of adult donors. A novel missense mutation (R243C) with damaging effect on Fs sythetase has been detected in a Palestinian donor.

Table of Contents

Lists of Tables

List of Figures

List of Appendices

List of abbreviations

5-Aza:	(5-Azacytidine) treatment
Agarose LE:	Agarose Low Electroendosmosis
AHG:	Antihuman globulin
BBC:	Boiled beef cell
BLAST:	Basic Local Alignment Search Tool
CDS:	Complete DNA sequence
CE:	Capillary electrophoresis
DOPE:	Dioleoylphosphatidylethanolamine
Fc :	Fragment crystallizable
FC:	Flow cytometry
FITC:	Fluorescein isothiocyanate
FORS:	Forssman blood group system (System 31)
FORS1:	The sole antigen of the FORS blood group system also the Forssman pentasaccharide, GalNAcα3GalNAcβ3Galα4Galβ4Glc
FS:	Forssman glycolipid synthetase
Fsdi:	Forssman disaccharide or GalNAcα3GalNAcβ
FSL:	Function-spacer-lipid KODE technology construct
GalNAc:	N-acetylgalactosamine
GBGT1:	GloBoside alpha-1,3-N-acetylGalactosaminyTransferase 1
GbO4:	Globoside
GBS:	Guillain-Barré syndrome.
GlcNAc:	N-acetylglucosamine
GPK:	Guinea pig kidney
GSL:	Glycosphingolipid

GT6:	Glycosyltransferase Family 6
HA:	Hemagglutination
HDFN:	Hemolytic disease of the fetus and newborn
HGP:	The Human Genome Project
HPA:	Lectin Helix pomatia
HPA:	Helix pomatia agglutinin
HPTLC:	High performance thin-layer chromatography
IM:	Infectious mononucleosis
ISBT:	International Society of Blood Transfusion
LISS:	Low ionic strength solution
MPC:	Master Pure™ Complete
NTC:	No template control
OGT:	O-linked N-acetylglucosamine (O-GlcNAc) transferase
PBS:	Phosphate-buffered saline
PCH:	Paroxysmal cold hemoglobinuria
PCR:	Polymerase chain reaction
PSA:	Prostate-specific antigen
Rpm:	Revolutions per minute
RT:	Room temperature
SNP:	Single Nucleotide Polymorphism
STRs:	Short tandem repeats
TE:	TE is derived from its components: Tris and EDTA (ethylenediaminetetraacetic acid disodium salt dehydrate)
UDP- sugar:	Uracil-diphosphate-sugar

Chapter One

Introduction

1.1 Background

Transfusion is an important tool for preventive and curative therapy in case of hemorrhage or blood loss due to traumatic injury or surgery. Several adverse health effects from blood transfusion can occur, such as an increased risk of cancer and transfusion-transmitted diseases [1]. Still the main risk of transfusion is blood mismatching due to presence of different blood group antigens on the donor and recipient red blood cells [2]. Dangerous consequences of blood transfusion for recipients ranges from hemolytic transfusion reactions to death [3]. The antigens are not restricted to the surface of erythrocytes, they are also found in other body fluids such as semen and saliva, as well as on other cells, tissues and organs. So, it is essential to take different blood groups into account in transfusion and transplantation [4]. Indeed, knowledge gained from studying blood groups have contributed to the development in several aspects in genetics, evolution, and biology [5].

The ABO and Rh blood group systems are the most common blood groups but they are just two of more than 30 blood group systems known so far. At present more than 350 blood group antigens has been recognized, and the list of these antigens is continuously

updated at online: http://www.ncbi.nlm.nih.gov/projects/gv/rbc/xslcgi.fcgi?cmd=bgmut [6]. All antigens fall into one of four classifications; 297 antigens within 35 systems, 50 antigens within collections (200 series), low incidence antigens (700 series), and high incidence antigens (901 series) [7, 8].

Nowadays, new subgroups and weak subgroups have been discovered are basically due to recognized genetic mutations in most cases [6] . Weak ABO subgroups are defined by individuals whose erythrocytes give weaker reactions or are nonreactive serologically with antisera than those of common subgroups [9]. They result from the respective mutated ABO gene due to the expression of an alternate weak allele present at the ABO loci. Even though the weak subgroups are rare phenotypes, and account for a minority of individuals, still we need to define them by confirmatory tests other than ABO forward and reverse typing and may include ABO genotyping [4]. On one hand, these subgroups give weak reactions, but they still can have serious consequences after multiple transfusions of patients with blood disorders, on the other hand, identification of these subgroups is important because they are keys for the discovery of their respective blood group systems and may be mistyped as group O [9].

A_{pae}, a rare phenotype was classified in 1987 as an A subgroup, "where "p" designates its reaction with the lectin Helix pomatia and "ae" reflects the absorption and elution of only certain human anti-A serum" [10]. It was discovered in three unrelated UK families who were blood group O and had the genotype O^{I}/O^{I} [11, 12]. It was distinguished from other A variants by several methods, such as its agglutination with only certain human anti-A and anti-A,B sera and their red cells reacted with anti-A lectin prepared from Helix pomatia [10]. Two decades later, this A-like expression on the RBCs was reinvestigated using genomic and biochemical methods [12].

In July 2012, A_{pae} blood type was the key to the discovery of a Forssman antigen (FORS1) in normal human hematopoietic tissue in Sweden, by researchers at Gothenburg University and Lund University [12, 13]. This was initiated because the A_{pae} phenotype was shown to be homozygous for common deletional O alleles, despite the expression of

the A-like antigen. Single heterozygous polymorphism c.887G > A (R296Q) was found in the Globoside alpha-1,3-N-acetylGalactosaminyl Transferase 1 (GBGT1) gene that makes the human Forssman synthase active [12]. This mutation causes substitution of the amino acid Arginine at codon 296 by Glutamine, the same amino acid observed in Forssman antigen positive animals.

Immunochemical and mass spectrophotometric studies demonstrated the presence of the Forssman glycolipid on these RBC. Thus, A_{pae} became an old terminology and it was abolished and replaced by the International Society of Blood Transfusion (ISBT) by newly formed 31st blood-group system given the name FORS [7, 12].

All the criteria for a blood group system which include: independency of other blood group antigen, be inheritable, be expressed on red blood cells, and presence of naturally-occurring antibodies were achieved for the FORS 1 antigen [7, 14]. So, the FORS system will now be a further factor to consider in blood transfusions and may lead to serious reactions in transfusions/transplantations, if anti-FORS1 positive plasma is donated to a FORS1 positive patient.

Until now, 35 blood group systems were discovered. Five of them were discovered in the past 2 years and ratified by the ISBT meeting in Cancun, July 2012; FORS (#31), JR (#32), LAN (#33), Vel, and the complement protein, CD59 [15, 16]. Figure 1.1 shows the date of discovery of blood group systems according to the date of discovery of the first antigen in the system. See also blood group systems in appendix A.

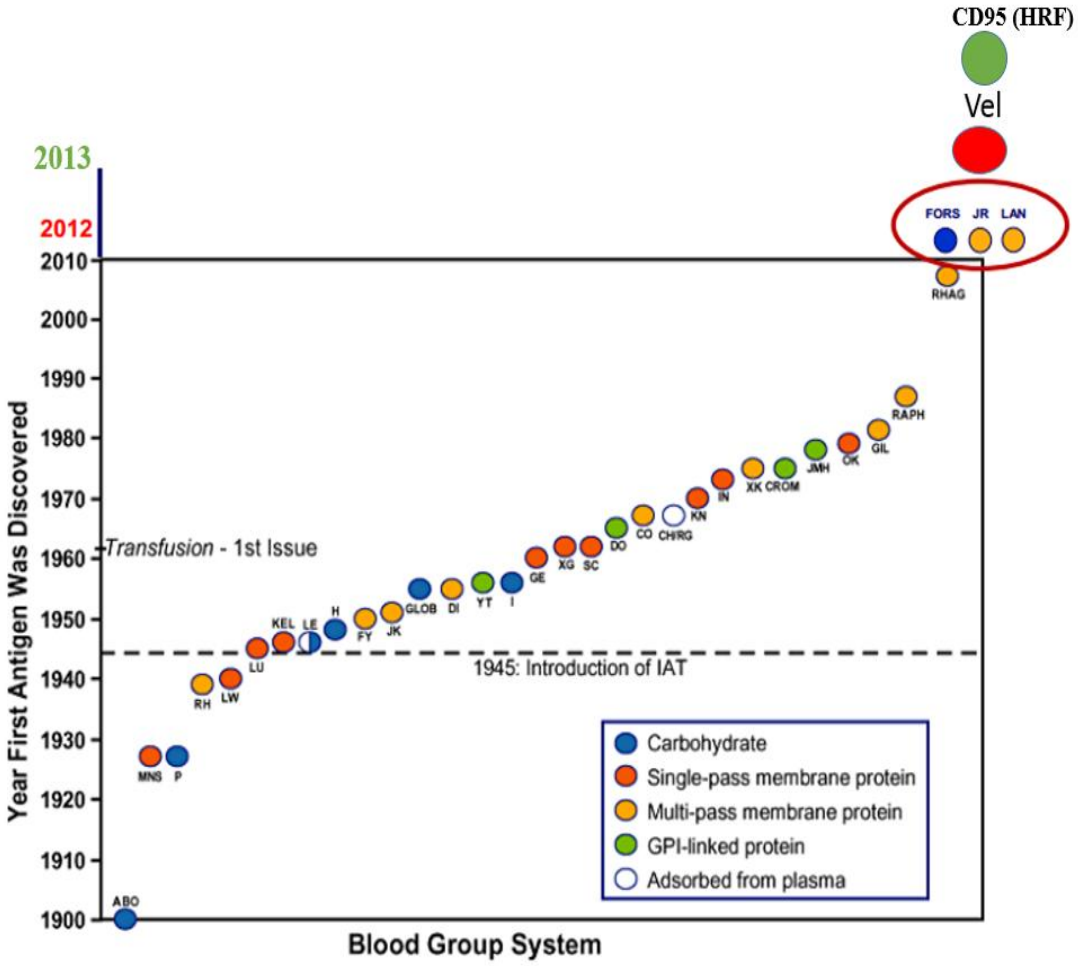

Figure 1.1 Date of discovery of blood group systems according to the date of discovery of the first antigen in the system [17]. The figure was modified by adding Vel and CD95**.** HRF; homologous restriction factor and is a synonym for the complement regulation protein CD59**.**

1.2 Forssman Antigen Story

The Forssman (FORS1) antigen was first described in 1911 by a pioneer Swedish Pathologist John Frederick Forssman (1868 – 1947) [18], so the discovery recently celebrated its 104th birthday. It was acknowledged after injection of rabbits with a

suspension of kidney tissue from guinea pig and the rabbits produced antibodies that were capable of hemolysing sheep erythrocytes [19].

FORS1 antigen is one of a set of 13 glycosphingolipids (GSLs), all of which have in common a core of galactosyl-(a1,3) galactose moiety [20]. They include the FORS1, P1PK and the GLOB blood group collection and are also closely related with respect to their structure and biosynthesis [13, 21]. FORS1 is a low-prevalence antigen of the FORS blood group system [22] and it is considered one of the carbohydrate antigens with the structure GalNAcα1→3GalNAcβ1→3Galα1→4Galβ1→4Glc→ceramide [23, 24].

Forssman antigen can be rapidly taken up or passively acquired by cells in medium containing serum from Forssman positive animals [25]. Chemically, Forssman antigen is distinguished from other antigens by their solubility in lipid solvents and stability to boiling, as it has a glycosphingolipid structure [26].

The Forssman antigen has been previously found only in lower mammals, it present in various species but not in others, so they were categorized depending on the antigen expression [19]. Guinea pigs, horses, hamsters, sheep and chickens are Forssman-positive while ox, pigeon, rat and rabbit are Forssman- negative [19, 27]. It also has been found in some bacteria, such as dysentery bacilli and pneumococci [27].

It has been found with unequal distribution within the same tissue of the same species. Mainly it is expressed in tissues (as in guinea-pig, horse, cat, and dog) [26, 28], on the erythrocytes (as in sheep), or in both (as in chicken) [29]. Thus, the rejection in transplantation by Forssman antibodies is depending on tissue expression of FORS antigen [19]. Svensson and her colleagues found that the FORS glycolipid is also expressed on human erythrocytes in a minority of individuals [12]. Thus individuals are grouped into FORS1 antigen negative individuals who produce the naturally occurring anti-FORS antibodies (most common) or FORS1 antigen positive individuals who do not produce the respective antibodies.

1.3 Biosynthesis of FORS Antigen

In general, a blood group system consists of one or more antigens controlled at a single gene locus, or by two or more very closely linked homologous genes with little or no observable recombination between them [30].

Normally, human cells do not produce FORS glycolipid but produce its biosynthetic precursor [31]. Both Pk (a biosynthetic precursor of P) and P blood group antigens, known as globotriose (Gb3) and globotetraose (Gb4), respectively are precursors for Forssman glycolipid [32, 33]. All the antigens produced based on these precursors are glycosphingolipid (oligosaccharide linked to sphingolipid on the cell membrane) [34].

The biosynthesis of FORS antigen requires multiple glycan-processing enzymes. A terminal step - transfer of N-acetylgalactosamine (GalNAc) to the terminal GalNAc residue of globoside via an α1,3-linkage [13] - is catalyzed by GBGT1 gene product, Forssman synthetase [12, 19]. The GBGT1 gene was first isolated in canine kidney cell in 1996 by using monoclonal antibodies against Forssman antigen [35] and three years later cloned in human [36]. GBGT1 is located on chromosome 9 (9q34) and consists of 347 amino acids [6]. It is homologous to the alpha 1,3 GalNAc ABO A transferase (45% amino acid sequence identity) [6]. The human GBGT1 gene has also about 83% identical amino acids to the animal GBGT1 gene [14]. The whole gene consists of 7 exons spanning more than 8 kb of DNA - of which the last one is the largest- with the coding region shared between all species positive for FORS 1 [12]. Figure1.2 represents Genomic organization of the human Forssman synthetase or GBTG1 gene, exon 1 through exon 7.

Figure 1.2 Genomic organization of the human Forssman synthetase gene or GBTG1 gene. The seven exons are drawn to scale whereas the introns are not. The numerals above the boxes represent the number of nucleotides in each exon. Grey, vertical lines in exon 2 and 7 indicate the start and stop codons, respectively [37].

It is a GT6 enzyme (the glycosyltransferase gene family 6) and it also called Forssman glycolipid synthase (Fs) [12, 19]. GBGT1 mRNA expression has been observed in a variety of human tissues including small and large intestines, placenta, and ovary, but as a result of differential methylation, GBGT1 nonsense and inactivating missense mutations, a truncated or enzymatically inactive enzymes are produced [36].

GBGT1 is also inactive due to point mutations or exon loss (cow, rat, primates) [13]. In all humans, the GBGT1 gene which can give rise to the Forssman antigen is present and mRNA has been found in several tissues in human, but an active enzyme is not observed [31]. A single point mutation in the GBGT1 gene makes the human Forssman synthase active and give rise to the blood group FORS, which was previously misclassified as an ABO subgroup. The details of GBGT1 gene are summarized in **Table 1.1**.

Table 1.1 GBGT1 Gene Details.

Gene symbol	GBGT1
Full nomenclature	Globoside alpha-1,3-N-acetylgalactosaminyltransferase 1
Designation	Forssman glycolipid synthase (Fs) Forssman glycolipid synthase- like protein
Protein family	GT6. Glycosyltransferase Family 6.
Chromosome	9 (136028335 – 136039309)
Map Location	9q34.13-q34.3
Exon count	7
Size	347 amino acids; 40127 Da
Cofactor	Manganese Mn^{++}
Subcellular location	Golgi apparatus membrane , Single-pass type II membrane protein
Similar genes	ABO*, GLT6D1*, A3GALT2*, and GGTA1P*
Expression (Tissue specificity)	Widely expressed, but usually expressed at higher levels in placenta, ovary and peripheral blood leukocyte.
Function	Catalysis the terminal step of Forssman antigen biosynthesis: (transfer of GalNAc to the terminal GalNAc residue of globoside via an α1,3-linkage)
First isolation date	In canine kidney cell in 1996 and three years later cloned in human.

***A3GALT2**: Alpha 1,3-galactosyltranferase 2; **GLT6D1**: Glycosyltransferase 6 Domain Containing protein 1; **GGTA1P:** glycoprotein, alpha-galactosyltransferase 1 pseudogene; **ABO**: transferase A, alpha 1-3-N-acetylgalactosaminyltransferase; transferase B, alpha 1-3-galactosyltransferase.

1.4 FORS1 antigen similarity with other blood group systems

Forssman antigen has a glycosphingolipid structure which is composed of hydrophilic carbohydrates responsible for the antigenic specificity. They are also linked to the hydrophobic sphingolipid (ceramide) which is anchored in the outer leaflet of the membrane [38]. Forssman epitope, composed of 1 mole of glucose, 2 moles of galactose,

and 2 moles of acetylgalactosamine, is similar to blood group antigens and in contrast to that of globoside which contains a less galactosamine (1 mole instead of 2 moles) [39]. Thus, Forssman antigen was shown to cross react with some blood antigens like A, para-Forssman and PX2 [12]. In human, ABO transferase and Fs related genes have the same evolutionary origin [40] and the hypothesis that they arose by gene duplication and subsequent divergence supported by presence of both genes on the same chromosome 9q34 [35].

Immunologically FORS antigen cross reacts with blood group A antigen as a result of presence of terminal non-reducing alpha linked N-acetyl D-galactoseamine residue in both [41]. It is interesting to note that, only those carbohydrate antigens which have a non-reducing terminal, such as Gal or GalNAc, and greater steric rigidity show strong antigenicity and thus have greater immunogenic clinical significance [39]. Forssman glycolipid is capable of inhibiting A-hemagglutination, and A-antigen inhibits hemolysis of sheep erythrocytes by anti-Forssman antibody [42]. FORS1-positive RBCs were agglutinated by polyclonal anti-A (3 of 18) and Anti-AB (8 of 18) but not with monoclonal anti-A and anti-B reagents [22, 43] .

There is structural relatedness between FORS1 and blood group A antigens. The H antigen which is the natural precursor of A and the P antigen is also the natural precursor for FORS. A antigen is produced by addition of α3-N-acetylgalactosamine (GalNAc) residue by a 1-3 glycosidic linkage by A transferase (1,3-N-acetyl-Dgalactosaminyltransferase) which catalyses the last step on the synthesis of A antigen to a precursor chain, H antigen. While, FORS antigen is produced by the addition of GalNAc to P (globoside) substance [4, 44]. **Figure 1.3** shows the structure of H, A, P, and FORS antigens and their chemical composition.

Figure 1.3 H, A, P, and FORS antigens structure are schematically drawn showing their chemical composition. GalNAc and GlcNAc stand for N-acetylgalactosamine, N-acetylglucosamine respectively. When α3-GalNAc binds to the H-substance will give blood group A and when α3-GalNAc binds to the P antigen will give FORS antigen.

The difference between GLOB and FORS blood group systems may be described as "what is common and what is rare″. In the GLOB system, the P antigen is common and anti-P antibody is very rare, whereas in the FORS system, the FORS1 antigen is extremely rare and most individuals are FORS negative and naturally occurring antibodies are common [13].

1.5 Clinical Significance of Forssman Antigen

There are no data about the influence of FORS antigen on human health because only few humans expressing the FORS1 antigen are currently known [13]. However, it may have as yet unknown biochemical functions.

In general, previous studies suggested that variability in glycolipid synthesis between species is an important determinant of microbial tropism [31]. As we mentioned before, FORS antigen is one of globo series glycosphingolipid (GSL). GSL play roles in many biological processes. They associate with malignant diseases, angiogenesis, multidrug resistance, and serve as receptors for human pathogens [36]. In some species, the conversion of a Gb4 precursor into the FORS will have an impact on the adherence of pathogenic organisms, directly affecting microbial ecology and modifying host susceptibility to infectious diseases [45]. On the other hand, Gb4, the precursor of the FORS glycolipid, serves as an initial attachment site for bacteria, viruses, and toxins in humans and other species [31] and the adherence of microbes to host cells is the first step in colonization and pathogenesis process of most infectious diseases [46].

It is possible that FORS1 expression on human uroepithelial cells could make such individuals more susceptible to infection by E. coli (especially P-fimbrinated strains which enhance early bacterial colonization of the urinary tract [47, 48] that are believed to reflect microbial selection. Other organisms known to bind globoseries glycolipids including the Shiga toxins (Stx2e) and parvovirus B19, the causative agent of erythema infectiousum [31]. The Stx2e variant of Shiga toxin which causes piglet edema disease and responsible for hemolytic uremic syndrome has recently been shown to bind Forssman glycolipid, which makes this subtype unique among the Stx subtypes [49]. On the other hand, the expression of Forssman glycolipid protects host cells against some microbial diseases, they are less susceptible to the effect of Shiga toxin 1 (Stx1) [50], as FORS1 synthesis decreases the amount of Pk (a Shiga toxin (Stx) receptor) [13, 19, 51]. FORS antigen like blood type A antigen have an essential role in the anti-depressive effects of glycolipids in a mouse model of depression but the mechanism responsible for such a process is still unclear [52].

1.6 FORS Antibodies; Indications and Clinical Significance

Forssman antibodies (anti-Fs) are naturally occurring and arise against the missing antigen in plasma of humans who lack active GBGT1 gene [12]. They react strongly (agglutinate and hemolyze) with Forssman positive cells in human [12]. The majority of normal human sera possessed anti-Fs with hemolytic activity against sheep erythrocytes, whereas a minority had weak or undetectable levels of such antibodies [53]. The incidence of FORS antibodies is more than 75% in random healthy individuals [54], whereas in cancer patients is only 35–40% [55]. Most of them belong to the IgM and some IgG classes and usually reactive at 37°C [13, 56].

The level of anti- Fs titer is influenced by age and the titer decreases gradually with advancing age in normal individuals [53]. By sex, the titer is higher in females than male patients and by blood group, the titer is higher in blood group AB than other blood groups. Although one study indicated that FORS antibodies in sera is independent of blood group [53]. In addition, anti-Fs antibody production is influenced in cancer patients by the histologic type of cancer, the titer is lower in differentiated adenocarcinoma and by the stage of the disease. The Anti-Fs titer also showed variations in both Igs classes, IgG and IgM [57].

The clinical significant of anti-Fs is unknown. It could be considered as one of the heterophilic antibody barriers in clinical organ transplantation [38, 58]. Many years ago, scientific researchers studied the relationship between complement-dependent hemolytic activity and the amount of the Forssman antisera hemolysin by measuring the lesion on the surface of a sheep erythrocyte, IgM was more effective [59].

The activity of FORS immunoglobulins were measured by haemolysis and cytolysis. For IgG antibodies were similar, but the IgM were showed to have much greater activity by haemolysis than by cytolysis compared to IgG antibodies, because it is more efficient than IgG antibody in activating complement due to its ability to place several Fc

(fragment crystallizable) receptor sites in close proximity [60]. Thus the IgM anti-FORS antibody is inefficient in lysing nucleated cells [61].

The anti-Fs present in sera may bind complement and could possibly cause intravascular lysis of transfused Fs-positive RBCs [12, 62]. So, these antibodies might have implications in transfusion medicine, organ transplantation and during pregnancy [12]. Furthermore, FORS antibodies could be - like most of naturally occurring anti carbohydrate antibodies - implicated in hemolytic disease of the fetus and newborn (HDFN) [13, 63]. The severity of graft rejection due to FORS antibodies may diminish when the levels of such antibodies are decreased by mechanisms of adaptation like immunosuppression, neutralization, or absorption of antibodies and plasmapheresis [38].

Anti-Fs antibodies have the ability to disrupt tight junction formation, cell adhesion, and apical-basal polarization, suggesting that glycosphingolipid molecule may participate in recognition and communication processes [64-66]. They may also function as anti-tumor antibodies [67]. As we mentioned before, their titer in patients with colonic and lung cancers would be low compared with healthy individuals. This decrease in Forssman antibodies titer in cancer patients was ascribed to the anti-Fs-Fs immune complexes when Fs antigen will be shed from the tumor and absorbed by anti-Fs as "self-non-self' cancer concept. The antigen-antibody complexes are then removed from the circulation by phagocytes [53, 68]. In addition, it may be the result of a number of factors, including nonspecific effects such as debilitation (tiredness or loss of energy) and immunosuppression. [53]. Thus, serum level of Forssman antibodies could be used clinically, as an index to determine the cancer recurrence in post-surgically cancer patients [57]. It was also noted a decrease in the level of heterophilic Forssman antibodies in the sera of females with malignant and benign genital tumors compared with healthy ones [69].

Fs antibodies also can be used to diagnose infectious mononucleosis (IM), a disease caused by the Epstein-Barr virus (EBV), both have activity against Forssman antigen [70]. The Davidsohn Differential Test was used to differentiate between heterophile

sheep cell agglutinins in human serum due to Forssman antigen, serum sickness, and Infectious Mononucleosis [71] **(Table 1.2).**

Table 1.2. Davidsohn differential test for differentiation between IM, anti-Fs and Serum Sickness

Antibody	**Absorbed by GPK***	**Absorbed by BBC***
Infectious Mono antibody	NOT absorbed	Antibody removed
Forssman antibody	Antibody removed	NOT absorbed
Serum Sickness	Antibody removed	Antibody removed

*GPK: guinea pig kidney and BBC: boiled beef cell.

Donath-Landsteiner autoantibodies that cause paroxysmal cold hemoglobinuria (PCH) syndrome are also inhibited by Forssman glycolipid more effectively than by globoside [70]. High Fs autoantibody titers were also found in Graves' disease and Hashimoto's thyroiditis [36, 72]. These results suggest that anti-Forssman antibody may be involved in the pathogenesis of these autoimmune diseases [72]. In contrary, low Fs antibodies were found in sera of patients with Guillain-Barré syndrome (GBS) [73].

Fs antibodies were also used as a new biomarker and early indicator for postvaccination immune responses in poxvirus-based prostate cancer vaccine. The vaccine was designed to stimulate T-cell responses to prostate-specific antigen (PSA) by comparing serum antiglycan IgG, IgM, and total Ig profiles in patients before and after initiating treatment with PROSTVAC-VF [74]. **Figure 1.4** summarize the advantages and disadvantages according to Forssman antibodies and antigen.

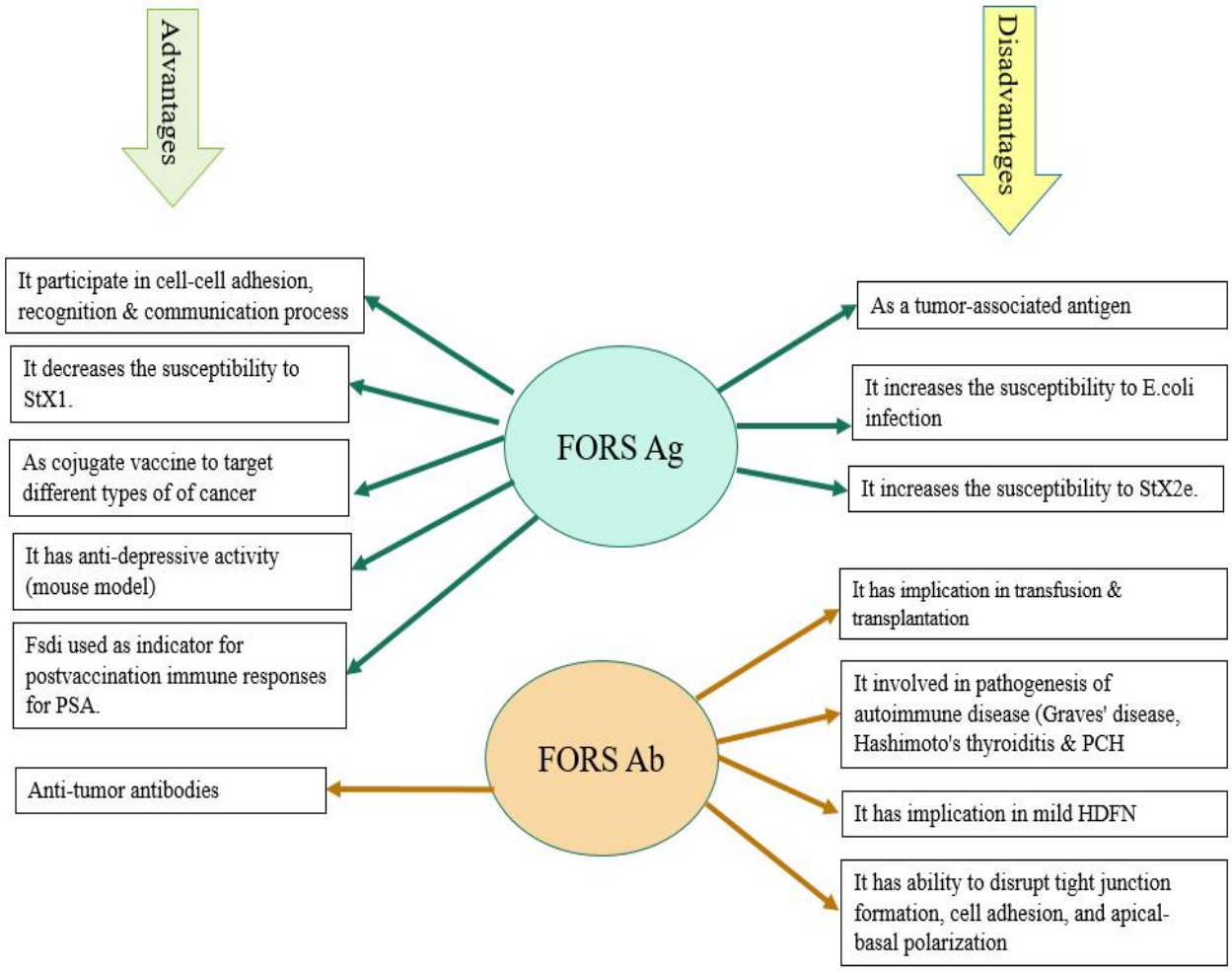

Figure 1.4. Summary of the advantages and disadvantages of Forssman antigen and antibodies. PCH; paroxysmal cold hemoglobinuria, HDFN; hemolytic disease of the fetus and newborn, StX1: Shiga toxins, Stx2e; Shiga toxin 2e, Fsdi; is used as an indicator for disaccharide of the FORS antigen (Fsdi=GalNAcα1-3GalNAcβ), PSA; prostate-specific antigen.

1.7 Detection of FORS Antigen and Antibodies

There is no routine diagnostic serologic reagent being used to detect this red blood cell phenotype, although Forssman reagents are available [43]. All blood groups antigens can

be detected serologically by reacting RBCs with specific antibodies that are prepared specifically against those blood groups' antigens [75].

Currently, direct crossmatching, lectin screening or DNA screening for 887G>A are used to detect Fs-positive donors [12]. RBCs expressing FORS1 antigen are agglutinated strongly by Helix pomatia lectin (HPL), a lectin from Helix pomatia agglutinin [76]. The lectin reacts with both Fsdi-kodecytes and FORS1-kodecytes but it slight preferentially binds Fsdi (GalNAcα1-3GalNAc-R), the terminal disaccharide of Fs antigen over the pentasaccharide [43, 77]. It recognizes (αGalNAc) containing epitopes which are present in cancer cell lines in the very early stages of the metastatic process. HPA staining was used and served as a "surrogate marker" for Fs antigen expression [36]. So, elevated HPA staining in the tissues could be a result of the increased GBGT1 expression and Fs synthesis. Thus, it is a determinant for poor prognosis in some carcinomas and malignancies, if GBGT1 gene expression increased in those tissues [36, 76].

In the past, sheep erythrocytes were used to detect FORS antibodies as they contain great amounts of the Forssman antigen. Recently, Kodecytes with synthetic disaccharide (Fsdi –kodecytes) and pentasaccharide Forssman (FORS1-kodecytes,) function-spacer-lipid (FSL) constructs were used to detect the anti-FORS [43]. The FSL-FORS1 is synthesized to be similar to the natural glycolipid of Forssman with some differences.

1.8 Mechanisms for FORS Antigen Positivity and Negativity in Human

There have been studies aimed to understand why human tissues do not express Forssman glycolipid. The absence of Forssman glycolipid (FG) in normal human tissues indicates either that this molecule is dispensable in terms of physiological functions [35] or there is another glycolipid that has the same function as FG [31]. Previous studies showed that the human FS mRNA was translated less efficiently than other species. Thus, protein synthesis was absent rather than the absence of functional Fs enzyme.

Some studies have reported the presence of the FORS antigen in certain human embryonic tissues [45]. Small amounts of FORS antigen were also found in some specimens of normal human gastric and colonic epithelium and in gastric [42, 53], colonic, and pulmonary carcinomas from cells originated from Forssman negative tissues [56, 66, 67]. The Forssman pentaglycosylceramide has also been characterized in normal human kidney [78] and in human lung [79].

Many mechanisms were hypothesised and contributed in GBGT1 gene changes and thus reactivate Forssman synthase in humans, but they are extremely rare [74]. Those mechanisms include overexpression of the GBGT1 gene mRNA, which might acquire altered enzyme specificity, increased stability of mRNA and protein, splicing variations, aberrant glycosylation by another GT, and post-translational protein modifications [19].

Recently, the basis of the Forssman antigen negativity in humans was clarified. Two inactivating missense mutations were identified and produced a truncated or enzymatically inactive enzyme and are very common in almost all individuals. A c.887G.A [p.Arg296Gln] polymorphism confers Forssman antigen positivity in the A_{pae} individuals, and Yamamoto suggested that either this or c.688A.G [p.Ser230Gly] polymorphism alone in human may produce GBGT1 gene protein with weak Fs activity [19]. The presence of FORS1 on erythrocytes in the A_{pae} individuals may be produced by those cells or adsorbed from tissues like the Lewis antigens [19].

Thus, Gln296 is responsible for activating Fs and present in lower mammals, whereas Arg296 is responsible of inactivating Fs (wild type) and found in primates, including humans [12, 22]. The explanation of that, the exchange of arginine by glutamine permitted the enzyme to make contact with the UDP- sugar (uracil-diphosphate-sugar) donor then catalyses synthesis of the terminal 3-a-N-acetylgalactosamine to its globoside acceptor [37, 80].

1.9 FORS Antigen & Cancer

In general, the change in the antigen expression is not constant, it could undergo variations during cellular differentiation, development, and aging as well as pathologic conditions, mostly in carcinogenesis [81]. Several reports have described the presence of blood group antigens in tumor tissues. The appearance of incompatible blood group antigens foreign to the host may occur in some human tumors as a result of functional and morphological differentiation of the cell [42, 82].

In the past, Forssman antigen was only detected in malignant tissues- but not detectable in the adjacent normal tissue although contained its globoside precursor substance- including gastric, biliary adenocarcinoma in liver, cervical cancer [83], colonic mucosa and lung carcinoma [84]. It was detected in tumors derived from Forssman-negative tissues whereas in Forssman-positive individuals this antigen was lost if tumors develop [12, 53]. On the other hand, some human tissues neither the normal nor malignant did not contain the Forssman antigen such as breast tissues [85], although a recent study reported the presence of FORS1 in breast cancer tissues [76]. Thus, the appearance of Forssman antigen in tissues is not associated with conversion of normal cells into tumor cells [85] but it has implication in cell adhesion, migration, proliferation and tumour growth [86].

In human, the mechanism of how Forssman antigen emerge in some tumors and malignancies is unknown. Some factors related to tumors may be reactivation of Forssman synthase, or other N-acetylgalactosaminyltransferases may be activated and lose their substrate specificity and thus may synthesize Forssman antigen [35]. Certain tumor tissues exhibit higher concentration of UDP-GalNAc (uracil-diphosphate- N-acetylgalactosamine) and Gal-GlcNAc (Galactose-N-acetylglucosamine) than threshold levels in the Golgi apparatus. Thus, the O-GlcNAc tranferase (OGT) will be activated and O-GlcNAcylation of target protein enhanced [19] or tumor induction of other enzymes having a similar GBGT1 enzymatic activity [28].

GBGT1 is one of glycosyltransferase-encoding genes that is regulated by DNA methylation. Previous studies explained one mechanism by which GBGT1 expression is regulated on the transcriptional level in ovarian cancer. They showed that GBGT1 expression is silenced through DNA hypermethylation in ovarian cancer cells but was re-activated by 5-Aza (5-Azacytidine) treatment [36].

As we mentioned before, the FORS1 antigen expression has been shown in many types of malignancy and carcinomas, so it could be recognized as a tumor-associated antigen [42]. The anti-Fs lectin (Helix pomatia) was used to recognize αGalNAC containing epitopes and the tumor tissues exhibiting higher concentrations of UDP-GalNAc and UDP-GlcNAc, which showed increased level of OGT [19]. So the detection of αGalNAc epitopes in cancer cell lines in the early stages of the metastatic process can be used as a tumor marker and used to follow treatment and disease prognosis. In addition, it might be possible immunologically to suppress tumors in O or B individuals by immunization with FORS glycolipid suitably arranged on a carrier macromolecule [42].

All these results confirming unusual enhancement of activity of FORS antigen in human tissues was strongly related to carcinogenesis and indicated that assay of the antigen would be of value to detect cancer [87]. Since the Forssman antigen is a tumor-specific antigen, it could be possible to incorporate it into conjugated vaccines used to target different types of cancer to raise an immune response against the Forssman pentasaccharide expressed on the surface of such cancerus cells [88].

Herein, we investigate the prevalence of FORS1 antigen in a Palestinian donors and a smaller European donors from Sweden and Portugal. We also aimed to determine the molecular basis for Forssman antigen negativity in those populations and to develop a method for routine testing of donor blood for the anti-Fs antibodies.

1.10 Problem Statement

The expression of FORS1 antigen has been demonstrated in several human tumors and FORS1 antibodies might have implications in both transfusion medicine and organ transplantation. The negativity of FORS1 antigen in humans has also been recently found to be due to two missense mutations in the GBTG1 gene. The global frequency of the FORS blood group is still unknown. The frequency of FORS1 antigen is probably very low and thus, most individuals are genetically FORS1 antigen negative. There are no previous studies about the prevalence of antigen and antibodies of Forssman in general, and its molecular basis neither in Palestine, nor in some other populations.

Recently in Sweden, Lund University has screened 256 blood donor for the c.A887G (Gln296Arg) and all of them were mutant [12, 37]. In general, the present study aimed to contribute to a better understanding of the Forssman antigen and antibodies in healthy individuals in the selected populations, the subject of this study.

1.11 Study Aims

The general aim of this study was to investigate the FORS blood group antigen (FORS1) and antibody (anti-FORS1) in specific populations (Palestine, Sweden and Portugal) and to determine the molecular basis of Forssman antigen negativity in these populations.

The specific aims were :

- To study the prevalence of Forssman Antigen in Palestinian population and some European populations in Sweden and Portugal.
- To validate the presence of anti-FORS by developing a method for routine testing of blood donors.
- To investigate the genetic mutations responsible for FORS1 antigen negativity in those populations.

Chapter Two

Materials and Methods

2.1 Materials

All materials used in this study are listed in **Table 2.1**. All reagents and chemicals used were of analytical grade.

Table 2.1. **Instruments and reagents used in the study.**

Item	Components	Supplier/Company
Blood collection tubes	EDTA Vacutainer® blood tubes.	
Blood grouping reagent	Commercial sera: Anti-A, anti-B and anti-D	
Fixative solution	Formaldehyde	

MasterPure™ DNA Purification Kit for Blood Version II	1- Red Cell Lysis Solution 2-Tissue and Cell Lysis Solution 3-MPC Protein Precipitation Reagent 4-RNase 5-TE Buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA)	Epicentre Chicago, USA.
PCR machine	DNA Engine PTC-200 Peltier Thermal Cycler (Dual-Block Models)	SDS (Scandinavian Diagnostic Services) Carlsbad, USA
PCR tubes	0.5 ml thin-walled reaction tubes with flat caps, Lot. AB-0533.	Thermo Scientific Waltham, USA
PCR components	Colorless Master Mix 1.1 X	Thermo Scientific Waltham, USA
GeneJET PCR purification kit	Binding Buffer, concentrated Wash Buffer, Elution Buffer (10 mM Tris-HCl, pH 8.5) and GeneJET Purification Columns (preassembled with collection tubes).	Thermo Scientific, Waltham, USA
ABI PRISM® 310 Genetic Analyzer	10x EDTA buffer, Polymerase	Applied Biosystems, California, USA
Cycle sequencing kit	BigDye® Terminator v1.1 Sequencing kit (BigDye Terminator v1.1 Matrix Standards)	Applied Biosystems, California, USA
Injection solvent for genetic analyzer	Hi-Di™ Formamide	Applied Biosystems (PN 4311320)
Agarose LE	EEO 0.12	PRONAROSE

Nucleic Acid Gel Stain	GelStar	Lonza, Basel, Switzerland
PCR product Dye	1- 6x Loading Dye Solution 2-BFB-glycerol	
Gel agarose standard	Gel agarose standard O GeneRuler 100 bp (green color).	Fermentas, Waltham, Massachusetts, USA.
Power Supplier	E443 (100-150 voltage)	CONSORT, Hertfordshire, England.
RBC Preservative/stabilization solution	CellStap	Bio rad, Hercules, California, USA.
Precipitation solution for anti-FORS1.	Ammonium sulfate (C=0.18 g/ml)	
Gel cards	1-NaCl, Enzyme Test & Cold agglutinins for IgM. 2-Coombs Anti IgG (rabbit) for IgG.	DiaMed, GmbH, Cressier FR, Switzerland)
PBS	Buffered isoton NaCl (pH=7.1)	
Gel card centrifuge	ID- Centrifuge 12 SII Micro Typing System	DiaMed-ID
Gel card incubator	ID-Incubator 37C	DiaMed, Ghaziabad, India.
Secondary antibodies	Fluorescein isothiocyanate (FITC) goat anti-mouse IgG (H+L, Heavy and Light chains) and IgM secondary antibodies.	Zemed, Invitrogen, Carlsbad, CA)

Penta-kodecyte	FSL (Function Spacer Lipid) construct 20 µg/mL penta Fs solution	(KODE technology, Auckland, New Zealand;http://www.kodebiotech.com).
Anti-Fs culture supernatant	monoclonal anti-Fs IgM which was concentrated from hybridoma tissue culture supernatant, rat cell culture (M1/22.25.8.HL (ATCC® TIB. 121™)	ATCC, Manassas, Virginia.
HPA (Helix pomatia agglutinin)	Lectin from Helix pomatia	SIGMA – ALDRICH, USA

2.2 Methods

2.2.1 Study Population

A convenient sample of 211 Paletinian adults, aged 19- 35 years (mean 20.5 ± 2.5 SD) who were students at Al-Quds University, main campus in Abu Dies were recruited to participate in this study. Study donors were from different geographical cities in Palestine **(Figure 2.1)** and from different colleges **(Figure 2.2)**. The details of each Palestinian donor are present in Appendix B and sample collection method in Palestine is present in appendix C.

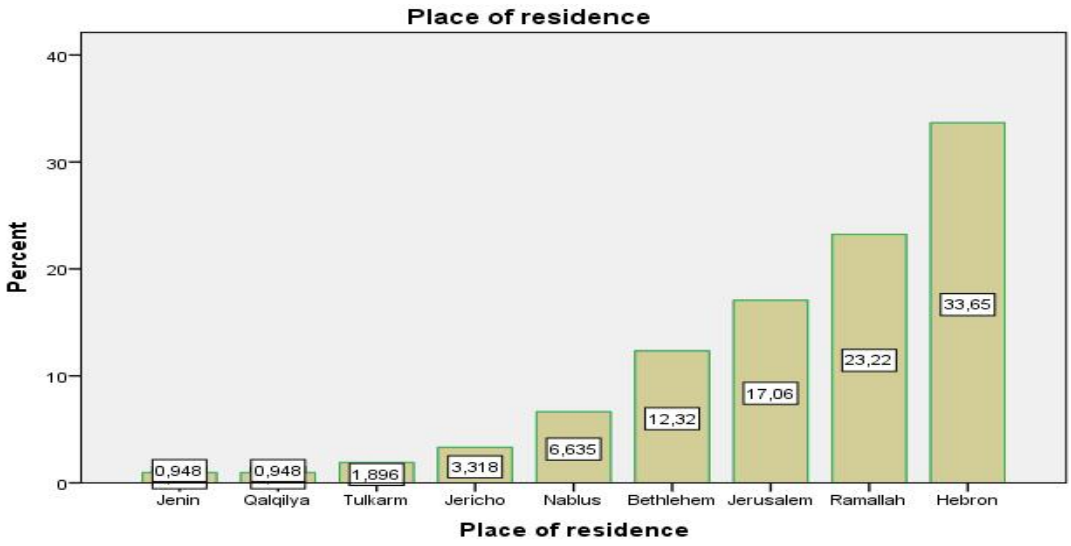

Figure 2.1. Regional distribution of Palestinian participants. Data are expressed as percentage (%).

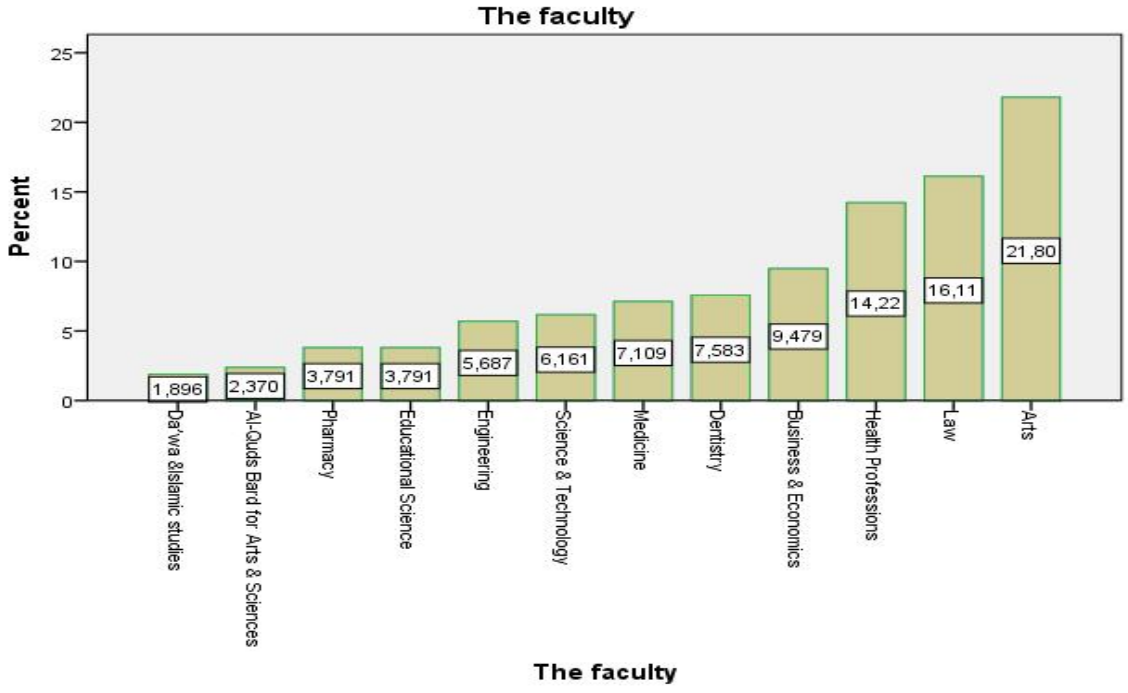

Figure 2.2. Distribution of Palestinian donors based on the faculty they were attending at time of sampling. Data are expressed as percentage (%).

Additionally, DNA samples from 47 Swedish and 18 Portugease blood donors as the Euoropean population were included in the study. All Swedish DNA samples were from randomly picked blood group O donors, thus the age or gender of them were unknown. Portuguese donors baseline characteristics are summarized in Table 2.2.

Table 2.2. Portuguese donors (n=18) baseline characteristics. Results are expressed as mean ± SD for age variable, and as frequency for the gender and blood group.

Gender	M: 9	F: 9
Age	Mean= 28.5	SD ±12.96
Blood group	Unknown 1	
	A Rh- 1	A Rh+ 5
	AB Rh- 0	AB Rh+ 2
	B Rh- 1	B Rh+ 1
	O Rh- 3	O Rh+ 4

2.2.2 Study Questionnaire

An interview-based questionnaire was prepared for this study and aimed to collect general demographic data (gender, age, college, residence place [city)]) and general health status. A copy of the study questionnaire is shown in Appendix D (study questionnaire in Arabic and English languages).

2.2.3 Sample Collection and Preparation

Subjects who accepted to participate in the study were asked to donate 5 ml of blood. Blood samples were collected using a syringe and dispensed into two EDTA tubes. One for blood typing and preparation of buffy coat for genomic DNA purification and the second tube for antibody testing. Blood samples were centrifuged at 3000 rpm for 5 minutes in a Heraeus Labofuge (model 200, UK) and the following components were collected:

- From the tube intended for blood typing and DNA preparation, the buffy coat (200 µL) was transferred to a new tube, stored at -20°C and used for preparation of genomic DNA as described in a later section; RBCs were collected, stored 4-8°C and used later for blood typing.
- From the tube intended for antibody testing, plasma was collected and stored at -20°C until used.

2.2.4 Ethical considerations

All subjects who agreed to participate in the study were briefed about the study aims and asked to sign an informed consent (See the study questionnaire in Appendix D). The principles of Helsinki declaration for scientific research were applied.

2.2.5 ABO Grouping and Rh Typing

ABO grouping and Rh typing were done by standard tube method. A small quantity of whole blood was washed 3 times by normal saline. Three drops of RBC deposit were suspended in 5 mL of normal saline to give 3-5% suspension of red cells. Three tubes labelled A, B and D were used for grouping as shown in **Table 2.3**. The tubes were mixed briefly and let to stand for 10 minutes at room temperature. Tubes were then centrifuged for 1 minute at low speed, 1000 rounds per minute (rpm) by using Heraeus

Labofuge 200,UK. The tubes were mixed, and the deposits of red cells were examined for agglutination. Agglutination of tested RBCs indicated a positive result or presence of the respective antigen. While lack of agglutination of tested RBCs indicated a negative result or absence of the respective antigen.

Table 2.3. ABO standard tube procedure.

	Tube A	Tube B	Tube D
Anti-serum	One drop (Anti-A)	One drop (Anti-B)	One drop (Anti-D)
3% red cell suspension	One drop	One drop	One drop

2.2.6 FORS1 antigen typing:

Grouping for FORS antigen was performed using monoclonal anti-Fs IgM which was concentrated from a hybridoma cell culture supernatant, rat cell culture (M1/22.25.8.HL (ATCC® TIB. 121™). A small quantity of whole blood was washed 3 times by normal saline. An equal volume from 3% suspension of washed RBCs and anti-Fs were added in a tube and incubated for 1 hr at room temperature. The tubes were then centrifuged for 1 minute at low speed (1000 rpm) by using Heraeus Labofuge 200,UK. They were graded macroscopically according to standard blood bank practice and the result was interpreted as described for the ABO typing method. Some samples were picked and examined microscopically to verify the negative reaction.

2.2.7 FORS Antibodies

In the absence of natural FORS1-positive RBCs, more than one method were tested for anti-Fs antibodies detection. Then, sheep red blood cells protocol (**2.2.7.4**) was used to test plasma from study subjects.

2.2.7.1 Ammonium sulfate precipitation for FORS1 antibodies

A suspension of Forssman antibodies was obtained from the respective cell culture. The cells used in this culture were derived from early mouse embryo, hybridoma B lymphocytes and rat IgM isotype. The suspension was concentrated by ammonium sulfate precipitation. A solution of 0.18 g/ml ammonium sulfate was prepared by diluting 9 g of ammonium sulfate in 50 ml of distilled water. An equal volume of ammonium sulfate was added slowly to antibody supernatant to give a final concentration of 50% saturation. The cells were incubated at 4°C for 2 hrs and then centrifuged at 3000 rpm for 10 minutes in a Thermo Scientific Multifuge 1S-R, Woburn, MA. The supernatant was removed and the pellet was suspended in phosphate-buffered saline (PBS) to give 10%-20% of the starting volume.

2.2.7.2 Kodecyte- based serological test for FORS1 antibodies

For penta Kodecyte preparation, the synthetic glycolipids were adsorbed onto blood group O RBCs which are then called Kodecytes and used for testing plasma for anti-FORS1 antibodies. Briefly, FSL (Function Spacer Lipid) construct for FORS1 pentasaccaride was obtained from KODE Biotech Materials Ltd (KODE technology, Auckland, New Zealand;http://www.kodebiotech.com). Blood group O RBCs were washed 6 times with PBS buffer. In every washing step, RBCs were suspended in at plenty of PBS buffer, centrifuged at 3000 rpm for 5 minutes and supernatant was discarded. The latter process was repeated 6 times and RBC pellet was suspended in PBS

to give an RBC suspension with a PCV of about 80%. Next, 100 µL of 100µg/mL penta Forssman FS- FSL solution were diluted by PBS buffer to make a concentration of 25µg/mL. A 100 µL of the washed group O RBC were added to 200 µl of the 25µg/mL modification solution (1:3). The solution was then incubated at 37°C for 2 hrs with occasional mixing and then cell suspension was incubated at 4°C overnight. After that, the treated RBCs were washed 6 times with PBS buffer and re-suspended in RBC preservative (CellStab) solution to 0.8%. Also, untreated O RBC was prepared with RBC preservative (CellStab) and used as a negative control. Treated cells (synthetic glycolipids adsorbed onto RBC, Kodecytes) and untreated RBC were tested using Gel cards and Flow cytometry methods.

Additionally, a second preparation of Kodecytes was prepared by incubating O cells in 50 µg/ml of FS-FSL solution instead of 25 µg/mL and then suspended in CellStab to give a 1% suspension of kodecyets. The second preparation of kodecytes aimed to investigate the effect of FS-FSL concentration in preparation of kodecytes. The kodecytes were tested by two methods:

Penta Kodecyte gel card method*:* The Kodecytes were tested in two gel cards; NaCl and cold agglutinins (DiaMed) for IgM and Coombs Anti IgG, rabbit (DiaMed) for IgG. As a positive control, a 50 µL of treated RBCs (Kodecytes) were crossed with 25µL of 1:100 Helix pomatia lectin and anti-Fs culture. As a negative control, CellStab, A_{pae} W plasma, and A_{pae} B plasma were used (two FORS1 positive individuals). The same steps were done by untreated RBCs as a negative control. The cards were incubated at room temperature for 15 minutes for IgM and at 37°C for IgG using ID-Incubator (DiaMed, Cressier FR, Switzerland). Then, gel cards were centrifuged at 1030 rpm for 10 minutes using ID- Centrifuge 12 SII Micro Typing System (DiaMed-ID, Cressier FR, Switzerland). Gel cards were then examined visually and interpreted according to the manufacturer's directions.

Penta Kodecyte Flow Cytometry method*:* For RBC fixation, a 50 µL of RBCs (treated and untreated) were fixed by the addition of 100 µL of paraformaldehyde to reduce agglutination of antigen-positive cells. The cells were then incubated at room temperature

for 20 minutes under constant mixing. Cells were centrifuged at 300 x g for 1 min and the supernatant was discarded. The fixed RBCs were used in the flow cytometry method.

As primary antibody, a 25 µL and 20 µL of anti-Forssman and plasma from A_{pae} W and A_{pae} B, respectively were added to fixed RBCs. As a negative control, 25 µL of PBS buffer was used. PBS was added to a total volume of 50 µL to each tube. Tubes were incubated at room temperature for 10 minutes under constant mixing. After that, incubation continued at at 4°C for an additional 50 minutes. The cells were washed twice with 150 µL PBS. The RBC pellet was re-suspended in 50 µL PBS. A 5 µL from FITC-goat anti-mouse IgG (H+L) and IgM (Zemed, Inviitrogen, Carlsbad,CA) secondary antibodies were added. After incubation at room temperature for 10 minutes in the dark under constant mixing, the samples were washed twice with 150 µL PBS. Then, the RBCs were re-suspended in 300 µL PBS. The results were analysed by BD FACSCalibur Flow Cytometry System using FACSflow sheath fluid. In total, 10,000 events were collected at a flow rate of 60 µL/min.

2.2.7.3 Using 50µg/ml FSL-Fs pent saccharides with diluted plasma- Gel card method

Different dilutions of plasma titres were prepared, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256 for Apae W, Apae B, and random sample (it was negative for FORS1 antigen). The protocol in part 2.2.7.2 was done except that the 0.8% kodecytes were replaced with the 1% kodecytes.

2.2.7.4 Using sheep red blood cells

Sheep RBCs –as Forssman antigen source- were washed 4 times with PBS buffer and suspended in **L**ow Ionic Strength Solution (LISS) to a final concentration of 0.8%. For sheep RBCs treatment, bromelain (Br) enzyme was used to remove antigens that mask the FORS1 antigen. Sheep RBC were incubated with bromelain at room temperature for

10 minutes.. Additionally, sheep RBCs were treated with bromelain without washing to look their reaction. The cells were then tested with plasma from A_{pae} W and A_{pae} B individuals as well as with anti-Fs antibodies (IgM obtained from cell culture supernatant). A serial dilution of plasma titres, 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 also were performed and tested with sheep RBCs by gel cards.

For tube method, sheep RBCs were washed four times with PBS and suspended in PBS to give a 0.8% suspension. Two drops of plasma sample were mixed with 1 drop of 0.8% suspension of sheep RBC. The tubes were incubated for 1 hr at room temperature (for saline testing) and at 37°C (for AHG testing). Each sample was also tested in duplicate and tested in saline to assess IgM agglutinins and with AHG for testing for IgG agglutinins.

For Gel card method, a 50μL of 0.8% suspension of sheep RBCs were transferred into each of two gel cards (Bio Med): the first card contained NaCl, Enzyme & Cold agglutinins for IgM and the second card contained rabbit anti-human IgG (AHG reagent). A 25 μL of each plasma sample were added to each gel card. The cards were incubated for 15 minutes at room temperature for first card (saline and enzyme card) or at 37°C (for the AHG card). Then, gel cards were centrifuged at 1030 rpm for 10 minutes at 900/1000 rpm using ID- Centrifuge 12 SII Micro Typing System (DiaMed-ID, Cressier FR, Switzerland). Gel cards were then examined visually and interpreted according to the manufacturer's directions.

2.2.8 DNA Analysis

Our aim in this part was to sequence and analyse the exon 7 of GBGT1 gene and more specifically the 3' end of exon where most gene-inactivating mutations occur such as c.688G.A [p.Gly230Ser] and c.887A.G [p.Gln296Arg mutations. Preparation of Genomic DNA was performed from buffy coat which was collected from 2 ml of EDTA whole blood and used for the DNA purification processes.

2.2.8.1 Preparation of genomic DNA

For Palestinian samples: Genomic DNA was prepared from buffy coat using the MasterPure™ DNA Purification (Epicentre) according to the manufacture instructions for Blood Version II kit. Briefly, a 2 ml EDTA whole blood was centrifuged at 1,000 x g for 15 minutes. A 200 µl of buffy coat (the white interface between the plasma and the red blood cells) was transferred carefully to a new tube. The buffy coat was either processed immediately or stored at 4°C for 1-7 days. The buffy coat sample was vortexed and was transferred to 1.5 ml micro centrifuge tube. A 600 µl of Red Cell Lysis Solution was added. The tube was inverted three times and then the bottom of the tube was flicked to suspend any remaining material. Then, it was incubated at room temperature for 5 minutes, inverted three times and it was flicked as outlined above. Then, it was incubated at room temperature for an additional 5 minutes, inverted three times and then it was flicked. The white blood cells were pelleted by centrifugation for 25 seconds. The supernatant was removed and ~25 µl of liquid was left. The tube was vortexed to suspend the pellet. The white blood cells were re-suspended in 300 µl of Tissue and Cell Lysis Solution by pipetting several times. A 1 µl of RNase was added and was mixed thoroughly, and then it was incubated at 37°C for 30 minutes. The samples were placed on ice for 3-5 minutes. A 175 µl of MPC (MasterPure™ Complete) Protein Precipitation Reagent was added to 300 µl of lysed sample and vortexed vigorously for 10 seconds. The debris was pelleted by centrifugation for 10 minutes at 13 rpm. The supernatant was transferred to a clean centrifuge tube and the pellet was discarded. A 500 µl of isopropanol was added to the recovered supernatant and mixed by inverting the tube 30-40 times. The DNA was pelleted by centrifugation at 4°C for 10 minutes in a microcentrifuge. The isopropanol was poured off carefully without dislodging the DNA pellet. Then, the pellet was rinsed twice with 70% ethanol. All of the residual ethanol was removed. The DNA was re-suspended in 35 µl of TE Buffer. The extracted DNA was stored at –20°C.

For Swedish samples, genomic DNA was prepared using Qiagen EZ1 DNA Blood 200 µl Kit (Robot) for automated purification of genomic DNA from whole blood samples.

Procedure details are provided in Appendix E. The BioRobot EZ1 DSP system (Qiagen, Germany) is a fully automated machine and uses magnetic-particle technology.

For Portuguese samples, genomic DNA was prepared using Wizard Genomic DNA Purification Kit (Promega). The procedure details are provided in Appendix F.

2.2.8.2 DNA Quantification

DNA concentration for Palestinian samples was measured spectrophotometrically by (Nanodrop 2000c; Thermoscientific) and for Swedish samples, the BioPhotometer Plus (Eppendorf) was used. Distilled water was used as a blank. Most readings were done twice to check the accuracy and precision. Spectral measurements at 230nm, 260nm, and 280nm were also done and the ratios (260/280 nm and 260/230 nm) were calculated to assess the purity of DNA samples. DNA concentration was adjusted to 50-150 ng/μL) in each 25 μL PCR reaction. Concentrated DNA samples were diluted with TE buffer. In contrary, the volume of template DNA was increased for samples with low concentration. DNA samples with good purity and A260/A280 ratio about 1.8 (1.7 to 1.9) were used.

2.2.8.3 Primer Design

We planned initially to sequence the whole GBGT1 gene. So, five pairs of primers were designed covering exons (2-7) using the primer design software in UCSC database from University of California, Santa Cruz Genome Bioinformatics. The sequence, length, melting temperature, nucleotide position and sequence are listed in **Table 2.4**. Our primers were 18-22 bases in length. They were selected with an average GC content around 52.4%-66.7% with random base distribution. Because exon 7 is long and it has high GC content, three primer pairs A, B and C were used and designed to cover the whole exon 7. Exon 7C primers were designed and used for all the samples. It covers from 970-1361 nucleotide (395bp) positions in mRNA according to EMBL (the

European Molecular Biology Laboratory) accession no. ENST00000372040 (position of 220-347 amino acids) which include the main SNPs reported to cause inactivation of the enzyme (namely Gln296Arg & Gly230Ser).

Table 2.4. Primers used for analysis of GBGT1 gene.

Primer	Oligo[1] Name	Sequence (5'->3')	Length (bp)	T_m[2]	GC-content
1	Exon 2 F[3]	CTCAGAGCCTGACATCCCTG	20	61.4	60%
2	Exon 2 R[4]	AGTCCTGGGTGGGGATAGAG	20	61.4	60%
3	Exon 3 F	GTTTTCAGCCTCTGGTGCAG	20	59.4	55%
4	Exon 3 R	CTCTCCTGTCTCCCCAACTG	20	61.4	60%
5	Exon 4-5 F	TCCTCCTTCTTCCTGTGCTG	20	59.4	55%
6	Exon 4-5 R	TAGCACCTCCACTACCCCAC	20	61.4	60%
7	Exon 6 F	AGAGATGGGGACGAAGCTG	19	58.8	57.9%
8	Exon 6 R	CCCAACTATAAACTCCTGTGGC	22	60.3	50%
9	Exon 7Af	CACACAGTGGGGACCCTG	18	60.5	66.7%
10	Exon 7aR	GATGGGGATGGAGCTGAG	18	58.2	61.1%
11	Exon 7bF	TTCTCAGCTCCATCCCCATC	20	59.4	55%
12	Exon 7bR	CCCACCATAATAGAAGTCCCC	21	59.8	52.4%
13	Exon 7cF	TTGGGAGACCTGGTGGCTGC	20	63.5	65%
14	Exon 7cR	CTCCGTGGTCAGCTCCTCAG	20	63.5	65%

[1]Oligo: Oligonecleotide; [2]T_m: melting temperature, [3]F: forward primer; [4]R: reverse primer.

2.2.8.4 DNA Amplification by PCR

The PCR mixture with final 1X reaction was used for DNA amplification. A final volume of 25µl was used and included 1 µL genomic DNA (~100 ng), 20 µL PCR buffer (buffer: 75 mM Tris-HCl, pH 8.8 at 25°C, 20 m*M* ammonium sulfate,1.5 m*M* $MgCl_2$ and 0.625 Units ThermoPrime *Taq* DNA Polymerase) and 1 µL of each amplification primer (10µM each). PCR amplification was carried out in a PCR system (DNA Engine, PTC-200, Peltier Thermal Cycler; Scandinavian Diagnostic Services (SDS) Company). A no template control (NTC) reaction was performed as negative control. An A_{pae} DNA was also sequenced for exon 7 B and C as positive control for the wild type unmutated GBTG1 gene. Thermal cycling was performed as shown in **Table2.5.**

Table 2.5. Thermal cycling program for amplification of Exon 7c.

PCR program	**Exon 7 amplification**		**Cycle sequencing**	
.	Temp	Time.	Temp	Time
Initial denaturation	95 °C	1min	…….	……..
Denaturation	94 °C	30 sec	96 °C	30 sec
Annealing	57 °C	30 sec	50 °C	15 sec
Extension	72 °C	1 min	60 °C	4 min
Final extension	72 °C	20 min	……..	……..
Keep in machine	6 °C	∞	6 °C	∞
No. of cycles	30		25	

2.2.8.5 Agarose gel electrophoresis

For visualization, Agarose gel electrophoresis was used for separating and analysing DNA. A 1.5% agarose gel was prepared by mixing 0.9 gm Agarose LE (Low Electroendosmosis), PRONAROSE with 60 ml of 0.5TBE (54g Tris, 27,5g borate, 20ml 0.5 EDTA) buffer. Then, the DNA was visualised in the gel by addition of 4 µL Nucleic Acid Gel Stain, Gelstar, Lonza after leaving it to cool down to about 60°C. A 5 µL of the PCR product were electrophoresed on agarose gel. A PCR product from colourless PCR Master Mix was mixed with 1.5µL of Gel loading Dye (6x) and then loaded on the gel. In the first lane, 3 µL of 100 bp DNA OGeneRuler Gel agarose ladder was added and used as a guide. All amplification products were separated by high-voltage electrophoresis (100 voltage for 45 min). Then, the gel was placed on a UV light transilluminator to visualize the DNA fragments.

2.2.8.6 Purification of PCR Product

Purification for PCR product was done using (GeneJET PCR purification kit) from Thermo Scientific Company which uses a silica-based membrane technology in the form of a spin column. A 1:1 volume of Binding Buffer was added to complete PCR mixture then was mixed thoroughly. A 30 µL of the solution from step 1 was transferred to the GeneJET purification column (preassembled with collection tubes). It was centrifuged for 30-60 s. Then, the flow-through was discarded. A 700 µL of a concentrated Wash Buffer was added (it was diluted at first with the ethanol) to the GeneJET purification column. Then it was centrifuged for 30-60 s. After that the flow-through was discarded and the purification column was placed back into the collection tube. The empty GeneJET purification column was then centrifuged for an additional 1 min to completely remove any residual wash buffer. The GeneJET purification column was transferred to a clean 1.5 mL microcentrifuge tube. A 50 µL of Elution Buffer (10 mM Tris-HCl, pH 8.5) was added to the centre of the GeneJET purification column membrane and then was centrifuged for 1 minute. The GeneJET purification column was discarded and the

purified DNA was stored at -20°C [89]. The quality and quantity of purified PCR product was assessed by agarose gel electrophoresis.

2.2.8.7 Sequencing of PCR products

Sequencing was done by dye terminator cycle sequencing chemistry. A 4 µL pure PCR product, 8 µL ddH2o (double distilled water), 1 µL Primer, 2 µL BigDye Terminator v1.1/3.1 Sequencing Buffer and 6 µl BigDye Sequencing Buffer were mixed in a PCR tube. This mixture was subjected to 25 three-temperature cycles (See Table 2.6, Cycle sequencing program).The sequencing reaction is summarized in **Table 2.6**.

Table 2.6. Cycle sequencing reaction.

Reagent	**Concentration**	**Volume µL.**
BigDye Terminator v1.1/3.1 Sequencing Buffer	5X	2
BigDye Sequencing Buffer	5X	6
Primer	10X	1
Template		4
dd water		8
Final volume		20
Mix well and spin briefly. Then, the tubes were placed in a thermal cycler and the volume was set to 20 µL.		

2.2.8.8 Ethanol/EDTA Precipitation

To precipitate the 20 µL sequencing reactions, a 100 µL of 99% ethanol and 5 µL of 125 mM EDTA were added to each PCR tubes. The mixture was incubated for 30 min to 4

hours at room temperature, and centrifuged for 20 min at 12000g. The next step followed immediately and if this was not possible, the tubes were spined for an additional 2 min before performing the next step. Ethanol was discarded and the samples were washed with 200 µL of 70% Ethanol. Then centrifuged for 5 min at the same speed and air dried. After that, 20 µL HiDi formamide was used to resuspened the single-stranded sequencing amplicons.

2.2.8.9 Sequencing

Sequencing was performed using DNA sequencing machine (ABI PRISM, 310 Genetic analyser, Applied Bio systems, U.S) which is based on Capillary electrophoresis (CE) and used POP-6 performance optimized polymer 6.

2.2.8.10 Analysis of DNA sequence

BLAST (**B**asic **L**ocal **A**lignment **S**earch **T**ool) searches /CDS feature (complete DNA sequence) was used to compare our DNA sequences with RefSeq, (Homo sapiens globoside alpha-1,3-N-acetylgalactosaminyltransferase (GBGT1), RefSeqGene on chromosome 9, Accession NG033868). Sequences were first examined for 2 polymorphisms in exon7; (c.688G.A [p.Gly230Ser] and (c.887A.G [p.Gln296Arg]. As a positive control we sequenced DNA from an individual positive for FORS1 antigen (A_{pae} variant individual) and looked for Homo sapiens globoside alpha-1,3-N-acetylgalactosaminyltransferase (GBGT1) (Forssman expressing variant A_{pae} #1, Accession HE583597). Other programs were used such as Exome Variant Server (EVS gene); to look for all GBGT1 variants discovered before and the role of each variant (polyphenotyping) in pathogenicity was examined using the Reference SNP (Reference Single Nucleotide Polymorphism) identifier and Ensemble Genomes Browser; to provide complete genome assembly.

2.2.10 Newborn Samples

Some studies have reported the presence of the FORS antigen in certain human embryonic and normal gastric mucosa in fetal gastrointestinal tissues. In addition, previous Swedish study showed that FORS antigen is absent in adult and present in 2 out of 8 newborns (unpublished data), so we aimed to investigate the expression of Forssman antigen in newborns in Palestine. Seventy three samples were collected from healthy newborns from different hospitals in Hebron and Beit-Jala cities (Table 2.7), West Bank, in order to investigate Forssman antigen expression in newborns. The samples were from 1-5 days old babies, except one sample that was collected from a 10-days old baby. Twenty five of these samples were venous blood collected by venipuncture and the other forty eight samples were cord blood samples that were collected immediately after delivery from the umbilical cord. All samples were withdrawn by experienced medical technicians at the respective medical centers in Hebron and Bethlehem. The RBC's that have been separated from cord blood were washed using the same steps used earlier for adults samples but for five times instead of three because to get rid of Wharton's jelly, to be able to use them for blood grouping. The DNA for all samples was extracted from the separated buffy coat using the Genomic DNA Mini Kit (Blood/Cultured Cell) by five steps: sample preparation, cell lysis, DNA binding, washing and DNA elution. An Amplification Refractory Mutation System (ARMS) PCR primers were used to detect the presence of A887G mutation at codon Gln269Arg in 30 samples. DNA sequencing was done for three samples to verify the ARMS PCR results.

Table 2.7: List of medical centers from which the study samples were collected.

Medical Center Name	Number of collected samples
Beit Jala governmental hospital-Beit Jala city	8
PRCS *specialized hospital-Hebron city	22
Alia Governmental Hospital- Hebron city	10
Al -Ahli hospital – Hebron city	33
Total	73

*PRCS: The Palestine Red Crescent Society

2.2.11 Statistical analysis

Descriptive statistics including mean and SD were calculated for all data. The correlation between presence/absence of anti-Fs antibodies and polymorphisms of GBGT1 gene was investigated by Spearman Coefficient correlation. IBM SPSS statistical software version 22 was used for all statistical calculations. Significance was set at $P < 0.05$.

Chapter Three

III Results

The general characteristics of all participants (Palestinian donors) obtained from the study questionnaire were analyzed and summarized in **Table 3.1**.

Table 3.1. Palestinian donors (n= 211) baseline characteristics. Results are expressed as mean ± SD for age variable, and as percentage (%) for the other variables.

Gender	M: 51.7%	F: 48.3%
Age (year)	Mean = 20.52	SD ± 2.471
Previous history of blood transfusion	Yes = 1.4%	No = 98.6%
Pregnant before	Yes = 2.8%	No = 97.2 %
Previous surgery or medical operation	Yes = 19.9%	No = 80.1 %
Chronic or hereditary diseases	Yes = 3.3%	No = 96.7 %

3.1 ABO grouping and Rh typing

The results of ABO and Rh typing for Palestinian samples are summarized in **Table 3.2**. Those for Portuguese Blood samples are summarized in **Table 2.3** (in chapter 2). All Swedish blood samples were blood group O type. Blood groups A and O were the most common blood groups among Palestinian subjects.

Table 3.2. Phenotypes of ABO blood groups and Rh (D) antigen for the Palestinian donors. Results are expressed as frequency.

		Rh(D)		
		Negative (n)	Positive (n)	Total count
Blood grouping	A	8	70	78
	AB	1	21	22
	B	5	31	36
	O	5	70	75
Total		19	192	211

3.2 FORS1 Antigen typing

All the samples were negative for FORS1 antigen by serological testing using anti-Fs antibodies.

3.3 Anti-Fs antibodies

More than one method were used for anti-Fs antibodies detection. Then, sheep red blood cells protocol was used (see section **2.2.7.4**) to test plasma of study subjects. A summary of the results of all anti-Fs testing methods are shown in **Table 3.3.**

Table 3.3 Summary of all anti-Fs testing methods.

Method used for anti-Fs detection			Positive Control		Negative Control			
FORS1 Ag Source	**Conc.**	**Technique**	**1:100 HPL**	**Anti-Fs culture**	**CellStap.**	**A_{pae} W****	**A_{pae} B****	**Untreated O RBC**
FSL-Fs- Kodecytes	**25 µg/mL**	**Gel card**	Agglutination	No agglutination	No agglutination	No agglutination	Agglutination	No agglutination
	50 µg/mL	**Gel card**	Agglutination	Agglutination	No agglutination	Agglutination	Agglutination	No agglutination
		Flow cytometry	Agglutination	Agglutination	No agglutination	Agglutination	Agglutination	No agglutination
Sheep RBC	**0.8%**	**Gel card**	Agglutination	Agglutination	No agglutination	Agglutination	Agglutination	…….

*Unexpected results are shown by red colour.

**A_{pae} W and A_{pae} B are both FORS1 positive individuals.

FSL-Fs: Function-spacer-lipid KODE technology construct for Forssman glycolipid.

For investigation of anti-Fs antibodies, first we had used 25µg/ mL penta- FORS Kodecytes-FSL technology and the gel card system based on hemagglutination principle (protocol 2.2.7.2). In this regard, as a positive control, Helix pomatia lectin (diluted 1:100) gave positive reaction (agglutination) with penta- FORS Kodecytes. On other hand, a negative result was observed when these kodecytes were reacted with anti-Fs antibodies (harvested from cell culture supernatant. RBC preservative solution (CellStap)

and A_{pae} W plasma (one of the FORS1 positive individuals) gave a negative results as expected and were used as negative controls. Unexpectedly, A_{pae} B plasma (another FORS1 positive individual) gave positive result. To exclude possible cross reactions that may be caused by other red cell antigens, all positive and negative controls were tested also with untreated type O red blood cells as another negative control and the result was negative, as shown in **Figure 3.1**.

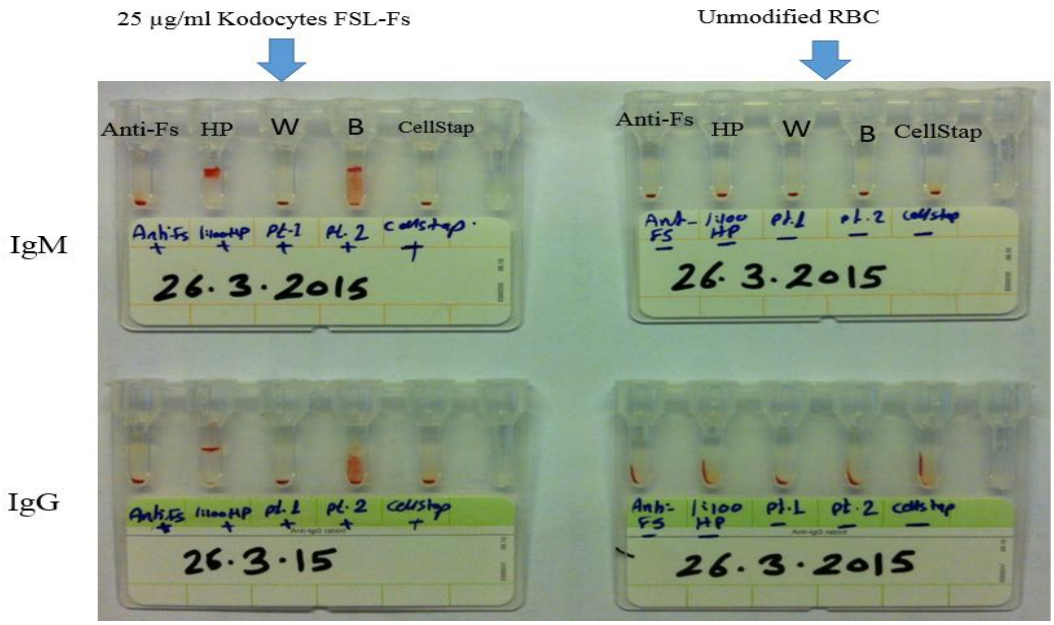

Figure 3.1. **Results of 25µg/ml kodecytes FSL-FORS 5 (penta Forssman kodecytes) by using gel card method.** Anti-Fs; anti-Forssman antibodies without precipitation, HP; 1:100 Helix pomatia, W; A_{pae} W plasma, B; A_{pae} B plasma and CellStap; cell stabilization solution.

We made precipitation for anti-Fs antibodies culture to concentrate the antibodies by using ammonium sulfate **(protocol 2.2.7.1)**. Same results were obtained from antibodies without precipitation in heamagglutination test by gel card method, see **Figure 3.2**.

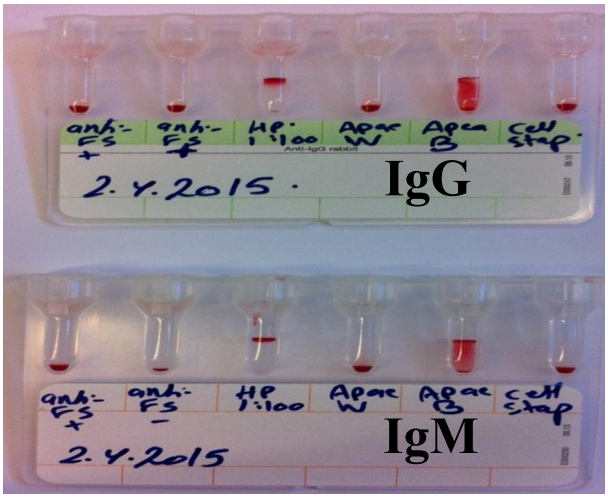

Figure 3.2. Results of precipitated anti-Fs antibodies with 25µg/ml kodecytes FSL-FORS by using gel card method. Anti-Fs +; anti-Forssman with precipitation, Anti-Fs - ; anti-Forssman without precipitation HP; 1:100 Helix pomatia, W; A_{pae} W plasma, B; A_{pae} B plasma and CellStap; cell stabilization solution

Since plasma from both A_{pae} W and B individuals should be negative for anti-Fs antibodies, new kodecytes were prepared with higher concentration of FSL penta sugars to resolve this anomaly. In the second part of **2.2.7.2 protocol**, the concentration of FSL was increased from 0.25 to 0.50 µg/mL. With an FSL concentration of, 50µg/ml, a positive reaction (agglutination) was observed with A_{pae} W, A_{pae} B and anti-Fs antibodies, as shown in **Figure 3.3**.

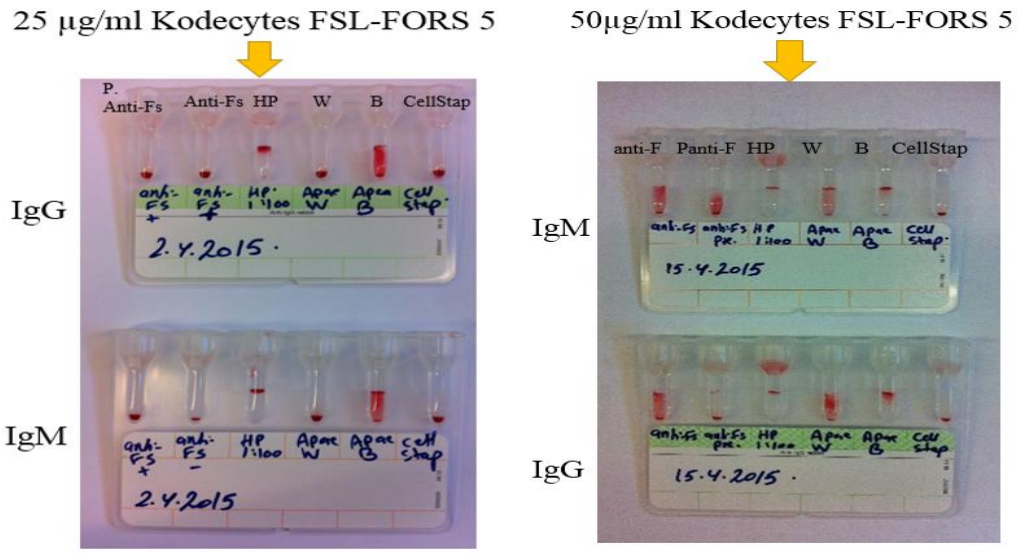

Figure 3.3. Comparison between the result of 25µg/ml and 50µg/ml kodecytes FSL-FORS 5 (penta Forssman kodecytes) gel card method. Anti-Fs; anti-Forssman without precipitation, HP; 1:100 Helix pomatia lectin, W; A_{pae} W plasma, B; A_{pae} B plasma and CellStap; cell stabilization solution.

These results were confirmed by flow cytometry analysis **(2.2.7.2 protocol, flow cytometry part)**, see **Figure 3.4.**

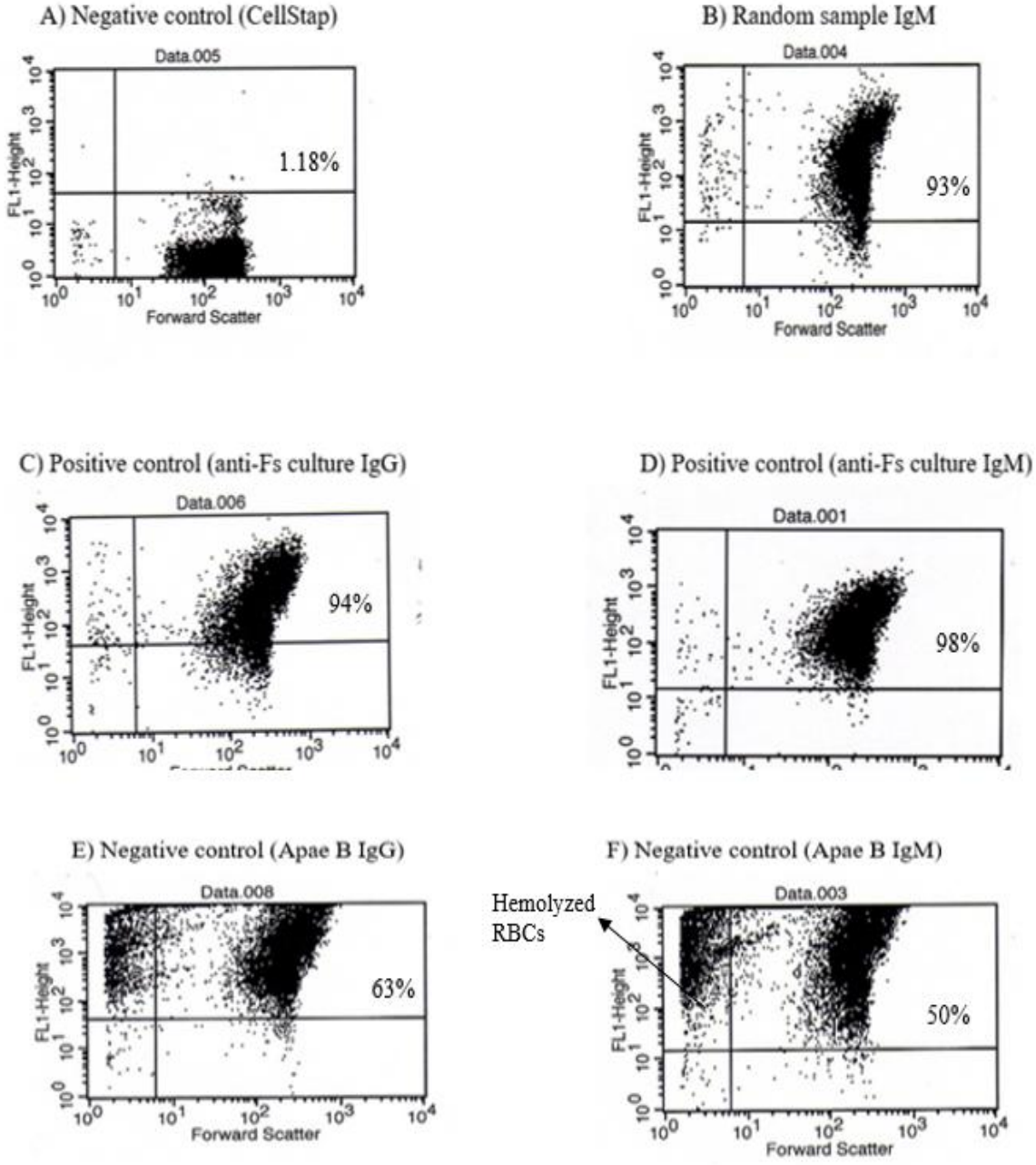

Figure 3.4. Results of anti-Fs testing using 50µg/ml kodecytes FSL-FORS and flow cytometry method. A) Negative control (cellStap), B) Random sample IgM, C) Positive control (anti-Fs culture IgG), D) Positive control (anti-Fs culture IgM), E) Negative control (A_{pae} B IgG) and F) Negative control (A_{pae} B IgM). The percentage in each figure represents the percent of gated population of interest (anti-Fs antibodies population).

To resolve this discrepancy in typing for anti-Fs antibodies, we tested diluted plasma against kodecytes prepared with 50µg/ml FSL-Fs penta-saccharides to test the effect of dilution of agglutination for kodecytes.(see section 2.2.7.3) Overall, the results observed with diluted plasma didn't give a large difference from those with undiluted plasma. One note was, with 1:8 dilution of plasma, the results of the gel card correlated well with the results of flow cytometry analysis using undiluted plasma (**Figure 3.4).** An A_{pae} B IgG and IgM tests gave 2 populations of RBCs (mixed field reaction) in both methods while with gel card and undiluted A_{pae} B plasma they gave positive result with one population. We observed also most of diluted plasma gave negative results with IgG after dilution. **Table 3.4** summarizes these results.

Table 3.4 Reaction pattern for detection of anti-Fs antibodies in diluted plasma from FORS1 antigen positive individuals (A_{pae} W and A_{pae} B) and FORS1 negative individual (PAM10).

Dilution titer	A_{pae} W		A_{pae} B		PAM10	
	IgG	IgM	IgG	IgM	IgG	IgM
1:2	w+	+	+	+	+	+
1:4	-	+	+	+	+	+
1:8	-	w+	MF	MF	w+	+
1:16	-	-	+	+	-	w +
1:32	-	-	+	+	-	w +
1:64	-	-	-	+	-	-
1:128	-	-	-	+	-	-
1:256	-	-	-	-	-	-

*w +: weak positive reaction (agglutination), +; positive reaction (agglutination), _; negative reaction (no agglutination), MF; mixed field and PAM: one of the Palestinian samples.

Using sheep RBCs as platform for testing for anti-Fs (2.2.7.4 protocol) and gel cards are summarized in **Table 3.5**. The anti-Fs and plasma from the individual A_{pae} B agglutinated with the sheep RBCs, while the plasma from the individual A_{pae}W didn't agglutinate in the NaCl Card (IgM) but reacted weakly in the AHG (IgG) card. In some cases, the reactions were stronger when unwashed RBCs were used.

The reaction pattern for plasma from A_{pae}B and A_{pae}W individuals and Anti-Fs with sheep RBC suspended in saline were as follows: plasma of A_{pae}B and Anti-Fs gave positive reaction while with plasma of A_{pae} W individual gave negative reaction and thus remained consistent with previous test (2.2.7.2).

The reaction patterns for diluted plasma with sheep RBCs were; negative reaction with A_{pae} W plasma, positive reaction was observed with A_{pae} B plasma (titer 1:2) and positive reaction with IgM anti-Fs (titre 1:4).

Table 3.5. Reaction patterns of sheep RBCs reaction with A_{pae} W plasma, A_{pae} B plasma and anti-Fs antibodies.

Suspension solution	A_{pae} W				A_{pae} B				Anti-Fs			
	Washed sheep RBC		Unwashed sheep RBC		Washed sheep RBC		Unwashed sheep RBC		Washed sheep RBC		Unwashed sheep RBC	
	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM
LISS	W+	-	W+	-	+	+	+	+	+	+	+	+
Bromelain Enzyme	W+	-	W+	-	+	+	+	+	+	+	+	+
Saline	-				+				+			
Serial dilution	- at all dilutions				+ at 1:2 dilution - at 1:4 and higher dilutions				+ at 1:4 dilution - at 1:8 and higher dilutions			

+: positive reaction (agglutination), - : negative reaction (no agglutination), W+: weak positive reaction, Br enzyme is bromelain and LISS: Low Ionic Strength Saline.

3.3.1 Results of anti-Fs antibodies in Palestinian samples (using sheep RBCs)

The results showed that 6.2% of samples were negative for IgG anti-Fs, 4.3% of samples were negative for IgM anti-Fs and 3.8% (8/211) of samples were negative for both IgM and IgG anti-Fs. The absence of anti-Fs (both IgG and IgM) was more common among males compared to females, where seven of the eight samples which were negative for both IgG and IgM anti-Fs were males. However, stronger reactions (agglutinations) were observed also in females (**Table 3.6).**

Table 3.6 Frequency of IgG and IgM anti-Fs antibodies in Palestinian subjects according to gender.

Gender	IgG anti-Fs antibodies						IgM anti-Fs antibodies						IgG and IgM
	Neg.	V	+1	+2	+3	+4	Neg.	V	+1	+2	+3	+4	Neg.
Female	3	1	13	26	31	25	1	7	19	21	28	24	1
Male	10	6	18	29	27	16	8	5	27	23	29	14	7
Total	13	7	31	55	58	41	9	12	46	44	57	38	8

* Neg.: negative and V: visible.

The majority of samples showed consistent results when tested for both IgG and IgM anti-Fs antibodies. Additionally, the majority of samples gave similar reaction strength when tested for both IgG and IgM anti-Fs (**Table 3.7)**. However, seven samples which tested negative for anti-Fs antibody in the tube method, showed positive reactions in gel cards (+2, +1, weak reaction or double populations).

Table 3.7.Comparison between the reaction patterns in IgG & IgM anti-Fs antibodies. Results are expressed as frequency.

Reaction strength for IgM	Reaction strength for IgG						
		+1	+2	+3	+4	V	Neg.
	+1	21	17	4	0	1	3
	+2	6	16	19	2	0	1
	+3	1	17	24	14	1	0
	+4	2	4	6	22	2	1
	V	0	1	5	3	3	0
	Neg.	1	0	0	0	0	8

*Neg.: negative and V: visible.

There was no correlation between presence/absence of anti-Fs IgM and IgG antibodies and GBGT1 polymorphisms (p= 0.408) and (p=0.433), respectively.

The frequency of samples negative for anti-Fs antibody showed variations in Ig's classes, among blood groups **(Table 3.8)**. The highest percent of samples negative for anti-Fs was in AB blood group, it was 13.6% in AB individuals and 1.4% in all individuals.

Table 3.8. Correlation between anti-Fs antibody negativity and blood groups.

Blood group	N	No. of individuals negative for both IgG and IgM (n)	% of individuals negative for both IgG and IgM in specific group	% of individuals negative for both IgG and IgM in all samples
A	78	1	1.3 %	0.47 %
B	36	2	5.6 %	0.95 %
AB	22	3	13.6 %	1.4 %
O	75	2	2.7 %	0.95 %
Total	211	8	--	3.8 %

Stronger agglutination reactions (grades +3 or +4) for IgG anti-Fs were observed in A and O blood groups, 53.8% and 53.3%, respectively. With IgM anti-Fs antibody stronger reactions were also observed in A and O blood groups, 51.3% and 48% of samples, respectively. The frequency of samples that showed strongest reaction in the IgG reaction (4+) is higher among group A compared to group O. This also applies for IgM reaction. In contrast, weaker agglutination reactions (+1 or visible) for IgG and IgM anti-Fs were observed in B blood group, 25% and 36.1% for IgG and IgM, respectively. Furthermore, in AB blood group, it was 18.1% and 36.3% for IgG and IgM, respectively **(Table 3.9).**

Table 3.9. Reaction strength for IgG & IgM anti-Fs antibodies in relation to blood group.

		Reaction in IgM test tube						Total
		+1	+2	+3	+4	V	Neg.	
Blood group	A	13	19	22	18	5	1	78
	AB	7	2	5	3	1	4	22
	B	11	6	9	6	2	2	36
	O	16	17	23	13	4	2	75
Total		47	44	59	40	12	9	

		Reaction in IgG test tube						Total
		+1	+2	+3	+4	V	Neg.	
Blood group	A	11	19	20	22	3	3	78
	AB	4	5	6	3	0	4	22
	B	9	11	9	4	0	3	36
	O	12	16	26	14	4	3	75
Total		36	51	61	43	7	13	

*Neg.: negative and V: visible

The results of anti-Fs antibodies testing according to receive a blood transfusion history are shown in **Table 3.10.**

Table 3.10: The FORS antibody results according to receive a blood transfusion. Results are expressed by a frequency**.**

		received blood transfusion		Total
		No	Yes	
FORS antibody test	Negative	8	0	8
	Positive	200	3	203
Total		208	3	211

3.4 GBGT1 sequencing results

After serological characterization of the blood samples, the next step aimed at testing samples for the Gln296Arg and Gly230Ser mutations that are responsible for the FORS1 antigen negativity in human. For this purpose, six primer pairs were designed to sequencing exons 2 to 7 of the GBTG1 gene. Exons 3 to 7 of the GBTG1 gene were sequenced for eight samples (6 from Palestine and 2 from Sweden). Seventy samples were sequenced for whole exon 7 (using primer pairs B and C). The remaining samples were sequenced only for exon 7 and specifically the sequencing targeted the 3'end of exon 7 covering the sequence flanking the two main mutation, Gln296Arg and Gly230Ser (using primer pair C) (**Figure 3.5)**. The respective exons were amplified using PCR and then PCR products were purified from agarose gel (**Figure 3.6)** and used for DNA sequencing.

10 20 30 40 50
MHRRRLALGL GFCLLAGTSL SVLWVYLENW LPVSYVPYYL PCPEIFNMKL
60 70 80 90 100
HYKREKPLQP VVWSQYPQPK LLEHRPTQLL TLTPWLAPIV SEGTFNPELL
110 120 130 140 150
QHIYQPLNLT IGVTVFAVGK YTHFIQSFLE SAEEFFMRGY RVHYYIFTDN
160 170 180 190 200
PAAVPGVPLG PHRLLSSIPI QGHSHWEETS MRRMETISQH IAKRAHREVD
210 220 230 240 250
YLFCLDVDMV FRNPWGPETL GDLVAAIHPS YYAVPRQQFP YERRRVSTAF
260 270 280 290 300
VADSEGDFYY GGAVFGGQVA RVYEFTRGCH MAILADKANG IMAAWREESH
310 320 330 340
LNRHFISNKP SKVLSPEYLW DDRKPQPPSL KLIRFSTLDK DISCLRS

Figure 3.5 Amino acids for GBGT1 protein that covered by exon 7C sequenced in this study (by red color). The picture modified from the reference: UniProtKB – ID: Q8N5D6 (GBGT1_HUMAN).

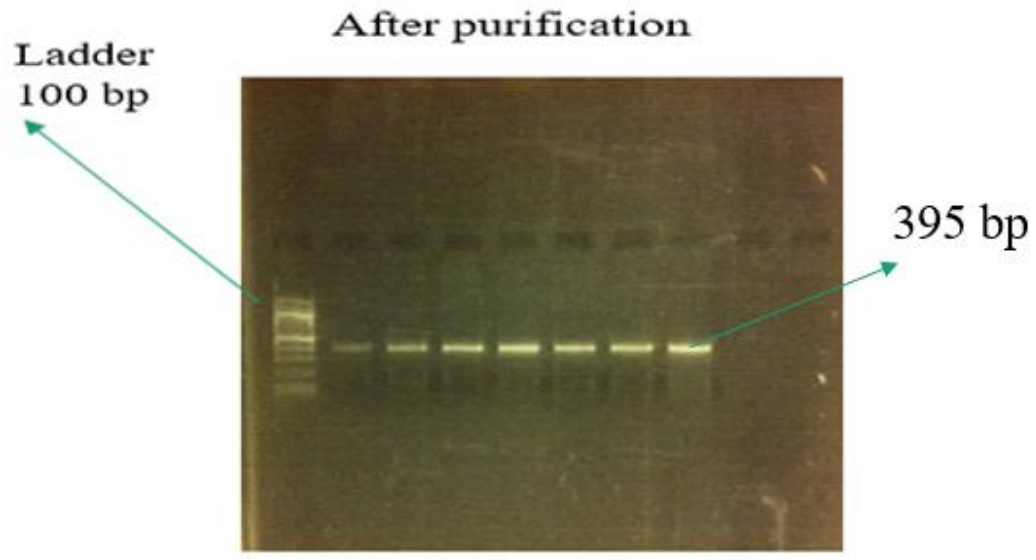

Figure 3.6 Agarose gel electrophoresis for PCR products of GBTG1 gene exon 7 following purification of PCR amplicons from agarose gel.

3.4.1 DNA sequence variants

Exon 7 was sequenced in all study samples and A_{pae} #2 individual was used as a positive control for active GBGT1 (R296Q polymorphism). DNA sequencing revealed that all samples have Gln296Arg and Gly230Ser polymorphisms.

Regarding Palestinian samples, an additional four polymorphisms were reported, one of them was a novel polymorphism (c.727 C→T p. R243C). Two SNPs were silent mutations (c.696 C→T p.Y232=, c.870 C→T p. G290=) and two SNPs were missense mutations (c.727 C→T p. R243C, c.728G→A p. R243H) (**Table 3.11**). To check for validity of DNA sequence, samples showing one of the aforementioned polymorphisms were sequenced using forward and reverse primers and/or the sequence was repeated twice. The novel SNP (c.727 C→T p. R243C) was found in Palestinian female, 19 years old.

Table 3.11 Summary of frequency and percent of each SNP in Palestinian subjects.

SNP	Frequency (n=211)			Allele frequency (n=422 chromosomes) (%)
	N	Heterozygote	Homozygote	
c.727 C→T p. R243C	1	1	0	0.2
c.728G→A p. R243H	4	4	0	1.0
c.696 C→T p.Y232=	2	2	0	0.5
c.870 C→T p. G290=	14	13	1	3.6

Regarding the Swedish samples, all 47 samples were homozygous for the two common mutations (Gln296Arg & Gly230Ser). Two samples were hetrozygous for the polymorphism (c.870 C→T, p. G290=).

Only eighteen Portuguese random donors were tested for exon 7C sequencing. All samples were homozygous for the two common mutations (Gln296Arg & Gly230Ser) (**Figure 3.7)**. Only one sample was heterozygous for the polymorphism (c.870 C→T, p. G290=). **Table 3.12** summarizes all SNPs/variants observed in the study samples.

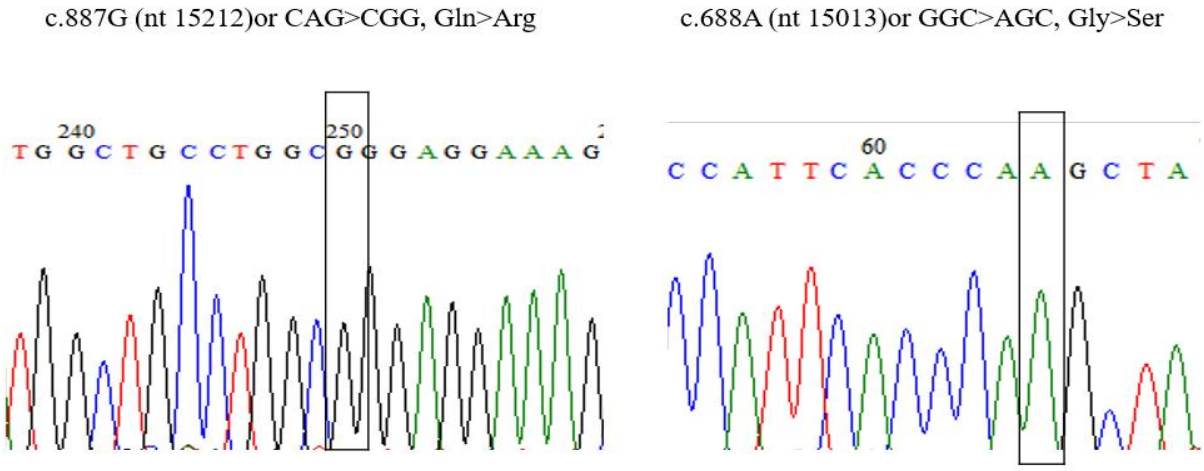

Figure 3.7 The two common mutations (Gln296Arg & Gly230Ser) GBGT1 exon 7 in human.

Table 3.12 DNA variants that were observed in the study samples.

Variant position*Chr:bp	rs ID*	Alleles *	cDNA change	Protein change	PolyPhen*	All Allele # *
9: 136029280	rs143563851	A+G	c.728G→A	p.(R243H)	probably-damaging	T=10/C=12996
9: 136029279	rs759428295	C+T	c.727C→T	p.(R243C)	probably-damaging	Novel
9:136029312	rs34691037	C+T	c.696C→T	p.(Y232=)	Unknown	A=374/G=12632
9:136029138	rs35902535	C+T	c.870C→T	p.(G290=)	Unknown	A=818/G=12188

***Variant Pos**.: is the SNV (Single Nucleotide Variant) location on the chromosome, **rs ID** (rsids): is an accession number used by researchers and databases to refer to specific SNP and it stands for (Reference SNP cluster ID), sometimes called dbSNP (Single Nucleotide Polymorphism Database) reference SNP identifier, **Alleles**: it refers to a change from a reference allele to an alternate allele, **All Allele #:** is the observed allele counts in all populations (European American population (EA) and in African American population (AA). **PolyPhen:** is the prediction of possible impact of an amino acid substitution on protein structure and function based on Polymorphism Phenotyping (PolyPhen2) program. All the data in this table were extracted from Exome Variant Server program.

The four SNPs listed in **Table 3.12**, were found in the heterozygote state, except one sample (Palestinian sample) was found to be homozygous for the "p.G290=" SNP, **(Figure 3.8).** The prevalence of SNPs relevant for this study are shown in **Table 3.13**

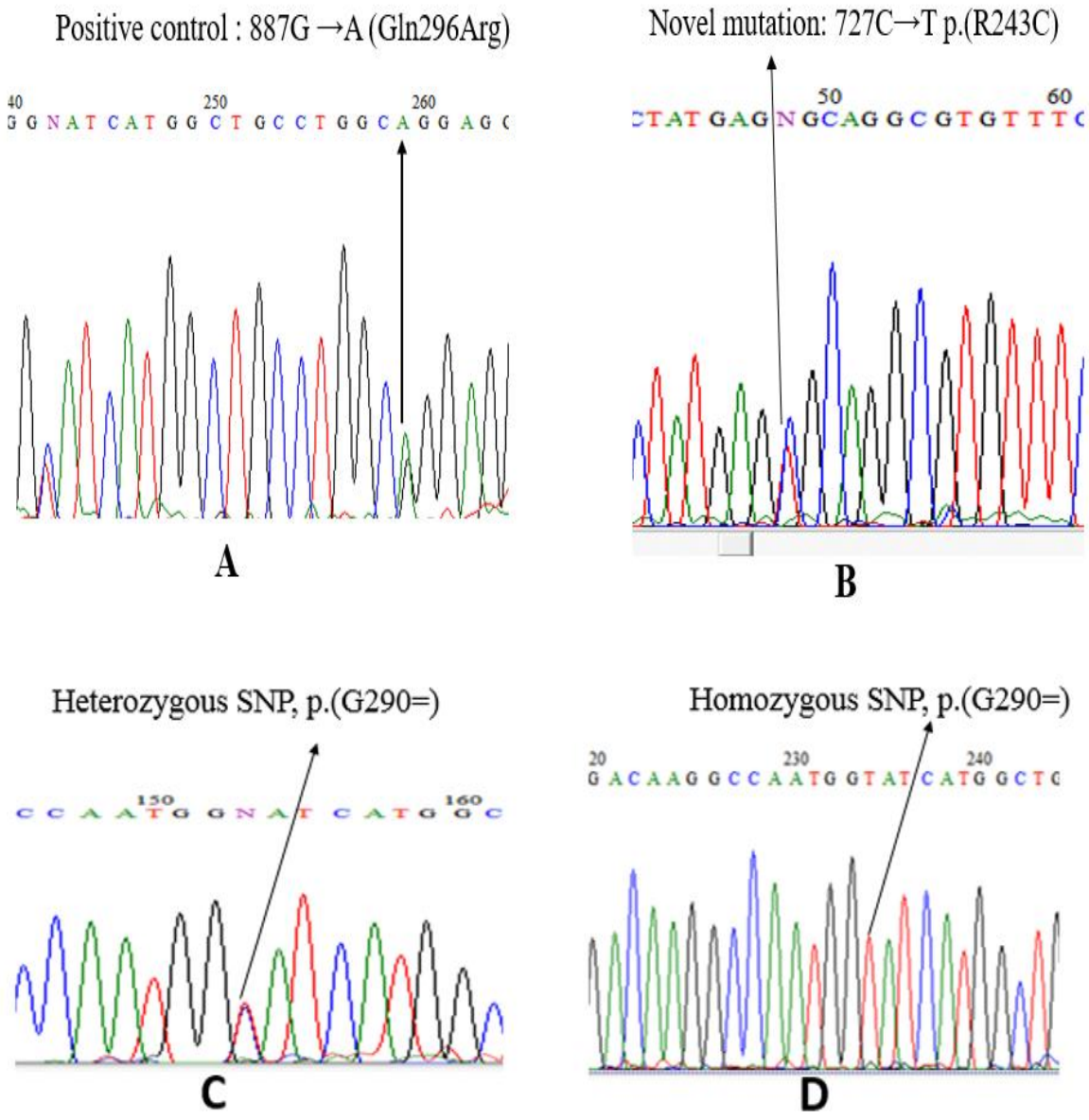

Figure 3.8 Representative sequence chromatograms. A) Positive control Gln296Arg (Apae DNA), B) Novel mutation R243C, C) heterozygous SNP G290= and D) Homozygous SNP G290=.

Table 3.13 Prevalence of SNPs relevant for this study. Values are given as the percentage of tested alleles in random donors.

SNP ID				
	c.870 C→T p. G290=	c.696 C→T p.Y232=	c.728G→A p. R243H	c.727 C→T p.R243C
Study populations (%)				
Palestinian samples (n=211)	6.6%***	0.95%	2%	0.5% (Novel)
Swedish samples (n=47)	4.3%	0	0	0
Portuguese samples(n=18)	5.6%	0	0	0
ENSEMBL Genetic Variation*/MAF	0.042	0.027	0.001	Not listed
EVS**/MAF	6.2894	2.7856	0.0769	Not listed

*ENSEMBL Genetic Variation available at: http://asia.ensembl.org/Homo_sapiens/Gene/Variation_Gene/Table?db=core;g=ENSG00000148288;r=9:133152948-133163945. Accessed on April 24th, 2016.

**EVS: Exome Variant Server: available at: http://evs.gs.washington.edu/EVS/ServletManager?variantType=snp&popID=AfricanAmerican&popID=EuropeanAmerican&SNPSummary.x=35&SNPSummary.y=10. Accessed on May 2nd, 2016.

MAF: minor allele frequency.

*** One of screening-positive samples was homozygous for c.870 C→T.

3.4.2 Result for newborn sample testing

Fifty newborn samples were collected from Palestinian hospitals and tested them for FORS1 antigen. Furthermore, 30 samples were tested for the Gln296Arg mutation using ARMS PCR. Serological grouping of newborn RBCs for Forssman antigen (FORS 1) revealed that all newborns were negative for this antigen. The serological results of newborn samples support serological grouping of our adult samples. ARMS PCR showed that the 30 samples tested all having the Gln296Arg mutation, which have been reported previously to cause inactivation of the Fs synthetase. To confirm the ARMS PCR results, exon 7 of GBGT1 gene was amplified and sequenced completely for three samples. DNA sequencing revealed the presence of the two common mutations (Gln296Arg & Gly230Ser) and the C allele at the SNP (c.363 C>A described earlier by Svensson et al. 2010) was normal in all 3 samples analysed by sequencing.

Chapter Four

IIII Discussion

Forssman antigen, one of the most well-known heteroantigens, was recently reported to be present in normal human hematopoietic tissue. Present on glycolipids, it is a normal constituent of fetal and various normal tissues and it was elevated in various cancer tissues [90, 91]. The study of FORS blood group has many beneficial aspects. First, the ability whether it express the Forssman glycolipid has implications for the host tropism of several infectious diseases. Second, natural anti-Fs antibodies, present in high concentration in many humans could be of immunological barrier to transfusion/ transplantation therapy. The sera of cancer patients displayed a decreased anti-Fs reactivity as compared with sera of a control group [53], so they also can be used as anti-tumor marker and to determine the recurrence in patients with cancer postsurgically. In addition, GBGT1 gene encoding the Forssman synthetase is evolutionarily related to the ABO gene. Sequencing of that gene to detect the mutation (Gln296Arg) is the preferable test for FORS1 human positivity. Moreover, different mutations in GBGT1 gene could be found in different populations and some relation between the Fs gene mutations and different diseases.

4.1 Blood grouping

Monoclonal anti-Fs IgM from rat cell culture supernatant was used to test FORS1 grouping of the samples. It was one step, simple, fast and appropriate method for all blood group types. In contrast, the classical test for FORS1 antigen using the Helix pomatia (HP) lectin has many advantages but it needs two steps. By using HP lectin for FORS grouping, only for non-A blood groups could be tested. Helix pomatia lectin has been used to recognizes N-acetylgalactosaminylated residues and it has been used in cancer research as an indication of metastasis behavior and poor patient prognosis [92] because of its specificity for cancer cells in the very early stages of the metastatic process. The binding preference was established to be Forssman antigen (strongest preference first), blood group A substance and Tn antigen [76]. Moreover, when various human cancer cell lines were transplanted into severe combined immune deficient mice, only the HPA positive cell lines metastasized. So the GalNAc-containing cancer epitopes on the surface of cancer cells could be serve as cancer- vector agent in tranplantation process [93]. Both the Tn antigen present on mucins and the Forssman antigen present on glycolipids are good candidates .In 1993, Oriol et al. used HP lectin to stain porcine tissues for the Forssman antigen [94].

In this study, we have tested 211 Palestinians, 47 Swedish and 18 Portuguese individuals for FORS1 antigen. All samples tested in this study were negative for the FORS1 antigen. These findings are consistent with previous reports that revealed that the prevalence of FORS1 antigen positivity is <0.1% [51]. On other hand, previous reports stated that 75% of healthy men and women have anti-Fs antibodies. This would suggest also that the majority of humans are Forssman antigen-negative [95], although the lack of antibodies cannot be used to deduce the phenotype of FORS1 antigen.

Different ABO and Rh blood groups were observed by reacting red blood cell suspension of the samples with monoclonal antibodies for these antigens. Forward or cell grouping for FORS1 antigen results showed that there is no relationship between ABO, Rh blood group system and FORS blood group system since all study subjects were negative for

FORS1 antigen regardless of the ABO and Rh phenotype. Reverse or serum grouping for anti-Fs antibody cannot be performed on plasma samples due to the absence of FORS positive human cells, therefore, alternative cells were used for this purpose as discussed below.

4.2 Anti-Fs antibodies testing

According to the study by Levine (1978), the incidence of anti-Fs in the normal population is 75%. Levine has analysed anti-Fs by age groups, a lower incidence of anti-Fs was found in the adult population [95]. These results can perhaps be attributed to the accumulated effect of immune responses to infections and infestations with increasing age [95]. As we mentioned before, FORS1 antigen is also expressed in some normal tissues such as gastrointestinal epithelial, lung and kidney tissues [90]. Thus, it was suspected that some samples will be negative for anti-Fs antibodies, as many previous studies confirmed [95]. This was formulated by Burnet (1969), as the body fails to produce antibodies to its own normal tissues [96].

Furthermore, the absence of Forssman antibodies in some individuals can be attributed to two factors. First, some persons could not synthesize anti-Fs, as their immune response against Forssman antigen could be under genetic control. Second, Forssman antibodies might be produced in all persons but would be absorbed onto some normal tissues containing the Forssman antigen. Alternatively, Fs antigen may be shed from the tissue resulting in the formation of anti-Fs-Fs immune complexes. [53]. In addition, some of them could have very low anti-Fs antibodies titer, and they cannot be detected by the method used in this study, as it was not sensitive enough to detect this antibody.

4.2.1 FSL-FS Kodecytes

In the absence of natural FORS1-positive RBCs, kodecytes were created using a synthetic pentasaccharide, Forssman glycolipid function-spacer-lipid (FSL-FS-5) constructs. These FSLs were used to upload synthetic FORS antigen onto normal group O RBCs (so-called kodecytes). These synthetic FORS RBCs were evaluated by heamagglutination test by two methods, gel card and flow cytometry methods, to asses if they have similarity to naturally FORS positive RBC or not.

We aimed to examine if kodecytes can be used as an effective tool to study the FORS antibodies and then to incorporate it in all serologic practical laboratory sessions. In the study by Hult (2013), the kodecytes were created and designed for ABO subgroups to replace the flow cytometry and manual tube serology [37, 97]. Group O RBCs were modified with synthetic blood group A or B- FSL constructs [98] and they were used as weakly positive controls for ABO reagents in some countries [97].

A Penta Kodecyte gel card method based on the hemagglutination (HA) for FORS antibodies detection had the disadvantages of relatively poor specificity and do not offer fine discrimination between positive and negative FORS1 individuals. So, by using FSL-FORS1 constructs we were not able to replace the natural FORS RBC by synthetic one. The Forssman kodecytes are artificial cells, so they are modified and cannot be compared with ordinary red cells. Thus, we have prepared kodecytes as described previously by Svensson et al. (2012), with one difference; she tested anti-Fs positive plasma against FORS positive (A_{pae} W and B) red cells. As a result, this discrepancy is almost certainly due to the difference in the way antigens are presented on the natural RBC membrane versus solid-phase assays and indirectly validates the importance of using natural RBCs in antibodies testing in routine laboratory [99].

Furthermore, FSL-FORS1 is a synthetic analogue of natural FORS1 but both do not have the same structure. The natural FORS1 glycolipid is a pentasaccharide linked to a ceramide lipid tail while the FSL-FORS[43]1 has identical antigen (pentasaccharide)

structure but with dioleoylphosphatidylethanolamine (DOPE) as the lipid tail. Another difference between them is the presence of approx. 2-nm spacer (equivalent in spacing to about two monosaccharides) in the FSL-FORS1 but not in natural FORS 1 [43] . The spacer in FSL is selected to provide a construct that is dispersible in water, yet will be spontaneously and stably incorporated into the RBC membrane [100]. Thus, the longer spacer can increase the affinity of the antigen when attached to RBC membranes [43].

Based on our previous observations, we cannot depend on FSL-Fs kodecytes construct to detect specifically anti-Fs antibodies. In the future, it is possible that kodecytes treated with the right amounts of FSL-Fs constructs or modified to be closer in structure to natural FORS1-positive RBCs which are difficult to access for many laboratories **(Figure 4.1).**

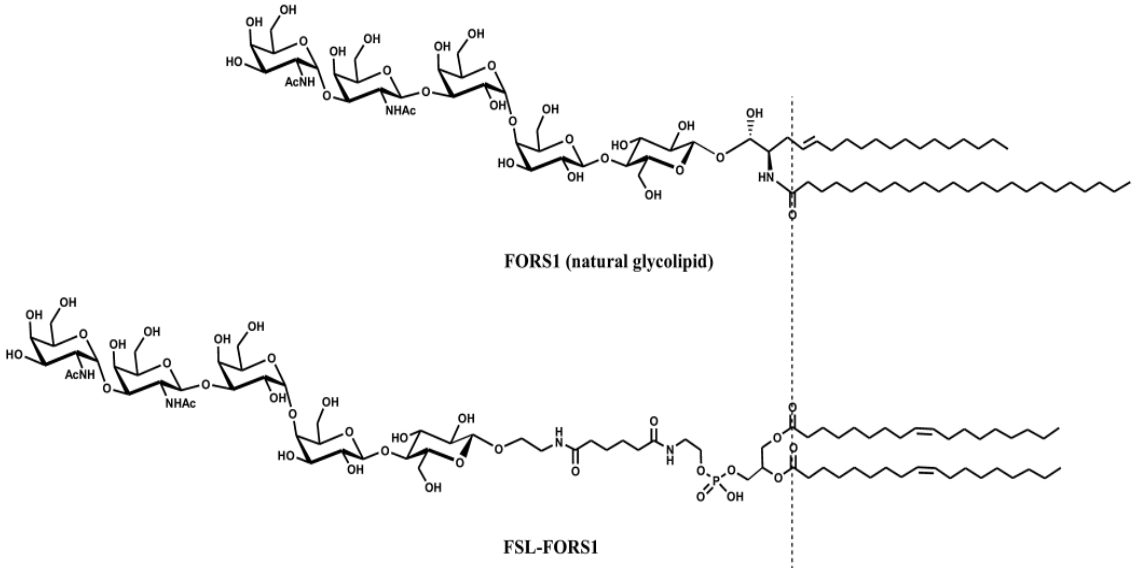

Figure 4.1. Schematic diagram of the natural Forssman (FORS1) glycolipid (with a ceramide lipid tail) and its synthetic analogue FSL-FORS1 (with DOPE lipid tail) [43] .

Flow cytometry (FC) methodology was also used as a highly sensitive method developed to detect human antibodies. It was used by Vyas (1998) for antibodies semi quantitative

discrimination of the amount of IgG and IgM antibodies in plasma by using a fluorescent labelled antibodies [101]. Flow cytometry overcome some of the shortcomings of gel card method such as reproducibility and quantification, whereas hemagglutination titers are discreet values [102]. It is well known to be a good tool to investigate the double cell populations or to identify weakly expressed antigens [8, 37].

For the flow cytometry (FC) method, we used fixed RBCs in the Fs antibodies testing because most Fs antibodies are IgM which have high ability to agglutinate RBCs, mostly as a pentamer but also as a hexamer.

The use of diluted cells in FC method instead of using fixation solution was recommended by Krishnan et al. (2008), to overcome the tendency of sensitized RBCs to be agglutinated. It was simplified for the FC assay and it has an advantage because the lower cell concentration in the assay increases sensitivity [102]. As we mentioned before, Fs antibodies mainly belong to IgM class, so most diluted plasma containing IgG, gave negative results in the gel card method after the dilution step. Furthermore, if FORS1 antigen is actually a human tumor-associated antigen, the outlook for specific immunotherapy may not be promising for cancer patients due to the IgM nature of human anti-Fs, as it consider the common problem of carbohydrate-based vaccines [53]. Still the FC method gave more sensitive results. It gave more clear differences between the two cell populations in the same sample without plasma dilution, the dilution step was necessary in hemagglutination by gel card to give the cell population differentiation.

The level of FSL antigen required to give a positive result depends on the quality and level of antibody being detected. However, based on a similar study of carbohydrate antigens, a FSL solution of 100 μg/mL will typically result in a strong positive reaction [100]. For A blood type, a 100 μg/mL FSL was used and a 300 μg/mL FSL for B type RBC [97]. Here, the concentration of FSL used to create kodecytes was selected to give strong positive reactions. We increased the concentration of FSL from 0.25 to 0.50μg/mL, according to Hult study that advised the labs to use appropriate concentrations of FSL construct and Hult suspected that this is the crucial point in the

testing procedure using the Kodecytes [98]. We found that 50 μg/mL FSL for penta-FORS give more accurate result than the concentration 25 μg/mL.

The synthetic pentasaccharide Forssman function-spacer-lipid (FSL) constructs, FORS1-kodecytes were agglutinated with anti-Fs monoclonal antibodies and Helix pomatia lectin dilutes 1:100. Thereafter, both were used as positive controls for anti-Fs antibodies testing. In contrast, both anti-Fs and H. pomatia lectin did not agglutinate untreated RBCs. Kodecytes did not agglutinate when suspended in PBS and thus used as a negative control. The strange was the positive reaction (agglutination) of these kodecytes with both FORS1 positive individuals.

Flow cytometry (FC) methodology was also used as a highly sensitive method developed to detect human antibodies. FC method was used by Vyas (1998) for semi-quantitative analysis of the amount of IgG and IgM antibodies in plasma using fluorescent labelled antibodies [101]. FC method overcomes some of the shortcomings of gel card method such as reproducibility, which be relevant with Nithya S. Krishnan study [102]. Additionally, the FC method is well known as a good method to investigate double cell populations such as most of Lutheran antibodies (bit like an A3 type reaction) or identifying weakly expressed antigens [8, 37].

The results of A_{pae} W and A_{pae} B plasma and kodecytes by gel card and flow cytometry methods led to the hypothesis that the positivity showed is caused by an antibody other than the anti-Fs, that is probably common in humans but at different concentrations. In fact, the exact biochemical reason for this is not known yet. Possible explanations for such irregular results is that agglutination of kodecytes was caused by some plasma components such as other immunoglobulin classes like IgA, many platelets, too much plasma proteins or fibrin. Additionally, agglutination of kodecytes with A_{pae} W and A_{pae} B plasma may be attributed to unspecific antibodies cross-reacting with FSL synthetic pentasaccharide structure.

As we mentioned before, FORS1 antigen is a heterophile antigen, an antigen shared by unrelated species [58]. Also Fs antibodies are hetrophilic in nature reacting with substances found in unrelated species [103]. However, the presence of autoantibodies cross reacting with FORS antigen is not applicable here, because a previous report by Svensson (2012) showed that these plasma gave negative result when tested using direct antiglobulin test (DAT) (Svensson et al, 2012; 104). Another expectation, presence of Fs antigen might stimulated or induced presence of unknown specific antibody of foreign origin.

Since 1956, Tanaka and Leduc have found that sheep RBCs gave brighter and more distinct reaction than other cells when tested with fluorescein-labeled antibody. For this reason, the sheep RBCs became the best choice for testing for anti-Fs antibodies in the later times [27]. In addition, Young (1979) suggested that the anti- sheep hemolysin present in the majority of human sera could be an alloantibody specific for Forssman alloantigen [53].

4.2.2 Sheep RBCs

Since natural human FORS1 antigen positive RBCs are not available, we used sheep RBCs as a natural source of Forssman antigen for forward testing for the anti-Fs antibodies in the plasma of study subjects. Several reports indicated that the majority of healthy humans have antibodies in their sera that can agglutinate sheep RBCs. They used sheep RBCs but against serum levels of sheep RBCs hemolysin (xenoantibodies) or Forssman-like antibodies [57] not against Forssman antibodies specifically. Strokan (1998), tested pooled human AB sera against sheep RBCs except for binding to forssman and Galalpha1-3. They eliminated The Forssman and Galal- reactivity in human sera by immunoadsorbtion using Forssman and Galal -3Gal-immunoadsorbent columns, respectively [104]. Campbell (2014), confirmed that anti-Fs and anti–α-Gal antibodies may act through similar mechanisms [74]. The Forssman antigen is similar to the Gal

antigen and it has been suggested to be a possible porcine non-Gal target for human natural antibodies [105].

Furthermore, the FORS Ag-Ab reaction may be enhanced using specific conditions like optimum pH and temperature [60]. However, the fine specificity and cross-reactivity of monoclonal antibodies are often unknown, particularly against synthetic antigens [99].

We used the sheep RBCs, although the level of Forssman antigen on the sheep RBCs is very strong compared to the natural FORS1 [43]. In contrary, the density of FORS antigen on canine RBCs and the 30 to 40μmol/L FORS1-kodecytes are equivalent to those of FORS1 humans. The human synthase cDNA has >80% amino acid sequence identity to the canine orthologue [43, 55]. The 3-5% suspension of sheep RBCs is probably a heavy cell suspension and thus contributed to the false negative result with anti-Fs antibodies and HP lectin due to post-zone phenomenon [106]. So we used 0.8% sheep RBC suspension. On the other hand, a previous study by Barr, (2014) showed that animal RBCs cannot be reliably used to validate the presence of anti-FORS in a human serum, as Anti-Galili, the most abundant natural antibody in humans [107] and other anti-xenoantigens are also present. In general, natural anti-a-galactosyl antibodies, present in high concentration in all humans [38]. Table 4.1 shows the main carbohydrate antigens which may have human naturally occurring antibodies.

Table 4.1 Known carbohydrate antigens against which humans may have naturally occurring antibodies [55].

1.	Blood type A: GalNAcα1,3(Fucα1,2)Galβ1,4GlcNAcβ-R
2.	Blood type B: Galα1,3(Fucα1,2)Galβ1,4GlcNAcβ-R
3.	Hanganutziu-Deicher: e.g. NeuGcα2,3Galβ1,4Glcβ1-R
4.	Thomsen-Friedenreich (T or TF): Galβ1,3GalNAcα1-R
5.	Tn (TF precursor): GalNAcα-R
6.	Sialosyl-Tn: NeuAcα2,6GalNAcα1-R
7.	Forssman: e.g. GalNAcα1,3GalNAcβ1,3Galα1,4Galβ1,4Glcβ1-R
8.	Rhamnose-containing oligosaccharides
9.	Sulphatide I: SO_4-3Gal-R
10.	P antigens
11.	I or i antigens

Testing sheep RBCs by gel card method protocol gave good results with diluted plasma. We used 2% of LISS and bromelain enzyme treated sheep RBCs instead of 2% of papain treated sheep RBBCs in Sellt (1966) study [61] to improve their agglutinability by anti-Forssman sera with both IgG and IgM antibody.

Based on our results, it would be expected that the sheep RBCs have the FORS1 Ag. The A_{pae} B individual plasma was agglutinated with the sheep RBCs, and didn't look like a non-specific reaction because the pattern is similar to the monoclonal anti-Fs antibodies. Sheep RBCs can be used to test for the anti-Fs in humans because the A_{pae} W individual (without anti-Fs) was negative in all testing procedures. If humans had some antibody nonspecific against the Sheep RBC all the testing will be positive.

We therefore used a simple and rapid method for detection of Fs antibody, which is the gel card method. Furthermore, the gel card test is more sensitive, some samples that were

negative in the tube method, showed positive reactions in gel cards. So, we confirmed the negative result in tube method by using a gel cards. In addition, the FSL-kodecytes could be used to confirm the negative results found in the tube test and/or the gel cards.

We have some explanations for unexpected results with A_{pae} W and A_{pae} B plasma. In general, the natural antibody is clearly linked either to carbohydrates which the host can produce naturally but which are normally hidden, or to carbohydrates that are environmentally common to it. We were interested in absence and presence of natural anti-carbohydrate antibodies because they have higher affinity to their host [108].

Furthermore, Nowinski (1980), observed that influenza virus and influenza vaccine both contain a number of glycolipids, including the highly immunogenic Forssman antigen and blood group A substances, so the specificity of monoclonal anti-Influenza antibody parallels that observed with antibodies to Forssman glycolipid found naturally in human sera [109]. The availability of the anti-influenza human monoclonal antibody against FORS antigen permits a more detailed examination of this possibility when testing for the human Fs antibodies, because the influenza vaccine became very common in many countries in last few years.

In 1998, Strokan et al. noted that human anti-sheep Abs, which are bound to Forssman epitopes, did not bind pig kidney glycolipids which completely lacked Gal and Forssman glycolipid antigens [104].

In addition, we also should think about the relationship between T and Tn antigens and the presence and absence of the corresponding antibodies. Tn (T, Tn and Sialyl-Tn) antigen like Forssman antigen, both have terminal GalNAc residues, behave as A-like structures and described as tumor markers [110, 111]. In Lescar (2007), study, the HP lectin was complexed with two GalNAc containing epitopes, the Tn (alphaGalNAc-Ser) and Forssman (alphaGalNAc1-3GalNAc) antigens [76]. In the HPA-Forssman (Helix pomatia agglutinin) complex, the nonreducing GalNAc establishes the same hydrogen bond network as in the HPA/Tn complex.

In addition to Forssman antigen, several other carbohydrate antigens have been identified that could act as targets for human natural antibodies, and these include Gal alpha 1-3Le(x), Hanganutziu-Deicher, Tn, and Forssman antigens [105]. Anti-HD (Hanganutziu-Deicher) and Paul-Bunnell antibodies are also hetrophilic, as Forssman antibodies [112]. The prevalence of anti-HD Abs in healthy humans was originally thought to be <4%, so it has low incidence. The Forssman antigen has been suggested to be a possible porcine non-Gal target for human natural Abs [55].

Our data showed that the frequency of anti-Fs was higher in female subjects compared to males. The later results showed good correlation with Hirayama et al. (1989) study [57]. For reasons which are as yet unclear, females are more susceptible to the development of anti-Fs [113]. Among our study subjects, there are only 6 females with a history of pregnancy, 2 females with a history of blood transfusion and 18 were exposed to surgery or medical operations. In contrast, one male has a history of blood transfusion and 24 were exposed to surgery or medical operations. Additionally, women in general have a stronger immune system/ responses than men. This gender difference is believed to be controlled by differences in the blood levels of gonadal steroid hormones including the female hormone, estrogen, which stimulates immune responses, and the male hormone, testosterone, which has an immunosuppressive effect [113]. In Tarlach (2013) study, they isolated a cluster of genes regulated by testosterone and also associated with the amount of antibodies and cytokines produced. The researchers found that male participants with the highest levels of the hormone testosterone had the lowest antibody response [114].

The anti-Fs frequency also showed variations in Igs classes, among blood groups, although Young (1979), previousely indicated that presence of Fs antibodies in sera is independent of blood group type [53]. Furthermore, the highest frequency of subjects lacking anti-Fs antibodies was among AB blood group, where 13.6% of AB individuals and 1.4% of all individuals were found to lack Fs antibodies. These results may be partially explained by the fact that individuals who lack naturally occurring anti-A and anti-B has also less anti-Fs present, but more convincing reasons are yet to be uncovered.

Stronger reaction was shown in our study in IgG test compared to IgM. Humphrey and Dourmashkin (1965) observed that with sheep RBCs, the maximum number of anti-Fs antibody molecules that will bind to respective FORS antigens is about 600 000 for IgG and 120 000 for IgM [115]. Thus, FORS antigen binds fewer IgM molecules compared to IgG molecules. The explanation for such finding could be that the antigen sites are so closely packed that, at saturation, IgG molecules cover the whole surface; while the IgM molecules are much larger, and thus the maximum number that could bind would clearly be less [116].

In addition, weaker reactions with IgG and IgM anti-Fs was seen in B blood group among our donors. This result contradicts with Hirayama et al. (1989) study, which showed higher titer of Fs antibodies in blood group AB than other blood groups.

4.3 GBGT1 sequencing

Multi-step glycosylation are required for biosynthesis of FORS antigen and deficiency or defect in any step may result in the failure to synthesize this antigen. For the FORS1 antigen negativity in humans we assumed that different missense mutations might be responsible in addition to the previous discovered mutations. To identify them, we did sequence analysis of the specific part of GBGT1 gene. GBGT1- likes ABO gene consists of seven exons, with most of the coding sequence localized in exon 7. Thus, we have screened exon 7 partially by DNA sequencing to detect the two most common mutations responsible for the FORS1 antigen negativity.

Our results are consistent with previous studies that humans usually do not express the FORS1 antigen. Previous published studies have shown that FORS1 antigen expression in human is very rare, due to many missense mutations that affect its catalytic domain encoded by exon 7 of GBGT1 gene which disturb Fs synthetase and inactivate it. The most important mutations are c.688G>A [p.Gly230Ser] and c.887A>G [p.Gln296Arg]).

These glycine and glutamine residues are conserved among functional GBGT1 genes in FORS1 antigen positive species [19].

In this study, we found the inactivating missense mutation Q296R present in all study subjects, which in turn confirms the FORS1 antigen negativity determined by serological testing. In addition, we found other missense and coding-synonymous polymorphisms. All these polymorphisms were found in the heterozygote state except one, c.870C>T p. (G290G) that was found in the homozygote state in one subject. On the other hand, we found these polymorphisms in unrelated subjects (all the study participants were from unrelated families).

Most alleles coding for most blood group antigens result from SNPs encoding amino acid substitutions in either a glycosyltransferase or extracellular domain of a red cell membrane protein. The allele frequency for the c.870 C>T (G290G) and c.696 C>T (Y232Y) variants detected among our Palestinian subjects, were 3.6% and 0.5% respectively, which were lower than the frequency of these alleles reported in Exome Variant Server (allele frequency reported for African Americans and European Americans), viz., 6.7% and 3%, respectively [117]. While the allele frequency of the c.728 G>A (R243H) variant was 1% among our Palestinian subjects and was higher than the frequency reported in the Exome Variant Server, viz., 0.008% [117].

The R243H has a damaging effect on the GBGT1 gene product if it occurs apart from the two known mutations for FORS1 antigen negativity in humans, as predicted by PolyPhen-2 software. Of the aforementioned three SNPs, only the G290G SNP was detected in a two Swedish subjects and in a single Portuguese subject. The variation in allele frequency observed between Palestinian subjects and that reported in EVS probably reflects the genetic variations among different populations. Concerning the Swedish and Portuguese subjects, the sample number included in this study is small and probably and cannot be used to deduce any conclusion about the allele frequency in these populations.

The SNPc.727C→T p. (R243C) is a novel mutation and was detected in one Palestinian subject (allele frequency 0.2%) and has not been reported earlier. This SNP has a damaging effect on the GBGT1 gene product if it occurs apart from the two known mutations for FORS1 antigen negativity in humans, as predicted by PolyPhen-2 software. This SNP was not detected in any of the Swedish or Portuguese subjects included in this study.

Taken together, the DNA analysis of exon 7 of the GBGT1 gene revealed that FORS1 antigen negativity in our study subjects from three different populations is most probably caused by the Q296R mutation which has been earlier reported in as the cause for Fs α1.3-GalNac transferase synthetase inactivation in other populations [12]. Also another two mutations R243H and R243C has been detected in a small number of our study subjects and can cause inactivation of Fs α1, 3-GalNac transferase when they occur in apart from the Q296R mutations. Since the Q296R mutation is almost ubiquitous in humans, it seems that the R243H and R243C have occurred after the Q296R mutation.

4.4 Conclusion

All study populations included in this study were negative for the FORS1 antigen by forward grouping of RBCs and DNA analysis. Naturally occurring anti-Fs antibodies are present in the serum of majority of study donors (96.2 %), they are predominantly IgM. DNA sequencing supports FORS1 negativity in our samples. Alignment of generated sequence with GBGT1gene reference sequence confirmed the presence of 887A>G [p.Gln296Arg] and 688G>A [p.Gly230Ser] mutations. For c.727C→T p. (R243C) SNP, a novel missense mutation was detected in a Palestinian subject and has a damaging effect on Fs synthetase.

Overall, the results of our study indicated that the prevalence of FORS blood group system is low at least among our studied populations. Nonetheless, care must be taken when interpreting the results especially when dealing with such very low frequency antigens in multiple transfusion patients. To date, our experience with FORS antigen/antibodies tests as a method for FORS1 antigen detection suggests that serology should still be complemented by GBGT1 gene sequencing.

Finally, further studies are needed to investigate the association between FORS1 antigen and or Fs antibody and physiological or pathological processes. Also further investigations are needed to further improve the Kodecyte system for testing for anti-Fs antibodies and explore its use as a screening method for certain tumors.

4.5 Weaknesses or limitations in the current study plan

Full gene sequencing of GBGT1 using 7 primer pairs has the ability to provide additional information regarding FORS1 human negativity and to reveal all possible or novel mutations when compared to targeted genotyping approaches.

The sample size of subjects from each target population was not representative to the respective population. Our data also used a limited genotyping samples based on

ethnicity, and cannot detect all polymorphisms carriers in all populations. Most genotyping of our samples are based on Palestinian population and small sample volume from Sweden and Portugal. We might assume that different populations could have different results regarding presence/absence of FORS system and GBGT1 genotyping.

For anti-Fs antibodies testing, we wanted to replace the sheep RBCs by synthetic RBC Function-spacer-lipid (FSL) constructs which is supplied by KODE Biotech Materials (Auckland, New Zealand), but they were not suitable for all negative controls.

4.6 Recommendations

Now, much research has focused on the challenges facing the tissue and organ transplantation such as unavailability of acceptable human donor organs, the low rate of long term success and the serious risks of infection and cancer. In that field, normal and cancer tissues FORS expression research are needed, because certain normal tissues and malignancies in humans have been shown to express this particular antigen.

In addition, our data prompted us to reinvestigate the full gene sequencing in large screening population samples to detect many variants, some of them known to be common, some rare and some not yet described. We need also to modify synthetic Fs and FSL- kodecyte construct for anti-Fs testing as new technology and more specific method.

So, further studies will be needed to completely understand FORS blood group system as an area of interest, since having positive FORS1 RBCs would be problematic in blood transfusion and transplantation.

4.7 Funding

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Appendix A

Blood group systems v3.0 1210228. *As recognized by the HUGO Gene Nomenclature Committee http://www.genenames.org/†MIC2 product, Updated October 2012.

No.	System name	System symbol	Gene name(s)*	Chromosomal location	CD numbers
001	ABO	ABO	*ABO*	9q34.2	
002	MNS	MNS	*GYPA, GYPB, GYPE*	4q31.21	CD235
003	P1PK	P1PK	*A4GALT*	22q13.2	
004	Rh	RH	*RHD, RHCE*	1p36.11	CD240
005	Lutheran	LU	*LU*	19q13.32	CD239
006	Kell	KEL	*KEL*	7q34	CD238
007	Lewis	LE	*FUT3*	19p13.3	
008	Duffy	FY	*DARC*	1q23.2	CD234
009	Kidd	JK	*SLC14A1*	18q12.3	
010	Diego	DI	*SLC4A1*	17q21.31	CD233
011	Yt	YT	*ACHE*	7q22.1	
012	Xg	XG	*XG, MIC2*	Xp22.33	CD99†
013	Scianna	SC	*ERMAP*	1p34.2	
014	Dombrock	DO	*ART4*	12p12.3	CD297
015	Colton	CO	*AQP1*	7p14.3	
016	Landsteiner-Wiener	LW	*ICAM4*	19p13.2	CD242
017	Chido/Rodgers	CH/RG	*C4A, C4B*	6p21.3	
018	H	H	*FUT1*	19q13.33	CD173
019	Kx	XK	*XK*	Xp21.1	
020	Gerbich	GE	*GYPC*	2q14.3	CD236

No.	System name	System symbol	Gene name(s)*	Chromosomal location	CD numbers
021	Cromer	CROM	*CD55*	1q32.2	CD55
022	Knops	KN	*CR1*	1q32.2	CD35
023	Indian	IN	*CD44*	11p13	CD44
024	Ok	OK	*BSG*	19p13.3	CD147
025	Raph	RAPH	*CD151*	11p15.5	CD151
026	John Milton Hagen	JMH	*SEMA7A*	15q24.1	CD108
027	I	I	*GCNT2*	6p24.2	
028	Globoside	GLOB	*B3GALT3*	3q26.1	
029	Gill	GIL	*AQP3*	9p13.3	
030	Rh-associated glycoprotein	RHAG	*RHAG*	6p21-qter	CD241
031	FORS	FORS	*GBGT1*	9q34.13	
032	JR	JR	*ABCG2*	4q22	
033	LAN	LAN	*ABCB6*	2q36	

Appendix B

Palestinian donor's characterizations

No.	Sex	Age	College	Residence place	received blood transfusion	surgery or medical operation before	chronic or hereditary diseases	ABO group	Rh(D)
1	M	21	Health Professions	Hebron	No	no	No	AB	positive
2	M	23	Health Professions	Qalqelia	No	no	No	O	positive
18	M	21	Health Professions	Hebron	No	no	No	AB	positive
19	F	18	Health Professions	Ramallah	No	no	No	B	positive
20	F	18	Health Profession	Jerusalem	No	no	No	A	positive
21	F	18	Health Professions	Hebron	No	no	No	O	Positive
22	F	18	Health Professions	Hebron	No	no	No	O	Positive
23	F	21	Al-Quds Bard for Arts & Sciences	Ramallah	No	no	No	A	Positive
24	F	20	Al-Quds Bard for Arts & Sciences	Hebron	No	no	No	A	Positive
25	M	23	Al-Quds Bard for Arts & Sciences	Bethlehem	No	no	No	O	Positive
26	M	23	Al-Quds Bard for Arts & Sciences	Ramallah	No	no	No	O	Positive
27	F	20	Al-Quds Bard for Arts & Sciences	Nablus	No	no	No	B	Positive
28	F	19	Health Professions	Bethlehem	No	Yes	No	A	Positive
29	F	19	Health Professions	Ramallah	No	no	No	AB	Positive
30	F	21	Arts	Al-Ezaria	No	no	No	A	Positive
31	F	21	Arts	Abu Dis	No	no	No	O	Positive
32	F	18	Health Professions	Abu Dis	No	no	No	B	Positive
33	F	18	Health Professions	Abu Dis	No	no	No	O	Positive

34	F	18	Arts	Al-Ezaria	No	no	No	O	Positive
35	F	21	Educational Science	Hebron	No	Yes	No	O	Positive
36	F	21	Educational Science	Hebron	No	no	No	O	Positive
37	F	20	Arts	Bethlehem	No	no	No	A	Positive
38	M	19	Dentistry	Ramallah	No	no	No	A	Positive
39	M	20	Dentistry	Ramallah	No	no	No	O	Positive
40	M	22	Pharmacy	Hebron	No	no	No	O	Positive
41	M	21	Pharmacy	Bethlehem	No	no	No	O	Positive
42	M	23	Pharmacy	Hebron	No	no	No	O	Positive
43	M	23	Arts	Bethlehem	No	no	No	A	Positive
44	M	24	Business & Economics	Hebron	No	Yes	No	AB	Positive
45	F	21	Pharmacy	Nablus	No	no	No	AB	Positive
46	F	21	Pharmacy	Hebron	No	no	No	A	Positive
47	F	20	Dentistry	Bethlehem	No	no	No	B	Positive
48	F	21	Pharmacy	Ramallah	No	no	No	A	Positive
49	M	19	Business & Economics	Hebron	No	no	No	A	Positive
50	M	20	Science & Technology	Hebron	No	no	No	A	Negative
51	M	19	Business & Economics	Ramallah	No	Yes	No	O	Positive
52	M	24	Business & Economics	Hebron	No	no	No	O	Positive
53	M	18	Business & Economics	Hebron	No	no	No	B	Negative
54	M	18	Arts	Hebron	No	no	No	A	Positive
55	M	21	Arts	Bethlehem	No	no	No	AB	Positive
56	M	20	Arts	Abu Dis	No	no	Yes	B	Positive
57	M	21	Science & Technology	Bethlehem	No	no	No	B	Positive
58	M	22	Arts	Bethlehem	No	no	No	AB	Negative
59	M	20	Business & Economics	Ramallah	No	no	No	A	Positive
60	M	18	Business & Economics	Bethlehem	No	Yes	No	O	Positive

61	F	18	Business & Economics	Hebron	No	no	No	A	Positive
62	F	18	Business & Economics	Hebron	No	no	No	A	positive
63	M	20	Law	Ramallah	No	no	No	O	positive
64	M	20	Law	Jerusalem	No	no	No	AB	positive
65	M	23	Law	Ramallah	No	no	No	A	Negative
66	M	21	Law	Nablus	No	Yes	No	B	positive
67	M	19	Law	Nablus	No	no	No	A	positive
68	M	18	Law	Ramallah	No	no	No	O	positive
69	M	21	Law	Ramallah	No	no	No	AB	positive
70	F	19	Medicine	Hebron	No	no	No	O	positive
71	F	19	Engineering	Ramallah	No	no	No	B	positive
72	F	19	Medicine	Hebron	No	no	No	O	positive
73	F	19	Medicine	Hebron	No	Yes	No	A	positive
74	M	21	Science & Technology	Bethlehem	No	Yes	No	A	positive
75	M	18	Medicine	Nablus	No	no	No	A	positive
76	F	19	Medicine	Al-Naserah	No	no	No	o	positive
77	F	18	Medicine	Hebron	No	no	No	A	positive
78	F	18	Medicine	Jerusalem	No	Yes	No	AB	positive
79	F	19	Dentistry	Nablus	No	no	No	O	positive
80	F	21	Arts	Jericho	No	no	No	A	positive
81	F	19	Arts	Jericho	No	Yes	No	A	positive
82	F	19	Pharmacy	Jericho	No	no	No	O	positive
83	F	19	Dentistry	Hebron	Yes	no	No	B	Negative
84	F	19	Law	Hebron	No	no	No	O	positive
85	M	18	Medicine	Abu Dis	No	Yes	No	A	positive
86	F	19	Dentistry	Qalqelia	No	no	No	o	positive
87	M	21	Dentistry	Nablus	No	Yes	No	AB	positive

88	M	23	Medicine	Abu Dis	No	no	No	O	Positive
89	M	24	Medicine	Jerusalem	No	Yes	No	A	Positive
90	M	22	Medicine	Nablus	No	no	No	O	positive
91	M	21	Arts	Hebron	No	Yes	No	A	positive
92	M	20	Arts	Jenin	No	no	No	A	positive
93	M	23	Arts	Abu Dis	No	no	No	A	positive
94	M	22	Arts	Jericho	No	no	No	B	Negative
95	M	22	Arts	Ramallah	No	no	No	A	positive
96	F	19	Arts	Bethlehem	No	Yes	No	A	positive
97	F	19	Arts	Bethlehem	No	no	No	B	positive
98	M	23	Arts	Hebron	No	no	No	O	positive
99	M	18	Arts	Hebron	No	no	No	B	positive
100	F	18	Educational Science	Al-Ezaria	No	no	No	B	positive
101	M	22	Arts	Nablus	No	no	No	O	positive
102	F	22	Arts	Jerusalem	Yes	no	No	B	positive
103	F	20	Arts	Abu Dis	No	Yes	No	B	Negative
104	F	19	Business & Economics	Ramallah	No	no	No	O	Negative
105	F	20	Science & Technology	Nablus	No	Yes	No	O	positive
106	F	19	Science & Technology	Ramallah	No	no	No	AB	positive
107	M	20	Business & Economics	Ramallah	No	no	No	AB	positive
108	M	20	Business & Economics	Ramallah	No	no	No	O	Negative
109	M	22	Arts	Hebron	No	Yes	No	A	positive
110	M	20	Science & Technology	Al-Ezaria	No	no	No	O	positive
111	F	18	Arts	Ramallah	No	no	No	A	positive
112	M	21	Engineering	Hebron	No	no	No	A	positive
113	F	18	Business & Economics	Ramallah	No	Yes	No	B	positive
114	M	19	Arts	Hebron	No	no	No	A	positive

115	M	21	Engineering	Ramallah	No	no	No	O	positive
116	F	21	Science & Technology	Jerusalem	No	no	No	A	positive
117	F	20	Science & Technology	Jerusalem	No	no	No	A	positive
118	M	22	Arts	Hebron	No	Yes	No	A	positive
119	M	19	Dentistry	Ramallah	No	no	No	O	Negative
120	M	18	Dentistry	Ramallah	No	no	No	AB	positive
121	M	18	Engineering	Tulkarm	No	no	No	A	positive
122	F	21	Arts	Jericho	No	no	No	A	positive
123	F	21	Arts	Ramallah	No	Yes	No	O	positive
124	M	26	Arts	Ramallah	No	no	No	B	positive
125	M	26	Arts	Hebron	No	no	No	O	positive
126	M	23	Educational Science	Abu Dis	No	no	No	O	positive
127	M	22	Educational Science	Jerusalem	Yes	Yes	No	A	positive
128	F	22	Arts	Jerusalem	No	no	No	B	positive
129	F	19	Arts	Jericho	No	no	Yes	A	positive
130	F	20	Arts	Jerusalem	No	no	No	A	positive
131	F	19	Arts	Ramallah	No	no	No	A	positive
132	F	21	Da'wa &Islamic studies	Ramallah	No	no	No	O	positive
133	F	20	Business & Economics	Jerusalem	No	Yes	Yes	O	positive
134	F	20	Engineering	Ramallah	No	no	No	AB	positive
135	F	20	Engineering	Bethlehem	No	no	No	A	Negative
136	F	20	Engineering	Bethlehem	No	no	No	B	positive
137	F	20	Engineering	Bethlehem	No	no	No	AB	positive
138	F	20	Engineering	Bethlehem	No	no	No	A	Positive
139	M	23	Law	Hebron	No	no	No	A	Negative
140	M	21	Law	Jericho	No	Yes	No	B	positive
141	M	22	Law	Hebron	No	no	No	B	positive

142	F	20	Business & Economics	Ramallah	No	no	No	A	positive
143	F	20	Business & Economics	Jerusalem	No	no	No	O	positive
144	F	22	Law	Bethlehem	No	no	No	A	positive
145	F	20	Business & Economics	Ramallah	No	Yes	No	A	positive
146	F	21	Business & Economics	Ramallah	No	no	No	B	positive
147	M	18	Pharmacy	Ramallah	No	Yes	No	A	positive
148	M	19	Engineering	Bethlehem	No	no	No	O	positive
149	M	20	Law	Hebron	No	no	No	AB	positive
150	M	21	Da'wa &Islamic studies	Hebron	No	no	No	O	positive
151	M	24	Business & Economics	Hebron	No	no	No	O	positive
152	M	19	Health Professions	Hebron	No	no	No	AB	positive
153	M	20	Health Professions	Hebron	No	no	No	O	positive
154	F	18	Dentistry	Jerusalem	No	no	No	O	positive
155	M	19	Health Professions	Hebron	No	no	No	O	positive
156	F	19	Law	Jerusalem	No	no	No	A	positive
157	F	19	Arts	Abu Dis	No	no	No	A	positive
158	M	19	Law	Al-Ezaria	No	Yes	No	B	positive
159	M	25	Science & Technology	Nablus	No	no	No	A	positive
160	F	18	Science & Technology	Nablus	No	no	No	O	positive
161	M	23	Law	Jerusalem	No	no	No	B	positive
162	F	21	Dentistry	Hebron	No	no	No	B	positive
163	F	21	Dentistry	Tulkarm	No	Yes	No	O	positive
164	M	22	Health Professions	Hebron	No	no	No	B	positive
165	F	19	Law	Ramallah	No	Yes	Yes	O	positive
166	F	20	Law	Bethlehem	No	no	No	B	positive
167	F	23	Law	Hebron	No	no	No	O	positive
168	F	19	Law	Hebron	No	no	No	A	Negative

169	M	18	Law	Hebron	No	no	No	O	positive
170	M	20	Medicine	Hebron	No	Yes	No	O	positive
171	M	19	Dentistry	Nablus	No	no	No	A	positive
172	M	19	Dentistry	Hebron	No	Yes	No	O	positive
173	M	20	Dentistry	Hebron	No	no	No	O	positive
174	M	23	Science & Technology	Ramallah	No	no	No	B	positive
175	M	21	Science & Technology	Ramallah	No	Yes	Yes	A	Negative
176	M	20	Arts	Nablus	No	no	Yes	O	positive
177	M	18	Educational Science	Bethlehem	No	no	No	B	Negative
178	M	22	Dentistry	Tulkarm	No	Yes	No	A	positive
179	M	26	Arts	Bethlehem	No	no	No	B	positive
180	M	24	Medicine	Hebron	No	Yes	No	O	positive
181	M	23	Medicine	Hebron	No	no	No	A	positive
182	M	23	Medicine	Hebron	No	no	No	O	positive
183	M	19	Law	Hebron	No	no	No	A	positive
184	M	21	Law	Ramallah	No	no	No	B	positive
185	M	23	Arts	Bethlehem	No	no	No	AB	positive
186	F	21	Arts	Jerusalem	No	Yes	No	O	positive
187	M	20	Da'wa &Islamic studies	Jerusalem	No	no	No	A	positive
188	F	19	Science & Technology	Jerusalem	No	Yes	No	B	positive
189	M	25	Engineering	Hebron	No	no	No	AB	positive
190	M	19	Business & Economics	Ramallah	No	Yes	No	A	positive
191	F	20	Law	Tulkarm	No	no	No	O	positive
192	F	20	Law	Ramallah	No	no	No	O	positive
193	F	30	Arts	Hebron	No	no	No	A	positive
194	F	37	Law	Hebron	No	no	No	AB	positive
195	F	26	Law	Al-Ezaria	No	no	No	A	positive

196	M	33	Engineering	Hebron	No	no	No	O	positive
197	F	19	Health Professions	Hebron	No	no	No	A	positive
198	F	19	Health Professions	Hebron	No	no	No	A	positive
199	F	21	Health Professions	Ramallah	No	no	No	O	positive
200	F	20	Health Professions	Ramallah	No	no	No	B	positive
201	F	21	Law	Hebron	No	Yes	No	O	positive
202	F	18	Law	Ramallah	No	Yes	No	O	positive
203	F	26	Arts	Jerusalem	No	no	No	O	Negative
204	F	19	Law	Jerusalem	No	no	No	B	positive
205	F	18	Educational Science	Hebron	No	no	No	O	positive
206	F	19	Law	Hebron	No	no	No	A	positive
207	F	24	Law	Hebron	No	no	No	A	Negative
208	F	18	Law	Hebron	No	no	No	A	positive
209	F	22	Law	Bethlehem	No	no	No	A	positive
210	F	18	Da'wa &Islamic studies	Hebron	No	no	No	A	positive
211	F	18	Educational Science	Hebron	No	no	No	B	positive

Appendix C

Population study-Palestine

Collection of Study sample: A convenient sample with a total of 211 samples will be collected from all colleges of Al-Quds University at Abu-Dies campus. The distribution of study sample among the colleges is shown in the table.

Collage name	No. of students per college*	Study sample		
		No. of samples to be collected from college	No. of Males	No. of Females
Arts	2183	46	24	22
Da'wa &Islamic studies	188	4	2	2
Health Professions	955	30	15	15
Engineering	531	12	6	6
Science & Technology	592	13	7	6
Pharmacy	357	8	4	4
Business & Economics	817	20	10	10
Medicine	580	15	9	6
Dentistry	583	16	9	7
Law	1546	34	17	17
Educational Sciences	371	8	4	4
Al-Quds Bard for Arts & Sciences	211	5	2	3
Total	**8914**	**211**	**109**	**102**

*The number of students per each college as for the Fall Semester 2014-2015.

Appendix D

Study Questionnaire/ English

Sample #: …….

Investigation of Forssmann antigen in a cohort of Palestinian and Swedish population

Dear Student: We are conducting a research project to investigate Forssman antigen among Palestinian population. This study aims to investigate this antigen in a cohort of Palestinian subjects and to determine the genetic mutations causing Forssman antigen negativity among the study subjects.

Therefore, we kindly ask you to participate in this study by providing the information necessary to fill out this questionnaire and to donate 5 ml of blood. The results of the study will be used for purpose of scientific research only.

Researcher: Wafa' Abu-Siba'

.....

- Gender: Male Female
- Age : ……. years
- College…
- Residence place…
- Have you received blood transfusion in the past three months? Yes No
- For married females: have you ever been pregnant before? Yes No
- Have you ever done a surgery or medical operation before? Yes No
- Do you suffer from any chronic or hereditary diseases? Yes No
- If your answer for the previous question is yes, please specify the disease ……..
- Do you know your blood group (ABO/Rh)? Yes No
- If you know your blood group, specify it: ……

……..... .....

Consent form:

Here I certify that I have voluntarily participated in this study by filling up this questionnaire and donating a blood sample and authorize the researcher to use the above data and blood sample for scientific research only.

Signature: Date:

Study Questionnaire/Arabic

رقم العينة: .....

استبانة

أخي الطالب /أختي الطالبة: نقوم في جامعة القدس بجمع عينات لأجل عمل دراسة ماجستير حول Forssman (Antigen) ومعرفة نسبة وجود هذا الأنتيجن في فلسطين والطفرات الوراثية المتعلقة به, وقد اخترنا عينة عشوائية من طلاب الجامعة لأجل هذا الغرض, راجين منكم ملء هذه الاستبانة.

وشاكرين لكم حسن تعاونكم

.....

الجنس: ذكر أنثى

العمر: سنة

الكلية:

مكان الاقامة :

هل سبق وأن احتجت إلى وحدة دم في الثلاث شهور الماضية نعم لا

للطالبات المتزوجات : هل سبق وكنت حامل نعم لا

هل سبق وأجري لك عملية جراحية نعم لا

هل تعاني من أي مرض مزمن نعم لا

هل تعرف فصيلة دمك نعم لا

ماهي :

.....

أقر أن العينات التي سيتم سحبها وكذلك ما يرد في هذه الاستبانة من معلومات سيحفظ بسرية تامة ولن يستعمل إلا لأغراض البحث العلمي الطالبة : وفاء أبو السباع

Appendix E

Swedish DNA extraction protocole

Wizard® Genomic DNA Purification Kit

INSTRUCTIONS FOR USE OF PRODUCTS A1120, A1123, A1125 AND A1620.

Quick PROTOCOL

Isolation of Genomic DNA from Whole Blood

Sample Size	Lysis Solution: Cell	Lysis Solution: Nuclei	Protein Precipitation Solution	Isopropanol	DNA Rehydration Solution
300µl	900µl	300µl	100µl	300µl	100µl
1ml	3ml	1ml	330µl	1ml	150µl
3ml	9ml	3ml	1ml	3ml	250µl
10ml	30ml	10ml	3.3ml	10ml	800µl

As little as 20µl can be processed with this system. Please see Technical Manual #TM050, Section 3.C.

Red Blood Cell Lysis

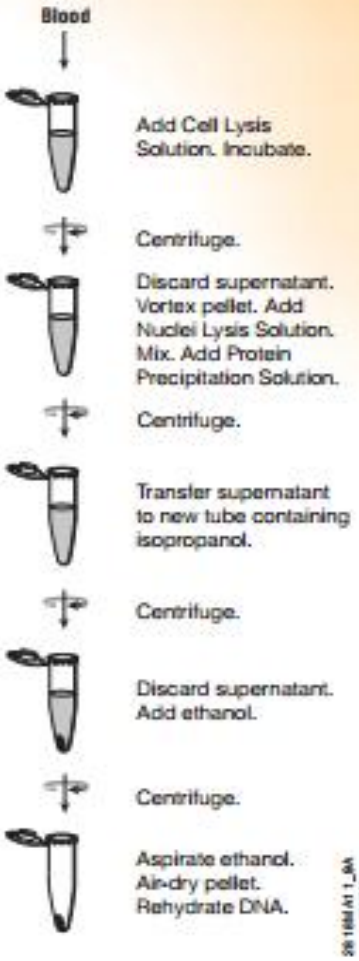
1. Using volumes from the table above, combine the appropriate volumes of Cell Lysis Solution and blood. Mix by inversion.
2. Incubate for 10 minutes at room temperature.
3. Centrifuge:
   ≤300µl sample 13,000–16,000 × *g**; 20 seconds
   1–10ml sample 2,000 × *g*; 10 minutes
4. Discard supernatant. Vortex pellet.

Nuclei Lysis and Protein Precipitation

5. Using volumes from the table above, add Nuclei Lysis Solution and mix by inversion.
6. Add Protein Precipitation Solution; vortex for 20 seconds.
7. Centrifuge:
   ≤300µl sample 13,000–16,000 × *g**; 3 minutes
   1–10ml sample 2,000 × *g*; 10 minutes

DNA Precipitation and Rehydration

8. Transfer supernatant to a new tube contaning isopropanol (using volumes from table above). Mix.
9. Centrifuge:
   ≤300µl sample 13,000–16,000 × *g**; 1 minute
   1–10ml sample 2,000 × *g*; 1 minute
10. Discard supernatant. Add 70% ethanol (same volume as isopropanol).
11. Centrifuge as in Step 9.
12. Aspirate the ethanol and air-dry the pellet (10–15 minutes).
13. Rehydrate the DNA in the appropriate volume of DNA Rehydration Solution for 1 hour at 65°C or overnight at 4°C.



Appendix F

Portuguese DNA extraction protocol

Procedure

1. **Pipet 200 µl or 350 µl whole blood (depending on protocol and kit used) into 2 ml sample tubes.**

   Thawed whole blood samples should be thoroughly resuspended prior to pipetting.

2. **Insert the appropriate EZ1 DNA Blood Card completely into the EZ1 Card slot of the EZ1 instrument.**
3. **Switch on the EZ1 instrument.**
4. **Press "START" to display the "Protocols" menu.**
5. **Press "1" or "2" to start worktable setup for the 200 µl protocol or the 350 µl protocol, respectively.**
6. **Press "1" to select an elution volume of 50 µl, "2" to select an elution volume of 100 µl, or "3" to select an elution volume of 200 µl.**
7. **Press "2" to select the ethanol wash step, optimized for MLPA assays.**
8. **Press any key to proceed through the text displayed in the LCD and start worktable setup.**

   The text summarizes the following steps which describe loading of the worktable. Wear gloves when loading the required items on the worktable.

9. **Open the instrument door.**
10. **Invert reagent cartridges 4 times to mix the magnetic particles. Then tap the cartridges to deposit the reagents at the bottom of their wells.**
11. **Load the reagent cartridges into the cartridge rack.**

    Note: After sliding a reagent cartridge into the cartridge rack, ensure that you press down on the cartridge until it clicks into place.

    If there are fewer than 6 reagent cartridges for the EZ1 Advanced or BioRobot EZ1, or fewer than 14 reagent cartridges for the EZ1 Advanced XL, you can load them in any order on the rack. However, when loading the other labware in steps 12–15, ensure that they also follow the same order. When using the data tracking option on the EZ1 Advanced or EZ1 Advanced XL, always start loading samples in position 1 and place the remaining samples one after the other into the next available positions on the worktable.

12. **Add 1800 µl of 80% ethanol to an empty 2 ml Sarstedt tube, and place the filled tube into the third row in the tip rack. Repeat for each reagent cartridge added to the workstation.**
13. **Load opened elution tubes into the first row of the tip rack.**
14. **Load tip holders containing filter-tips into the second row of the tip rack.**

15. **Load opened sample tubes containing 200 µl or 350 µl blood into the back (fourth) row of the tip rack.**
16. **Close the instrument door.**
17. **Press "START" to start the purification procedure.**
18. **The automated purification procedure takes 17–23 min depending on the protocol version and the EZ1 instrument.**
19. **When the protocol ends, the LCD displays "Protocol finished". Open the workstation door.**
20. **Retrieve the elution tubes containing the purified DNA. The DNA is ready to use, or can be stored at 2–8°C or –20°C.**

    If the purified DNA is to be analyzed by real-time PCR, tubes containing eluate should first be applied to a suitable magnetic separator and the eluate transferred to a clean tube (see the appendix of the *EZ1 DNA Handbook*) in order to minimize the risk of magnetic-particle carryover.

21. **To run another protocol, press "ESC", prepare samples as described in step 1, and follow the procedure from step 5 onward. Otherwise, press "STOP" twice to return to the first screen of the LCD, close the workstation door, and switch off the EZ1 instrument.**
22. **Clean the EZ1 instrument.**

    Follow the maintenance instructions in the respective EZ1 instrument user manuals.

بحث وجود المستضد فورسمان في كريات الدم الحمراء بين السكان الفلسطينيين

اعداد : وفاء علي عبدالمنعم ابوسباع

مشرف اول : د. محمود عبد سرور

مشرف ثان : د. كاميلا هيس

الملخص

مقدمة : وُصف الأنتجين فورسمان (FORS1) مؤخرا في الأنسجة المكونة لخلايا دم الإنسان الطبيعية ، وبالتالي تم التعرف على فورس (FORS) كأحد أنظمة الدم ويحمل رقم نظام الدم *31*st .

الأهداف : للبحث حول تعبير المستضد فورس1 في كريات الدم الحمراء في الانسان وتمييز الاجسام المضادة له بين السكان الفلسطينيين ومقارنة ذلك مع مجموعة من السكان الاوروبيين.

الطرق والمواد : شملت هذه الدراسة 211 فرداً بالغا و73 مولودا جديداً من فلسطين وكذلك 65 فرداً من أوروبا ، حيث تم فحص كريات الدم الحمراء لهؤلاء الأفراد لوجود الأنتجين فورسمان باستخدام الأجسام المضادة الوحيدة النسلية (monoclonal anti-Fs)، وكذلك تم فحص البلازما لوجود الأجسام المضادة للأنتجين فورسمان باستحدام كريات الدم الحمراء للأغنام ، وأيضا تم التحقق من البيولوجيا الجزيئية لهذا الأنتجين عن طريق عمل تسلسل للحمض النووي من اكسون 7 للجين GBGT1 والذي يعمل ترميزا للأنزيم Fs-synthetase فيحفّز الخطوة النهائية في تصنيع الأنتجين فورسمان (FORS1) .

النتائج : جميع عينات الدراسة كانت سلبية لوجود الأنتجين فورسمان باستخدام الكشف المصلي ، الأجسام المضادة للانتجين فورسمان بنوعيها IgG و IgM وجدت في 96.2% من عينات الدراسة ، وكان غياب هذه الأجسام المضادة بنوعيها أكثر شيوعا بين الذكور مقارنة بالاناث ، والتفاعل الأقوى لوحظ أيضا في الاناث ، بالنسبة لفصائل الدم : كان التفاعل الأقوى (درجة +3 و +4) للأجسام المضادة سواء IgG أو IgM لوحظ وجوده في فصائل الدم A و O (50% و 50.6% على التوالي) مقارنة مع فصائل الدم الأخرى ، وكانت أعلى نسبة لسلبية الأجسام المضادة في فصيلة الدم AB (كانت 13.6% في الأفراد الذين يحملون فصيلة الدم AB و 1.04% في جميع أفراد الدراسة .

بالنسبة لتسلسل الحمض النووري من اكسون 7 للجين GBGT1 فقد كانت جميع العينات تحمل الطفرتين الوراثتين (887A>G [p.Gln296Arg] و688G>A [p.Gly230Ser]) التي ُأبلغ عنها سابقا لتكون مسؤولة عن سلبية الأنتجين فورسمان في الانسان ، بالاضافة إلى ذلك تم الكشف عن أربع SNP (اختلاف الأشكال في نيوكليوتيدة واحدة) ، كانت منهم طفرتين صامتتين وطفرتين مغلطتين مع تردّد للأليل يتراوح بين 0.2% إلى 3.6% . واحدة من

الطفرات المغلطة (R2443C) كانت جديدة ووجدت في أنثى فلسطينية من عينات الدراسة ، هذه الطفرة لها تأثير ضار على أنزيم (Fs synthetase) كما كان متوقعا من قبل برنامج PolyPhen 2 ، أيضا لم يكن هناك فرق كبير بين وجود أو غياب الأجسام المضادة للأنتجين فورسمان ($p=0.408$ و $p=0.433$) وتعدد الأشكال الجينية للجين GBGT1 .

الاستنتاج : هذه هي الدراسة الأولى للتحقيق FORS نظام فصيلة الدم في فلسطين بشكل عام نتائجنا لهذه الدراسة تشير إلى أن نظام الدم FORS هو نظام منخفض الانتشار على الاقل في السكان الذين شملتهم الدراسة ، كذلك الأجسام المضادة للأنتجين فورسمان هي موجودة في مصل غالبية البشر الأصحاء البالغين . كما تم اكتشاف طفرة وراثية جديدة (R243C) في واحدة من العينات الفلسطينية ويعتقد أن لها تأثير ضار على الأنزيم Fs-synthetase .