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Vitamin D and Recurrent Abortion: Inference through several Vitamin D Receptor (VDR) gene polymorphisms and vitamin D status among the inflicted Palestinian women

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Thesis Approval

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Declaration

I, Maysa'a Sawabteh, hereby declare that I am the author of this thesis and that it has not been submitted in any way to any other university or institution for the award of any degree. This thesis does not include any previously published content except in cases where proper acknowledgment has been provided.

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Date: July 25, 2024

Dedication

To the martyrs of Gaza

To my father Saleh, who taught me not to overthink things, that nothing will ever be perfect, so just keep moving and do your best

To my mother Naeema, a teacher of compassion, love, and fearlessness

To my beloved brothers and sisters for their endless support and love

To my scientific advisor Prof. Hisham Darwish for his invaluable advice on the research subject

Acknowledgment

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In conclusion, I am deeply appreciative of the support and guidance I have received from Professor Hisham Darwish and my family. Their unwavering support has been crucial in helping me navigate the challenges of my research and academic pursuits. I am truly grateful for their contributions to my success.

Abstract

Background and objectives: Recurrent pregnancy loss (RPL) is a complicated medical status affecting 1–4% of women during reproductive age. It occurs when a woman repeatedly loses her fetus before it reaches 20 weeks of gestation. In Palestine, the prevalence of RPL is not determined among Palestinian females due to the absence of any study regarding this matter. Recently, selected variants in the VDR gene have been linked to RPL in some populations. Hence, our study aimed to investigate whether two maternal SNPs of the VDR gene, specifically (rs2228570) and (rs1544410), and the level of vitamin D status may be involved in the etiology of RPL among inflicted Palestinian women.

Methods: A total of 131 females were enrolled in this study. The case group consisted of 51 women who experienced at least three consecutive miscarriages in the second trimester, while the control group consisted of eighty women with two or more full-term successful pregnancies. DNA samples were obtained and genotyped for VDR rs2228570 and rs1544410 polymorphisms using PCR- restriction fragment length polymorphism (PCR-RFLP). The serum 25-hydroxyvitamin D3 level was measured. Additionally, within the case group, two females had a familial history of RPL and were subjected to whole exom sequencing. The confirmed variants were analyzed by In Silico bioinformatic tools.

Results: Significant differences in allele and genotype frequencies were observed among study subjects in the VDR rs1544410 polymorphic site (G vs. A p-value = 0.05, GG vs. GA p-value = 0.01, GG vs. AA p-value = 0.02). The risk of developing RPL increased under the dominant genetic model (GG vs. total A, p-value = 0.006). No statistically significant differences in allele and genotype frequencies were observed among study

subjects in the VDR rs2228570 polymorphic site. Since, the T-G haplotype was more frequent in the control group than the case group therefore it was associated with RPL prevention (P-value = 0.023). No statistically significant association was observed between serum 25-hydroxyvitamin D3 levels and study subjects since the majority of subjects in the study suffer from VD deficiency. No statistically significant difference between serum 25-hydroxyvitamin D3 levels and both VDR SNPs (rs2228570 and rs1544410) genotypes was observed among study subjects. Regarding family analysis, two variants were identified in each family. In the first family, the FGA *787T>C and HLA-G Leu154fs*60 variants follow autosomal dominant inheritance and were positively confirmed and segregated well within the family, while in the second family, the F12 Arg55Trp failed to segregate within the family.

Conclusions: The data revealed significant correlation between rs1544410 variant and RPL with no significant correlation with rs2228570 variant in the VDR gene. Serum VD levels did not alter this result which needs further assessment due to the VD deficiency status of all participating subjects. WES analysis of two RPL familial cases showed specific variants in FGA (*787T>C) and HLA-G (Leu154fs*60) are linked with the risk of recurrent abortion. This could be through influencing fetus stability, growth, and proceeding to full term, which needs further testing and confirmation.

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List of Abbreviations

Abbreviations	Definitions
RPL	Recurrent pregnancy loss
SM	Sporadic miscarriage
Apl	Antiphospholipid antibodies
FV	Factor five (thrombin)
F2	Factor two (prothrombin)
VD	Vitamin D
UV	Ultraviolet
DBP	Vitamin D binding protein
CYP450	Cytochrome P450
VDR	Vitamin D receptor
RXR	Retinoid X receptor
VDRE	Vitamin D response elements
NCBI	National Center for Biotechnology Information
ESHRE	European Society of Human Reproduction and Embryology
DNA	Deoxyribonucleic acid
Kb	Kilo base
5'UTR	The 5′ untranslated region
3'UTR	The 3′ untranslated region
SNP	Single nucleotide polymorphism
Rs	Restriction site
Bp	Base pare
mRNA	Messenger Ribonucleic acid
LD	Linkage disequilibrium
VNTR	Variable number of tandem repeats
hCG	Human chorionic gonadotropin
EDTA	Ethylenediaminetetraacetic acid
Rpm	Round per minute
TAE	Tris Acetate EDTA
PCR	polymerase chain reaction

RFLP	Restriction fragment length polymorphism
NGS	Next-Generation Sequencing
WES	Whole exom sequencing
HLA-G	Human leukocyte antigen G
FGA	Fibrinogen A alpha chain
F12	Factor twelve
HLA-DQB1	Human leukocyte antigen DQ beta 1
BDRR	Big Dye Terminator
PROVEAN	Protein Variation Effect Analyzer
FATHMM	Functional Analysis through Hidden Markov Models
SIFT	Sorting Intolerant from Tolerant
GVGD	Grantham Variation, Grantham Difference
SPSS	Statistical Package for the Social Sciences
OR	Odds ratio
95%CI	95% confidence interval
NTC	No template control
HWE	Hardy-Weinberg equilibrium
Del	Deletion
Fs	Frame-shift
Ins	Insertion
ACMG	American College of Medical Genetics
MHC	Major histocompatibility complex
EVT	Extravilloustrophoblast
sHLA-G	Soluble HLA-G
mHLA-G	Membrane-bound HLA-G
NK	Natural Killer cell
KIR2DL4	killer cell immunoglobulin-like receptor-2 Igdomainsand long cytoplasmic tail 4
ILT2	Immunoglobulin-like transcript-2
DNA-PKcs	DNA-dependent protein kinase
NF-κB	Nuclear factor kappa B
IL	Interleukin

TNF- α	Tumor necrosis factor-alpha
MIP	macrophage inflammatory proteins
HP1-γ	Heterochromatin protein 1-γ
β-gal	β-galactosidase
SASP	Senescence-associated secretory phenotype
PI3K	Phosphatidylinositol 3-kinase
dNK	Decidual natural killer cell
PBX1	Pre-B-cell leukemia transcription factor 1
siRNA	Small interfering RNA
GPFs	Growth-promptingfactors
PTN	Pleiotrophin
OGN	Osteoglycin

Chapter One

Introduction

1.1 Recurrent Pregnancy Loss

Recurrent pregnancy loss (RPL), also referred to as recurrent miscarriage (RM), idiopathic recurrent miscarriage (iRM), recurrent spontaneous abortion (RSA), or unexplained recurrent spontaneous abortion (URSA), is a complicated medical status that affects 1–4% of women during reproductive age (Dimitriadis et al., 2020). This condition represents pregnancy termination that occurs when a woman repeatedly loses her fetus before it reaches 20 weeks of gestation or delivers a fetus weighing less than 400 g if the gestational age is undefined (Zegers-Hochschild et al., 2009). This has detrimental psychological effects on both parents and may lead to social stigma. The precise characterization of recurrent miscarriage remains a topic of discussion within the scientific community, with variations in definition depending on the source. The European Society for Human Reproductive and Embryology (ESHRE) and the Royal College of Obstetricians and Gynaecologists (RCOG) define RPL as three or more successive pregnancy losses (Kolte et al., 2015; Regan et al., 2023), while the American Society for Reproductive Medicine (ASRM) describes it as two or more non-consecutive clinical pregnancy losses confirmed by ultrasound or histopathologic examination (Lir, 2012).

1.2 Epidemiology

Estimating the prevalence of recurrent pregnancy loss (RPL) poses challenges and exhibits significant variability across studies due to several factors. These factors include the timing at which women recognize their pregnancies, the characteristics of the study

population, the absence of a standardized definition for RPL, and the utilization of different criteria (Magnus et al., 2019). However, the available data from USA and Europe suggest that the incidence of RPL among women attempting to conceive is estimated between 1% and 4% (Dimitriadis et al., 2020; Lir, 2012; Rasmark Roepke et al., 2017). In the Palestinian community, recurrent abortion affects approximately 4% to 8% of Palestinian women attending prenatal care clinics (Hussein et al., 2010). It is important to note that reported cases of RPL are likely underestimated compared to stillbirths, as only few countries require medical providers to document miscarriages in official registers. Additionally, due to social and cultural influences, only a limited number of women feel comfortable openly discussing their medical condition related to RPL (Magnus et al., 2019).

1.3 Classification

There are three main categories in which RPL can be classified, considering woman's reproductive history and the potential risk factors associated with each category (Christiansen et al., 2004; Wramsby et al., 2000). These categories are as follows:

- Primary RPL: refers to women who had multiple miscarriages without any successful pregnancies.
- Secondary RPL: refers to women who had several miscarriages after giving birth to a live baby.
- Tertiary RPL: refers to women who had three non-consecutive pregnancy losses.

1.4 Risk Factors

Each pregnancy entails the development of a freshly regenerated endometrium and a distinct embryo with its own genetic composition. While the specific causes may vary among women, a combination of certain pathological processes probably contributes to pregnancy loss. Various risk factors for recurrent pregnancy loss (RPL) have been identified as illustrated in Figure1 and Table 1. These are elaborated upon in the subsequent section.

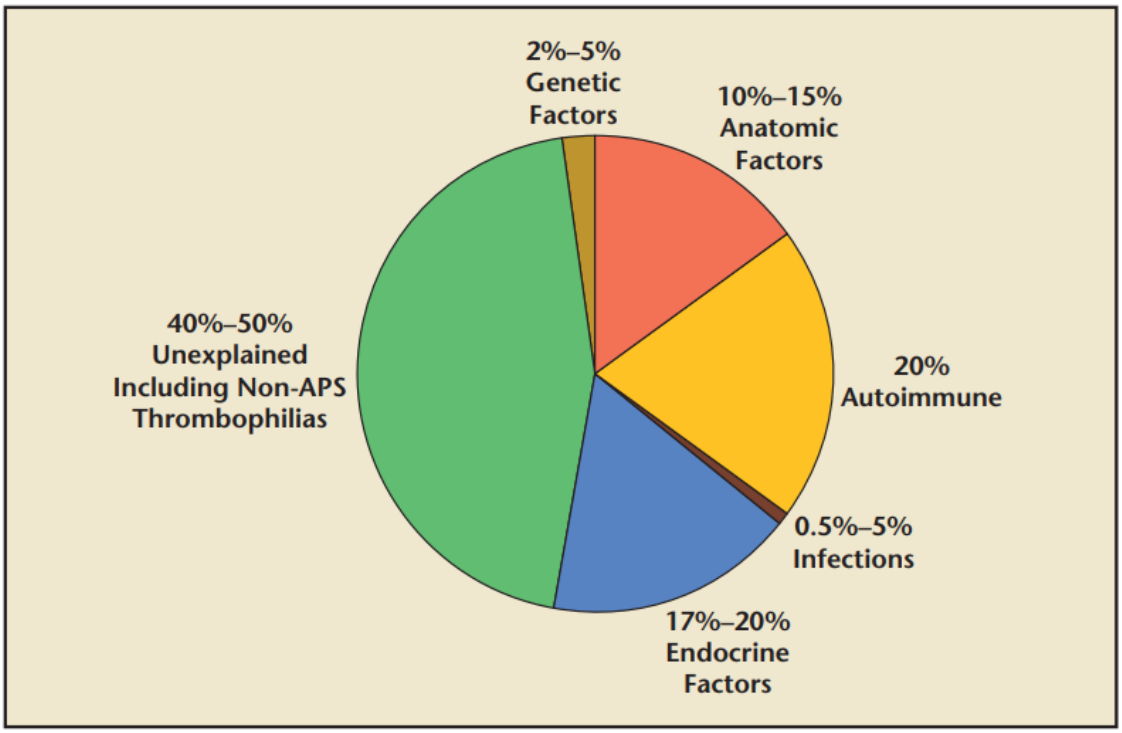


Figure 1: Risk factors for recurrent pregnancy loss (as described by Ford & Schust, 2009).

Table 1: Major Risk Factors Associated With Miscarriages And Their Association Strength

Risk Factors	Association level	Evidence	Strength
Maternal age	Increased risk of SM	2++	B
Paternal age	Increased risk of SM	2++	B
Consanguineous	No association	2-	D
Black ethnic background	Increased risk of SM	2+	D
Previous live birth	No association	2+	C
Previous miscarriages	Increased risk of subsequent miscarriage	2++	B
Smoking	Increased risk of SM	2+	D
Caffeine consumption	Increased risk of SM	2++	B
Alcohol consumption	Increased risk of SM	2+	D
Obesity	Increased risk of SM	2++	B
Environmental factors	Inconclusive	2-	D
Antiphospholipid antibodies	Increased risk of RPL	2++	B
Inherited thrombophilias	Weak association with RPL	2++	C
Parental chromosome rearrangements	Increased risk of RPL	2+	C
Chromosome anomaly of the pregnancy	Increased risk of SM and RPL	2++	B
Congenital uterine anomalies	Increased risk of miscarriage	2++	B
Acquired uterine anomalies	Inconclusive	3	D
Cervical insufficiency	Increased risk of second trimester miscarriage	2-	C
Well managed diabetes and thyroid disease	No association	2+	C

subclinical hypothyroidism	Increased risk of RPL	2-	C
Thyroid autoantibodies	Increased risk of RPL	2++	B
Luteal phase defect	Inconclusive	2-	D
Prolactin imbalances	Increased risk of RPL	2-	D
Polycystic ovary syndrome	Inconclusive	2-	D
Peripheral immune factors	Inconclusive	2++	C
Uterine NK cells	Inconclusive	2-	C
Genital tract infections	Inconclusive	2+	C
Sperm DNA fragmentation	Increased risk of RPL	2++	C

***Classification of evidence levels**: 1++: High-quality meta-analyses, systematic reviews of randomised controlled trials (RCTs) with a very low risk of bias, 1+: Well-conducted meta-analyses, systematic reviews of RCTs with a low risk of bias, 2++: High-quality systematic reviews of case–control or cohort studies or high-quality case–control or cohort studies with a very low risk of confounding, bias or chance and a high probability that the relationship is causal, 2+: Well conducted case–control or cohort studies with a low risk of confounding, bias or chance and a moderate probability that the relationship is causal, 2–: Case–control or cohort studies with a high risk of confounding, bias or chance and a significant risk that the relationship is not causal, 3: Non-analytical studies, 4: Expert opinion. **Grades**: A: At least one meta-analysis, systematic reviews or RCT rated as 1++, and directly applicable to the target population; or a systematic review of RCTs or a body of evidence consisting principally of studies rated as 1+, directly applicable to the target population and demonstrating overall consistency of results, B: A body of evidence including studies rated as 2++ directly applicable to the target population, and demonstrating overall consistency of results; or extrapolated evidence from studies rated as 1++ or 1+, C: A body of evidence including studies rated as 2+ directly applicable to the target population, and demonstrating overall consistency of

results; or extrapolated evidence from studies rated as 2++, D: evidence level 3 or 4; or extrapolated evidence from studies rated as 2+ (Regan et al., 2023).

1.4.1 Epidemiological Factors

Multiple epidemiological factors have been identified contributing to a higher risk of recurrent pregnancy loss (RPL), with maternal age being one such factor as described in Table 1. It has been observed that as maternal age increases, the quantity and quality of the oocytes decrease, leading to an elevated rate of aneuploidy in fertilized embryos (Hassold & Chiu, 1985). A comprehensive study based on registry data highlighted that the risk of miscarriage associated with age follows a J-shaped curve, as depicted in Figure 2. Interestingly, women between the ages of 25 and 29 exhibit the lowest likelihood of experiencing pregnancy loss (9.8%), while those aged 30-35 face modest risk. The risk increases significantly for women aged 40 and above, reaching up to 53% (Magnus et al., 2019). Apart from age, other potential factors such as smoking (A. Nielsen et al., 2006), alcohol consumption (Kesmodel et al., 2002), caffeine intake (Chen et al., 2016), obesity (Metwally et al., 2010), and exposure to environmental substances including air pollution and chemicals have also been associated with higher rates of miscarriage (Gaskins et al., 2019).

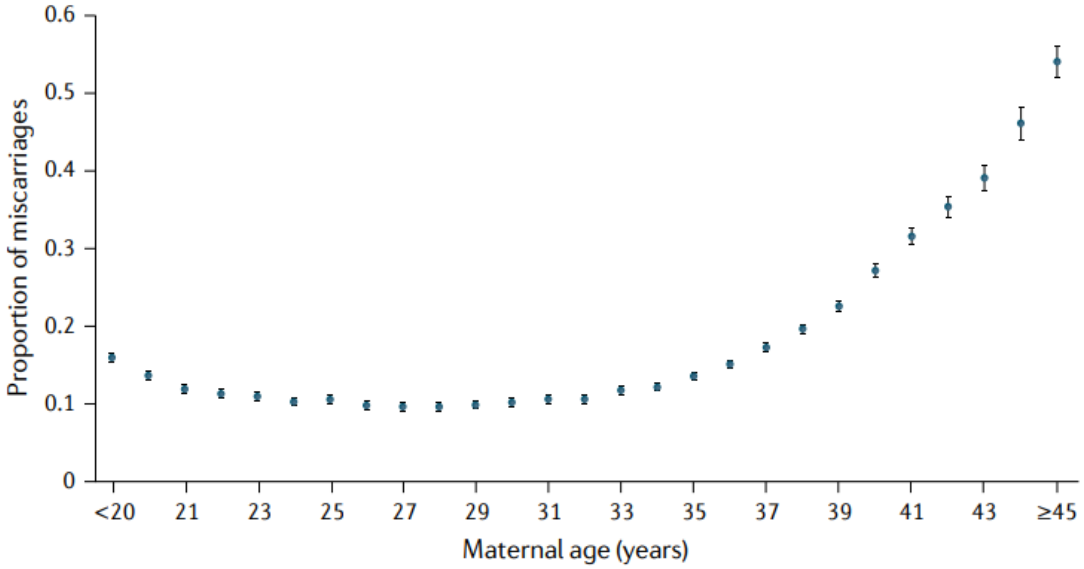


Figure 2: The association between maternal age and risk of pregnancy loss. The bars represent 95% confidence intervals (Magnus et al., 2019).

1.4.2 Thrombophilia

Thrombophilia comprises a range of conditions that can hinder blood coagulation and elevate the likelihood of thrombosis. It can be divided into two categories; inherited and acquired. Acquired factors encompass anti-phospholipid antibodies (aPL), anti-cardiolipin, anti-beta2 glycoprotein I antibodies, and lupus anticoagulants, while inherited factors include single mutations in FV gene or F2 prothrombin (G20210A) gene, as well as deficiency in protein S, C, or anti-thrombin (Greer, 2003). However, thrombophiliais associated with both SM and RPL through an increased risk of clot formation in the utero-placental circulation (Simcox et al., 2015).

1.4.3 Anatomical Factors

Abnormalities in uterine structure, detected during fertility exams, represent an established risk factor associated with 10–15% of RPL cases (Y. Y. Chan et al., 2011; Lin, 2004). These abnormalities can be either congenital or acquired. The first group, include didelphis, septate, bicornate, arcuate, and unicornate uteri, are often linked to miscarriages occurring in the late first and second trimesters (Saravelos et al., 2010). On the other hand, acquired factors including Asherman syndrome, intrauterine adhesions, myomas or fibroids, and endometrial polyps can also contribute to RPL. The exact mechanism by which uterine abnormalities lead to recurrent miscarriages remains unclear. There is some evidence suggesting that they may disrupt the vascular supply of the endometrium, consequently affecting the attachment and development of the placenta resulting in miscarriages (D & Rackow, 2018).

1.4.4 Endocrine Factors

RPL has been linked to systemic maternal endocrine abnormalities including thyroid disease and diabetes mellitus in 17% to 20% of cases (Stephenson, 1996). However, well-controlled diabetes mellitus and effective treatment of thyroid dysfunction do not heighten the risk of RPL (Abalovich et al., 2002; Mills, 2010). Furthermore, some evidence suggests a correlation between RPL and vitamin D deficiency, which is prevalent among women attempting to conceive (Chu et al., 2019; Gonçalves, 2018). Although the precise mechanism through which vitamin D deficiency may lead to miscarriage remains unknown, however, low vitamin D levels could elevate autoantibody concentrations, such as anti-phospholipid and anti-thyroid antibodies (Ota et al., 2014).

1.4.5 Immunological Factors

For long time, scientists have been intrigued by the mystery of how the implanting embryo and trophoblast manage to evade rejection by the maternal immune system in the uterus, even though they carry allogeneic proteins encoded by paternal genes. Indeed, normal implantation and pregnancy rely on maternal immune system's ability to tolerate the fetus. This involves activating regulatory T cells and the induction of anti-inflammatory Th-2 profile. Disruptions in the functioning of CD4 T-helper cells, uterine natural killer (NK) cell activity, and an imbalance in Th cells within the endometrium may result in failed implantation and pregnancy loss (Mekinian et al., 2016).

Compelling evidence supporting the significance of the immune system in RPL is derived from genetic and epidemiologic research, which revealed that genetic biomarkers linked to immunologic dysregulation during pregnancy are more prevalent in women with RPL. These biomarkers have a detrimental effect on prognosis. Some examples of these genetic biomarkers include maternal homozygosity for a 14 base-pair insertion in exon 8 of the human leukocyte antigen (HLA)-G gene, maternal possession of HLA class II alleles that confer a predisposition to immunity against male-specific minor histocompatibility antigens (HY antigen), which are encoded by the Y chromosome and present on male embryos, and specific combinations of maternal NK cell receptor genotypes and fetal HLA-C genotypes could potentially lead to abnormal recognition of the trophoblast by maternal NK cells (Ole B. Christiansen et al., 2012; Hiby et al., 2008; H. S. Nielsen et al., 2009).

1.4.6 Genetic Factors

Genetics is strongly considered as possible risk factor for recurrent pregnancy loss (RPL). The exact prevalence of genetic factors in the development of RPL is a wide topic of discussion. However, studies have indicated that genetic factors play a role in approximately 5% of RPL cases (Ford & Schust, 2009). These factors include:

1- Parental chromosomal rearrangements are responsible for approximately 2-4% of recurrent pregnancy loss (RPL) cases which include various alterations such as copy number variations, insertions, mosaicism, inversions, and translocations including reciprocal or Robertsonian rearrangements (Fan et al., 2016; Stephenson & Sierra, 2006).

2- Fetal chromosomal anomalies are common factors in up to half of cases involving sporadic and early recurrent miscarriages (Zhang Ting, 2016). These anomalies arise due to meiotic non-disjunction (improper separation of the chromosomes), resulting in conditions like trisomy, polyploidy, and monosomy, along with structural chromosomal rearrangements such as balanced translocations or inversions (Kaser, 2018).

3- Single gene defect: Numerous polymorphisms and mutations in nearly 90 different genes have been extensively investigated in their correlation with RPL as described in Table 2. These genes primarily play a role in regulating the implementation process, fetal and placental development, immunotolerance and inflammation, maternal metabolism and adaptation to pregnancy, as well as blood coagulation, however, most studies investigating the correlation between these genes and RPL yielded negative or conflicting results (Rull et al., 2012).

1.5 Vitamin D

Cholecalciferol, often known as vitamin D3, is primarily obtained through the process of cutaneous biosynthesis. This biosynthesis is initiated when the skin is exposed to ultraviolet light from the sun which triggers photochemical conversion of 7-dehydrocholesterol to vitamin D_3 (Webb et al., 1988).Vitamin D_3 undergo two conversion steps into its active form. Initially, vitamin D binding protein (DBP) transports vitamin D_3 to the liver, where it is

hydroxylated by vitamin D 25-hydroxylases to form the major circulating form of vitamin D,25(OH)D3. Subsequently, this form is transported to the proximal renal tubule of the kidney, and undergoes further hydroxylation to form the biologically active form of vitamin D, known as1α,25-dihydroxyvitamin D3 as illustrated in Figure 3 (Cheng et al., 2004; Omdahl et al., 2002).Vitamin D plays crucial role in maintaining calcium homeostasis for

Table 2 Correlation Between Various Genes Involved In Different Mechanisms And Recurrent Pregnancy Loss

Function	Gene Name	Results
Inflammation	IL21	Positive correlation
	IL1R1, IL4, IL12B, TNFR1, TNF-β	Negative correlation
	IFNG, IL1B, IL1RN, IL6, IL10, IL18, TNF-α	Contradictory results
Thrombosis and Cardiovascular system	ACHE, ANXA5, GPIa, HMOX1, SELP, TAFI, ZPI	Positive correlation
	AGT, APOB, FGB, F12, JAK2, MTHFD1, TGFB1, TM, TSER	Negative correlation

	ACE, APOE, AT1R, EPCR, F2, F5, F13A, GPIIIa, MTHFR, PAI-1, PZ, VEGF	Contradictory results
Detoxification system	-	Positive correlation
	AHR, ARNT, CYP1B1, NAT2	Negative correlation
	GSTT1, GSTP1, GSTM1, CYP2D6, CYP1A1, CYP1A2	Contradictory results
Chromosomal segregation	SYCP3	Contradictory results
Immune response	CTLA4, CX3CR1, HLA-DPB1	Positive correlation
	INDO, MBL	Negative correlation
	CCR5, HLA-A, B, HLA-C, HLA-E, HLA-G, HLA-DQA1, HLA-DQB1, HLA-DR, KIR	Contradictory results
Hormonal regulation	hCG beta (CGB5/8), CYP19A1	Positive correlation
	-	Negative correlation
	AR (and XCI), CYP17A1, PROGINS, ESR1/2	Contradictory results
Placental function	ACP1, ADA, EG-VEGF, PKR1, PKR2, IGF-2, KDR, PAPPA, PGM1, STAT3	Positive correlation
	TPH1, MMP9, MCP, H19, CD14, ANGPT2, ADRA2B	Negative correlation
	NOS3, P53	Contradictory results
Mitochondrial function	Mutational burden	Contradictory results

Rull, K., Nagirnaja, L., & Laan, M. (2012). Genetics of recurrent miscarriage: Challenges, current knowledge, future directions. Frontiers in Genetics .

optimal bone health. However, its functions extend to several other functions, including cell proliferation and differentiation, immune system function, brain development, fetal

development, and hormone secretion. Furthermore, it has been suggested that 1α,25-dihydroxyvitamin D3 is involved in hair follicle cycling, blood pressure regulation, and the development of mammary gland (Bikle, 2011, 2016; Christakos & DeLuca, 2011; Eyles, 2021).

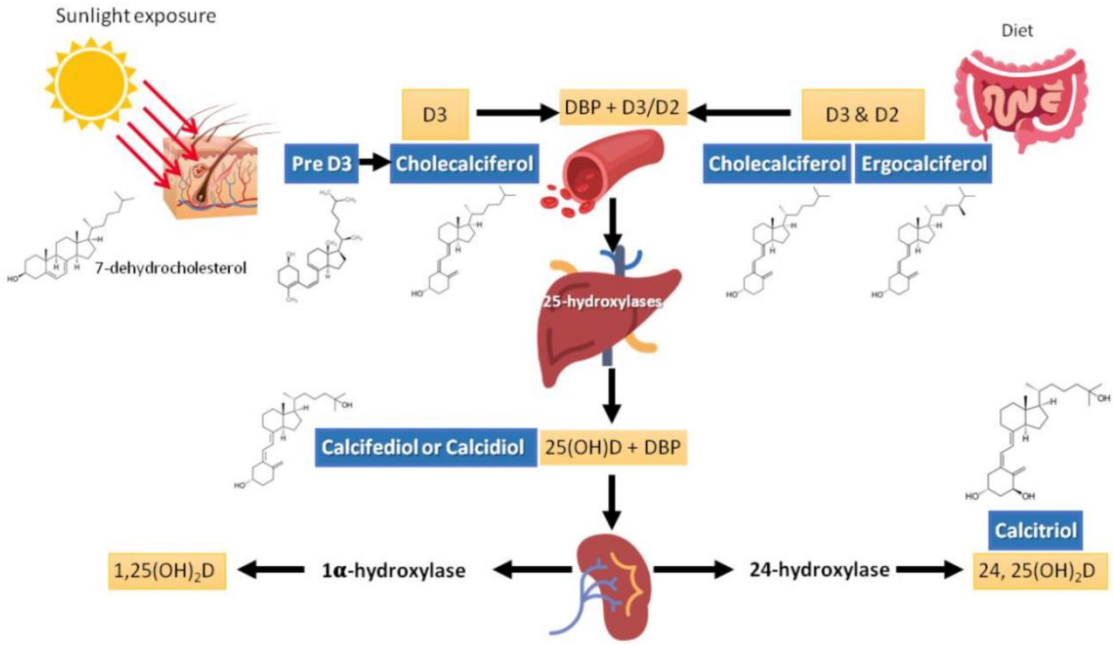


Figure 3: The Metabolic Pathway of Vitamin D (as Described by Dominguez et al., 2021).

1.6 Vitamin D receptor (VDR)

The biological functions of VD is evidently mediated through the vitamin D receptor (VDR), which is a member of the nuclear receptor family and functions as a ligand-activated transcription factor (Strugnell & Deluca, 1997). VDR serves as the mediator for the genomic effects of vitamin D. When VDR binds to 1,25-dihydroxy vitamin D3 (1,25(OH)2D3) hormone, it forms a heterodimer with the nuclear retinoid X receptor (RXR). This complex binds to vitamin D response elements (VDRE) in the promoter

regions of vitamin D-responsive genes and recruits various co-factors to regulate gene expression as illustrated in Figure 4 (Freedman & Arce, 2015; Karras et al., 2017). The VDR gene is located on chromosome 12q13.11 (accession # NG_008731.1), spans approximately 75kb of genomic DNA, and consists of 14 exons: 6 alternatively spliced untranslated exons (1a–1f) located in the 5'UTR, and eight protein-coding exons (II–IX) as described in Figure 5 (L. A. Crofts et al., 2010; G. Uitterlinden et al., 2004).The VDR gene is highly expressed in multiple human tissues including the reproductive tissue (i.e., ovaries, uterus, placenta, and endometrium), osteoblasts, chondrocytes, skin epithelial cells and immune cells (Shahrokhi et al., 2016; Y. Wang et al., 2012).

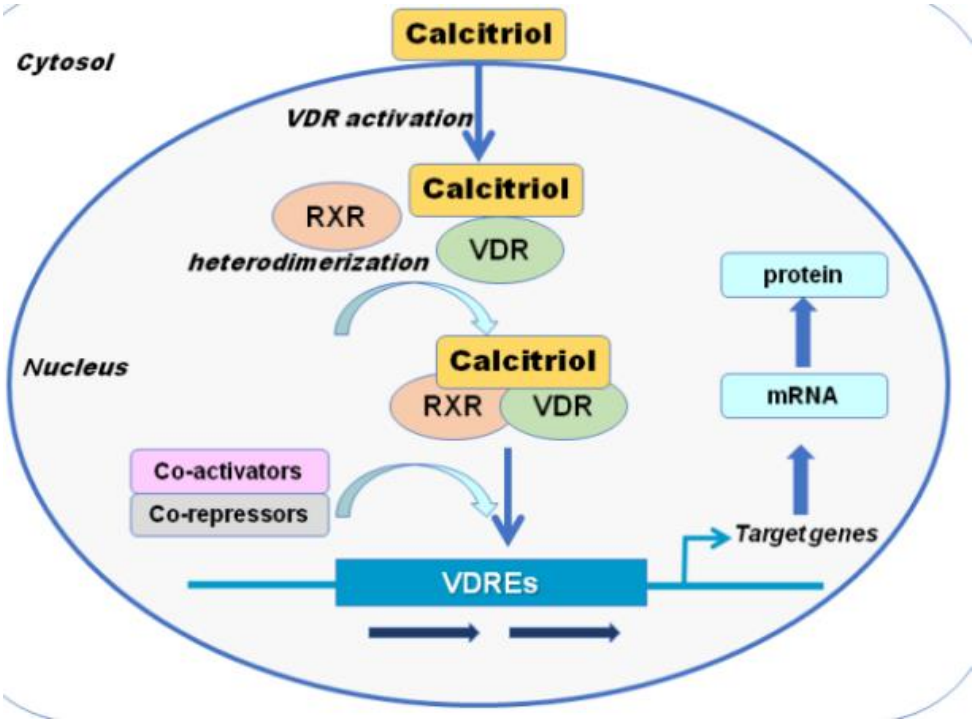


Figure 4:Gene Expression by VDR in Response to Calcitriol (1,25-Dihydroxyvitamin D3) Binding (as Described by Dominguez et al., 2021).

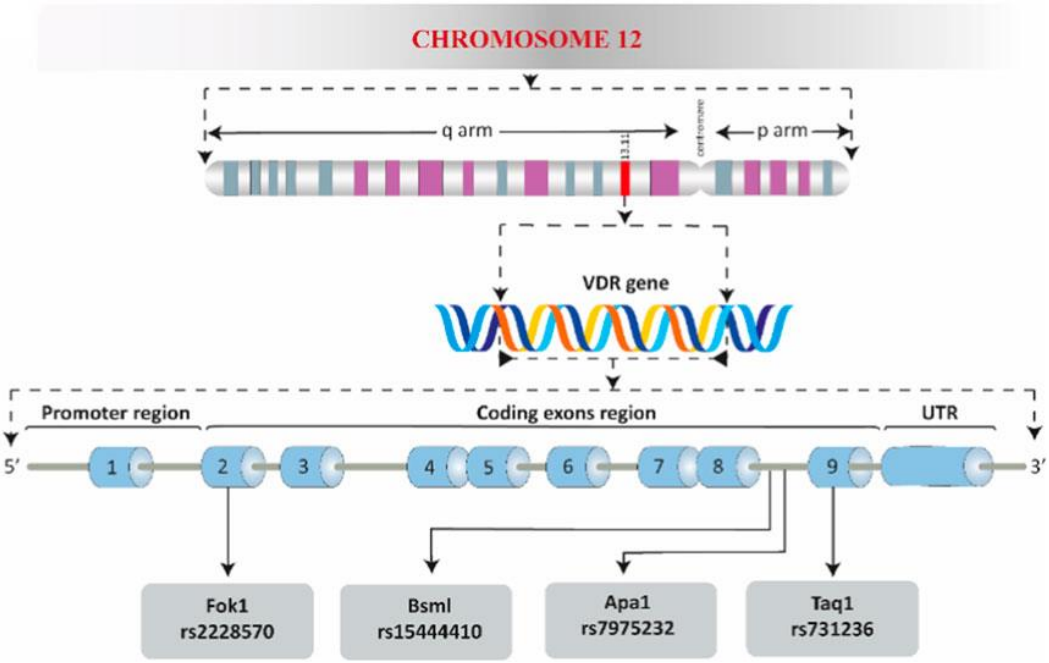


Figure 5: A schematic Overview of the Human VDR gene (as Described by Yao et al., 2024).

1.7 VDR Gene Polymorphisms

The existence of polymorphism variations in the VDR gene can affect VDR activity (Hidekazu Arai et al., 1997). Studies have demonstrated the significance of VDR genetic variations in a number of pregnancy-related problems, including preeclampsia, gestational diabetes, preterm birth, and recurrent abortion (Farajian-mashhadi et al., 2019; Javorski et al., 2018; Wolski et al., 2021). More than 470 single nucleotide polymorphisms (SNPs) have been identified in the VDR gene. The most frequently studied VDR polymorphisms are FokI (rs2228570), BsmI (rs1544410), ApaI (rs7975232), and TaqI (rs731236) (G. Uitterlinden et al., 2004). Table 3 summarize the most common VDR genetic polymorphisms and their genomic locations.

Table 3: The Most Common Snps Of The VDR Gene With Their Position And Nucleotide Variations

Polymorphisms	rs	Location	Codon	Nucleotide Variant	Key References
FokI	2228570	Exon 2	ATG (1st codon)	T/C	(Hidekazu Arai et al., 1997)
BsmI	1544410	Intron 8	_	G/A	(N. A. Morrison et al., 1992)
Tru 9I	757343	Intron 8	_	G/A	(Ye et al., 2000)
Eco RV	4516035	Intron 8	_	G/A	(N. A. Morrison et al., 1992)
CdX2	11568820	1e promoter	_	G/A	(H. Arai et al., 2001)
ApaI	7975232	Intron 8	_	G/T	(Faraco et al., 2010)
TaqI	731236	Exon 9	Codon 352	T/C	(Nigel A. Morrison, 1994)

*rs: restriction site

1.7.1 FokI Polymorphism (rs2228570 T>C)

As shown in Table 3, FokI polymorphism is the only one that has been shown to have an impact on amino acids sequence in the VDR protein (André G. Uitterlinden et al., 2004). The FokI polymorphism (rs2228570) is located in exon 2 and results in the thymine-to-cytosine transition (ATG>ACG) at the first start codon (ATG) of the VDR gene. Under

normal circumstances, the VDR gene contains two ATG translation initiation sites separated by nine bp; hence, in individuals with the T variant (referred to as the f allele), the VDR gene has two translation start codons, and the translation of VDR mRNA will commence from the first initiation site, not the second. Conversely, in case of the C variant (referred to as the F allele), the VDR gene will lose the first start codon and the second start codon will be utilized to initiate the translation of the VDR mRNA as illustrated in Figure 6. Such a difference will alter the VDR protein structure and significantly affect protein function and activity by shortening the amino acid sequence (424 amino acids (M4 isoform)) instead of 427 amino acids (M1 isoform) and making it more transcriptionally active (Hidekazu Arai et al., 1997; Jurutka et al., 2000). Interestingly, FokI polymorphism is the only one among other VDR polymorphisms not linked to any other polymorphisms in the VDR gene, which may give it a unique role (G. Uitterlinden et al., 2004). Few studies have discussed the possible functional role of FokI polymorphism on recurrent pregnancy loss (Barišić et al., 2021; Salari et al., 2021).

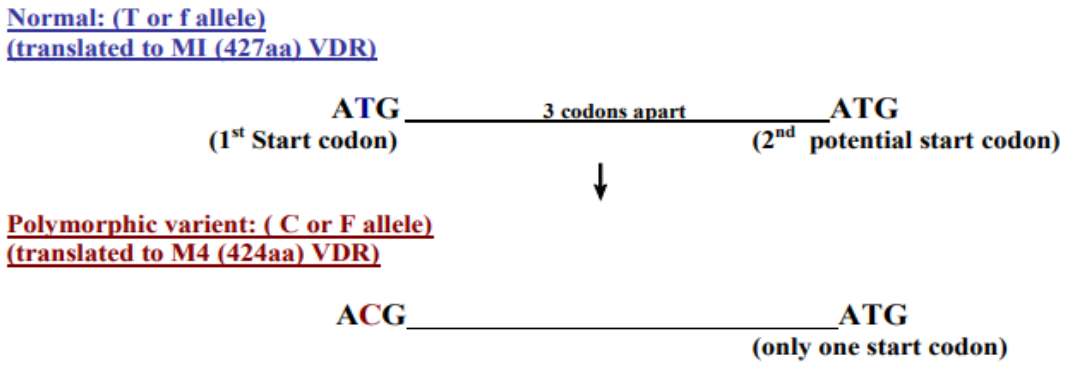


Figure 6: Structural Consequence of Fok I Polymorphism on VDR Protein.

1.7.2 BsmI Polymorphism (rs1544410 G>A)

The BsmI polymorphism (rs1544410) is located in intron eight and results in the substitution of guanine to adenine (G>A) (N. A. Morrison et al., 1992). Since this polymorphism is located in the regulatory area rather than the exon coding area, it does not alter the amino acid sequence of the VDR gene; instead, it affects the level of gene expression through altering VDR mRNA stability (Nigel A. Morrison, 1994). Since the BsmI SNP is located in the non-coding region, it is assumed that the association between a specific diseases and BsmI polymorphism can be explained by linkage disequilibrium with one or more truly functional polymorphisms in other regions of the VDR gene (S A Ingles et al., 1997; Andre G Uitterlinden et al., 1996). Several studies have examined the degree of linkage disequilibrium (LD) throughout the VDR gene with strong LD observed between BsmI polymorphism and the nearby polymorphisms at the 3' end of the VDR gene, including ApaI, Eco RV, and TaqI. In addition, BsmI polymorphism is closely linked to the poly-A variable number of tandem repeats (VNTR) in the 3'UTR. Nevertheless, only one study demonstrated the potential role of BsmI polymorphism in the etiology of RPL (Wolski et al., 2021).

1.8 The impact of Vitamin D and its Receptor on Pregnancy Outcome

Vitamin D has significant impact on the development of pregnancy and due to its potential antiproliferative and immunomodulatory roles, it seems to influence the course and outcome of pregnancy (Cyprian et al., 2019). Vitamin D plays potential role in feto-placental development as well as the regulation of several placental hormones, including progesterone, estradiol, human placental lactogen, and human chorionic gonadotropin (hCG). These hormones are essential for the proper growth and development of the

placenta (Barrera et al., 2007, 2008). Additionally, a study in vitro showed that vitamin D metabolites (1,25-D3 and 25-D3) have a direct effect on the placental function and promote trophoblast invasion (S. Y. Chan et al., 2015). Moreover, VD plays vital role in modulating the immune system's response, affecting both the innate and adaptive immune systems by regulating cytokine synthesis and suppressing the proliferation of pro-inflammatory cells (Brannon, 2012). Vitamin D acts directly to stimulate T helper 2 lymphocyte differentiation, increasing the synthesis of anti-inflammatory cytokines such as IL-4, 6, 9, 10, and 13, while at the same time inhibiting T helper 1 lymphocyte differentiation and decreasing the synthesis of pro-inflammatory cytokines such as interleukin 2, IL-12, interferon-gamma, and tumor necrosis factor-alpha (Chun et al., 2014; Essen et al., 2010). However, during pregnancy, the maternal innate immune system is suppressed while the maternal adaptive immune system is activated. These transitions in the maternal-fetal space in response to vitamin D produce immunological tolerance, reduce fetal rejection risk, and result in successful pregnancy (Liu et al., 2015; Tamblyn et al., 2015). On the other hand, disruptions in this system caused by VD insufficiency result in several pregnancy complications, including preterm delivery, preeclampsia, gestational diabetes mellitus, low birth weight as well as recurrent pregnancy loss (Gilani & Janssen, 2019; Gonçalves, 2018; Taneja et al., 2020).

Several studies discussed the role of impaired vitamin D complex, including vitamin D, vitamin D receptor (VDR), vitamin D binding protein (DBP), and vitamin D activating enzymes, on the pathogenesis of recurrent pregnancy loss. Most studies suggested reduction both in VD and VDR expression, is associated with a decrease in enzyme activity in the placentas of women experiencing recurrent miscarriages. An in vitro study reported that the placental and decidual expression of VDR, DBP, and CYP24A1 (the

enzyme responsible for the degradation of vitamin D metabolites 25-OHVD and 1,25(OH)2D3) were higher in cases with spontaneous miscarriages compared to control cases, whereas the expression of CYP27B1 (the enzyme responsible for converting 25OHD-hydroxyvitamin D3 into its active form, 1,25-dihydroxy vitamin D3 was reduced (Hou et al., 2020). Another study conducted on women who experienced at least two consecutive pregnancy losses and forty women with a normal pregnancy concerning the expression of VDR in the first trimester of pregnancy (7-10 gestational weeks) to investigate the potential role of VDR in recurrent pregnancy loss. The study has revealed that the expression level of VDR in villous and decidual cells was reduced among cases with RPL compared to the control group (46% and 52% reduction of VDR expression in villi and decidua cells, respectively, with a p-value <0.0001) and serum VDR level were also lower in the RPL group compared to the control group with a p-value of 0.003. In addition, the VDR expression level was significantly lowered in stromal cells, villous cytotrophoblasts, and decidual glandular epithelial and stromal cells. According to the study, women with RPL have lower levels of VDR expression in their chorionic villi, decidua, and serum compared to healthy pregnant women, indicating that lower VDR expression levels during the first trimester of pregnancy may be linked with RPL (Yan et al., 2016). Since adequate VDR expression is needed during pregnancy, the consequences of altered VDR expression, especially in reproductive tissue, are still unknown, they could be due to local immune tolerance of the fetal-maternal interface or altered downstream signaling pathway of the target genes. Nevertheless, studies have highlighted the impact of vitamin D metabolism disturbance on the production of cytokines during the local immune response while also suggesting a potential role in the pathogenesis of RM. Such that was proved by Li et al. who investigated the levels of vitamin D

(25(OH)D), IL-17, IL-23, transforming growth factor (TGF-β), and the expression levels of VDR and 1-α-hydroxylase (CYP27B1) in the decidual tissues of thirty women with recurrent spontaneous abortions (RSA group) and thirty women with elective abortions (control group). The study showed that levels of VD, TGF-β, as well as the expression level of VDR were dramatically reduced in the RSA group compared to the control group, whereas the concentrations of IL-17 and IL-23 were significantly elevated. These results imply a possible connection between vitamin D levels in the decidua and the synthesis of inflammatory cytokines as well as immune responses, suggesting at the same time that vitamin D and VDR may be involved in the occurrence of RSA (N. Li et al., 2017).

Equally interesting are studies on the associations of common placental genetic variations (SNPs) involved in VD metabolism and pregnancy outcomes. One study focused on maternal-infant pairs to investigate the association between different SNPs related to VD metabolism genes and baby birth weight. Eight SNPs in five genes, including LRP2 (rs4667591 and rs2229263), VDR (rs2228570), GC (rs2282679), CYP2R1 (rs10741657), and CUBN (rs1801231, rs1801224, and rs1801222) were examined. Within the cohort of studied infants, correlation was evident between the rs2282679 variant and increase in birth weight. In addition, significant association was demonstrated between the rs4667591 (LRP2) variant with both sex and birth weight of the newborns (p value< 0.001). This study offers insights into the pleiotropic impact of vitamin D on fetal development, along with a potential link between genetic variations in the placenta related to vitamin D metabolism and birth weight (Workalemahu et al., 2017). Moreover, the role of various VDR polymorphisms in the development of recurrent pregnancy loss or spontaneous miscarriages has been relatively understudied. However, the majority of the available data highlighted the significance of specific VDR genetic variants, such as FokI

and BsmI SNPs, in influencing VDR function. As a consequence, these genetic variations may contribute to disruptions in vitamin D metabolism, potentially playing a role in the pathogenesis of these conditions (Salari et al., 2021; Wolski et al., 2021).

It seems that current available evidence support the notion that vitamin D supplementation can improve the course and outcome of pregnancy supported by the results of randomized clinical trials regarding the effect of vitamin D supplementation on mother-infant health during pregnancy. Clearly, vitamin D supplementation led to significantly higher level of 25 (OH) D concentrations both in the mother and infant compared to the control group, reduced the maternal insulin resistance and increased birth weight of the infant (Gallo et al., 2019). Further, a meta-analysis of 15 randomized and quasi-randomized clinical studies revealed that vitamin D treatment during pregnancy decreased the risk of preeclampsia, with no effect of vitamin D supplementation on the risk of gestational diabetes (Cristina et al., 2016). Moreover, meta-analysis study which involved 22 trials (3725 pregnant women) showed the preventive role of vitamin D supplementation in reducing the risk of preeclampsia, gestational diabetes, postpartum hemorrhage, as well as low birth weight of the infant (Palacios et al., 2019). In addition, a double-blind randomized controlled clinical trial was conducted on 80 patients concerning the effect of vitamin D intake on unexplained recurrent spontaneous abortion. The findings revealed that vitamin D supplementation reduces serum level of cytokines such as IL-23 as well as the prevalence of abortion among women with the condition (Samimi et al., 2017).

1.9 Study Problem

Recurrent pregnancy loss is a complicated and multi-factorial condition, and several genetic and non-genetic factors have been identified to increase the risk of RPL. Globally, about 50–60% of RPL cases remain with no defined cause. Likewise, in Palestine, recurrent abortion is detected among several Palestinian females. This could be due to several factors, including single gene mutations and polymorphisms. Previous studies identified the factor V gene as a major risk factor for recurrent abortion in only 40% of the studied cases. Recently, selected variants in the vitamin D receptor (VDR) that affect its function have been linked to recurrent abortion in some populations. In addition, the level of vitamin D status, which reflects the hormonal form of the vitamin, may significantly contribute to abortion.

1.10 The Aim of the Study

Many single nucleotide polymorphisms (SNPs) have been identified in the VDR gene, including BsmI (rs1544410), ApaI (rs7975232), TaqI (rs731236), and FokI (rs2228570). These genetic variations appear to have a significant impact on the function and expression of VDR, with some being implicated in the development of recurrent abortion. Hence, the primary objective of this research is to assess the correlation between two maternal single nucleotide polymorphisms of the VDR gene, specifically FokI (rs2228570) and BsmI (rs1544410), the haplotypes of these SNPs, the level of vitamin D status, and their link to recurrent pregnancy loss among affected Palestinian women.

1.11 The Significance of the Study

- This research is the first of its kind regarding the association between different VDR genetic variants and recurrent pregnancy loss in both Palestinian and Arabs populations.
- Detection of VDR genetic variants among Palestinian subjects will extend our knowledge and understanding of how those particular genetic variants may affect the occurrence of recurrent pregnancy loss in our population.
- The results may provide a potential medical intervention approach to help the inflicted females in their efforts to have successful pregnancy to full term and reduce the time needed to achieve a successful pregnancy.
- The findings can be used in genetic counselling to advice women who are at high risk for recurrent pregnancy loss.
- The findings may be significant for prenatal care in terms of a woman's overall health and life, as well as in terms of preventive medicine.
- Our study could help create new best practice recommendations in the future.
- Regarding long-term physiological effects on couples, our findings may offer some answers for the medical care of pregnant women who are at high risk of RPL.

Chapter Two

Materials and Methods

2.1 Study Subjects

A case-control study was conducted on 131 women who attended prenatal care clinics on the West Bank of Palestine. The case group consisted of 51 women who experienced at least three primary consecutive idiopathic miscarriages (between 10 and 20 weeks of gestation). Moreover, the exact obstetric history was collected, including the number of miscarriages and gestational weeks at the time of the pregnancy's termination. The women were recruited to the case group in accordance with ESHRE guidelines (Bender Atik et al., 2018). The control group consisted of 80 women with two or more successful pregnancies, a regular menstrual cycle, and no history of recurrent abortion or any other pregnancy complications. Both groups' medical and family histories were obtained through a uniform questionnaire, a personal interview with the participants, governmental official records, and consultation with the medical team. The exclusion criteria for both groups were determined based on previous lab reports submitted by each subject and are as follows: women aged >= 35 years old, inherited thrombophilia (FV Lieden, F2), chromosomal anomalies, cigarette smoking, alcohol consumption, autoimmune diseases (anti-phospholipid antibodies, anti-cardiolipin antibodies, lupus anticoagulant, anti-nuclear antibodies and B2 glycoprotein 1), uterine abnormalities, infectious diseases (rubella, toxoplasma, HIV, HBV, HCV, and CMV), endocrine diseases (diabetes mellitus, thyroid diseases, and hyperprolactinemia), Rh blood group incompatibility, any women with any pregnancy complications other than recurrent pregnancy loss, such as those with a history of preterm birth or preeclampsia.

It is worth noting that within our case group, two participants have a familial background of recurrent pregnancy loss (RPL) as illustrated in Figure 7 A and B. To gain a better understanding of the underlying mechanisms behind RPL, we performed whole exome sequencing on their samples. The aim was to identify any potential novel gene(s) that may play a role in the occurrence of RPL. Further details regarding this investigation will be provided in the result section (chapter 3).

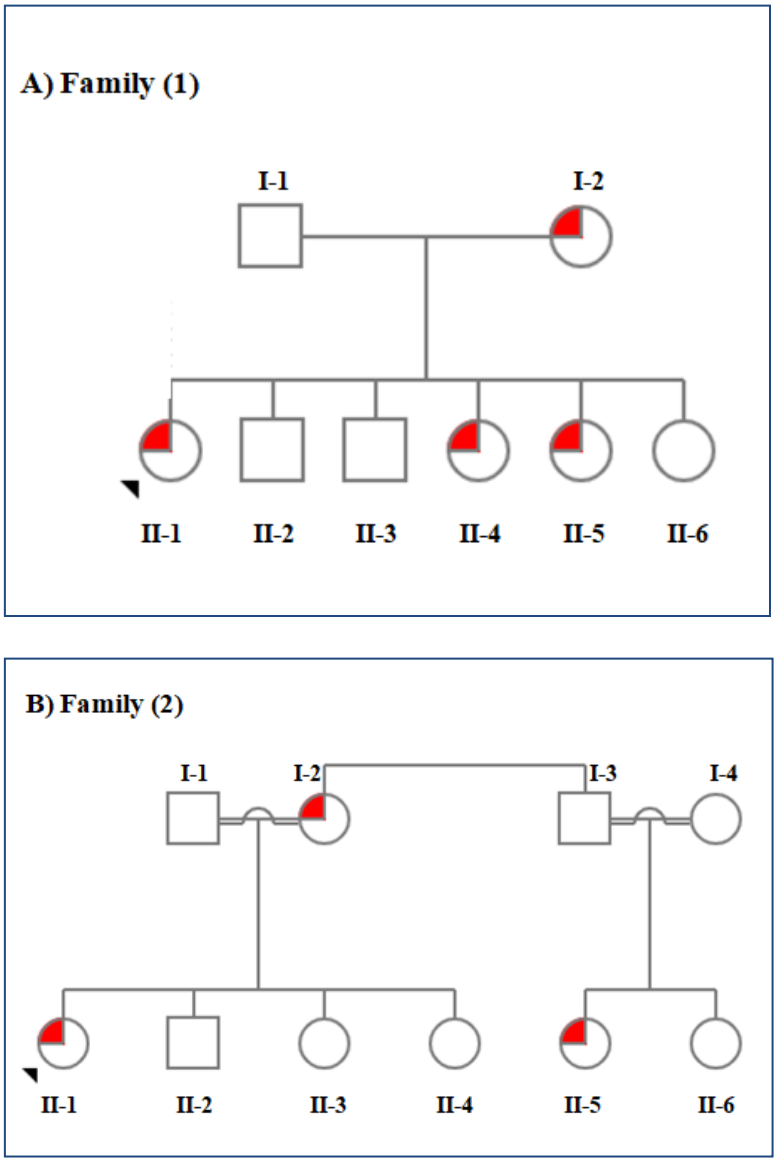


Figure 7: The family pedigree of the first participant(A) and the family pedigree of the second participant(B).

2.2 DNA extraction

Three ml of EDTA whole blood was collected from both control and case groups and centrifuged for 15 minutes at 1000 rpm. Then, the genomic DNA was extracted from the whole blood using a leukocyte rich buffy coat Following the MasterPure™DNA Purification Protocol (Epicentre Biotechnologies, Wisconsin, USA, Cat No. MG71100). The DNA extraction steps are summarized in Figure 8.

2.3 DNA Quantitation and Qualification

The concentration and purity of the extracted genomic DNA were evaluated using a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA).

2.4 Agarose gel electrophoresis

In order to detect the PCR products, the agarose gel electrophoresis was carried out. 2% (w/v) of agarose gel (SeaKem^{RLE}Agarose) was added to 100 ml of diluted TAE working buffer (Tris Acetate EDTA (1X)). The mixture was heated for 90 seconds to ensure that the agarose was completely dissolved in the working buffer, which was then allowed to cool to ~60 ºC. Afterward, 30 µl of Ethidium Bromide (10 mg/ml) was added to the mixture to stain and enhance the DNA visualization. 5-7 µl of the PCR product were loaded into a separate well of the gel, and 5 µl of ready-made DNA ladder (50 bp or 100 bp) was allowed to run alongside the samples to determine the size of the bands. Finally, the gel was run for 30-45 minutes at 120 Volt, and the PCR products were visualized under a UV transilluminator.

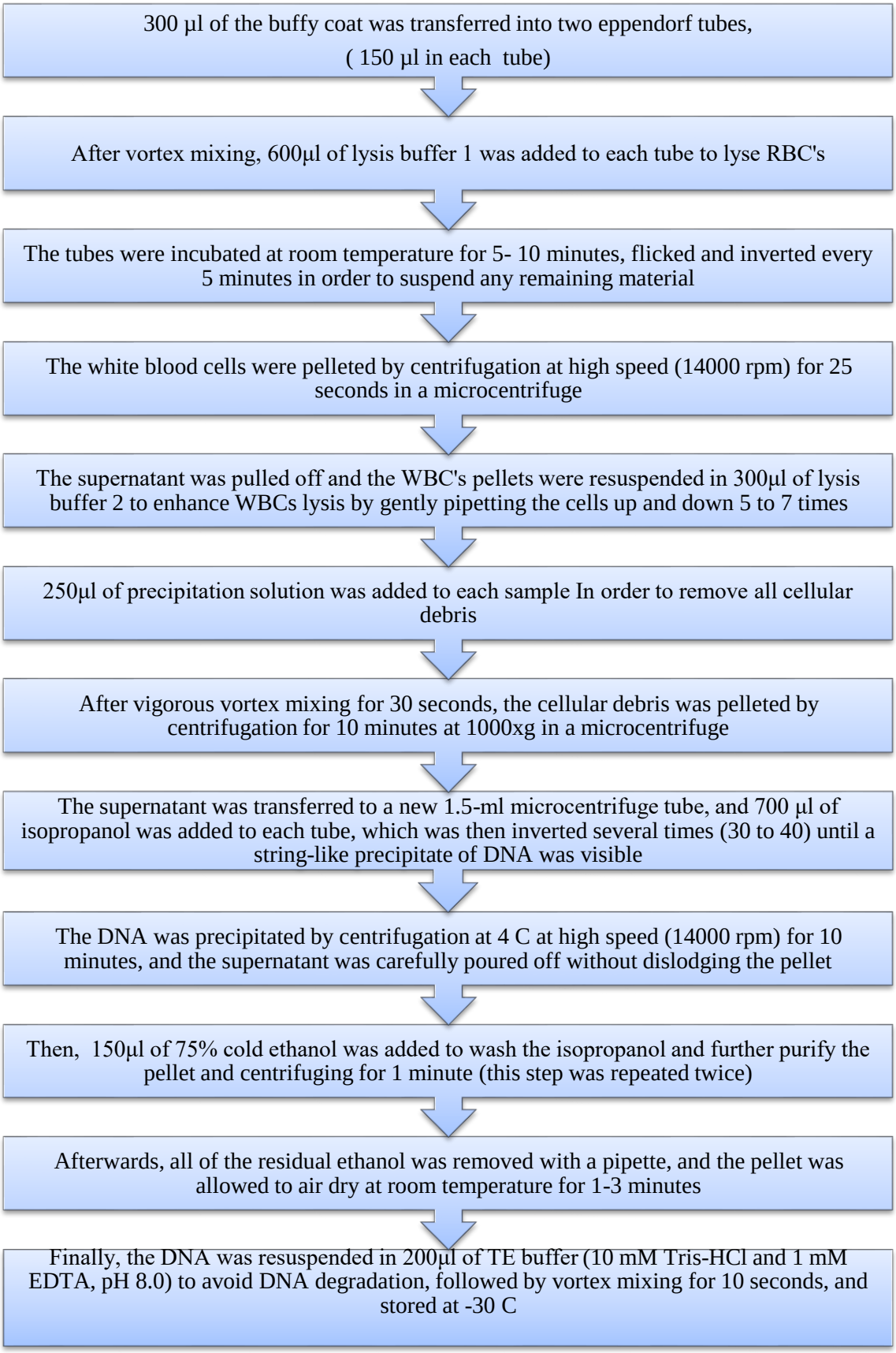


Figure 8: Flowchart illustrating the steps of DNA extraction.

2.5 VDR Primer design

According to the primer sequences published earlier for VDR, primer 3 plus web was used to design the primer sequences for amplifying DNA segments of 265 bp and 820 bp, which encompass the rs2228570 and rs1544410 polymorphisms. The details of the primer sequences can be found in Table 4.

Table 4: The Primer Sequences For Foki (Rs2228570) And Bsmi (Rs1544410) Polymorphisms Used In The Study

Gene	Polymorphism	Primer type	Primer Sequence 5'----------3'	Product size (bp)	Key references
VDR	FokI polymorphism **(rs2228570)**	**Forward**	**AGCTGGCCCTGGCACTGACTCTG**	265	(Tofteng et al., 2002)
		Reverse	**TCTTCTCCCTCCCTTTCCACTG**		
	BsmI polymorphism (**rs1544410)**	**Forward**	**AACCAAGACTACAAGTACCGCGTCAGTGA**	820	(Tofteng et al., 2002)
		Reverse	**AACCAGCGGGAAGAGGTCAAGGG**		

2.6 Polymerase Chain Reaction (PCR)

The DNA fragments containing the polymorphic sites of FokI and BsmI in the VDR gene were amplified using a polymerase chain reaction. The PCR reaction was carried out in 25µl reaction volumes containing 12.5µl Taq ^{R}PCR green master mix (2X), 1µl DNA (150 ng), 0.5µl forward primer (10 mM), 0.5µl reverse primer (10 mM), and 10.5µl

nuclease-free water. The thermal cycler was set according to the following conditions listed in Table 5.

Table 5: The PCR Conditions for VDR SNP's Including rs1544410 and rs2228570

PCR Conditions	FokI VDR SNP(rs2228570)		BsmI VDR SNP(rs1544410)	
Initial denaturation	94 C, 2min		94 C, 2min	
2nd Denaturation	94º C,30sec		95º C, 30sec	
Annealing	56º C,45sec	32 cycles	60º C,45sec	32 cycles
Extension	72 ºC,30sec		72º C,2min	
Final extension	72º C, 5 min		72º C, 5min	

2.7 Restriction Fragment Length Polymorphism (PCR-RFLP)

DNA samples were genotyped for VDR SNPs, including rs1544410 in intron 8 and rs2228570 in exon 2, using restriction fragment length polymorphism (PCR-RFPL). The fundamental idea behind RFLP is that it depends on the digestion of DNA fragments (PCR products) that contain a specific restriction site with an endonuclease restriction enzyme; thereby, if a single nucleotide variant is found among the DNA sequence, the enzyme will cleave it, resulting in an aberrant banding pattern that can be detected by gel electrophoresis (Nigel A. Morrison, 1994; Tao et al., 1998). For FokI digestion, the digestion mixture was performed in 15µl reaction volumes containing 10µl PCR product, 1.5µl buffer (10X), 0.3µl FokI restriction enzyme (5000 µ/ml source), and 3.2µl nuclease-free water. Then, the digestion mixture was incubated at 37 ºC overnight. The following genotypes were obtained: (Figure 9)

- Homozygotes uncut (FF or CC), resulting in one fragment of 265 bp.
- Heterozygotes (Ff or CT), resulting in three fragments of 265 bp, 196 bp, and 69 bp.
- Homozygotes cut (ff or TT), resulting in two fragments of 196 bp, and 69 bp.

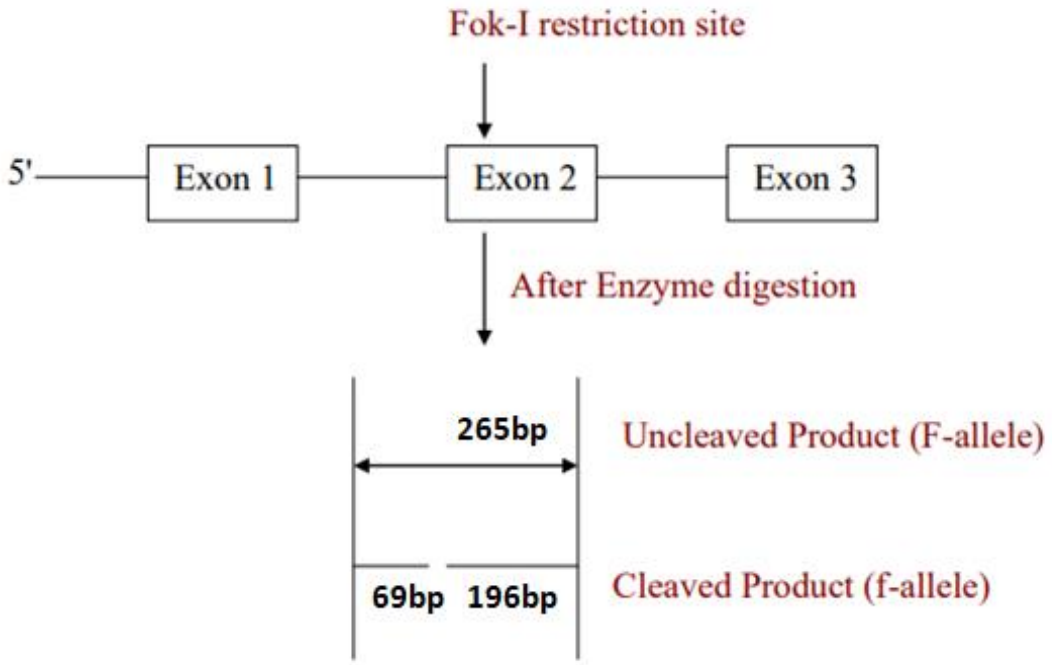


Figure 9: Restriction pattern of PCR products by using FokI restriction enzyme.

For BsmI digestion, the digestion mixture was performed in 15µl reaction volumes containing 10µl PCR product, 1.5µl buffer (10X), 0.2µl BsmI restriction enzyme (10000µ/ml source), and 3.3µl nuclease-free water. Then, the digestion mixture was incubated at 60 ºC overnight. The following genotypes were obtained: (Figure 10)

- Homozygotes uncut (BB or GG), resulting in one fragment of 820 bp.
- Heterozygotes (Bb or GA), resulting in three fragments of 820 bp, 650 bp, and 170 bp.
- Homozygotes cut (bb or AA), resulting in two fragments of 170 bp, and 650 bp.

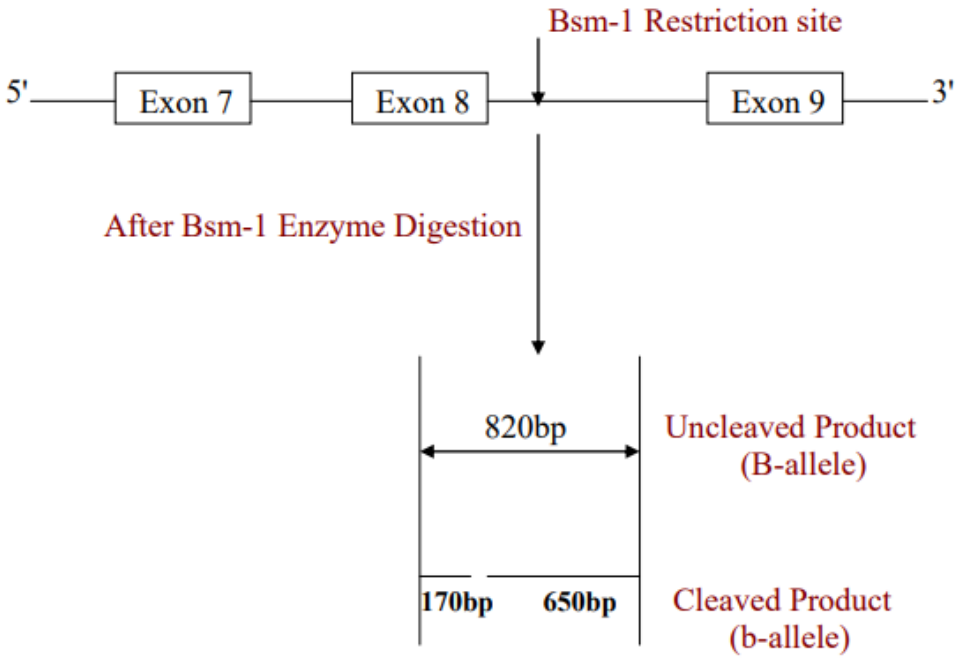


Figure 10: Restriction Pattern of PCR Products by using BsmI Restriction Enzyme.

2.8 Quantification of Serum Vitamin D level

One to three ml of whole blood was obtained from the study subjects and centrifuged at 4000 Xg for 10 minutes. The serum level of 25-hydroxyvitamin D3 was measured using the Elecsys Vitamin D Total III Kit (Ref No. 09038078160, Cobas, Roche, USA) according to the manufacturer's protocol (Roche, 2008). Depending on the total serum concentration of 25-hydroxyvitamin D3, our study subjects were classified into three major groups, as follows:

Status	Total concentration level of 25-hydroxyvitamin D3 (ng/mL)
Deficient	<10 ng/mL
In sufficient	10-20 ng/mL
Sufficient/Normal	>20 ng/mL

2.9 Next-Generation Sequencing

Each family had one patient selected, and the samples obtained from these patients were analyzed through whole-exome sequencing using Illumina 5500. The sequencing procedure was carried out in accordance with the manufacturer's instructions (Guide, 2021), as outlined below:

2.9.1 Genomic DNA Tagmentation

The process of fragmenting and tagging the DNA with adaptor sequences involved adding 30 µL (Final concentration = 500 ng) of each sample to separate wells within a 96-well PCR plate. Subsequently, the Tagmentation master mix, consisting of eBLT and TB1, was introduced into each well. To ensure proper containment, the plate was sealed using Microseal B and then placed on the thermal cycler (preheat lid 100 C, reaction volume 50 µL, 55 C for 5 minutes, Hold 10 C).

2.9.2 Post-Tagmentation Cleanup

To wash the adaptor-tagged DNA, 10 µL of Stop Tagment Buffer 2 (ST2) was introduced to the tagmentation reaction. The mixture was then shaken at 1600 rpm for 1 minute and subsequently placed on a magnetic stand. Following this, 100 µL of Tagment Wash buffer (TWB) was added to the beads and the samples were placed on the magnetic stand. The supernatant was discarded, and these steps were repeated twice.

2.9.3 Tagmented DNA Amplification

Amplification of the Tagmented DNA involved the addition of 40 µL of PCR master mix (EPM) to each well. Subsequently, pre-paired index 1 and index 2 adaptors, consisting of

10 base pairs, were introduced from the index adaptor plate to each well. The amplification of the Tagmented DNA was carried out using the eBLT PCR Program on the thermal cycler. (Preheat lid 100 C, reaction volume 50 µL, 72 C for 3 minutes, 98 C for 3 minutes, 9 cycles of: 98 C for 20 seconds- 60 C for 30 seconds- 72 C for 1 minute, 72 C for 3 minutes, Fold at 10 C).

2.9.4 Libraries Cleanup

To ensure the purity of the amplified libraries, 45 µL of supernatant from each well was carefully transferred to the corresponding well of a new MIDI plate. Following this, nuclease-free water (77µL) and AMPure XP beads (88µL) were added. A washing step was carried out using 200µL of fresh 80% Ethanol before the addition of 17µL RSB.

2.9.5 Pre-Enriched Libraries Qualification

The quality of each library was evaluated using Agilent Technology 2100 Bioanalyzer (DNA 1000 kit).

2.9.6 Pre-Enriched Libraries Pooling

By incorporating specific indexes for each library, the DNA libraries were combined into a single pool. To obtain a final volume of 30 µL (150 ng DNA), the volume required from each sample was calculated based on its concentration and subsequently combined in the PCR tube.

2.9.7 Probes Hybridization

Targeted regions of DNA were bound to capture probes. In a new tube, 50µL NHB2, 10µL Enrichment probe panel, and 10µL EHB2 were added to 30µL of the sample. The tube was then placed on the thermal cycler and the NF-HYB program was initiated as follows:

Preheat lid option 100 C, reaction volume 50 µL, 95 C for 5 minutes, 16 cycles of 1 minute starting with 94 C then decreasing 2 C per cycle. Hold for 24 hours at 62 C.

2.9.8 Hybridized Probes Capture

The sample underwent centrifugation at 280g for 30 seconds. In a separate tube, 250µL of Streptavidin Magnetic Beads (SMB3) were introduced to 100µL of the sample to capture the hybridized probes in the targeted regions. Subsequently, a washing step was performed using 200µL of Enhanced Enrichment Wash (EEW). For elution, a mixture of 342µL of Enrichment Elution Buffer 1 (EE1) and 18µL of 2N NaOH (HP3) was prepared. Then, 23µL of this elution mix were added to the sample and incubated at room temperature for 2 minutes. Afterward, the sample was centrifuged at 280g for 30 seconds and placed on a magnetic stand for 2 minutes. Finally, 21µL of the supernatant were combined with 4 µL of Elute Target Buffer 2 (ET2) in a new tube.

2.9.9 Enriched Library Amplification

To amplify the enriched library, 5µL of PCR primer Cocktail (PPC) and 20µL of Enhanced PCR mix (EPM) were included. Subsequently, the sample underwent centrifugation and was subsequently placed on thermal cycler, following the program outlined below:

Preheat lid option 100 C, Reaction volume 50µL, 98 C for 60 seconds, 10 cycles of: (98 C for 20 seconds, 60 C for 30 seconds, 72 C for 30 seconds), 72 C for 5 minutes, and finally Hold at 10 C.

2.9.10 Amplified Enriched Library Cleanup

For the purpose of library purification, AMPure XP Beads (45µL) were added to the sample tube. Subsequently, the sample was washed twice by adding 200µL of 80% Ethanol, followed by 32µL of RSB.

2.9.11 Purification and Quality Control

Measurement of the library concentration was carried out using the Qubit dsDNA HS assay (kit No.Q23850), while the mean fragment size was determined using the High sensitivity DNA kit (catalog No.5067).

2.10 Data Analysis

FastQ paired end reads were mapped to the reference human genome version GRCh38 using BWA-MEM software package that produced the mapped reads in bam format. The mapped reads were filtered for the following two criteria; first, we retained only paired reads for which both the forward and the reverse read have been mapped to the reference successfully using Samtools. Second, PCR duplicates were removed using RmDup tool. Filtered mapped reads were then used to call the variants using FreeBayes variant detector to identify SNPs (single-nucleotide polymorphisms) and indels (insertions and deletions). The list of variants produced in a VCF format have been filtered and prioritized with respect to their potential relevance for RPL. The SnpEff tool that annotate variants with

their calculated effects on known genomic features have been used and produced annotated VCF file containing annotations of variant effects. The VCF file contains chromosome number, position, reference and variant nucleotide, gene name, genomic context (whether the variant is in exon, intron, splice site, or others), synonymy (whether the variant is synonymous or not), amino acid change, the clinical significance of the variant (pathogenic or benign), phenotypes related to the variant according to HPO and OMIM, average frequency of the variant (frequency of occurrence in a population), quality by depth (QD), RMS mapping quality (MQ), predicted pathogenicity scores according to some tools like Polyphen2 and SIFT, and the Phenotype correlation score from exomiser. Finally, we filtered out variants that are unlikely to have a pathogenic effect like variants in UTRs, upstream/downstream the gene and deep intronic variants (but we leave variants that affect splice sites). Furthermore, variants with low QD (less than 3) or low scores of phenotype correlation were also sorted out.

We ended up with a small number of variants. We checked them in OMIM to prioritize likely causative variants based on the genotype-phenotype relationship. Since parents' samples were not whole-exome sequenced, unfortunately, we couldn't find a single candidate variant. Therefore, we selected the most suspected variants in each family (Highest correlation score) to do family segregation. Figure 11 illustrates a concise overview of the analysis steps.

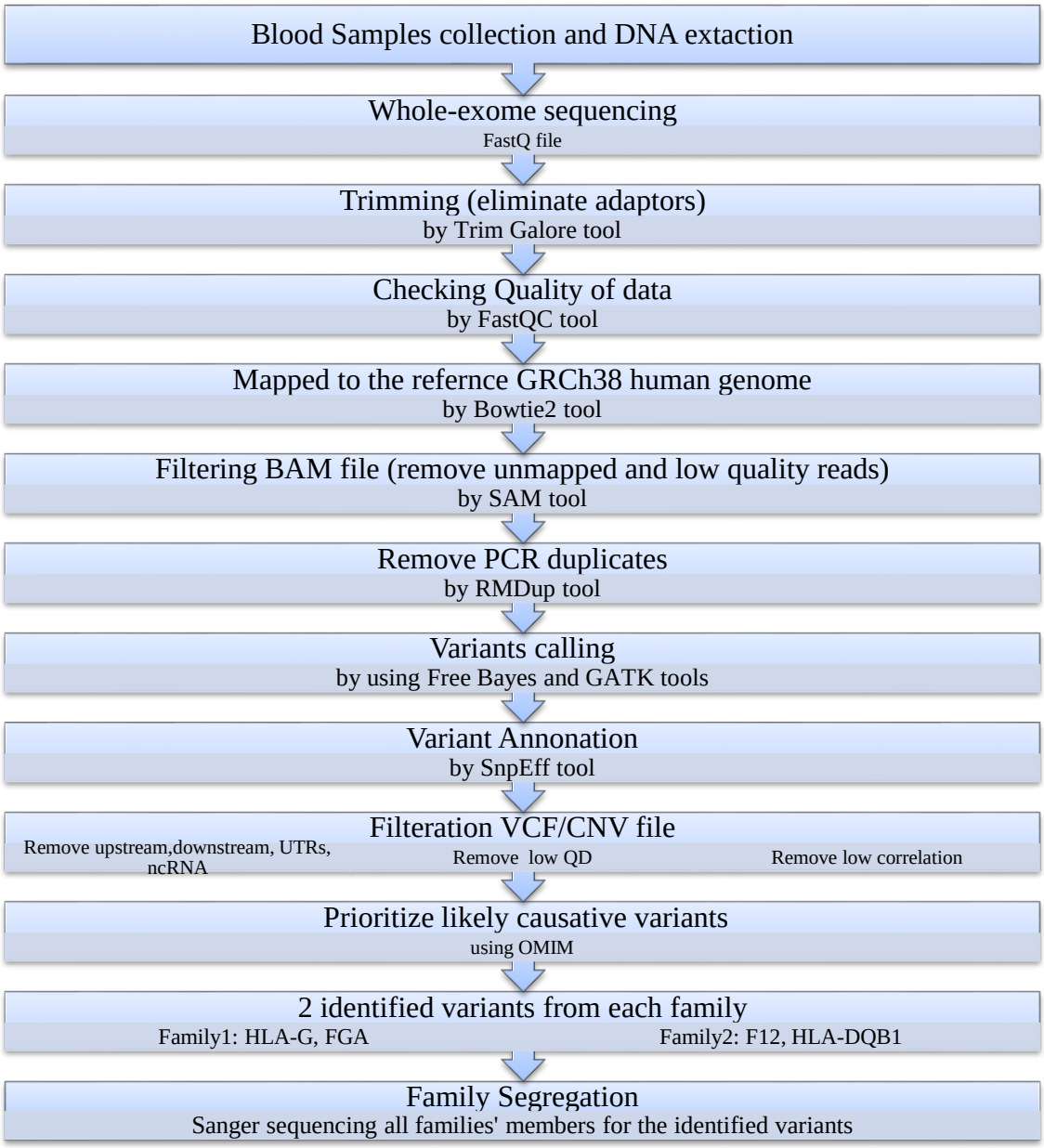


Figure 11: Flowchart illustrating the steps of WES analysis.

2.11 Family Segregation

In the first family, the HLA-G and FGA genes were identified as the most suspected variants, while in the second family, the F12 and HLA-DQB1 genes exhibited a higher level of suspicion. To target these specific variants, distinct primers were designed. The

subsequent steps involved PCR amplification and Sanger sequencing to thoroughly investigate the identified variants.

2.11.1 Primers Design

The primers for the identified variants in each family were designed by using Primer 3 plus (https://www.primer3plus.com/index.html/). Table 6 shows the primer sequences for each variant.

Table 6: The Primer Sequences For The Identified Variants In Each Family

Gene name	Primer type	Primer Sequence 5'----------3'	Product size (bp)	GC% content
HLA-G	Forward	GGTTCTCACACCCTCCAGTG	216	60
	Reverse	CCTCCAGGTAGGCTCTCCTT		60
FGA	Forward	AACCCTAAGAACAGAGCCCC	231	55
	Reverse	ATCAAGCTACCGGGATCCAG		55
F12	Forward	GAAGGACAGGCAGGGTACAT	244	55
	Reverse	CCTTTTCCTGACCAGACCCT		55
HLA-DQB1	Forward	GACGCTCACCTCTCCTCTGC	367	60
	Reverse	CGGTTCCACAGCTCCAGG		60

2.11.2 PCR Amplification

Genetic variant testing was conducted on each member of the first family for the HLA-G and FGA genes and on each member of the second family for the F12 and HLA-DQB1 genes. Table 7 provides a list of the required reagents and volumes for each PCR reaction.

Table 7: Reagents And Volumes Used For PCR Reaction

Reagent	Volume
PCR master mix (1X)	12.5 µl
Forward primer (10 picomole)	0.5 µl
Reverse primer (10 picomole)	0.5 µl
DNA sample (100 ng)	1.0 µl
Nuclease free H2O	10.5 µl
Total volume	25µl

Subsequently, PCR mixtures were set on the thermocycler according to the following conditions listed in Table 8. Finally, the PCR products were visualized under a UV transilluminator.

Table 8: PCR Program Used For Variants Amplification

PCR Conditions	**HLA-G gene**		**FGA gene**		**F12 gene**		**HLA-DQB1 gene**	
Initial denaturation	**94 C, 3min**		**94 C, 3min**		**94 C, 3min**		**94 C, 3min**	
2nd Denaturation	**94º C,20sec**		**94º C, 20sec**		**94º C,20sec**		**94º C,20sec**	
Annealing	**62.5º C,30sec**	**33X**	**58.5º C,30sec**	**33X**	**58.5º C,30sec**	**33X**	**61.5º C,30sec**	**36X**
Extension	**72 ºC,45sec**		**72º C,45sec**		**72 ºC,45sec**		**72 ºC,45sec**	
Final extension	**72º C, 5 min**		**72º C, 5min**		**72º C, 5min**		**72º C, 5min**	
Hold	**4°C, ∞**		**4 °C, ∞**		**4 °C, ∞**		**4 °C, ∞**	

2.11.3 Sanger Sequencing

Samples were sequenced using BigDye™ Terminator Cycle Sequencing Kit and Applied Biosystems Genetic Analyzer as manufacturer's instructions (Biosystems, 2010).To perform PCR clean-up, 5µL of PCR product was treated with 1µL of EPPIC-FAST reagent (Catalog #1021-100F A&A Biotechnology). This reagent is a combination of thermolabile nucleotide hydrolase and recombinant exonuclease I, which have been engineered to exhibit higher efficiency. Subsequently, the Products were placed on thermocycler at 37°C for 10 minutes followed by 1 minute at 80°C. Following this, the cycle sequencing was initiated by the addition of 18µL of Big Dye Terminator (BDRR) mix with 2 µL of cleaned PCR, as shown in Table 9. The resulting mixture was then placed on a thermocycler, following the conditions outlined in Table 10. Following a specific sequence, the sample underwent EDTA Ethanol precipitation. Initially, the sample was mixed with 60 µl of 100% cold ethanol and 5 µl of EDTA (12.5 mM). Subsequently, cold centrifugation was carried out for 30 minutes at 2200g. The supernatant was then removed, and 80 µl of 80% ethanol was introduced to the pellet. After a 15-minute centrifugation at 1600g, the supernatant was discarded, and the pellet was air dried. Finally, High dye was incorporated, and the sample was placed on a hot plate at 95°C for 5 minutes, followed by ice incubation for an additional 5 minutes.

Table 9: Reagents and volumes used to prepare BDRR mix

Reagent	**Volume**
Sequencing buffer (5x)	3.5 µl
H2O	11.5µl
Sequencing primer	2.0 µl
BDRR	1.0µl
Total volume	18 µl

Table 10: BDRR-PCR Protocol

Temperature °C	**Time**	**Cycle numbers**
96°C	20 seconds	1 cycle
96 °C	10 seconds	25 cycles
50 °C	5 seconds	
60 °C	4 minutes	
4°C	Hold	∞

2.11.4 In-Silico Analysis

A variety of bioinformatics tools were used to predict how amino acid substitutions or indels would affect the protein's structure and function. These tools are exclusively employed for confirmed and well-segregated variants, which include:

- COBALT Alignment tool which was used to detect the conservation of the variant locus https://www.ncbi.nlm.nih.gov/tools/cobalt
- MutPred-Indel https://mutpred2.mutdb.org/mutpredindel/index.html
- PROVEAN (Protein Variation Effect Analyzer) http://provean.jcvi.org/index.php
- FATHMM (Functional Analysis through Hidden Markov Models (v2.3)) http://fathmm.biocompute.org.uk/
- Mutation Taster https://www.mutationtaster.org/
- SIFT-Indel (Sorting Intolerant from Tolerant) https://sift.bii.a-star.edu.sg/
- GVGD (Grantham Variation, Grantham Difference) http://agvgd.hci.utah.edu/index.php
- LIST-S2 https://precomputed.list-s2.msl.ubc.ca/.

2.12 Statistical Analysis

Statistical analyses were performed using SPSS (V.20) and Microsoft Excel. For categorical and continuous data, the chi-square test and Student's t-test were used to compare the differences between the case and control groups. Data are presented as mean±SD, frequencies (%), odds ratio (OR), and 95% confidence interval when appropriate. Each SNP was tested for deviation from Hardy-Weinberg equilibrium using the chi-square test. The allele frequencies and genotype distributions of VDR SNPs, and the association between SNP genotypes and haplotypes with RPL under different genetic models were analyzed using the web tool SNPStats (https://www.snpstats.net/start.htm). P-values less than 0.05 were accepted as statistically significant.

2.13 Ethical Approval

All methods and procedures performed in this study including study participants were in accordance with the Arab American University's guidelines and regulations. Moreover, ethical approval was obtained from AAUP's graduate studies.

2.14 Consent Form

All participants signed an informed consent form before enrolling in this study.

Chapter Three

Results

3.1 Characteristics of Study Subjects

This study was performed on 51 women with a mean age ±SD of 30 ± 5.4 years who had experienced at least 3 RPLs. The mean ±SD number of abortions was 5±2.7 and ranged from 3 to 14. The control group consisted of 80 age-matched healthy women with a mean age ±SD of 29.7 ± 4.7 years, at least two full-term pregnancies (the mean ±SD number of successful pregnancies was 3±1.4), and without any history of abortion as described in the materials and methods section.

3.2 Detection of the VDR SNPs

Polymerase chain reaction (PCR) was used to amplify 265 bp and 820 bp of DNA fragments encompassing (rs2228570) and (rs1544410) polymorphisms, respectively. Figure 12 shows a representative gel of the amplified PCR product encompassing exon 2 variant (rs2228570) showing 265 bp DNA band, while Figure 13 shows a representative gel of the amplified PCR products harboring intron 8 variant (rs1544410) showing 820 bp DNA band. No other bands were observed, indicating reaction's specificity. The genotypes of the indicated SNPs were identified using restriction fragment length polymorphism (PCR-RFLP). Exon 2 amplified PCR product was digested with FokI endonuclease enzyme, and the following genotypes were obtained; detection of 265 bp DNA band indicates homozygous CC genotype while detection of two DNA bands (196 bp and 69 bp) indicates homozygous TT genotype; and detection of three DNA bands (265 bp, 196 bp, and 69 bp) indicates heterozygous CT genotype, as illustrated in Figure

14. Similarly, intron 8 amplified PCR product was digested with BsmI endonuclease enzyme and the following genotypes were observed; detection of 820 bp DNA band indicates homozygous GG genotype while detection of two DNA bands (170 bp and 650 bp) indicates homozygous AA genotype; and detection of three DNA bands (820 bp, 650 bp, and 170 bp) indicates heterozygous GA genotype, as shown in Figure 15.

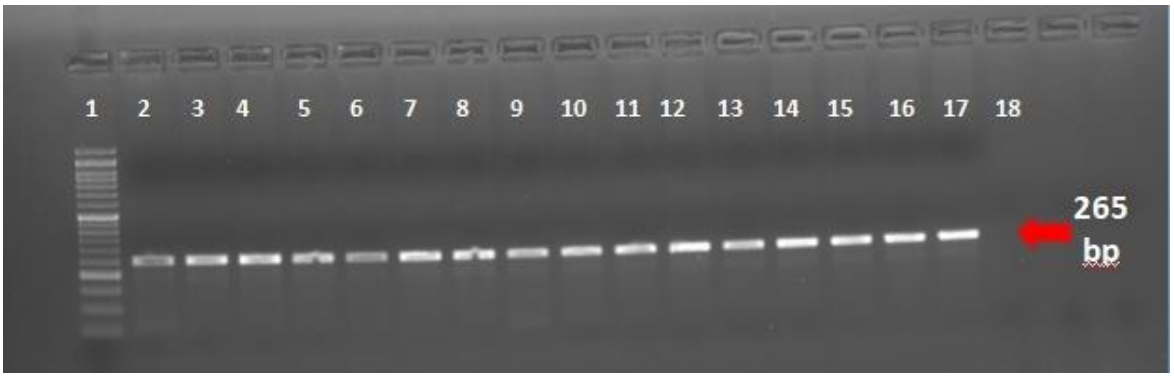


Figure 12: Representative gel electrophoresis illustrating the amplified DNA fragments encompassing FokI (rs2228570) polymorphism from exon 2 of the VDR gene with a band size of 265 bp (lanes 2-17). A 50-bp DNA ladder and NTC control were run alongside the samples (lanes 1 and 18, respectively).

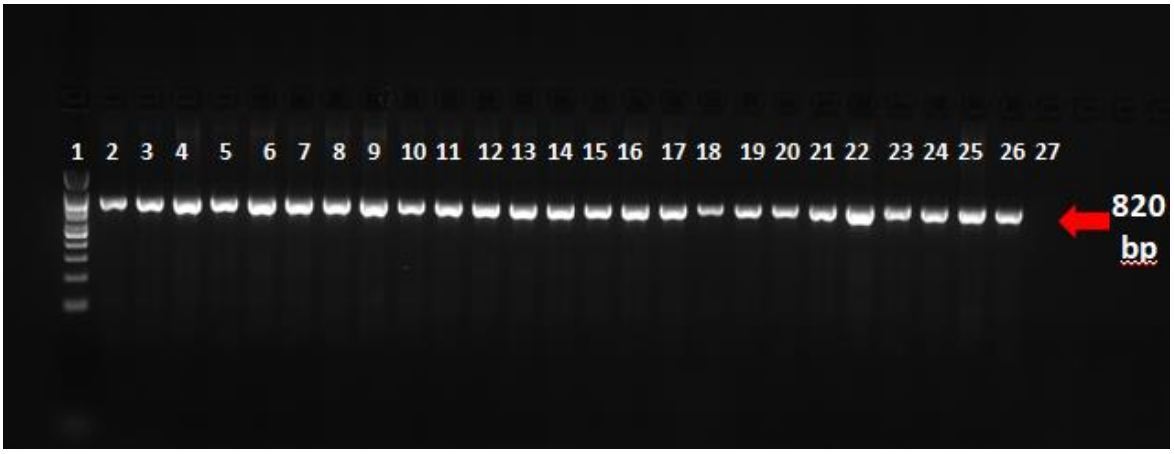


Figure 13: Representative gel electrophoresis illustrating the amplified DNA fragments encompassing BsmI (rs1544410) polymorphism from intron 8 of the VDR gene with a band size of 820 bp (lanes 2-26). A 100-bp DNA ladder and NTC control were run alongside the samples (lanes1 and 27, respectively).

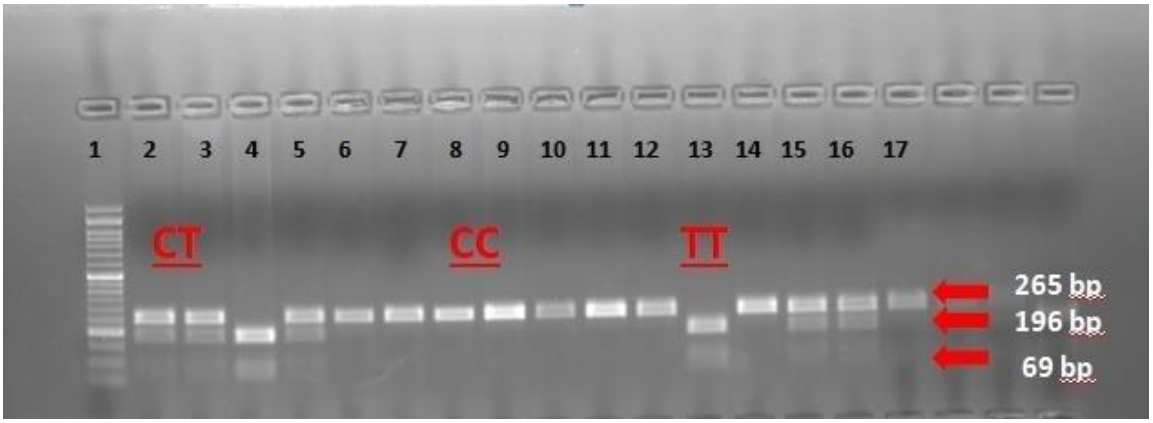


Figure 14: Representative agrose gel showing the observed genotypes following FokI enzyme digestion of the 265 bp amplified PCR products. CC genotype (lanes 6–12, 14, 17), CT genotype (lanes 2, 3, 5, 15, 16), and TT genotype (lanes 4,13). A 50-bp DNA ladder is shown in lane1.

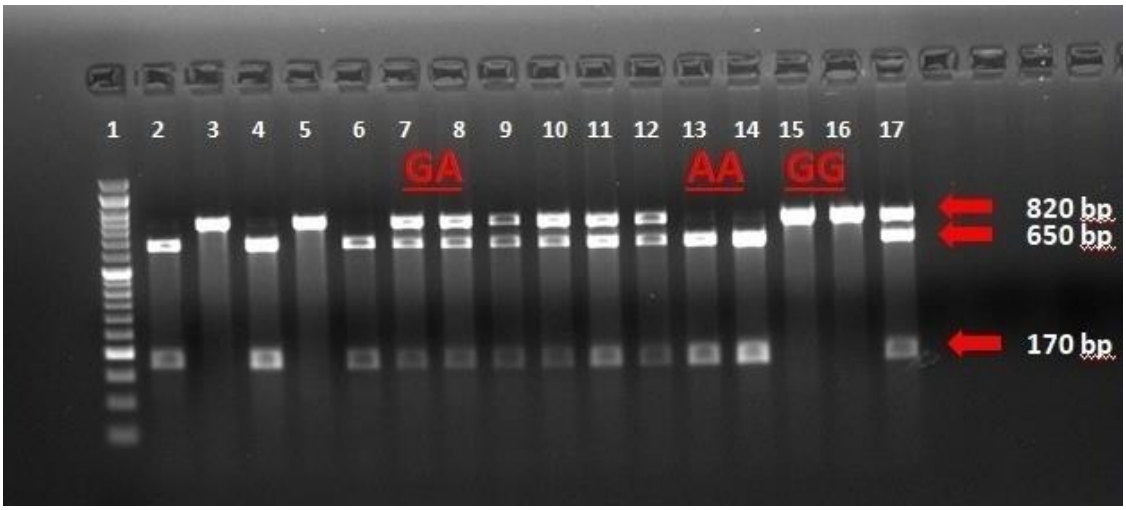


Figure 15: Representative gel electrophoresis showing the resulting genotypes following Bsm I enzyme digestion of the 820 bp amplified PCR products. GG genotype (lanes 3,5,15,16), GA genotype (lanes 7–12,17), and AA genotype (lanes 2,4,6,13,14). A 50-bp DNA ladder is shown in lane1.

3.3 Analysis of Hardy-Weinberg Equilibrium (HWE)

We have selected two SNPs in the VDR gene. HWE analysis of the selected SNPs (rs2228570) and (rs1544410) was used to calculate the number of heterozygous and homozygous variant carriers depending on their allele frequency in a given population (Wigginton et al., 2005). Table 11 shows the result of this analysis. The allele and genotype frequencies of the rs2228570 and rs1544410 polymorphisms were found to be consistent with HWE analysis among all RPL patients and healthy controls since the observed genotype frequencies were quite similar to the expected ones (P-value >0.05).

Table 11: The Analysis of HWE Parameters

VDR SNPS		RPL cases				Control group			
	Genotypes	Observed	Expected	X^2	P-value	Observed	Expected	X^2	P-value
rs2228570	CC	29	27	2.8	0.09	43	41.3	0.8	0.35
	CT	15	19.7			29	32.3		
	TT	6	4			8	6.3		
rs1544410	GG	7	8.8	1.1	0.29	27	24	2.3	0.13
	GA	28	24.4			33	39		
	AA	15	16.8			20	17		

* If X^2 test *P* value <0.05, not consistent with HWE.

3.4 Allele Frequencies of rs2228570 and rs1544410 SNPs Among RPL Patients and Control Subjects

The differences in allele frequencies of the two selected SNPs in the VDR gene are listed in Table 12. The frequencies of the C and T alleles of rs2228570 were as follows: C: 73%, T: 27%, and C: 72%, T: 28% among RPL patients and healthy controls, respectively. The allele frequencies of C and T alleles were similar between both groups with no significant

association between both allele and RPL (C vs. T: OR-1.05 (95% CI: 0.60-1.85) p-value = 0.79). The frequencies of the G and A alleles of rs1544410 were as follows: G: 42%, A: 58%, and G: 54%, A: 46% in RPL cases and healthy subjects, respectively. The G allele was higher in the control group, whereas the A allele was more frequent in the RPL group. The A allele of rs1544410 acts as a risk allele, with slightly statistically significant association with the risk of RPL (G vs. A: OR-1.64 (95% CI: 0.99–2.72), p-value = 0.05).

Table 12: The Allele Frequencies of the rs2228570 And rs1544410 SNP's of the VDR Gene Among The Study Subjects

VDR Gene	Alleles	RPL cases	Healthy controls	OR(95% CI)	P-value
rs2228570	C	73(73%)	115(72%)	1.05(0.60-1.85)	0.79
	T	27(27%)	45(28%)		
rs1544410	G	42(42%)	87(54%)	1.64(0.99-2.72)	0.05
	A	58(58%)	73(46%)		

*SNPStat software was used to calculate the associated chi-square P-value based on 2X2 contingency table.

*OR: Odds ratio, 95% CI: 95% confidence interval

3.5 Analysis of Genotype Frequencies of the VDR SNPs Among the Study Subjects According to Different Genetic Models

The estimated risk of subjects carrying one or two copies of risk alleles according to different genetic inheritance models (dominant and recessive) is listed in Table 13. The genotype distribution of rs2228570 was similar between RPL cases and healthy controls (CC vs. CT: OR-1.20 (95% CI: 0.54-2.68)) with p-value = 0.63 and (CC vs. TT: OR-0.78 (95% CI: 0.24-2.59)) with p-value = 0.69. In addition, rs2228570 genotypes and alleles did not show any statistically significant correlation with the risk of RPL in any genetic

model. In contrast, the frequency of AA and GA genotypes of rs144410 polymorphic site was higher in RPL cases compared to healthy controls (GG vs. AA: OR-3.48 (95% CI: 1.13-10.7) p-value = 0.02), and GG vs. GA: OR-0.96 (95% CI: 0.41-2.26) p-value = 0.01). This indicates that the risk of developing RPL was increased under using the dominant genetic model (GG vs. AA+GA: OR-3.57 (95% CI: 1.34-9.54) p-value = 0.006) suggesting the risk of developing RPL is 2.57 fold higher in women having at least one alternative A allele compared to women having GG genotype.

Table 13: Genotype Frequencies, Odds Ratios, and 95% Confidence Intervals for the Risk of RPL in Women According To Different Genetic Models

VDR SNPs	Model	Genotype	RPL cases	Healthy controls	OR(95% CI)	P-value
rs2228570		CC	27 (56.2%)	40(54%)	1.00	
		CT	15(31.2%)	27(36.5%)	1.20 (0.54-2.68)	0.63 0.69
		TT	6(12.5%)	7(9.5%)	0.78 (0.24-2.59)	
	Dominant	CC vs. CT+TT	27 (56.2%) 21(43.8%)	40(54%) 34(46%)	1.00 1.08(0.52-2.25)	0.83
	Recessive	CC+CT vs. TT	42 (87.5%) 6(12.5%)	67 (90.5%) 7(9.5%)	1.00 0.73(0.23-2.32)	0.59
rs1544410		GG	6(12.5%)	25(33.8%)	1.00	
		GA	27(56.2%)	31(41.9%)	0.96 (0.41-2.26)	*0.01
		AA	15(31.2%)	18(24.3%)	3.48 (1.13-10.7)	*0.02
	Dominant	GG vs. AA+GA	6(12.5%) 42(87.5%)	25(33.8%) 49 (66.2%)	1.00 3.57(1.34-9.54)	*0.006
	Recessive	GA+GG vs. AA	33(68.8%) 15(31.2%)	56(75.7%) 18(24.3%)	1.00 1.42(0.63-3.18)	0.37

The association between genotypes and the risk of developing RPL was measured using logistic regression analysis. The results were adjusted for covariates (VD level). (*) indicates a significant correlation.

3.6 The association Between Haplotypes and Study Subjects

Four haplotypes interaction between the two selected SNPs in the VDR gene were indicated among the RPL cases and healthy controls as shown in Table 14. The most frequent haplotype association in both groups was the C-G combination, with a 41% and 40% frequency, respectively. Interestingly, the T-G haplotype combination seems more associated with healthy controls than in RPL cases, providing the significant indication of correlation with RPL prevention (P-value = 0.023).

Table 14: Interaction Between the Indicated Haplotypes of VDR SNPs and Study Subjects

rs2228570	rs1544410	Frequency (RPL cases)	Frequency (healthy controls)	Frequency (overall)	OR(95%CI)	P-value
C	**G**	0.390	0.418	0.404	1.00	---
C	**A**	0.339	0.316	0.327	0.86(0.40-1.82)	0.69
T	**A**	0.240	0.1052	0.1726	0.39(0.15-1.01)	0.057
T	**G**	0.029	0.1384	0.0837	3.97(1.22-12.86)	*0.023

*Indicates significant association.

3.7 Analysis of 25-Hydroxyvitamin D Level Among Study Subjects

The 25-hydroxyvitamin D (25(OH)VD) level was evaluated in RPL cases and healthy controls. The vitamin D levels were subdivided into three groups: deficient <10ng/ml, insufficient 10–20ng/ml, and normal >20 ng/ml. As shown in Table 15, 43.7% of RPL cases have deficient serum 25(OH)D level, 35.5% had insufficient level, and 20.83% had normal vitamin D serum level. In comparison, 39.2% of the control group have deficient

serum 25(OH)D level, 39.2% had insufficient level, and 21.6% had normal serum VD level. No statistically significant association was detected between the two study subjects and serum VD (P-value = 0.81). Moreover, the distribution of the various genotypes of the both indicated SNPs according to vitamin D status is shown in Tables 16 and 17. No statistically significant association was observed between serum levels of 25(OH)D status and study subjects and genotypes in both (P-values are 0.13 and 0.3, respectively).

Table 15: The Distribution of Study Subjects According to Serum Vitamin D Level (Status)

Vitamin D status	RPL cases	Controls	P-value
Deficient (<10 ng/ml)	21(43.7)	29(39.2)	0.81
Insufficient (10–20ng/ml)	17(35.5)	29(39.2)	
Normal (>20 ng/ml)	10(20.8)	16(21.6)	
Total	48(100)	74(100)	

*frequencies are presented as n (%).

Table 16: Distribution of rs2228570 Genotypes with Serum 25(OH)D Level

Vitamin D status	**RPL cases**			**Controls**			**Interaction p-value**
	CC	CT	TT	CC	CT	TT	
Deficient (<10 ng/ml)	13(27)	6(12.5)	2(4.2)	16(21.6)	10(13.5)	3(4)	0.13
Insufficient (10–20 ng/ml)	7(14.6)	8(16.6)	2(4.2)	17(23)	10(13.5)	2(2.7)	
Normal (>20 ng/ml)	7(14.6)	1(2.1)	2(4.2)	7(9.5)	7(9.5)	2(2.7)	
Total	48(100)			74(100)			

*frequencies are presented as n (%).

Table 17: Distribution of rs1544410 Genotypes with Serum 25(OH)D Level

Vitamin D status	**RPL cases**			**Controls**			**Interaction p-value**
	GG	GA	AA	GG	GA	AA	
Deficient (<10 ng/ml)	3(6.3)	13(27.1)	5(10.4)	9(12.2)	15(20.3)	5(6.7)	0.3
Insufficient (10–20 ng/ml)	2(4.2)	7(14.5)	8(16.7)	13(17.6)	9(12.2)	7(9.4)	
Normal (>20 ng/ml)	1(2.1)	7(14.5)	2(4.2)	3(4.1)	7(9.4)	6(8.1)	
Total	48(100)			74(100)			

*frequencies are presented as n (%).

3.8 Whole Exome Sequencing Analysis

Whole exome sequencing was performed on two female subjects with a familial history of RPL, as shown in the family pedigrees below. The aim was to identify additional genetic factors responsible for the idiopathic RPL observed in these families. Following the bioinformatic analysis of the generated NGS data, including variant filtration, two genetic variants were identified in each family, which were subsequently subjected to segregation analysis following direct sequence analysis.

3.8.1 Family 1

A 30-year-old female experienced seven times recurrent pregnancy loss at week 12 of gestation (member II-1 in the pedigree indicated by a black arrow). She does not suffer from classical risk factors associated with this condition (see Chapter 2). Her mother experienced two consecutive abortions (member I-2). Additionally, she had three sisters, two of whom experienced multiple miscarriages (members II-4 and II-5) while the third sister is not married. Two distinct variants were identified in this family (FGA*787T>C and HLA-G 459delC), as described in Table 18.

Table 18: Information and Details Regarding Family 1 Detected Variants

Gene	*FGA*	*HLA-G*
Chromosome	Chr.4	Chr. 6
Type of mutation	Missense	deletion
Nucleotide change	*787T>C	459delC
Protein change	N/A	Leu154fs*60
Sanger sequencing	Validated	Validated
Segregation in family	Confirmed	Confirmed

3.8.1.1 FGA Gene

The FGA gene is located on chromosome 4. The identified variant *787T>C is located in exon 5 which did not alter the amino acid sequence. The variant segregated well within the family as shown in Figure 16.A. The data shows that subjects I-1 and II-6 possess wild-type AA genotypes, while subjects I-2, II-1, II-4, and II-5 exhibit heterozygous GA genotypes. Data from II-2 and II-3 could not be verified due to in ability to obtain the needed samples. Figure16.B provides a representative sequence with AA and GA genotypes, respectively. Since the indicated variants did not affect the amino acid protein

sequence (silent mutation), it may have impact on mRNA stability and the amount of the relevent protein level and function which needs further investigation.

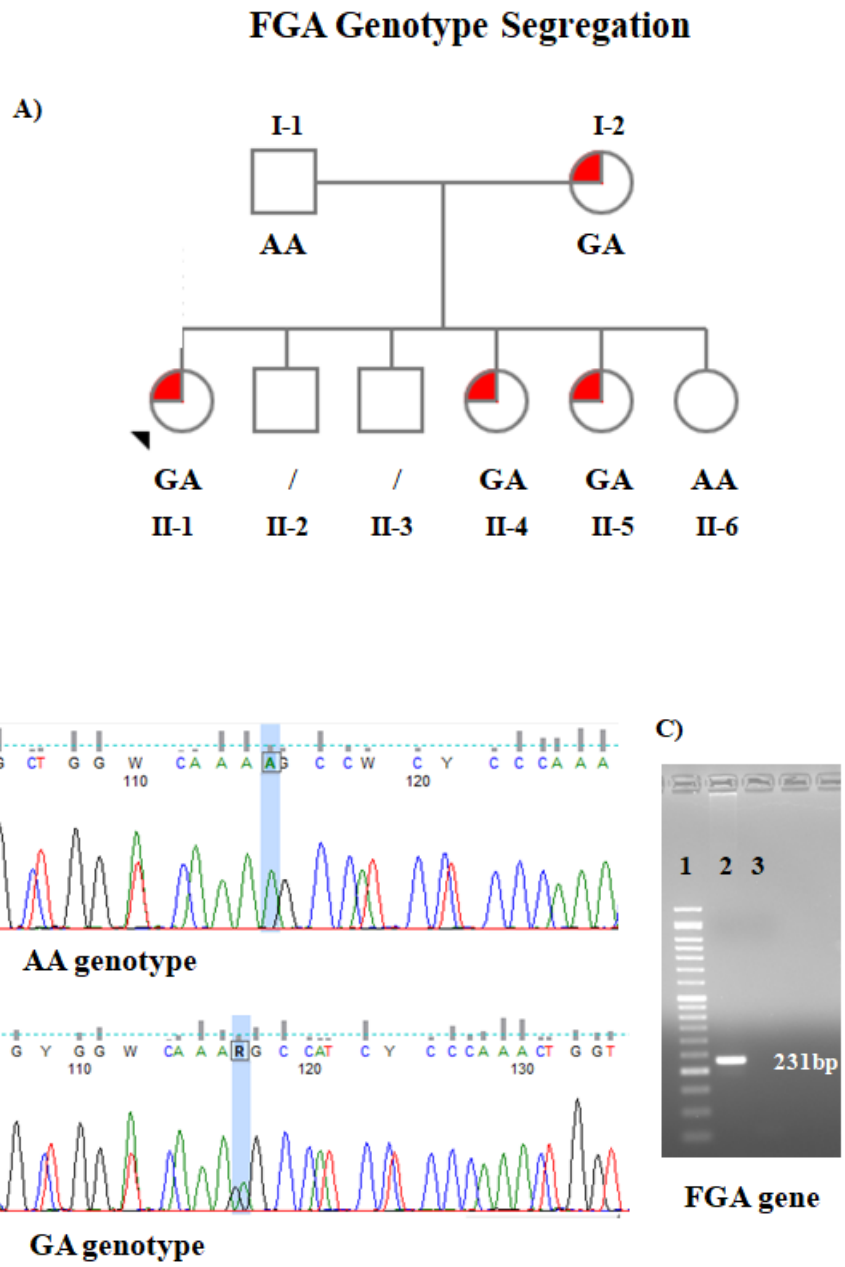
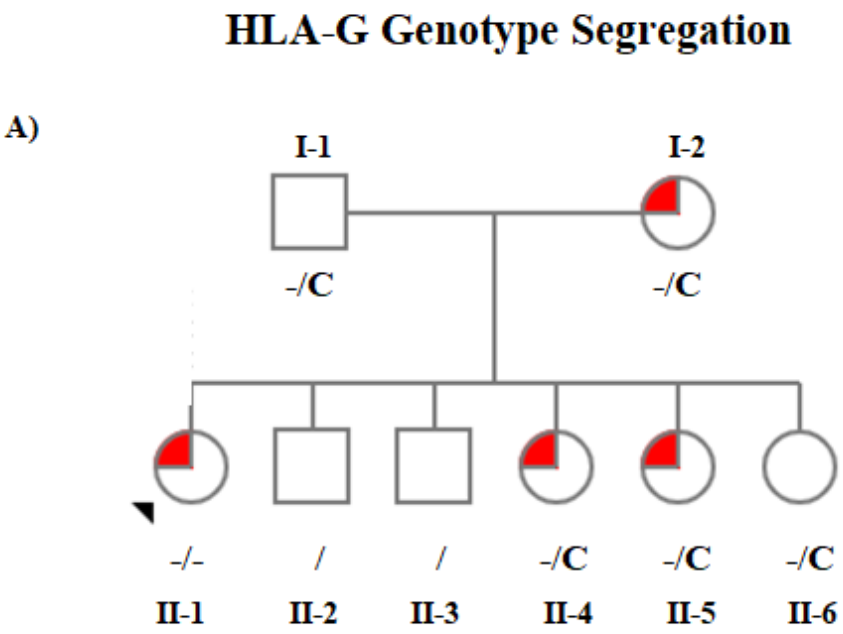


Figure 16: Segregation of FGA variant. A) The family pedigree with the genotype results of each member; the black arrow indicates the proband that was subjected to WES, and the red-filled circle indicates the affected females with RPL. B) Sanger sequencing results of the detected variant in this family. C) A representative gel electrophoresis of the FGA gene (lane 2), the NTC control (lane 3), and the 50-bp DNA ladder (lane 1).

3.8.1.2 HLA-G Gene

The HLA-G gene is located on chromosome 6. The identified variant 459delC is located in exon 3 which caused a frame-shift and resulting in the creation of a premature stop codon after 60 nucleotides (at the beginning of exon 4). The variant segregated well within the family as illustrated in Figure 17. A. The data shows that subjects I-1, I-2, II-4, II-5, and II-6 have heterozygous C deletions, subject II-1 has a homozygous C deletion, while samples from subjects II-2 and II-3 are not available. Figure 17.B provides a representative sequence with homozygous (-/-) and heterozygous (-/C) genotypes, respectively. The impact of this gene on RPL could be through its effect on fetal stability, immune tolerance, and blood vessel remodeling at the maternal-fetal interface.



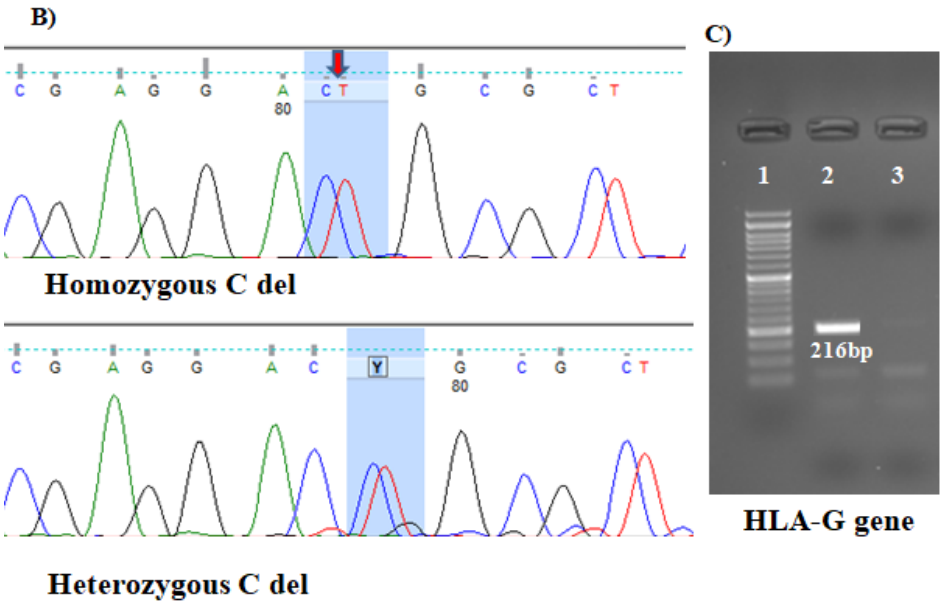


Figure 17: Family segregation of HLA-G variant. A) The family pedigree with the genotype results of each member: the black arrow indicates the proband that was subjected to WES; the red-filled circle indicates the affected females with RPL; (-/c) indicates a heterozygous C deletion; and (-/-) indicates a homozygous C deletion. B) Sanger sequencing results of the detected variant in this family. C) A representative gel electrophoresis of the HLA-G gene (lane 2), the NTC control (lane 3), and the 50-bp DNA ladder (lane 1).

3.8.2 Family 2

A 30-year-old female had experienced twelve repeated miscarriages at weeks 12–16 of gestation (member II-1 in the pedigree indicated by black arrow). She is free of the classical risk factors associated with this condition (see Chapter 2). Her mother had experienced three abortions (member I-2). Additionally, her cousin experienced six abortions (member II-5). In contrast, the other females have not experienced any miscarriages or other pregnancy complications. Two distinct variants were identified (F12163C>T and HLA-DQB1172_173delCTinsTA and 294_295delAGinsCA) in this family, as described in Table 19.

Table 19: Information and Details Regarding the Identified Variants In Family 2

Gene	*F12*	*HLA-DQB1*
Chromosome	Chr.5	Chr. 6
Type of mutation	Missense	Indels
Nucleotide change	163C>T	172_173delCTinsTA 294_295delAGinsCA
Protein change	Arg55Trp	Leu58Tyr GluVal98AspIle
Sanger sequencing	Validated	/
Segregation in family	Not confirmed	/

3.8.2.1 F12 Gene

The factor 12 gene is located on chromosome 5. The identified variant 163C>T is located in exon 3. The missense mutation 163C>T involves the substitution of Cytosine with Thymidine at position 163, resulting in the replacement of Arginine with Tryptophan at codon number 55. However, the segregation pattern does not align with the disease expression among family members. As depicted in Figure 18.A, members I-2, I-3, I-4, II-2, II-3, II-4, II-5, and II-6 have wild-type GG genotypes, while members I-1 and II-1 exhibit heterozygous GA genotypes. Figure18.B provides a representative sequence with GG and GA genotypes, respectively.

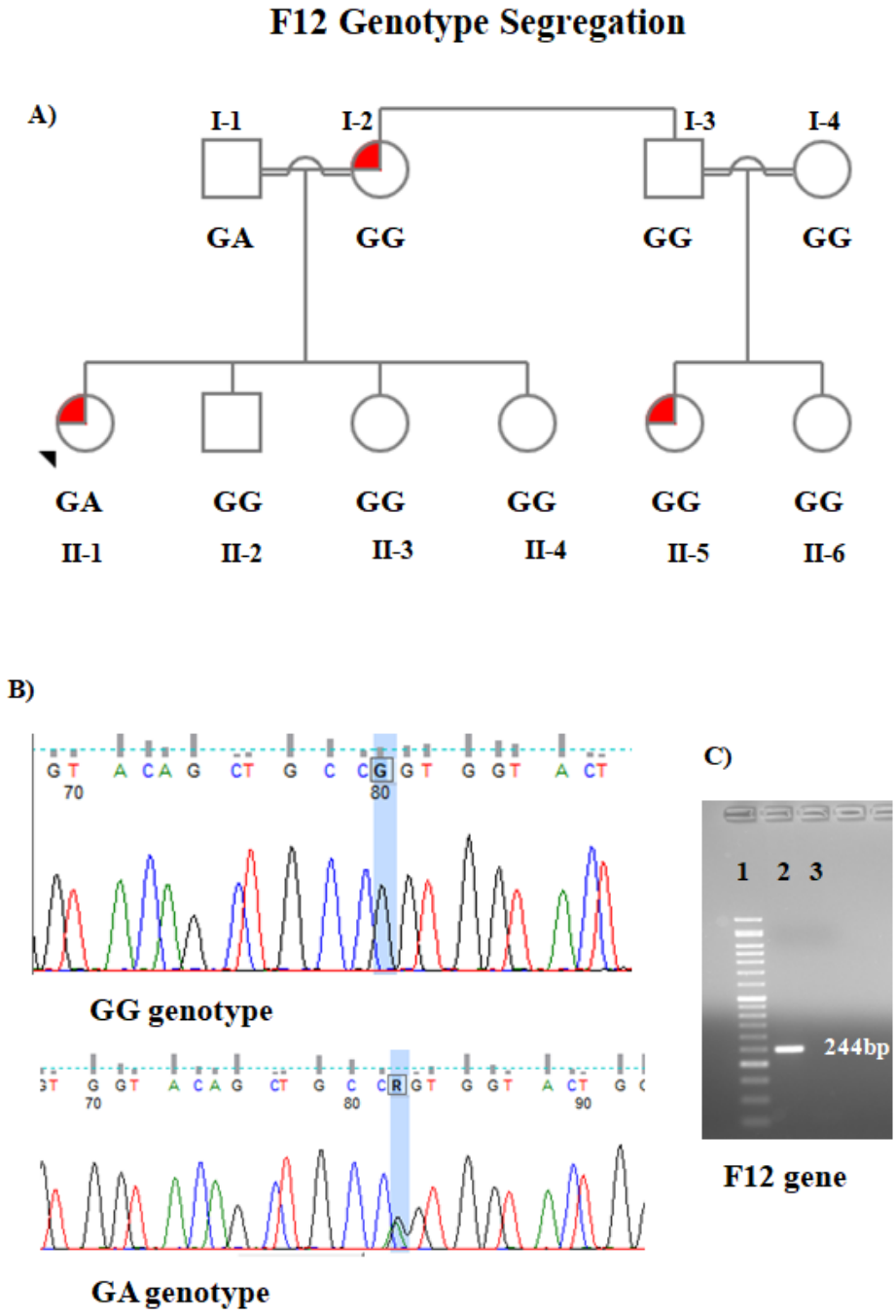


Figure 18: Family segregation of F12 variant**.** A) The family pedigree with the genotype results of each member; the black arrow indicates the proband that was subjected to WES, and the red-filled circle indicates the affected females with RPL. B) Sanger sequencing results of the detected variant in this family. C) A representative gel electrophoresis of the F12 gene (lane 2), the NTC control (lane 3), and the 50-bp DNA ladder (lane 1).

3.8.2.2 HLA-DQB1

The genotype segregation of the HLA-DQB1 gene among the indicated family members could not be verified due to the presence of deletion and insertion in close proximity which caused technical difficulties in analyzing the sequencing data of the gene region following designing several primers for this purpose. One apparent complication seems to be the similarity of this region with comparable region in the HLA-DQB2 gene. This variant needs further investigation to clarify its potential link with RPL in this family.

3.9 In Silico Analysis

Several bioinformatics tools were used to assess the potential impact of HLA-G (Leu154fs*60) on protein function and structure. According to the COBALT alignment tool, Leu154 is located in a highly conserved region, as depicted in Figure 19.The majority of the prediction tools identified this variant as disease-causing or pathogenic, suggesting that this variant has a drastic effect on protein function and structure, as illustrated in Table 20.

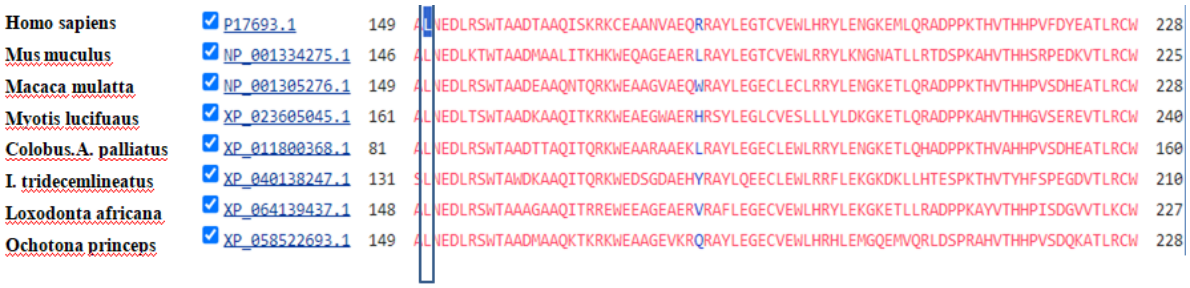


Figure 19: Conservation Analysis of HLA-G Leu154 Locus According to COBALT Alignment Tool.

Table 20: In Silico Analysis of the HLA-Gleu154fs*60 Variant

Prediction tools	Results
PROVEAN	Deleterious
MutPred-indel	Pathogenic
FATHMM	Tolerated
Mutation Taster	Disease causing
GVGD	Pathogenic
SIFT	Damaging
LIST-S2	Deleterious

Chapter Four

Discussion

Recurrent pregnancy loss is a complicated medical status that affects 1–4% of women during reproductive age. It occurs when a woman repeatedly loses her fetus before it reaches the 20th week of gestation (Abdel-razik et al., 2014; Zegers-Hochschild et al., 2009). Despite the complexity and multi-factorial origin of recurrent pregnancy loss, around half of the cases have no clear explanation (Ford & Schust, 2009; Toth et al., 2010). This highlights the need to investigate other genetic and no-genomic factors that may contribute to RPL. In recent years, there has been a growing interest in the potential impact of vitamin D and its receptor (VDR) on the development of RPL due to their diverse effects on human cells. Hence, the major aim of this study was to investigate whether the two maternal single nucleotide polymorphisms of the VDR gene, specifically rs2228570 and rs1544410, and the level of vitamin D status may be involved in the pathogenesis of recurrent pregnancy loss among affected Palestinian women.

In the present study, we first checked for Hardy-Weinberg equilibrium (HWE)which states that allele and genotype frequencies must stay constant across generations under random mating and in the absence of disturbing forces including mutations, genetic drift, natural selection, and inbreeding (Lachance, 2016). Based on this definition, an HWE test was evaluated that compares the observed genotype frequencies with the expected ones to test for the null hypothesis. Our data showed the allele and genotype frequencies of the two selected SNPs were consistent with HWE among all subjects since their P-values rejected the null hypothesis (p-values > 0.05), indicating at the same time that the observed allele and genotype frequencies are quite similar to the expected frequencies.

In our study, we genotyped the two indicated SNPs of the VDR gene, measured the serum level of 25 (OH)D among RPL and control subjects, and analyzed the correlations between the various haplotypes and genotypes of the two SNPs in addition to serum vitamin D levels and the risk of developing RPL under different genetic models. The results revealed statistically significant higher frequencies of the A allele (p-value = 0.05) as well as the genotypes of the A allele (AA and GA with p-values = 0.02 and 0.01, respectively) of the rs1544410 polymorphism among RPL subjects compared to the healthy control women. In addition, the risk for RPL with rs1544410 polymorphism was significantly increased under the dominant genetic model for GG vs. total A, with a p-value of 0.006. These results suggest a correlation between the expression level of VDR influenced by this variant and its consequent impact of the protein biological functions leading to disturbances in vitamin D signaling mechanism, and the pathogenesis of RPL. These results are in agreement with Wolski et al., who investigated the association of several VDR polymorphisms, including rs1544410, with the etiology of recurrent miscarriages (RM) in Polish-inflicted women. These authors showed the A allele of rs1544410 was significantly more frequent among women with RM compared to healthy controls (p-value = 0.035). Moreover, the risk for RPL was also increased under the dominant genetic model (p-value = 0.036), suggesting that these genetic variants have a great impact on VDR function (Wolski et al., 2021). Evidently, the rs1544410 polymorphism is located within the 8^{th} intron and consists of a G-A transition which seems to be involved in VDR gene expression by altering the stability of mRNA (Nigel A. Morrison, 1994). The A allele is associated with decreased mRNA stability and half-life, thus significantly affecting VDR gene expression leading to a decrease in VDR synthesis, which in turn affects subsequent vitamin D biological activity (Al-daghri et al.,

2017; Rosenfeld et al., 2017). Recent studies mentioned that lower level of VDR expression may be linked with RPL. Yan et al., who investigated the potential role of VDR in recurrent pregnancy loss and showed that expression of VDR gene in villous and decidual cells was significantly reduced among cases with RPL compared to the control group (46% and 52% reduction of VDR expression in villi and decidua cells, respectively, p-value <0.0001), and serum VD level was also lower in the RPL subjects compared to the control group during the first trimester of pregnancy (Yan et al., 2016). The same findings were reported by multiple researches, indicating the potential role of vitamin D and VDR in the etiology of RM (N. Li et al., 2017; L. Wang et al., 2016). These findings support our results indicating higher frequency of AA genotype and the A allele in women with RPL compared to the healthy control, thus affecting mRNA stability leading to decreased VDR expression.

Regarding the rs2228570 polymorphism (with T>C transition), it is located in the 2nd exon of the VDR gene, which affects the amino acid sequence and results in a 3 amino acid shorter VDR protein associated with lower transcriptional activity (Hidekazu Arai et al., 1997). Our results did not show any statistically significant differences between the two variants under any genetic models with the occurrence of RPL suggesting that the rs2228570 polymorphism is not involved in the pathogenesis of RPL in our population. The same finding was reported by Wolski et al., who showed that this polymorphism is not associated with RPL in the Polish population (Wolski et al., 2021). In contrast, a study conducted on Slovenian and Croatian women revealed that the CC genotype and the C allele in rs2228570 were more frequent in the case group compared to the control group (CC genotype, p-value = 0.036 and C allele, p-value = 0.015), and the risk of RPL was increased with the recessive CC vs.CT +TT (P-value = 0.015) and codominant genetic

models (CC vs. TT: P-value = 0.029; CC vs.CT: P-value = 0.036), suggesting that genetic variations in this site to be associated with RPL in this particular population (Barišić et al., 2021). Furthermore, another study reported the potential role of rs2228570 polymorphism in the etiology of RPL in the Iranian population (Salari et al., 2021). This discrepancy can be justified by several factors including different control groups used in various studies, differences in RPL definitions and criteria and focusing on RPL women rather than RPL couples or stillbirth, the impact of environmental factors and lifestyle on the course and outcomes of the pregnancy, ethnic differences or population heterogeneity in the risk variants, the allele and genotype frequencies may differ among different populations, and the small sample size which may result in inadequate statistical power (Iinuma et al., 2002; Topalidou et al., 2009).

Our data holds significant value due to the haplotype analysis conducted, which highlights the preventive effects of T-G haplotype (p-value 0.023) against RPL. In the present results, we identified the mutated A allele of rs1544410 as a risk allele in RPL, while the G allele of the same SNP could exert an antagonistic effect against RPL. Our findings were consistent with those of Wolski et al., who shed light on the protective effect of several haplotypes in the VDR, including TGT with rs7975232, rs1544410, rs2228570, TTG with rs731236, rs7975232, rs1544410, TG with rs7975232, rs1544410, and TT with rs731236, rs7975232, and TTGT haplotype (p-value 0.0024) against RM (Wolski et al., 2021). Our data and that of other groups emphasize the importance of considering haplotype analysis in genetic studies, which allows for more comprehensive examination of how genetic variations interact with one another and exert impact on disease susceptibility. This information is crucial for developing personalized treatment strategies that take into account individual's unique genetic makeup.

In addition, we also investigated the potential contribution of serum level of 25 (OH)D on RPL among the study subjects. No statistically significant association was observed among RPL subjects compared to the control group since the majority of subjects in the study suffer from VD deficiency. This finding is consistent with the widespread occurrence of vitamin D deficiency in Palestine and other Middle Eastern countries (Abdeen et al., 2014; Bassil et al., 2013). It is important to note that the level of vitamin D in women is influenced by various factors including their dietary choices, feeding patterns, exposure to sunlight, age, genetic predispositions, metabolic processes, and other environmental influences (Lips, 2007). In Palestine, similar to other Middle Eastern nations, there is an abundance of sunshine all year round, however women face limitations in their exposure to sunlight due to cultural and social influences may have major impact on this factor (Abdeen et al., 2014; Gharaibeh & Stoecker, 2009; Hekimsoy et al., 2010). Furthermore, lack of knowledge and awareness about the importance of VD with absence of screening programs, and insufficient guidance on the regular consumption of dairy products and VD-rich foods, plays a significant role in the high prevalence of vitamin D deficiency among women in our region (Kharroubi et al., 2017).

Our data revealed no statistically significant association between the VD levels and the VDR SNPs genotypes among study subjects (interaction P-values = 0.13 and 0.3 for rs2228570 and rs1544410 genotypes, respectively). This is the first study that correlates serum level of VD with participant's genotypes and the development of RPL. However, further investigations in this area are necessary and could lead to new treatment options and improved outcomes for women experiencing RPL. By taking steps to optimize vitamin D levels, healthcare providers can potentially help improve the chances of a successful pregnancy for women struggling with recurrent miscarriages.

Interestingly, within our case group, two participants had a familial background of recurrent pregnancy loss (RPL). DNA samples from these subjects were analysed by WES to uncover additional genetic factors that may be involved in the occurrence of RPL in these families. In one family, two variants could be identified, with one variant seems to have an influence on the relevant protein structure and function. The HLA-G p.Leu154fs*60 variant (which follows autosomal dominant inheritance) was confirmed by Sanger sequencing and segregated within the family, suggesting its possible role in the etiology of RPL. This variant was not reported in ClinVar and was classified as uncertain significance according to the ACMG classification (American College of Medical Genetics). No previous studies reported the association between this variant and RPL. The identified variant is located within a highly conserved region, which disrupts the reading frame from position 154 until 174, and results in a premature stop codon, indicating a detrimental effect on protein function and structure. This is also confirmed by InSilico analysis since the majority of bioinformatics tools, including PROVEAN, MutPred-indel, Mutation Taster, GVGD, SIFT, and LIST-S2, predict the variant as pathogenic or disease-causing. The HLA-G gene belongs to the non-classical HLA class 1 gene family located within the major histocompatibility complex (MHC) on the short arm of chromosome 6 (6p21.3). HLA-G molecule consists of a heavy chain that contains alpha domains (extracellular domains), which are bound non-covalently to the light chain β2-microglobulin. The HLA-G gene consists of seven introns and eight exons, and exon 2 is responsible for encoding the last part of the 5'UTR, which also plays crucial role in encoding the HLA-G leader peptide. This particular exon contains the primary translation start site, exons 3, 4, and 5 encode the alpha domains (alpha 1, alpha 2, and alpha 3) of HLA-G, exon 6 encodes the transmembrane domain, and exon 7 encodes the cytoplasmic

tail. The presence of a premature stop codon in exon 7 results in a cytoplasmic tail that is shorter in length compared to other class I molecules. The region following the stop codon in exon 7, which extends up to exon 8, forms the HLA-G 3' UTR. Additionally, the binding of peptides occurs within the α1 and α2 domains, which together form the peptide binding cleft, while the α3 domain functions as a site for co-receptor binding. Furthermore, the HLA-G gene can generate a minimum of seven protein isoforms through the process of alternative splicing of the primary transcript. Among these isoforms, four are membrane-bound and exhibit both a transmembrane domain and a short cytoplasmic tail. HLA-G1 represents the full-length membrane-bound isoform, HLA-G2 isoform is characterized by the absence of the alpha-2 domain, while HLA-G3 lacks both the alpha-2 and alpha-3 domains. Lastly, the HLA-G4 isoform is distinguished by the absence of the alpha-3 domain. On the other hand, three isoforms exhibit solubility as a result of lacking the transmembrane domain. The soluble isoforms HLA-G5 and HLA-G6 share the same extracellular domains as HLA-G1 and HLA-G2, respectively. However, both HLA-G5 and HLA-G6 transcript variants maintain intron 5, which causes a stop codon to occur before the translation of the transmembrane domain. Additionally, a 21-amino acid tail is present in these isoforms, contributing to their solubility. The HLA-G7 transcript variant retains intron 3, leading to a premature stop codon. Consequently, the HLA-G7 isoform only displays the alpha-1 domain, connected to two amino acids that are encoded by intron 2 (Castelli et al., 2014; Sciences et al., 2002; Vauvert & Sine, 2003).

It has been shown that HLA-G gene is highly expressed in the extra villous trophoblasts (EVTs) at the maternal-fetal interface (Bernards et al., 1989), with soluble HLA-G (sHLA-G) being found in the maternal blood (Hunt et al., 2000), cord blood (Fausta et

al., 2017), the culture medium of in vitro-fertilized (IVF) embryos (Pfeiffer et al., 2000), and amniotic fluid (Hackmon et al., 2004), suggesting the important role of HLA-G in modulating the immune tolerance, remodeling of spiral arteries, as well as fetal growth. During pregnancy, the nourishment and oxygenation required for the development of the fetus within the uterus relies on the maternal blood supply. To facilitate an efficient and consistent flow of blood to the placenta, the remodeling of spiral arteries plays a vital role. This remodeling process commences with the restructuring of the vascular smooth muscle associated with the decidua, resulting in its enlargement and disorganized arrangement followed by vascular endothelium undergoes liquefaction, and the elastic membrane disintegrates. These significant changes are predominantly triggered by angiogenic growth factors, which are secreted by decidual Natural Killer cells (NK) and macrophages (Brosens et al., 2019; X. F. Li et al., 2001). NK cells make up less than 20% of the lymphocytes found in the peripheral blood. However, their presence is significantly higher in the first-trimester decidua, where they account for approximately 70% of the lymphocytes present (Zhang et al., 2021). NK cells expressed two major inhibitory receptors that can interact with HLA-G, which include killer cell immunoglobulin-like receptor-2 Ig domains and long cytoplasmic tail 4 (KIR2DL4) and immunoglobulin-like transcript-2 (ILT2) receptors. It has been shown that the KIR2DL4 receptor, in response to soluble HLA-G binding, interacts with the DNA-dependent protein kinase (DNA-PKcs) and induces phosphorylation of Akt at Ser473, which in turn activates the NF-κB pathway. This will induce the secretion of various pro-angiogenic and pro-inflammatory cytokines and chemokines, such as IL-6, IL-1β, IL-8, IL-23,tumor necrosis factor–α (TNF-α),macrophage inflammatory proteins (MIP-1-α, and MIP-3-α) (Rajagopalan et al., 2010). Moreover, the activation of DNA-PKcs signaling triggers the expression of cyclin-

dependent kinase inhibitor p21 and the phosphorylation of heterochromatin protein 1-γ (HP1-γ) at Ser 83. Thus are hallmarks of cell senescence (the cells become enlarged and exhibit a higher β-galactosidase (SA-β-gal) activity). Further, the NK cells stimulated with the KIR2DL4 agonist antibody could increase the vascular permeability and tube formation of human umbilical vein endothelial cells. These data suggest that sHLA-G can induce the production of a senescence-associated secretory phenotype (SASP) in response to KIR2DL4 binding at the surface of NK cells, enhance vascular permeability and angiogenesis, and result in spiral artery remodeling (Rajagopalan & Long, 2012). Nevertheless, HLA-G can facilitate fetal development through activation of the Phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway. A study has reported that when dNK cells are co-cultured with EVT cells, HLA-G binds to the ILT2 receptor and enhances the expression of PDK2, which in turn induces the phosphorylation of AKT at Serine 473 and increases the expression of the transcription factor PBX1. Additionally, when the culture system was treated with small interfering RNA (siRNA) that targeted both PDK2 and AKT1 or with HLA-G and ILT2 blocking antibodies, an impairment in AKT1 activation as well as decreased PBX1 expression was observed. In the same study, they also revealed that PBX1 (which is a transcription factor that plays an important role in embryonic development) could enhance the release of growth-prompting factors (GPFs) such as pleiotrophin (PTN) and osteoglycin (OGN) by binding directly to their promoter and driving their expression. In the decidual NK cells of RSA patients with mutant PBX1 (G21S), a decrease in PBX1 expression was noticed along with PTN and OGN expression. Further, in PBX1 knocked-out mice (Pbx1NK-KO), they found that the expression levels of PTN and OGN were down-regulated, and the mice exhibited a fetal resorption phenotype (a decrease in birth rate), and they also exhibited severe fetal growth

restrictions, including low weight, short length, and weak bone formation. These data suggest that the HLA-G-ILT2-AKT1 axis drives PBX1 expression, which in turn facilitates fetal growth by up-regulating the expression of GPFs (Zhou et al., 2020). In light of all this evidence, the HLA-G p.Leu154fs*60 is predicted to be a loss of function mutation that hinders the binding of HLA-G to NK cell receptors, affecting the release of pro-angiogenic and pro-inflammatory cytokines and chemokines as well as the release of growth-promoting factors at the maternal-fetal interface and therefore resulting in abortion.

The association between decreased HLA-G level in RPL is evident. A study was conducted on 55 Tunisian women who had experienced multiple abortions, regarding the association between soluble HLA-G isoforms (particularly G1, and G5) and multiple abortions. The study revealed that pregnant women displayed elevated levels of sHLA-G in comparison to non-pregnant women or women who had experienced abortion. Within the group of women who experienced spontaneous abortion, those with recurrent spontaneous abortions (RSA) exhibited lower levels of sHLA-G compared to those who experienced a single abortion, indicating its potential association with the occurrence of repeated abortion events (Zidi et al., 2016). Another study was conducted on examining the relationship between serum level of HLA-G5 isoform and RPL. The findings revealed the patient group had a lower serum level of the HLA-G5 isoform compared to the group of healthy pregnant women, suggesting that the HLA-G5 isoform may plays significant role in maintaining pregnancy and that a decrease in its level may be involved in the occurrence of RPL (Madduru, 2021). Recent meta-analysis that examined the occurrence of HLA-G 14 bp polymorphism both with homozygous (insertion/insertion) and compound heterozygous (insertion/deletion) genotypes among

European women diagnosed with RPL revealed that women affected by RPL exhibited a greater frequency of HLA-G with 14-bp insertion/insertion genotype compared to the control group. However, there was no statistically significant variance in the prevalence of the HLA-G ins/del 14 bp genotype between the two groups. These findings suggest that the homozygous 14-bp insertion/insertion genotype of the HLA-G gene may be involved in the etiology of RPL (Monti et al., 2019). Furthermore, another study was conducted on Brazilian women with RPL regarding the association between major SNPs found in the 3'UTR region of the HLA-G gene, including 14-bp insertion/deletion, +3003T/C, +3010C/G, +3027C/A, +3035C/T, +3142G/C, +3187A/G, and +3196C/G, and the risk of RPL showed strong association between +3010CC, +3142GG, and +3187AG HLA-G 3'UTR genotypes and the risk of RPL. This study shed light on the protective effect of the UTR-1 DelTGCCCGC haplotype against RPL, suggesting that HLA-G 3'UTR plays a significant role in RPL (Tomoya et al., 2016). Moreover, another study highlighted the significance of seven polymorphisms of the HLA-G locus that define twelve HLA-G alleles demonstrated a significant connection between the occurrence of HLA-G*0104 or HLA-G*0105N alleles (that are characterized by the presence of polymorphisms in the α-2 domain) in either partner and increased susceptibility to miscarriage, indicating the α-2 domain of HLA-G1 isoform may harbor allelic variation associated with recurrent miscarriage (Aldrich et al., 2001).

Another variant identified in the first family is the FGA*787T>C variant. This variant follows autosomal dominant inheritance and was confirmed by Sanger sequencing and segregated well within the family, suggesting its possible role in the pathogenesis of RPL. This variant was not reported in ClinVar and was classified as uncertain significance according to the ACMG classification. No previous studies reported the association

between this variant and RPL. The fibrinogen alpha chain (FGA) gene consists of six exons and encodes the alpha subunit of the coagulation factor fibrinogen, which plays an essential role during pregnancy by supporting trophoblast proliferation and spreading, the development of the placenta, as well as maintaining the development of fetal-maternal circulation (Iwaki et al., 2002; Snir et al., 2013). However, the presence of mutations in the FGA gene has been associated with coagulation pathologies, including afibrinogenemia and dysfibrinogenemia, which can lead to miscarriage. For example, homozygous deletion in exon 5 of the FGA gene can result in afibrinogenemia, which is a rare bleeding disease; therefore, women with this disease are at high risk of developing bleeding complications and miscarriages due to the lack of fibrinogen protein (Robert-ebadi et al., 2009). In the present study, the FGA*787T>C variant did not alter the amino acid sequence and is located outside the exon – intron border indicating no effect on the protein activity or splicing. However, the indicated variant may affect mRNA stability which needs further investigation and testing. Evidently, our data could not confirm the association between the identified variants (F12 and HLA-DQB1) with RPL occurrence in the second family. The F12 Arg55Trp variant failed to segregate with the disease status among the affected family members, while in case of HLA-DQB1 (Leu58Tyr) and HLA-DQB1 (GluVal98AspIle) variants, the nature and location of the identified variants (deletion and insertion) presented technical complexity for that purpose which required further analysis.

Conclusion

In conclusion; the present case control study focused on investigating potential correlation between specific selected variants in the VDR gene (rs2228570 and rs1544410) with direct impact on the receptor biological activity and expression level and RPL among Palestinian subjects. The data revealed no significant correlation between the variant that affects the protein activity (exon 2 variant) while positive correlation was evident with the variant in intron 8 that affects VDR gene expression and RPL. Serum levels of VD did not seem to alter these results, however this could be masked by the fact that all participants in the study suffer from VD deficiency which requires further investigation. Two subjects in the study revealed familial cases of RPL. WES analysis revealed two variants could be identified in each family including the FGA*787T>C variant affects fibrinogen mRNA stability and HLA-G Leu154fs*60 variant which plays an important role in the immune system. Both variants followed autosomal dominant inheritance and segregated well within one family. However, the F12 Arg55Trp variant failed to segregate within the second family and the HLA-DQB1 (Leu58Tyr) and HLA-DQB1 (GluVal98AspIle) variants require further investigation for probable confirmation.

Study Limitations

Relatively small sample size compared to previous study due to limited collaboration and assistance by health care providers in governmental and privet clinics. In addition, both groups in the study (case and control) suffer from Vitamin D deficiency similar to the majority of population which hinders the evaluation of the serum hormonal effect on RPL.

Recommendations

1) Since the results indicate significant links between the level of VDR expression and RPL, an expanded study on the molecular mechanism of VDR-dependent genes and successful pregnancy is warranted.
2) A long-term study on the management of vitamin D levels through supplementation and its implications for successful pregnancy will provide clear evidence of the potential contribution of this vitamin.
3) The whole exom study revealed the potential positive correlation of a major gene (HLA-G) linked to immune system activity and pregnancy. This suggests that the immune system plays a major role in the process which is currently under investigation in our group.

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Appendices

Appendix 1: Consent Form

دراسة حول فيتامين د والإجهاض المتكرر: الاستدلال من خلال تعدد الأشكال الجينية لمستقبلات فيتامين د وحالة فيتامين د لدى النساء الفلسطينيات.

هذه الدراسة يُجريها فريق البحث تحت إشراف أ.د هشام درويش من الجامعة العربية الأمريكية.

ما الهدف من هذه الدراسة؟

الهدف من هذا المشروع هو تحديد العلاقة بين تعدد الأشكال الجينية لمستقبلات فيتامين د (VDR SNPs) و حالة فيتامين د مع الإجهاض المتكرر لدى السيدات الفلسطينيات للمساعدة في التشخيص المبكر و توفير التدخل الطبي المناسب و مساعدة السيدات على الإنجاب و تجاوز فقدان الأجنة.

كم عدد الأشخاص المشاركين في هذه الدراسة؟

من المتوقع مشاركة 100 سيدة تعاني من اجهاض متكرر تم تشخيصهن من قبل الأطباء الإختصاصيين في الأمراض النسائية و 100 سيدة تمثل العينة الضابطة في هذه الدراسة.

ما فكرة الدراسة؟

دراسة العلاقة بين فيتامين د والإجهاض المتكرر و الاستدلال من خلال تعدد الأشكال الجينية لمستقبلات فيتامين د وحالة فيتامين د لدى النساء الفلسطينيات.

ما المطلوب مني في هذه الدراسة؟

نحن نطلب إذنك أيضا بأخذ عينة دم (3-5ml) من أحد أوردة ذراعك. لذا قد نضطر لوخزك بإبرة صغيرة لأخذ هذه العينة، كما و نطلب إذنك لمراجعة ملفك الطبي الأن و ربما في المستقبل، للحصول على معلومات قد تساعدنا في البحث. و سيتم ذلك مع المحافظة على سرية و خصوصية هذه المعلومات.

حول استعمال عينة الدم لأغراض بحثية

البحث الذي يستند على عينة الدم مهم لفحص أمراض البشر. بعض الباحثين يستخدمون هذه العينات لمحاولة فهم سبب الأمراض. بعض الباحثين قد يقومون بتطوير فحوصات جديدة لكشف و مراقبة الأمراض، بينما قد يقوم آخرون بتطوير طرق جديدة للوقاية و علاج بعض الأمراض. في المستقبل، بعض الباحثين قد يقومون بتطوير منتجات جديدة و أدوية جديدة. معظم عينات الدم الذي سيتم جمعه، سيحفظ في مختبر الوراثة الجزيئية في حرم الجامعة العربية الأمريكية في ضاحية الريحان برام الله.

كم المدة التي سأبقى فيها في الدراسة؟

إذا اخترت المشاركة، تتم عملية سحب الدم في نفس اليوم. علما بأنه يمكنك اختيار التوقف عن المشاركة في أي وقت مستقبلا بعد إعلام الباحثين شخصيا بذلك.

ما هي المخاطر المترتبة على المشاركة بهذه الدراسة؟

المخاطر المترتبة على سحب عينة دم من ذراعك تتضمن انزعاج لحظي مع / دون تكوّن كدمة. من المستبعد جدا، أن تصابي بإلتهاب، أو نزيف، أو تجلط، أو إغماء أثناء سحب عينة الدم. عندما يسحب لك عينة دم لإجراء فحوصات كجزء من الإجراء الروتيني لرعايتك الصحية فإن المخاطر المترتبة على مشاركتك في البحث تكون مشابهة تماما.

الخصوصية و السرية

ستحفظ المعلومات البحثية و الطبية الخاصة بهذه الدراسة تحديداً في ملف خاص بالبحث منفصل عن الملفات الطبية الأخرى. للمحافظة على الخصوصية، فإن اسمك او أية معلومات قد تقوم بالتعريف بك، ستزال من عينة الدم ، و من كل المعلومات عن وضعك الطبي و سيرتك المرضية التي ترافق عينتك. كل المعلومات الخاصة بالتعريف بك ستكون عن طريق إعطاء رمز خاص. هذا الرمز سيكون معروفا فقط لدى المسؤولين عن جمع العينات.

هل هناك فوائد للمشاركة في هذه الدراسة؟

إنه من الممكن أن يكون لك فائدة مباشرة من خلال استعمال عينة الدم الخاصة بك في الأبحاث. على الرغم من ذلك، فإنه يوجد فوائد ممكنة للمجتمع نتيجة هذه الأبحاث، منها معرفة مسببات الأمراض، كيفية الوقاية منها، و كيفية علاجها و التعامل مع الآثار الناتجة عنها.

هل هناك بدائل عن المشاركة في هذه الدراسة؟

يمكنك اختيار عدم إعطائنا عينة من دمك لهذه الدراسة. في هذه الحالة، سيستعمل دمك لرعايتك الطبية فقط.

ما هي التكاليف؟

لن يكون هناك أية تكاليف إضافية عليك إثر مشاركتك بهذه الدراسة.

ماذا عن التعويضات ؟

لن نقوم بالدفع لك لمشاركتك بهذه الدراسة.

ماذا عن حقي في رفض المشاركة بهذه الدراسة او الإنسحاب منها؟

المشاركة بهذه الدراسة هي مشاركة طوعية بحتة. يمكنك رفض المشاركة في هذه الدراسة أو في حال وافقت، يمكنك الإنسحاب منها إن رغبت في أي وقت. قرارك بعدم المشاركة أو الإنسحاب من المشاركة بهذه الدراسة لن يترتب عليه أية عقوبة أو خسارة لأية امتيازات، و لن يؤثر ذلك على مقدرتك على الحصول على الرعاية الصحية.

بمن اتصل في حال كانت لدي أسئلة أو واجهتني مشاكل؟

للسؤال عن الدراسة أو إذا كان لديك أية مشاكل، مخاوف، أسئلة أو استفسارات تتعلق بالبحث او عن حقوقك كمشارك يمكن التواصل مع الباحثة ميساء سوابتة على العنوان التالي: maysaa1996.mat@gmail.com

نص الموافقة :

تم شرح لي الهدف من هذه الدراسة، الخطوات التي سيتم إتباعها، المخاطر و الفوائد المترتبة على المشاركة بها. لقد تم السماح لي بسؤال الأسئلة، و تمت الإجابة عن تساؤلاتي لحد يرضيني. لقد تم إخباري بمن أتصل إذا كانت لدي تساؤلات, أو لمناقشة مشاكل، أو مخاوف، أو اقتراحات متعلقة بالبحث، أو للحصول على معلومات أو إعطاء أية إضافات حول البحث. لقد قمت بقراءة وثيقة الموافقة هذه و عليه، أوافق على المشاركة بهذه الدراسة، مع العلم أنه بإمكاني الإنسحاب أنّى شئت.

☐ أوافق على أن يتم أخذ عينة دم مني لفحص المادة الوراثية.

الاسم الثلاثي للمشارك :_____ التوقيع_____ التاريخ _____

الاسم الثلاثي للباحث: _____ التوقيع_____ التاريخ _____

Appendix 2: Questionnaire

Patient's Name: _____

Age:_____

Patient's Number: _____

Please answer the following questions:

- How many times she had experienced recurrent pregnancy loss?

  _____

- In which trimester? _____

Please clearly state if the patient suffers from any of the following conditions:

- Factor V mutations Yes ◯ No ◯ NA ◯
- Factor 2 prothrombin gene (PT G20210A) mutation Yes ◯ No ◯ NA ◯
- Protein S deficiency Yes ◯ No ◯ NA ◯
- Protein C deficiency Yes ◯ No ◯ NA ◯
- Anti prothrombin antibody Yes ◯ No ◯ NA ◯
- Chromosomal anomalies Yes ◯ No ◯ NA ◯
- Cigarette smoking Yes ◯ No ◯ NA ◯
- Alcohol consumption Yes ◯ No ◯ NA ◯
- Uterine abnormalities Yes ◯ No ◯ NA ◯
- Autoimmune diseases Yes ◯ No ◯ NA ◯
- Infectious diseases Yes ◯ No ◯ NA ◯
- endocrine diseases (diabetes mellitus, thyroid diseases) Yes ◯ No ◯ NA ◯
- Rh blood group incompatibility Yes ◯ No ◯ NA ◯
- Any history of preterm birth or preeclampsia Yes ◯ No ◯ NA ◯
- Successful pregnancy Yes ◯ No ◯ NA ◯

  -If yes, how many times? _____

الملخص

الخلفية والأهداف: يعد فقدان الحمل المتكرر (RPL) حالة طبية معقدة تؤثر على 1-4٪ من النساء خلال سن الإنجاب. ويحدث ذلك عندما تفقد المرأة جنينها بشكل متكرر قبل أن يصل إلى الأسبوع العشرين من الحمل. في فلسطين، يعدالإجهاض المتكررشائعًا نسبيًا بين الإناث الفلسطينيات بسبب عدة عوامل، بما في ذلك الطفرات الجينية المفردة وتعدد الأشكال(mutations and SNPs). في الآونة الأخيرة، تم ربط متغيرات مختارة في جين VDR بـفقدان الحمل المتكرر في بعض المجموعات السكانية. وبالتالي، هدفت دراستنا إلى التحقق مما إذا كان اثنان من تعدد الأشكال SNPsالأمومي لجينVDR ، على وجه التحديد (rs2228570) و(rs1544410) ، ومستوى حالة فيتامين د قد يكونان احد العوامل المسببة لفقدان الحمللدى النساء الفلسطينيات المصابات بهذه الحالة.

الطرق و المواد: تم تسجيل ما مجموعه 131 أنثى في هذه الدراسة. تتكون مجموعة الحالات (case group) من 51 امرأة تعرضن على الأقل لثلاث حالات إجهاض متتالية في الثلث الثاني من الحمل، في حين تتكون المجموعة الضابطة (control group) من ثمانين امرأة مع حالتي حمل ناجحة أو أكثر و بدون اي اجهاضات. تم الحصول على عينات من الحمض النووي وتنميطها وراثيًا لأشكال VDR rs2228570 وrs1544410 باستخدام تقنية تعدد أشكال طول جزء التقييد (PCR-RFLP) و من ثم تم قياس مستوى 25-هيدروكسي فيتامين د3 في المصل باستخدام مجموعة Elecsys Vitamin D Total III. تم استخدام برنامجي SPSS وSNPStat لتحليل البيانات الناتجة. بالإضافة إلى ذلك، ضمن مجموعة الحالات، كان هناك حالتان لديهن تاريخ عائلي من الإجهاض المتكرر و بالتالي تم فحص عيناتهم باستخدام تقنية التسلسل الوراثي الكامل (WES). تم تحليل الجينات المؤكدة في هذه العائلات بواسطة أدوات المعلومات الحيوية InSilicobioinformatic tools.

النتائج: لوحظت اختلافات كبيرة في ترددات الأليل والنمط الوراثي بين الاشخاص الخاضعينللدراسة في موقع متعدد الأشكال VDR rs1544410 (Gمقابل A، p-value = 0.05، GG مقابل GA، p-value = 0.01، GG مقابل AA، p-value =0.02). كما انه يزداد خطر الإصابة بـالإجهاض المتكرر في ظل النموذج الجيني السائد (GG مقابل المجموع A، p-value = 0.006). لم يلاحظ أي فروق ذات دلالة إحصائية في ترددات الأليل والنمط الوراثي بين الاشخاص الخاضعينللدراسة في موقع متعدد الأشكال VDR rs2228570. بالإضافة الى ذلك. ارتبط النمط الفرداني (T-G haplotype) بالوقاية من الاجهاض المتكرر، حيث أنه كان أكثر شيوعًا في المجموعة الضابطة

من مجموعة الحالات. لم يلاحظ أي فروق ذات دلالة إحصائية بين مستويات المصل 25 هيدروكسي فيتامين D3 والأنماط الجينية VDR rs2228570 وrs1544410 بين الأشخاص الخاضعين للدراسة. وفيما يتعلق بتحليل الأسرة، تم تحديد متغيرين في كل عائلة. في العائلة الأولى، تتبع متغيرات FGA *787T>C وHLA-G Leu154fs*60 التوريث الجسدي السائد وتم تأكيدها بشكل إيجابي وفصلها جيدًا داخل العائلة، بينما في العائلة الثانية، فشل جين F12 Arg55Trp في التوريث داخل العائلة.

الملخص: في هذه الدراسة كشفت البيانات عن وجود ارتباط كبير بين متغير rs1544410 و فقدان الحمل المتكرر مع عدم وجود ارتباط مع متغير rs2228570 في جين VDR. لم تغير مستويات فيتامين د في الدم هذه النتيجة بسبب وجود نقص في مستويات فيتامين د في الدم لدى جميع المشاركين في هذه الدراسة و بالتالي هذه النتيجة تحتاج إلى مزيد من التقييم. كشف تحليل WES لحالتين لديهم تاريخ عائلي من فقدان الحمل المتكرر عن بيانات مثيرة للاهتمام تربط متغيرات محددة في الجينات (FGA *787T>C وHLA-G Leu154fs*60) التي يمكن أن تؤثر على استقرار الجنين ونموه والانتقال إلى فترة الحمل الكاملة والتي تحتاج إلى مزيد من الاختبار والتأكيد.