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Identification of gene mutations associated with Type 1 Diabetes by Next Generation Sequencing in Inflicted Palestinian Families.

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Declaration:

I certify that the work provided in this thesis, unless otherwise referenced, is the researcher's own work and has not submitted for a higher degree to any other university or institution.

Abrar Bawatneh

Dedication

I would like to dedicate this work and effort to my great parents, the first and last love.

Abrar Bawatneh

Acknowledgement

First and foremost, thanks be to Allah, the lord of all worlds, before and after. Then, I would like to thank the two families who participate in this study, wishing them a life full of health and happiness. Thanks to Dr. Hasan Eideh and Khalida Mathloum from Layan Medical center for their help in sample collection. Many thanks to Dr. Alaa Darwish for everything she gave us and the valuable things she taught me. Thanks to all my teachers and doctors and Special thanks to my wonderful supervisor, Prof. Hisham Darwish, for his support. My sincere thanks to the members of the thesis committee, Professor Zaidoun Salah and Dr. Fawaz Awad, for their remarks and careful reviewing. Thanks to our lab team; Rua Thawabtah, Husam Sallam, and Nadeen Balqis. Thanks also to my beautiful family and my sisters Raya and Tasneem.

Abstract

Diabetes Mellitus is a group of metabolic disorders that are characterized by the presence of hyperglycemia secondary to insulin resistance or deficiency. It is considered a major health problem worldwide. It is classified into several subgroups including Type 1 diabetes mellitus, type 2 diabetes mellitus, gestational diabetes, and monogenic diabetes such as maturity-onset diabetes of the young (MODY). T1DM results due to a combination between genetics, epigenetics, and environmental factors. Several genes have been associated with T1DM including *HLA, INS, CTLA4,* and *PTPN22*. However, none of them is based on linkage analysis because it's rare to find families with several diabetic individuals. Two Palestinian families having several affected members with variations in the mode of inheritance were identified and selected for this study. We aimed to clearly identify the causative gene(s) responsible for T1DM development in these families, in order to improve the understanding of the molecular genetics of the disease. One inflicted member from each family was selected for Whole-exome sequencing. Data were mapped to the reference human genome and the resulting VCF file containing many thousands of variants was filtered. The variants with the highest phenotype correlation score (four variants in each family) were checked by Sanger sequencing in all the family members. The confirmed variants were *in-silico* analyzed by bioinformatics tools. In family I, variants in *INS, KCNJ11*, *HNF1A,* and *IGF1R* genes were identified and tested. Only the IGF1R p.V579F variant follows autosomal dominant inheritance was confirmed and segregated in the family. In family II, variants in *ABCC8, NEUROD1, RYR1,* and *CTRC* genes were identified and checked. NEUROD1 p.P197H variant which follows autosomal recessive inheritance was positively confirmed and

segregated. In conclusion, IGF1R p.V579F and NEURID1 p.P197H variants were associated with T1DM development in the two inflicted families in our study. Further analysis and functional assays should be done to fully confirm these findings and unravel their specific role in the disease development. This data will be valuable in the screening and diagnosis of patients in addition to the potential significance of these genes to serve as therapeutic targets.

بسم الله الرحمن الرحيم

الملخص

داء السكري هو عبارة عن مجموعة من الاضطرابات العضوية التي تتميز بوجود ارتفاع السكر في الدم نتيجة لمقاومة الانسولين أو نقصه. يشكل السكري مشكلة صحية أساسية على مستوى العالم. تم تصنيف المرض إلى عدة أنواع مثل السكري النوع الأول، السكري النوع الثاني، سكري الحمل، وسكري الشباب الناضجين(MODY). النوع الأول من السكري ينتج من تفاعل بين العوامل البيئية والجينية. تم إيجاد بعض الجينات التي ترتبط وتسبب مرض السكري مثل جين *HLA, INS, CTA4,* و *PTPN22*. من الصعب العثور على عائلات تضم عدة أفراد مصابين بالسكري النوع الأول، لهذا معظم الجينات المرتبطة نتجت من دراسات عشوائية وليست تحليل ارتباطي تعتمد على الوراثة. تم التعرف واختيار عائلتين فلسطينيتين لديها العديد من الأفراد المصابين بالسكري النوع الأول، مع اختلاف بين نمط الوراثة بين العائلتين. في هذه الدراسة، نهدف إلى تعريف واضح للجينات أو الطفرات المسؤولة عن تطور مرض السكري النوع الأول عند هذه العائلات من أجل تحسين فهم الوراثة الجزيئية للمرض. مريض سكري واحد من كل عائلة تم اختياره لإجراء فحص تسلسل اكسوم كامل (whole exome sequencing). تمت مقارنة النتائج مع الجينوم المرجعي، ثم تصفية وترشيح الطفرات والمتغيرات الناتجة في ملف VCF. اخترنا المتغيرات التي لها أعلى درجات ارتباط مع النمط الظاهري(السكري) ليتم لاحقا فحصها بواسطة تسلسل سانجر (Sanger sequencing)لنتأكد من وجودها في العائلة. تم اختيار أربعة متغيرات في كل عائلة للفحص، ثم عمل تحليل للطفرة بواسطة عدة أدوات في المعلوماتية الحيوية. في العائلة الأولى، تم تحديد متغيرات في الجينات التالية: *IGF1R, INS, KCNJ11, HNF1A*. أوجدنا الارتباط بين الطفرة V579F الموجودة بجين IGF1R والتي تتبع الوراثة السائدة مع السكري النوع الأول. في العائلة الثانية، المتغيرات التي وجدت كانت في الجينات التالية:*ABCC8, CTRC,NEUROD1, RYR1* . تم تأكيد ارتباط المتغير NEUROD1 P197H الذي يتبع الوراثة المتنحية مع السكري في الدراسة. في الخلاصة، ارتبطت المتغيرات IGF1R V579F و NEUROD1 P197H مع الإصابة بمرض السكري

النوع الأول. يجب إجراء المزيد من التحليل والفحوصات الوظيفية لتأكيد نتائجنا والتحقيق في دورها وتأثيرها المحدد في المرض .هذه البيانات ستكون ذات قيمة في فحص وتشخيص المرضى، بالإضافة لقدرة هذه الجينات على ان تكون اهدافا علاجية.

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Chapter 4:

List of Abbreviations

T1DM	Type one diabetes mellitus
T2DM	Type two diabetes mellitus
MODY	Maturity onset diabetes of the young
HLA	Human leukocyte antigens
INS	Insulin
CTLA4	Cytotoxic T-Lymphocyte Associated Protein 4
PTPN22	Protein Tyrosine Phosphatase Non-Receptor Type 22
VCF	Variant Call Format
PolyPhen-2	Polymorphism Phenotyping v2
SIFT	Sorting Intolerant from Tolerant
PROVEAN	Protein Variation Effect Analyzer
FATHMM	through Hidden Markov Models Functional Analysis
GVGD	Grantham Variation Grantham Deviation
KCNJ11	Rectifying Channel Subfamily J Member 11 Potassium Inwardly
HNF1A	factor-1 alpha hepatocyte nuclear
IGF1R	Factor 1 Receptor Insulin Like Growth
ABCC8	Subfamily C Member 8 ATP Binding Cassette
NEUROD1	Differentiation 1 Neuronal
RYR1	Ryanodine Receptor 1
CTRC	Chymotrypsin C
NEUROD1 P197H	differentiation 1 Neuronal

T1D	Type 1 diabetes
IL2RA	receptor subunit alpha interleukin 2
IFIH1	with helicase C domain 1 interferon induced
LDL	lipoprotein low-density
KCNQ1	voltage-gated channel subfamily Q member 1 potassium
KCNK16	domain channel subfamily K member 16 potassium two pore
SLC30A8	member 8 30 solute carrier family
SRR	serine racemase
ADAMTS9	with thrombospondin type 1 motif 9 ADAM metallopeptidase
MTNR1B	melatonin receptor 1B
CAMK1D	calcium/calmodulin dependent protein kinase ID
CENTD2 / ARAP1	Ankyrin Repeat and PH Domain 1
DUSP9	Dual specificity phosphatase 9
BCL11A	BAF chromatin remodeling complex subunit BCL11A
PRC1	Protein regulator of cytokinesis 1
CHCHD9	Coiled-coil-helix-coiled-coil-helix domain containing 9
CF7L2	Transcription Factor-7-Like 2
TCF1	Transcription factor T cell factor 1
TCF2	Transcription factor-2
HHEX / IDE	Hematopoietically expressed homeobox - insulin-degrading enzyme
IGF2BP2	Insulin like growth factor 2 mRNA binding protein 2
CDKAL1	CDK5 regulatory subunit associated protein 1 like 1
GLIS3	GLIS family zinc finger 3

NOTCH2	Notch receptor 2
PPARG	Peroxisome proliferator activated receptor gamma
IRS1	Insulin receptor substrate 1
GRB14	Growth factor receptor bound protein 14
PTPRD	Protein Tyrosine Phosphatase Receptor Type D
SPRY2	Sprouty RTK Signaling Antagonist 2
TCF7L2	Transcription factor 7 like 2
Pro12Ala	polymorphism of peroxisome proliferator activated receptor gamma 2
PPAR-Gamma 2	Peroxisome Proliferator Activated Receptor Gamma 2
WHO	World Health Organization
GCT	Glucose Challenge Test
GTT	Glucose Tolerance Test
FBG	Fasting Blood Sugar
GCK	Glucokinase
HNF1A	Hepatocyte nuclear factor 1-alpha
HNF4A	hepatocyte nuclear factor 4 alpha
HNF1B	hepatocyte nuclear factor-1 beta
PAX	Paired box
T2D	Type 2 diabetes
GAD	Glutamic acid decarboxylase
NDM	Neonatal Diabetes Mellitus
TNDM	Transient neonatal diabetes mellitus
PNDM	Permanent neonatal diabetes mellitus

6q24	Chromosome 6q24-related diabetes mellitus
GATA6	GATA-binding factor 6
EIF2AK3	Eukaryotic translation initiation factor 2 alpha kinase 3
PTF1A	Pancreas associated transcription factor 1a
FOXP3	Forkhead box P3
ATP	Adenosine triphosphate
DNA	Deoxyribonucleic acid
tRNA	Transfer ribonucleic acid
OGTT	Oral glucose tolerance test
HbA1c	Glycated hemoglobin
IAA	Insulin Autoantibodies
ZnT8A	Tetraspanin-7, zinc transporter protein 8
IA2	Islet Antigen 2 Antibody
APCs	Antigen-presenting cells
CD	cluster of differentiation
NK	Natural killers
DC	Dendritic cells
MHC	Major histocompatibility complex
ROS	Reactive Oxygen Species
AFF3	AF4/FMR2 family member 3
RGMA	Repulsive guidance molecule BMP co-receptor A
MCTP2	multiple C2 and transmembrane domain containing 2
ANKS1A	ankyrin repeat and sterile alpha motif domain containing 1A

COL4A2	collagen type IV alpha 2 chain
APOE	Apolipoprotein E
MAPK14	Mitogen-activated protein kinase 14
IDF	International Diabetes Federation
AFR	International Diabetes Federation Africa Region
EUR	International Diabetes Federation Europe Region
MENA	International Diabetes Federation Middle East and North Africa
NAC	International Diabetes Federation North America and Caribbean
SACA	International Diabetes Federation South and Central America
SEA	International Diabetes Federation South East Asia Region
WP	International Diabetes Federation Western Pacific Region.
LIC	low-income countries
LMIC	lower-middle-income countries
UMIC	upper-middle-income countries
HIC	High income countries
CAR	Coxsackie- adenovirus receptor
RNA	Ribonucleic acid
PKR	protein kinase R
MDA5	melanoma differentiation- associated protein 5
MxA	myxovirus resistance protein
HLA- I	human leukocyte Antigen- I
IFN)- I	Interferon type I
CVB6	Coxsackievirus group B

CVB3	Coxsackievirus B3
CVB6	Coxsackievirus B6
CMV	Cytomegalovirus
TLR4	Toll-like receptor 4
DBP	D–binding protein
VDR	Vitamin D receptor
VDBP	Vitamin D binding protein
CYP2R1	Cytochrome P450 Family 2 Subfamily R Member 1
CYP27B1	Cytochrome P450 Family 27 Subfamily B Member 1
BMI	Body mass index
miRNAs	Micro Ribonucleic acid
FOXP3	Forkhead box protein P3
HLA-A	Major histocompatibility complex, class I, A / human leukocyte Antigen
HLA-B	Major histocompatibility complex, class I, B / human leukocyte Antigen
HLA-C	Major Histocompatibility Complex, Class I, C / human leukocyte Antigen
HLA-DP	human leukocyte Antigen class II, DP
HLA-DQ	human leukocyte Antigen (heterodimer of type MHC class II)
HLA-DR	Human Leukocyte Antigen – DR isotype
IPD-IMGT/HLA	Immuno Polymorphism Database
HLA W15	Human Leukocyte Antigen – W15
SSO	Single sign-on
NGS	Next Generation Sequencing
IDDM2	The insulin-dependent diabetes mellitus 2 gene

VNTR	variable number of tandem repeats
CTLA4	Cytotoxic T-Lymphocyte Associated Protein 4 gene
SLE	systemic lupus erythematosus
SNPs	Single nucleotide polymorphisms,
Lyp	lymphoid-specific tyrosine phosphatase
TCR	T cell receptor
BCR	B cell receptor
RA	Rheumatoid Arthritis
IBD	inflammatory bowel disease
BD	Behcet's disease
TCR/CD3	T cell receptor/CD3 complex
ZAP70	Zeta chain of T cell receptor associated protein kinase 70
MS	Multiple sclerosis
IDDM10	Insulin-dependent diabetes mellitus type 10
RBM17	RNA Binding Motif Protein 17)
IFIH1	Interferon-induced with helicase C domain 1
MDA5	Melanoma differentiation-associated gene 5
MAVS	Mitochondrial Antiviral Signaling Protein
OMIM	Online Mendelian Inheritance in Man
IDDM19	Insulin-dependent diabetes mellitus susceptibility genes 19
ITPR3	Inositol 1,4,5-Trisphosphate Receptor Type 3
IPEX	Immunodysregulation polyendocrinopathy enteropathy x-linked
CLEC16A	C-Type Lectin Domain Containing 16A

GWAS	Genome-wide association studies
KIAA0350 gene	Now called CLEC16A
Nrdp1/Parkin	RING finger ubiquitin E3 ligase
USP8	Ubiquitin Specific Peptidase 8
ERBB3	Erb-B2 Receptor Tyrosine Kinase 3
EGFR	epidermal growth factor receptor
WTCCC	Wellcome Trust Case Control Consortium
PIK3	Phosphoinositide 3-kinases
mTOR	Mammalian target of rapamycin
SH2B3	Src Homology 2B Adaptor protein 3
LNK	lymphocyte adapter protein
SH2B	The Src homology 2B family
G1-ILCs	group 1 innate lymphoid cells
PTP	protein tyrosine phosphatase
STAT	Signal transducer and activator of transcription
JAK	Janus Kinase
MAPK	mitogen-activated protein kinases
UBASH3A	Ubiquitin-associated and SH3 domain-containing protein A
STS-2	suppressor of T cell signaling 2
TULA	T-cell ubiquitin ligand
CLIP4	CAP-Gly Domain Containing Linker Protein Family Member 4
IKK	IκB kinase
IL2	Interleukin 2

WT	Wild type
PPIL2	Peptidylprolyl Isomerase Like 2
NF-κB	Nuclear factor kappa B (NF-κB)
TEDDY	The Environmental Determinants of Diabetes in the Young
UBE2L3	ubiquitin conjugating enzyme E2 L3
MAPK1	Mitogen-Activated Protein Kinase 1
YDJC	YdjC chitooligosaccharide deacetylase homolog
YPEL1	Yippee like 1
CCDC116	Coiled-coil domain containing 116
SDF2L1	Stromal cell derived factor 2 like 1
MIR301B	MicroRNA 301b
MIR130B	MicroRNA 130b
EDTA	Ethylenediaminetetraacetic acid
RBCs	Red Blood Cells
WBCs	White Blood Cells
RPM	Round Per Minute
dsDNA	double stranded Deoxyribonucleic acid
TAE	Tris-acetate- Ethylenediaminetetraacetic acid
PCR	Polymerase chain reaction
eBLT	Enrichment Bead-Linked Transposomes
TB1	teosinte branched1
TWB	Tagment Wash buffer
EPM	Enhanced PCR mix

RSB	Resuspension Buffer
SMB3	Streptavidin Magnetic Beads
EEW	Enhanced Enrichment Wash
EE1	Enrichment Elution Buffer
HP3	2N NaOH
ET2	Elute Target Buffer 2
PPC	PCR primer Cocktail
EtOh	Ethanol
HS	High Sensitivity
GRCh38	Genome Reference Consortium Human Build 38
BWA-MEM	Burrows-Wheeler Aligner
Indels	insertions and deletions
HPO	Human Phenotype Ontology
QD	quality by depth
MQ	RMS mapping quality
UTRs	Untranslated region
NTC	No template control
dNTPs	Deoxynucleoside triphosphate
BDRR	Big Dye Terminator
IRS	Insulin receptor substrate
CRK	CT10 Regulator of Kinase
SHC	Src homology and Collagen
Erk	extracellular-signal-regulated kinase

AKT	Protein kinase B
PI3K	Phosphoinositide 3-kinases
BRCA1	Breast Cancer gene 1
ECM	Extracellular matrix changes
MDR1	Multidrug resistance 1
ACMG	The American Collage of Medical Genetics
MKL	Multiple kernel learning
3D	Three dimensions
bHLH	Basic helix-loop-helix
CANNTG	Containing An E-box
HEB	HeLa E box-binding
HLH	Helix-loop-helix
POMC	Proopiomelanocortin
BETA2	Beta-2-adrenergic receptor
PDX1	Pancreatic And Duodenal Homeobox 1
PNDM	Patients with Monogenic permanent neonatal diabetes
CREB	Cyclic AMP-responsive element-binding protein
PP2A	Protein phosphatase 2
SHP	Small heterodimer partner
IGRP	Islet-specific glucose-6-phosphatase catalytic subunit-related protein
SUR1	Sulfonylurea receptor 1

Chapter 1: Introduction

1.1 Diabetes Mellitus

Diabetes Mellitus is a group of metabolic disorders that are characterized by the presence of hyperglycemia secondary to insulin resistance or deficiency. It is associated with abnormalities in lipid and protein metabolism and electrolyte disturbances[1]. Diabetes is considered a serious health problem worldwide, with about 425 million adults suffered from it in 2017 [2]. Patients with Diabetes mellitus most commonly have hyperglycemia with polydipsia, polyuria, and polyphagia..[3,4] Figure (1) shows the main symptoms of Diabetes Mellitus and Type 1 Diabetes Mellitus (T1DM). [5]

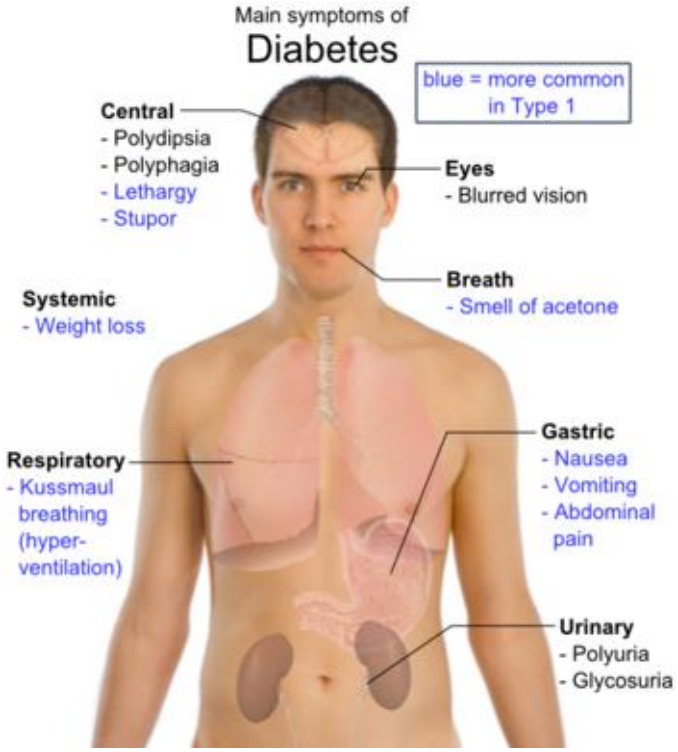

Figure (1): Symptoms of Diabetes Mellitus. [5]

1.2 Classification of Diabetes Mellitus

1.2.1 Type 1 Diabetes Mellitus

It is considered an autoimmune disease in which insulin is deficient due to the destruction of pancreatic beta cells. Therefore, T1DM patients are insulin-dependent and need daily injections of insulin. T1DM is the most common form in children and whites, where for example it accounts for 80% of childhood diabetes in the united states [6]. However, it can be diagnosed in all races and ages. It is considered a multifactorial disease that results from the interplay of genetic, epigenetic, and environmental factors, and can be influenced by age, ethnicity, race, geography, and socioeconomic status.[2]

Genetics is responsible for 80% of hereditary T1DM, and the HLA region on chromosome 6p21 accounts for 40% to 50% of the familial T1DM. It is correlated with the autoimmunity role of T1DM and can increase its risk or be protective. The insulin gene which located on chromosome 11 (locus 11p15) is the next strongest genetic factor in T1D. The third associated locus is the cytotoxic T lymphocyte-associated protein 4 (*CTLA4*) gene[7]. The protein tyrosine phosphatase, non-receptor type 22 (*PTPN22*) gene was reported to be the fourth locus in 2004 [8]. Later, the interleukin 2 receptor alpha (*IL2RA*) gene[9] and the interferon-induced with helicase C domain 1 (*IFIH1*) gene on chromosome 2q24.3 [10] were reported as the fifth and sixth associated loci, respectively.

1.2.2 Type 2 Diabetes Mellitus

It is the most common type of diabetes with more than 90% of all diabetes cases worldwide.[11] It is characterized by insulin resistance, relative insulin deficiency, and adult-onset (after 35 years of age). Unlike T1DM, T2DM patients don’t depend on

insulin, don't usually develop ketoacidosis, and don't have beta cells' autoimmune destruction[12]. The major risk factors for T2DM are obesity, hyperlipidemia (high triglycerides and LDL levels)[13], smoking[14], physical inactivity, vitamin D deficiency[15], aging [16], genetics and family history.

Several genes have been associated with T2DM. First, genes that are related to beta-cell failure including *KCNJ11, KCNQ1, ABCC8, KCNK16, SLC30A8, SRR, ADAMTS9, MTNR1B, CAMK1D, CENTD2, DUSP9, BCL11A, PRC1,* and *CHCHD9*. Second, genes that are involved in pancreas development and affect the capacity of pancreas to secrete insulin including *CF7L2, TCF1, TCF2, HHEX / IDE, IGF2BP2, CDKAL1, GLIS3,* and *NOTCH2*[17]. Third, genes that are related to insulin resistance including *FTO* and *PPARG* genes which increase the risk for T2DM by affecting adiposity and obesity[18–20]. Furthermore, *IRS1, GRB14, PTPRD, DUSP9,* and *SPRY2* genes have been linked to the disease development through the insulin receptor signaling pathway.[21]

In Palestinian population, previous studies showed the significant association between the rs7903146 variant in the transcription factor 7 like 2 gene (TCF7L2) and Pro12Ala Polymorphism of the PPAR-Gamma 2 gene and T2DM.[22,23]. A more recent study reported that variant rs9939609 of the FTO gene increases the risk for T2DM[24].

1.2.3 Gestational Diabetes:

It is defined as any glucose intolerance or hyperglycemia that occurs during pregnancy. It results from inability to overcome insulin resistance, which happens due to placental secretion of diabetogenic hormones like progesterone, growth hormone, and corticotropin-releasing hormone as a result of pancreatic B-cell hyperplasia.[25] It is

considered the most common metabolic disorder in pregnancy. Its prevalence range from 1-45% of all pregnancies depending on the area.[26]

The risk factors of this type include obesity, physical inactivity, maternal age, hypertension, history of cardiovascular disease, Vitamin D deficiency, presence of polycystic ovarian syndrome, family history of diabetes, and a previous history of gestational diabetes. Gestational diabetes increases the risk for preeclampsia, macrosomia (Birth weight more than 4000 grams), shoulder dystocia, neonatal hypoglycemia, respiratory distress, and Polyhydramnios.[25,27,28]

1.2.4 Maturity-Onset Diabetes of the Young (MODY)

It is a rare type of diabetes (about 1% of all cases) that is characterized by autosomal dominant inheritance and non-dependence on insulin[29]. The age of onset is usually between 6 months and 35 years.[30] It results from several mutations in the enzymes involved in glucose sensing of pancreatic beta cells such as Glucokinase (GCK) that decreases glucose phosphorylation and sensitivity in beta cells, or mutations in transcription factors responsible in beta cells development, including hepatocyte nuclear factors HNF1A, HNF4A, and HNF1B that lead to decrease insulin secretion, and PAX4 which affect apoptosis and proliferation of β-cells. Most MODY cases are mistakenly diagnosed as T1DM or T2DM. The major differences in laboratory tests between T1DM and MODY are the absence of β-cell antibodies (as glutamic acid decarboxylase) and the normal levels of C-peptide in MODY patients compared to presence of autoantibodies and very low or absence of C-peptide in T1DM patients. [31,32]

1.2.5 Neonatal Diabetes Mellitus (NDM)

It typically occurs before 6 months of age due to a monogenic defect. Its prevalence is approximately 1 in 90,000-160,000 live births. It can be either transient (TNDM), permanent (PNDM), or syndromic[33]. The characteristics of patients usually include low birth weight, intrauterine growth restriction, and β-cell failure.[30] There are over 20 known single-gene causes of NDM including *KCNJ11, ABCC8, 6q24, INS, NEUROD1*[34], *GATA6, EIF2AK3, GCK, PTF1A,* and *FOXP3*. Activating mutations in *KCNJ11* and *ABCC8* genes are the most common. These genes encode the inner and the outer subunits of ATP-sensitive potassium channel of the pancreatic beta-cell, respectively. Their closures help in depolarizing the cell membrane and activate calcium influx through voltage-gated calcium channels and insulin exocytosis. Therefore, mutations in these genes will keep K channels open and insulin will not be released from beta-cell.[35,36]

1.2.6 Mitochondrial Diabetes

Point mutations in mitochondrial DNA are associated with endocrinopathies as Diabetes[37]. It is transmitted via mother inheritance and occurs in 20/100 000 adults[38]. The most common mutation where found at position 3243 in the tRNA leucine gene [39]

1.2.7 Syndromic Diabetes Mellitus

Many genetic syndromes are associated with diabetes including Down syndrome, Klinefelter syndrome, Turner syndrome and Wolfram syndrome. [39]

1.2.8 Known Causes of Diabetes [39,40]

Several non-genetic factors can cause diabetes including the following:

1. Disease of the exocrine pancreas can cause damage to the β cells of the pancreas leading to diabetes. This includes pancreatic carcinoma, pancreatitis, infection, pancreatectomy, trauma, cystic fibrosis, and hemochromatosis.

2. Many chemicals and drugs can impair insulin secretion leading to diabetes especially in people with insulin resistance.

3. Excess amounts of some hormones that antagonize insulin action can cause diabetes. Examples include growth hormone in acromegaly, cortisol in Cushing's syndrome, glucagon in glucagonoma, and epinephrine in pheochromocytoma.

4. Some viruses are associated with Diabetes such as Rubella, coxsackievirus B, cytomegalovirus, adenovirus, and mumps.

1.3 Diagnosis Criteria of Diabetes Mellitus Types

Diabetes mellitus is diagnosed using either plasma glucose (Fasting plasma Glucose, oral glucose tolerance test) or HbA1c. Estimation of the cut off values for glucose and HbA1c is based on the association of FPG or HbA1c with retinopathy. Fasting plasma glucose of ≥ 126 mg/dL (7.0 mmol/L), plasma glucose after 2-hour OGTT ≥ 200 mg/dL (11.1 mmol/L), HbA1c ≥ 6.5% (48 mmol/mol) or random plasma glucose ≥ 200 mg/dL (11.1 mmol/L) along with symptoms of hyperglycemia will be the confirmatory diagnostic of diabetes mellitus [39]. Table (1) shows the differences of Diabetes Mellitus types based on several common parameters.

Table (1): Differences between Diabetes Mellitus types based on common parameters.

	FBS	HbA1C	C-peptide	Anti-GAD	Onset	Insulin	Age of onset	Ketoacidosis	Inheritance
T1DM	≥ 126 mg/dL	≥ 6.5%	low	Present	Sudden	Dependent	Mostly young	Common	Polygenic
T2DM	≥ 126 mg/dL	≥ 6.5%	Normal to high	Absent	Gradual	Independent	Mostly adults	Rare	Polygenic
MODY	≥ 126 mg/dL	≥ 6.5%	normal	Absent	-	Independent	Mostly young <25 years	Rare	Autosomal dominant

1.4 Etiology and Pathophysiology of Type 1 Diabetes

The mechanisms of developing T1DM are not fully understood, but likely results from a complex interaction between genetic, environmental, immune, metabolic factors[41]. Type 1 diabetes mellitus develops in three stages. Stage 1 is asymptomatic since the individuals have normal fasting glucose and normal glucose tolerance, but have two or more pancreatic autoantibodies. Stage 2 is characterized by presence of two or more autoantibodies with dysglycemia and without symptoms. Stage 3 is the stage of diagnosis where pancreatic autoantibodies are associated with hyperglycemia and clinical symptoms[3,42]. Figure (2) summarizes the factors involved in T1DM progression. Pancreatic autoantibodies include antibodies against glutamic acid decarboxylase (GAD), insulin (IAA), tetraspanin-7, zinc transporter protein 8 (ZnT8A), and protein tyrosine phosphatase (IA2 and IA2β).[43]

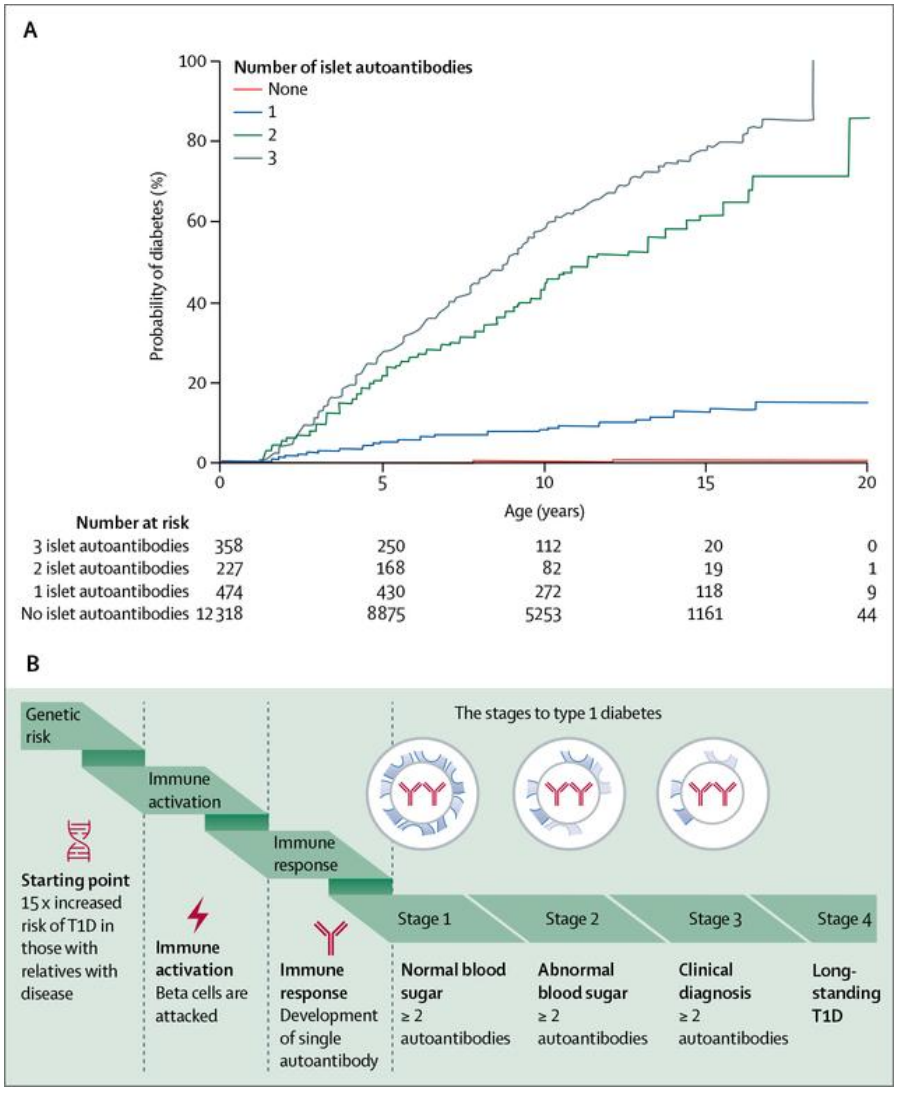

Figure (2): Factors involved in Type 1 Diabetes Mellitus progression.[3,42]

The pathogenesis of type 1 diabetes results from an intensive interaction between pancreatic β-cell with innate and adaptive immune systems including antigen-presenting cells (APCs), B cells, T cells (CD4+ and CD8+), macrophages, natural killers (NK), and dendritic cells (DC). The initiation of T1DM begins when β-cell peptides are presented by an antigen-presenting cell (APCs) (Figure 3A). The autoantigens will be migrated to pancreatic lymph nodes and interacted with autoreactive CD4+ T lymphocytes. CD8+ T cells will be then activated and lyse the beta cells expressing self-antigens on MHC

(Figure 3B). Inflammatory cytokines and reactive oxygen species (ROS) released from immune cells will increase beta cells destruction (Figure 3C). Moreover, defects in regulatory T lymphocytes lead to inability to suppress autoimmunity effectively (Figure 3D). Consequently, autoantibodies against β-cell proteins will be released (Figure 3E). [42]

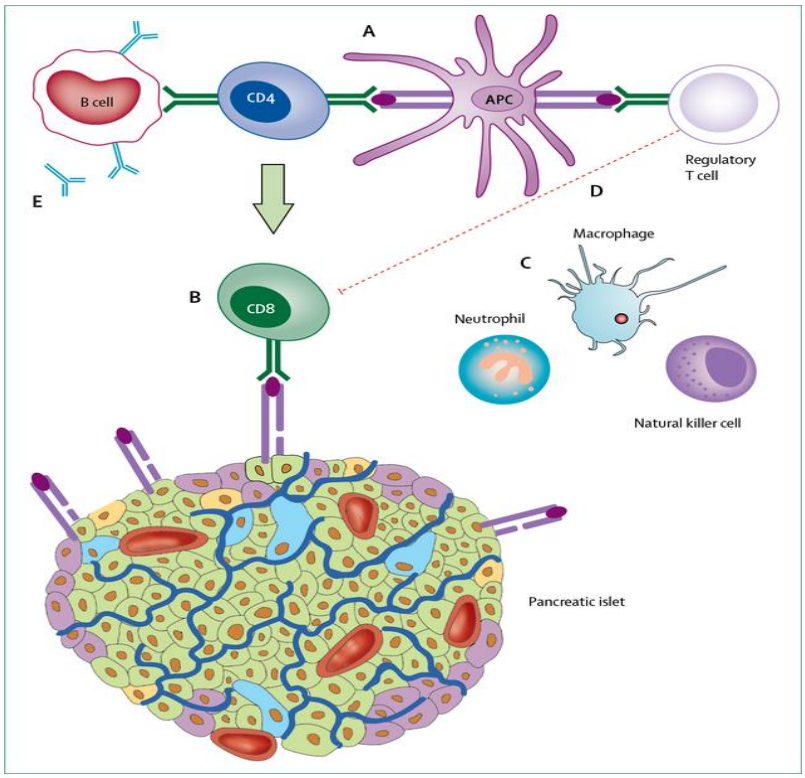

Figure (3): The immunopathogenesis of type 1 diabetes mellitus. [42]

1.5 Subtypes of Type 1 Diabetes

1.5.1 Autoimmune Type 1 Diabetes (Type 1 A)

This type includes 5-10% of total diabetic patients. It results from autoimmune destruction of pancreatic beta cells (as discussed above). It is characterized by the presence of pancreatic autoantibodies, absence of insulin secretion, and dominance in children and adolescents. This type has high association with genetics (especially the

HLA gene), and environmental factors (diet, viruses, pollutants). Patients with this type are also prone to other autoimmune disorders such as Graves' disease, Hashimoto's thyroiditis, Addison's disease, vitiligo, celiac sprue, autoimmune hepatitis, myasthenia gravis, and pernicious anemia. [2,40,44–46]

1.5.2 Idiopathic Type 1 Diabetes (Type 1 B)

It is a rare form of diabetes that results from unknown causes. It is less severe than Type 1A and characterized by severe insulin deficiency with lacking autoimmunity evidence or *HLA* association[47]. One study showed that type 1B diabetes have higher risk to cardiovascular diseases compared to type 1A.[48]

1.5.3 Fulminant Type 1 Diabetes

It was first diagnosed in Japan in the year of 2000. It is considered a severe disorder with rapid onset and high fatal risk. The condition is characterized by complete beta cells destruction, Ketoacidosis, non-immune mediated, absence of pancreatic autoantibodies, absence of C-peptide, and increase in serum pancreatic enzymes. Both genetic and environmental factors are associated with Fulminant type 1 diabetes especially viral infections. [49–51]

1.6 Complications of Type 1 Diabetes:

The most life-threatening complications are hypoglycemia and ketoacidosis. They are associated with problems on cognitive function, loss of consciousness or seizure (with a rate of 2-8/100person per year), and death (responsible for 4-10% of type 1 diabetes-related deaths)[52–54]. Other T1DM-related complications are classified as Microvascular (neuropathy, retinopathy, nephropathy) or Macrovascular (atherosclerosis, thrombosis in

heart and brain, cardiovascular diseases, cerebrovascular accidents, and peripheral vascular disease)[55]. Cardiovascular diseases are the major reason for the premature morbidity and mortality and can shorten the life expectancy of about 8-13 years[41,56]. These complications increase with significantly high HbA1c levels, hypertension, and hyperlipidemia.

Some genetic loci associated with T1D-related complications have been reported. For example, in diabetic Kidney disease, which is the leading cause of end-stage renal disease, the *AFF3* gene locus and an intergenic region on 15q26 between the *RGMA* and *MCTP2* genes were found to have significant association. With Coronary artery disease, *ANKS1A, COL4A2,* and *APOE* loci are associated[57]. Moreover, the *MAPK14* gene is associated with diabetic foot ulcers.

1.7 Epidemiology of Type 1 Diabetes

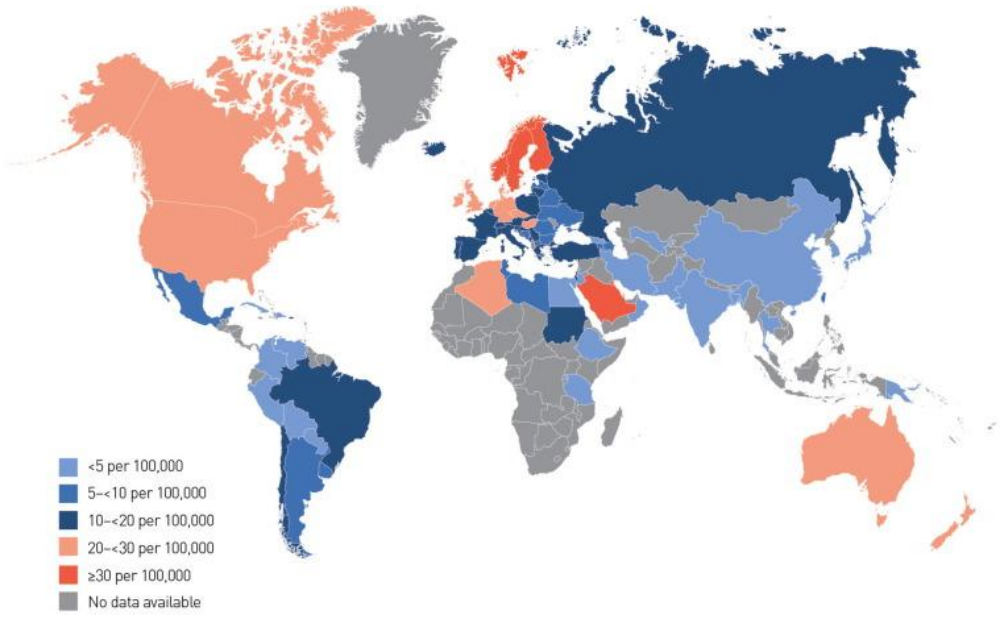

Figure (4): Map of age-sex standardized incidence rates (per 100,000) from publications of type 1 diabetes in children aged under 15 years.[58]

Worldwide: The incidence of type 1 diabetes is 15 per 100,000 with prevalence of 9.5% according to a systematic review that included 193 studies[59]. The disease incidence increases by 2-3% annually [41]. In children and adolescents, about 128,900 cases under age of 20 develop T1DM every year and 1,110,100 cases are alive with the disease. The most common cases are in Europe region, followed by North America and Caribbean Region, South East Asia, Middle East, and North Africa, South and Central America Region, Western Pacific, and African regions (shown in Figure 4 and 5). Interestingly, the most prevalent cases are among high-income countries while the highest mortality rate is in low-income and lower-middle-income countries, as illustrated in Figure (6) [58].

In Palestine: According to the health annual report Palestine in 2018, the fifth cause of death was the complications of diabetes (about 7.5 % of all deaths). The 2018 report registered 5,555 new cases of Diabetes Mellitus with an incidence rate of 210.7 per 100,000 individuals; 2,420 cases among males and 3135 among females. About 4.4% of these diabetic patients are diagnosed with type 1 diabetes. The mortality rate was 20.4 per 100,000 in the West Bank. [60]

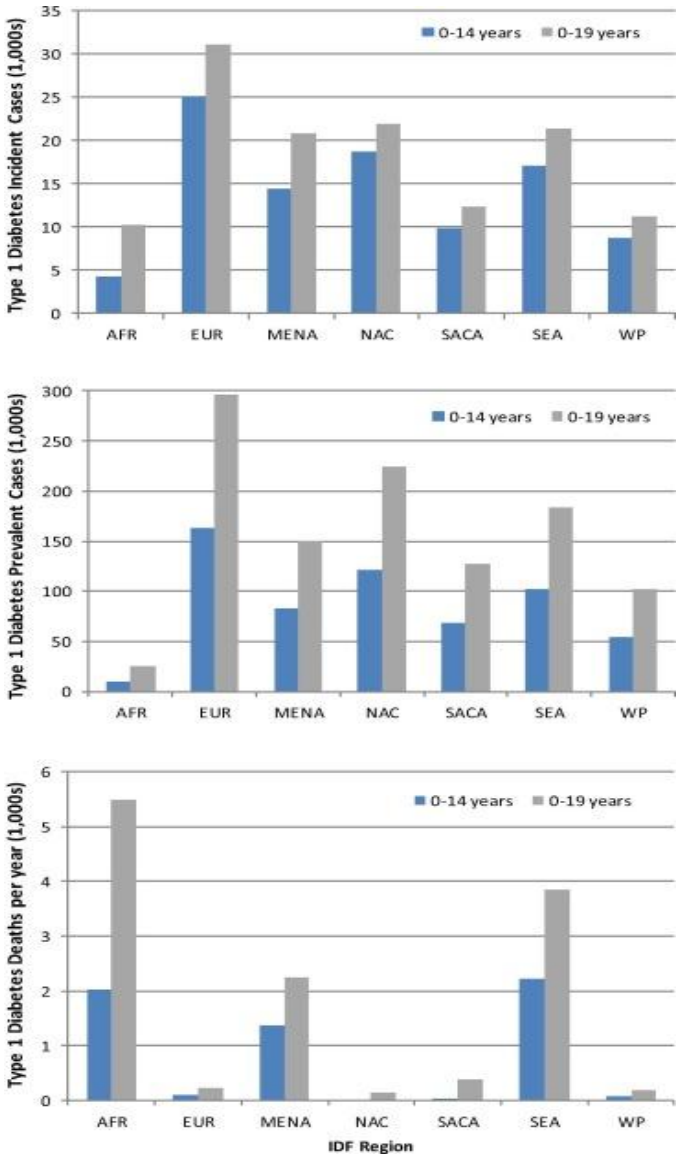

Figure (5): Comparison of type 1 diabetes incident and prevalent cases and deaths by International Diabetes Federation for 0–14 year and 0–19-year age-groups. Regions: AFR, International Diabetes Federation Africa Region; EUR, International Diabetes Federation Europe Region; MENA, International Diabetes Federation Middle East and North Africa Region; NAC, International Diabetes Federation North America and Caribbean Region; SACA, International Diabetes Federation South and Central America Region; SEA, International Diabetes Federation South East Asia Region; WP, International Diabetes Federation Western Pacific Region. [58]

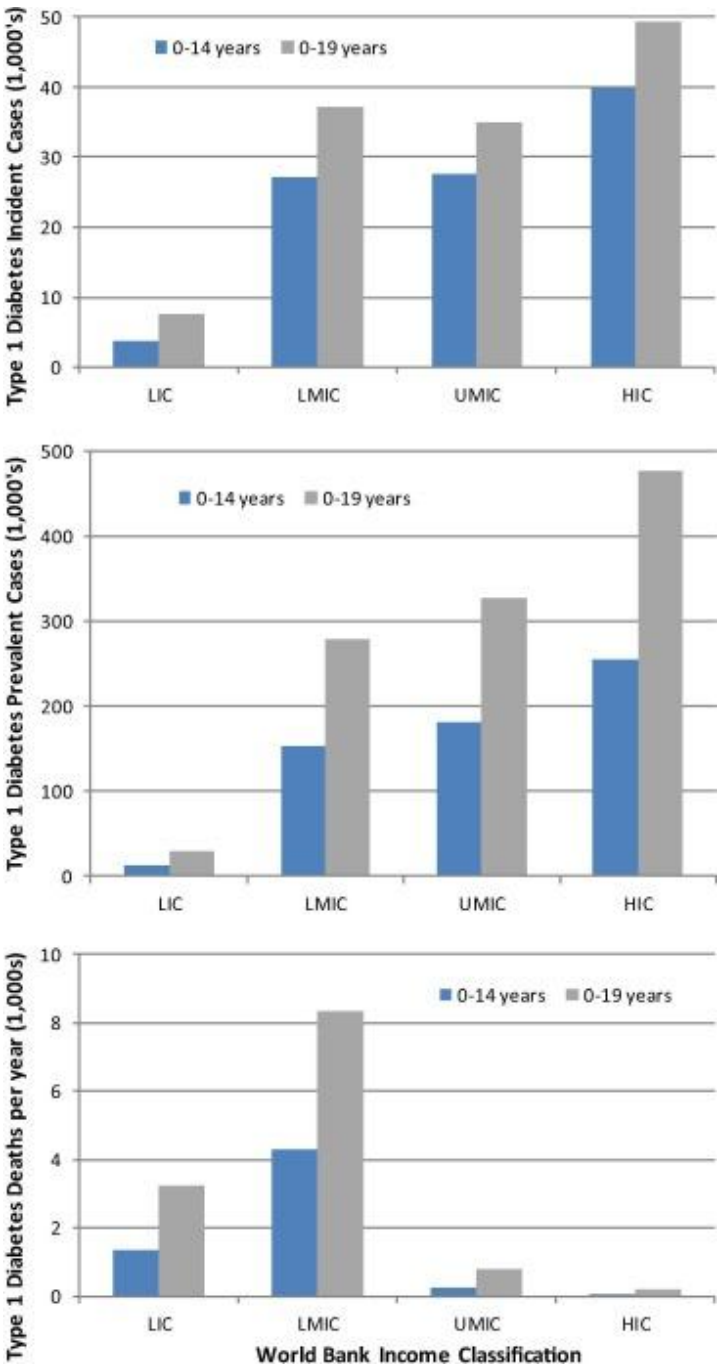

Figure (6): Comparison of Type 1 Diabetes incident and prevalent cases and deaths by World Bank Income Classification for 0–14 year and 0–19 year age-groups (LIC – low-income countries, LMIC – lower-middle-income countries, UMIC – upper-middle-income countries, HIC—High income countries).[58]

1.8 Risk factors for Type 1 Diabetes

1.8.1 Viral Infections:

It was noticed that T1DM is seasonal with increased incidence in winter-autumn and decreased in summer-spring. This indicates seasonal viral infections that could be correlated. [61] Enteroviruses resemble the majority of viruses associated with T1DM. A significant association between T1DM and enterovirus infection (OR 12.7) was reported according to a systemic review of 26 studies.[62] The etiological role of this association includes the ability of enteroviruses to destroy beta-cell by killing them or by creating an inflammatory response and attracting autoreactive T cells. Enteroviruses enter beta cells through the Coxsackie- adenovirus receptor (CAR) which is expressed in islet cells in the pancreas. Its RNA is then sensed by protein kinase R (PKR) and melanoma differentiation- associated protein 5 (MDA5) which in turn increase the myxovirus resistance protein (MxA) and islet human leukocyte Antigen- I (HLA- I) expression. Production of Interferon (IFN)- I will be induced and all this will enhance the presentation of beta-cell antigens and destruction of beta cells by CD8 T cells.[63] One study showed that coxsackievirus group B (CVB)1 antibodies increase the risk to autoimmune T1DM, whereas antibodies to CVB3 and CVB6 reduce the risk.[64]

Rotavirus can be a trigger of T1DM due to the similarity between its peptide sequence and T1DM autoantigen peptide sequence. Therefore, molecular mimicry is one of its etiological mechanisms. It can also cause direct beta-cell damage or immune response involve autoreactive T cells. [65,66]

Congenital rubella is also associated with T1DM. It was reported that T1DM develops in 12%–20% of patients with congenital rubella infection. The etiological role is still unknown. [67] Other viruses have been linked to T1DM without further confirmation including mumps, CMV, and others.[61]

1.8.2 Intestinal Microbiota:

In addition to viruses, bacterial infections can affect T1DM onset. Gut microbiota, which contains more than 500 species of microorganisms, can influence health and disease status in the body and changes in its composition (dysbiosis) cause several diseases including T1DM. Its etiological role in increasing the risk is through affecting glucose and lipid metabolism or by inducing an inflammatory state. Endotoxins, which is a component of bacteria and derived from gut microbiota, can increase cytokines levels and impair beta cell functions leading to diabetes. Endotoxins also activate Toll-like receptor 4 (TLR4) causing metabolic inflammation.[68,69]

1.8.3 Diet:

Dietary factors act as cofactors for inflammation or gut infection or trigger autoimmunity.[62] These factors include:

Breastfeeding: Low duration of breastfeeding is associated with a higher risk for T1DM.[61,70]

Cow's milk: Many studies showed no significant association between cow's milk and increased risk of T1DM. However, some concluded that it is a significant contributor to the disease[71].

Polyunsaturated fatty acids: Long-chain polyunsaturated fatty acids, especially omega-3 fatty acids, can contribute to T1DM development by affecting inflammatory responses. A Finnish study showed higher risk of islet autoimmunity and T1DM in children with lower omega-6 (linoleic acid) concentrations. An American study reported protective effect of high omega-3 consumption in developing T1DM.[72,73]

Meat is also a possible trigger for T1DM. A previous study reported that high consumption of meat especially in early life, in addition to maternal intake of meat during lactation promotes the development of T1DM in a dose-response manner.[74]

Gluten: Some studies reported a dose-dependent relationship between maternal gluten intake and T1DM risk. Higher intake of gluten showed a higher risk of developing T1DM in infants. Moreover, the gluten intake by children themselves can also increase the risk. A previous study showed that early introduction (before 3 months) of gluten-containing cereals is associated with early islet autoimmunity. [75–77]

1.8.4 Vitamin D:

Vitamin D plays a role in immune system regulation, making it a possible protective factor for T1DM. A previous study showed significant decrease risk of T1DM in children whose mothers' diet is rich with vitamin D[62]. Another study showed that higher vitamin D–binding protein (DBP) in mothers at delivery and higher 25(OH)D levels in infants at birth will decrease the risk of T1DM[78]. One more study in India showed higher prevalence of vitamin D deficiency in T1DM patients compared to healthy people[79]. However, other studies showed no association between vitamin D status and incidence of T1DM[72,80]. This reflects the complicated association between vitamin D and T1DM that

depend not only on diet, supplementation, or sunlight exposure, but also on genetic polymorphisms of certain genes including vitamin D receptor (VDR), vitamin D binding protein (VDBP), vitamin D 25-hydroxylase (CYP2R1) and 1α-hydroxylase (CYP27B1)[71].

1.8.5 Weight:

The incidence of T1DM is associated with increasing BMI and weight. This can be due to insulin resistance that occur from excess weight which leads to autoimmunity and apoptosis of beta cells. Some studies showed that risk of T1DM increases in infants with high birth weight (> 4.5 kg). Moreover, the rapid increase in weight during the first year of age is associated with T1DM. [61]

1.8.6 Psychological Stress:

Studies reported an association between high stress in patients with serious life events and the presence of anti-GAD at the age one year.[61]

1.8.7 Genetics:

It was noticed that the risk of diabetes increases in relatives and siblings, and can exceeds 70% in identical twins[81]. A child born to an affected family has much higher risk to develop T1DM at age of 20 compared to a child born to an unaffected family (5% vs 0.3%)[82]. Genetics are responsible for 80% of hereditary of T1DM, and the HLA region on chromosome 6p21 accounts for 40% to 50% of familial T1DM.

1.8.8 Epigenetics:

It represents the link between environmental and genetics factors that lead to T1DM development. It includes aberrant DNA methylation, histone modification, and miRNAs. It plays a role in regulating some genes' expression as FOXP3, and thus it can affect T cell activation. Recent studies showed the ability of using microRNA and DNA methylation as early markers to predict beta cells death[83,84]. Figure (7) demonstrates the interplay between genetic, epigenetic, and environmental factors in T1DM development.

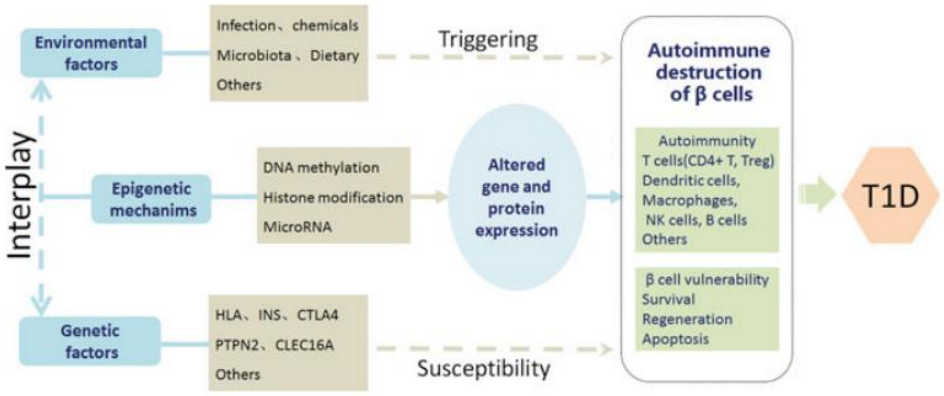

Figure (7): Genetic, environmental and epigenetic factors contribute to T1DM development.[83]

1.9 Genetic Susceptibility of Type 1 Diabetes

1.9.1 Candidate Genes:

1.9.1.1 *HLA*

HLA is the human MHC that plays a major role in adaptive immunity, innate immunity, and autoimmunity. *HLA* region maps to chromosome 6p21.31, as shown in Figure (8).

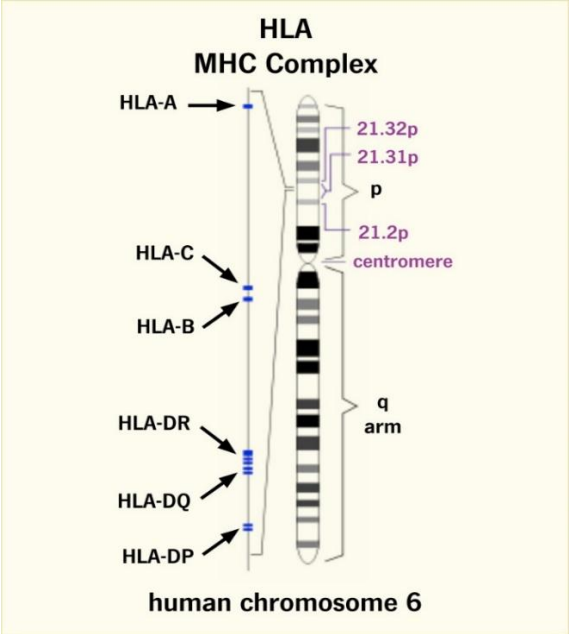

Figure (8): Human Leukocyte Antigen Major Histocompatibility Complex. [85]

Role in T1DM:

The role of MHC in T1DM is represented by its function in presenting peptide antigens on cell surfaces to be recognized by T cells. Thus it is associated with the autoimmunity of T1DM [86,87]. Polymorphisms of class II HLA genes (DQ and DR), in addition to class I HLA-B gene alleles, are the major contributors to T1DM [88]. The first reported risk association was HLA W15 antigen which is now called HLA-DRB1*04:01, followed by HLAB*08:01[89]. Among DR-DQ haplotypes, DRB1*03:01-DQA1*05:01-DQB1*02:01 (called DR3) and DRB1*04:01/02/04/05/08-DQA1*03:01-DQB1*03:02/04 (DR4) have the highest risk. Interestingly, the heterozygote of these haplotypes has a much higher risk compared to homozygous individuals (OR 16.59 vs 6.32 DR3/DR3, 5.68 DR4/DR4). Moreover, DRB1*15:01-DQA1*01:02-DQB1*06:02 (DR2; OR = 0.03) is the most protective haplotype. Among HLA-B alleles, allele B*39:06 is the strongest risk factor

(OR=10.31), and HLA-B*57:01 is the strongest protective factor (Or=0.19).[88] Table 2 shows the Odds ratio of most susceptible and protective DRB1-DQA1-DQB1 haplotypes.

Table (2): Selected susceptible and protective DRB1-DQA1-DQB1 haplotypes from the Type 1 Diabetes Genetics Consortium data. [89]

haplotype	DRB1	DQA1	DQB1	OR	p value
DR3	03:01	05:01	02:01	3.64	2 x10-22
DR4	04:05	03:01	03:02	11.37	4 x10-5
DR4	04:01	03:01	03:02	8.39	6 x10-36
DR4	04:02	03:01	03:02	3.63	3 x10-4
DR2	15:01	01:02	06:02	0.03	2×10^{-29}
DR6	14:01	01:01	05:03	0.02	1×10^{-6}
DR7	07:01	02:01	03:03	0.02	3×10^{-4}
DR7	07:01	02:01	02:01	0.32	2×10^{-9}
DR4	04:03	03:01	03:02	0.27	0.017

Intriguingly, HLA genotypes and haplotypes frequencies and effects on T1DM can vary among populations. For example, the DR3 haplotype is susceptible in Europe but it was found to be protective in African Americans. Similarly, the DR7 haplotype is protective in Europe and risk factor in African Americans, as shown in table (2).[89–91]

Table (3): Comparison of T1D susceptibility effects for DR3 and DR7 haplotypes in two populations. [89]

A. DR3				
Population	**DRB1**	**DQA1**	**DQB1**	**OR***
European	03:01	05:01	02:01	4.64
African American	03:02	04:01	04:02	0.16
B. DR7				
European	07:01	02:01	02:01	0.34
African American	07:01	03:01	02:01	3.96

In Mexico, a study conducted on recently diagnosed T1DM Mexican pediatrics aimed to study the association between HLA haplotypes, beta-cell functions, and autoantibodies. They showed that DRB1*04/DQA1*03/DQB1*03:02 haplotype increases the risk and severity of T1DM, and together with positive antibodies can be a predictor for insulin deficiency. Moreover, they found one more risk haplotype which is DRB1*03:01/DQA1*05/DQB1*02, and one protective which is DRB1*14/DQA1*05/DQB1*03:01 [92].

In Morocco, HLA-DRB1*03 (p = 0.0001), DRB1*04 (p = 0.0001), DQB1*02 (p = 0.0001, OR = 4.4) and DQB1*03 (p = 0.002) alleles were found to be associate with T1DM risk, while HLADQB1*06 allele (p=0.008) is a protective factor. Regarding haplotypes, Moroccans share some susceptible and protective haplotypes with other populations as Tunisians, Algerians, Spanish, and French, including DRB1*08-DQA1*0401-DQB1*0402, DRB1*03-DQB1*02, DRB1*04- DQB1*03 as susceptible, and DRB1*04-DQB1*02 and DRB1*13- DQB1*06 and DRB1*15-DQB1*06 as protective haplotypes. [93] Several other haplotypes were recently found to associate with T1DM including HLA~DRB1*03:01~DQA1*05:01 g~DQB1*02:01 (OR 5.8, p < 0.00001) in Brazilian population.[94]

1.9.1.2 Insulin (*INS)*

INS gene is located on the short arm of chromosome 11 at position 15.5 (11p15.5), as shown in figure (9), and mostly expressed in pancreatic β-cell. It produces insulin hormone and thus its expression is regulated by glucose levels in the body. [95,96]

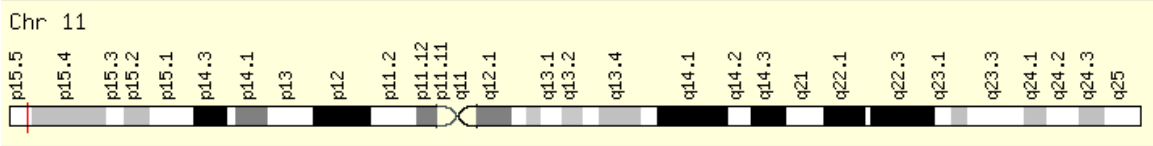

Figure (9): Location of *Insulin* gene on the human genome.

Role in T1DM:

INS is also called IDDM2. It confers 10% of genetic risk to T1DM. Its role in the pathogenesis is directed mainly by acting as autoantigens rather than affecting beta cells. The variable number of tandem repeats (VNTR), which located 0.5 kb upstream of *INS*, have been associated with T1DM especially. Its class I alleles have risk effect, whereas Class III alleles have protective effects[97,98]. Previous study detected the insulin gene single nucleotide polymorphisms among Finnish and Swedish populations. They showed a significant strong protective role of VNTR class III alleles in T1DM (p= 0.02). They also showed that heterozygotes of class III haplotype and class I haplotype decrease the risk of T1DM compared to homozygotes of class I haplotypes[99,100].

1.9.1.3. Cytotoxic T-Lymphocyte Associated Protein 4 (*CTLA4*)

CTLA4 gene is located on the long arm of chromosome 2 (2q33.2) as shown in figure (10), and consists of 4 exons. It encodes a protein that inhibits T-cells. Therefore, it is associated with autoimmune diseases including celiac disease, Graves' disease, systemic lupus erythematosus (SLE), type 1 diabetes mellitus (T1DM), Hashimoto thyroiditis, and thyroid-associated orbitopathy.

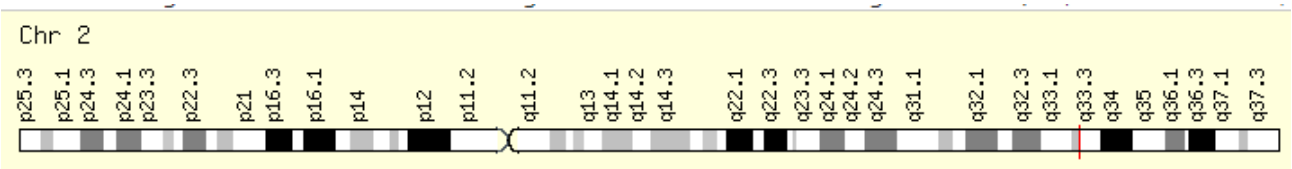

Figure (10): Location of *CTLA4* gene on the human genome.

Role in T1DM:

Many studies reported the association of CTLA4 polymorphisms; C(-318)T at the promoter region, (A49G) at exon 1, and an (AT)n dinucleotide repeat polymorphism in the 3′ untranslated region of exon 4; in T1DM susceptibility[101,102]. A systemic review and meta-analysis had shown that CTLA4 c.A49G (p.T17A, rs231775) polymorphisms can highly increase T1DM risk in children and can be used as a genetic marker.[103] The presence of the G allele affects CTLA4 protein characteristics including hydrophobicity, signal peptide polarity, and α-helix propensity.[104] Similar results were shown in meta-analysis of 52 studies involving 11,017 T1DM patients.[105,106]

Interestingly, a microarray analysis reported many deregulated miRNAs in newly diagnosed T1DM pediatric patients. *CTLA4* gene was found to be repressed by miR-487a-3p by binding to its 3'UTRs. This can significantly increase T1DM risk.[107]

Several Arab countries revealed a positive correlation between CTLA4 gene and T1DM development, including rs3087243, rs231775, rs5742909, rs11571316, rs1427676, and rs231727 SNPs. [104,108–112]

1.9.1.4. Protein Tyrosine Phosphatase Non-Receptor Type 22 (*PTPN22*)

PTPN22 gene is located on the short arm of chromosome 1 (1p13.3–13) as shown in figure (11), and contains 25 exons. It encodes a lymphoid-specific tyrosine phosphatase

(Lyp) which has a role in autoimmunity by inactivation and inhibition of T-cell. It is considered a negative regulator of proximal T cell receptor/B cell receptor (TCR/BCR) signaling. It is consequently associated with autoimmune diseases as rheumatoid arthritis (RA), Graves' disease, systemic lupus erythematosus (SLE), type 1 diabetes mellitus (T1DM), Behcet's disease (BD), inflammatory bowel disease (IBD), Sjögren's syndrome, Hashimoto thyroiditis, celiac disease, and others.[113]

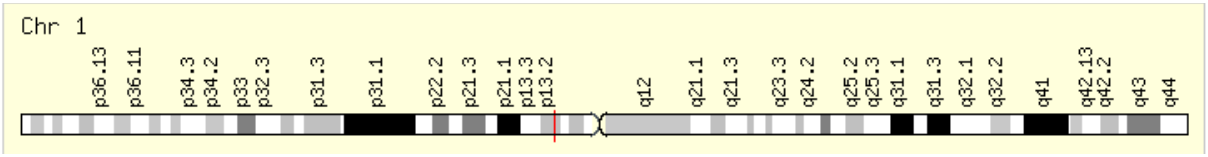

Figure (11): Location of *PTPN22* gene on the human genome.

Role in T1DM:

Several SNPs have been identified in *PTPN22* gene; PTPN22 c.C1858T (rs2476601) in exon 14 is the most important one in autoimmunity. This SNP change cytosine to thymidine at 1858 nucleotide leading to the change of arginine to tryptophan at codon 620 (p.R620W) of polyproline-binding motif P1. Therefore, it damages the ability of PTPN22 to bind to c-Src kinase, leads to an increase in the activity of LYP protein, and thus inhibits the signaling of T-cell. Many studies confirm the association between c.C1858T polymorphisms and T1DM, while only a few did not[114]. Table (4) summarizes this association in several populations.[8,111] A Japanese study showed another SNP to be associated with T1DM risk, which is rs1310182.[116]

In Emirates, a recent study was conducted on T1DM patients to determine the role of four genes; *INS*, *CTLA4*, *IL2-RA*, and *PTPN22* in the disease risk. Two SNPs of *PTPN22* gene

rs2476601 and rs1310182 were studied. Results showed the association of both these SNPs in T1DM risk. The A allele and AG genotype of rs2476601 (c.C1858T) significantly increase T1DM risk (OR= 6.25 and 6.27, respectively). Of rs1310182 SNP, G allele and GG genotype increase the risk (OR=2.41 and 4.72, respectively). Similar results were found in Columbian and Brazilian studies.[117,118]

Interestingly, a previous study in Tunis evaluated the role of genes involved in the T cell activation pathway in T1DM development including *TCR/CD3, CD28, ZAP70,* and *PTPN22* genes. They showed a positive correlation of these genes with T1DM. This results from problems in the lineage-commitment decisions during T cell development leading to autoimmunity[119]. Moreover, PTPN22 rs2476601 allele A was found to be associated with increased frequencies of T regulatory cells (Which has an important role in immune homeostasis maintenance) in T1DM patients. The association was observed by low CD4+CD25+CD127.[120]

Table (4): Association of PTPN22 c.C1858T polymorphism with T1DM susceptibility.[115]

Population	Case/controls	Genotype/allele/ polymorphism	Association
Saudi	372/372	T-allele	Susceptible
German	220/239	C1858T	Susceptible
Egyptian	150/165	T-allele	Susceptible
Egyptian	120/120	T-allele	Susceptible
Kuwaiti Arabs	253/2014	T-allele	Susceptible
Chinese	202/240	C1858T	Susceptible
Chinese	364/719	C1858T	No association
Brazilian	612/792	C1858T	Susceptible
Brazilian	205/308	C1858T	Susceptible
Polish	215/236	C1858T	Susceptible
Polish	147/327	C1858T	Susceptible
Russian	27/62 families	C1858T	Susceptible
Croatian	102/193	T-allele	Susceptible
Caucasian	140/100	T-allele	Susceptible
Caucasian	8677	C1858T	Susceptible
Caucasian	113	C1858T	Susceptible
Czechs	372/400	T-allele	Susceptible
Iranian (Azeri)	160/271	T-allele	Susceptible
Iranian	99/100	C1858T	Susceptible
Iranian	144/197	C1858T	No association
Estonian	170/230	T-allele	Susceptible
Italian	271/89	C1858T	Susceptible
Spanish	316/554	T-allele	Susceptible
Colorado	753/662	CT, TT	Susceptible

1.9.1.5. Interleukin 2 Receptor Subunit Alpha (*IL2RA*)

IL2RA gene is located on the short arm of chromosome 10 (10p15.1) as shown in figure (12) and consists of 8 exons. It is also called CD25. It encodes the α-chain of the interleukin 2 receptor that has high affinity to interleukin 2. *IL2RA* is associated with Multiple sclerosis (MS), Rheumatoid arthritis (RA), Graves' disease, T1DM, and lung cancer.[121,122]

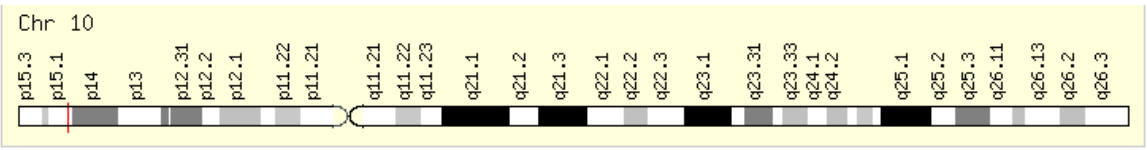

Figure (12): Location of *IL2RA* gene on the human genome.

IL2RA is also called IDDM10. It has a critical role in immune regulation; its expression on regulatory T cells is important for development, homeostasis, and suppressing T-cell immune responses. Therefore, it is associated with the autoimmunity role of T1DM.[123]

IL2RA was first reported in 2005 as the fifth T1DM genetic locus.[9] Among IL2RA polymorphisms, two (rs706778 and rs3118470) had a significant association with T1DM. These SNPs are located in the 5' end of intron 1.[124] Previous study revealed the causal role of IL2RA in T1DM and showed the association between reduced circulatory concentrations of IL2RA and T1DM-SNPs in intron 1 and the intergenic region between IL2RA and RBM17.[125] A meta-analysis had reported additional SNPs associated with T1DM including rs2104286 (in intron 1), rs11594656, and rs41295061 (both mapping to the 5' flanking region)[126]. A recent study in Emirates (mentioned above) studied rs12251307 SNP of IL2RA only. The T allele, TC, and TT genotypes were found to have a significant association with T1DM (OR=3.82, 3.8, and 16.53, respectively).[112]

1.9.1.6. interferon-induced with helicase C domain 1 (*IFIH1*)

IFIH1 gene is located on the long arm of chromosome 2 (2q24.2) as shown in figure (13) and contains 16 exons. It encodes MDA5 protein which is a cytoplasmic viral RNA receptor that play role in innate immunity and activates interferon I signaling through the Mitochondrial antiviral-signaling protein (MAVS). It is associated with Graves' disease, immunoglobulin A deficiency, SLE, T1DM, and others. [127]

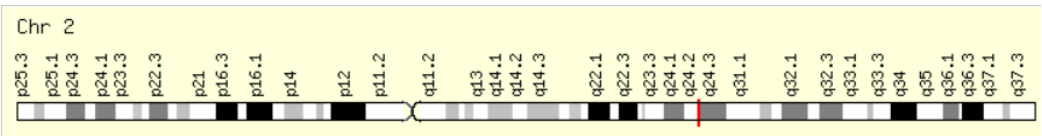

Figure (13): Location of *IFIH1* gene on the human genome.

Role in T1DM:

IFIH1 is also called IDDM19. It was first reported in 2006 as the sixth genetic locus associated with T1DM. [10] Since IFIH1 has a critical role in antiviral activity, its genetic polymorphisms affect viral infections fate by weakening the defense system. This results in excessive inflammation and death of beta cells which can trigger T1DM.[114,123,128] Four SNPs of IFIH1 (rs1990760, rs3747517, rs2111485, rs13422767) were found to be associated with T1DM in many populations[129–131]. Additional SNP rs10930046 was reported in a Columbian study[117]. Amusingly, a previous study showed a significant association of IFIH1 SNPs in increasing the frequency of Enterovirus RNA in blood. This can elucidate the role of this candidate gene in T1DM development. [132]

1.9.1.7. Inositol 1,4,5-Trisphosphate Receptor Type 3 (*ITPR3*)

ITPR3 gene is located on the short arm of chromosome 6 (6p21.31) as shown in figure (14), and 500 kb centrometric to HLA class II genes. It encodes a receptor for inositol 1,4,5-trisphosphate which is a second messenger that leads to intracellular calcium release. [133]

Figure (14): location of *ITPR3* gene on the human genome.

Role in T1DM:

ITPR3 protein regulates both insulin and pancreatic excretion via calcium metabolization. A Swedish case-control study mapped the HLA region had revealed a peak of association of ITPR3 gene in T1DM, independently of HLA class II effects.[134] Therefore, a Canadian study replicated this association in a family-based study. However, they found an association between rs2296336 SNP of ITPR3 and T1DM but in linkage disequilibrium (LD) with HLA DQB1.[135]

1.9.1.8. Forkhead Box P3 (*FOXP3*)

It is located on the short arm of chromosome X (Xp11.23) a shown in figure (15). It encodes a member of the forkhead/winged-helix family of transcriptional regulators. It is associated with enteropathy, immunodeficiency polyendocrinopathy, Hashimoto thyroiditis, and X-linked syndrome (IPEX).

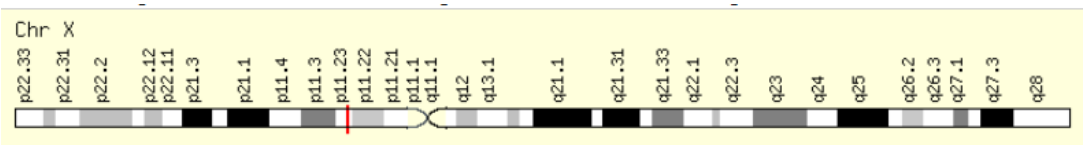

Figure (15): Location of *FOXP3* gene on the human genome.

Role in T1DM:

FOXP3 plays role in immune homeostasis and regulation of T-cell activation thus can mediate autoimmunity of T1DM. [136] A Japanese study reported two regions with microsatellite SNPS; one under LD in intron 5, and other (GT)n in intron zero with significant susceptibility in T1DM. [137] However, other studies showed no association[138,139].

1.9.2 Genome Wide Association Study

In 2007, genome-wide association studies were started to search for further loci associated with T1DM. They confirmed the previous loci and found new ones.

1.9.2.1 C-Type Lectin Domain Containing 16A (*CLEC16A*)

CLEC16A is located on the short arm of chromosome 10 (16p13.13) as shown in figure (16) and contains 24 exons. It encodes a member of the C-type lectin domain-containing family. It is associated with diseases like Multiple Sclerosis (MS), Crohn's disease, Primary Adrenal Insufficiency, Diabetes Mellitus, and Rheumatoid arthritis.

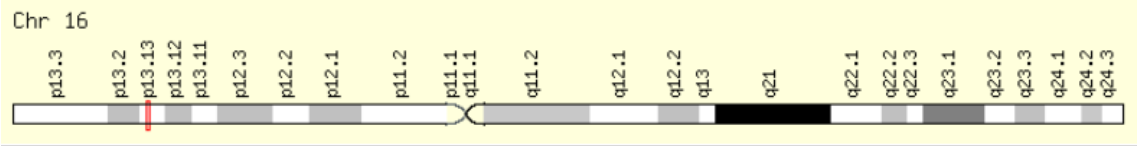

Figure (16): Location of *CLEC16A* gene on the human genome.

Role in T1DM:

In a GWAS in a large European pediatric cohort, 500000 SNPs were genotyped. A significant association was found in a new variation near 16p13 that contains the KIAA0350 gene (now called CLEC16A). Three SNPs (rs2903692, rs725613, and rs17673553) were in a strong association.[140] KIAA0350 gene was independently replicated and confirmed to associate with T1DM and other autoimmune diseases.[141–143] In China, 205 T1DM patients were genotyped to investigate whether *CLEC16A* gene is associated with T1DM. They showed a strong association with the intron SNP rs725613.[144] Similar results were found in Spanish, Japanese, and US Caucasian populations. [145–147]

The protein encoded by CLEC16A plays role in glucose homeostasis by regulating mitophagy of beta cells[148,149]. This achieved via upstream regulation of the Nrdp1/Parkin pathway. Deletion of CLEC16A increases Parkin which is the major regulator of mitophagy. Patients with CLEC16A T1DM risk allele (rs12708716) G showed reduced islet CLEC16A expression and insulin secretion. Moreover, Deletion of CLEC16A in mice resulted in hyperglycemia and insulin secretion defections.[150] Another study reported that CLEC16A can form ubiquitin-dependent complex with NRDP1 and USP8 to regulate β-cell mitophagy. [151]

Interestingly, CLEC16A Knockout was performed in mice to examine its role in autoimmunity. The results showed disrupted mitophagy and upregulated inflammatory cytokine response. Thus this can increase the risk of autoimmunity.[152]

1.9.2.2. Erb-B2 Receptor Tyrosine Kinase 3 (*ERBB3*)

ERBB3 gene is located on the long arm of chromosome 12 (12q13.2) as shown in figure (17). It encodes a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases. It is associated with Lethal Congenital Contracture Syndrome 2 and Erythroleukemia.

Figure (17): Location of *ERBB3* gene on the human genome.

Role in T1DM:

It was first reported to associate with T1DM in the Wellcome Trust Case–Control Consortium (WTCCC) genome-wide study. They found a locus surrounded ERBB3

gene.[153] Later, it was studied and reported in several populations. They detected many SNPs in ERBB3 including rs773107, rs2271189, rs2292239 and rs10876864, and rs1701704[154–159]. ERBB3 has a role in glucose metabolism by interacting with PIK3, which in turn activates the downstream mTOR signaling pathway and regulates insulin production. [160,161]

1.9.2.3. Src Homology 2B Adaptor Protein 3 (*SH2B3*)

SH2B3 gene is located on the long arm of chromosome 12 (12q24.12) as shown in figure (18). It encodes lymphocyte adapter protein (LNK) which is a member of the SH2B adaptor family of proteins. LNK is involved in many signaling pathways. It is associated with cardiovascular and autoimmune diseases including celiac disease, myocardial infarction, type 1 diabetes, hypercholesterolemia, hypertension, and thromobocythemia.

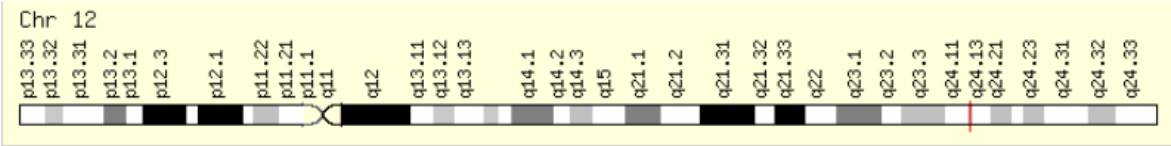

Figure (18): Location of *SH2B3* gene on the human genome.

Role in T1DM:

Todd et al reported 12q24 as a new locus associated with T1DM. rs3184504 is the most common SNP of SH2B3. It encodes the change from Arginine to Tryptophan at codon 262 (p.R262W). [145,162]

SH2B3 has a role in regulatory T-cells. It also affects glucose tolerance and insulin response. A recent study showed that loss of SH2B3 in mice leads to insulin resistance

rather than insufficiency. This was linked to impaired activation of IL-15-dependent adipose G1-ILCs that LNK regulates[163]. Another study showed the role of SH2B3 in increasing myeloid cells and expansion of CD8+ T cells.[164]

1.9.2.4. Protein Tyrosine Phosphatase Non-Receptor Type 2 (*PTPN2*)

PTPN2 gene is located on the short arm of chromosome 18 (19p11.21) as shown in figure (19). It encodes PTPN2 protein which is a member of the protein tyrosine phosphatase (PTP) family. It is associated with Inflammatory bowel disease, RA, T1DM, and metabolic syndromes.[165]

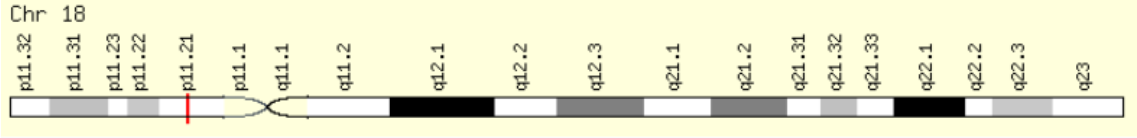

Figure (19): Location of *PTPN2* gene on the human genome.

Role in T1DM:

PTPN2 is expressed in immune cells and upregulated by cytokines. It is a negative regulator of STAT, JAK, STAT, MAPK, EGFR, and insulin receptors. Knockdown of PTPN2 leads to beta-cell apoptosis[166–168]. Another study revealed the role of PTPN2 in T1DM pathogenesis by modifying the β-cell responses to a viral infection. Defectives in the gene lead to an exaggerated inflammatory response to viral infections and excessive beta-cell death.[169] PTPN2 is reported to be associate with T1DM in several studies. Its major SNP (rs1893217) causes aberrant T-cell activation. [98,159,170]

1.9.2.5. Ubiquitin-Associated and SH3 Domain-Containing Protein A (*UBASH3A*)

UBASH3A gene is located on the long arm of chromosome 21 (21q22.3) as shown in figure (20). It is also called STS-2, TULA, and CLIP4. It encodes a member of the T-cell ubiquitin ligand (TULA) family that plays role in the negative regulation of T cell signaling, thus it is primary expressed in T cells. It has two domains; SH3 histidine phosphatase.[171]

Figure (20): Location of *UBASH3A* gene on the human genome.

Role in T1DM:

The Type 1 Diabetes Genetics Consortium study found a significant SNP (rs876498) to increase T1DM risk. This SNP was mapped to intron 6 of the UBASH3A gene.[172,173] UBASH3A mediate T1DM by inhibiting the NF-κB signaling pathway through stimulation of T-cell receptor (TCR) and suppressing the IKK complex. A previous study showed that T1DM-risk SNPs (rs11203203 and rs80054410) increase the expression of UBASH3A in CD4+ T cells, inhibit NF-kB pathway, and decrease the expression of IL2 gene.[171] In contrast, in a recent study, mice had been targeted of UBASH3A using zinc-finger nuclease mediated mutagenesis. They showed significant insulitis in targeted mice compared to WT mice. Interestingly, these UBASH3A-defecient mice developed T1DM and exhibited accumulation of autoreactive T cells in spleen and pancreatic lymph node.[174]

1.9.2.6. Peptidylprolyl Isomerase Like 2 (*PPIL2*)

PPIL2 gene is located on the long arm of chromosome 22 (22q11.21) as shown in figure (21). It belongs to the cyclophilin family of peptidylprolyl isomerases. It plays role in immunosuppression and protein folding. It is highly expressed in testis, thymus, and pancreas.

Figure (21): Location of *PPIL2* gene on the human genome.

In the TEDDY study (The Environmental Determinants of Diabetes in the Young), they aimed to identify genes associated with autoimmunity and T1DM. It included 5806 subjects. They found an rs428595 SNP mapped to a novel region near PPIL2 gene associated with islet autoantibodies[175,176]. Other genes mapping to the locus are *UBE2L3*, *MAPK1*, *YDJC*, *YPEL1*, *CCDC116*, *SDF2L1*, and two microRNAs (MIR301B and MIR130B).[57]

1.9.2.7 Other Loci

Several other loci were reported to in a meta-analysis to have a significant association with T1DM risk (shown in Figure (22)).

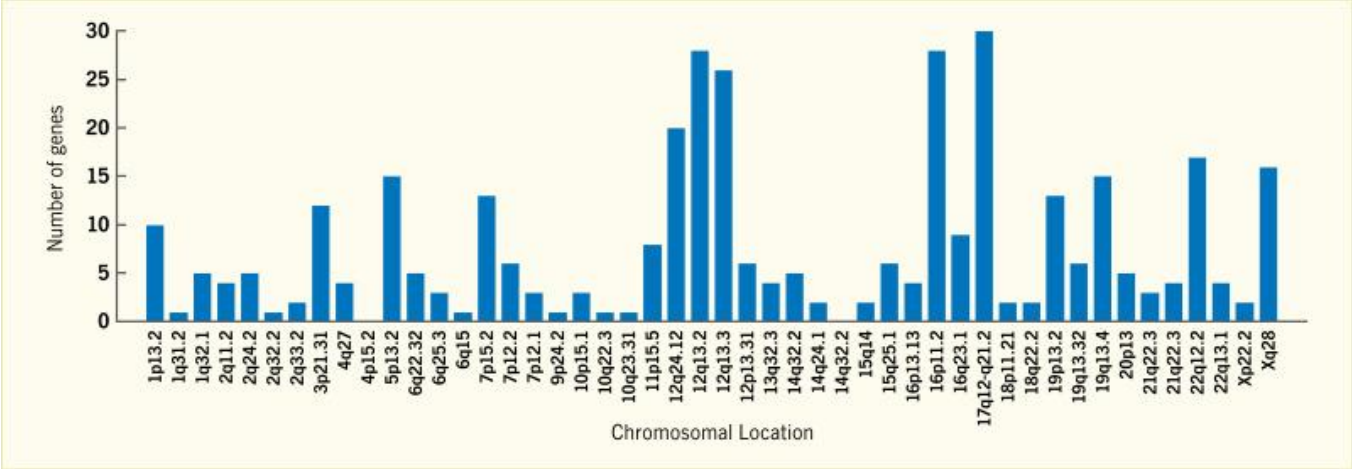

Figure (22): Type 1 Diabetes Susceptibility Loci Identified by Genome-Wide Association Study Meta-Analysis.[85]

1.10 Study Problem and Objectives:

In Type 1 Diabetes, genetic factors play a central role in its development during fetal life and the early years after birth. Several previous studies reported several genetic factors in different populations that are associated with the disease. However, the accumulated data is widely sporadic and the genetic basis of the disease is poorly understood. Very limited studies based on linkage analysis are available. One major obstacle is the limited availability of familial cases that can provide strong tool for that purpose. We have identified two Palestinian families having several affected members with clear variation in the inheritance pattern of the disease. We hypothesized the presence of putative gene mutations that have a direct role in developing T1DM in these families. Therefore, our objective is to clearly identify causative gene(s) responsible for T1D development in these patients using WES by NGS technology. The identified genes will provide solid valuable information to increase understanding of the molecular genetics basis of Type 1 diabetes. They represent potentially candidate gene targets for diagnosis and therapy.

Chapter 2: Methodology

2.1 Study Subjects:

The study was conducted on two Palestinian families inflicted with Type 1 Diabetes. They were confirmed to have T1DM in Layan Medical Center based on specific parameters illustrated in Table (5). Family I is composed of diabetic parents, 6 diabetic offspring, and one non-diabetic daughter indicating the presence of a potentially dominant gene factor. Family II is composed of non-diabetic parents, 3 diabetic offspring, and a non-diabetic daughter, indicating the presence of a potentially recessive gene mutation. Figure (23) shows the pedigree of both families. Written informed consent was obtained from all subjects to participate in the study. (Enclosed in Appendix)

Table (5): Diagnostic characteristics of diabetic patients in Family I and II.

Parameter	Patients' results
HbA1C level	> 7%
C-peptide level	Low
Insulin dependence	Dependent
Autoantibodies	Present

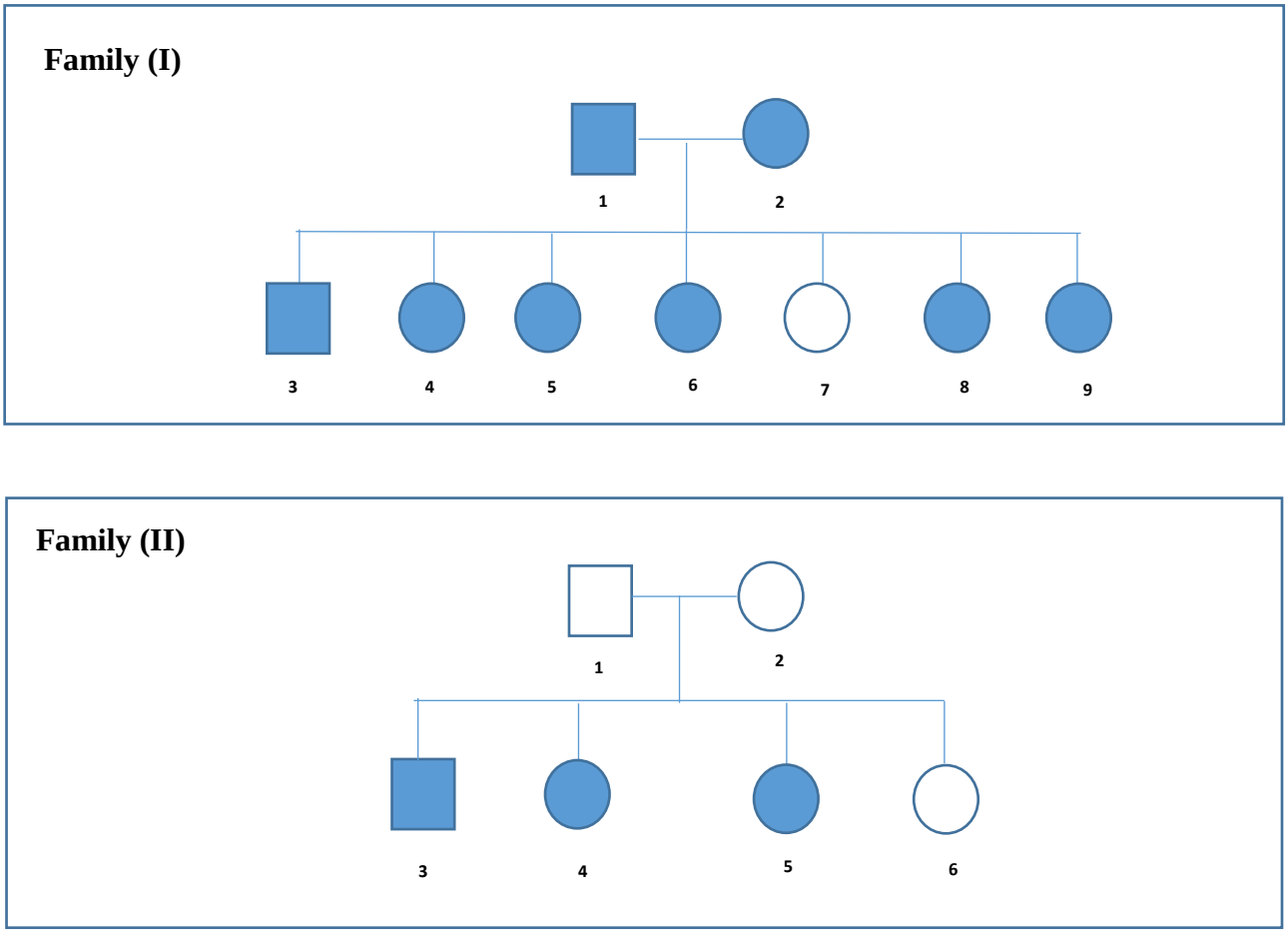

Figure (23): Families' Pedigrees. Family I is composed of diabetic parents, 5 diabetic offspring, and two non-diabetic daughters. Family II is composed of non-diabetic parents, 3 diabetic offspring, and a non-diabetic daughter.

2.2 Genomic DNA Preparation:

2.2.1 DNA Extraction:

Whole EDTA blood samples (3 mL) were collected and centrifuged for 10 minutes at 3000 rpm. Buffy coat was used to extract DNA according to a commercially available kit (Master PureTM Genomic DNA Purification Kit, Epicenter Technology Co. Cat.No.MG71100)[177] according to the manufacture guidelines with some modifications.

First, 150 µl of the buffy coat was mixed with 600 µl of Cell Lysing Solution in a sterile microcentrifuge tube. The tube was inverted several times and incubated at room temperature for 5 minutes. This was repeated X1 time. The sample were centrifuged at high speed (14000 rpm) for 25 seconds. The Supernatant was discarded leaving 50 µl lysate, and the remaining white pellet was vigorously vortexed and resuspended in 300 µl of lysis buffer 2. After the addition of 250 µl of a precipitation solution, the mixture was vortexed for 45-60 seconds and the sample was centrifuged at 14000 rpm for 10 minutes. The supernatant was poured into a new Eppendorf tube and 700 µl of ice cold isopropanol was added. The tube was then inverted 30-40 times until a stringy precipitate of DNA was visible. The DNA was pelleted by centrifugation for 10 minutes at 14000 rpm and the supernatant was discarded. The DNA pellet was rinsed twice with 200 µl of 75% cold ethanol to wash the remaining isopropanol. After that, ethanol was aspirated and the tube was inverted for air dry. Finally, 200 µl of TE buffer were added. The DNA pellet was dissolved and the samples were stored frozen at-30C until use.

2.2.2 DNA Quantification:

Nanodrop spectrophotometer was used to check the purity and concentrations of extracted DNA. The absorption was measured at 260 nm for nucleic acid, 280 nm for proteins, and 230 nm for salts. DNA concentration can be calculated in µg/mL by multiplying the absorbance of DNA at 260nm by dilution factor and 50 µg/mL (represent that 1 OD = 50 g/mL for dsDNA).

2.2.3 DNA Qualification

DNA was run in the Agarose Gel Electrophoresis to test its size and integrity. 0.8 grams of agarose were added to TAE buffer and boiled, and then mixed with ethidium bromide.

2.3 Next-Generation Sequencing

One patient from each family was selected. Their samples were subjected to whole-exome sequencing using Illumina 5500 according to manufacturer's instructions [178], briefly as follows:

1. **Genomic DNA Tagmentation**

In order to fragment and tag the DNA with adaptor sequences, 30 µL (Total amount = 500 ng) of each sample were added to separate wells in a 96-well PCR plate. Tagmentation master mix which consists of eBLT and TB1 was added to each well. The plate was sealed with Microseal B and placed on the thermal cycler. (preheat lid 100 C, reaction volume 50 µL, 55 C for 5 minutes, Hold 10 C).

2. Post-Tagmentation Cleanup

For washing the adaptor-tagged DNA, 10 μL of Stop Tagment Buffer 2 (ST2) was added to the tagmentation reaction, followed by shaking at 1600 rpm for 1 minute and placed on magnetic stand. Samples were then washed using 100 μL of Tagment Wash buffer (TWB) added to the beads and placed on the magnetic stand. Supernatant was then discarded. These steps were repeated 2X times.

3. Tagmented DNA Amplification

To Amplify the Tagmented DNA, 40 μL of PCR master mix (EPM) was added to each well. Pre-paired 10 base pairs Index 1 and index 2 adaptors were added from the index adaptor plate to each well. Tagmented DNA was amplified using 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).

4. Libraries Cleanup

To purify the amplified libraries, 45 μL supernatant from each well was transferred to the corresponding well of a new MIDI plate. Nuclease-free water (77 μL) and AMPure XP beads (88 μL) were added. Fresh 80% Ethanol (200 μL) was used for washing before the addition of 17 μL RSB.

5. Pre-Enriched Libraries Qualification

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

6. Pre-Enriched Libraries Pooling

DNA libraries were combined with specific indexes for each library into one pool. For a final volume of 30 µL (150 ng DNA), specific volume from each sample was calculated according to its concentration and then added together onto the PCR tube.

7. Probes Hybridization

Targeted regions of DNA were bound to capture probes.

50 µL NHB2, 10 µL Enrichment probe panel, and 10 µL EHB2 were added to 30 µL of the sample in a new tube, and then placed on the thermal cycler with the NF-HYB program 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.

8. Hybridized Probes Capture

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

9. Enriched Library Amplification

To amplify the enriched library,5 μL PCR primer Cocktail (PPC) and 20 μL Enhanced PCR mix (EPM) were added. The sample was centrifuged and then placed on the thermal cycler for processing with the following program:

Preheat lid option 100 C, Reaction volume 50 μL, 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.

10. Amplified Enriched Library Cleanup

AMPure XP Beads (45 μL) were added to the sample tube for library purification. Then, the sample was washed two times by adding 200 μL 80% EtOh followed by 32 μL RSB.

11. Purification and Quality Control

The concentration of library was measured by Qubit dsDNA HS assay kit #Q23850 kit. Whereas, mean fragment size was measure by High sensitivity DNA kit catalog #5067.

2.4 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 Diabetes. 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 other), 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 (24) shows a summary of the analysis steps.

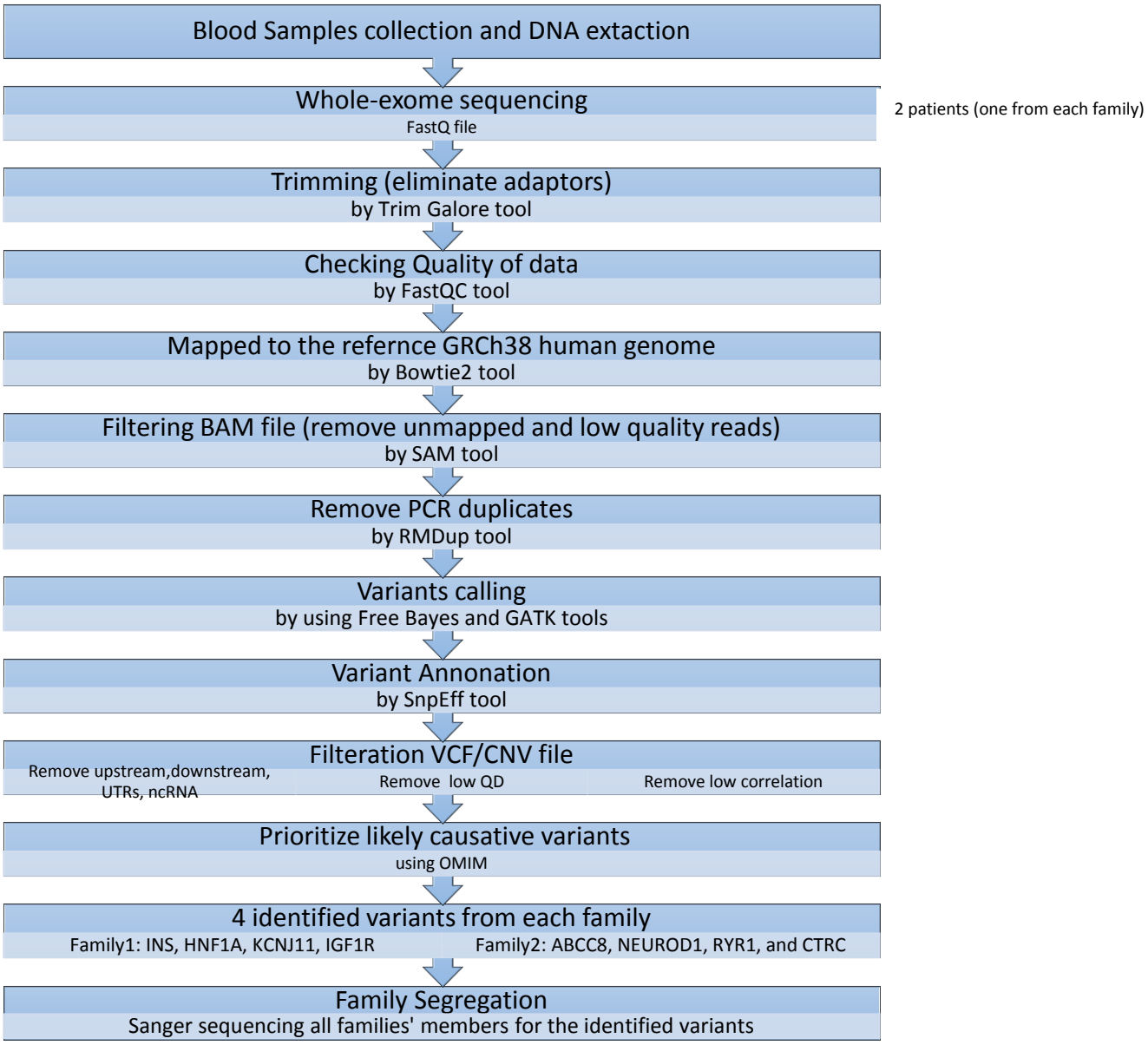

Figure (24): Flowchart of analysis steps.

2.5 Family Segregation

The most suspected variants in the first family were in genes *INS*, *HNF1A*, *KCNJ11*, and *IGF1R*. Whereas in the second family they were in *ABCC8*, *NEUROD1*, *RYR1*, and

CTRC genes. Specific primers were designed for each variant. PCR amplification followed by Sanger sequencing were then done.

2.5.1 Primers Design

Primer 3 web (https://primer3.ut.ee/) was used to design the primers listed in Table (6) below:

Table (6): The sequences and characteristics of the variants' primers.

Gene name	Sequence of primer	Primer length	GC% content	Tm °C
INS	Forward: TGGCAGAAGGACAGTGATCT	20	50	55.8
	Reverse: TCAGAAGAGGCCATCAAGCA	20	50	56.3
KCNJ11:	Forward: AGAGATGCTGAACTTGGGCT	20	50	56.4
	Reverse: ATTGTAGCTGAGGAGGACGG	20	55	56.3
HNF1A:	Forward: CAGTGAGTCCGGGCTTCA	18	61.11	57.2
	Reverse: GTGACGGACAGCAACAGAAG	20	55	56.1
ABCC8:	Forward: TTTGAAGAAGCTGACGTGGC	20	50	55.9
	Reverse: CCATGAGATGACTGTGGGGA	20	55	56.4
NEUROD1:	Forward: ACTGGTAGGAGTAGGGGTGT	20	55	56.9
	Reverse: ACCTGGTCTCCTTCGTTCAG	20	55	56.4
RYR1:	Forward: GTTGACGCTTCCTTCATGCA	20	50	56
	Reverse: TGAGGTTCCAAGGCTCCATT	20	50	56.4

IGF1R	Forward: GAGCCCGGCATCTTACTACA	20	55	56
	Reverse: TTGCGAAGAAGTGTGGATGC	20	55	56
CTRC	Forward: CTGAGAAGCCAACCCACATC	20	55	55.9
	Reverse: AGCTGACGATGCCAAACAC	19	52.6	56

2.5.2 PCR Amplification

Each family member of the first family were tested for variants in the following genes: *INS, HNF1A, KCNJ11,* and *IGF1R*. Similarly, each family member of the second family were tested for variants in *ABCC8, NEUROD1, RYR1,* and *CTRC* genes. The Reagents and volumes needed for each PCR reaction are listed in the Table (7):

Table (7): Reagents and volumes used for PCR reaction.

Reagent	Volume
PCR master mix (1X)	10 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	8.0 µl
Total volume	20 µl

After that, PCR mixtures were placed on the thermocycler in a program of the following conditions listed in Table (8).

Table (8): PCR program used for amplification variants.

Temperature °C	Time	Cycle numbers
99 °C	Preheat lid	-
95 °C	5 min	1 cycle
95 °C	30 seconds	35 cycles
56 °C	30 seconds	
72 °C	30 seconds	
72 °C	5 min	1 cycle
4 °C	Hold	∞

Finally, PCR products were visualized on 2% agarose gel (2g of agarose with 100 ml TAE buffer). NTC and DNA ladder (50 bp) were also run.

2.5.3 Sanger Sequencing

Samples were sequenced using BigDye™ Terminator Cycle Sequencing Kit and Applied Biosystems Genetic Analyzer as manufacturer's instructions.[179] For PCR clean-up, 1 µL of EPPIC-FAST reagent (Catalog #1021-100F A&A Biotechnology), which is composed of mixtures of thermolabile nucleotide hydrolase and recombinant exonuclease I with increased efficiency, was added to 5 µL of PCR product. Products were then placed on thermocycler at 37°C for 10 minutes followed by 1 minute at 80°C. After that, cycle sequencing was started by adding 18 µL of Big Dye Terminator (BDRR) mix with 2 µL of cleaned PCR and then placed on thermocycler according to the following conditions shown in table (9). Table (10) shows the reagents needed to prepare BDRR mix. Sequenced sample was then followed by EDTA Ethanol precipitation; First, the sample was mixed with 60 µl of 100% cold ethanol and 5 µl EDTA (12.5 mM). Second, cold centrifugation for 30 minutes at 2200g was done. Third, the supernatant was discarded and 80 µl of 80% ethanol was added to the pellet. Fourth, 15 minutes of centrifugation at

1600g was done. Fifth, the supernatant was also discarded and the pellet was air dried. Sixth, High dye was added and then the sample was put on a hot plate at 95°C for 5 minutes followed directly by ice incubation for another 5 minutes.

Table (9): BDRR PCR program used.

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	∞

Table (10): Reagents and volumes used to prepare BDRR mix.

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

2.6 *In-Silico* Analysis

The confirmed and segregated variants, IGF1R p.V579F and NEUROD1 p.P197H, were analyzed using several bioinformatics prediction tools including:

-COBALT Alignment tool was used to detect the conservation of the variant locus. https://www.ncbi.nlm.nih.gov/tools/cobalt

- PolyPhen-2 (Polymorphism Phenotyping v2) http://genetics.bwh.harvard.edu/pph2/

- 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 (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/

These tools are used to predict the impact of amino acid substitution or indels on the protein structure and function.

Finally, haploR tool from R package was used to analyze any motif changing in response to NEUROD1 p.P197H variant. R package is a genome-wide association (GWA) analysis for SNPs that displays results about variants including their phenotypes (traits) tested and, alternative allele, and motifs changed.

Chapter 3: Results

To identify candidate gene variants associated with T1DM, two Palestinian families inflicted with Type 1 diabetes mellitus were included in this study. One diabetic member from each family was selected for next-generation sequencing (NGS) by performing whole exome sequencing (WES). After NGS data analysis and variant filtration, four variants in each family were identified and subjected for direct confirmation by family segregation.

3.1 Family I

The first family is composed of diabetic parents, 6 diabetic offspring, and one non-diabetic daughters. Four variants were identified and information and details about each variant are listed in Table (11). The family segregation is shown in the family pedigree for each variant. Samples from Member I-3 were not available.

Table (11): Information and details about the identified variants in the first family.

Gene	*INS*	*HNF1A*	*KCNJ11*	*IGF1R*
Chromosome	Chr.11	Chr. 11	Chr. 12	Chr. 15
Type of mutation	Missense	Missense	Missense	Missense
Nucleotide change	T176A	A1720G	G1009A	G1735T
Protein change	Intronic	Ser574Gly	Val337lle	Val579Phe
Sanger sequencing	Validated	Validated	Validated	validated
Segregation in family	Not confirmed	Not confirmed	Not confirmed	confirmed

3.1.1 *INS* Gene

The INS gene is located on chromosome 11. The identified variant T176A is located in intron where Thymidine is replaced by Adenine at position 176. This variant was tested and confirmed by sanger sequencing. However, it failed to segregate in the family as expected. Members I-1, I-6, I-7, and I-8 have homozygous TT genotypes, while members I-2, I-4, I-5, and I-9 have heterozygous TA genotypes. This indicates the affected and non-affected individuals have similar genotypes for this gene, as shown in Figure (25).

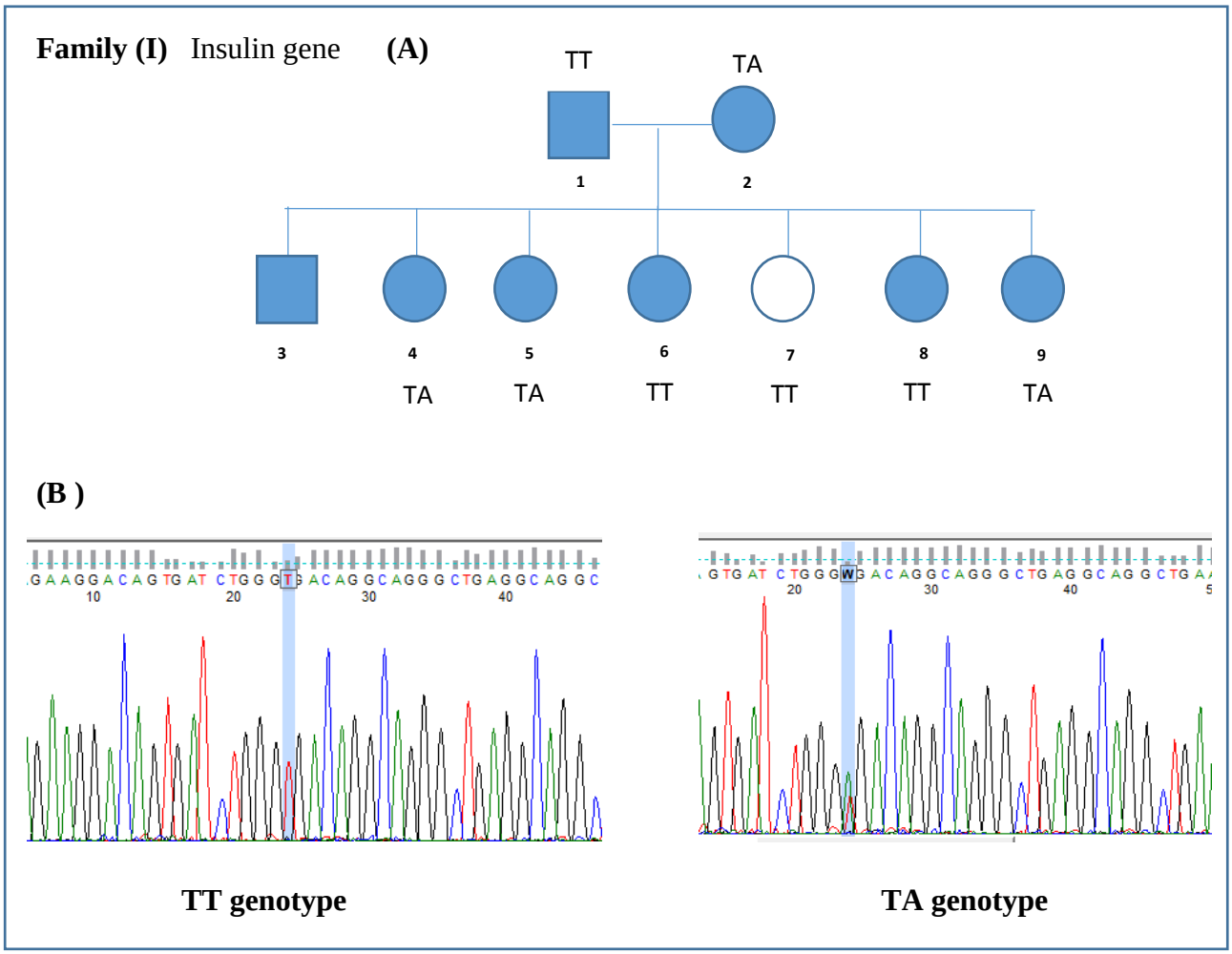

Figure (25): Family segregation of INS variant. (A) Genotype results of each family member shown in the family pedigree. (B) Sanger Sequencing results of the detected variant in Family I.

3.1.2 *KCNJ11* Gene

The KCNJ11 is located on chromosome 11. The identified variant G1009A is a missense mutation where Cytosine is replaced by Thymidine at position 1009. This leads to isoleucine instead of Valine at codon number 337. This variant is also validated by sanger sequencing. However, its segregation didn't match the observed expression of the disease among the family members as described in Figure (26). All affected and non-affected members of family I have similar genotype of this variant; they are homozygous TT.

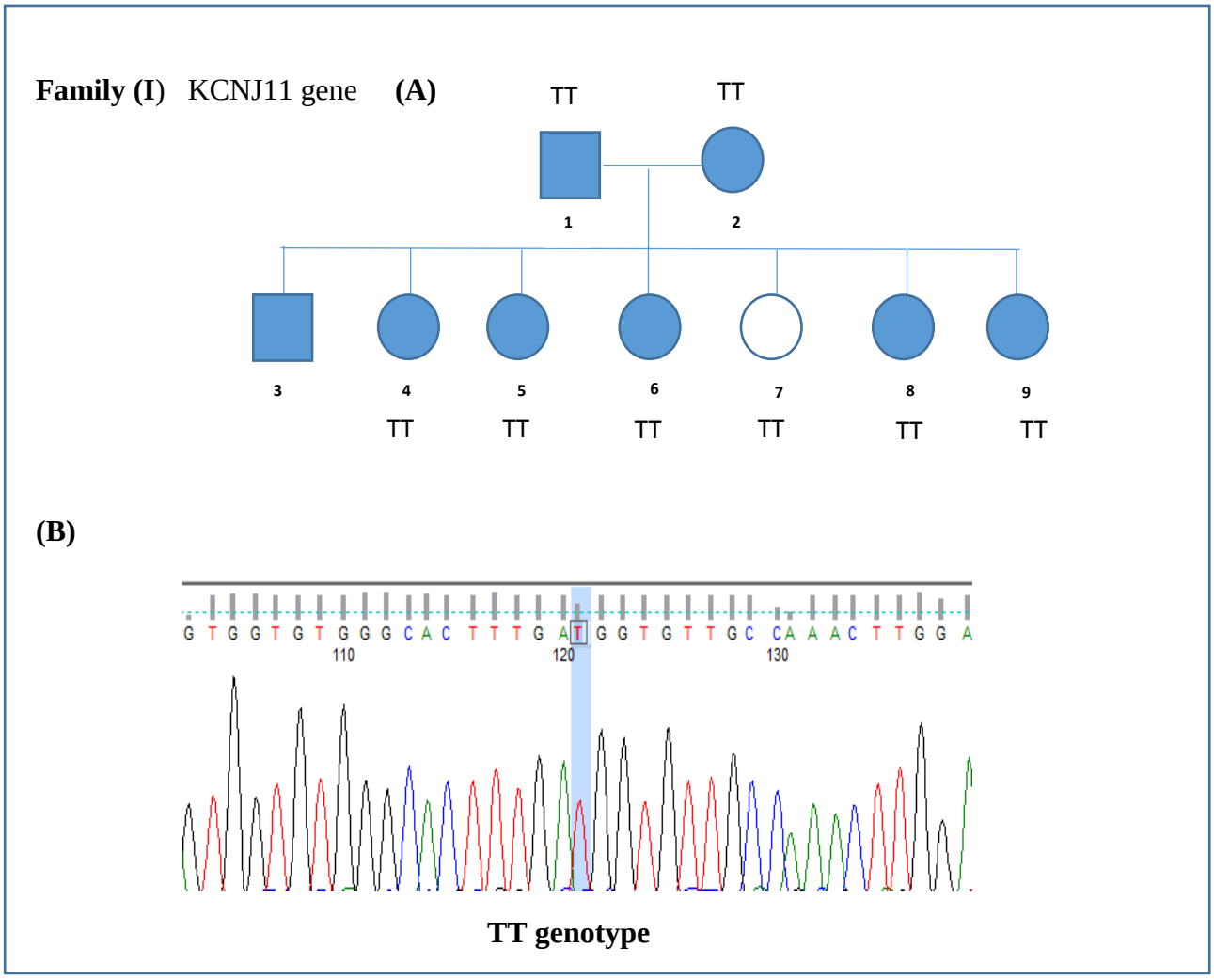

Figure (26): Family segregation of KCNJ11 variant. (A) Genotype results of each family member shown in the family pedigree. (B) Sanger Sequencing results of the detected variant in Family I.

3.1.3 *HNF1A* Gene

The HNF1A is located on chromosome 12. The identified variant A1720G causes a missense mutation where Adenine is replaced by Guanine at position 1720 resulting in Glycine replacing serine at this location. This variant was validated by sanger sequencing, but not segregated since the segregation didn't provide evidence it is the disease causing variant. All members in Family I have homozygous GG genotypes for this variant, as shown in Figure (27).

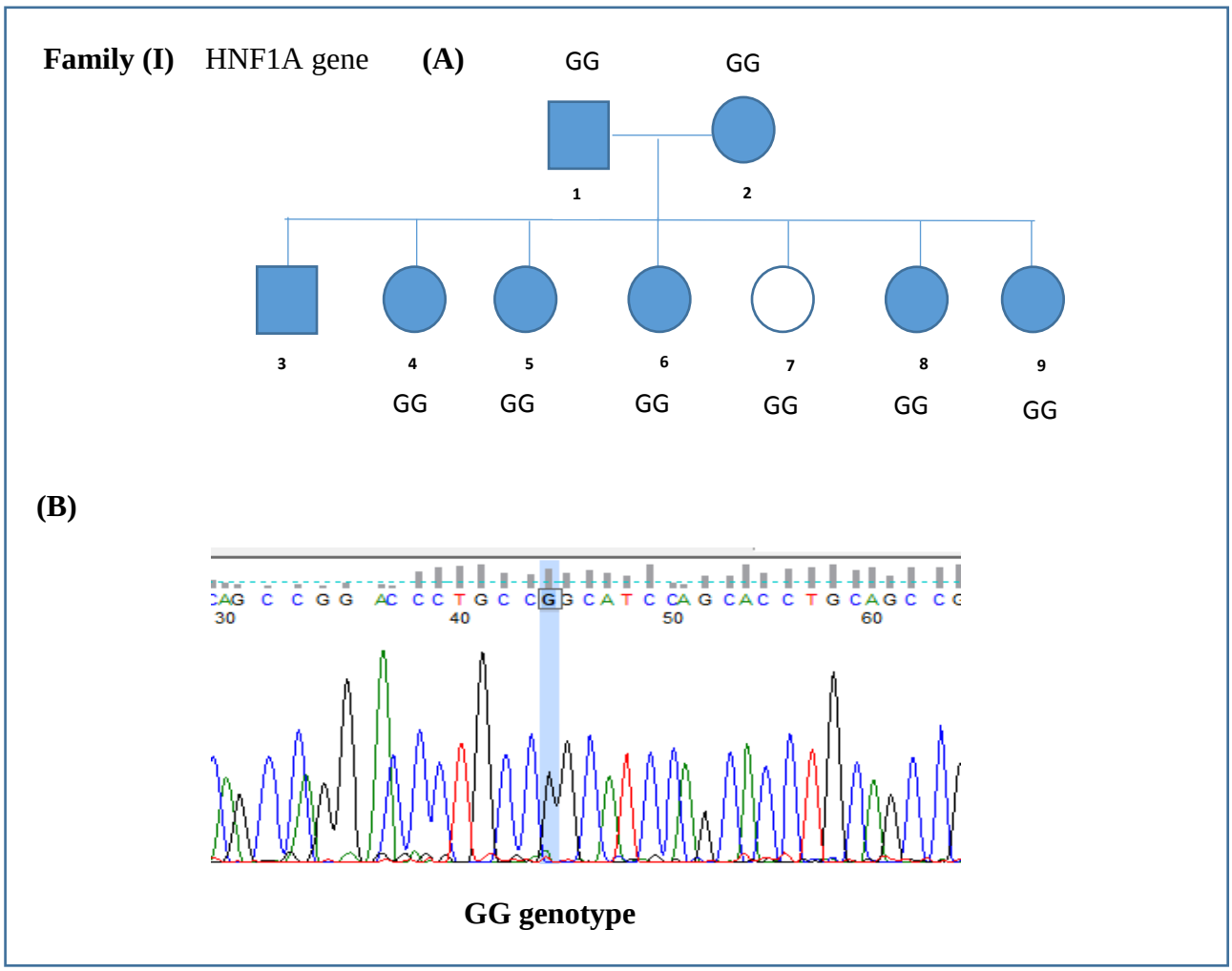

Figure (27): Family segregation of HNF1A variant. (A) Genotype results of each family member shown in the family pedigree. (B) Sanger Sequencing results of the detected variant in Family I.

3.1.4 *IGF1R* Gene

The IGF1R is located on chromosome 15. The identified variant G1735T is a missense mutation where Guanine is replaced by Thymidine at position 1735 leading to convert Valine to Phenylalanine at codon number 579. This variant was confirmed by sanger sequencing and segregated in the family (genotypes match the phenotype). Members I-1, I-2, I-4, and I-9 are heterozygous GT, non-diabetic member I-7 is wild-type GG, and members I-5, I-6, I-8 are homozygous TT, as shown in Figure (28).

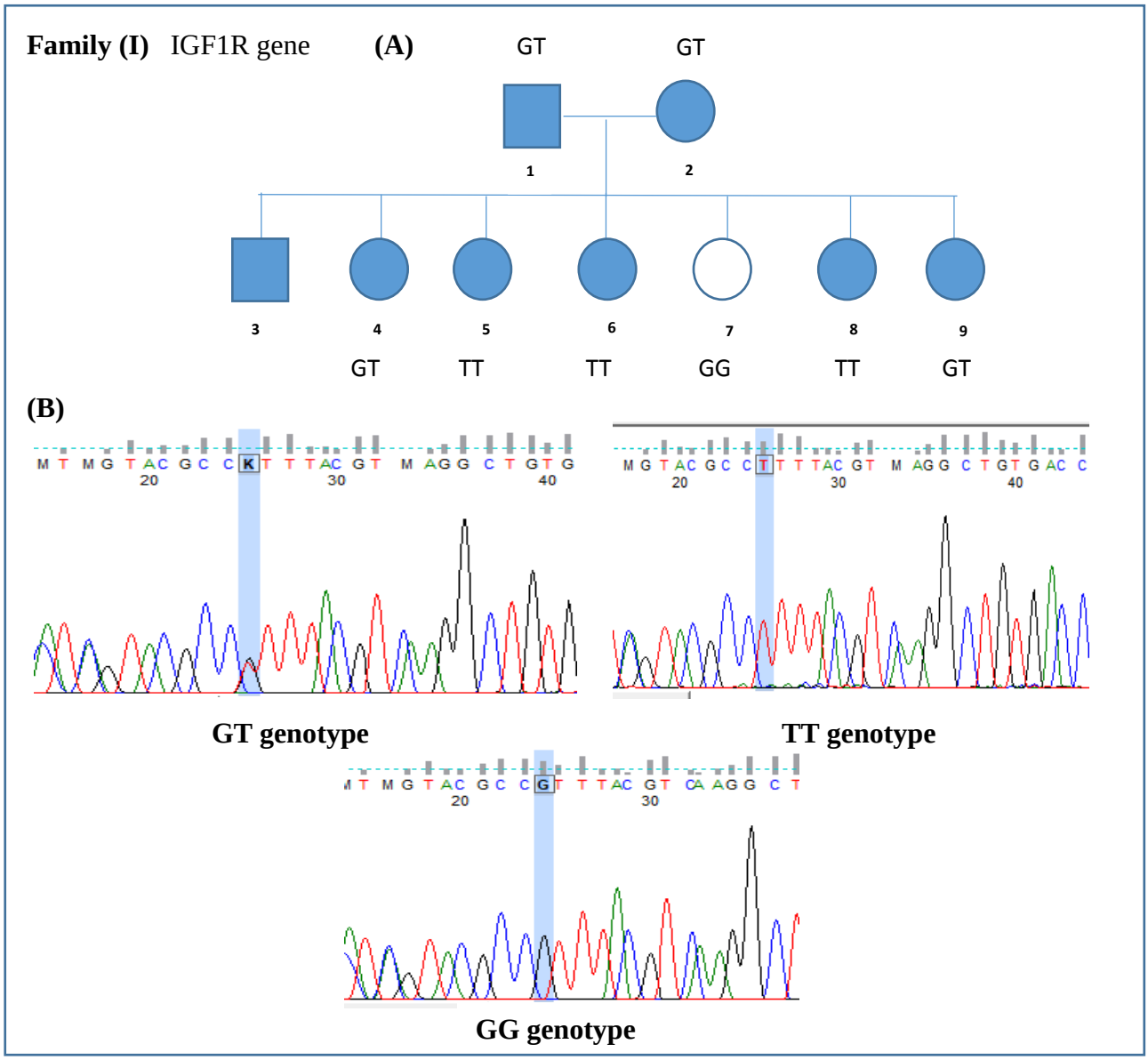

Figure (28): Family segregation of IGF1R variant. (A) Genotype results of each family member shown in the family pedigree. (B) Sanger Sequencing results of the detected variant in Family I.

3.2 Family II

The second family is composed of non-diabetic parents, 3 diabetic offspring, and a non-diabetic daughter. In this family, similarly, four variants were identified and checked. Information and details about each variant are listed in Table (12). The family segregation is also shown below in the family pedigree for each variant.

Table (12): Information and details about the identified variants in the second family.

Gene	*ABCC8*	*NEUROD1*	*RYR1*	*CTRC*
Chromosome	Chr. 11	Chr. 2	Chr. 19	Chr. 1
Type of mutation	non-coding transcript variant	Missense	Insertion	Missense
Nucleotide change	C1686T	G590T	A7835+5G	G649A
Protein change	His562His	Pro197His	-	Gly217Ser
Sanger sequencing	Not validated	Validated and confirmed	Not validated	Validated
Segregation in family	Not confirmed	Confirmed	Not confirmed	Not confirmed

3.2.1 *ABCC8* Gene

The ABCC8 gene is located on chromosome 11. The identified variant C1686T is a non-coding synonymous where Cytosine is replaced by Thymidine with no effects in codon. This variant was not confirmed by sanger sequencing and thus not segregated. All members of Family II are wild-type GG, as shown in Figure (29).

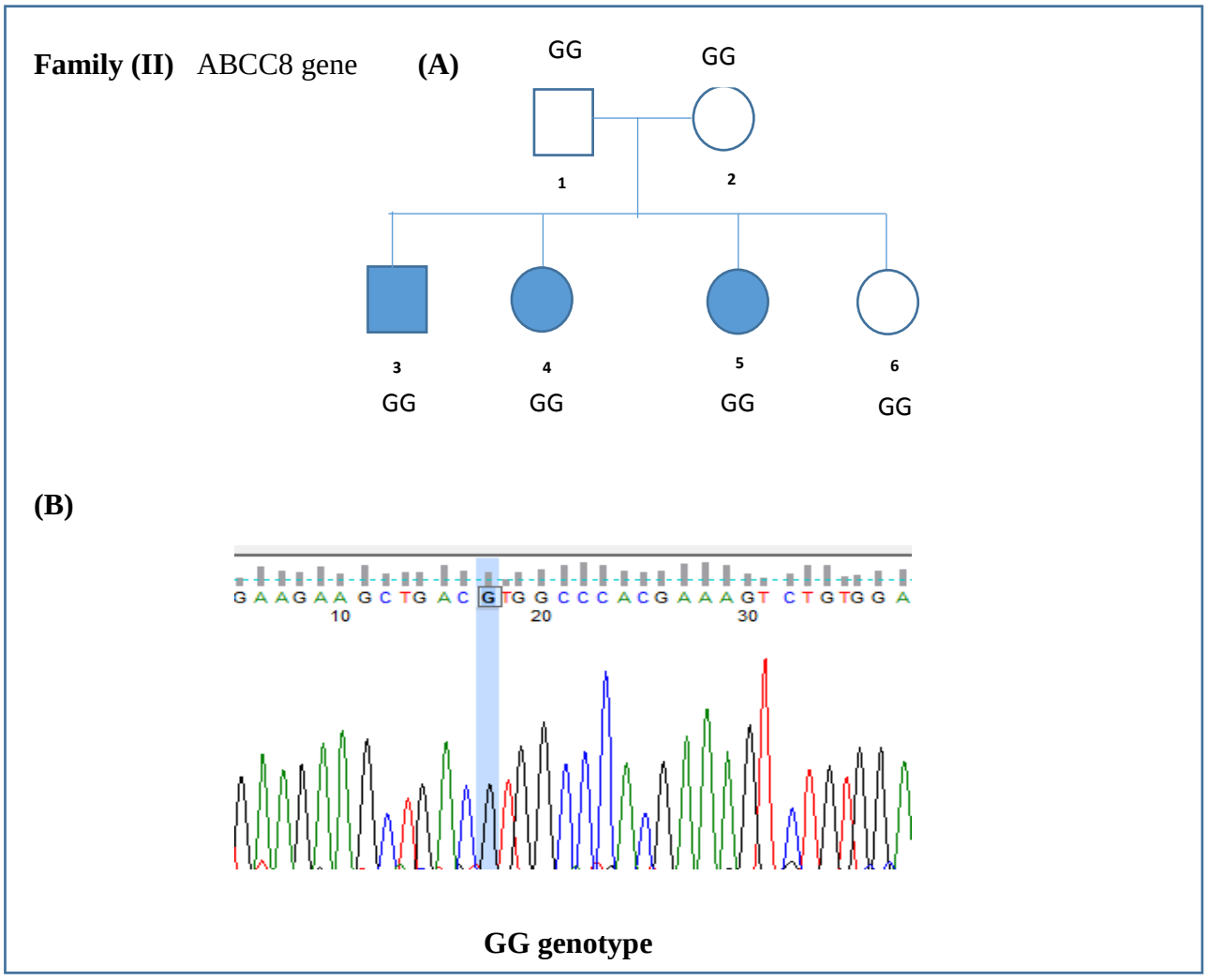

Figure (29): Family segregation of ABCC8 variant. (A) Genotype results of each family member shown in the family pedigree. (B) Sanger Sequencing results of the detected variant in Family II.

3.2.2 *RYR1* Gene

The RYR1 gene is located on chromosome 19. The identified variant A7835+5G is located in intron. This variant was not validated nor segregated in family II. All members have wild-type AA genotype, as shown in Figure (30).

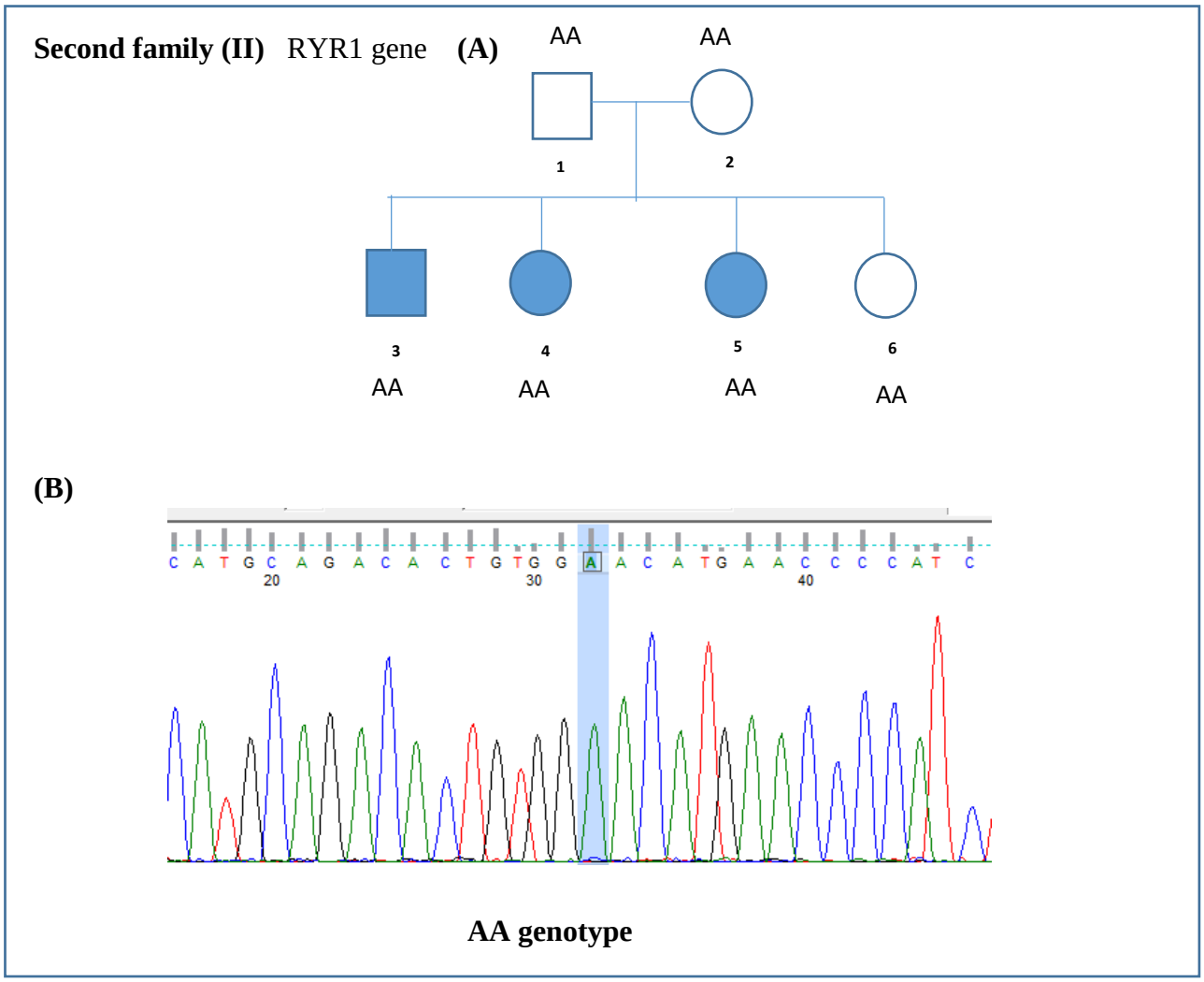

Figure (30): Family segregation of RYR1 variant. (A) Genotype results of each family member shown in the family pedigree. (B) Sanger Sequencing results of the detected variant in Family II.

3.2.3 *CTRC* Gene

The CTRC is located on chromosome 1. The identified variant G649A is a missense mutation where Guanine is replaced by Adenine at position 649 leading to Serine amino acid instead of Glycine at codon 217. It was confirmed and validated by sanger sequencing. However, its segregation didn't match the observed expression of the disease among the family members as described in Figure (31). Members II-1, II-3, and II-4 have heterozygous GA genotype. On the other hand, members II-2, II-5, and II-6 are wild-type GG.

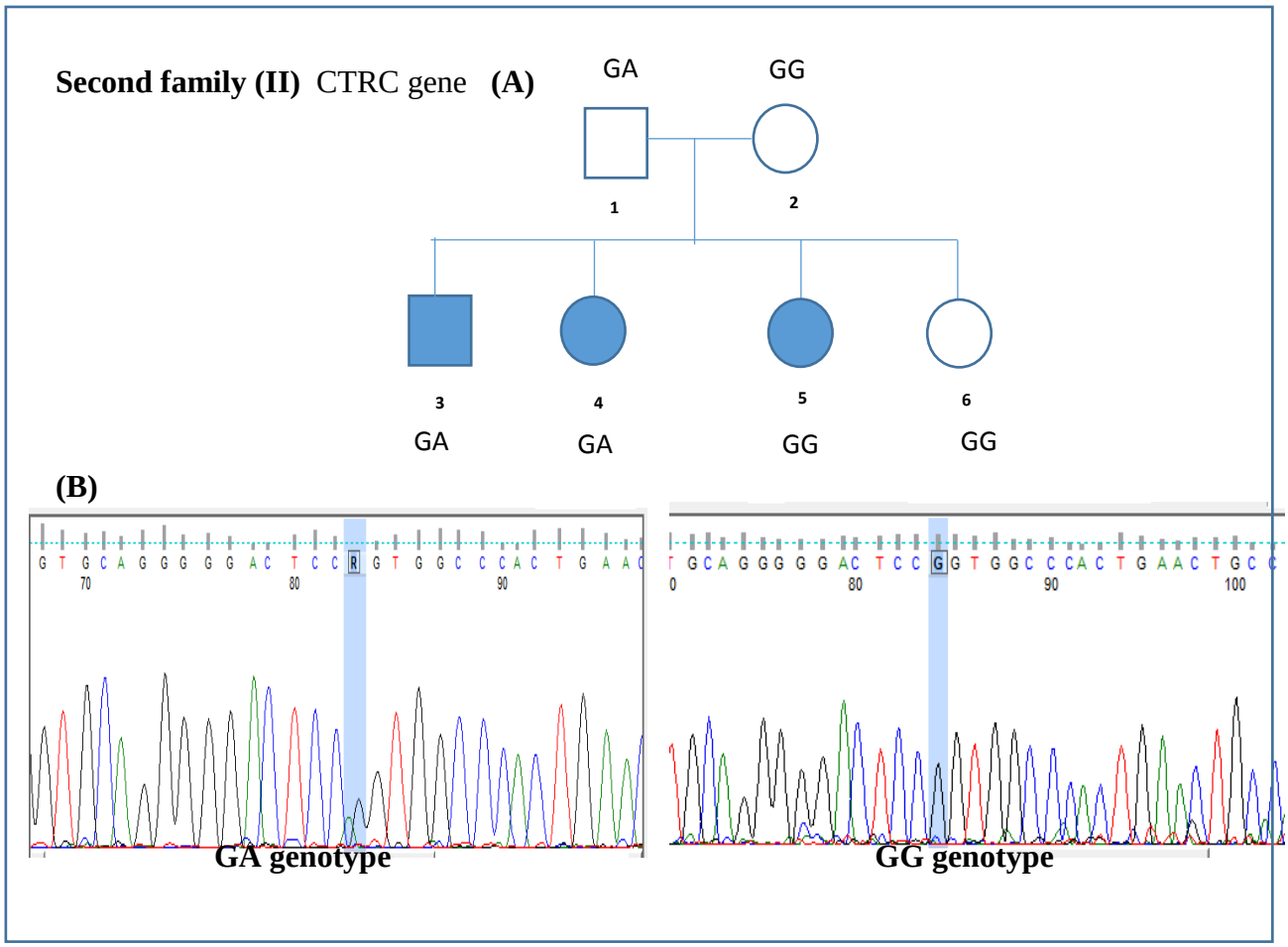

Figure (31): Family segregation of CTRC variant. (A) Genotype results of each family member shown in the family pedigree. (B) Sanger Sequencing results of the detected variant in Family II.

3.2.4 *NEUROD1* Gene

The NEUROD1 gene is located on chromosome 2. The identified variant G590T is a missense mutation where Cytosine is replaced by Adenine at position 590. This leads to replace Proline amino acid by Histidine. This variant was validated by sanger sequencing. It was also segregated in Family II, as shown in figure (32). Members II-1 and II-2 (Parents) have heterozygous GT genotype, whereas members II-3, II-4, and II-5 (diabetic offspring) have homozygous TT genotypes. Non diabetic daughter (member II-6) has a wild-type GG genotype. Therefore, the segregation matches the observed expression of the disease.

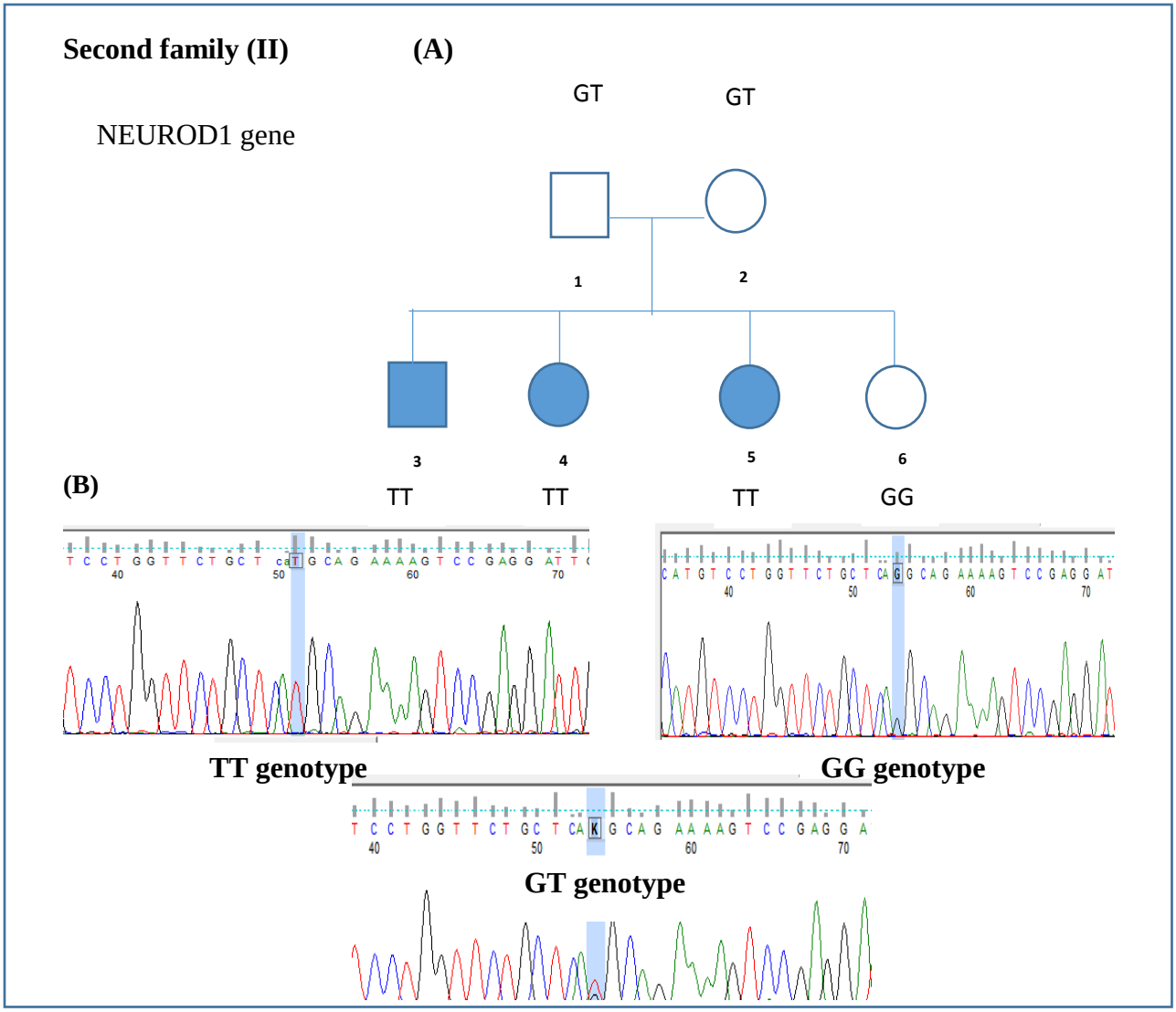

Figure (32): Family segregation of NEUROD1 variant. (A) Genotype results of each family member shown in the family pedigree. (B) Sanger Sequencing results of the detected variant in Family II.

3.3 *In-silico* Analysis:

To predict the effects of the amino acid substitution on the protein structure and function for the confirmed and segregated variants, several bioinformatics tools were used as summarized in the Table (13):

Table (13): Summary of *in-silico* analysis for IGF1R p.V579F and NEUROD1 p.P197H variants.

Variant/Tool	Conservation	PROVEAN	PolyPhen-2	FATHMM	SIFT	Mutation Taster	GVGD	LIST-S2
IGF1R V579F	Highly conserved	Neutral	Benign	Tolerated	Tolerated	Disease causing	Pathogenic	Damaging
NEUROD1 P197H	Highly conserved	Deleterious	Possibly damaging	Tolerated	Damaging	Disease causing	Pathogenic	Damaging

3.3.1 *In-silico* Analysis for IGF1R p.V579F Variant:

3.3.1.1 COBALT Alignment Tool

To investigate whether IGF1R V579F locus is conserved, COBALT alignment tool was used. The results in Figure (33) showed the IGF1R V579 locates at highly conserved region.

Figure (33): Conservation analysis of IGF1R V579 locus using COBALT alignment tool.

3.3.1.2 Functional Location

IGF1R protein is composed of 5 domains including two Receptor-L domains, Furin-like cysteine rich region, Fibronectin type III domain, and Protein tyrosine kinase domain. IGF1R p.V579F is located between Receptor-L domain and Fibronectin type III domain, as shown in Figure (34).

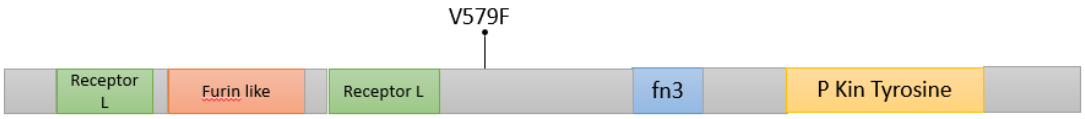

Figure (34): Location of IGF1R p.V579F variant on IGF1R protein.

3.3.1.3 Prediction Tools:

Several prediction tools were used to investigate whether the IGF1R p.V579F variant has impact on the biological function and structure of the protein. The variant is predicted to be neutral and tolerated according to PROVEAN and SIFT tools. It is also predicted to be benign by PolyPhen-2 tool. Based on FATHMM prediction tool, IGF1R p.V579F will be tolerated. However, by FATHMM-MKL and LIST-S2 tools, it is predicted to be damaging. The IGF1R p.V579F variant is a disease-causing based on Mutation Taster tool, and is classified in C45 class of Align-GVGD classes, which means pathogenic.

3.3.2. *In-silico* Analysis for NEUROD1 p.P197H Variant

3.3.2.1 COBALT Alignment Tool

To investigate whether NEUROD1 p.P197H locus is conserved, COBALT Alignment tool was used. The results showed the NEUROD1 P197 locates in highly conserved region, as shown in figure (35).

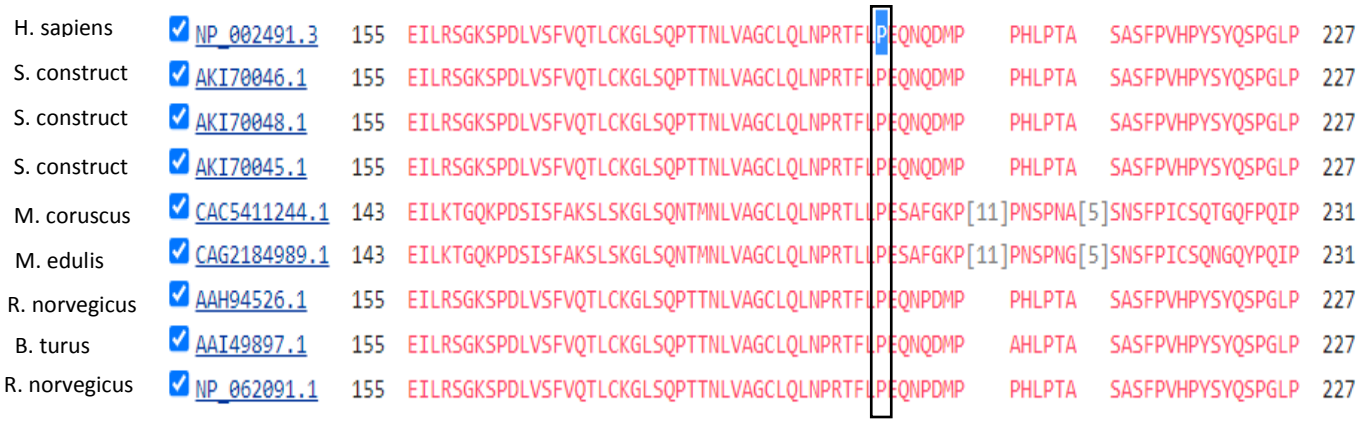

Figure (35): Conservation analysis of NEUROD1 P197 locus using COBALT alignment tool.

3.3.2.2 Functional Location

NEUROD1 protein is composed on two domains; helix loop helix domain and transactivation domain. NEUROD1 p.P197H variant is located at the transactivation domain of NEUROD1 protein.as shown in figure (36).

Figure (36): Location of NEUROD1 p.P197H variant on NEUROD1 protein.

3.3.2.3 Prediction Tools

The NEUROD1 p.P197H variant is predicted to be damaging according to PROVEAN, LIST-S2, and FATHMM-XF tools. It is also predicted to be possibly damaging according to PolyPhen-2 tool. Based on GVGD and Mutation Taster tools, the variant is predicted

to be disease-causing and pathogenic, respectively. On the other hand, the NEUROD1 p.P197H variant is predicted to be tolerated according to the FATHMM, and FATHMM-MKL tools.

3.3.2.4 HaploR Tool:

To detect whether NEUROD1 p.P197H variant affect the binding motif of any transcription factor, HaploR tool was used. Figure (37) shows the variant locates within PAX motif.

Regulatory motifs altered

Position Weight Matrix ID (Library from Kheradpour and Kellis, 2013)	Strand	Ref	Alt	Match on: Ref: GGTGGGGGGGCATGTCCTGGTTCTGCTCAGGCAGAAAAGTCCGAGGATTGAGTTGCAGG Alt: GGTGGGGGGGCATGTCCTGGTTCTGCTCATGCAGAAAAGTCCGAGGATTGAGTTGCAGG
Pax-8_1	-	7.2	10.1	BDNHYCAYKCDWNDVDSH
Pax-8_2	-	7.7	10	DHYCAYBCDDNDNDV

Figure (37): *In-silico* analysis for NEUROD1 p.P197H variant by HaploR tool (motifs alteration).

Chapter 4: Discussion

Despite several gene mutations have been linked with type 1 diabetes mellitus, the molecular mechanisms of the disease are not fully understood. This hampers the presence of specific genetic factors that need expanded efforts to unravel the transmittance of the disease within generations. This work was initiated to screen families with T1DM inherited between consecutive generations.

Two Palestinian families with several inflicted members were identified to have T1DM based mainly on the insulin dependence and the presence of autoantibodies. One diabetic patient from each family was selected for whole-exome sequencing. Following data analysis using bioinformatics approaches, some putative candidate variants were prioritized. These variants were checked for confirmation by sanger sequencing and family segregation.

In the first family who follows autosomal dominant inheritance, the variants *in INS*, *HNF1A*, and *KCNJ11* genes were confirmed by sanger sequencing but not segregated in the family because both diabetic patients and non-diabetic individuals have similar genotypes. However, IGF1R p.V579F variant was associated with T1DM in our study. It was confirmed by sanger sequencing and segregated in the family with the phenotype. At position 1735, Guanine is replaced by Thymidine resulting in Phenylalanine amino acid instead of Valine. It is a variant with uncertain significance according to ACMG classification (The American Collage of Medical Genetics). No previous studies reported the association between this variant and T1DM, or other diseases. However, the Val579 residue is highly conserved indicating its importance and pathogenicity possibility. Moreover, several *in-silico* prediction tools including FATHMM-MKL, LIST-S2,

GVGD, and Mutation Taster predict the variant to be damaging and disease causing. While other tools predict the variant to be benign including PolyPhen-2, SIFT, and PROVEAN. This shows the necessity for further analysis and studies including the effects of IGF1R p.V579F variant on the 3D structure of the protein, and its functional implications.

The insulin growth factor 1 receptor (IGF1R) gene is located in chromosome 15. It encodes insulin growth factor 1 receptor which bind with high affinity to insulin growth factor (IGF1) and with lower affinity to insulin. Structure of IGF1R and insulin receptor are very similar, as shown in Figure (38); they consist of a tetramer of two α subunits representing extracellular ligand-binding domains, and two β subunits representing transmembrane domain with tyrosine kinase activity. They are linked together with disulfide bonds[180].The IGF1 binds to the α subunits of IGF1R causing conformational changes and stimulation of β subunits activity. This leads to autophosphorylation and transphosphorylation of tyrosine (especially tyrosine 1131, 1135, 1136 and 1221) increasing its kinase activity, and thus activating and recruiting Insulin receptor substrate (IRS), CT10 Regulator of Kinase (CRK), and Src homology and Collagen (SHC) adaptor proteins. The latter will transduce and activate downstream signaling pathways including MAPK/RasRaf-Erk pathway, phosphatidylinositol-3-kinase/AKT/mTOR (PI3K/AKT) pathway, and Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway resulting in stimulation cell proliferation, survival, and differentiation[181]. Moreover, activated AKT can activate mTOR and inhibit FOXO1 and GSK3 molecules. These downstream molecules are involved in cellular processes and glucose homeostasis resulting in gluconeogenesis inhibition and increasing glycogen and protein synthesis.[182]

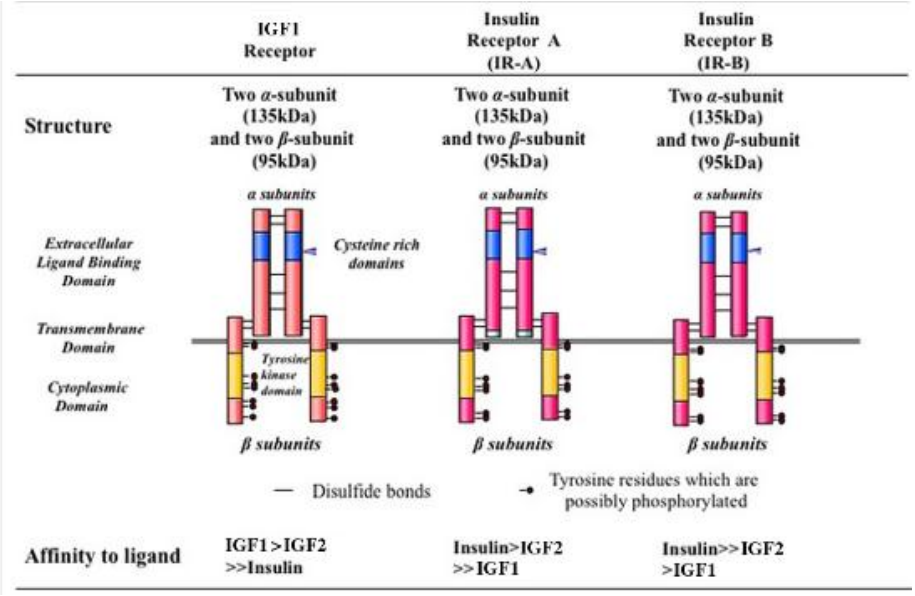

Figure (38): Structure of Insulin growth factor 1 receptor and insulin receptors.[180]

The IGF1R gene is highly expressed in 9% [183] of cancer cells including lung adenocarcinoma, cutaneous melanoma, breast cancer, and colon adenocarcinoma. It plays an important role in phenotypic and oncogenic transformation through activating signaling pathways downstream. IGF1R can then interact with oncogenes as Ras, c-myc, and epidermal growth factor receptor (EGFR) causing an increase in cell migration, invasion, poor prognosis, and shorter survival rate[184].

Several studies suggest close link between IGF1R function and glucose metabolism. They showed that IGF1R gene plays several physiological roles in glucose metabolism and tissue development including growth, heart ventricular development, neutrophils differentiation, and brain development[185–188]. Mouse models with IGF1R knockout showed a significant decrease in their weight after birth. These animals suffer from reduced glucose tolerance in addition to several organs failure leading to earlier death. [180]

The significant association between IGF1R expression and diabetes is evident[189]. Previous study found that absence of IGF1R in beta cells in mice caused glucose intolerance and deficiency in insulin secretion[190]. Another study showed that loss of one allele of IGF1R is associated with glucose intolerance and later insulin resistance, resulting in low birth weight and growth[191]. Additionally, osteopathy defects which is a serious complication of T1DM and T2DM result from interruption of insulin and IGF1R[189].

Noncoding RNAs (LncRNA and Micro RNAs) have a major role in disease development including diabetes and cancer through regulation of IGF1R or other IGF signaling molecules[192]. Interestingly, Knockout of miR-375 in mice model revealed insulin resistance and decreased glucose homeostasis leading to diabetes, and this was associated with upregulation of IGF1R[193].

The IGF1R p.V579F is predicted to be a loss-of-function mutation affecting insulin release from beta cells. We showed previously the significant association between IGF1R and cancer. However, cancer will result from IGF1R gain-of-function mutations. Therefore, diabetic patients from family I are not expected to develop cancer. In contrast, we cannot exclude that some IGF1R gain-of-function mutations are associated with diabetes as previous studies showed[193,194]. The possible explanation is that upregulated IGF1R may suppress insulin signaling through direct effect on insulin receptor itself or its signaling mechanisms. Therefore, the collective indication of these studies and the present study indicate significant correlation between IGF1R signaling pathway and disruption in glucose metabolism leading to diabetes.

In the second family who follows autosomal recessive inheritance, the variants in *ABCC8* and *RYR1* genes were suggested to be associated with T1DM. However, the mutations could not be confirmed by sanger sequencing. One possible reason to explain this discrepancy is the relatively low coverage and correlation score reported by the bioinformatics data analysis concerning these variants. The CTRC variant was confirmed by sanger sequencing but the segregation didn't match the phenotype. Interestingly, the NEUROD1 p.P197H variant was found to be associated with T1DM in our study. It was confirmed and segregated in the family. NEUROD1 p.P197H is a missense mutation where Cytosine is replaced by Adenine at position 590 resulting in Histidine residue instead of Proline at codon 197 which can lead to a major change in the protein structure. This variant is locating in Neuronal helix-loop-helix transactivation domain, and its locus is well conserved in evolution which means that this site is having an important functional significance. It is also predicted to have deleterious functional consequences by several bioinformatics prediction tools including PolyPhen-2, PROVEAN, SIFT, GVGD, LIST-S2 and FATHMM-XF.

The Neurogenic differentiation 1 (NEUROD1) gene is located in chromosome 2 and encodes basic helix-loop-helix (bHLH) transcription factors. These factors regulate the expression of genes containing the consensus sequence CANNTG, known as E-box, by binding them. NEUROD1 combined with E2A-encoded proteins and HEB-encoded proteins. The HLH region of NEUROD1 is responsible for the dimerization between bHLH protein. On the other hand, the basic region mediates protein-DNA interactions. The NEUROD1 gene is expressed in intestine, pituitary, some central and peripheral nerves, and pancreatic islet cells. In the intestine, NEUROD1 gene stimulates the secretin

hormone releas[195]. In the pituitary gland, it regulates the expression of proopiomelanocortin (POMC) which is a precursor of some important hormones[196]. In the nervous system, it motivates the formation of neurite therefore its name derived from this activity. This gene is also known as BETA2 due to its activity to activate the insulin gene in beta cells. Moreover, it has an important role in the development of the pancreas[197]. Previous study showed that a mice lacking NEUROD1 -/- had serious effects on the pancreas, causing a decline in beta cells leading to severe diabetes. [198]

NEUROD1 form heterodimer with a bHLH transcription factor called E4 which binds to E-box binding site in the promoter region of insulin (INS), glucokinase (GCK), sulfonylurea receptor 1 (SUR1), Paired box (PAX), and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) genes and activate their expression[199]. These genes are known to play an important role in glucose homeostasis besides differentiation and development of pancreatic cells[200].This explains the connection between NEUROD1 gene and diabetes. NEUROD1 is also involved in Beta cells dysfunction during chronic hyperglycemia through two mechanisms; First, High glucose concentration will activate the expression of a small heterodimer partner (SHP) gene which in turn inhibit p300-mediated pancreatic duodenal homeobox factor 1 (PDX1) and NEUROD1 resulting in insulin gene downregulation. Second, decreased Protein phosphatase 2 (PP2A) level will activate cyclic AMP-responsive element-binding protein (CREB) leading to NEUROD1 and insulin repression. [201,202] In our analysis, NEUROD1 P197H variant is found within motif sequence specific for PAX transcription factor. This can explain its role in developing diabetes. Further validation should be done using CHIP-sequencing.

NEUROD1 gene contains two exons; the first one is not translated, while the second encodes for a protein with several motifs. Four variants have been identified in exon 2 as shown in Figure (39). The first variant is p.Ala45Thr, the second one is p. Pro197His, and the third one is p.Arg111Leucine which is located in the proximal basic portion of the basic HLH domain. The last one is insertion of a cytosine in codon 206, identified as c.206+C, leading to nonsense mutation and premature stop codon as shown in Figure (39C). Malecki M. et al studied these variants in autosomal dominant families inflicted with T2DM. The first two variants, p.Ala45Thr and p.Pro197His, were not associated with T2DM., whereas the latter two variants, p.Arg111Leu and c.206+C, were associated with T2DM development through affecting NEUROD1 activity to bind E-box and to active CBP/p300, respectively. [203,204]

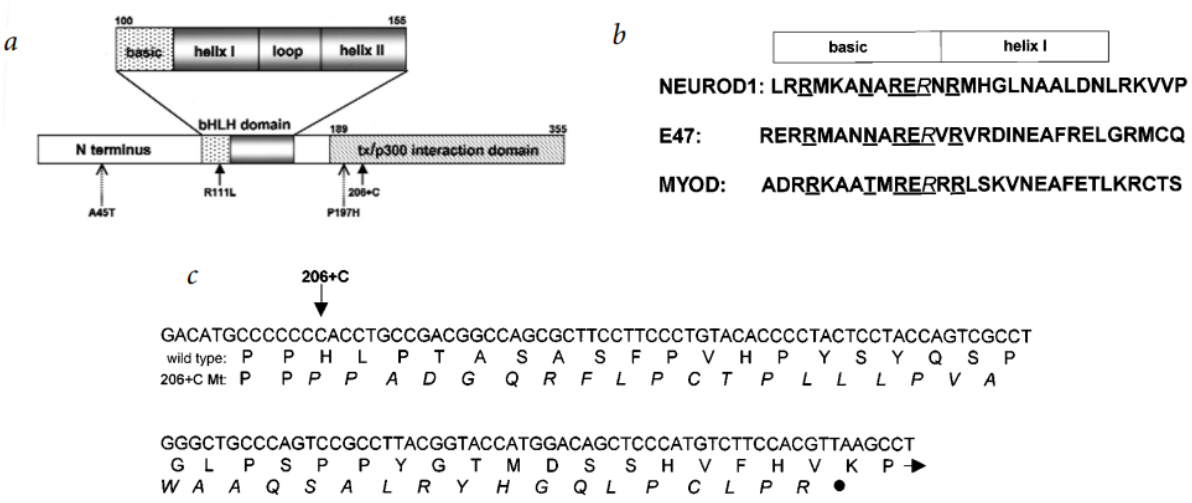

Figure (39): NEUROD1 variants. [203]

Previous studies found that NEUROD1 p.Ala45Thr variant, which is produced from nucleotide G-to-A transition, was associated with T1DM, but not T2DM.[205,206] In another case-control Japanese study, the researchers hypothesized that NEUROD gene can affect

the development and onset of T1DM since it acts on the initial islet precursors[207]. They studied NEUROD1 polymorphisms in 105 T1DM patients and 122 non diabetic controls. The diabetic patients were classified intro groups according to their onset pattern. Interestingly, they found a significant difference in NEUROD1 polymorphisms between cases and control in the acute-onset group[207]. This reveal the association of NEUROD1 gene in T1DM development.

A study in 2010 aimed to examine the effects of NEUROD1 mutations on patients with Monogenic permanent neonatal diabetes (PNDM) found two rare homozygous mutations. The first was a duplication of single base pair (c.364dupG), while the second has deletion of two base pairs CT (c.427_428del). Both mutations lead to the absence of transactivation domain from the protein by causing premature truncation of the C terminus (p.Asp122Glyfs*12 and p.Leu143Alafs*55, respectively), as shown in Figure (40) .These two patients had also some neurological abnormalities, including weakening of vision and hearing, cerebellar hypoplasia, and development delay. This leads to the conclusion that NEUROD1 plays an important role in the pancreas as well as the nervous system.[199]

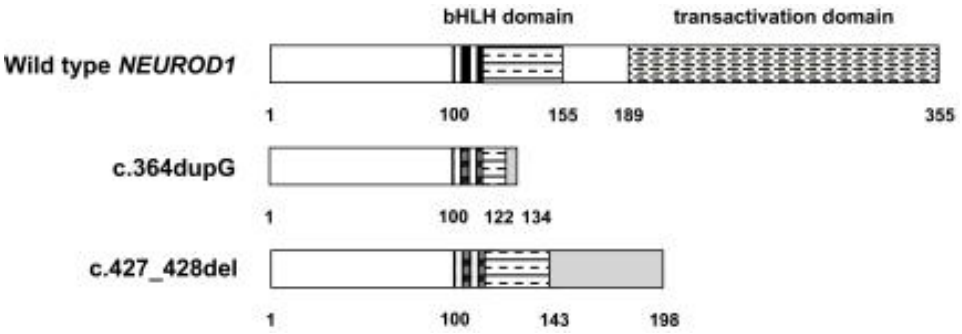

Figure (40): The effects of c.364dupG and c.427_428del mutations on NEUROD1 protein structure.

In maturity onset diabetes of the youth (MODY), several studies demonstrated some NEUROD1 candidate mutations including NEUROD1 p.Pro197His, p.Asp202Glu, p.Leu157Arg and p.Arg103Pro.[208–212] Recently, another heterozygous mutation NEUROD1 p.Met114Leu (c.340A > C) was reported in an Italian patient with MODY6.[213] This variant is predicted to be pathogenic based on many prediction tools and was confirmed in a French family.[214] In Latin America, a novel frameshift deletion (p.Phe256Leufs*2) in NEUROD1 was reported in MODY6 family.[215] Horikawa Y. and Enya H. concluded that heterozygous mutation in NEUROD1 gene are associated with MODY6 whereas homozygous mutations cause neonatal diabetes. [202,216]

Diabetic members of Family II (II-2, II-3, and II-4) don't have neurological disorders. Similarly, some patients in previous studies have homozygous mutations in NEUROD1 gene but with no neurological abnormalities[217]. The reasons are not clear, however, it is possible that out missense mutations P197H don't affect the domain needed for neurological functions compared to the mutation shown in Figure (40).

A previous study aimed to determine if genetic variation in MODY genes can affect the response to insulin sensitizing interventions. The study included individuals with high risk to develop diabetes (high fasting glucose, overweight). They were divided into three groups; the first group took placebo, the second group took metformin drug twice a day, and the third group with life style intervention. Many variants in several MODY genes including *HNF4A, GCK, HNF1A, PDX1, HNF1B,* and *NEUROD1* were genotyped in all participants. There was a significant association between HNF4A, HNF1B, and NEUROD1 variants and treatment response to metformin and life style intervention. One minor allele of rs6719578 in NEUROD1 gene showed increase in insulin secretion in the

metformin group[218]. Therefore, NEUROD1 variants can also be used in treatment programming. Its genotypes can predict the treatment response, thus it affects the selection of the drug and the doses given.

Further studies should be done to confirm the causative relationship of NEUROD1 p.P197H and IGF1R p.V579F in T1DM and its mechanisms. We recommend performing human in-vitro modeling studies by using embryonic stem cells transfected with the mutation (by CRISPR-CAS9) and then differentiated into pancreatic beta-like cells. Several functional assays will be performed on the cells including glucose-stimulated insulin secretion and extracellular flux assay. These studies, if give positive results, can be followed by mouse models studies. Moreover, this variant can be screened in several families with T1DM.

Chapter 5: Conclusion

The data presented in this study revealed the potential role of two new putative genes in the development of Type 1 diabetes mellitus. The first variant, IGF1R p.V579F, follows autosomal dominant inheritance. It is a variant with uncertain significance but well conserved and predicted to be pathogenic by some bioinformatics prediction tools, including LIST-S2 and Mutation Taster. The IGF1R gene plays a role in cell growth, survival, and development through activation signaling pathways. It is associated with glucose homeostasis and insulin secretion but needs further confirmation and explanation. The second variant, NEUROD1 p.P197H, follows autosomal recessive inheritance. It is located in the transactivation domain of the NEUROD1 protein in a highly conserved region. It is predicted to be pathogenic and disease-causing by several prediction tools including PROVEAN, PolyPhen-2, LIST-S2, GVGD, and Mutation Taster. The association of the NEUROD1 gene in diabetes is derived from its role in beta cell activation through regulation expression of important genes known to have roles in pancreatic development including INS, GCK, and PAX. The NEUROD1 p.P197H variant is also found within the motif sequence of the PAX transcription factor, affecting its binding and function. Human in-vitro models are recommended to confirm our results and investigate the causative mechanisms of IGF1R p.V579F and NEUROD1 p.P197H in T1DM. Chip sequencing is also recommended to validate the effects of NEUROD1 p.P197H on the PAX binding motif. These variants will be very valuable for the screening and diagnosis of patients and potential carriers. Moreover, the data will provide valuable tools for the treatment and development of further therapeutic approaches to compensate for the loss of the specific gene function and/or its clinical consequence. Also, it can help the affected family members to have children free of the disease.

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Appendix

Consent form

<u>موافقة للمشاركة في دراسة بحثية</u>

تهدف هذه للبحث عن متغير جيني مسؤول عن الإصابة بالنوع الأول من السكري في العائلات التي تنقل المرض وراثيا، من أجل فهم الوراثة الجزيئية الخاصة بالمرض.

هذه الدراسة تُجريها الطالبة أبرار بواطنة من برنامج ماجستير الوراثة الجزيئية والسمية الجينية تحت إشراف أ.د. هشام درويش في الجامعة العربية الأمريكية.

ما الهدف من هذه الدراسة؟
الهدف من هذا المشروع هو تجميع عينات الدم من عائلات مصابة بالنوع الأول من السكري من أجل البحث عن جين مسؤول عن الإصابة بالمرض ومعرفة تأثيراته.

كم عدد الأشخاص المشاركين في هذه الدراسة؟
من المتوقع مشاركة 5 عائلات تنطبق عليها شروط الدراسة، وهي وجود أكثر من شخص في العائلة تم تشخصيه بالنوع الأول من السكري.

ما المطلوب مني في هذه الدراسة؟
نحن نطلب إذنك أيضا بأخذ عينة دم (3-5 ml) من أحد أوردة ذراعك. لذا قد نضطر لوخزك بإبرة صغيرة لأخذ هذه العينة، كما ونطلب إذنك للحصول على بعض المعلومات التي ستساعدنا في تقييم نتائج البحث. وسيتم ذلك مع المحافظة على سرية وخصوصية هذه المعلومات.

حول استعمال عينة الدم لأغراض بحثية
البحث الذي يستند على عينة دم مهم لفهم الوضع الصحي وأمراض البشر. بعض الباحثين يستخدمون هذه العينات لمحاولة فهم سبب الأمراض ويقومون بتطوير فحوصات جديدة لكشف ومراقبة الأمراض، بينما قد يقوم آخرون بتطوير طرق جديدة للوقاية وعلاج بعضها او كلها في المستقبل، بينما يقوم بعض الباحثين بتطوير منتجات جديدة وأدوية جديدة. جميع عينات الدم الذي سيتم جمعه، سيحفظ في مختبر الوراثة الجزيئية للأبحاث في حرم الجامعة الأمريكية في حي الريحان قرب رام 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 من دون تكوّن كدمة. قيامنا بسحب عينة الدم مشابه تماما عندما يسحب لك عينه دم لإجراء فحوصات طبيه كجزء من الإجراء الروتيني لرعايتك الصحية لذلك فإن الازعاج المترتب على مشاركتك في البحث تكون ضئيلة.

الخصوصية والسرية

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

هل هناك فوائد للمشاركة في هذه الدراسة؟

هناك فوائد أساسيه للمجتمع نتيجة هذه الأبحاث، منها معرفة مسببات الأمراض، كيفية الوقاية منهم، وكيفية علاجهم والشفاء منهم. تحديداً، نحن نطمح لمعرفة كيفية الوقاية وعلاج الخلل في المادة الوراثية.

هل هناك بدائل عن المشاركة في هذه الدراسة؟

يمكنك اختيار عدم إعطائنا عينة من دمك لهذه الدراسة.

ما هي التكاليف؟

لن يكون هناك أية تكاليف عليك من خلال مشاركتك بهذه الدراسة

ماذا عن التعويضات؟

لن نقوم بالدفع لك لمشاركتك بهذه الدراسة.

ماذا عن حقي في رفض المشاركة بهذه الدراسة أو الانسحاب منها؟

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

بمن أتصل في حال كانت لدي أسئلة أو واجهتني مشاكل؟

للسؤال عن الدراسة أو عن أو إذا كانت لديك مشاكل، مخاوف، أسئلة أو اقتراحات حول البحث، الاتصال ب أ.د. هشام درويش على العنوان التالي: hisham.darwish@aauj.edu

نص الموافقة

تم شرح لي الهدف من هذه الدراسة، الخطوات التي سيتم اتباعها، المخاطر والفوائد المترتبة على المشاركة بها. لقد تم السماح لي بسؤال أسئلة، وتمت 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 وأوافق على المشاركة بهذه الدراسة، مع العمل أنه بإمكاني الانسحاب أنّى شئت.

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

الاسم الثلاثي للمشارك _____

توقيع المشارك _____ التاريخ _____

الاسم الثلاثي للباحث/ة: أبرار بلال بواطنة

توقيع الباحث/ة _____ التاريخ _____