Association Analysis of *STAT-4* Gene Polymorphism in Rheumatoid Arthritis & Type 2 Diabetes Mellitus in Pakistani Patients

MARIA SANA

00000205497

MS Healthcare Biotechnology

Supervisor

DR. ATTYA BHATTI

ATTA-UR-RAHMAN SCHOOL OF APPLIED BIOSCIENCES (ASAB)
NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY (NUST)

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BY

MARIA SANA

(Fall 2017-MS HCB- 00000205497)

Supervisor

DR. ATTYA BHATTI

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2020

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Healthcare Biotechnology,
ASAB, NUST

DEDICATED TO

To my wonderful parents, for always loving & supporting me & for raising me to believe that anything is possible.

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LIST OF ABBREVIATIONS

T2D	Type 2 diabetes
RA	Rheumatoid arthritis
STAT4	Signal transducer & activator of transcription
GWAS	Genome wide association studies
HbA1C	Hemoglobin A1C
HLA	Human leukocyte antigen
CDC	Centre for disease control
TNF	Tumor necrosis factor
Th17	T helper 17 cell
TLR	Toll like receptor
NLR	Nod like receptor
FLS	Fibroblast like synoviocytes
NSAIDs	Non-steroidal anti-inflammatory drugs
DMARD	Disease modifying antirheumatic drugs
IR	Insulin resistance
NK	Natural killer cells
IFN	Interferon
JAK	Janus kinases
EDTA	Ethylene diamine tetraacetic acid

DAS28	Diseases activity score of 28 joints
WHO	World Health Organization
ANOVA	Analysis of variance
SDS	Sodium dodecyl sulphate
ARMS PCR	Amplification refractory mutation system polymerase chain reaction

ABSTRACT

Diabetes mellitus is defined as reduced insulin synthesis and action. It is marked by gradual loss of β-cell function and persistent insulin resistance. Lately, there is an overwhelming mystery that chronic low-grade inflammation and activated immune system are responsible for the pathogenesis and initiation of diabetes mellitus type 2. Rheumatoid arthritis is a persistent, systemic autoimmune disorder, which causes inflammation and joint damage linked to structural, bone and metabolic comorbidity. This study was carried out in 274 participants (T2D = 111, RA = 110, Controls = 53) in total to test *STAT-4* prevalence (*rs7574865*). In order to enforce the target SNPs and examine their polymorphism in the Pakistani population, allele-specific polymerase chain reaction was performed. Between *STAT-4* (*rs7574865*), T2DM and RA a significant correlation was noticed. The GT genotype indicates susceptibility of developing both RA and T2DM, in RA vs. control with OR= 2.97, 95% CI= 2.00 (1.20-3.33), P= <0.0001 whereas in T2DM with OR= 2.52, 95% CI= 1.84 (1.09-3.07), P= 0.0004. It indicates that the strong association of *STAT4 rs7574865* with T2DM and RA shows that both systemic conditions can be present as co-morbidities. The *STAT4 rs7574865* allelic distribution reveals that there is a significant distribution of T allele in RA, T2DM, and Control which indicates that T2D and RA patients who are homozygous for the T allele are susceptible to have increased disease activity and severity. However, more studies are still needed for the same population on larger scale to prove the relation of this polymorphism and both the severity and activity of RA and T2D.

CHAPTER # 1 INTRODUCTION

1.1 Type 2 Diabetes Mellitus

1.1.1. Introduction

Type 2 diabetes mellitus is a non-communicable metabolic disease which results from elevated blood glucose level. The major reason regarding this severe hyperglycemia is due to either insufficient insulin secretion from pancreas or insensitivity to insulin action or both. This condition may lead to secondary impairments like nephropathy, diabetic neuropathy, ocular damage especially to the retina and cardiovascular disorders. The level of blood glucose in patients with T2D can be controlled through diet and drugs, they don't require exogenous insulin until and unless it is not under control (Asif, 2014; Goyal & Jialal, 2019).

1.1.2. Epidemiology

Non-insulin dependent diabetes mellitus accounts for at least 90 percent of all cases of diabetes mellitus (González, Johansson, Wallander, & Rodríguez, 2009). In developing nations, the prevalence increase is estimated to be much higher as compared to the developed ones (69% vs. 20%) (Shaw, Sicree, & Zimmet, 2010).

People between the ages of 40 and 60 years in emerging nations (usually the work era) opposed to those over 60 years in advanced nations are impacted most (Shaw *et al*., 2010). Such an inescapable increase in T2D is related to a transition to the Western culture (elevated fat diet and sedentary lifestyle) in developing nations, so the increase in overweight and obesity prevails (Chan et al., 2009; Colagiuri, 2010). About 1.4 million people in the UK are diagnosed with T2D (Bennett, Dodd, Flatley, Freeth, & Bolling, 1995). With age, the incidence of diabetes raises and most cases remain undiagnosed before 40 years of age. This corresponds to a lifelong threat of 1 in 10 to develop diabetes (Neil *et al*., 1987).

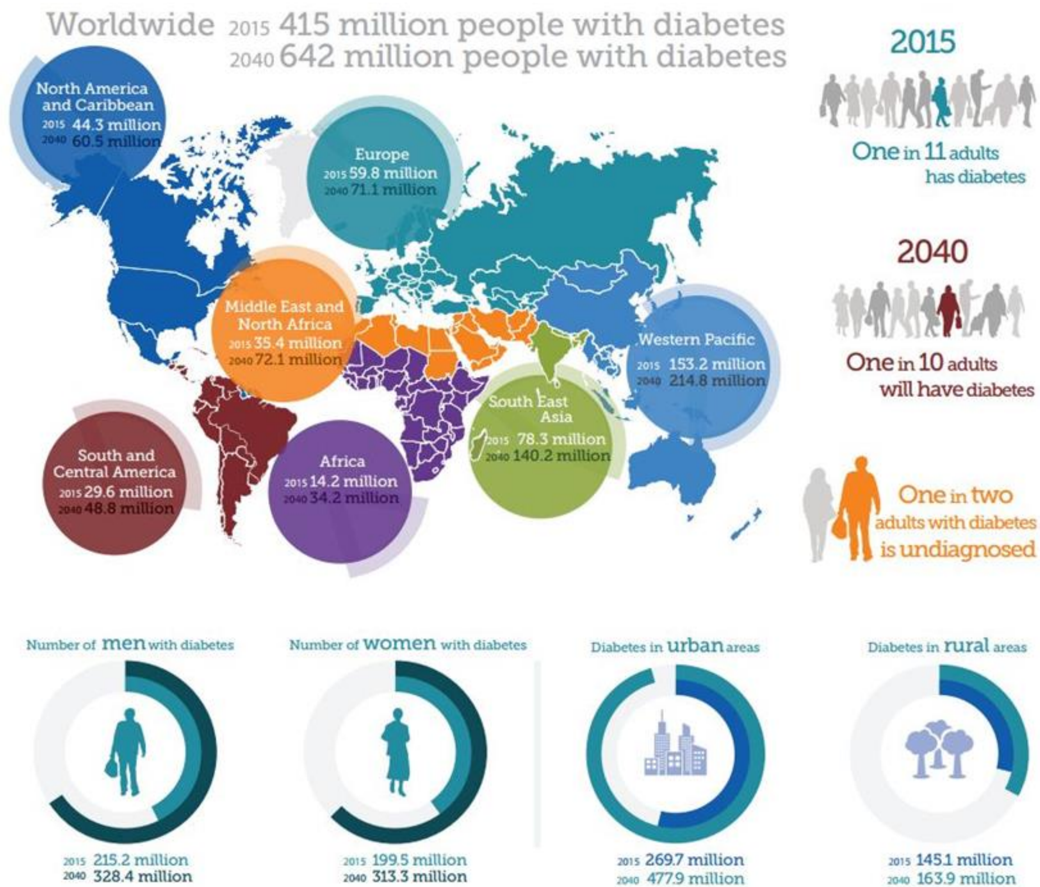


Figure 1. 1 Diabetes mellitus type 2 epidemiology and demographics ("IDF Diabetes Atlas 7th Edition," 2015)

Presently Pakistan has a prevalence of 11.77% of T2D. The prevalence in men is 11.20% and in women 9.19%. The usual occurrence in the Sindh is 16.2 percent in men and 11.70 percent in women; 9.83 percent in women and 12.14 percent in men in the province of Punjab. In the region of Baluchistan, 13.3% of men, 8.9% of women, while in the region of Khyber Pakhtunkhwa (KPK), 9.2% of men and 11.60% of women are type 2 diabetic. In urban regions of Pakistan, the prevalence of T2D is 14.81% and in rural regions is 10.34%. It is more prevalent in men than women and in urban zones than in rural zones (Meo, Zia, Bukhari, & Arain, 2016).

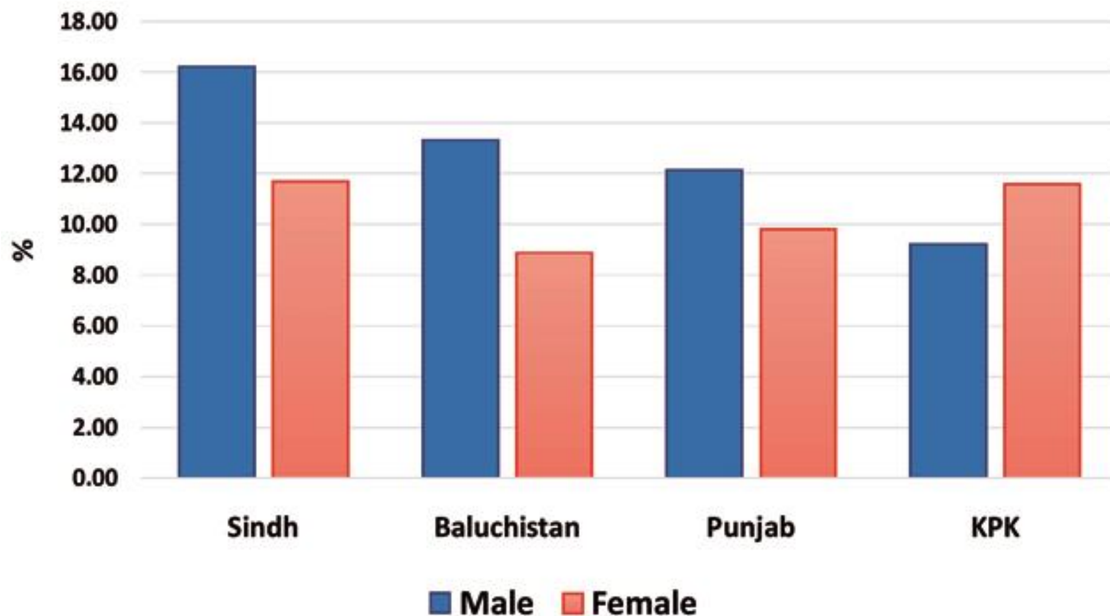


Figure 1. 2 Prevalence of type 2 diabetes mellitus in various regions of Pakistan (Meo et al., 2016)

1.1.3. Etiology

Diabetes of Type 2 is a heterogeneous disease triggered by a blend of genetic factors associated to impaired insulin resistance and excretion and external variables, including obesity, eating too much, anxiety and aging, etc (Kohei, 2010). This disease generally includes various genes and diverse degrees of exogenous variables (Holt, 2004).

The prevalent type of idiopathic diabetes is T2D and is distinguished by the absence of insulin necessary for ketoacidosis prevention. This isn't an autoimmune disorder and yet most people have not seen prone genes that increase the risk to NIDDM. This might be owing to the heterogeneity of the NIDDM sensitive genes.

Factors that can boost your T2D include:

a. **Weight:** The primary risk factor for T2D is being overweight. However, to develop T2D, you do not have to be obese.

b. **Fat distribution**: You are at higher danger of T2D if you accumulate fat primarily in the abdomen than you do if you hoard fat elsewhere, such as in your legs and hips. If you're a man with more than 40 inches (101.6 cm) waist or if a female with more than 35 inches (88.9 cm) waist your danger for T2D increases.

Figure 1. 3 Risk factors for T2D ("Risk factors for type 2 diabetes - Rxvalet Blog - Online Pharmacy USA," n.d.)

c. **Inactiveness:** The more inactive you become, the higher the risk of T2D. The physical activity enables you to regulate weight and makes energy from glucose, making your cells more insulin-sensitive.

d. **The history of the family:** If your parent or sibling has T2D, the danger of T2D rises.

e. **Race:** Although it is not clear why certain races of people— including individuals from Black, Hispanic, American Asian and Indians — are more probable than whites to develop T2D.

f. **Age:** Type 2, particularly when you're 45, raises the danger of diabetes as you become older. Probably because individuals tend to work less and lose their muscle mass as

they age and gain weight. However, type 2 diabetes among kids, adolescents, and younger adults have also dramatically increased.

g. **Prediabetes:** Prediabetes is a disease in which your blood sugar levels are greater than usual but not high enough for diabetes. Prediabetes, which if left untreated, often leads to T2D.

h. **Diabetes in gestation:** You are in danger of developing T2D if you acquire gestational diabetes when you are pregnant. You are also vulnerable to T2D when you give birth to a child that weighs more than 9 pounds (4 kg).

i. **Polycystic ovarian syndrome:** The risk of diabetes is increased for females who have polycystic ovarian syndrome – a prevalent condition described by uneven, surplus hair development, menstrual periods and obesity.

j. **Darkened skin areas, generally in the axes and neck:** Often this condition shows resistance to insulin ("Type 2 diabetes - Symptoms and causes - Mayo Clinic," n.d.).

k. **Genetic factors:** While the genetic factors of type 2 diabetes are of little concern, it has to be taken into account that this disease is highly heterogeneous. Consequently, the findings of genetic research were very varied. In general, 3 methods for studying the genetic factors of a particular disease are used: the candidate gene approach, Linkage studies, and the genome-wide approach.

i. Linkage studies

Linkage is tending to be inherited together by genes and other genetic markers because of their proximity on the same chromosome. Where these processes succeeded considerably in detecting rare variations of big impact such as classical one-gene diseases, the detection of genes that are engaged in complicated polygenic diseases was comparatively ineffective.

- ***CAPN10,*** calpain 10; CAPN10 codes cysteine proteases, which belong to an enormous family of ubiquitous genes that perform several roles in post-receptor signaling,

intracellular restructuring, and other intracellular functions, which are part of the calpain family.

- ***TCF7L2*,** transcription factor 7-like 2; After a robust linkage signal is plotted to the chromosome 10q in the Mexican-American population, CF7L2 was found as a T2D susceptibility gene. Since then, in several Genome-Wide Association Studies (GWAS), the association between the T2D and a number of (SNPs) of the TCF7L2 gene has been highly verified (Ali, 2013).

ii. Candidate gene studies

Genes assumed of playing a role in T2D pathogenesis have been studied in candidate gene studies through focused sequencing efforts. The approach usually focused on already recognized genes engaged in post-receptor, signaling insulin receptors, glucose metabolism, and secretion of insulin and lipid metabolism.

- ***PPARG*,** peroxisome proliferator-activated receptor gamma; PPARG gene has been an appealing T2D entrant because it translates the molecular aim for a common anti-diabetic class of thiazolidinediones. The proline to an arginine change in position 12 in the PPARG gene reveals that the risk of diabetes has increased by 20%.
- ***IRS1* and *IRS-2*,** insulin receptor substrate; Peptides that play a major role in insulin signal transduction, are encrypted in IRS-1 and IRS-2 genes. In some populations, polymorphisms of these genes were correlated with reduced insulin susceptibility.
- ***KCNJ11*,** potassium inwardly-rectifying channel, subfamily J, member 11; The KCNJ11 gene encodes a potassium channel of Kir 6.2 that is sensitive to ATP and plays a major part in the control of beta-cell insulin secretion. The odds ratio of T2D development is approximately 1:2 in risk allele carriers and this allele has also been discovered to be linked to reduced insulin secretion in various populations.
- ***WFS-1*,** Wolfram syndrome

1. It seems the WFS1 gene is engaged in beta-cell function and

2. WFS-1 SNPs have substantially been discovered to be linked with T2D. These studies have shown that beta-cell dysfunction is critical to T2D and have pointed towards novel genes that play a previously unfamiliar part in the function and survival of beta cells, but remain minor in terms of worldwide liability of diabetes.

- ***HNF1A*, *HNF1B*, and *HNF4A***, homeobox; These genes play a part in liver growth, hepatic metabolic function regulation and the growth and function of beta cells (Ali, 2013).

iii. GWAS

A small number of T2D risk genes were recognized by candidate gene research and linkage analyzes, but their overall impact to the heritability of T2D remained low. Additional techniques were evident in order to find variations that were not readily recognized with these methods. Here we concentrate on genes which, regardless of obesity, specifically boost the risk of T2D. Among them the most significant are:

- ***TCF7L2,*** This is still the significant and most continuously replicated T2D gene. TCF7L2 encrypts the transcription factor recognized to be active in beta cells that is part of the Wnt signal pathway. Early research disclosed a higher TCF7L2 protein level in beta cells and was linked with the weakened secretion of insulin, incretin impacts, and an increased frequency of production of hepatic glucose. In homozygotes, TCF7L2 expression in human islet was amplified five times in T2D and glucose-stimulated insulin secretion decreased with TCF7L2 overexpression in human islets.
- ***HHEX*,** hematopoietically expressed homeobox; This gene is also in the homeobox family at chromosome 10q and encodes a transcription factor for Wnt signaling.

- ***SLC30A8,*** Solute carrier family 30 (zinc transporter), member 8; This gene encodes a protein, which is used for storing and secreting insulin granules and which is only expressed in the pancreas at an elevated rate, especially in Langerhan islets.
- ***CDKN2A/B,*** Cyclin-dependent kinase inhibitor 2A/B; These genes are situated at 9p21 chromosome and produce multiple transcript variants. At minimum three split-up versions of CDKN2A have been revealed encoding separate proteins, of which two are known to operate as CDK4 kinase inhibitors. In the same region, CDKN2B also produces a minimum of 2 slice versions. Both genes have a significant function in tumor suppression as cell cycle regulators.
- ***IGF2BP2,*** insulin-like growth factor 2 mRNA binding protein 2; The protein is encoded and binds to 5'UTR of insulin-like mRNA growth factor 2, which control the translation of IGF2. Alternate transcriptional variants have been defined which encode various isoforms (Ali, 2013).

1.1.4. Pathogenesis

The most common type of diabetes is T2D. It is characterized by resistance of insulin that results from obstruction in insulin action on its target tissues such as liver, fat and muscle. The complications occur in understanding its cause where insulin secretory capacity of beta cells get damaged progressively. It has also been found out that T2D Mellitus patients become obese due to insulin resistance (Ergun-Longmire & Maclaren, 2000).

T2D pathogenesis is associated with peripheral insulin resistance, compromised hepatic glucose synthesis regulation, and decreasing β-cell function, ultimately leading to β-cell dysfunction.

The underlying events are thought to be an intrinsic insulin secretion decline and in many patients a comparative insulin deficiency paired with the resistance of peripheral insulin (Olefsky, 1989; Reaven, 1988).

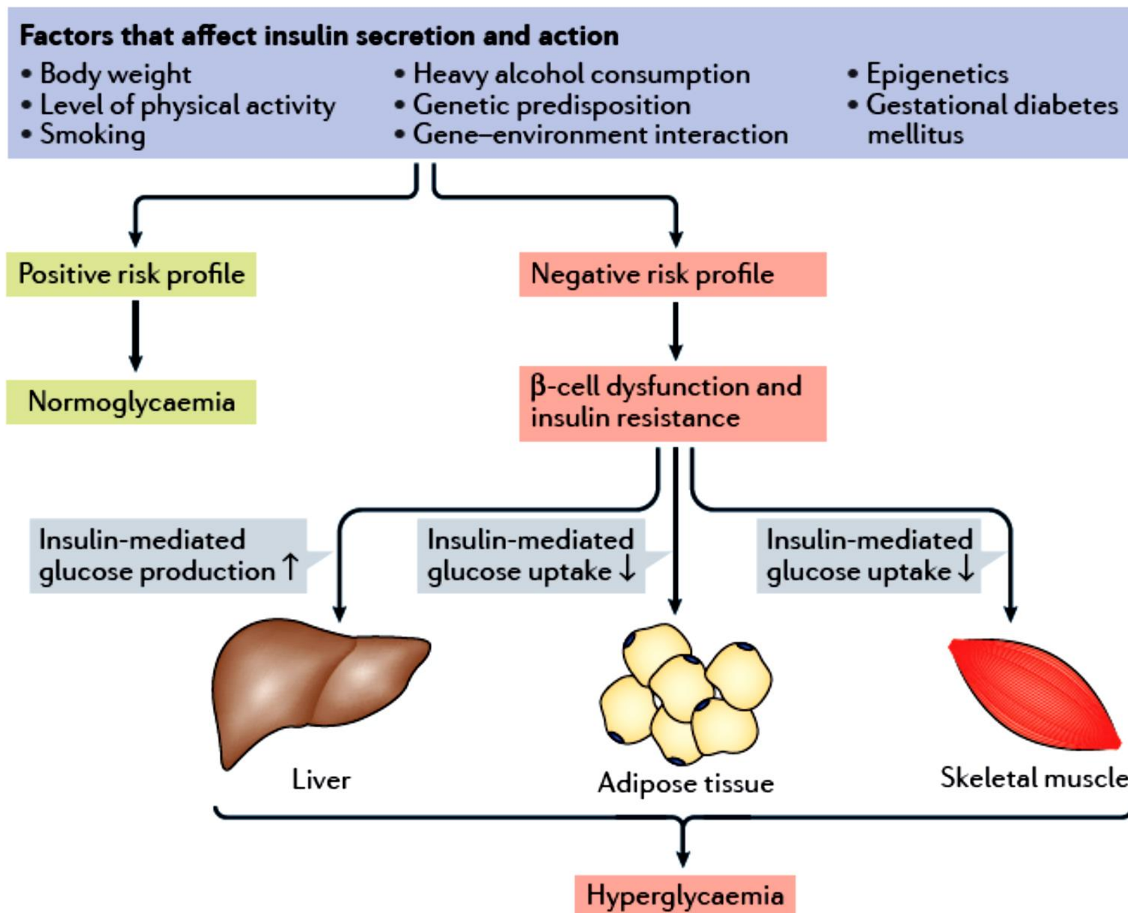


Figure 1. 4 Pathophysiology of hyperglycemia in T2DM (Zheng et al., 2018)

The β-cell insulin production in the pancreas usually decreases the liver's glucose release, and raises skeletal muscle and adipose tissue glucose consumption. when β cell dysfunction in the pancreas or liver insulin resistance, the skeletal muscle or adip osal tissue arise, Hyperglycemia, due to an elevated volume of blood glucose, is produced The different factors at the top influence the secretion of insulin and its effect (Zheng *et al*., 2018).

a. Insulin Resistance

Insulin Resistance occurs when both the glucose elimination in skeletal muscles and the inhibition of endogenous glucose manufacturing mainly in the liver have a below-anticipated biological influence of insulin (Dinneen, Gerich, & Rizza, 1992). In a fasting state, however, the muscle accounts for a tiny percentage of glucose eliminations (less than 20 percent). In patients with T2D or with compromised fasting glucose production, endogenous glucose is enhanced (Meyer, Stumvoll, …, & 1998, n.d.; Weyer, Bogardus, Diabetes, & 1999, n.d.).

Since this rise happens in the presence, at least in early and middle phases of hyperinsulinemia, hepatic insulin resistance is the leading force behind T2D hyperglycemia.

b. β-cell Dysfunction

The first stage of impairment in secretion of insulin during glucose prompt is defined as β-cell dysfunction and can be declared as glycemic intolerance in T2D (Ward, Beard, & Porte, 1986). Insulin reaction initiation will depend on glucose transmembrane transport and glucose coupling with the glucose sensor. The complex of the glucose/glucose sensor then stimulates an increase in glucokinases through the maintenance and impeding the degradation of the protein. The first stage in the direct association of intermediary metabolism to the insulin secretory system is the introduction of glucokinase. Glucose transmission in patient β cells suffering from type 2 diabetes seems significantly reduced, switching the insulin secretion control point from glucokinase to the glucose transport system (J. L.-D. care & 1990, n.d.; Diabetes & 1991, n.d.). Afterward, the second stage release of freshly synthesized insulin is impaired. Such an impact may be reversed, partly in a few patients, by restoring rigid control of serum glucose. The secondary phenomenon, known as desensitization or β-cells glucotoxicity, is a paradoxical effect of glucose limiting insulin release and could be caused by glycogen retention within the β-cell due to persistent hyperglycemia (reviews & 1996, n.d.).

Other alternatives suggested are the aggregation of sorbitol in β-cells or non-enzymatic β-cell glycation. The impaired glucose efficacy in reaction to non-glucose insulin secretagogues, the nonparallel release of insulin and a reduced transformation of proinsulin into insulin are also included in β cell dysfunction in type 2 diabetes mellitus (O'Rahilly *et al.*,1988; Porte etal.,1989).

c. Usual Insulin Secretion

The pancreatic β cell quickly takes glucose through the glucose transporter 2 (GLUT2), where phosphorylation of glucokinase takes place which is the rate-limiting phase for the metabolism of β-cell glucose. Additional degeneration results in pyruvate formation that is then absorbed by the mitochondria in which additional metabolism leads to the formation of ATP. In order to supply energy for the release of insulin, ATP is essential but also engaged in the depolarization of the cell membrane. The sulphonylurea 1 receptor 1 (SUR1) protein activated by the ADS-ATP ratio contributes to the termination of an adjacent channel of potassium (potassium inward rectifier [KIR] 6·2 channel). The shutdown of potassium channels will change the membrane potential and open calcium channels, resulting in the release of insulin-containing granules. (Stumvoll, Goldstein, & van Haeften, 2005).

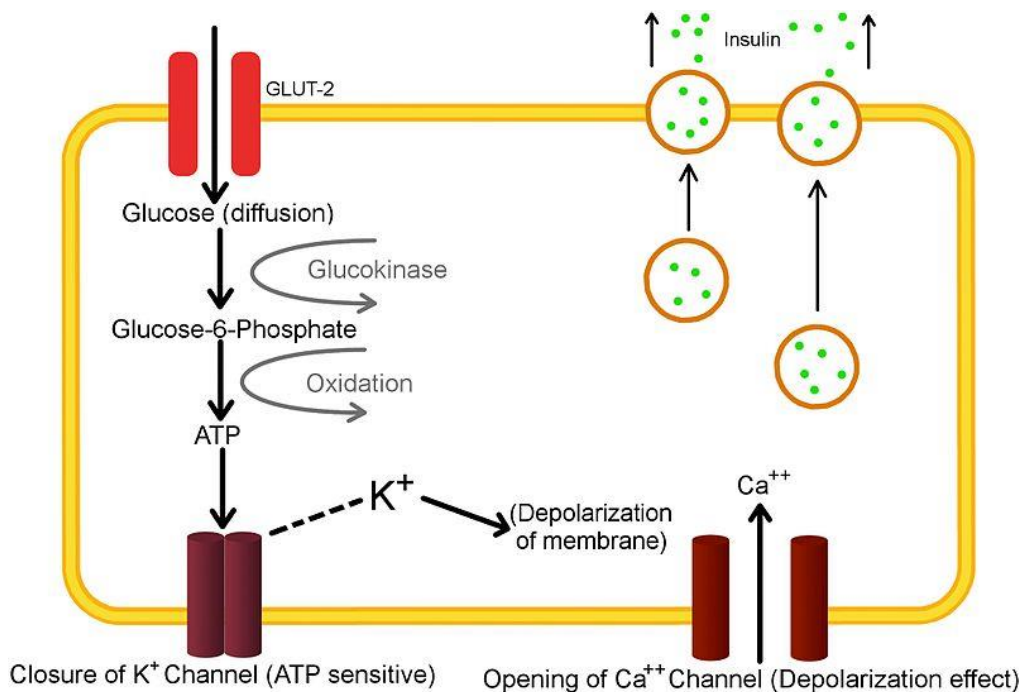


Figure 1. 5 Normal Glucose Induced Insulin Secretion (Stumvoll et al., 2005)

1.1.5. Treatment

a. Dietary Therapy

If hyperglycemia co-exists with obesity, the principal purpose of nutritional treatment ("Weight control: metabolic and cardiovascular effects," n.d.) is the decrease in weight for most people with type 2 diabetes. Conventional suggestions highlight reducing to 50%–55% of nutritional calories both complete and saturated fat content and substitutes for complex carbohydrates. Such diets can trigger marked postprandial hyperglycemia in T2D patients. Since the rate of absorption of glucose is considerably varied by the patient to patient, careful attention is taken to track the post-prandial glucose and the adding of high fiber content in the diet. As the glycemic response of the di*et al*so relies on the texture and content of other food items, and also on the rate of gastric motility, diabetes type 2 diabetes must be considered individually as well as stage and duration. (Franz *et al*.,1994; Holt *et al*., 2004)

b. Exercise

Exercise has proved useful in preventing the development of T2D and also in improving control of glucose due to the increased sensitivity to insulin (Helmrich *et al.*,1994). The processes through which exercise reinstates insulin sensitivity appear to be responsible for the reduction of intra-abdominal fat, an increase of insulin-sensitive glucose transporter (GLUT-4) in muscle, increased blood flow in insulin sensory tissue and lowered free fatty acid levels (Eriksson *et al.*, 1997). Exercise also has the advantages to lower the blood pressure, increase myocardial efficiency and reduce serum triglycerides, while increasing cholesterol concentrations of high-density lipoproteins.

c. Insulin Therapy

In the management of T2D, insulin therapy is considered as initial therapy for drastic hyperglycemic conditions after oral failure or for perioperative or other acute hyperglycemic conditions. Multiple combinations have been employed in type 2 diabetes with the use of latest insulin analogs (Burge & Schade, 1997). Lispro insulin is the first insulin analog available. When injected sc, lispro insulin does not produce aggregates and makes the onset faster and shorter than regular insulin (Howey, Bowsher, Brunelle, & Woodworth, 1994).

While these characteristics can reduce postprandial glycemic progression and reduce the risk of early hypoglycemia, both qualitative and quantitative issues are not overlooked when using insulin in type 2 diabetes. In obese Type 2 diabetic insulin therapy can further increase the weight gain, and hypoglycemia risk (although less frequently than in type 1). Furthermore, a risk factor for cardiac diseases (Després, Lamarche, …, & 1996, n.d.) can be the peripheral hyperinsulinemia obtained with exogenous treatment with insulin.

d. Pharmaco-Therapy

Type 2 diabetes can be treated with the available current therapeutic agents such as biguanides, sulfonylureas & related compounds, insulin, a-glucosidase inhibitors and thiazolidenediones as shown in the figure shown below. Moreover, there will be other classes of therapeutic agents. As the nature and stage of the disease progressing, a rational approach would be to begin as combination therapy. Pharmacological agents are helpful in improving glucose values with minimal side effects (Mahler & Adler, 1999).

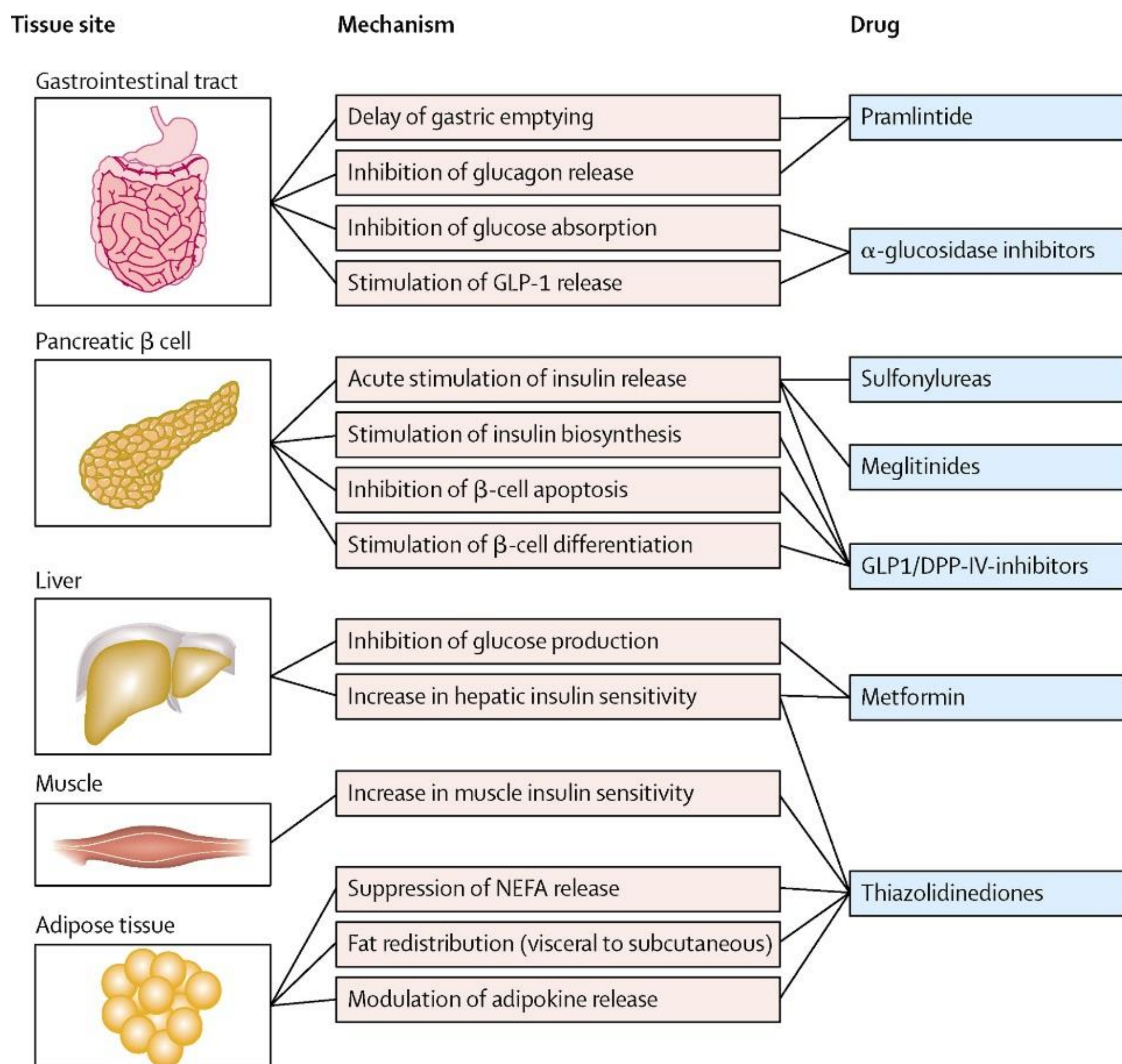


Figure 1. 6 Pharmacological therapy and its mode of action (Stumvoll et al., 2005)

- **Thiazolidinediones**

Drugs that increase sensitivity to insulin are mainly thiazolidinediones medications that not only decrease glycemia but also increase the vascular function and improve type 2 diabetes dyslipidemia and inflammatory conditions.

- **Metformin**

Metformin is an efficient, pancreatic-free antihyperglycaemic that spares insulin. It reduces the production of hepatic glucose and has shown that it benefits cardiovascular results.

α-glucosidase inhibitors

Acarbose has shown that the alpha-glucosidase inhibitor reduces glycaemic consumption and protects against diabetes and cardiovascular disease.

- **Sulfonylurea derivatives**

Sulfonylurea derivatives function in the closure of potassium pancreatic cell channels that lead to increased insulin secretion.

- **Exogenous insulin**

Substituting circulating insulin levels is vital for supporting the clinical impacts of the ineffective metformin and thiazolidinediones without sufficient insulin accessibility.

- **Glucagon-like peptide 1**

This incretin hormone has strong insulinotropic glucose-dependent characteristics, trophic effects on β cells, and an inhibitive effect on intestinal motility, which all decrease plasma glucose (Stumvoll *et al*., 2005).

1.1.6. Screening Criteria & Diagnostic Tests

According to the American Diabetes Association (ADA), T2DM can be screened and detected by the following criteria. The same criteria are also adopted by the World Health Organization (WHO).

Table 1. 1 Standards for screening tests of T2DM (American Diabetes Association, 2017; Goldenberg, Cheng, Punthakee, & Clement, 2011)

	FPG mmol/L (mg/dL)		**2hPG in the 7-g OGTT mmol/L (mg/dL)**		**Hb $A1_c$**
Pre-Diabetes: IFG (Isolated)	6.1-6.9 (110-12)	and	<7.8 (<140)	-	-
Pre-Diabetes: IFT (Isolated)	<6.1 (<110)	and	7.8-110 (140-199)	-	-
Pre-Diabetes: IFG and IGT	6.1-6.9 (110-125)	and	7.8-110 (140-199)	-	-
Diabetes	≥7(≥126)	or	≥11.1 (≥200)	or	≥6.5% (0.06)

a. HbA1C Test

Hemoglobin (Hb) bonded covalently to glucose is known as glycated hemoglobin. HbA1C is an assessment test that estimates a mean level of glycated hemoglobin over a period of 2-3 months ("A1c Blood Test Identifies Diabetes, Heart Risk," n.d.). In the last two to three months this blood test shows your average blood sugar level. Normal concentrations are less than 5.7%, resulting in prediabetes between 5.7% and 6.4%. Diabetes implies if you have an A1C rate of 6.5% or more on two distinct tests. If the A1C test is not accessible, or you are experiencing certain circumstances — such as a rare type of hemoglobin (so-called hemoglobin variant) — that interfere with the A1C test, your physician will diagnose diabetes using these tests.

b. Fasting Blood Glucose Level

After an overnight fast, a blood sample is taken. It is normal to read at less than 100 mg/dL (5.6 mmol/L). Prediabetes are considered to be between 100 and 125 mg/dL (5.6 to 6.9 mmol/L). If you have 126mg/dL (7mmol/L) or higher blood sugar fasting on two different tests, your diabetes tends to occur.

c. Random Blood Glucose Level

Sugar values of the blood are expressed in milligrams (mg/dL) or millimoles (mmol/L) per liter. Whether you last ate, a blood sample showing your blood sugar levels at or above 200 mg/dL, especially if you have signs and symptoms of diabetes, such as frequent urination and extreme thirst. It may be a sign that you have diabetes.

d. Oral Glucose Tolerance Test (OGTT)

Except during pregnancy, this exam is less common than other tests. You must quickly drink sugar in the doctor's office after fasting overnight. For the next two hours, blood sugar levels will be checked regularly.

A blood glucose content of below 140 mg/dL is normal (7.8 mmol/L). Prediabetes can be seen in readings from 140 to 199 mg/dL (7.8 mmol/L and 11.0 mmol/L). Diabetes can be found after two hours at or greater than 200 mg/dL (11.1mmol/L) (“Type 2 diabetes - Symptoms and causes - Mayo Clinic,” n.d.).

1.2. Rheumatoid Arthritis

1.2.1. Introduction

Rheumatoid arthritis is an escalating, chronic and enfeebling autoimmune disorder that is distinguished by the inflammation, hyperplasia, destruction of cartilage and bone, production of autoantibodies and pain of synovial joints. It can cause immobility and deformity due to

inflammation of the joint that can lead to the demolition of joints. Since it is a systemic disease so, it can affect the heart and lungs as well as tiredness (Warren, 2011).

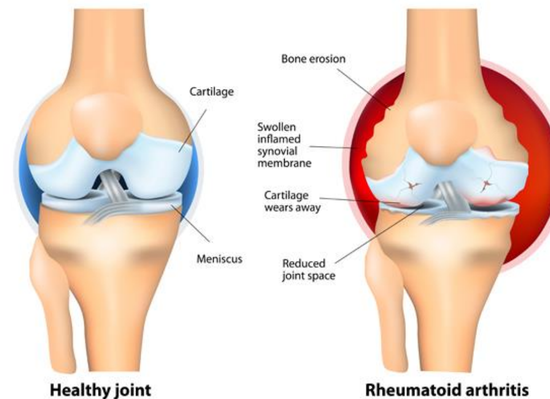


Figure 1. 7 Normal and RA joint (“Learn about rheumatoid arthritis & treatments - MSK,”)

In the course of the blood, the immune cells move to the joints. After reaching the joints, the joints start to cause irritation that causes inflammation and then weaken the cartilage between the joints. If the cartilage erodes entirely, the bones might bend and deform gradually.

1.2.2. Epidemiology

There are no records of regions or ethnic groups where this disease is not discovered and its incidence does not tend to differ considerably between the communities surveyed. Rheumatoid arthritis (RA) is spread widely. RA was common in the United States in 2005 in approximately 1.3 million adolescents, with approximately 1.5 million people impacted two years ago (Viatte, Plant, & Raychaudhuri, 2013).

Table 1. 2 RA incidence and distribution (Viatte *et al*., 2013).

Sociodemographic Epidemiology of RA	**Trends**
Women vs Men	2:1 to 3:1
Caucasian from North America	100/100,000
Rural and urben Africans	20-90/100,000
Native Americans	500/100,000
Asians	20-45/100,000
Caucasian from Europe	5-89/100,000
Latin America	10-50/100,000
Middle east countries	10-50/100,000

The recent analysis shows that the current RA incidence is around 40/100,000 globally, where females from 2 to 3:1 are more susceptible than males to be impacted (Viatte *et al*., 2013). The risk of adults with RA is 3.6% (1 in 28) for women and 1.7% (1 in 59) for men overall for life (Lajas *et al*., 2003).

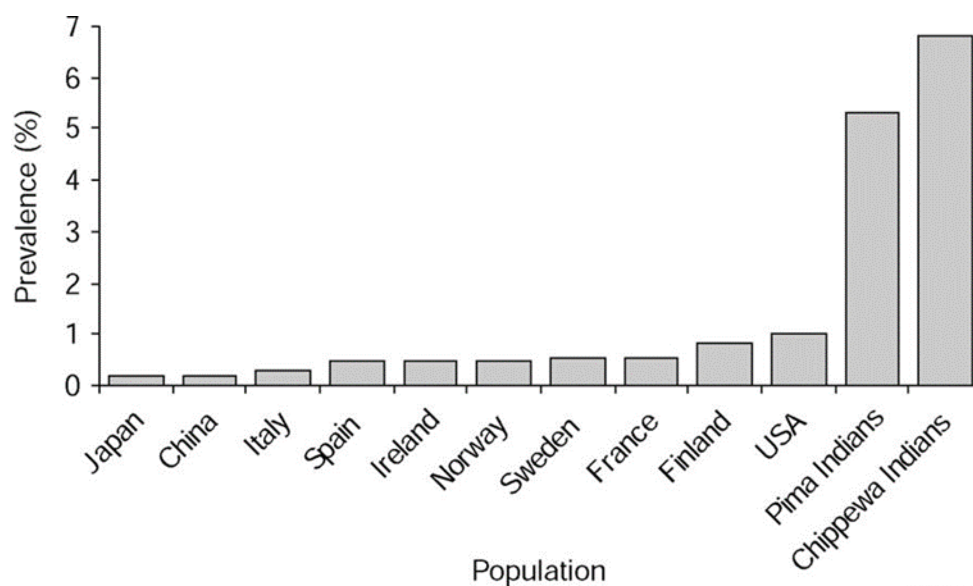


Figure 1. 8 Prevalence of RA in different populations (Alan J Silman et al., 2002)

In Pakistan, the incidence was 0.5%, while in India it was 0.2%–1%. The enormous interval in the incidence of Indians can be due to their population variety. In Pakistan, the proportion of women to men was 8:3 and in India 3:2. In India, the larger proportion of patients have a familial history (24%) compared to Pakistan, where only 14% were found (Akhter, Bilal, & Haque, 2011). A positive family history increases the risk of rheumatoid arthritis by about three to five; twins have increased concordance rates, which entails genetic pathogenesis (Silman *et al*., 1993). Currently, the heritability of rheumatoid arthritis is predicted to be 40% to 65% in seropositive rheumatoid arthritis but less (20%) in seronegative conditions (Jiang *et al*., 2015).

1.2.3. Symptoms

- Pain in several joints.
- Rigidity in several joints.
- Tenderness and inflammation in several joints.
- On each side of the body the same symptoms (for instance in both hands or knees).
- Loss of weight.
- Fever.
- Tiredness, Exhaustion

1.2.4. Etiology

Rheumatoid arthritis occurs if the synovium is take on by your immune system— the membrane lining that surrounds your joints. The inflammation caused thickened synovium, which can abolish the cartilage and bone inside the joint ultimately.

The tendons and ligaments, which weaken and stretch the joint together. The joint is progressively losing its shape and symmetry (“Rheumatoid arthritis - Diagnosis and treatment - Mayo Clinic,” n.d.)

Several problems can enhance the likelihood of RA development. Some are inevitable, but someone can act to avoid others.

- **Genetics**

Our knowledge of the genetics of the disease has been enhanced by modern gene technology incorporated with big, characterized cohorts. In association studies with single nucleotide polymorphisms, more than one hundred loci have been characterized by rheumatoid arthritis vulnerability, mostly involving immune mechanisms some of which are commonly connected with other chronic inflammatory illnesses as shown in figure 1.2 (Roberson & Bowcock, 2010).

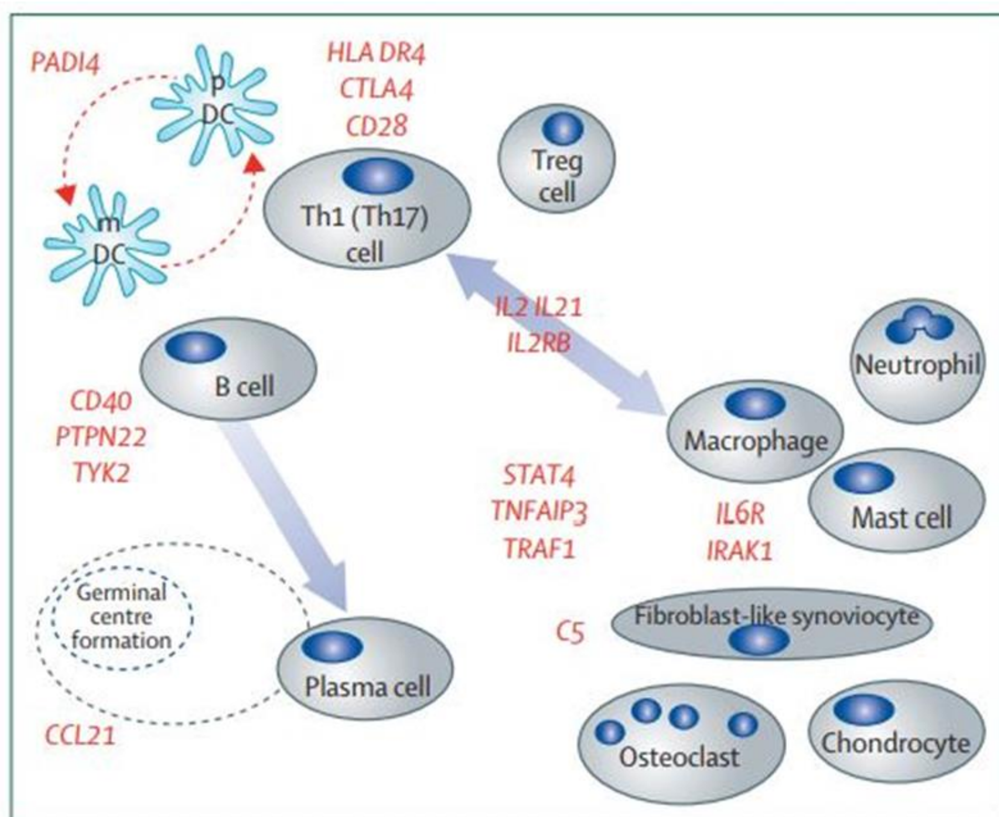


Figure 1. 9 Significant loci linked with rheumatoid arthritis risk and development (Roberson et al., 2010).

The dominant influence remains the *HLA* system (especially HLA-dreb1), which implies a strong binding of the self-peptide in pathogenesis (Okada *et al*., 2014). Alleles with diseases

share common sequences of amino acids within the peptide-binding groove (the so-called common epitope) (Gregersen, Silver, & Winchester, 1987). In addition, certain *HLA* genotypes have a particular effect on the more invasive erosive disorder and increased death, indicating a critical function in peptide binding (Viatte *et al.*, 2015). Smaller, presumably individual and cumulative behavioral impacts may be associated with other genetic loci, for instance through modified costimulation mechanisms (eg, CD28, CD40), lymphocyte receptor activation threshold (e.g, *PTPN22*), cytokine signaling and activation of innate immune, respectively. In individuals with mutual epitopes, the enhanced threat of rheumatoid arthritis is related to autoantibodies against citrullinated peptides (ACPAs) and IgG (rheumatism receptor [RF]) autoantibodies. These distinctive self-antibodies for rheumatoid arthritis occur in about half to seventy percent of the diagnosed individuals with significant stabilization throughout the entire course of their diseases (Barra, Pope, Bessette, Haraoui, & Bykerk, 2010). The common epitope has little connection to ACPA-negative and RF-negative rheumatoid arthritis (Viatte *et al.*, 2015).

Epigenetics, perhaps through integrating environmental and genetic effects, can contribute to pathogenesis (Klein & Gay, 2015). In a recent epigenome study, ten distinct methyl positions that can promote genetic risk in rheumatoid arthritis are identified (Liu *et al.*, 2013). The biology of synovial fibroblast and leukocyte can be regulated by modified histone acetylation and by DNA methylation (Klein & Gay, 2015). Another epigenetic aspect of microRNAs is to focus on mRNA for degradation and thus to fine-tune cellular responses (Baxter, McInnes, & Kurowska-Stolarska, 2012). Many microRNAs are known to be important lymphocyte, macrophage and synovial fibroblasts (e.g. miR146a or miR155) controls (Blüml *et al.*, 2011). The medicinal use of microRNAs for rheumatoid arthritis still remains uncertain (Klein & Gay, 2015).

- **Age**

RA can start at any era, but with age, the probability rises. In their 60s, RA occurs most frequently in adolescents.

- **Gender**

New instances of RA are usually 2-3 times greater for females in comparison with males.

- **Smoking**

Multiple studies indicate that the risk of developing RA in a person is increased by cigarette smoking and can worsen the disease.

- **Live Birth History**

The risk of RA development is higher for women who never have given birth.

- **Exposures to Early Life**

Early life exposures can boost the risk of adult RA. One research discovered, for instance, that kids whose mothers smoked have double the danger of adult RA. The risk of developing RA as adults are increased for children of lower-income parents.

- **Obesity**

Obesity may raise the risk of RA development. Studies that investigate the role of obesity also found that more the overweight, the higher the risk of developing RA ("Rheumatoid Arthritis (RA) | Arthritis | CDC," n.d.)

1.2.5. Pathogenesis

Synovitis happens when the synovial compartment is infiltrated by leukocytes. Accumulation of leukocytes mainly represents migration rather than local proliferation.

Endothelial activation in synovial microvessels enables cell migration, which improves the activity of adhesion molecules (including integrins, selectins, and representatives of the

superfamily of immunoglobulins) and chemokines. Neoangiogenesis is, therefore, a feature of premature and developed synovitis caused by the local hypoxic circumstances and cytokines as well as inadequate lymphangiogenesis that reduces cellular evacuation (Polzer *et al*., 2008). These microenvironmental, coupled with the severe synovial restructuring and stimulation of the local fibroblast, enable the growth of synovial inflamed tissue in the region.

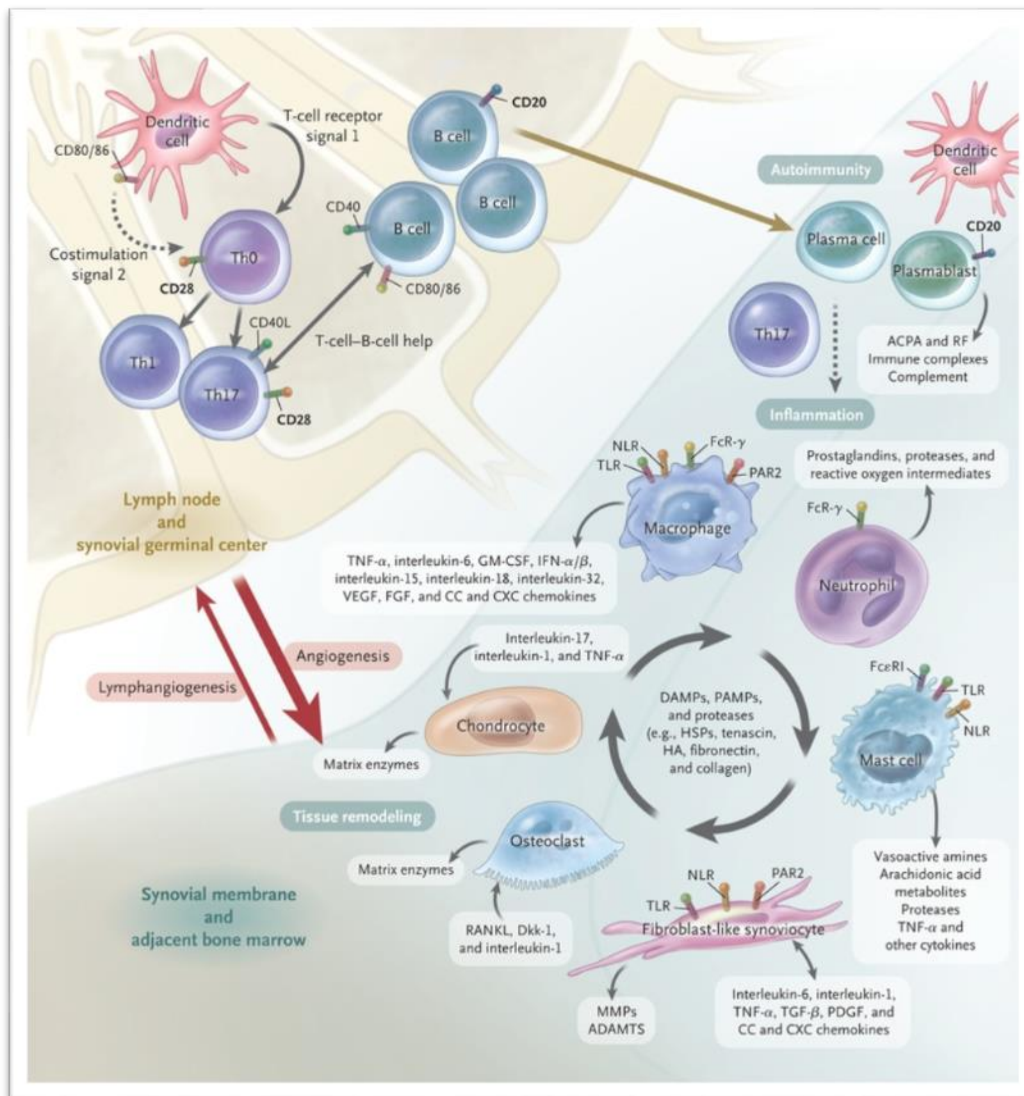


Figure 1. 10 Pathogenesis of Rheumatoid arthritis with reference to adaptive and innate immunity (Mcinnes & Schett)

Adaptive & Innate Immune Processes in Rheumatoid Arthritis Joint

It is shown in the figure that costimulation-dependent associations between dendritic cells, B cells, and T cells happen mainly in the lymph node; these outcomes develop an autoimmune response to self-proteins holding citrulline. Adaptive and innate immune mechanisms are incorporated in the synovial membrane and adjoining bone marrow to facilitate tissue remodeling and disruption. Positive feedback loops caused by the interfaces shown between leukocytes, synovial fibroblasts, chondrocytes, and osteoclasts, along with molecular injury products, fuel the chronic stage of rheumatoid arthritis pathogenesis (McInnes & Schett, 2007).

I. Role of Adaptive Immunity Pathway

The genetics of rheumatoid arthritis and auto-antibodies obviously emphasize the early pathogenesis mostly with adaptive immunity. Although T cells abound in the synovial environment, however, T cells' vital function appears poorly known. Cyclosporine or T-cell depleting treatment, directly blocking the T cells, has shown restricted or no effectiveness (Panayi, 2006). This finding may indicate the deletion of regulatory and effector T cells through a "wide spectrum," and states that a T-cell subset should be targeted. Rheumatoid disease comprises of extensive myeloid and dendritic plasmacytoid that are vital in the T-cell formation and the antigen delivery. They express cytokines (interleukin-12, 15, 18, and 23), HLA type II molecules, and costimulatory molecules (Lebre et al., 2008; Schroder, Greiner, Seyfert, & Berek, 1996). Autoreactive T proteins have been recognized against citrullinated autoproteins. The persistent local T-cell-mediated B cell assistance is recommended by synovial T-cell oligoclonality, germ center responses, and B-cell hypermutation. While traditionally rheumatoid arthritis is regarded as a Type 1 helper T cell-mediated illness, the focus has become highly oriented on the role of 17-helper T-cells (Th17), which produces interleukin-17A, 17F, 21, 22 & TNFα (Chabaud, Fossiez, Taupin, & Miossec, 1998; Miossec, Korn, & Kuchroo, 2009). The transforming growth factor β & interleukin-1β, 6, 21, and 23 derived from

macrophages and dendritic cells establishes an environment for Th17 differentiation and suppress the regulatory T cells differentiation, by doing so switching the homeostasis of T-cells to arthritis (Genovese *et al*., 2010). This discrepancy between Th17 and regulatory T cells can also be embodied in local TNF-α, restricting the functioning of regulatory T cells. Additional pathogenic pathway included antigen nonspecific, T-cell contact-mediated triggering of fibroblasts and macrophages by interaction with the CD40 and CD40 ligand, the CD200 and the CD200 ligand and the leukocyte function-associated antigen 1 & intracellular adhesion molecule 1 (McInnes, Leung, & Liew, 2000; Nadkarni, Mauri, & Ehrenstein, 2007). Humoral adaptive immunity is an essential aspect of rheumatoid arthritis. The B cells of synovial are located primarily in T-cell-B cell aggregates— certain organs have ectopic lymph follicles. The synovium and juxta-articular bone marrow have more commonly spread plasma cells and plasmablasts (Seyler *et al*., 2005). The effectiveness of rituximab in rheumatoid arthritis reveals the pathogenic significance of CD20 + B cells (Edwards *et al*., 2004). Based on clinical observation, the function of B cells and their progeny in rheumatoid arthritis pathogenesis extends beyond auto-antibody production, to include the progression of autoantibody and cytokine (e.g. interleukin-6, TNF- α, and lymphotoxin-β) because plasma cells are not affected by anti-CD20 antibodies and autoantibody post-treatment concentrations differ.

II. Innate Immune System Activation

A number of innate effector cells are identified in the synovial membrane, including macrophages, mast cells, and natural killer cells, though neutrophils exist primarily in synovial fluid. The colony-stimulation factor for macrophages, the granulocyte-colony stimulation factor and the colonization factor for granulocytes-macrophages (GM-CSF) boost the germination of these cells, and their migration from bone marrow to the synovium (Cornish, Campbell, McKenzie, Chatfield, & Wicks, 2009). Macrophages, in specific, are key synovitis effectors; clinically efficient biological agents continuously decrease macrophage synovium

infiltration (Haringman *et al*., 2005). Macrophages operate by releasing cytokines (e.g., TNF-α and interleukin-1, 6, 12, 15, 18, and 23), reactive nitrogen and oxygen intermediates, prostanoids production, matrix deteriorating enzymes, phagocytosis, and presentation of antigen. This paradigm of proinflammatory cytokines and inducible synthase of nitric oxide reveals a predominant macrophage M1 phenotype. Macrophages are triggered by toll-like receptor TLRs & NLRs. They identify a variety of pathogen-related molecular patterns and molecular damage-related patterns that possibly contain bacterial, viral and putative endogenous ligands (Seibl *et al*., 2003).

Neutrophils synthesize prostaglandins, proteases, and intermediate reactive oxygen and cause synovitis (Cascão, Rosário, Souto-Carneiro, & Fonseca, 2010). These results demonstrate that triggering the innate immune pathway can make contributions to synovitis, which can lead to treatment modulation that can be TLR, NLR, and inflammatory dependent pathways.

III. Intracellular Signaling Pathways & Cytokines

The pathogenesis of rheumatoid arthritis works on the generation of cytokine, resulting in multiple synovial cell groups. Cytokine patterns may be amended with the moment. Early rheumatoid arthritis has a supposedly separate cytokine pattern, comprising interleukin-4, 13 and 15 genes (Karim Raza et al., 2005) that eventually develops in chronic disease. TNF-α performs a major part by activating cytokine activity and chemokine activity, expression of endothelial cell-adherence molecules, synovial fibroblast defense, the advancement of angiogenesis, structural removal of regulatory T cells, and pain stimulation. In the same way, interleukin-6 activated local leukocyte and autoantibody production and mediated the systemic effects that encourage acute phase responses, anemia, cognitive dysfunction, and lipid-metabolism dysregulation. A successful membrane and soluble TNF-α, and interleucine-6 receptor therapy has been shown to be central to these two cytokines. They encourage

leukocytes, endothelial cells, chondrocytes, and osteoclasts to become active (Brennan & McInnes, 2008; McInnes & Schett, 2007).

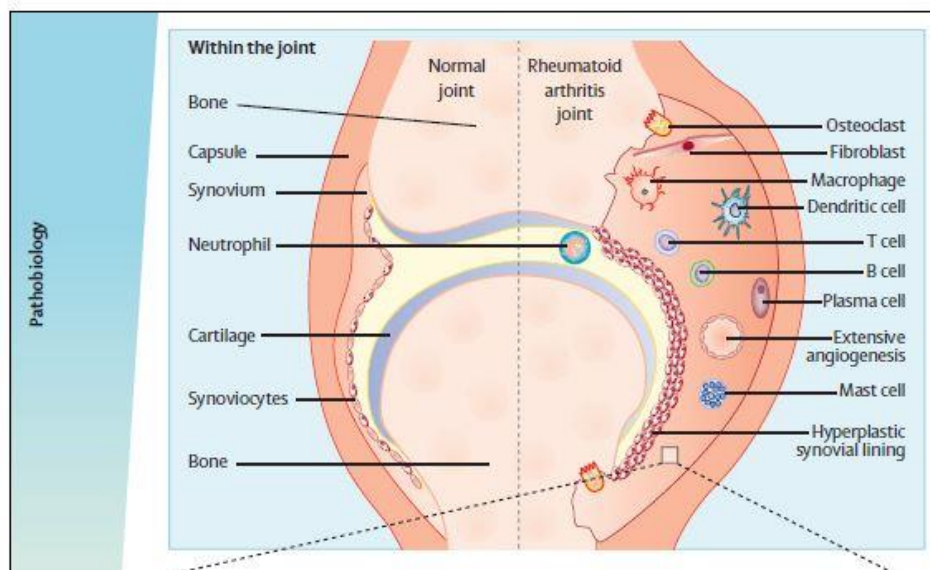
IV. Responses to Mesenchymal Tissue

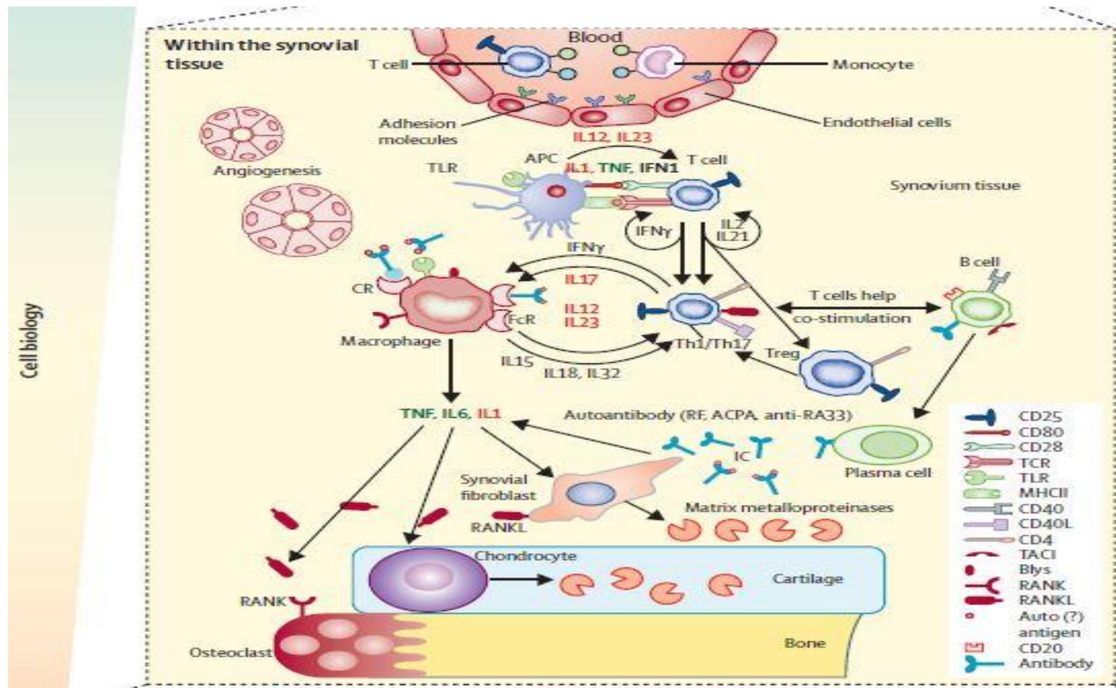
The natural synovium includes macrophages derived from mesenchymal and fibroblast-like synoviocytes (FLSs). In the case of rheumatoid arthritis, the membrane coating is extended and the FLSs hold a semi-autonomous phenotype defined by the separation of the anchorage, the loss of contact inhibition and expressions of cytokines and chemokines, adherence molecules, metalloproteinase matrix (MMPs) and metalloproteinase tissue inhibitors (TIMPs) at elevated rates (Bradfield *et al*., 2003). FLSs lead thus directly to local cartilage decomposition and synovial inflammatory chronicity and maintain a permissive microenvironment supporting the sustainability of T and B cells and adaptive immune organizations (Filer *et al*., 2006). Incomprehensible is molecular mechanisms supporting synovial hyperplasia. More probably, changed apoptosis resistance, which is influenced by various means, among many others are tumor suppressor p53 genes mutation (Aupperle *et al*., 1998) and expression of shock protein (Schett *et al*., 1998) that promotes the longevity of FLSs. Synoviolin controls p53 activity and its biological roles negatively. Moreover, cytokine-induced NF-κB-path activity in FLSs promotes longevity after TNF-α receptor ligation. The increase in the flow of mesenchymal proteins could also represent synovial hyperplasia. FLSs have shown to migrate and thereby encourage articular involvement in a mouse model of arthritis with serious combined immunodeficiencies (Lefèvre *et al*., 2009).

V. Inflammation

Rheumatoid arthritis joint swelling represents inflammation of the synovial membrane as a result of immune activation, with leucocyte infiltration into the commonly sparsely populated synovial area. Innate immune cells and adaptive immune cells are the cellular compositions of synovitis in rheumatoid arthritis. A strong reaction to tissue promotes joint destruction

(Mcinnes & Schett, n.d.). Cytokines and chemokines cause an inflammatory response to be induced or exacerbated by endothelial cells activation and the attraction of immune cells to accumulate inside the synovial compartment. In addition to cells T and B, monocytes, and macrophages, activated fibroblasts eventually cause osteoclasts to be generated by means of nuclear receptor activator т B-Ligand (RANKL), which is expressed on T-cells, B-cells, and fibroblasts, with their receptor RANK on dendritic cells, macrophages and preosteoclasts (Redlich *et al*., 2002).





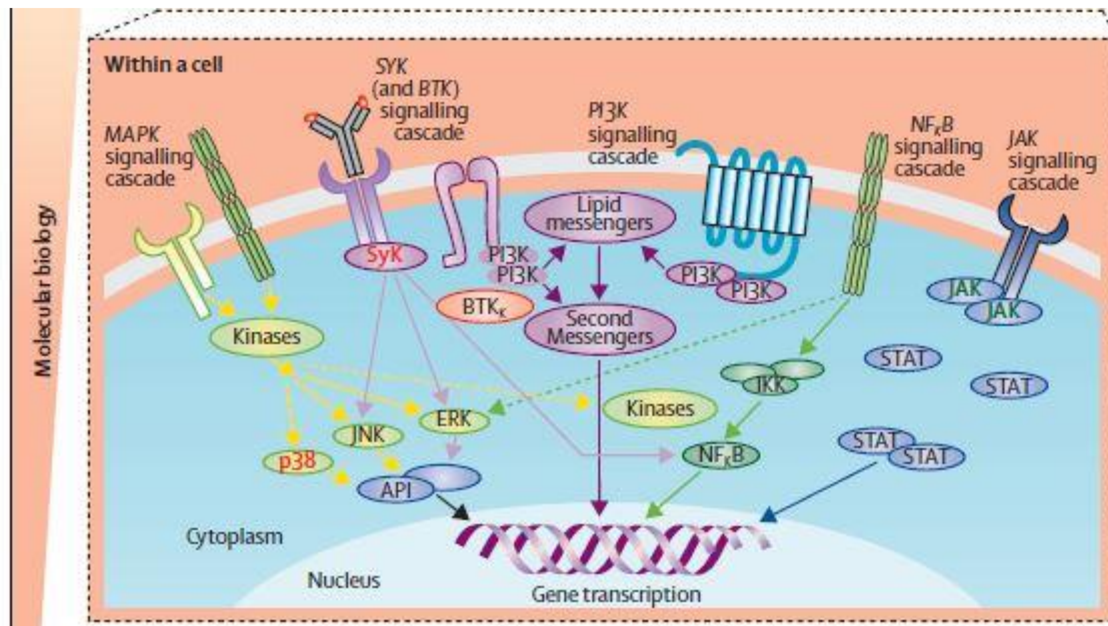


Figure 1. 11 Pathogenetic pathways in rheumatoid arthritis (Smolen et al., 2007)

Bony erosions occur at the intersection between cartilage, periosteal synovial membrane formation and bone from the so-called bare region. After the stimulation by cytokines, cartilage is damaged by catabolic activities in chondrocytes.

The matrix of cartilage is degraded by metalloproteinases and other enzymes. Cytokines bind cognate receptors to trigger several transductive intracellular signal events between extracellular events and activation of a range of genes that can lead to or exacerbate inflammatory response and damage (Martel-Pelletier, Welsch, & Pelletier, 2001).

1.2.6. Diagnosis

In the early phases of rheumatoid arthritis, early signs and symptoms are similar to those of numerous other illnesses. The diagnosis cannot be confirmed through a single blood test or physical finding. Your doctor will check for swelling, redness, and warmth during your physical examination. He or she can inspect your muscle power and reflexes, too ("Rheumatoid arthritis - Diagnosis and treatment - Mayo Clinic," n.d.).

i. Physical Examination

A physical exam is one of the first diagnostic techniques. The purpose of the assessment is to assess the attributes of joint pain and swelling to differentiate better from other causes of joint pain such as osteoarthritis.

Table 1. 3 Difference between rheumatoid arthritis and osteoarthritis ("Rheumatoid Arthritis or Osteoarthritis: How Can You Tell? | HealthCentral,")

	Osteoarthritis	**Rheumatoid Arthritis**
Age of onset	Usually begins after age 40	May begin at any age, but usually before age 50
Location of joint pain	Usually affects weight-bearing joints, such as the knees and hips, but affects the finger joints; pain is often on one side of the body only	Usually affects small joints, such as hand, foot, wrist, elbow, shoulder or ankle, usually on both sides of the body
Joint appearance	Usually cool, not red or swollen	Inflammation causes joints to worm, red and swollen
Morning joint stiffness	Lasts only a few minutes	Lasts for at least 30 minutes and can persist for hours
Symptoms besides joint pain stiffness	Usually does not affect overall health	May be accompanied by fatigue, weight loss and fever
Disease progression	Symptoms gradually worsen over a period of years	Symptoms worsen over a period of weeks or months
What eases pain or stiffness	Pain subsides with rest and worsens with activity	Stiffness decreases with activity

ii. Laboratory Tests

Laboratory tests are used to diagnose rheumatoid arthritis for two main reasons: to diagnose your serostatus and measure / monitor your body inflammation.

a. Serostatus

Two tests are used to determine your serostatus:

- **Rheumatoid factor (RF)** is a form of autoantibody that is discovered in 80% of diseased individuals. Autoantibodies are body-produced proteins that attack healthy cells. Although high levels of RF strongly suggest rheumatoid arthritis, other autoimmune disorders such as lupus or non-auto-immune disorders, such as cancer and chronic infections may occur.

- **Anti-cyclic citrulline peptide (APCC)** is another form of autoantibody found in most rheumatoid arthritis patients. In contrast with RF, a favorable anti-CCP test outcome happens in rheumatoid arthritis almost exclusively. A favorable outcome could also recognize family members at risk for the disease.

b. Inflammatory Markers

Tests are performed to assess inflammation level by examining important blood markers.

- **Erythrocyte sedimentation rate (ESR) test** is used to measure the rate at which red blood cells settle down in an hour at the bottom of a lengthy upright tube known as a Westergren tube. When inflammation is present, the red blood cells stick together and sink faster. It is a non-specific inflammation assessment but it provides important insights towards a diagnosis.
- **C-reactive protein (CRP)** is an inflammatory-produced protein of the liver. Although ambiguously defined, the inflammatory reaction is a more direct measure.

iii. Imaging Tests

Imaging exams are used to define the indications of joint harm in rheumatoid arthritis, such as bone and cartilage erosion and joint narrowing. Each exam can give various and particular perspectives:

- X rays are particularly helpful for bone erosion and joint damage identification.
- Magnetic resonance imaging (MRI) can look beyond the bone, spot soft tissue changes, and even positively identify early disease joint inflammations.
- Early joint erosion may also be better detected by ultrasounds and certain joint inflammatory regions can be identified (Aletaha et al., 2010; Anderson et al., 2012; Bykerk & Massarotti, 2012; Smolen, Aletaha, & McInnes, 2016).

1.2.7. Complications

- **Heart problems**: People with rheumatoid arthritis will die of heart problems 50 percent more likely. They have a heart attack or a stroke twofold more likely.
- **Overall eye issues**: The chance of eye conditions, such as dry eyes, pain, blurred vision or vision loss and eye inflammation, rises with rheumatoid arthritis.
- **Osteoporosis:** This condition is also called bone loss in people who have rheumatoid arthritis. People with RA need to be checked every two years for their bone density.
- **Blood Vessel Disease**: This disease is unusual in patients, but can influence the blood flow in the body and consequently affect all of the vital organs.
- **Lung problems**: rheumatoid arthritis inflammation occurs, which can influence lung lining, causing fluid collection.
- **Forming nodules:** Strong tissue lumps start to thrive below joints, like fingers and elbows in the body. These nodules can grow in every part of the lungs.
- **Carpal tunnel syndrome**. The nerve supplying to your fingers and your hand can be greatly impacted if your writs are influenced by rheumatism.

1.2.8. Treatment

Rheumatoid arthritis is not cured. Clinical research indicates that symptom remediation is more probable if medicines known as anti-rheumatic disease-modifying medicines (DMARDs) therapy begin early.

a. Medications

- **NSAIDs.** Anti-inflammatory drugs (NSAIDs) that are not steroidal may alleviate pain and lower inflammation. The NSAIDs includes ibuprofen and naproxen sodium (Aleve), over-the-counter (Advil). The prescription for stronger NSAIDs is accessible. Neither stomach irritation, heart problems or renal damage are part of the side effect.

- **Steroids:** Corticosteroid drugs like prednisone decrease swelling and pain and slow joint injury. Side impacts may include bone erosion, increase in weight and diabetes.
- **Disease-modifying antirheumatic drugs (DMARDs):** These medications can slow rheumatoid arthritis progression and prevent prolonged harm to the joints and other tissues. Common DMARDs include sulfasalazine, methotrexate & hydroxychloroquine.

These medicines could target immune system components that cause inflammation causing damage to joint and tissue. Such medicines also improve the risk of infection. Higher doses of tofacitinib can boost the risk of blood coagulation in the lungs in individuals suffering from rheumatoid arthritis. When combined with nonbiological DMARD, such as methotrexate, biological DMARD becomes generally most efficient.

b. Therapy

Your doctor may ask a physical or occupational therapist to teach you workouts that assist to maintain your joints flexible.

c. Surgery

One or more of the following processes may include rheumatoid arthritis surgery:

- Synovectomy. Operation to remove the inflamed joint lining.
- Tendon repair
- Joint fusion
- Replacement of total joint

d. **Alternative medicine**

- Fish oils: Preliminary studies have discovered that supplements of fish oil may decrease pain and rigidity of rheumatoid arthritis. Nausea, rashes and a fishy smell in the mouth

can include as side effects. Medications may interfere with fish oil, so first check with your physician.

- Plant oil. In the seeds of primrose, borage, and black currant, the fatty acid is present, which can help to heal rheumatoid arthritis and stiffness in the morning. Headache, diarrhea, and gas may also have side impacts. Some vegetable oils can harm the liver or interfere with medicines, first check with your physician ("Rheumatoid arthritis - Diagnosis and treatment - Mayo Clinic," n.d.)

CHAPTER # 2 LITERATURE REVIEW

The complicated and chronic systemic conditions of (T2DM) and rheumatoid arthritis (RA) are both main causes of disease and death, particularly in association with each other (Consortium *et al*., 2010; Maradit-Kremers *et al*., 2005), and the risk of cardiovascular disease (CVD). As is well established, RA is an autoimmune disorder with inflammation of various joints and gradual and chronic immune system activation (Julia, chemistry, & 2013, n.d.; Solomon et al., 2003). T2D is a metabolic disorder with glucose uptake defects in response to insulin (Johansen Taber & Dickinson, 2015). While T2D is commonly not labeled as autoimmune disease, its condition is related to modified immune response, including low-level inflammation (J. P.-D. care & 2004, n.d.). Moreover, several studies have verified that RA patients are at elevated danger for developing T2D (Han, Robinson, …, & 2006, n.d.; Simard, rheumatology, & 2007, n.d.). The association between RA and T2D has not however been excluded, and whether the association is linked with the immune response is still not explained, in short, because the molecular association and mechanisms that are commonly shared among these two disorders are not yet studied in depth (rheumatology & 2007, n.d.).

The disruption of inflammation and immune response signaling pathways have been regarded as one of the key factors against RA and T2D. There was evidence that in visceral adipose tissue and insulin receptor signaling impaired insulin resistance (IR), an overproduction of pro-inflammatory cytokines TNF-α and IL-6, which were the main arbitrators of inflammation in RA (Bastard, Maachi, Lagathu, …, & 2006, n.d.; Dessein, Joffe, Arthritis, & 2005, n.d.). Some inflammatory cytokines were found to be corresponding with both RA and T2D and linked to insulin sensitivity in RA patients (Öncül, Top, Özkan, Çavuşlu, & Danaci, 2002; K Raza, Falciani, …, & 2005, n.d.), for example, high IL-2 concentrations in both serum and synovial tissue (Llorens *et al*., 2011). The complex relationships in RA and T2D among cytokines had not yet been fully understood.

2.1. Signal Transducer & Activator of Transcription-4 (*STAT-4)*

Initially, *STAT-4* was isolated by two groups using degenerative PCR or low-stringency hybridizing, both based on SH2 homology of the other *STAT* proteins (Yamamoto, Quelle, biology, & 1994, n.d.). *STAT-4* is a critical anti-inflammatory immune response mediator. A family of factors involved in a variety of biological processes are *STAT* proteins. Latent *STAT-4* molecules are triggered to form homodimers by cytokine stimulation. Then the dimers shift to the nucleus, bind DNA and modulate the transcription of genes. This mechanism provides a direct link in the nucleus between the stimulation of cytokine / growth factor surface cells and the activation of genes. Interleukin 12 (*IL-12*), a hallmark of *STAT4* activator, is a pléiotropical cytokine found in macrophages and dendritic cells (DCs) (Immunology & 2003, n.d.). *IL-12*'s biological impacts include interferon-γ expression to NK and activated T-cells, increasing cytotoxic response in both T and NK cells, induced T-cell proliferation and stimulation to fully functioning Th1 cell development. Initial studies in *STAT-4* deficient mice have shown that most *IL-12* biological responses, including development of *IFN-α*, require *STAT-4* (Kaplan, 2005).

The receptor *IL-12* consists of two chains, named *IL-12Rβ1* and *IL-12Rβ2*, and ligand binding results in the activation and forming of the JAK kinase, JAK2 and TYK2 heterodimer receptors. These tyrosine kinases phosphorylate *STAT-4*, homodimers are formed through the SH2 domain and moves to nucleus where conventional N3 *STAT* target sequences can be recognized in *rIL-12* responsive genes (Jacobson, Szabo, Medicine, & 1995, n.d.).

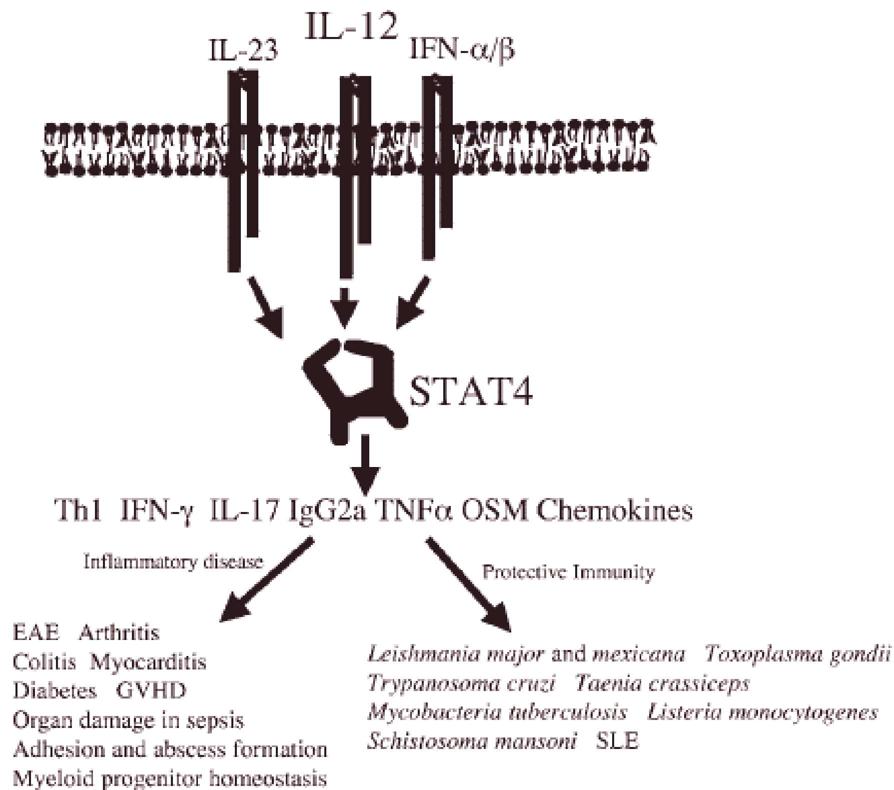


Figure 2. 1 Functional in vivo summary of *STAT-4*. Cytokines that activate *STAT-4* lead to biological responses that may protect in response to different pathogens but if uncontrolled, lead to tissue destruction and organ damage (Kaplan, 2005)

2.2. Differentiation of T-cells & *STAT4*

While *STAT-4* is expressed in both Th1 and Th2 cells, *STAT-4* can only be phosphorylated in Th1 cells by *IL-12* due to *IL-12Rβ* downregulation specifically in Th2 cells. This indicated that *STAT-4* plays a major role in the activity or growth of Th1 cells (Szabo, Dighe, …, & 1997, n.d.). *STAT-4* is found in both CD4 + T cells and CD8 + T cells, both of which, although these cells have distinct roles, can generate IFN-γ. A recent report has shown that the need for *STAT-4* is unique in these cells (Carter & Murphy, 1999; Wurster, Tanaka, & Grusby, 2000).

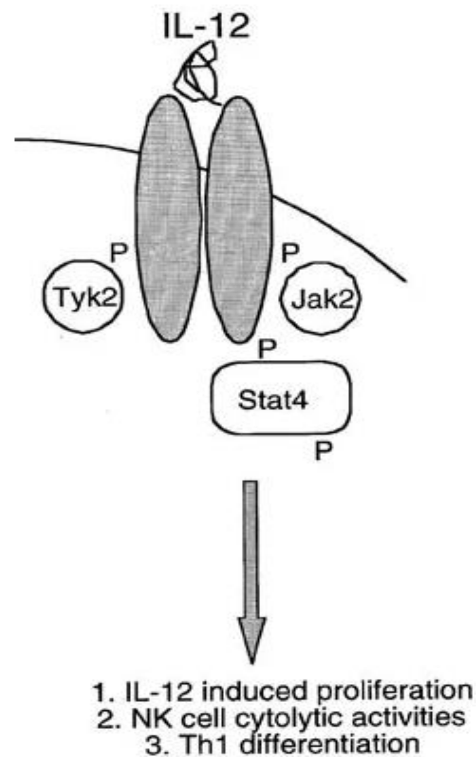


Figure 2. 2 Biological outcomes of *STAT4* activation (Wurster et al., 2000)

2.3. *STAT-4* & Diabetes

STAT-4 is a key mediator for inflammation in protective and immune-mediated diseases. Since *STAT-4* is required to develop fully functional Th1 cells, *STAT-4* deficient mice are protected from the impacts of autoimmune diseases mediated by T-cells. *STAT-4* deficient mice show less disease in experimental diabetes models and lower inflammation parameters compared to WT mice. *STAT-4* could therefore play a significant role in diabetes (Yang et al., n.d.). The *STAT-4* signaling pathway for autoimmune diabetes is examined using a virally induced autoimmune diabetes model rat via insulin promoter lymphocytic choriomeningitis virus. The abolition of the signal from *STAT-4* significantly reduced CD4 +-T cell-dependent but not CD4 +-T cell-independent diabetes, which revealed the kinetics that have been great-tuned in autoimmunity pathogenesis. But without *STAT-4* the generation of autoreactive cell responses to Th1/Tc1 T and the protection of antiviral immunity was not prevented. A new and attractive

approach to preventing clinical insulin-dependent diabetes mellitus in the prediabetic individual could be a selective inhibition of the *STAT-4* signal transduction pathway (Holz et al., n.d.).

2.4. *STAT-4* & Rheumatoid Arthritis

The *STAT-4*, a key player in both sides, has been shown to have a key role in experimental autoimmunity models. Mice with a *STAT-4* deficiency are usually resistant to autoimmune disease models, such as arthritis. Furthermore, inhibitory oligodeoxynucleotides or antisense oligonucleotides may be used to improve the diseases of *STAT-4* in models of arthritis, suggesting the use of *STAT-4* as a therapeutic target (Remmers *et al*., 2007). Only *HLA* genes have been identified as genetically confirmed genetic risk factors for rheumatoid arthritis for 30 years. Recently, a second, more modest link with the *PTPN22* gene has been identified. In recent study, the *STAT-4 rs7574865* polymorphism is identified as a new susceptibility marker for rheumatoid arthritis. Interestingly, the systemic lupus erythematosus is also associated with this SNP (Orozco & Martín, 2008).

STAT in RA synovium is also overexpressed. Previous studies have found chromosome linkages 2q33 in RA and have shown that the polymorphism at 2q33 of the *STAT-4* gene is the liable marker for the contact signal at 2q33. *STAT-4 rs7574865* polymorphism was found to be associated with RA patients in Europe, Asia and Latin America (Korczowska, 2014).

2.5. *STAT-4* SNP *rs7574865*

The most common type of genetic variation in the human genome was single nucleotide polymorphism (SNP) (Park *et al*., 2011). Some potentially working SNPs, for example cancer, schizophrenia and stroke, have been reported to be associated with the risk of complex conditions in humans. A SNP (*rs7574865* G/T) in *STAT-4* is noteworthy in the third intron of the gene. Although the *STAT-4* expression can be affected, it is not located in the promoter or

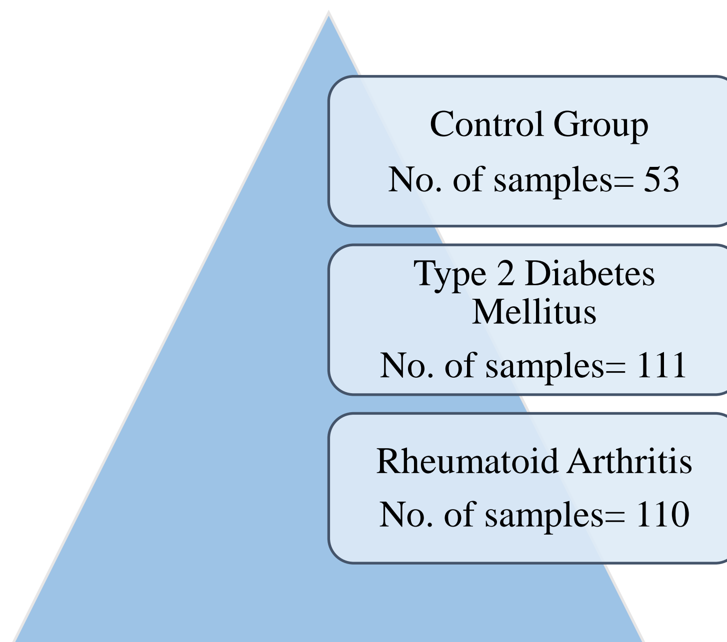
3′-untranslated region. Compared to *rs7574865* G, *STAT-4* mRNA transcription and protein expression may be significantly improved by the presence of T allele (Lamana *et al*., 2015).

CHAPTER # 3 METHODOLOGY

3.1. Sample Size & Collection

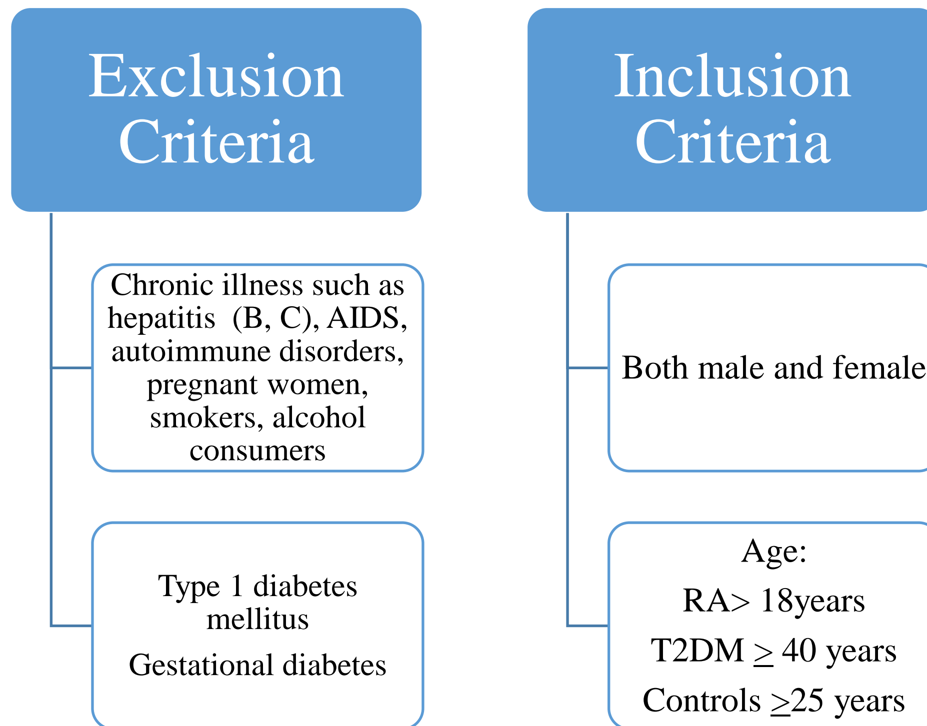
This underlying study was conducted to analyze the prevalence of *STAT-4* (*rs7574865* G/T) in the population of patients enduring with T2DM and RA in comparison with healthy controls. Total of 274 samples was collected for this study along with thorough history taking and clinical and laboratory data. The sampling was done from PIMS, Islamabad, MH Rawalpindi and DHQT hospital, DIK over the period of 3 to 4 months.

The study was categorized into the following groups:



3.2. Study Subjects

This involved below-mentioned inclusion and exclusion criteria for collecting samples.



3.3. Blood Sample Collection

The blood was collected from RA and T2DM patients with the assistance of phlebotomist in appropriately labeled EDTA vial of 5ml with name, age & identification number. The informed consent of concerned patients was retrieved before blood was drawn. The required above mentioned criterias were duly assessed during blood sampling. The samples were immediately placed in an ice box and brought back to the lab. The samples were later stored at 4°C.

Table 3. 1 List of materials required for blood sampling

S.No.	Materials Required for Blood Sampling
1.	Tourniquet
2.	5ml EDTA Vials
3.	Syringes
4.	Alcohol Pads
5.	Adhesive Bandages
6.	Gloves
7.	Ice Box

3.4. Isolation of Genomic DNA

After bringing back samples to ASAB Immunogenetics lab, NUST, Islamabad, isolation of genomic DNA from blood samples was performed employing the Phenol-Chloroform Protocol extending over for two days.

All the materials and glass wares used for extracting DNA were washed, cleaned and autoclaved at 121°C and 15psi pressure. The subsequent are the tables for materials and recipes used for the extraction.

3.4.1. Reagents

Table 3. 2 Solution A (lysis of blood cell)

S.No.	Components	Molarity (mM)	Quantity (g/1000ml)
1.	Sucrose	0.32	109.44
2.	Tris	10	1.21
3.	$MgCl_2$	5	1.01
4.	Distilled Water	-	Upto 1000

Before using the autoclaved solution A, Triton 100X (1% V/V) was added.

Table 3. 3 Solution B (Precipitation of DNA & Protein)

S.No.	Components	Molarity (mM)	Quantity (g/1000ml)
1.	Tris	10	1.21
2.	NaCl	400	23.37
3.	EDTA	2	0.58

Table 3. 4 Solution C (DNA isolation)

S.No.	Components	Quantity (µl)
1.	Phenol	400

Table 3. 5 Solution D (DNA Purification)

S.No.	Components	Quantity (ml/500ml)
1.	Chloroform	480
2.	Isoamyl Alcohol	20

Table 3. 6 20% SDS solution

S.No.	Components	Quantity (g/100ml)
1.	SDS	20
2.	Distilled Water	Upto 100ml

20g of SDS was dissolved in 100ml of distilled water.

3.4.2. Protocol

3.4.2.1. DAY 1 of Isolating DNA Protocol

Blood samples kept in EDTA tubes were incubated at room temperature and mixed it well by inverting the tube for several times and then 750µl of the blood sample was added into the 1.5ml centrifuge tube (Axygen, California, USA). 750µl of solution A was first added in a centrifuge tube which was then closed and inverted 4-6 times and then incubated at room temperature for 5-10 minutes. The mixture was centrifuged for 1 minute at 13000 rpm in a microcentrifuge. The supernatant was discarded and the pellet was re-suspended twice in 400µl of lysing solution A. Re-suspended pellet was again centrifuged for 1 minute at 13000 rpm. The supernatant was then discarded and the pellet was re-suspended in 400µl of a solution B

for precipitation. 20µl of 20% SDS (Sodium Dodecyl Sulphate) and 5µl of proteinase K were added and the mixture was incubated at 37°C overnight.

3.4.2.2. DAY 2 of Isolating DNA Protocol

A freshly prepared solution C and solution D was made and 500µl was added into the previously incubated sample. Afterward, these tubes were centrifuged at 13000 rpm for 10mins. The aqueous phase was collected in a new tube. 500µl solution D was added to the aqueous layer and it was then centrifuged at 13000 rpm for 10 min. In a new tube, the aqueous layer was collected. DNA was precipitated out by adding 55µl of sodium acetate (3M, pH 6) and the equal volume of isopropanol and inverted tube several times.

Centrifuged for 10 minutes at 13000 rpm. A volume of 200µl of 100% ethanol was added to the DNA pellet and centrifuged at 13000 rpm for 7 min. Then 200µl of 70% ethanol was added and centrifuged at 13000 rpm for 7 minutes. Ethanol was discarded and DNA pellet was then dried for 30 min by keeping the microfuge tubes at 37°C and then dissolved the pellet in 200µl of TE buffer.

3.5. 1% Agarose Gel Electrophoresis

It is an efficient method to separate DNA or protein in the agarose matrix. 1% (w/v) agarose gel was resorted to analyzing the quality of DNA. The following table depicts reagents used for agarose gel electrophoresis.

3.5.1. Reagents

Table 3. 7 10 X TBE Buffer

S.No.	Components	Quantity (g/1000ml)
1.	Tris Base	108
2.	Boric Acid	55
3.	EDTA	7.5
4.	Deionized Water	Upto 1000

The buffer was diluted up to the volume of 1000ml. White chunks were dissolved by using magnetic stirrer on vortex machine.

NOTE: For the preparation of 1 X TBE buffer, 50ml TBE buffer was dissolved in 450ml distilled water to make the volume of 500ml

Table 3. 8 1% agarose gel

S.No.	Components	Quantity (g or µl/1000ml)
1.	1X TBE	100ml
2.	Agarose	1g
3.	1X Ethidium Bromide	25

3.5.2. Protocol

1g of agarose powder was weighed appropriately on an electronic balance. It was then dissolved in 100ml of 1X TBE buffer in the microwave for nearly 2 minutes. After letting the

solution to cool down for a while and steam was evaporated, ethidium bromide was mixed in the gel solution for the purpose of staining. Afterward, the gel solution was cascaded into the gel-casting tray and accredited it to solidify at room temperature. The gel was loaded after placing it in the gel tank containing 1 X TBE buffer. The mixture of loading dye and genomic dye was settled well into the wells of the gel. The electrophoresis was initiated at 80 volts for 40 minutes. Afterward, the gel was analyzed in Omnidoc for obtaining electrogram of the gel.

3.6. Quantification of DNA

The isolated genomic DNA was quantified with the help of ThermoScientific Nanodrop 2000 UV-Vis Spectrophotometer and NanoDrop ^{TM} 2000 software at ASAB laboratory, NUST. At first, the TBE buffer was used for blank and then the sample was loaded to arbitrate the absorbance ratio. The absorbance wavelength 260nm was kept as standard and optimum absorption of nucleic acid was recorded at this wavelength. The absorbance ratio of 260/280nm was suggestive towards purity of DNA.

3.7. Primer Designing

Manually, one forward and two reverse primers are designed for allele-specific polymerase chain reaction (ASPCR) utilizing tools such as dbSNP, Ensemble, Oligocalc and UCSC genome explorer.

FASTA SNP sequence is retrieved by entering SNP ID in dbSNP, a database for identifying gene variations. Hair-pin formation and self-complementarity are verified by entering that SNP sequence in Oligocalc, a software for calculating oligonucleotides. Subsequently, in-silico PCR in reference to UCSC genome browser is done to verify the specific binding of primers through target allele, as it calculates the hypothetical effects of PCR and gets to know the amplicon size. Forward and reverse primers are entered to amplify the target DNA sequence, and computational amplification is tested.

Table 3. 9 Forward & reverse primers sequences of *STAT-4* rs7574865 for ARMS PCR

S.No.	Gene *STAT-4*	Sequence	Melting Temp	Base Pairs	Amplicon Size
1.	*rs7574865* Forward T	GACTCTGTGTACCTGCAACT	52.7 °C	20	500
2.	*rs7574865* Reverse G	GAAAAGTTGGTGACCAAAATGTG	60.6 °C	23	500
3.	*rs7574865* Reverse T	GAAAAGTTGGTGACCAAAATGTT	59.3 °C	23	500

3.8. ARMS PCR

Allele specific amplification refractory mutation system polymerase chain reaction was carried out on T2DM and RA samples for amplifying the target SNPs and analyzing the polymorphism in study groups. Tri primers i.e. one forward & two reverse were designed for the given SNP. A set of forward and reverse primer was added in a single tube hence two sets of tubes for each sample were utilized. Amplification of the target primer indicated the presence of target SNP in the sample. Total reaction mixture of 25µl was made in each PCR tube in the Biosafety cabinet and vortexed for 30 seconds to homogenize the mixtures properly and removed the air bubbles. The reaction mixture was then loaded in Thermocycler 2720 (Applied Biosystems, Foster City, USA).

Table 3. 10 Reaction mixture profile for ARMS PCR of *STAT-4* rs7574865

S.No.	Components	Quantity (µl)
1.	Nuclease Free Water	15.7
2.	$MgCl_2$	2.0
3.	*Taq* Buffer	2.5
4.	2mM dNTPs	1.5
5.	Forward Primer	1.0
6.	Reverse Primer	1.0
7.	Genomic DNA	1.0
8.	*Taq* DNA Polymerase	0.3

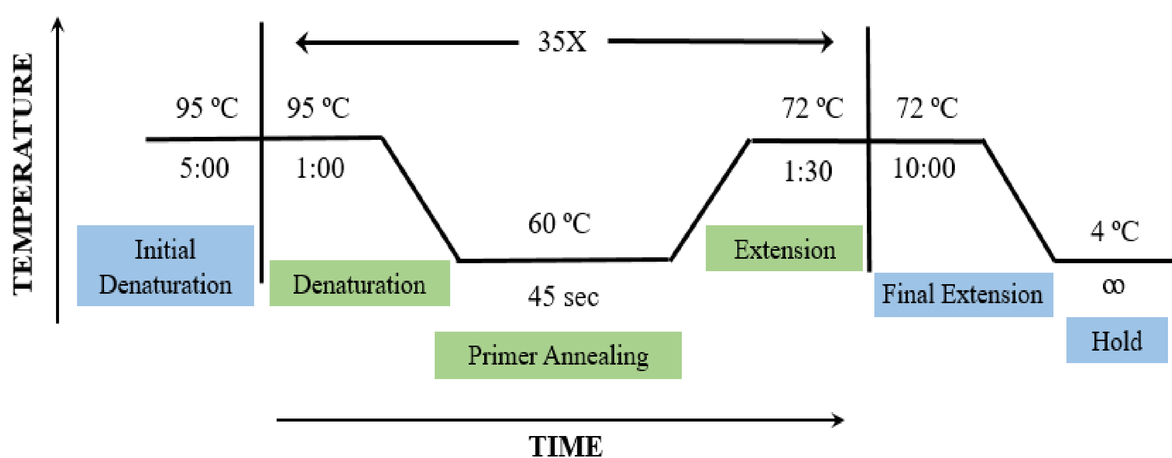


Figure 3. 1 PCR cycle of *STAT-4* rs7574865

3.9. 2% Agarose Gel Electrophoresis (w/v) for PCR Products

For the analysis of PCR products, 2% agarose gel electrophoresis was carried out. It is an effective method to visualize PCR products.

Table 3. 11 Recipe for 2% agarose gel

S.No.	Components	Quantity
1.	1X TBE Buffer	100ml
2.	Agarose	2g
3.	1X Ethidium Bromide	8µ/ml

3.9.1. Protocol

2g of agarose powder was weighed appropriately on an electronic balance. It was then dissolved in 100ml of 1X TBE buffer in the microwave for nearly 2 minutes. After letting the solution to cool down for a while and steam was evaporated, ethidium bromide (8µ/ml) was mixed in the gel solution for the purpose of staining. Afterward, the gel solution was cascaded into the gel-casting tray and accredited it to solidify at room temperature. The gel was loaded after placing it in the gel tank containing 1 X TBE buffer. The mixture of loading dye and the PCR product was loaded in the ratio of 2:5 with the help of micropipette. Along with this, the 100bp ladder was loaded as well to classify the size of the PCR product. The loading dye, PCR product, and ladder was settled well into the wells of the gel. The electrophoresis was initiated at 80 volts for 40 minutes. Afterward, the gel was analyzed in Omnidoc for obtaining electrogram of the gel.

3.10. Statistical Analysis

The results were statistically analyzed using "GRAPHPAD PRISM" and online 2 X 2 contingency table. One way ANOVA, Chi-square, Odds ratio, Relative risk, Genotypic Frequency & Allelic Frequency were calculated. P values; *P: <0.05, **P: <0.01, ***P: <0.001 were considered as significant.

CHAPTER # 4 RESULTS

Research was conducted to ascertain the association of *STAT-4* gene polymorphism with the vulnerability and severity of rheumatoid arthritis and type 2 diabetes mellitus. Blood sample collection was done from PIMS Islamabad, MH Rawalpindi and DHQ Hospital D.I.Khan. The overall samples obtained were 274, of which 110 were of RA, 111 were of T2DM and 53 were controls.

4.1. DNA Extraction & Quantification

The Phenol-Chloroform ® approach was used to extract DNA from the blood samples of all groups and the process lasted for two days. To assess the quantity and quality of extracted genomic DNA, Thermo Scientific NanoDrop 2000 Spectrophotometer and NanoDropTM 2000 software were used. The 260/280 ratio was established to ensure genomic DNA purification.

4.2. Analysis of *STAT-4 rs7574865* Polymorphism

For the analysis of *rs7574865 STAT-4* polymorphism, allele specific polymerase chain reaction was done to amplify DNA. All blood samples were screened to determine the existence and lack of a specific allele. The PCR products were visualized via agarose gel electrophoresis after amplification of DNA by allele-specific PCR.

STAT-4 rs7574865 is an SNP variant present in the third intron at chromosome 2q32.2 position. It has G>T variation, where G is the ancestral allele and T is the risk allele. The RA, T2DM and control genotypes are shown in the figure 4.2, whereas an allele frequency graph is given for the control and experimental groups in figure 4.3. Moreover, the electropherogram of 2% agarose gel stained with ethidium bromide was set up for the visualization under gel doc of the SNP i.e. *STAT-4 rs7574865* as given in the figure 4.1.

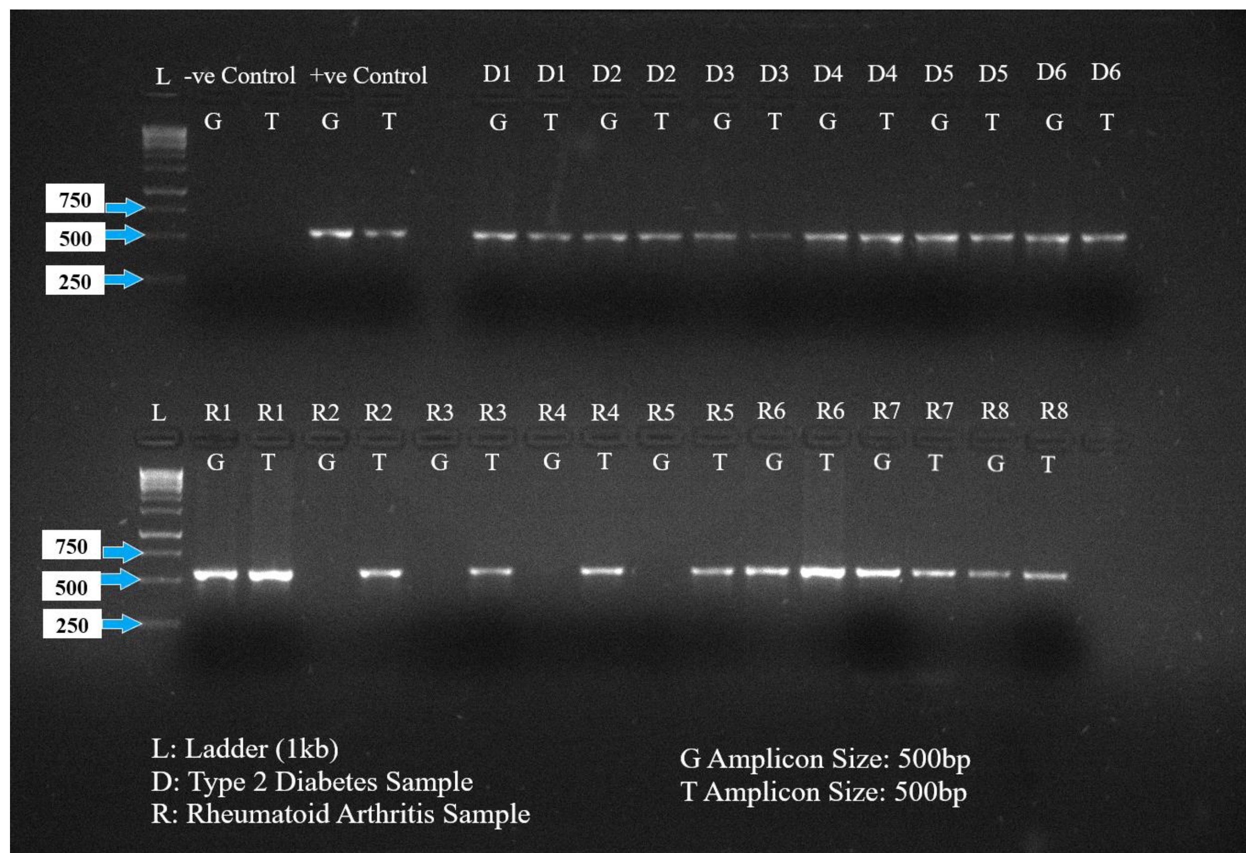


Figure 4. 1 *STAT-4* rs7574865 electropherogram of PCR products on 2% agarose gel electrophoresis

2% agarose gel electrophoresis showed the amplified PCR product of *STAT-4 rs7574865*. The bands were observed under the gel doc, which was visualized under the ultraviolet light. The 100bp ladder was run along the products to determine the size of the enhanced product, as well as the positive and negative control. The amplicon size of ancestral allele G in the first band which has a protective effect on disease was found to be 500bp while that of risk allele T in the second band was also at 500bp. L is designated for the ladder, D for the T2DM samples, R for the RA samples and C for the controls.

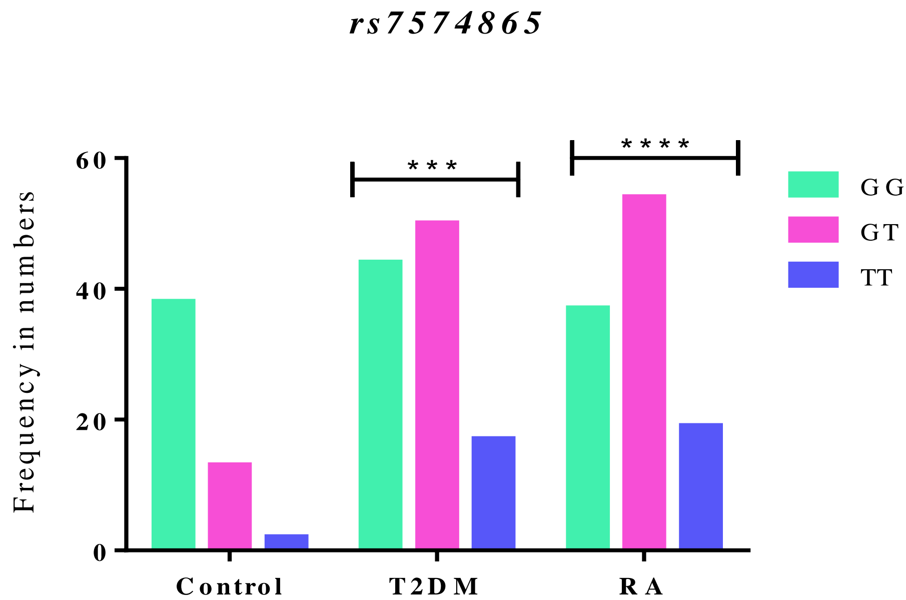


Figure 4. 2 Genotype distribution of *STAT-4 rs7574865* polymorphism in control vs experimental groups

The genotype frequency graph of *STAT-4 rs7574865* in figure 4.2 shows the higher prevalence of heterozygous GT and homozygous TT in disease groups i.e. RA & T2DM as compared to controls. While higher trends of genotype GG is seen in all the 3 groups.

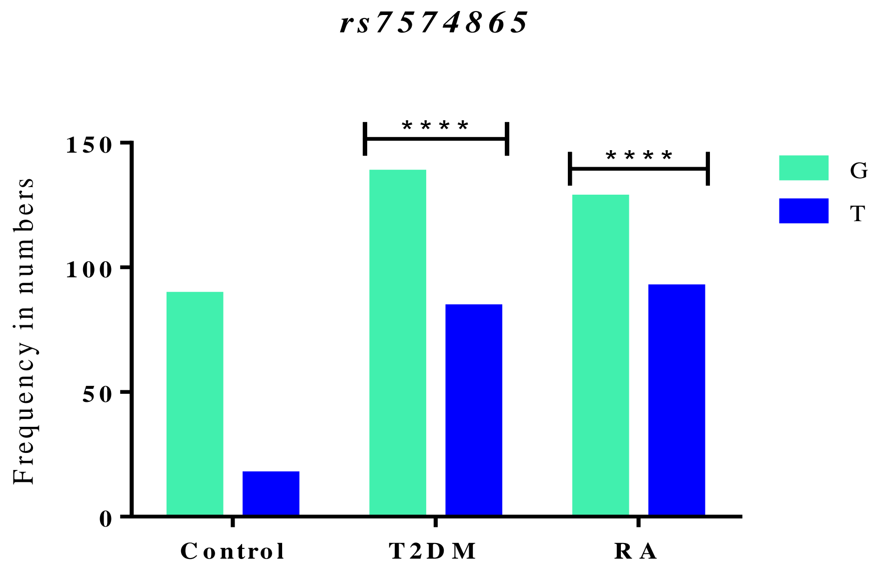


Figure 4. 3 Allele distribution of *STAT-4 rs7574865* polymorphism in control vs experimental groups

The allele frequency graph in figure 4.3 exhibits distribution of ancestral allele and risk allele in cases i.e. RA & T2DM and control group for *STAT-4 rs7574865*.

Table 4. 1 Genotype and allele frequencies of *STAT-4* rs7574865 polymorphism

Genotype Or Allele	Control n = 53 (%)	RA n = 110 (%)	T2DM n = 111 (%)	Control vs RA			Control vs T2DM		
				OR (95% CI)	RR (95% CI)	*p* value	OR (95% CI)	RR (95% CI)	*p* value
Genotype									
GG	38 (71%)	37 (34 %)	44 (40%)	0.20 (0.09-0.41)	0.47 (0.34-0.64)	<0.0001	0.26 (0.13-0.53)	0.55 (0.42-0.74)	0.0004
GT	13 (25%)	54 (49 %)	50 (45%)	2.97 (1.43-6.15)	2.00 (1.20-3.33)		2.52 (1.21-5.23)	1.84 (1.09-3.07)	
TT	2 (4%)	19 (17 %)	17 (15%)	5.32 (1.19-23.79)	4.58 (1.11-18.93)		4.61 (1.02-20.75)	4.06 (0.97-16.93)	
Allele									
G	89 (84%)	128 (58 %)	138 (62%)	0.27 (0.15-0.47)	0.69 (0.60-0.79)	<0.0001	0.31 (0.17-0.56)	0.74 (0.65-0.84)	<0.0001
T	17 (16%)	92 (42 %)	84 (38%)	3.76 (2.09-6.75)	2.61 (1.64-4.14)		3.19 (1.77-5.72)	2.36 (1.48-3.76)	

OR= Odds Ratio
RR= Relative Risk
95% CI= Confidence interval
Chi-square test (x^2) and Fisher's exact test for genotype and for allele frequencies
Controls: 53
T2DM: 111
RA: 110

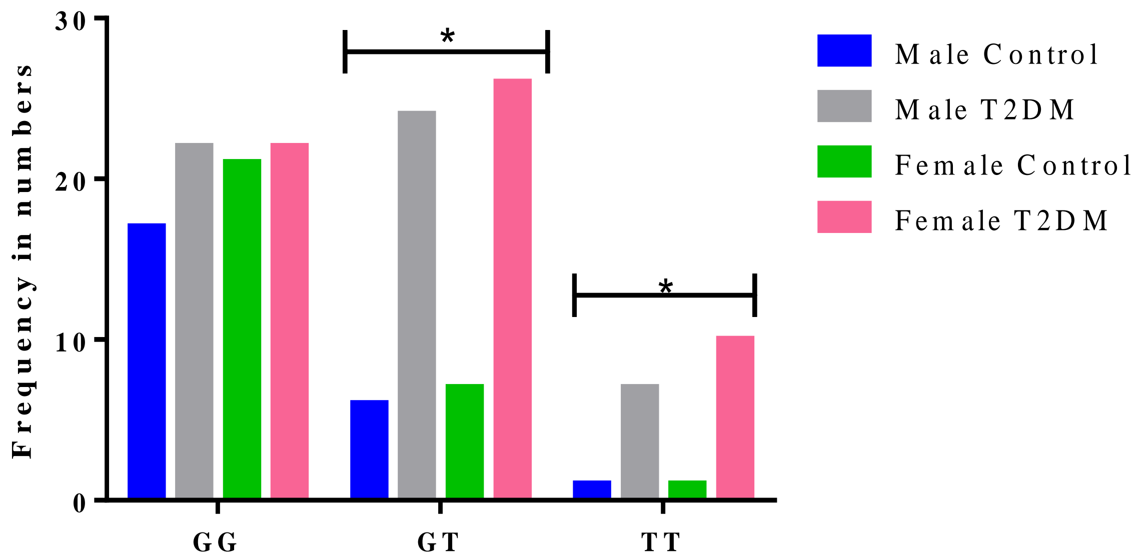


Figure 4. 4 Gender based genotype distribution of *STAT-4 rs7574865* polymorphism in control vs experimental groups

The findings of the correlation of the genotypic frequency of male and female control and T2DM group reveal a higher prevalence of GT and TT in T2DM patients than in the control group. The large disparity in prevalence connects heterozygous GT and homozygous TT with diabetes, while the GG genotype expression trends are not significant. The values of Chi square, df is 15.91,6 and p value is 0.0143*.

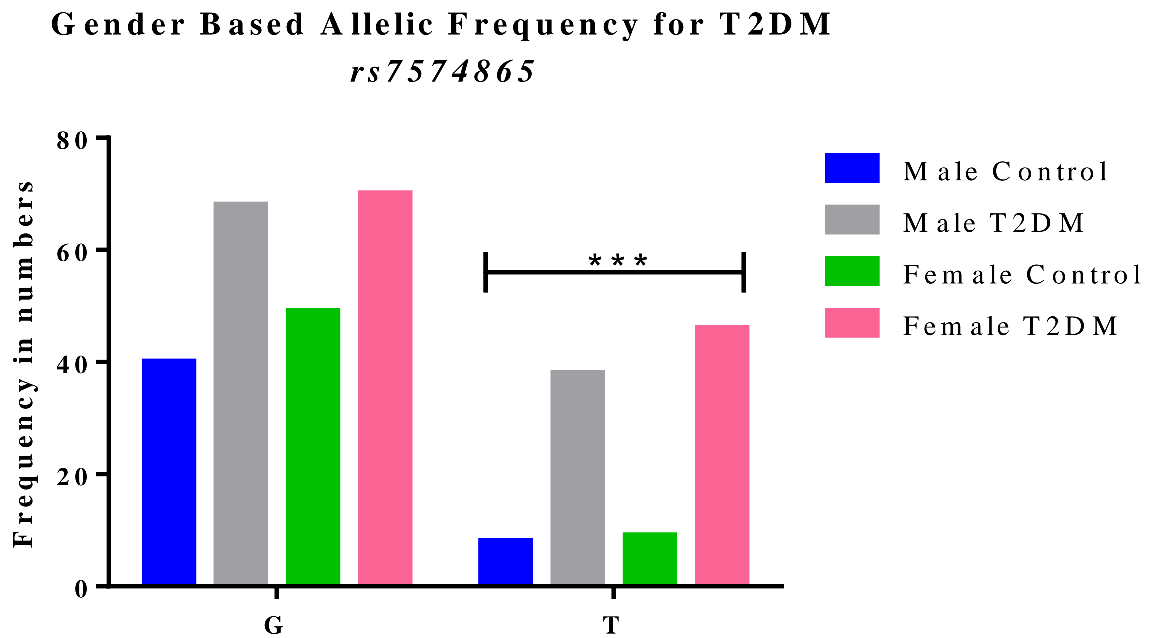


Figure 4. 5 Gender based allele distribution of *STAT-4 rs7574865* polymorphism in control vs experimental groups

The allele frequency graph illustrates the distribution of G and T alleles between the male and female controls and T2DM. In the pattern of distribution of the G allele, no significant difference is seen while the T allele in male and female T2DM is marginally higher than in male and female control group. 16.39,3 is the Chi square, df while *p* value is 0.0009***.

Table 4. 2 Gender based genotype & allele frequencies of control & T2DM along with Chi-square & p value

Genotype / Allele	Male Control n=24	Male T2DM n=53	Female Control n=29	Female T2DM n=58	Chi square, df	*p* value
rs7574865						
Genotype						
GG	17 70.8%	22 41.5%	21 72.4%	22 37.9%	15.91, 6	0.0143
GT	6 25%	24 45%	7 24%	26 45%		
TT	1 4.2%	7 13.2%	1 3.4%	10 17%		
Allele						
G	40 83.3%	68 64%	49 84.4%	70 60.3%	16.39, 3	0.0009
T	8 16.6%	38 35.8%	9 15.5%	46 39.6%		

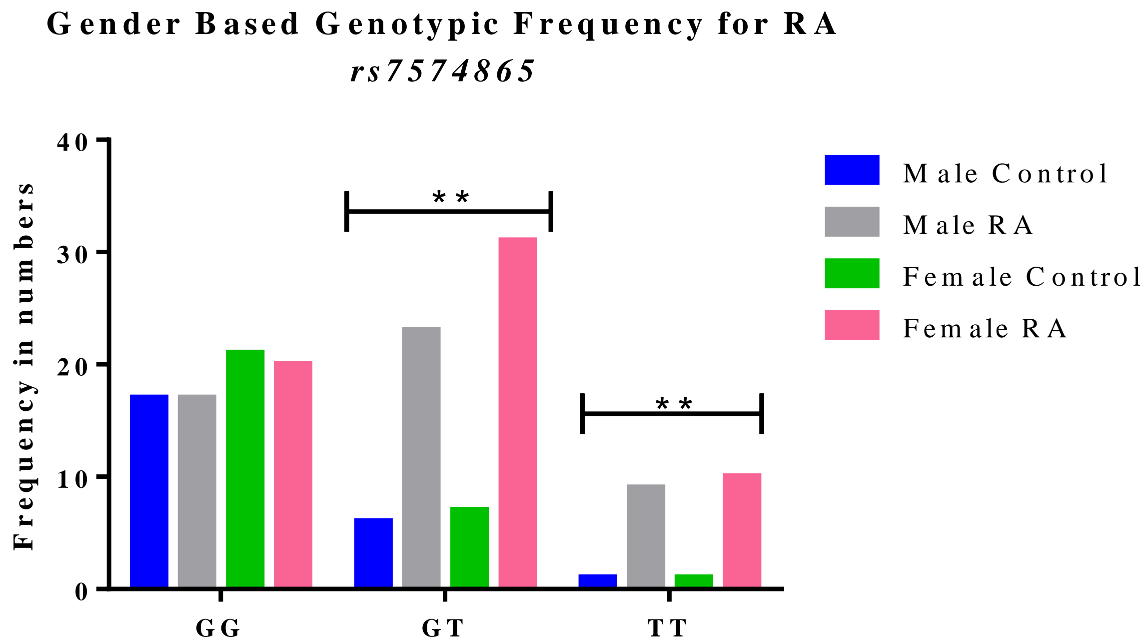


Figure 4. 6 Gender based genotype distribution of *STAT-4 rs7574865* polymorphism in control vs experimental groups

The graph in figure 4.6 shows genotype frequency distribution between males and females of control and RA group. It exhibits that in RA heterozygous GT is more prevalent in females than in male, while homozygous TT is equally significant in both genders. The clear difference in their frequency is strongly related to RA, whereas the trend for the homozygous genotype GG is approximately identical and thus not significant. The Chi square, df is 21.79,6 and *p* value is 0.0013^{**}.

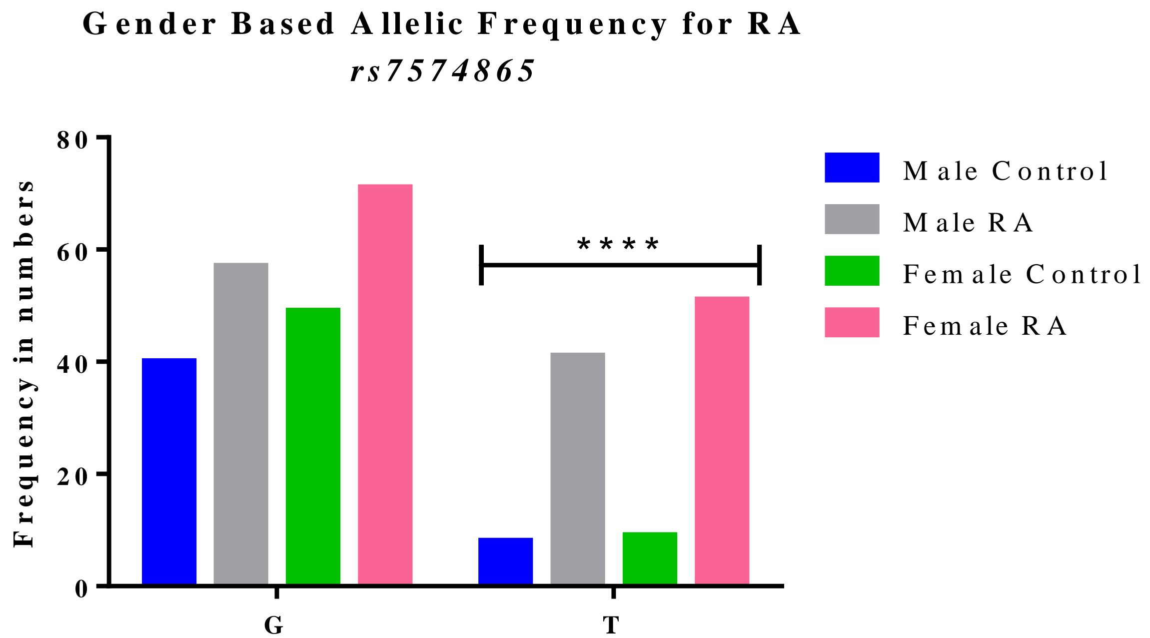


Figure 4. 7 Gender based allele distribution of *STAT-4 rs7574865* polymorphism in control vs experimental groups

The allelic graph shows the expression trends of the G and T alleles in males and females for both control and RA. For the male and female control groups, the G allele is not significantly different compared to RA and distribution is nearly identical. Furthermore, the T allele is viewed significantly higher for male and female RA than male and female control. Statistical value of Chi square, df is 21.38, 3 and p value is $<0.0001^{****}$.

Table 4. 3 Gender based allele distribution of *STAT-4* rs7574865 polymorphism in control vs experimental groups

Genotype / Allele	Male Control n=24	Male RA n=49	Female Control n=29	Female RA n=61	Chi square, df	*p* value
rs7574865						
Genotype						
GG	17 70.8%	17 34.7%	21 72.4%	20 32.7%	21.79, 6	0.0013
GT	6 25%	23 46.9%	7 24%	31 50.8%		
TT	1 4.2%	9 18.4%	1 3.4%	10 16.4%		
Allele						
G	40 83.3%	57 58%	49 84.4%	71 58%	21.38, 3	<0.0001
T	8 16.6%	41 41.8%	9 15.5%	51 41.8%		

4.3. Association of *STAT-4 (rs7574865)* Polymorphism with Disease Susceptibility

STAT-4 rs7574865 showed a significant association with both RA and T2DM. The GT genotype displayed the susceptibility to both RA and T2DM progression when compared to control group in RA vs control with OR = 2.97, 95% CI = 1.43-6.15, p = <0.0001****, and T2DM vs control with OR = 2.52, 95% CI = 1.21-5.23, p = 0.0004***. While TT genotype indicates a higher risk of developing RA and T2DM compared to controls in RA vs control with OR = 5.32, 95% CI = 1.19-23.79, p = <0.0001**** and T2DM vs control with OR = 4.61, 95% CI = 1.02-20.75, p = 0.0004***.

However, GG genotype specified a protective role in case of RA and T2DM in accordance to controls in RA vs control with OR = 0.20, 95% CI = 0.09-0.41, p = <0.0001**** and T2DM vs control with OR = 0.26, 95% CI = 0.13-0.53, p = 0.0004***. The Chi-square (x^2) value (22.82) with p value = 0.0001*** of RA vs T2DM reveals that there is a considerable association of *STAT-4 rs7574865* with the susceptibility of having RA and T2DM, both existing side by side. Also the allele frequency graph of *STAT-4 rs757865* shows that the distribution of alleles in all the three groups varies considerably.

4.4. Association of *STAT-4 (rs7574865)* Polymorphism with Severity of RA

GT & TT genotype of *STAT-4 rs7574865* polymorphism indicates a remarkable association with clinical features i.e. ESR, TJC, SJC, patient global health & DAS28 given in the table 4.4. The graph in figure 4.8 also represents a significant association of GT & TT genotype with DAS 28 of RA patients as compared to GG genotype. On the other hand, no significant difference was seen in the age and duration of the disease by any of the genotypes.

Table 4. 4 Association of clinical parameters of RA with *STAT-4* (rs7574865) polymorphism

Clinical Parameters	**Genotype in RA Patients (n = 110)**			***p* value**
	GG (n = 37)	**GT (n = 54)**	**TT (n = 19)**	
Age (years)	42.23 ± 12.23	46.28 ± 13.33	49.77 ± 11.58	0.1016
Duration of Disease (years)	6.85 ± 5.58	7.59 ± 7.71	7.67 ± 7.14	0.8614
ESR (mm/h)	17.55 ± 10.89	27.03 ± 23.42	35.67 ± 25.05	0.0057
Tender Joint Count (TJC)	1.82 ± 1.76	9.39 ± 5.64	18.05 ± 7.14	<0.0001
Swollen Joint Count (SJC)	0.74 ± 1.37	2.96 ± 3.33	5.83 ± 3.65	<0.0001
Patient Global Health	27.31 ± 16.01	43.32 ± 18.96	50.55 ± 25.06	<0.0001
DAS 28	3.00 ± 0.64	4.73 ± 0.47	5.99 ± 0.45	<0.0001

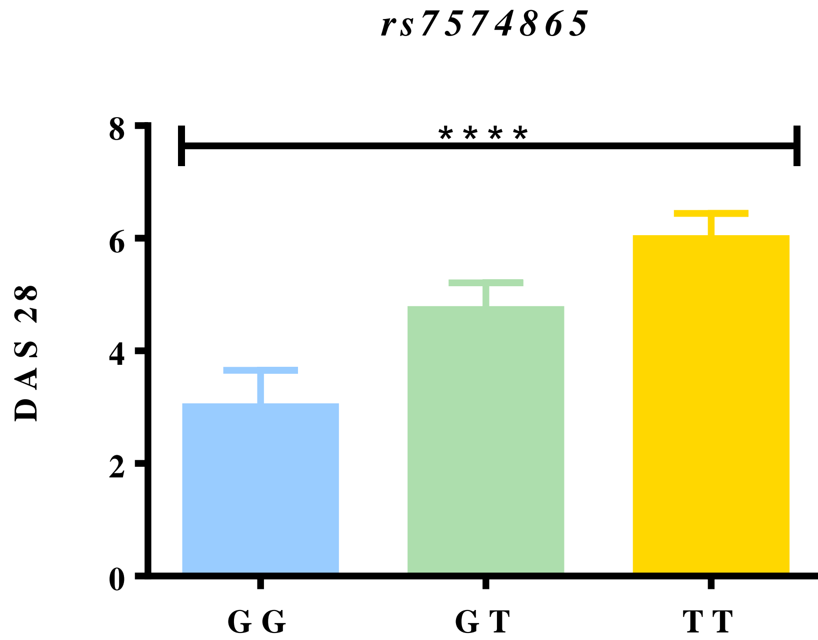


Figure 4. 8 Relation of DAS 28 in RA with *STAT-4* (*rs7574865*) polymorphism

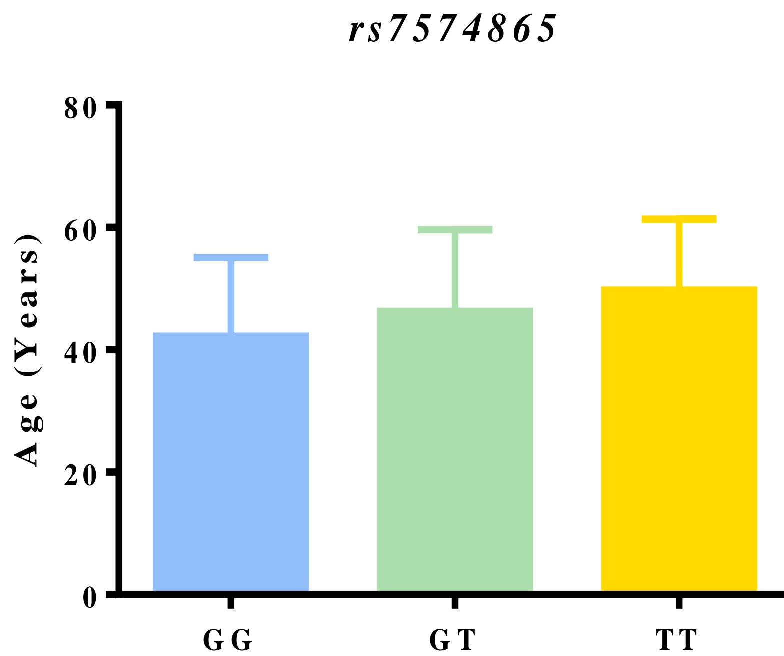


Figure 4. 9 Relation of age (years) in RA with *STAT-4* (*rs7574865*) polymorphism

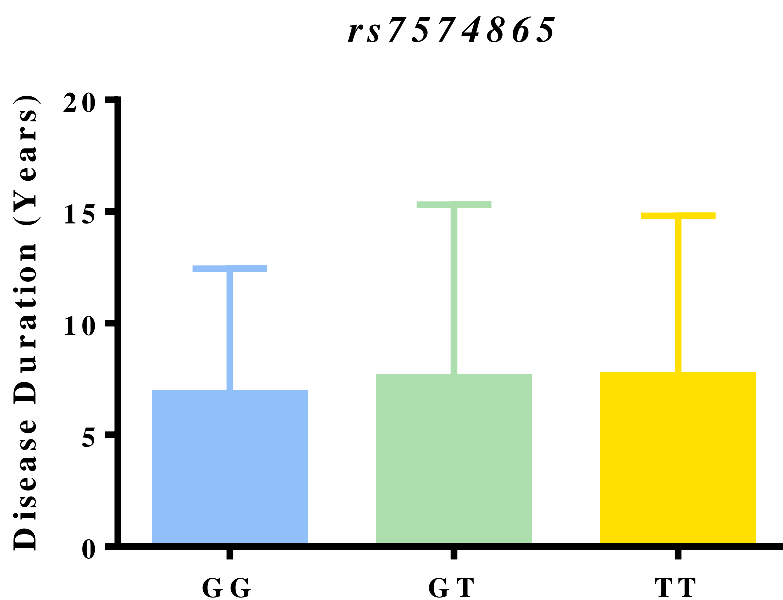


Figure 4. 10 Relation of duration of disease (years) in RA with *STAT-4* (*rs7574865*) polymorphism

4.5. Association of *STAT-4 (rs7574865)* Polymorphism with Severity of T2DM

Clinical data of T2DM patients in table 4.5 showed association with GT & TT genotype of *STAT-4 rs7574865* polymorphism. Graph in figure 4.9 validates that TT genotype has considerable association with fasting blood sugar level in comparison to GT & GG genotype. Similarly, graph in figure 4.10 ensures a significant association of GT & TT genotype with random blood sugar level. However, age and disease duration showed no significant difference.

Table 4. 5 Association of clinical parameters of T2DM with *STAT-4* (rs7574865) polymorphism

Clinical Parameters	Genotype in T2DM Patients (n = 111)			*p* value
	GG (n = 44)	**GT (n = 50)**	**TT (n = 17)**	
Age (years)	53.72 ± 11.23	56.00 ± 13.93	55.93 ± 12.21	0.5121
Duration of Disease (years)	8.23 ± 6.05	10.86 ± 7.95	12.13 ± 9.71	0.1276
Fasting Blood Sugar	173.75 ± 46.79	199.76 ± 58.76	274.94 ± 69.95	<0.0001
Random Blood Sugar	244.61 ± 41.10	348.72 ± 34.73	489.38 ± 58.00	<0.0001

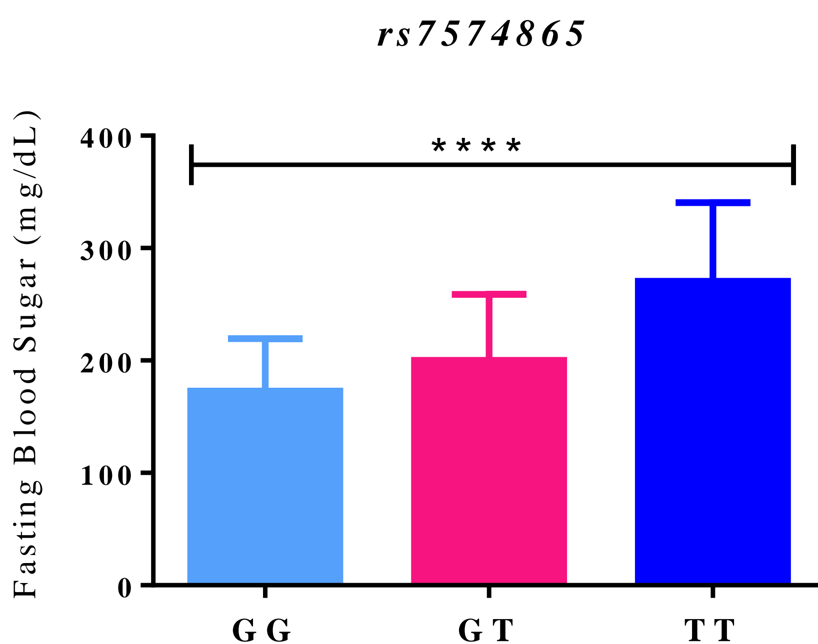


Figure 4. 11 Relation of fasting blood glucose level in T2DM with *STAT-4* (*rs7574865*) polymorphism

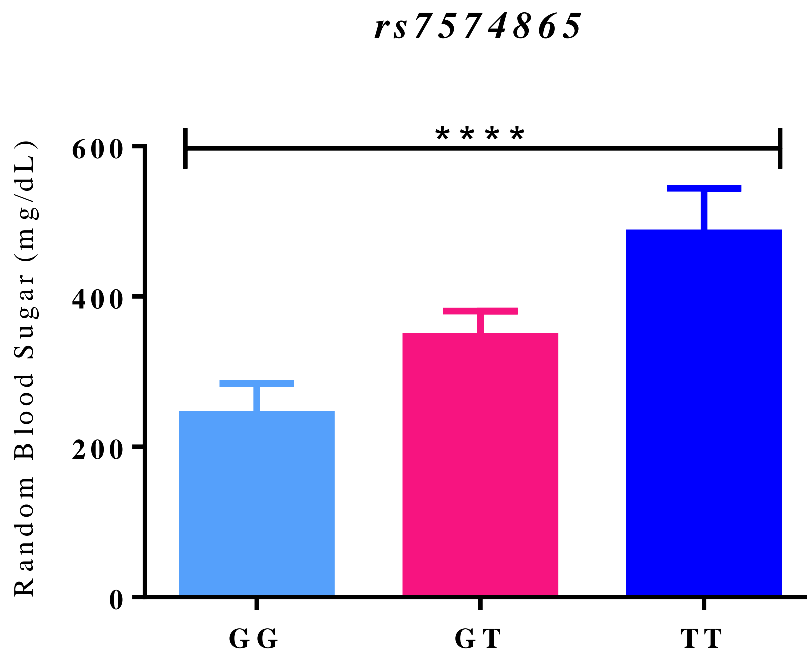


Figure 4. 12 Relation of random blood glucose level in T2DM with *STAT-4* (*rs7574865*) polymorphism

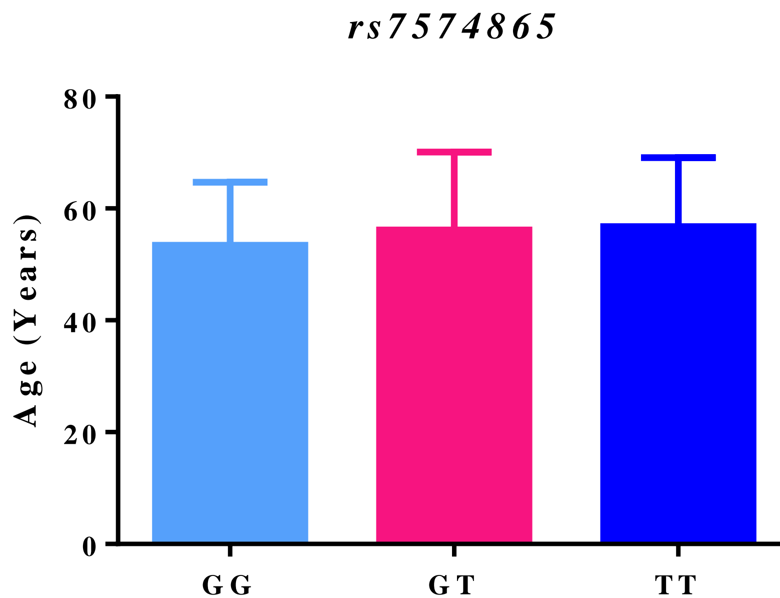


Figure 4. 13 Relation of age (years) in T2DM with *STAT-4* (*rs7574865*) polymorphism

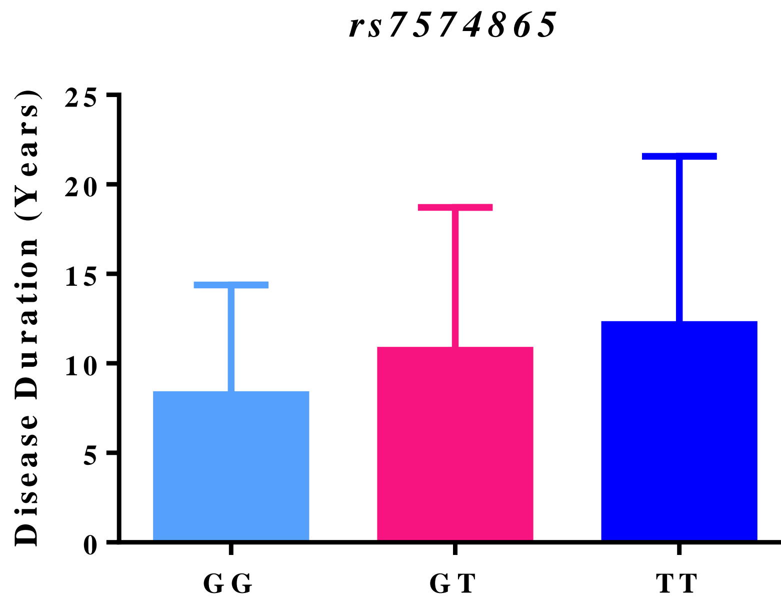


Figure 4. 14 Relation of duration of disease in T2DM with *STAT-4* (*rs7574865*) polymorphism

4.6 Insilico Analysis

HAPLOREG V4.1 RESULTS

Gene	dbSNP ID	SNP location	Chromosome Position	Motifs Changed by SNP	Enhancer Histone Markers	GWAS Hits
STAT-4	*rs7574865*	Intronic	2.191099907	2 (Foxj2, Foxj1)	BLD	13 hits

CHAPTER # 5 DISCUSSION

Rheumatoid arthritis is a leading cause of inability and is the most severe inflammatory arthritis. Recent theories regarding rheumatoid arthritis pathogenesis centered on autoantibodies and immune complexes (Firestein, 2003). Throughout recent decades, numerous occurrence and prevalence reports have been recorded for rheumatoid arthritis (RA), which show a considerable difference in the onset of disease among different populations. RA is related to increased mortality, with RA patients predicted to live for 3-10 years. Epidemiological evidence suggests that genetic factors are correlated with an increased RA risk Nevertheless, RA is a multi-factorial disease that is triggered by the association of genetic and environmental factors that lead to its expression and occurrence (Alamanos & Drosos, 2005).

The global health epidemic is Type 2 diabetes. The classic triad of β-cell dysfunction, overproduction of hepatic glucose, and insulin resistance is distinguished by the classic b-cell dysfunction identified as the insulin-based clearance to the Skeletal Muscle. Clinicians know well that type 2 diabetes is a genetic disease by how it develops in communities and by ethnicities with a high risk (Leahy, 2005).

The intracellular signal activation of *STAT-4* is regulated by cytokines, for example by the interferons *IL-12, IL-23, IL-27* and Type1 interferon. The T helper 1 differentiation and *IFN-c* expression were reported to be crucial for developing Th17 cells). *STAT-4* thus plays a crucial role in differentiating and dividing Th1 and Th17 cells that are the main effectors of RA and T2D (Liang *et al*., 2012; Thierfelder *et al.,* 1996; Watford *et al*., 2004).

Chromosome 2q33 gene maps with *STAT-4*. The polymorphism of *STAT-4 rs7574865* is found within the gene's third intron. Allelic gene frequencies often vary from one population to another. To date, there have been limited resources and research in the Pakistani population for the identification of *STAT-4* polymorphism.

In several autoimmune diseases, including SLE, type I diabetes, Crohn's disease, primary Sjogren syndrome, psoriasis, and juvenile idiopathic arthritis, the role of *STAT-4* polymorphism is known (El-Saadany, Amer, Khalil, Gaber, & Elshweikh, 2016). This study was conducted to evaluate the association in Pakistani patients of a *STAT-4 rs7574865* polymorphism with RA and T2DM and its relevance in terms of disease occurrence and severity.

In the current study, *STAT-4 rs7574865* gene polymorphism showed that the genotypes of GT and TT in patients with RA and T2DM were significantly higher as compared to controls. On the contrary, the genotype of GG was much less common. For RA and T2DM patients, however, there was an increased frequency of the T carrier and the T allele than in control, indicating that individuals with the T allele were at a significant positive risk of RA and T2DM relative to the G allele. Other studies have established a strong link between *STAT-4 rs7574865* polymorphism and RA in Egyptian (Mohamed, Pasha, immunology, & 2012, n.d.) and European populations (Orozco *et al*., 2008). Nevertheless, these results are not compatible with the research of Li *et al*., who noticed that *STAT-4 rs7574865* in the Chinese Han population was not correlated with RA (Li et al., n.d.). Also, the genetic risk factor for T1D was reported as *STAT-4* (*rs7574865*) G/T polymorphism in Crete. T1D is an organ-specific, autoimmune disease regulated by T-cells that is characterized by selective disruption of cells of the pancreas and is defined by a combination of genetic and environmental factors (Zervou, Mamoulakis, Panierakis, Boumpas, & Goulielmos, 2008).

The T-allele *STAT-4 rs7574865* with a high ESR rate, global medical care for the person, number of tender joints, number of swollen joints, and the *DAS 28* are significantly associated with these activities. (Shen *et al*., 2013) in accordance with *STAT-4 rs7574865* GT genotype, showed that the higher ESR and DAS28 levels compared to the GG genotype signify that such a polymorphism could be correlated with the active RA forms. Nevertheless, no significant

differences in genotypes with respect to these disease behavior parameters were reported by (Kobayashi *et al*., 2008) and (Mohamed *et al*., 2012). The T allele *STAT-4 rs7574865* G/T polymorphism is significantly associated with random blood sugar and fasting blood sugar in diabetic patients as compared to non-diabetic patients. The previous study found that a major association of T1D susceptibility was found in the minor allele T of *rs7574865*. In contrast, there was no association with T1D with another SNP (*rs3024866*) (Bi *et al*., 2013)

CONCLUSION

Current study depicted that T2DM and RA have a close link to *STAT-4* SNP *rs7574865*. While these findings must be replicated in more samples and in other population segments for affirmation. According to genotypic distribution the heterozygous trait is more prevalent. Similarly, in the case of experimental, the allelic distribution showed a slightly higher trend than in control. Moreover, the clinical and laboratory data also suggested a significant association of *STAT-4 rs7574865* polymorphism with T2DM and RA susceptibility and severity. Results of current study have the potential role of *STAT4* pathway in human immune responses to RA and progression to T2D. Identifying the downstream pathways of *STAT4* function in specific health and disease states may identify therapeutic strategies that inhibit harmful effects while preserving their beneficial effects.

FUTURE PROSPECTS

The nature of the *STAT-4* gene expression in T2DM and RA in relation to the control sample group would be estimated by expression analysis. To achieve wide-scale data that validate whether *STAT-4 rs7574865* polymorphism may be involved in T2DM and RA disease etiology, the larger sample size is essential. Huge-scale research data can be useful in designing targets of in-silico drugs for the disease. The studied SNP can be recognized as a forecast marker for disease development. The downstream pathway of genetic function may identify therapeutic strategies that inhibit harmful effects while maintaining their beneficial effects in specific health and diseases.

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Association Analysis of *STAT-4* Gene Polymorphism in Rheumatoid Arthritis & Type 2 Diabetes Mellitus in Pakistani Patients

MARIA SANA
00000205497
MS Healthcare Biotechnology

Supervisor
DR. ATTYA BHATTI

ATTA-UR-RAHMAN SCHOOL OF APPLIED BIOSCIENCES (ASAB)
NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY (NUST)
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