Investigating the Role of Interleukin 1 receptor antagonist (IL1RN) and Paraoxonase 1 (PON1) Susceptibility Factor with Type 2 Diabetes Mellitus

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DEDICATION

All my effort is dedicated to

“My beloved family”

Especially my Father and Mother

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I would like to express my gratitude and admiration to all those who offered me the opportunity to complete this thesis. Bounteous praise for **ALLAH Almighty**, the Magnificent and Supreme, Whose Blessing, Greatness and Magnificence flourish my thoughts and all the Praise and Gratitude for the **Holy Prophet (PBUH)**, who is the thriving city of knowledge. I feel much honour to express my deepest sense of appreciation and acknowledgment to my Honourable Supervisor **Dr. Attya Bhatti**, Professor ASAB, NUST, whose knowledge, help, motivation, affection, support, and loving behaviour helped me in all the time of research and thesis writing. I am very grateful to the members of my General Examination Committee, **Dr. Peter John**, Professor ASAB, NUST, **Dr. Muhammad Tahir**, Professor ASAB, NUST, and **Dr. Yasmeen Badshah**, Assistant Professor ASAB, NUST for their guidance to accomplish my work. I owe my sincerest gratitude to my external supervisory committee member **Dr. Amir Ali Abbasi**, Professor, National Centre for Bioinformatics, Quaid-e-Azam University, Islamabad. I am thankful to my **Parents** for their love, countless prayers, and moral support throughout my life. Thank you both for giving me strength to reach for the stars and chase my dreams. I am grateful to my **Siblings** whose endless efforts and best wishes encouraged me at all stages of my life. I extend my sincerest appreciation to my seniors, **Kashaf Rasool** and **Maria Sharif**, and Lab fellows, **Faria Shahid**, **Ameema Khan** and **Emaan Imtiaz**, who always help me in every situation. I am thankful to my friend **Nimra Riasat** for her encouragement and countless support.

Tayyaba Rehman

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LIST of ACRONYMS

DM	Diabetes Mellitus
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
OGTT	Oral Glucose Tolerance test
FFA	Free Fatty acids
GLUT 2	Glucose transporter 2
GLUT 4	Glucose transporter 4
IDF	International Diabetes federation
mL	milli Liter
WHO	World Health Organization
UPR	Unfolded Protein response
INSR	Insulin receptor
TGs	Triglycerides
G3P	Glucose 3 Phosphate
NGSP	National Glycohemoglobin standardization program
ROS	Reactive Oxygen Specie
HDL	High density lipoproteins
LDL	Low density lipoproteins

ABSTRACT

Type 2 diabetes mellitus is a chronic metabolic disorder that is associated with abnormal endocrine functioning and attributed to hyperglycaemia, defective insulin production and insulin resistance caused by many factors including genetic, environmental, lifestyle. Interleukin 1 receptor antagonist (IL1RN) protein, a member of the cytokine family encoded by the gene IL1RN located on the long arm of chromosome 2 expressed by the cells of immune system. It plays a key role in regulating uncontrolled inflammation by binding to the receptors of IL-1 and preventing it from activation. Inflammation is a crucial contributing factor of Type 2 Diabetes Mellitus (T2DM), Hence low levels of IL1RN or SNPs may lead to T2DM and its related complications. While Paraoxonase 1 enzyme encoded by PON1 gene, located on long arm of chromosome 7, perform metabolism of toxins as well as provide protection against oxidative stress and reactive oxygen species. Hence variation in PON1 leads to certain health conditions like T2DM and CVDs. Various abnormalities like SNPs in IL1RN and PON1 were found to be associated to T2DM in certain populations. This research study aimed to find out the missense pathogenic polymorphisms of IL1RN and PON1 using Insilico analysis and further invitro analysis of these polymorphisms were also performed. By using computational analysis, SNP rs201638660 (C94F), is reported as a high-risk non-synonymous SNP of IL-1RN because of its deleterious and disease associated nature and it causes loss interactions and destabilizes protein hence affecting protein function and five SNPs of PON1 (rs72552788 (L90P), rs138512790 (C42R), rs185623242 (S302L), rs368206333 (G344C), rs369422555 (W281C)) are shortlisted as deleterious, that will cause damage to protein structure and function. Association of rs854560 of PON1 and rs380092 of IL1RN is performed via HRM analysis which measures the change in fluorescence with gradual increase in temperature and detects variation. Using graph pad prism 10, p value comes out to be 0.0132 for rs380092 of IL1RN and 0.053 for rs854560 of PON1, and significant association is confirmed. Further invitro studies are required to validate the shortlisted pathogenic nsSNPs of PON1 and IL1RN sequencing is required to refine the data of these SNPs.

INTRODUCTION

Type 2 Diabetes Mellitus

Type 2 diabetes mellitus is the major form of Diabetes mellitus characterized by insulin resistance due to which a body experiences dysregulation of lipid, carbohydrate, and protein metabolism. T2DM is the most severe public health concern which is emerging as challenging epidemic globally. 90 % cases of diabetes mellitus are T2DM (El-Kebbi *et al*., 2021). Population of obese people affected by T2DM is more than the lean population (DeFronzo *et al*., 2015).

T2DM is associated with inefficient response of target tissues towards insulin which leads to insulin deficiency in the body, and hence, high blood glucose level. The average age for the induction of T2DM is 42.5 years but adults can develop earlier due to certain factors (Tao *et al*., 2015). Various genetic, environmental, behavioural and metabolic factors are involved in the pathophysiology of T2DM (Galicia-Garcia *et al*., 2020). Multiple genes play role in dysfunction of pancreatic beta cells and insulin resistance while environmental factors responsible for diabetes induction include improper diet sedentary lifestyle, aging, and obesity. The exposures causes the intermediary outcomes like disturbed blood lipid levels, prediabetes, hypertension and obesity (Dendup *et al*., 2018). Various other symptoms are associated with T2DM which involves polydipsia (thirst), polyphagia (overeating) and polyuria (frequent urination) and weight loss. T2DM leads to several complications which mainly includes cardiovascular diseases, kidney malfunctioning, diabetic retinopathy, and nephropathy. Due to emerging epidemic of T2DM, the financial burden is increased specifically on developing countries like Pakistan affecting the quality of life and productivity of youth (El-Kebbi *et al*., 2021).

Studies have shown that malnutrition, substance abuse, gestational diabetes, and xenobiotics exposure during intrauterine development of the fetus lead to perturbance in the maternal-fetal environment causing defective organogenesis. The results of which can be quite disturbing include reduced β cell mass, impairment in neuronal development, excessive lipid storage and decreased muscle mass. All these factors lead to the impairment of glucose tolerance, development of insulin resistance, metabolic syndrome and ultimately Diabetes Mellitus (Vaiserman & Lushchak, 2019).

Epidemiology

T2DM is alarming and fastest growing public health concern specifically in underdeveloped countries it is highly associated with mortality and other health complications. According to IDF report of 2021, diabetes has affected 537 million people worldwide and it is predicted that by 2030, 634 million people and by 2045, 783 million people will be affected with diabetes mellitus. Most of the people affecting with diabetes belong to low-income and middle-income countries. Diabetes is also associated with higher mortality rate as 6.7 million deaths were caused by diabetes in 2021. T2DM is responsible for up to 90% pf the total cases of diabetes. In the region of South-East Asia, 90 million are suffering from diabetes and around USD 10 billion has been spent on this disease in 2021 increasing the financial burden on these countries (Sun *et al*., 2022).

Figure 1 *Prevalence of Diabetes around the globe (https://idf.org/)*

South Asian countries like Pakistan, India, Bangladesh, Nepal, Bhutan, and Sri Lanka are at higher risk of developing T2DM. It has been demonstrated by several studies that South-Asians are more susceptible to T2DM as Asians may have comparatively higher fat mass, hence more abdominal obesity and consequently more prone to diabetes as compared to other populations with same BMI (Narayan *et al*., 2021). Lack of proper nutrition in early life compared to excessive nutrition in later years, combined with a sedentary lifestyle, can lead to the development of diabetes in an accelerated manner. Diabetes affects both genders differently. Men are more prone to developing diabetes than women (Nordström *et al*., 2016).

Pathophysiology of Type 2 Diabetes Mellitus

Normal physiology of β-cells

For the normal secretion of insulin, the integrity of β-cells must be assured by constantly regulating the cellular pathways and mechanisms responsible for the normal functioning of pancreatic β-cells. Under normal conditions, 30-70 units of insulin is released from β -cells per day and the main role is played by glucose in the release and enhanced production of insulin (Marchetti *et al*., 2020).

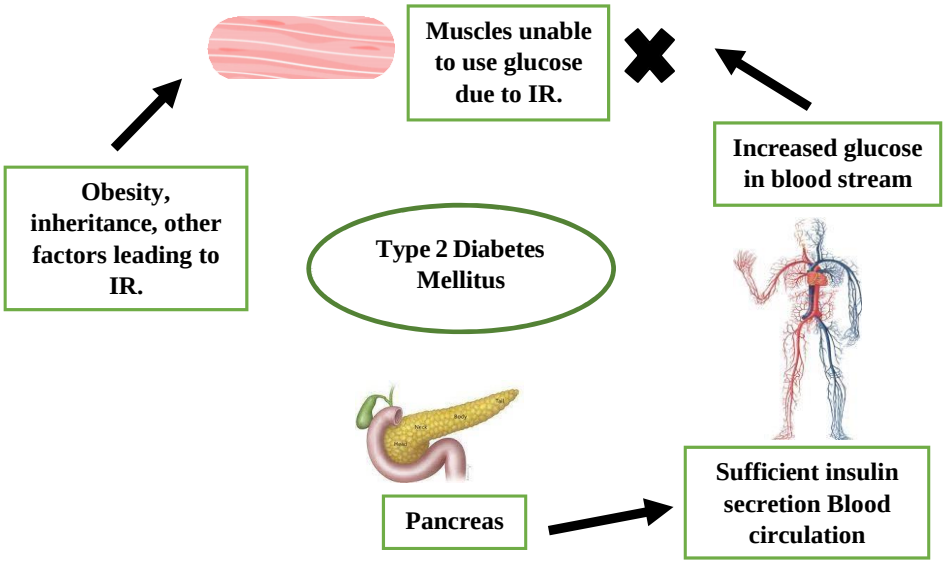


Figure 2 *Function of Pancreas in T2DM (Galicia-Garcia et al., 2020).*

Inulin is first produced in an inactive form, pre-proinsulin, which go through conformational changes and converted into proinsulin. It is then transported to Golgi apparatus where it is cleaved into c-peptide and insulin. This matured insulin is then stored into granules, and it is primarily released in response to higher concentration of glucose. Amino-acids, hormones and fatty acids are also responsible for the excretion of insulin from granules. The rise of glucose concentration in blood triggers the intake of glucose by Glucose transporter 2 (GLUT 2) located on the membrane of β-cells. This glucose is catalysed in the cell which results in membrane depolarization due to the increased concentration of ATP in cell. The calcium ions start entering the cells from voltage dependent Ca2+ channels. These Ca2+ ions fused with insulin granules releasing insulin from cells. The insulin then acts on liver and other body tissues for the regulation of plasma glucose (Galicia-Garcia *et al*., 2020).

Figure3 *Pictorial representation on insulin synthesis in normal conditions.*

β-cells dysfunction

The failure of β-cells in the production of insulin plays major role in development of T2DM. β-cells dysfunction is thought to be resulted from the degeneration and death of β-cells by apoptosis leading to reduction in the production and stimulation of insulin thus, increasing glucose concentration in blood plasma (Marchetti *et al*., 2020). The downstream signalling pathways for insulin secretion can also be affected by the reduced gene expression of glucose transporter and glucokinase in T2DM (Taneera *et al*., 2012).

The uncontrolled nutrition state i.e., obesity, hyperlipidaemia and hyperglycaemia, results in insulin resistance and chronic inflammation in pancreas. The pancreatic β-cells are then subjected to toxic environmental conditions including inflammation, oxidative stress, endoplasmic reticulum stress, and amyloid stress (Halban *et al*., 2014). Endoplasmic reticulum stress is generated by the presence of excessive free fatty acids (FFA) and higher glucose concentration. These FFA activates apoptotic unfolded protein response (UPR) pathways leading to apoptosis of β-cells (Yamamoto *et al*., 2019). Inflammation in β-cells occurs when macrophages are inducted by interleukin-1β due to the altered mobilization of calcium ions from endoplasmic reticulum (Galicia-Garcia *et al*., 2020).

Insulin Resistance

Insulin resistance is mainly the ineffective response of target tissues towards insulin hormone, and it is thought to be the major hallmark of T2DM as it is the first detectable defect. It is present in prediabetic condition in individuals with family history of type 2 diabetes (Goldstein, 2002). It can occur due to suppressed secretion of insulin from β-cells, presence of insulin antagonist in blood and ineffective response of target tissues. Skeletal muscles, adipose tissue and liver are the three main targets of insulin (Galicia-Garcia *et al*., 2020). The synchronization of glucose levels in the blood is controlled by the insulin and glucagon ratio. Insulin breaks down the glucose or converts it into glycogen for storage and lowers the glucose levels in the

blood. On the other hand, glucagon prevents insulin-based hypoglycaemia by converting glycogen into glucose (Galicia-Garcia *et al*., 2020).

Due to the impaired insulin action, β-cells are forced to increase the synthesis of insulin which leads to functional defects in β-cells. Insulin resistance is the leading cause hyperinsulinemia, hyperglycaemia, cardiovascular disease, and other metabolic disorders. Hyperinsulinemia results due to the over secretion of insulin from pancreas in order to overcome the defects of insulin resistance. In patients with T2DM, increased secretion of insulin helps to sustain euglycemia in body. Insulin resistance has an impact on a wide range of tissue functions and metabolic processes in the body. Exaggerated responses in insulin-sensitive tissues are generated by hyperinsulinemia. It stimulates the sympathetic nervous system, which can lead to hypertension, and it also boosts ovarian androgen production, which contributes to the hormonal abnormalities (Goldstein, 2002).Regarding impaired response from insulin-responsive cells, skeletal muscles, adipose tissue, and liver plays important role in the development of T2DM.

Skeletal Muscle

The most essential factor responsible for T2DM besides the pancreas is skeletal muscles. Under a normal environment, insulin increases glucose uptake and enhances the synthesis of glycogen from glucose. Three factors are crucial in this conversion: hexokinase, glucose transporter 4 (GLUT 4), and glycogen synthase. Muscles cells contain Insulin receptors (INSR) to which insulin binds which results in the translocation of GLUT 4 from the intracellular chamber to the plasma membrane which initiates glucose uptake lowering the levels of circulating glucose in the blood (Takahashi *et al*., 2014).

Insulin binds to the α-subunit of INSR which results in the phosphorylation of the β-subunit on tyrosine residues, activating the INSR tyrosine kinase for glucose intake. Any mutation in these subunits results in the inactivation of INSR tyrosine kinase that leads to impaired glucose intake by skeletal muscles. Environmental or nutritional factors are also responsible for IR besides genetic factors. A sedentary lifestyle and excessive nutritional conditions lead to chronic inflammation in skeletal muscles. Obesity contributes to the infiltration of macrophages and proinflammatory cytokines which eventually results in muscle inflammation and IR (H. Wu & Ballantyne, 2017).

Adipose Tissue

Adipose tissue is involved in a wide range of metabolic processes occurring in the body including the production of bioactive compounds, coagulation, angiogenesis, reproduction, immunity, appetite regulation, and glucose and lipid regulation (Rosen & Spiegelman, 2006). Two major mechanisms are involved in insulin action on adipose tissue: (i) increasing glucose intake and triglycerides (TGA) production, and (ii) mitigating hydrolysis of TGA and increasing FFA and glycerol uptake from blood circulation (Galicia-Garcia *et al*., 2020).

In normal conditions, circulating blood glucose is taken into the cell by GLUT-4. Once inside the cell, glycolysis is activated, and glucose-3-phosphate (G3P) is synthesized from glucose which is then utilized in lipogenic pathways. G3P then contributes to the formation of TGA by combining with fatty acids (esterification). TGA is then stored in lipid droplets. In metabolic stress conditions, TGA is broken down into free fatty acids that can be used as an alternative energy source for the body (Unuofin & Lebelo, 2020). Any abnormality in the above-mentioned process can lead to reduced glucose uptake, increased secretion of FFA and glycerol resulting in hyperglycaemia and IR (Medyczny, 2020).

Liver

The liver is responsible for glucose synthesis, utilization, and lipid metabolism. Under normal circumstances, glucagon, and Insulin both play a vital role in the regulation of hepatic glucose. Glucagon activates glucose synthesis while insulin lowers the level of glucose by converting it into glycogen for storage. With the rise in the level of circulating blood glucose, insulin is released from pancreatic β-cells. Insulin binds to INSR resulting in the autophosphorylation of the receptor. Insulin receptor substrates (IRSs) are thereby recruited and phosphorylated. After that, phosphatidylinositol 3- kinase (PI3K) is activated by IRSs which produce phosphatidylinositol (3,4,5)- triphosphate (PIP3) by phosphorylating phosphatidylinositol (4,5)-bisphosphate (PIP2). PIP3 then stimulates pyruvate dehydrogenase kinase-1 (PDK1), causing protein kinase B (AKT) to phosphorylate. AKT is also phosphorylated by mTOR Complex 2 (mTORC2). This completed activation of AKT performs several vital roles in metabolic pathways like glycogenolysis, gluconeogenesis, lipid production, and glycolysis (Unai, *et al*., 2020).

The other mechanism by which insulin reduces glucose production in the liver is activating fork head box O1 protein (FOXO1), a transcription factor. This factor identifies an insulin response element (IRE), a specific regulatory element, on the promoters of glucose-6-

phosphatase (G6Pase) and phosphoenolpyruvate carboxy-kinase (PEPCK) genes, which both play critical roles in maintaining glucose levels in starving situations. By inhibiting FOXO1, glucose storage in the form of glycogen is elevated and glucose production is stopped (Gorska-ciebiada, 2015). Under pathogenic conditions such as IR, glycogen synthesis is reduced, gluconeogenesis and lipogenesis are increased. The production of proinflammatory C-reactive protein (CRP) is also elevated (Leclercq *et al*., 2007).

Factors Affecting Type 2 Diabetes Mellitus

Genetic Factors

The pathophysiology of insulin resistance has not been clear yet, but it is investigated that genetic factors play role in onset of T2DM (Y. Wu *et al*., 2014). T2DM, a polygenic disorder proved by segregation analysis, is termed to have several susceptible loci located by genome wide association studies (GWAS) in 2007. After GWAS studies conducted on people belong to different countries and ethnicities has discovered more than 80 susceptible loci connected to T2DM 3. These genes include KCNJ11, TCF7L2, IRS1, MTNR1B, PPARG2, IGF2BP2, CDKN2A, HHEX and FTO are found to correlated with development of T2DM. There are still a lot of unsorted loci responsible for pathogenesis of T2DM. To fully comprehend and manage T2DM, we must thus enhance our present biological understanding (Y. Wu *et al*., 2014).

Environmental Factors

Sedentary lifestyles, unbalanced diet, physical inactivity, smoking, alcohol consumption, and obesity are the major environmental risk factors associated with T2DM pathogenesis. Other environmental factors like pollution, unbalanced sleep cycle and effect of different chemical has not clearly understood yet (Hills *et al*., 2018).

Obesity

Obesity is a primary cause of type 2 diabetes mellitus. The cell resistance to insulin increases as the development of fatty tissue increases. However, being overweight does not always lead to T2DM (Gress *et al*., 2000).

Fat Distribution

If the body stocks fat in abdomen, the chances of developing T2DM is higher than the fat storage anywhere else such as thighs or hips (Espeland *et al*., 2007).

Inactivity

Physical activity controls the body weight by utilizing glucose as a source of energy and make cells more sensitive to insulin. The more active a person is, less are the chances for developing T2DM and vice versa.

Family History

If a person has a family history of T2DM, he has more chances to acquire T2DM.

Age

The risk of T2DM increases with age, especially after 45 as people do not exercise much and gain weight thus losing muscle mass. Other than this T2DM is progressing among children and younger adults as well (Stratton *et al*., 2000) .

Polycystic Ovarian Syndrome

Women having polycystic ovarian syndrome (PCOS), conditions like excess hair growth, irregular menstrual cycle and obesity leads to the risk of diabetes (Inzucchi *et al*., 2015).

Screening & Diagnostic criteria for diabetes

The first step to check the progression and severity of any disease is diagnosis. According to World health organization and American diabetes association have set certain criteria and provided some guidelines for considering the patient as diabetic. There are various tests that are being used by the physicians for the diagnosis of diabetes.

Fasting Plasma Glucose test

For body, glucose is the primary energy source, any disturbance in it can leads to complications. Fasting referred to as no intake of calories for no less than 8 hours (Brown *et al*., 2023). Fasting plasma glucose tests estimate the body's blood glucose level at the minimum of 8 hours of fasting (overnight). Normal body's blood glucose level is 96mg/dL and if it ranges from 100-126mg/dL it can be a sign of pre-diabetes. If this level rises above 126mg/dL, the person will be considered as diabetic (Goldenberg & Punthakee, 2013). FPG test is used preferably because it is established as a standard and quick and easy to perform. Also, it can predict the associated microvascular complications. But it is observed that it shows high day to day variability (Goldenberg & Punthakee, 2013).

Random Glucose test

Regardless of the fasting, this is easy to perform and have high specificity. If the blood glucose level is 200mg/dL, the person will be diabetic, but person must have polyuria, polydipsia, like conditions (Zia *et al*., 2016).

Oral Glucose Tolerance test

Oral glucose tolerance test is performed before and after consuming glucose rich liquid (acc. To WHO, equivalent to 75g glucose dissolved in water). After liquid consumption, the glucose level rises. 140mg/dL is considered as Normal glucose level and if its higher than 200md/dL it will be impaired glucose tolerance in diabetic patients. OGTT is very sensitive test that is being used for the IGT detection (Goldenberg and Punthakee 2013, Zia *et al*. 2016, Noreen *et al*. 2018).

HbA1c test

HbA1c is glycosylated haemoglobin test, this test is performed according to NGSP certified method to measure the glucose that is attached to haemoglobin in blood (Goldenberg and Punthakee 2013). This test is being used to estimate the average amount of glucose for past three months, as the average life span of haemoglobin is 120 days. If level is 5.7% it is considered normal but if its higher than 6.5% the person is referred to as diabetic and if the level is from 5.7-6.5%, the person will be considered as prediabetic. Further tests can confirm it, but it can't be performed for the diagnosis of diabetes in expecting females and children (Goldenberg and Punthakee 2013, Spiller *et al*. 2017).

Prevention & treatment options

According to DPP, lifestyle modifications and switching from sedentary lifestyle towards a healthy lifestyle involving low fat diet and physical activity, significantly reduced the risk of T2DM. Metformin was found to be most effective in women with gestational diabetes and lifestyle interventions in populations with individuals older than 60 years of age (Chatterjee *et al*., 2017). The therapies currently used to treat diabetes in patients include metformin, glucokinase activators, antagonist of GLP-1 receptor, alpha-glucosidase inhibitors, SGLT2 inhibitors, thiazolidinediones, DPP-4 inhibitors, sulfonylureas etc (Chellappan *et al*., 2018).

They work by employing various mechanisms which include increasing insulin release from pancreas, increasing incretin effect in gastrointestinal tract, decreasing lipolysis in adipose tissue, decreasing glucagon secretion, increasing glucose uptake in muscle cells, decreasing hepatic glucose production in liver, etc.

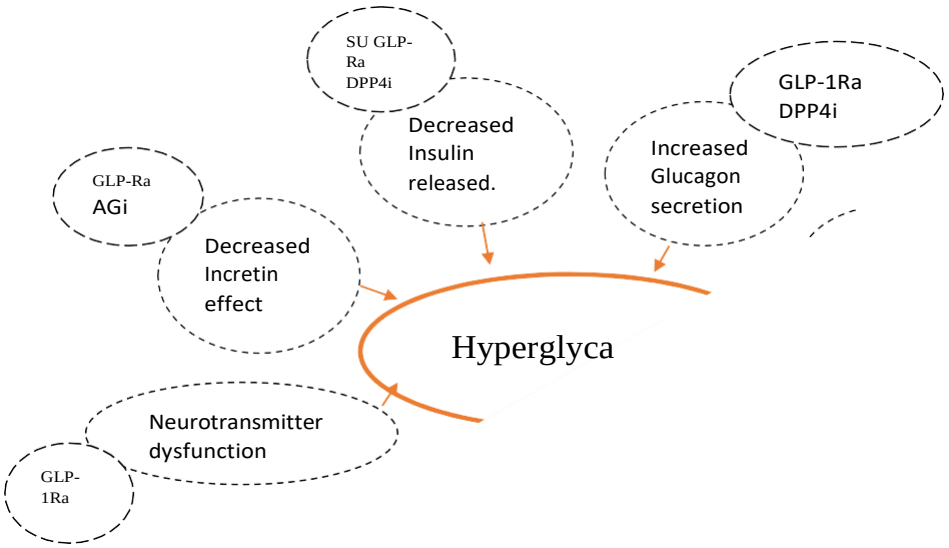


Figure 4 *Drugs for diabetes and route of action.*

Insulin therapy

Insulin is a primary choice of treatment for T1DM but in case of T2DM it is encouraged to use when other drugs become incapable of regulating glucose levels in the blood, it can be utilized in combination with other drugs. Insulin's limitation is that it must be supplied by injections (Capoccia *et al*., 2016).

Biguanides

The commonly used biguanides are metformin. Other biguanides like phenformin and buformin are withdrawn due to the risk of lactic acidosis (Capoccia *et al*., 2016). Metformin is the most prescribed diabetes medication, especially among obese and overweight people. For monotherapy, this medicine is still the best option. It works by improving insulin sensitivity, enhancing glucose uptake by phosphorylating GLUT enhancer factor, and inhibiting hepatic gluconeogenesis. Metformin helps in weight loss by lowering triglycerides and LDL

cholesterol levels. Abdominal discomfort and other gastrointestinal problems, such as diarrhea, are the most common side effects of metformin medication (Tahrani *et al*., 2016).

Sulfonylureas

Sulfonylureas are secretagogues that act by causing pancreatic beta cells to secrete natural insulin. It primarily targets beta cells' ATP-sensitive potassium channels and is only effective when residual pancreatic beta-cells are present. Sulfonylureas have no long-term protective effects on beta cells activities, and they may hasten beta cell death. There have been numerous reports of sulfonylureas causing hypoglycaemia, particularly with earlier generation medications (Tahrani *et al*., 2016).

Thiazolidinediones

Peroxisome proliferator-activated receptor g activators, such as thiazolidinediones (TZDs), improve insulin sensitivity in adipocytes, cardiac muscles, and the liver (Tahrani *et al*., 2016). They work on beta cells to regulate insulin secretion. As a result, its being utilized as a part of an insulin resistance therapy plan for T2DM patients, with results lasting up to 5 years. Increased body weight is a typical TZD adverse effect (Gaggini *et al*., 2013).

Dipeptidyl peptidase-4 (DDP4) inhibitors

Gliptins, commonly known as DDP4 inhibitors, are a novel class of therapeutic agents that function by blocking the enzyme dipeptidyl peptidase 4, inhibition of dipeptidyl peptidase4 delays the inactivation of incretin hormones including glucagonlike peptide 1 (GLP-1) and gastric inhibitory polypeptide (GIP), which are involved in physiologically maintaining glucose homeostasis. These medications have fewer reported side effects, reduced risk of hypoglycaemia, and are weight neutral (Crepaldi *et al*., 2007).

Glucagon-like peptide 1 (GLP-1) analogues

GLP-1 analogues are primarily incretin-based medicines that boost insulin release in a glucose-dependent manner, suppress glucagon secretion, and eventually inhibit hepatic glucose synthesis (S. *et al*., 2013). HbA1c levels have been seen to drop for up to three years. Even though these medicines are not as well handled as DPP4 inhibitors, they result in higher HbA1c reduction and weight loss (Stonehouse *et al*., 2012). There is some evidence that incretin-based

medications have beneficial effects on sleep, inflammation (via lowering reactive protein levels), the central nervous system, liver, and cardiovascular health (Inzucchi *et al*., 2015).

Sodium-glucose co-transporter-2 (SGLT2) inhibitors

By inhibiting glucose absorption in proximal renal tubules, SGLT2 inhibitors, also known as gliflozins, reduce sodium transport and promote glucose excretion via the kidneys, decreasing plasma blood glucose concentrations (Kalra, 2014). Canagliflozin, dapagliflozin, and empagliflozin are examples of pharmacological drugs in this class. Because they act independently of insulin, they can be utilized in individuals at any stage of diabetes (Inzucchi *et al*., 2015). These medications can help to improve b-cell activity, insulin sensitivity, and glucotoxicity caused by glucosuria. They can cut HbA1c levels by 0.5%, lose weight, and control blood pressure (Abdul-Ghani *et al*., 2011). Urinary tract infections, vaginal mycotic infections, particularly in females, and volume depletion-related symptoms have all been reported (Cherney *et al*., 2014). When prescribing this medication to the elderly and patients on diuretics, extra caution is required.

Combination Therapy

Combination therapy is initiated for more effective blood glucose control and dose reductions in individual drugs. When monotherapy fails to keep blood glucose levels under control, it is usually started. Exogenous insulin can be coupled with a variety of oral antidiabetic medications to reduce insulin dosage. Glycaemic control is improved when insulin is combined with metformin or TZD. HbA1c levels are reduced along with weight loss when basal insulin is coupled with GLP-1 receptor agonists. SGLT2 inhibitors are commonly used in combination with metformin or other drugs (Eng *et al*., 2014). It can also be used with DPP4 inhibitors to improve glycaemic management and weight loss while lowering the risk of hypoglycaemia (Min *et al*., 2018).

Ethno-botanical medication

Medicines Plants are the rich source of phytochemical such as phenols, alkaloids, terpenoids, flavonoids and other secondary metabolites. These phytochemicals possess antioxidant, anti-inflammatory, antimicrobial, and antidiabetic properties. Some phenolic compounds have shown to alter inflammatory activity, genes involved in the pathogenies of T2DM and transcriptional factor enzymes. Antioxidants found in phytochemicals have attained greater attention as they have potential for mopping up reactive oxygen species and lowering the

oxidative stress. The vast therapeutic potential of plants regarding antidiabetic is yet to be explored in detail (Unuofin & Lebelo, 2020).

Genetic Factors

Cytokines

Cytokines cover the communication gap between immune cells and cells at the site of inflammation and produce immune response and inflammatory response. Cytokines including interleukins, interferon and colony stimulating factors releases in the body when there is a need as in case of disease and activated by binding to the receptors. Cytokines are regulated by certain mechanisms like cytokine inhibition mechanism, competitive blocking of receptor and many more.

Interleukin-1 is a glycoprotein having molecular weight of 17kDa, which is expressed by monocytes and macrophages (Breder *et al*., 1988). Then thromboxane and prostaglandins are produced and which in return stimulate other cytokines (Oppenheim *et al*., 1986). Interleukin 1a and interleukin 1b are 26% similar in amino acid sequence in human and have some kind of similar activity. IL1R (interleukin receptor 1) is present at the proximal region of q arm of chromosome 2 (Modi *et al*., 1988). IL1RN is a receptor antagonist that binds in place of IL1

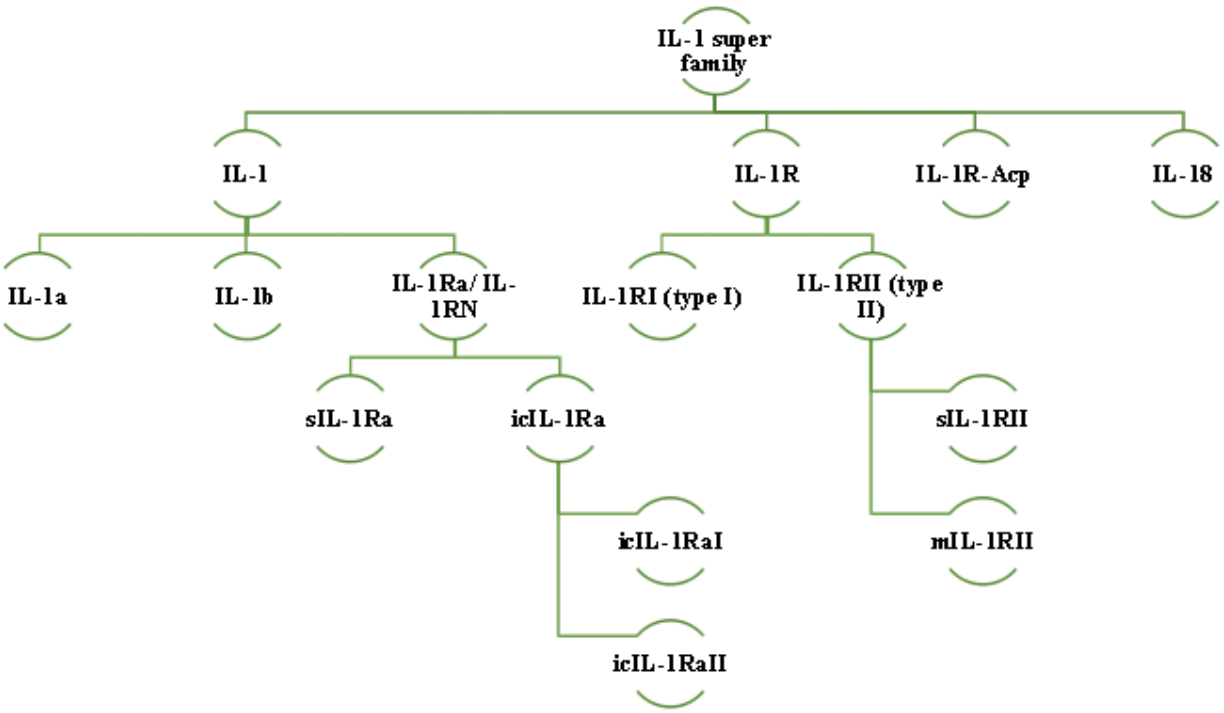


***Figure 6** IL-1 Super family*

receptor and inhibits the biological activity of IL1 and IL10. Interleukin 1a and interleukin 1 receptor antagonist have 18% similarity in amino acid sequence (Steinkasserer *et al*., 1992) . The exists three members of IL-1 family which includes IL-1a, IL-1b and IL-1RN that is the receptor antagonist (Greenfeder *et al*. 1995).

IL-1a and IL-1b as a ligand binds to different receptors but the receptors for these present serially at different target cells. Normal physiology is not clear yet however in case of certain diseases like Rheumatoid arthritis Interleukin-1 causes the tissue destruction and prevents the uncontrolled inflammation (Arend, 1998).

Interleukin-1 receptor antagonists

Interleukin-1 receptor antagonists designated as IL-1RN or IL-1RA, is a glycoprotein having molecular weight of about 22-25 kDa. Studies which were conducted on cultured immunoglobulin (IgG), showed that IL-1RN have inhibitory bioactivity and it was reported as first naturally occurring antagonist protein against cytokines (Dinarello, 1989).

Previous studies revealed that scientists were looking forward to the function of IL-1 and IL-1RN in monocytes and urine samples. They found immune complexes especially in diseases samples like Rheumatoid arthritis and nephritis. These were found in adherent form that travels to disease cells along with IgG and become macrophages there and release plasminogen activator and elastase enzyme (Hovi *et al*., 1981), (Chang *et al*., 1980). Studies revealed IL-1RN shows inhibitory activity and effect only IL-1 not IL-2. Partially purified IL-1 inhibitor showed similar properties as IL-1 as adherent monocytes complex. Purification and cloning showed non-glycosylated form weighed 18 kDa and glycosylated form weighed 22 kDa. Cloning was performed using T7 expression vector to pT5T make the recombinant IL-1RA (Chang *et al*., 1980). Another study reported the purification and cloning of IL-1RN named as IRAP obtained from human myelomonocytic leukemia cell line (Zahedi *et al*., 1994).

Serum Paraoxonase 1 (PON1)

PON1 belongs to multigene family. It has 3 members which are paraoxonase 1, paraoxonase 2 and paraoxonase 3. All these are present side by side on the same chromosome number 7 and have similar structure with slight difference that is PON1 have 3 more nucleotides at exon 4 as compared to PON2 and PON3.

Objectives of the study

I. Computational analysis of the polymorphisms of interleukin 1 receptor antagonist and serum Paraoxonase 1, to determine the most deleterious missense SNPs associated to disease.

II. Screening of genetic association of polymorphisms of genes IL-1RN (rs380092) and PON1 (rs854560) with type 2 diabetes mellitus.

REVIEW of LITERATURE

Type 2 Diabetes Mellitus

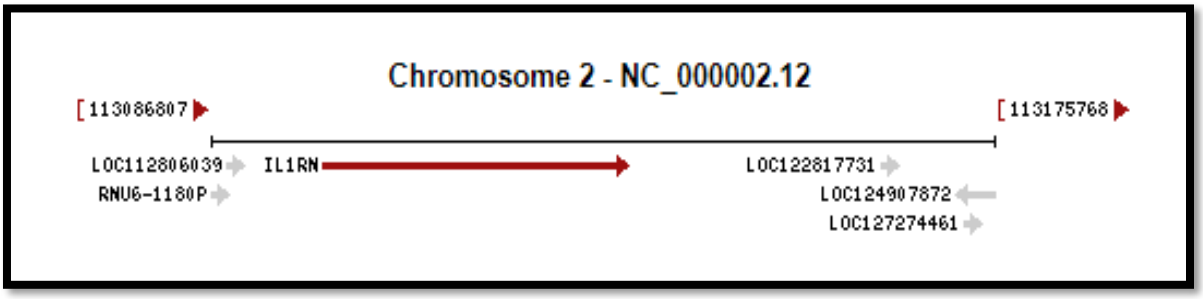
Type 2 diabetes mellitus is the major form of Diabetes mellitus characterized by insulin resistance due to which a body experiences dysregulation of lipid, carbohydrate, and protein metabolism. T2DM is the most severe public health concern which is emerging as challenging epidemic globally. 90 % cases of diabetes mellitus are T2DM (El-Kebbi *et al*., 2021). Population of obese people affected by T2DM is more than the lean population (DeFronzo *et al*., 2015). T2DM can be caused by both environmental factors and genetic factors.

Genetic Factors

There are a lot of genes which are involved in the onset of type 2 diabetes mellitus like KCNJ11, TCF7L2, IRS1, MTNR1B, PPARG2, IGF2BP2, CDKN2A, HHEX and FTO. But the main focus of this research is IL1RN and PON1 which plays an important role in the development of AGEs (advanced glycation end product), a major contributing factor of T2DM.

Interleukin 1 receptor antagonist (IL1RN)

Interleukin 1 receptor antagonist a protein which is encoded by IL1RN gene, was the earliest reported cytokine resembling a hormone, that occurs naturally. In consonance with genome



assembly GRCh38.p14, interleukin 1 receptor antagonist gene (IL1RN) is located at chromosome 2 corresponds to position 2q14.1 with genome coordinates 2:113,127,598-113,134,014, comprises of 13 exons. *IL1RN* and five other related cytokine genes make a cluster of about 400 kb (from NCBI). The *IL1RN* gene encodes for the protein interleukin 1 receptor antagonist that comprises of 177 amino acids (accession # P18510) and possesses a molecular mas of 20, 055 Da (from UniProt). This is secreted form of protein that is present outside the cell membrane.

IL1RN has three isoforms present in cytoplasm that vary in amino acid number as well as molecular mass. Isoform 2 “icIL-1ra” consists of 159 amino acids having molecular mass of 17,888 Da. Isoform 3 “icIL-1ra type II” having amino acid 180 and molecular mass 19,897. Isoform 4 having amino acid 143 and molecular mass 16,142.

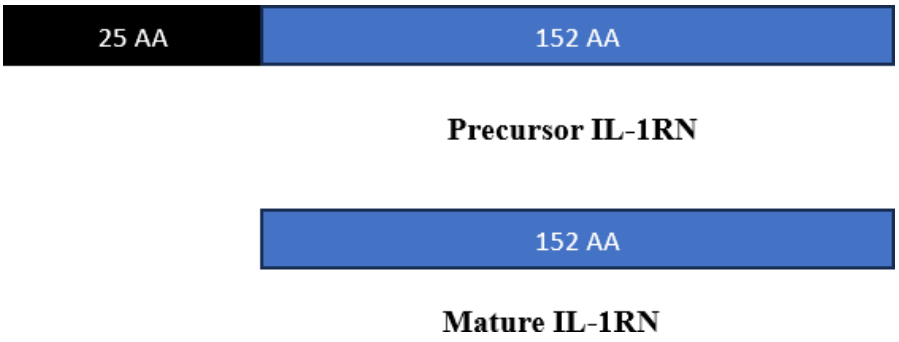


Figure 5 *Structure of IL-1RN*

Role of IL-1RN in Diabetes and associated Pathways

T2DM is characterised by high blood sugar level due to insulin resistance and defective insulin production by the cells of pancreas. Inflammation is the contributing factor in T2DM as occurs in Pathogenesis of type 2 diabetes mellitus and immune response is generated over there. Here cytokines perform an essential role as T2DM was acknowledged as the disease conciliated by immune response cause beta cells’ destruction (Bid *et al*., 2008).

There exist two types of receptors: type 1 receptor and type 2 receptor. As a naturally occurring competitive inhibitor, IL-1RN binds to the type 1 receptor and provide protection to the beta cells from apoptosis (O’Connor *et al*., 2007). Studies reported that there may exist the therapeutic correlation between IL1RN and Type 2 diabetes mellitus (Dinarello, 2000), (Maedler *et al*., 2002). Some studies proved the improvement in efficacy of beta cells due to IL-1RN in T2D (Pfleger *et al*., 2008).

Figure 7 *Mechanism for how diabetes is induced.*

When there is a high glucose level in body, proinflammatory mediators activated and hence it leads to the induction of Type 2 diabetes mellitus. In return this leads to insulin resistance and inflammation which mask down the secretion of insulin in beta cells of pancreas. All these contribute to T2DM.

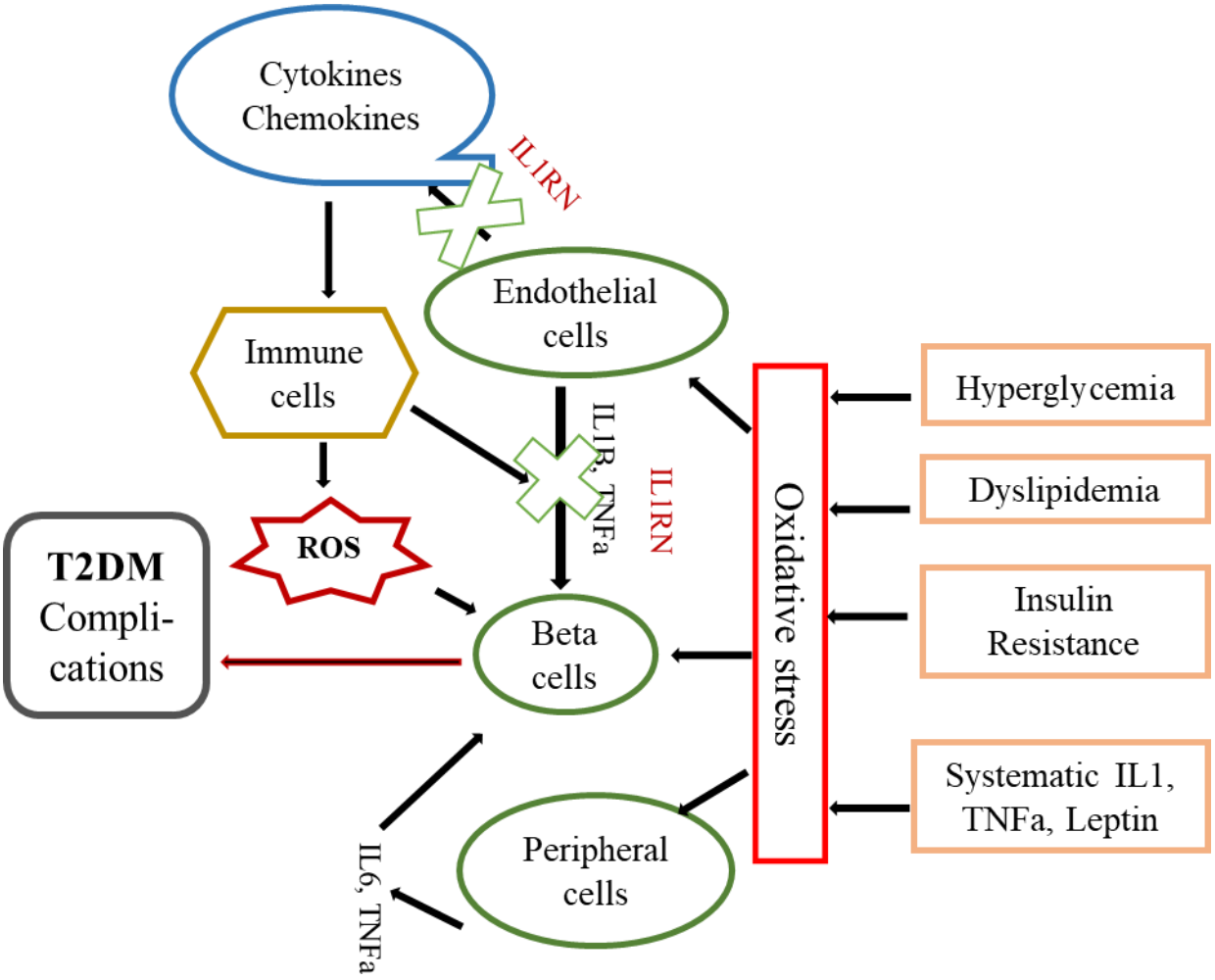


Figure 8 *Pathway of IL-1RN and T2DM*

Here is the description of type 3 diabetes mellitus that when beta cells are not functional properly and oxidative stress produced due to high glucose level and FFAs, causing insulin resistance and inflammation leads to the apoptosis of cells. It can also stimulate TNF-a and IL-6 and Leptin. Oxidative stress causes damage to the endothelial cells and islets of Langerhans by producing reactive oxygen species (ROS) (Akash *et al.*, 2012). Production of IL-1B and TNFa due to impaired function of endothelial cells causes damage to Beta cells which produces chemokines and cytokines and causes immune cells to produce ROS and cascade reaction is started. Reactive oxygen species hence increase the production of IL-1b and oxidative stress which leads to type 2 diabetes mellitus. The activation of impairment of endothelial cells and proinflammatory cytokines as well as signalling pathway of IL-1 and production of ROS are

blocked or suppressed by Interleukin-1 receptor antagonists due to its ability to block them (Akash *et al*., 2012).

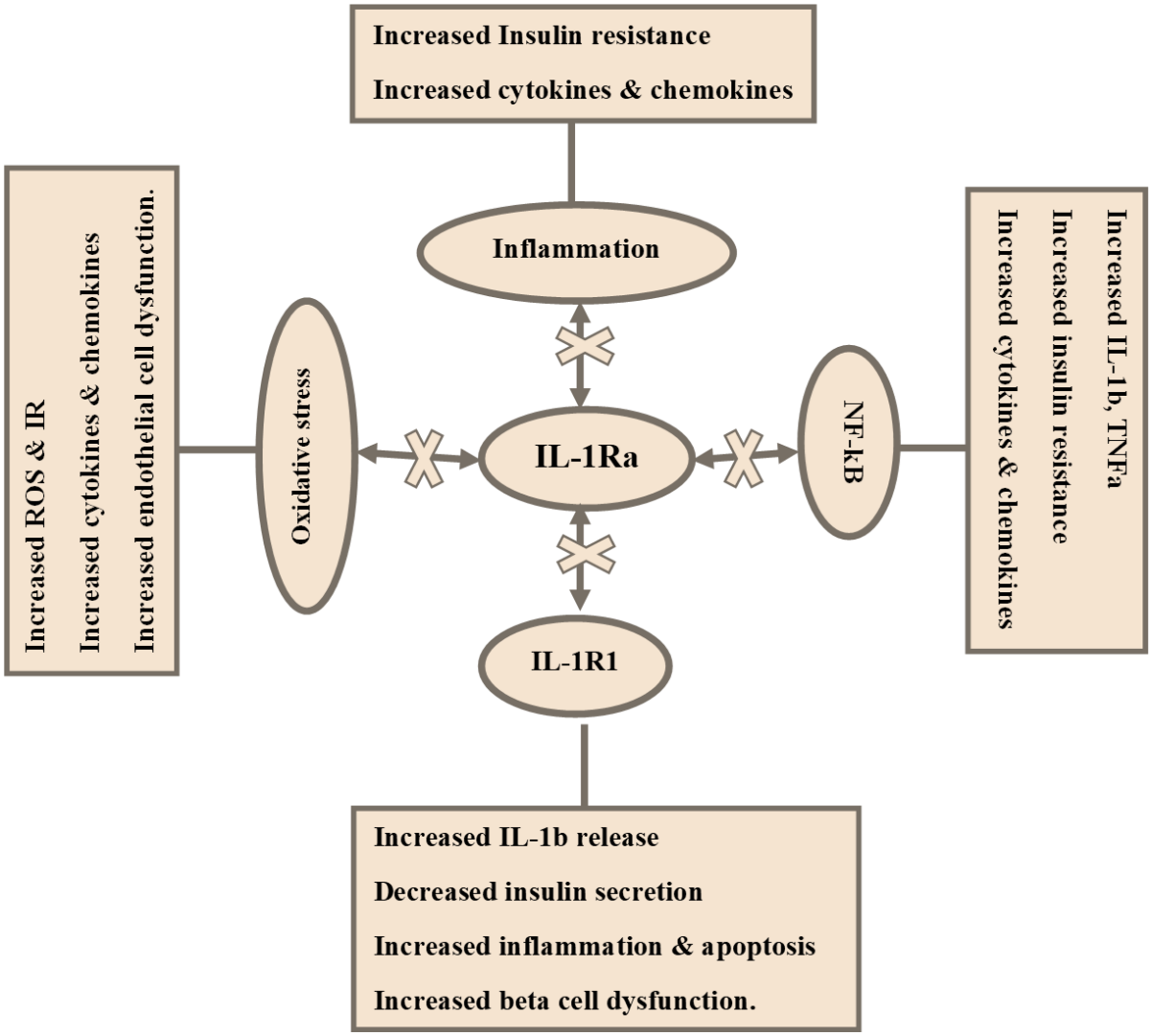


Figure 9*: *Inhibition pathway of IL-1RN

As described earlier beta cell function impairment, insulin resistance, decreased insulin production and oxidative stress with reactive oxygen species, also the activity of IL-1b and production of reactive oxygen species at the end of nuclear factor-kappa B (NF-KB) pathway which leads to type 2 diabetes mellitus, IL-1RN an interleukin -1 receptor antagonist provide protection against type 2 diabetes mellitus and increase the production of insulin (Maritim *et al*., 2003).

Interleukin-1 receptor antagonist as a therapeutic approach in T2DM

Studies were conducted to check the mediators as therapeutic solution for T2DM, the approach used was to block the passage of IL1R1 to refrain the activity of IL1R1. Upon testing other

antagonists like IL-1B and TNFa and salsalate, it was concluded that they are unable to treat T2DM as they couldn't improve the blood glucose level by insulin production, but Interleukin-1 receptor antagonists proved to be therapeutic approach for T2DM as it improves the glucose level in blood by improving insulin production and sensitivity, approved after giving for 13 weeks (Rosenvinge *et al*., 2007), (Dominguez *et al*., 2005). Interleukin-1 receptor antagonist have least limitations as compared to others like salsalate having side effects like bleeding, gastrointestinal and pregnancy issues also IL-1RN have greater affinity to bind as compared to others (Chyka *et al*., 2007), (Larsen *et al*., 2007).

Polymorphisms in interleukin- 1 receptor antagonists (IL-1RN)

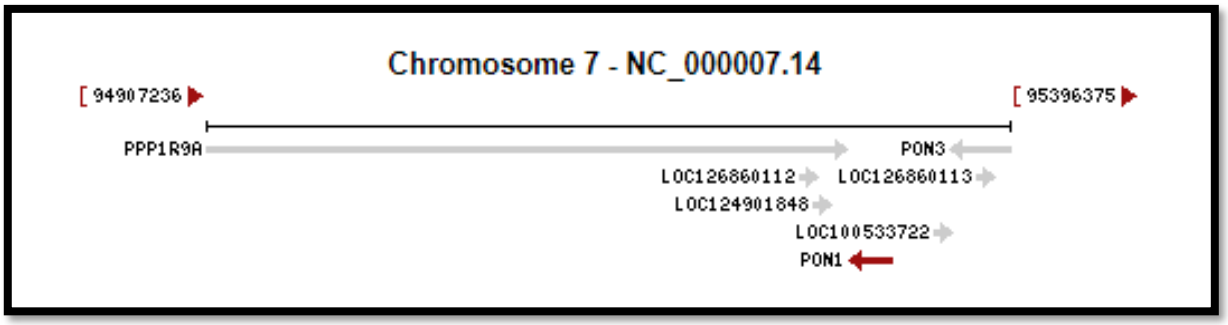
There exist about 14000 single nucleotide polymorphisms in interleukin 1 receptor antagonists. Out of these 12440 are intronic, 189 are missense and 6 others. Some of the variants are associated with T2DM and other diseases. IL-1RN, a naturally occurring antagonist of IL-1B, polymorphism in it leads to certain diseases involving inflammation as well as oxidation stress, for example diabetes and rheumatoid arthritis. Studies suggested that exonic SNP rs4251961 of IL1RN is associated to C- reactive protein level. SNP rs2232354 seemed to be associated with fibrinogen. Certain studies revealed that the variant of IL-1RA rs4251961 is associated to reduced synthesis of IL1RA ex-vivo (Reiner *et al*., 2008), (Cauci *et al*., 2010). According to certain studies genetic variant rs6759676 of IL-1RN seemed to be associated with glycaemic traits. C/T 511 substitution in intron 2 of Interleukin is associated with T2DM (Lee *et al*., 2004).

Serum Paraoxonase/ arylesterase 1 (PON1)

Paraoxonase Family

PON1 belongs to multigene family. It has 3 members which are paraoxonase 1, paraoxonase 2 and paraoxonase 3. All these are present side by side on the same chromosome number 7 and have similar structure with slight difference that is PON1 have 3 more nucleotides at exon 4 as compared to PON2 and PON3.

Serum paraoxonase/ arylesterase 1 is a protein (enzyme) that is encoded by PON1 gene. In consonance with genome assembly GRCh38.p14, Serum Paraoxonase 1 gene (IL1RN) is located at chromosome 7 corresponds to position 7q21.3 with genome coordinates 7: 95,297,676- 95,324,532 comprises of 9 exons. The *PON1* gene encodes for the protein serum paraoxonase 1 that comprises of 355 amino acids (accession # P27169) and possesses a molecular mass of 39,731 Da (from UniProt).



PON1 is produced in liver and secreted in blood stream. It is a cellular component associated with high density lipoproteins (HDL), that helps in metabolism of lipids including cholesterol, triglycerides, in blood or serum, supervise its antioxidant activity.

Structure and Catalytic Domains of PON1

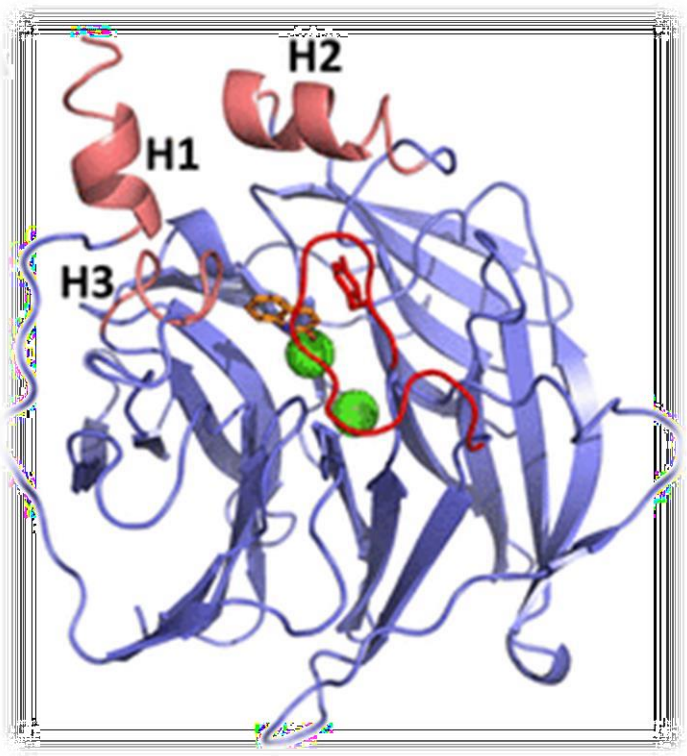


Figure 10 *Structure & catalytic domain of PON1*

X-ray crystallography disclosed the calcium dependent paraoxonase enzyme, a six bladed structure along with a space where calcium ions bind. Position of calcium plays important role in functioning of PON1. Deep down calcium ion is responsible for structural and confirmational properties of enzyme PON1 and the one in bottom is responsible for catalytic activity and bond activation (easter bond). X-ray crystallography shows helices present above active sites called H1, H2, H3. Out of these three, H1 and H2 are important for PON1 to interact with high density lipoproteins (Shunmoogam *et al*., 2018).

Genetics of Paraoxonase 1

The most researched member of the paraoxonase family, PON1 (EC 3.1.8.1), is classed as an aryldialkyl phosphatase. Because of its propensity to hydrolyse organophosphate compounds, it was initially identified in the 1940s (Mazur, 1946). PON1 is encoded by a gene on chromosome 7 (7q21.3-q22.1) in humans and the proximal region of chromosome 6 in mice (Sorenson *et al*., 1995), (Humbert *et al*., 1993) . The gene's coding region consists of nine exons and eight introns that include many polymorphic CA repeats. Surprisingly, it appears that the PON1 gene's 5-UTR region lacks a TATA box (Primo-Parmo *et al*., 1996). Specificity protein 1 (Sp1), protein kinase C (Arii *et al*., 2010),(Tan & Khachigian, 2009), and peroxisome proliferator-activated receptors (PPARs) (Moraes *et al*., 2006) all play important roles in PON1 transcription. Sp1, which has a binding site in the PON1 gene, is a positive regulator of PON1 (Brophy *et al*., 2001). It has been shown in hepatic cell lines that increasing Sp1 promotes PON1 transcription while decreasing Sp1 leads to reduced gene transcription (Osaki *et al*., 2004a). Furthermore, protein kinase C, particularly the zeta isoform, promotes Sp1 binding to the PON1 DNA promoter, hence influencing transcription (Osaki *et al*., 2004, Arii *et al*., 2010).

Polymorphisms in Paraoxonase-1 gene

More than 200 single nucleotide polymorphisms (SNPs) on the PON1 gene have previously been identified, contributing to interindividual variations in PON1 concentration and activity (Richter *et al*., 2010). There are two frequent polymorphisms in the PON1 gene's coding region that have differing biological implications. PON1 concentration is affected by the leucine to methionine substitution at position 55 (L55M): the PON1L allele has more mRNA than the PON1M allele, resulting in a rise in PON1 levels (Leviev *et al*., 1997), (Blatter Garin *et al*., 1997) . The glutamine to arginine substitution at position 192 (Q192R) on the other hand, corresponds with PON1 activity because it results in variances in their catalytic activity towards synthetic substrates. In this case, PON1R has more paraoxonase and arylesterase activity than

PON1Q, but PON1Q has higher lactonase activity than PON1R (Adkins *et al*., 1993), (Rainwater *et al*., 2009). Several additional variations in the PON1 gene's non-coding region have also been discovered. One of the most significant is the cytosine to thymidine substitution at position 107 (C107T), which results in greater blood PON1 levels in the PON1C allele (James *et al*., 2000). Diet, age, lifestyle, pharmacological treatments, and epigenetic variables, in addition to genetic determinants, influence PON1 activity and blood levels (Schrader & Rimbach, 2011). These variables, taken collectively, contribute to the observed interindividual heterogeneity of PON1 (Draganov & La Du, 2004).

Role of PON1 in Beta cells and synthesis of insulin

Beta cells produce insulin and store them (Kulkarni, 2004), hence essential for secretion of insulin according to the level of glucose (Anuradha *et al*., 2014). When there is high glucose level in blood and improper lipid metabolism, it results in the production of reactive oxygen species, which causes damage to beta cells and hence production of insulin as well as its secretion gets disturbed (Laybutt *et al*., 2001). The secretion of insulin from beta cells is inversely related to cellular oxidation (Koren-Gluzer *et al*., 2011). As PON1 has the antioxidant properties, it seems to be directly related with insulin secretion rom beta cells under hyperglycaemic condition (Koren-Gluzer *et al*., 2011). Experimental studies revealed injecting PON1 before inducing diabetes to models, results in decreased chances of getting diabetes and increased insulin (Koren-Gluzer *et al*., 2011). Hence this study revealed that in case of hyperglycaemia, PON1 improves cell survival by increasing insulin secretion from beat cells. As PON1 is linked with HDL, it also helps in insulin secretion but in case when SH groups of PON1 are available. Studies suggested that when SH groups are block due to mutation, it is unable to stimulate its secretion (Koren-Gluzer *et al*., 2011), (Aviram *et al*., 1998).

We can have an estimate of the importance of SH groups in this purpose as these express on the cell membrane of beta cells, granules containing insulin and GLUTs and plays essential role in structure as well as function. Catalytic activity of PON and N-terminus are not responsible for insulin secretion (Khersonsky & Tawfik, 2006), (Gaidukov & Tawfik, 2005). Hence, PON1 increases insulin synthesis from beta cells and cells viability.

Glucose homeostasis, PON1 and its pathways

PON1 plays important role in controlling glucose metabolism (Koren-Gluzer *et al*., 2013). Studies revealed that the expression of GLUT 4 is stimulated by PON1 in the muscles independent of insulin receptors (Koren-Gluzer *et al*., 2013). This upregulation of GLUT 4 and its translocation is done while inhibiting pathway p38MAPK which results in decreased substrate of insulin i.e., serine phosphorylation and increased substrate i.e., tyrosine phosphorylation. The leading character behind increase in the expression of GLUT 4 is SH group and lactonase activity at His 115, a catalytic site (Koren-Gluzer *et al*., 2013), (Khersonsky & Tawfik, 2006). Other than this, part of glycolysis is also stimulated by PON1 directly. These includes lactate, PEP, 3-PG. Besides this PON1 adversely affect Fru-1,6-P2 in glycolysis and some of the components in pentose phosphate pathway (Deakin *et al*., 2003). Whenever the PON1 is absent, glycolysis and Krebs cycle decreases their activity, and energy production in these pathways get reduced. In return, NADPH are produced in excess due the activation of pentose phosphate pathway (Deakin *et al*., 2003). This clearly tells us the value of this enzyme in lipid metabolism as well as glucose homeostasis, hence important for health sector and diseases.

HDL is synthesised in liver as well as in intestine. But PON1 is synthesized only in liver. It is present in two forms either as free form or associated form (with HDL). When synthesized, they are secreted in blood circulation. The main purpose of PON1 is prevention of oxidation of LDL & HDL to prevent production of ROS. HDL brings PON1 towards tissues where it performs its antioxidant activity and prevents body from diseases (Reichert *et al*., 2021), (Mohammed *et al*., 2022).

Antioxidant properties of PON1 and Type 2 diabetes mellitus

The aetiology of several illnesses, including liver disease, cancer, kidney disease, Alzheimer's disease, and diabetes mellitus, is influenced by oxidant-antioxidant imbalance. T2D is a silent, chronic, and progressive illness with a complicated pathophysiology that is influenced by both hereditary and environmental factors (Yki-Järvinen, 2014), (Sorenson *et al*., 1999). According to research, oxidative stress causes the creation of reactive oxygen species, which are implicated in the pathophysiology of T2D. Diabetes complications such as vascular disease are

also influenced by oxidative stress. The evaluation of oxidative stress indicators may be useful in assessing the risk of cardiovascular disease in T2D patients. The significance of this issue is highlighted by the fact that cardiovascular issues are the leading cause of death in these individuals. Diabetes appears to have a reciprocal interaction with PON1, in that diabetes lowers PON1 levels, and the PON1 genotype may also have a role in the risk of diabetes development.

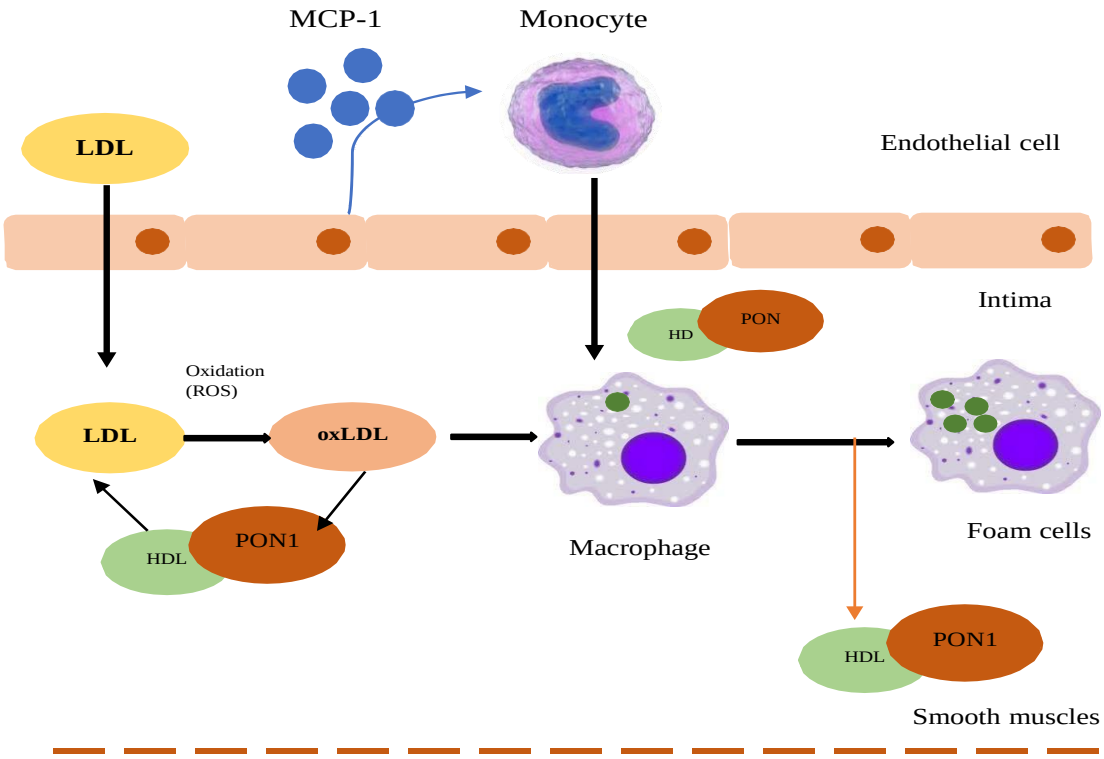


Figure 12 *PON1 & Lipid Metabolism*

Previous research has demonstrated that diabetes mellitus lowers the amount of PON1, an antioxidant and antiatherogenic enzyme. PON1 protein and paraoxonase activity are reduced by 2.8 and 1.7 times in HDLs taken from diabetic patients, respectively, as compared to HDLs extracted from controls. It has been demonstrated that diabetic individuals with heart failure are more prone to develop severe heart failure when their blood PON1 activity is low, possibly due to decreased antioxidant capacity (Seo *et al*., 2018). According to some studies, impaired PON1 activity contributes to HDL dysfunction, which may contribute to the development of atherosclerosis-related illnesses in T2D (Daimon *et al*., 2011). Scientists discovered that PON1 activity reductions lead to reduced HDL anti-inflammatory capability in T2D patients, increasing the risk of T2D-associated atherosclerosis (Won *et al*., 2014).

Role of PON1 in Type 2 Diabetes mellitus

Diabetes mellitus, chronic metabolic disorder results in high glucose level known as hyperglycaemia, insulin resistance and deficient production of insulin (Cnop *et al*., 2005). It is categorized in two forms, T1DM and T2DM. T1DM is autoimmune, caused due to beta cells damage and no or less insulin produced over there (Alam *et al*., 2014), so patients need to take insulin from outsources (Xia *et al*., 2019). T2DM results in insulin resistance whenever there is high blood glucose level due to abnormality in function of beta cells (Alam *et al*., 2014). Conversely of T1DM, unhealthy diet, sedentary lifestyle, and genetic factors all are the leading causes of T2DM (Merino *et al*., 2017). Prediabetes can help us to predict the onset of diabetes (de Vegt *et al*., 2001), by glucose tolerance test and fasting glucose test. hypertension, dyslipidaemia these can be the contributing factors as well (Shaw *et al*., 1999). Mechanisms behind diabetes mellitus are still required to be uncovered (Kahn *et al*., 2014).

Studies revealed that Type 1 and Type 2 diabetes, in both conditions, activity of PON1 is reduced (Craciun *et al*., 2016), (Boemi *et al*., 2001), (Letellier *et al*., 2002). This reduction results independent of the mutations of PON1 but PON1 polymorphism L55M comes out to be in association with diabetic retinopathy (MACKNESS *et al*., 2000)s. In healthy people, this mutation is assigned for dysfunction of beta cells, expanded insulin resistance and disability to get rid of extra glucose (Deakin *et al*., 2002), (Chiu *et al*., 2004). The mechanism behind this is not understood well. High glucose level in blood adversely affects the expression as well as activity of PON1. High level of glucose causes insulin resistance and enlarged accumulation of lipids (Nakajima *et al*., 2000). In return which results in the stimulation of Kinase C, a protein isoform, which is essential for conducting the phosphorylation of regulator of paraoxonase-1 i.e., SP1- specific protein 1. It will increase the transcription of PON1(Osaki *et al*., 2004b). Metabolic disorders producing oxidative stress, ROS, excess insulin, and lipid accumulation causes decrease in activity of PON1 including glycation of HDL associated PON1 (Mastorikou *et al*., 2008). To repay this, expression of mRNA of PON1 and its production both are enhanced (Ikeda *et al*., 2008).

Dyslipidaemia is related to diabetes in which lipid profiling is altered. The concentration of TGs and LDLs are more in patients suffering from T2DM as compared to normal people (Mastorikou *et al*., 2006), (Vergès, 2005), (Fonseca *et al*., 2017), (Adiels *et al*., 2006). As well as antioxidant activity also get reduced. HDL plays key role in glucose homeostasis by

increasing muscle uptake of glucose and release of insulin from beta cells (Drew *et al*., 2009). In Type 2 diabetic patients, externally administrated HDL help sin balancing plasma glucose by increasing insulin in plasma (Drew *et al*., 2009), (Patel *et al*., 2009).

Hyperglycaemia can also lead to the glycation of PON1, which inhibits its activity (Yu *et al*., 2017). Glycation also leads to dysfunction and catabolism of HDL, which then cannot metabolize lipid and balance cholesterol efflux (Witztum *et al*., 1982), (Mastorikou *et al*., 2008). Glycation of PON1 along with T2DM, results in many other diseases like obesity, cancer, neurodegenerative diseases and many more due to impaired function of endothelial and oxidative stress (Ozcan & Tabas, 2012). Hence summary of the story is in metabolic disorder, because of inactivation of oxidation and glycation of PON1 as well as HDL associated PON1, its activity is reduced. Consequently, it is the key player in pathophysiology of many diseases including Diabetes mellitus.

Lipid metabolism and atherosclerosis

Due to production of oxidative stress and reactive oxygen species, low density lipoproteins are converted to oxidized lipoprotein by oxidation. Monocytes release and the macrophages develops from them. These macrophages take the oxidized lipoproteins and convert them into foam cells. Paraoxonase-1 invert this process and convert the oxidized Lipoprotein into lipoproteins and stimulates the release of cholesterol from macrophages. Thus, inhibiting the chances of diseases such as atherosclerosis.

Effect of medication on PON-1

PON1 levels have been reported in certain studies to be affected by diabetes treatments. These impacts may show themselves by altering the expression or activity of the enzyme gene. It should be emphasised that the influence of a medicine on PON1 gene expression or protein function may occur in multiple ways. In other words, a medication that increases an enzyme's gene expression may also limit its protein function (B. N. La Du *et al*., 1999). Previous research has shown that rosiglitazone, an agonist of the peroxisome proliferator-activated c-receptor (PPAR-c), can boost PON1 activity (Tward *et al*., 2002), (Rozenberg *et al*., 2003). According to some findings, rosiglitazone therapy of rats can boost the liver activity of this enzyme by up to 67% (Shih *et al*., 2000). Coll *et al*. discovered that this medication stimulates PON1 activity

in HIV patients (Rozenberg *et al*., 2003). Studies investigated the combined impact of rosiglitazone and fenofibrate on PON1 activity, and their findings revealed that rosiglitazone and fenofibrate administration can considerably boost PON1 activity (Aviram & Rosenblat, 2004). It has also been shown that rosiglitazone may produce antiatherogenic effects by raising apo AI plasma concentrations and smaller HDL particles, in addition to enhancing plasma PON1 activity (Mackness *et al*., 2004). Furthermore studies in atherosclerotic rabbits, rosiglitazone can lower serum MPO activity while increasing PON1 activity, which might impact HDL quality and function (Rosenblat *et al*., 2005). Scientists investigated the effect of glimepiride and glibenclamide (sulfonylureas) on PON1 activity in rats (Navab *et al*., 2006). Their findings revealed that these two drugs can boost the enzyme's hepatic activity while having no influence on its plasma activity. Metformin, the most commonly used diabetes therapy, can influence PON1 levels (Watson *et al*., 1995), (Aviram *et al*., 1999). 1g of metformin administered daily for three months was shown to dramatically boost blood PON1 activity and lower oxidative stress indicators in diabetics (García-Heredia *et al*., 2013). One of the scientists discovered that metformin was more beneficial than just changing one's lifestyle in decreasing oxidative stress indicators like PON1 in a randomised clinical study (Mackness & Mackness, 2010). Metformin appears to exert its action on PON1 and the production of chemokine (C-C motif) ligand 2 (CCL2) via AMP-activated protein kinase (AMPK) (García-Heredia *et al*., 2013). Studies suggested that its long-term administration to diminish the activities of PON1, i.e., paraoxonase, arylesterase, and lactonase, which might be a favourable impact of metformin on diabetic problems (Farbstein & Levy, 2012).

MATERIALS AND METHODOLOGY

Insilico analysis of PON1 and IL1RN

The protein sequences were retrieved from UniProt (https://www.uniprot.org/) with accession number P27169 and P18510 for PON1 and IL1RN respectively. The sequences of both proteins are given below.

Length 355 **Last updated** 2010-10-05 v3
Mass (Da) 39,731 **Checksum**[1] 9B5895509166167E

```
        10         20         30         40         50         60         70         80         90        100        110
MAKLIALTLL GMGLALFRNH QSSYQTRLNA LREVQPVELP NCNLVKGIET GSEDLEILPN GLAFISSGLK YPGIKSFNPN SPGKILLMDL NEEDPTVLEL GITGSKFDVS

       120        130        140        150        160        170        180        190        200        210        220
SFNPHGISTF TDEDNAMYLL VVNHPDAKST VELFKFQEEE KSLLHLKTIR HKLLPNLNDI VAVGPEHFYG TNDHYFLDPY LQSWEMYLGL AWSYVVYYSP SEVRVVAEGF

       230        240        250        260        270        280        290        300        310        320        330
DFANGINISP DGKYVYIAEL LAHKIHVYEK HANWTLTPLK SLDFNTLVDN ISVDPETGDL WVGCHPNGMK IFFYDSENPP ASEVLRIQNI LTEEPKVTQV YAENGTVLQG

       340        350
STVASVYKGK LLIGTVFHKA LYCEL
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Length 177 **Last updated** 1990-11-01 v1
Mass (Da) 20,055 **Checksum**[1] D1690776A7394057

```
        10         20         30         40         50         60         70         80         90        100        110
MEICRGLRSH LITLLLFLFH SETICRPSGR KSSKMQAFRI WDVNQKTFYL RNNQLVAGYL QGPNVNLEEK IDVVPIEPHA LFLGIHGGKM CLSCVKSGDE TRLQLEAVNI

       120        130        140        150        160        170
TDLSENRKQD KRFAFIRSDS GPTTSFESAA CPGWFLCTAM EADQPVSLTN MPDEGVMVTK FYFQEDE
```

Identification of functional SNPs of the PON1 and IL1RN

SNP retrieval

All the information related to missense SNPs of the genes including their accession numbers, positions and amino acid change, were retrieved from NCBI dbSNP database (https://www.ncbi.nlm.nih.gov/snp/) (Irfan *et al*., 2022). Total 10990 SNPs of PON1 were found, out of which 306 missense SNPs were filtered. Details of 38 found missense SNPs were given in Annexure A.

Total 14196 SNPs were found and out of which 189 were filtered as missense SNPs. The details of 31 found missense SNPs are given in the annexure B.

Identifying the high-risk non-synonymous SNPs

For the prediction of functional impact of non-synonymous SNPs six different tools were used including SIFT, PolyPhen-2, PhD-SNP, SNP&GO, PANTHER, snap[2]. For further analysis only

those nsSNPs were considered, which came out to be deleterious or damaging by all six prediction tools. The details of the tools are discussed below.

SIFT (Sorting Intolerant from Tolerant)

SIFT is a bioinformatics tool that sort out the intolerant SNPs from tolerant SNP and predicts weather amino acid change have any impact on proteins function because of sequence homology and on proteins physical properties. Query sequence was submitted and nsSNPs were sorted out as tolerated or non-tolerated. It has various steps that is firstly it searches sequence having similarity with query sequence, secondly finds out the sequences closely related to the query that are somehow have similar functions. Thirdly procure alignments and lastly calculate the possible tolerance index of all the SNPs. (Arshad *et al*., 2018). The threshold value is 0.05, If the tolerance index (TI) will be less than 0.05, the SNP will be considered as deleterious or intolerant and if the value of tolerance index of SNP will be equal or greater than 0.05 it will be considered as tolerant or non-pathogenic. Lower the tolerance index greater will be the possibility of SNP to be deleterious and higher the tolerance index, lower will the possibility of SNP to be deleterious. (Kaur *et al*., 2017). rsIDs of the SNPs were submitted in SIFT tool (https://sift.bii.a-star.edu.sg/www/SIFT_dbSNP.html) on 03-02-2023 and the analysis was done based on the parameters set by default. the SNPs were sorted out as tolerant or intolerant. All the missense SNPs for both proteins were analysed using this tool.

PolyPhen 2 (polymorphism phenotyping v2)

PolyPhen is a bioinformatic tool that is used to predict the structural and functional impact of the amino acid substitution in a protein sequence based on physical and comparative considerations (http://genetics.bwh.harvard.edu/pph2/). Shortly this is used for annotating coding nsSNPs. For all 306 SNPs of PON1 and 189 SNPs of IL1RN, FASTA sequence of both genes were submitted on 07-02-2023 along with the positions of substitution and amino acid variants (Ramensky *et al*., 2002). PolyPhen gave the output in the form of score, sensitivity and specificity and it also calculated the PSIC (position specific independent count) for both the original and mutant amino acid. This score range is 0.0-1.00 that is proportional to the functional impact of the SNP on the protein, higher the score more will be the functional impact on the protein. There exist three categories, If the score ranges between 0.0- 0.15 it will be predicted as benign, if it ranges from 0.15 to 0.85, it will be possibly damaging and from 0.85 to 1.00 will be most probably damaging (Singh *et al*., 2023).

PhD-SNP (predictor of human deleterious single nucleotide polymorphism)

This bioinformatics tool uses support vector machine (SVM) method and predicts the effect of SNPs whether its neutral or diseased, based on three ways that are Hybrid, Sequence based and Sequence and Profile-based method. The sequence of both proteins was submitted on PhD-SNP (https://snps.biofold.org/phd-snp/phd-snp.html) on 07-02-2023 and position of mutations and new variants were submitted individually. All other parameters were used as by default (Mustafa *et al*., 2020).

PANTHER

PANTHER is a tool that gives the functional prediction of non-synonymous SNP on the protein. It estimates the length of time that from how long amino acid is preserved in lineage that leads to the protein of interest, a method known as PANTHER-PSEP (position-specific evolutionary preservation). More time, higher will be the functional impact of SNP on the protein (Tang & Thomas, 2016). Protein sequences for PON1 and IL1RN proteins along with the substitutions were submitted on 25-02-2023. Remaining parameters were used as by default and PANTHER gives results as possibly benign, possibly damaging and probably damaging along with the score pdel. SNPs will pdel 0.5 are considered as possibly damaging, less than 0.5 will considered to be possibly benign and others with score greater than 0.5 will be considered as probably damaging.

SNP & GO

SNP & GO is a bioinformatic tool that predicts disease associated variations by using gene ontology terms. It can predict via three ways; by raw sequence, sequence file and by Swiss-Prot code. The raw sequence of the proteins PON1 and IL1RN were submitted to this server (https://snps.biofold.org/snps-and-go/snps-and-go.html) on 07-02-2023 along with the mutations. If the probability of the variation is greater than 0.5, it will be predicted as diseased and if its less than 0.5 it will be considered neutral (Tarapara & Shah, 2022).

SNAP 2

SNAP 2 is a server (https://rostlab.org/services/snap2web/) used to predict functional effect of SNPs. On the simple submission of FASTA sequence of the proteins, the server will predict either the substitution will affect the function of protein or it will comes out to be neutral along with the heat map (Syed *et al*., 2023).

Identification of structural and functional properties

MutPred2

MutPred2 (http://mutpred.mutdb.org/) is a web-based tool that sort out the diseased or benign amino acid substitutions effectively. And gives the impact of substitutions on more than 50 various protein properties and hence enable us to infer the molecular mechanism of the pathogenicity. The web server gives us results based on SIFT and other 14 different functional and structural properties like gain of intrinsic disorders, gain of B-factor, loss of phosphorylation, gain of catalytic site, altered metal binding and transmembrane protein. (Pejaver *et al*., 2020).This server takes benign polymorphism from SWISS-PROT and deleterious from human gene mutation database. The FASTA sequence of both proteins PON1 and IL1RN were submitted to web server along with the substitutions and threshold 0.05 as by default. The substitutions were sorted out as neutral or deleterious along with their structural and functional impacts. If the p- value is less than 0.05 it will be considered confident and if its less than 0.01 its mean confidence is very high.

Analysing protein stability

I-MUTANT

I-mutant 2.0 is a software based on support vector based machine algorithms (**http://gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi**). This is used to predict the stability of proteins automatically when mutations occur in them (Capriotti *et al*., 2005). The change in the Gibbs free energies of the wild type and mutants can easily predict the change in the stability of the protein structure. ProTherm, an experimental data library on protein mutations. I-MUTANT gives data from this library as well. Gibbs free energy (DDG), if it ranges from -0.5≤ to ≤0.5, it will be neutral, if its greater than 0.5 kcal/mol protein structure will be largely stable and if its, -0.5 it will be considered as unstable (Hussein & Al-kazaz, 2021). I-MUTANT also gives reliability index, along with the results, ranging from 1-10. Reliability index closer to 10 means more reliable is the outcome. All the SNPs of the PON1 and IL1RN were submitted keeping the parameters by default i.e., pH 7.0 and temperature as 25℃.

Evaluating evolutionary conservation of the protein

ConSurf

ConSurf (https://consurf.tau.ac.il/consurf_index.php) is a bioinformatics tool was used to estimate and analyse the conservation profile of amino acid or nucleic acid positions in a certain protein or nucleic acid (Ashkenazy *et al*., 2016). This was performed because of phylogenetic relations among homologous sequences and multiple sequence alignment done by MUSCLE tool. This tool was preferrable because of its accuracy in computing evolutionary rate depending on the methods i.e., Bayesian method and maximum likelihood method (Yariv *et al*., 2023). The outcomes gave the conservation score ranging from 1 to 9 and tells us about the structural and functional properties of the protein either they were exposed or buried and if they are structurally important or functionally. If the score is close 1 it will depict positions most likely to variation along with a colour prediction (turquoise), score 5 represents average conserved positions, coloured white and conservation score 9 represents regions that are most conserved, coloured maroon. The SNPs which were positioned at highly conserved were also analysed.

HOPE (have your protein explained) analysis

HOPE (https://www3.cmbi.umcn.nl/hope/) is a bioinformatics user friendly web service that was used to predict to what extent structural changes occur due to point mutations in the sequence of protein. This software collects information from several sources including UniProt, Reprof's predictions (Bratti *et al*., 2023). The protein sequences for both PON1 and IL1RN were submitted as input along with mutant position and amino acids and HOPE gave us a lot of information on hydrophobicity, charge, size change due to substitution and a lot more. Its output also included protein structures, animations, and highlighted mutations.

Comparative Modelling of PON1 and IL1RN proteins and their mutants

The wild type sequences for both proteins PON1 and IL1RN were submitted, along with their sequences that were mutated, shortlisted being high-risk SNPs, to the modelling tools including Phyre2 (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index) (Kelley *et al*., 2016), I-TASSER (https://zhanggroup.org/I-TASSER/) (Yang *et al*., 2014) , trRosetta (https://yanglab.nankai.edu.cn/trRosetta/) (Z. Du *et al*., 2021) and the 3D structures were generated. Later PyMOL (https://pymol.org/2/) was used to visualise them and to superimpose

the wild and mutant structures (Warnecke *et al*., 2014; Yuan *et al*., 2017). These generated structures were validated by certain structure validating servers including ERRAT program (https://saves.mbi.ucla.edu/) (Colovos, C., & Yeates, 1993), PROCHECK (https://saves.mbi.ucla.edu/) and MolProbity (http://molprobity.biochem.duke.edu/) (Arshad *et al*., 2018) . Afterwards wild type and mutants' structures for the proteins PON1 and IL1RN were compared by using Tm-align (https://zhanggroup.org/TM-align/). It gives score ranging from 0-1, 1 means closest wild and mutant structure. It also provided us with RMSD values i.e., root mean square values which tell us how much mutant structures are deviated from the wild structures (Zhang & Skolnick, 2005). TM-score tells us the topological similarity of wild and mutant type structure while RMSD (root mean square deviation) tells us the average distance of alpha carbon backbone between wild type and mutant type. Higher RMSD values represents more deviation of mutant structure from wild type (Carugo & Pongor, 2008).

PTMs (post translational modifications)

Phosphorylation sites

GPS 3.0 that is group-based prediction system (http://msp.biocuckoo.org/online.php) and NetPhos 3.1 (https://services.healthtech.dtu.dk/services/NetPhos-3.1/) tools were used to predict the putative phosphorylation sites in PON1 and IL1RN proteins. NetPhos 3.1 web server uses neural network for the task completion. The residues which show scores greater than 0.5 corresponds to phosphorylated sites and vice versa. This tool predicted phosphorylated sites by using mentioned 17 kinases i.e., ATM, CKI, CKII, CaM-II, DNAPK, EGFR, GSK3, IN SR, PKA, PKB, PKC, PKG, RSK, SRC, cdc2, cdk5 and p38MAPK. GPS-3.0 predicted phosphorylation sites in PON1 and IL1RN based on kinases. It gives the threshold score, if the value of threshold is higher it corresponds to higher potential for residues to get phosphorylated. FASTA sequences of IL1RN and PON1 were submitted to web servers, GPS-3.0 and Netphos 3.1.

Methylation sites

The alleged sites of methylation in PON1 and IL1RN were predicted by using GPS-MSP 3.0-group based prediction system- methyl group specific prediction (http://msp.biocuckoo.org/online.php). FASTA sequence of both the proteins IL1RN and PON1 were submitted. It gives score along with the position of the residue being methylated. Also gives the information about type of methylation either mono, di or tri etc. if the score is

high, it will correspond to more potential of the residue to get methylated. Different cut off values below threshold means the residue vary in specificity, precision, and sensitivity.

Glycosylation site

Glycosylation sites of both proteins PON1 and IL1RN were predicted by using different web servers. NetOGlyc 4.0 (https://services.healthtech.dtu.dk/services/NetOGlyc-4.0/) , NetNglyc 1.0 (https://services.healthtech.dtu.dk/services/NetNGlyc-1.0/), NetCGlyc 1.0 (https://services.healthtech.dtu.dk/services/NetCGlyc-1.0/) were used for the prediction of mucin type glycosylation sites, c-monnosylation sites in mammalian proteins and N-linked glycosylation sites in human proteins respectively. These software uses neural networks for the prediction. FASTA sequences for both proteins PON1 and IL1RN were simply submitted, and outcomes predicted glycosylation sites in these proteins.

Ascertainment of Study Subjects

This study was planned according to case control manner. Also, this study was approved by ERB (ethical review board) of Atta Ur Rahman School of Applied Biosciences, NUST. The subject of study was diabetic patients (patients with Type 2 diabetes mellitus) from Ali hospital, Islamabad.

Informed consent was obtained from all patients. There are two groups, first group contains 100 samples of T2DM patients, and second group have 50 samples of controls. 3CC blood was drawn from all the patients. Those patients were included whose Hba1c (glycated haemoglobin) and BSR (blood sugar random) test values were higher than normal values. Values for other diagnostic tests were also mentioned on the questionnaire. The collected blood samples were stored at -20◦C until next procedure.

Inclusion and exclusion criteria

Inclusion criteria

People with type 2 diabetes mellitus were considered in this research. Patients includes both genders, male and female. While performing sampling patients of age group 25-60 were included.

Exclusion criteria

Patients with certain characteristics and diseases were excluded from the study. These diseases include HIV, AIDS, other organ severe diseases. Also Type 1 diabetes, gestational diabetes and brain disorders were excluded.

Materials

Micro centrifuge tube of *Eppendorf*, micro tips of *Extra GENE*, glass wares all were cleaned thoroughly and autoclaved to sterilize them before being used in experiments. Reagents used in the research are given in the tables along with the units.

Reagents required for DNA extraction

Mainly four different types of solutions are required for DNA extraction. This includes solution A, B, C and D. The making of solutions is given in the table below.

Making of Solution A

Chemical reagents	Amount for 1L (1000 mL)	Amount for 250 mL
Sucrose-0.32M by invitrogen	109.44 g	27.384 g
Tris (pH=7.5) by invitrogen	12.114 g	3.0285 g
$MgCl_2$ (magnesium chloride), (5mM) by Sigma Aldrich	0.476 g	119.014 mg
Triton X-100 (1% V/V) by Solarbio	10 mL	2.5 mL
Distilled water	Raised up to 1000 mL	Raised up to 250 mL

Making of Solution B

Chemical reagents	Amount for 1L (1000 mL)	Amount for 250 mL
Tris (pH=7.5) by invitrogen	12.114 g	3.0285 g
NaCl (sodium chloride), (400 mM) by Riedel-deHaen	23.37 g	5.844 g

EDTA (ethylene diamine tetra-acetic acid) 2 mM by invitrogen	0.58 g	141.12 mg
Distilled water	Raised up to 1000 mL	Raised up to 250 mL

Making of Solution C

Chemical reagents	Amount
Phenol- crystalline by DAEJUNG	20 g
Tris-HCl (50 mM) by invitrogen	20 mL

The pH was set to 8 and it was stored at 4℃.

Making of Solution D

Chemical reagents	Amount for 250 mL
Isoamyl Alcohol by MERCK	240 mL
Chloroform by RCI Labscan	10 mL

Making of 20% SDS (sodium dodecyl sulphate)

Chemical reagents	Amount for 100 mL
SDS	20 g
Distilled water	Raised up to 100 mL

Making of 50X TAE (Tris-acetate-EDTA) buffer

Chemical reagents	Amount
Tris Base	242 g
Glacial Acetic acid	57.1 mL
EDTA (ethylene diamine tetra-acetic acid) (0.5M) pH= 8.0 by invitrogen	100 mL
Double Distilled water	Raised up to 1000 mL

Making of 1X TAE (Tris-acetate-EDTA) buffer

Chemical reagents	Amount to make 100 mL 1X buffer
50X TAE buffer	2 mL
Distilled water	98 mL

Making of agarose gel (2%)

Chemical reagents	Amount to make 100 mL 1X buffer
Agarose by invitrogen	2
1X TAE buffer	100 mL
Ethidium bromide	2 µL

The measured agarose was added to 100 mL of 1X TAE buffer and heated in oven to get mixed until crystal clear and then let it cool down to bearable temperature. Add 2 µL of ethidium bromide and mix it well. pour the gel in casting tray and wait till it solidifies.

Sample Ascertainment

Blood samples from 100 diabetic patients (25-60 of age) were drawn and 100 blood samples were drawn from healthy individuals as control. The related information like name, age, gender, and family history were also collected from all the donors. Informed Consent was also taken from all the patients and healthy members. The blood sample was taken in EDTA vial and stored at 4℃.

Extraction of Genomic DNA from Blood samples by Phenol-Chloroform method

Extraction of genomic DNA from blood samples was performed using following protocol i.e., phenol chloroform method of DNA extraction.

Steps performed on DAY-1

1. Micro-centrifuge vials were labelled according to the sample number.
2. 750 µL of blood sample was added to the microcentrifuge tube and equal amount of solution A was added to the tube to each tube.
3. Micro centrifuge tubes were inverted several times and then the tubes were incubated at room temperature for more 10 minutes.

4. After 10 minutes incubation, the micro centrifuge tubes were subjected to centrifugation for 10 minutes at 13000 rpm (revolution per minute).
5. After centrifugation, the supernatant got separate, and pellet formed in the bottom. The supernatant was discarded, and process was proceeded with remaining pellet.
6. 400 µL of solution B was then added to the pellet formed in previous step, and pellet was dissolved completely in the solution.
7. The tubes were again subjected to the centrifugation for 10 minutes at 13000 revolution per minutes.
8. After performing second centrifugation step, the supernatant formed was discarded again and the pellet was again dissolved in 400 µL of solution B, 12 µL of 20% SDS (sodium dodecyl sulfate), and 5 µL of proteinase K.
9. Then the tubes were incubated for 3 hours at 60℃ or overnight at 37℃.

Steps performed on DAY-2

10. After incubation step, 250 µL of solution C and 250 µL of solution D was added in a new micro centrifuge tube separately and then these solutions were added to the tubes containing samples in process.
11. The tubes were subjected to centrifugation for phase separation at 13000 revolution per minute for 10 minutes.
12. Three layers of sample were formed after centrifugation. The upper most aqueous layer was separated to in a new microcentrifuge tube carefully for further processing.
13. 55 µL of sodium acetate and 500 µL of properly chilled isopropanol was added to it and the tubes were inverted 5-10 times for the DNA to get precipitated and the subjected to centrifugation at 13000 rpm for 10 minutes.
14. The supernatant was again discarded, and the remaining pellet was treated with 70% ethanol (200 µL) to make DNA more concentrated.
15. After adding ethanol, centrifugation was performed for 8 minutes at 13000 revolution per minute.
16. The supernatant after centrifugation was discarded and the pellet was dried in the air for about 1-2 hour.
17. After drying 100 µL of nuclease free water was added to the micro centrifuge tubes and the extracted DNA was stored at 4℃ until next experiment.

Agarose gel electrophoresis for extracted DNA visualization

After performing extraction, the genomic DNA was visualized on agarose gel electrophoresis. 2% agarose gel was prepared by adding 2 g agarose in a flask and the volume is raised up to 100 mL by adding 1X TAE buffer in it. Then the gel was mixed and heated in microwave oven until gel became crystal clear. Gel was cooled down to bearable temperature and then 2-4 µL of ethidium bromide was added in it and gel was poured in the caster and solidified in some time. Then 6 µL of sample along with 2 µL of tracking dye was added to the wells and gel was run for 40 minutes at 80 volts in 1X TAE buffer. 50 Kb ladder was run along, for comparison of DNA fragments. After this the gel was visualized in UV transilluminator and then in Gel Documentation system in UV light.

DNA quantification by Nanodrop

For the quantification of extracted genomic DNA, nanodrop was used which also tells about purity of DNA along with its concentration. At first, nuclease free water or PCR water or TE buffer was used to blank and then DNA sample was loaded to measure its absorbance. Absorbance ratio of DNA is 260/280, which tells the respective wavelength at which sample absorbed Ultraviolet light. The pure DNA corresponds to ratio about 1.8.

Interleukin 1 receptor antagonist gene (IL1RN) and Serum paraoxonase/arylesterase 1 (PON1) primers

Following primer set of IL1RN and PON1 were designed for the study by using Primer 3 and verified by UCSC genome browser and ordered from Eurofins.

Name of sequence	Sequence	OD	Length	Tm	GC content	Product size
IL1RNrs380092F	ATGCTGGGGTCACTTTGGAA	11.4	20	60.4	50	150 bp
IL1RNrs380092R	CTTTTGGAGTCAGCCGCC	9.5	18	62.2	61.1	

Name of sequence	Sequence	OD	Length	Tm	GC content	Product size
PON1rs854560F	TCTGTTCTCTTTTCTGGCA	10.2	19	55.5	42.1	59 bp
PON1rs854560R	GTCCATTAGGCAGTATCTC	9.5	19	58.5	47.4	

Genotyping of IL1RN and PON 1 by real time PCR using HRM software

Before performing experiments, the apparatus to be used was autoclaved properly. All the experiments were performed in Hood (laminar flow). The PCR reaction mixture was made on ice and PPE (personal protection equipment) were ensured throughout the experiments. Eva green PCR master mix of Solis BioDyne was used.

Optimization of IL1RN and PON1 primers

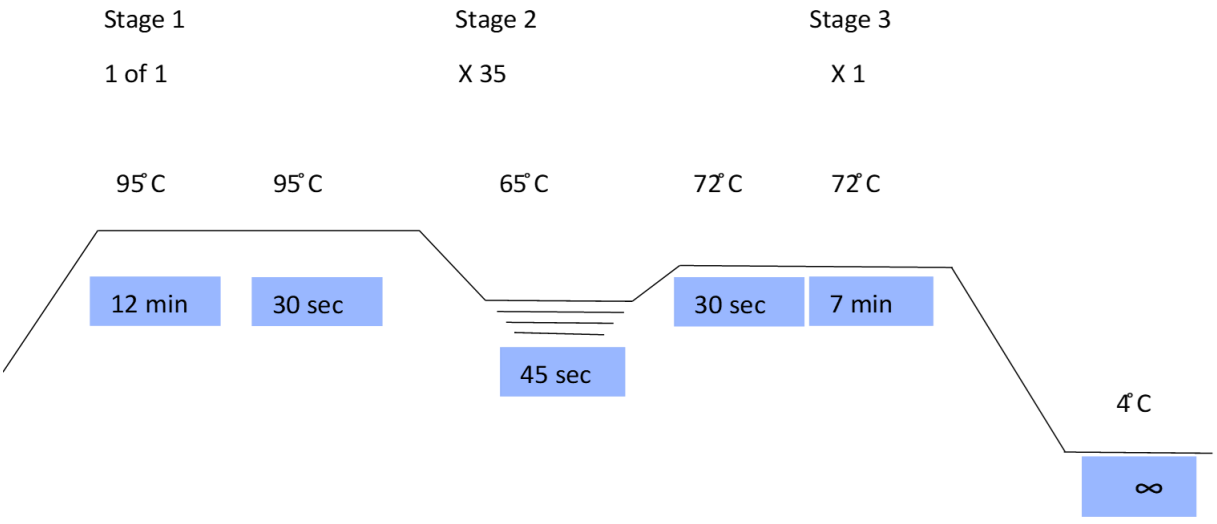
Gradient PCR of IL1RN and PON1 was performed several times at a range of temperatures to get the optimized temperature of the respective gene that is perfect for amplification.

PCR reaction mixture composition

The reaction mixture was made on ice and in hood. The composition of reaction mixture is given in table below.

Components	Amount
Master mix containing Taq Polymerase	10 µL
Forward primer	1 µL
Reverse primer	1 µL
DNA template	2 µL
PCR water	6 µL
Total volume	20 µL

PCR cycle



Stage 1 includes initial denaturation at 95 ℃ for 12 minutes (incubation to activate Taq polymerase) and denaturation at 95 ℃ for 30 seconds. Stage 2 includes annealing at different temperatures at 65 ℃ for 45 seconds and then extension at 72 ℃ for 30 seconds. Stage 3 includes final extension at 72 ℃ for 7 minutes and the storage for infinite time at 4 ℃.

For analysing polymorphism in all the selected patients, real time PCR was performed using HRM software for all the diabetic samples and controls in two batches. Reaction mixture was prepared containing volume of 20 µL. First batch contains forward and reverse primers of IL1RN and second batch comprises of forward and reverse primers of PON1 all other components were kept same. The PCR tubes containing reaction mixture were placed in thermocycler and reaction was run.

Agarose gel electrophoresis for visualization of PCR product

After performing real time PCR, the products were visualized on agarose gel electrophoresis. 2% agarose gel was prepared by adding 2 g agarose in a flask and the volume is raised up to 100 mL by adding 1X TAE buffer in it. Then the gel was mixed and heated in microwave oven until gel became crystal clear. Gel was cooled down to bearable temperature and then 2-4 µL of ethidium bromide was added in it and gel was poured in the caster and solidified in some time. Then 6 µL of sample along with 2 µL of tracking dye was added to the wells and gel was run for 40 minutes at 80 volts in 1X TAE buffer. 50 Kb ladder was run along, to compare the product size. After this the gel was visualized in UV transilluminator and then in Gel Documentation system in UV light.

Association/ statistical analysis

The controls and patient's results were compared with each other In order to analyse the association of Polymorphism in IL1RN and PON1 in diabetic patients. Further statistical analysis was performed using High resolution melt curve and u-analyse.

RESULTS

Insilico analysis of paraoxonase-1 (PON1) and interleukin 1 receptor antagonist (IL1RN) proteins

Retrieval of sequence of PON1 and IL1RN

The sequence of IL1RN and PON1 was retrieved from NCBI (National Centre for Bioinformatics Information) and further analysis were conducted.

Prediction of secondary structure of PON1 and IL1RN

Secondary structure of protein PON1 and IL1RN are predicted by PSIPRED tool. The results revealed clear allocation of B-strand, coil, and alpha helix of PON1 and IL1RN (figure 13 & 14). For PON1 B-strand and helix covers the major portion i.e., 44.78% each followed by coil that comprises the smallest portion in the whole structure i.e., 10.42%. For IL1RN, B-strand covers maximum portion i.e., 46.89% followed by 45.76% of coil. While helix is the smallest portion of the structure i.e., 7.34%.

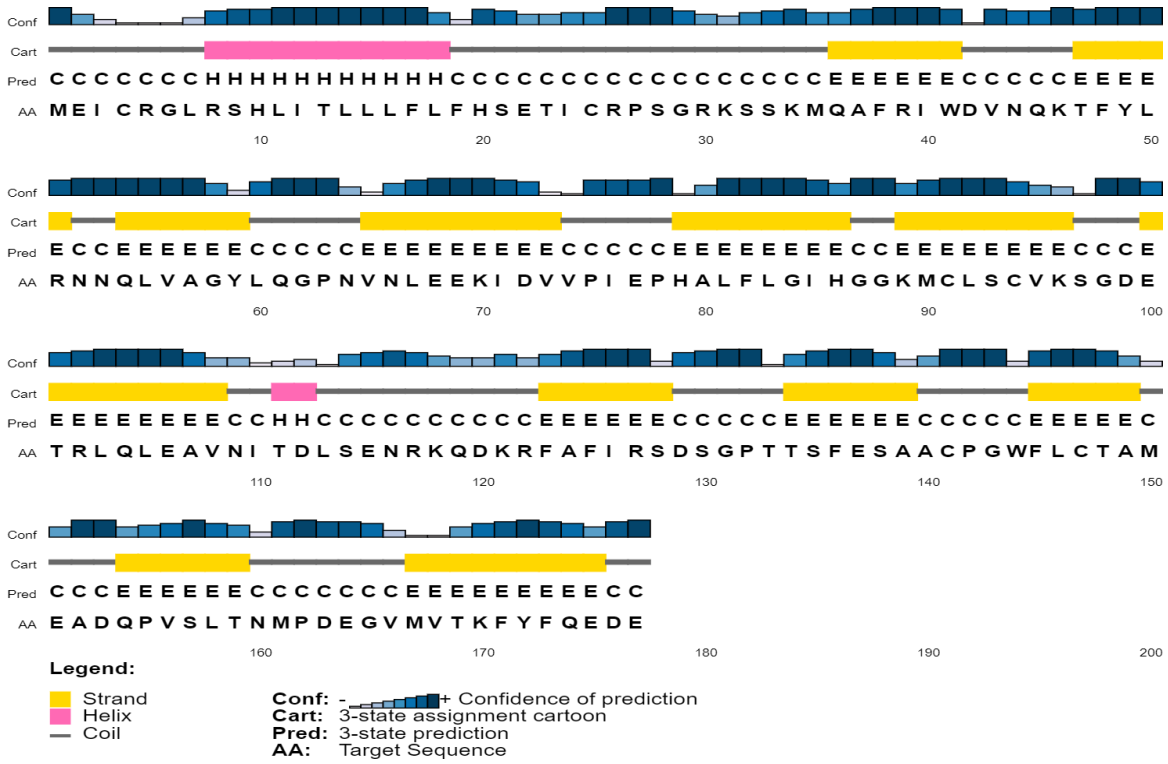


Figure 14 *Secondary structure of IL-1RN predicted by PSIPRED.*

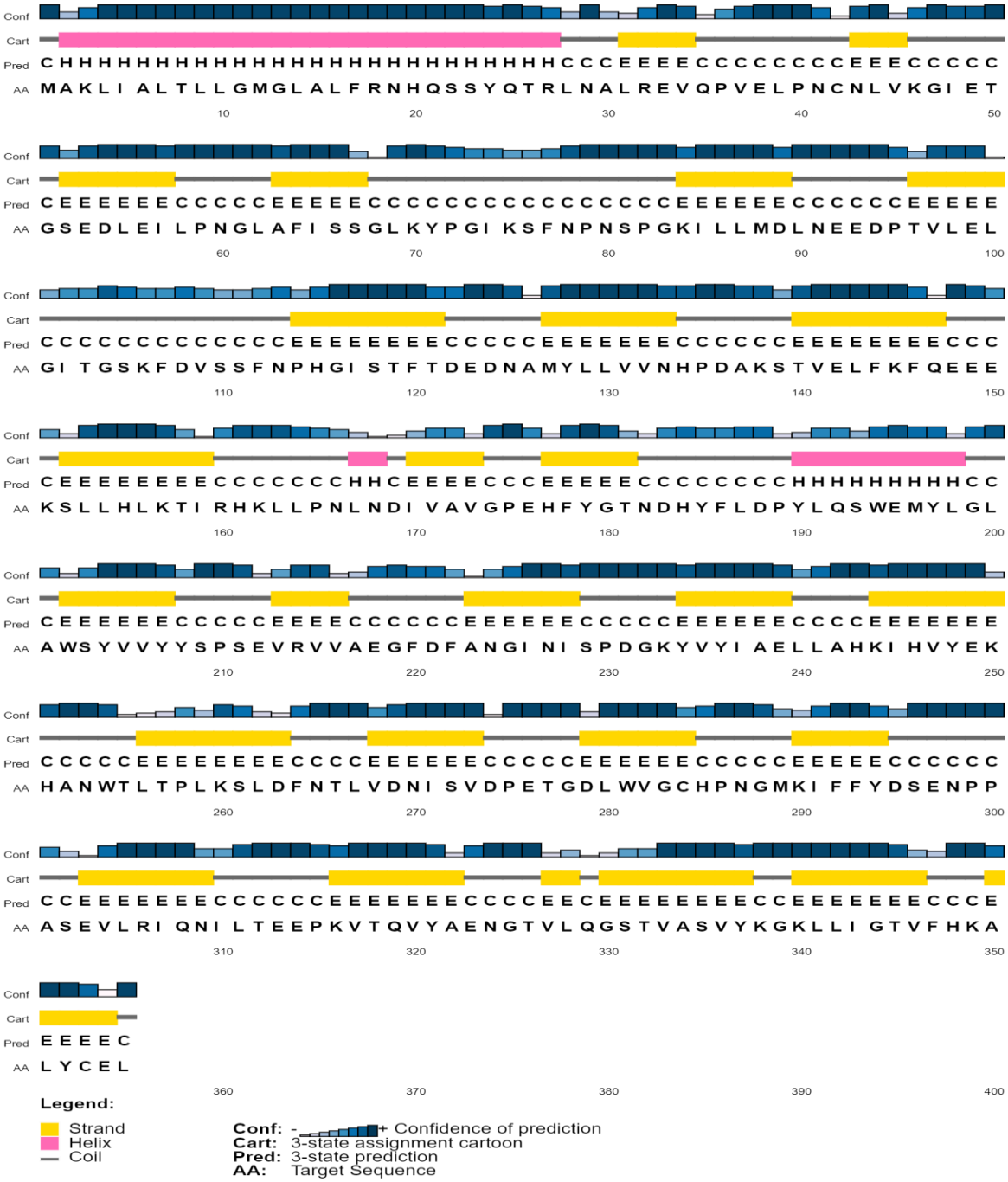


Figure 13 *Secondary structure of protein PON1 predicted by PSIPRED.*

Retrieval of missense SNPs of PON1 and IL1RN

Missense SNPs of bot genes were retrieved from two databases i.e., dbSNP and Ensembl because these are the most appropriate and widespread databases. PON1 contains total 10990 SNPs, out of which 306 were non-synonymous missense SNPs, 128 were synonymous SNPs,

52 lies in 5'UTR region, 895 lies in 3' UTR region and rest were included in other different types of SNPs. These are represented in the figure below.

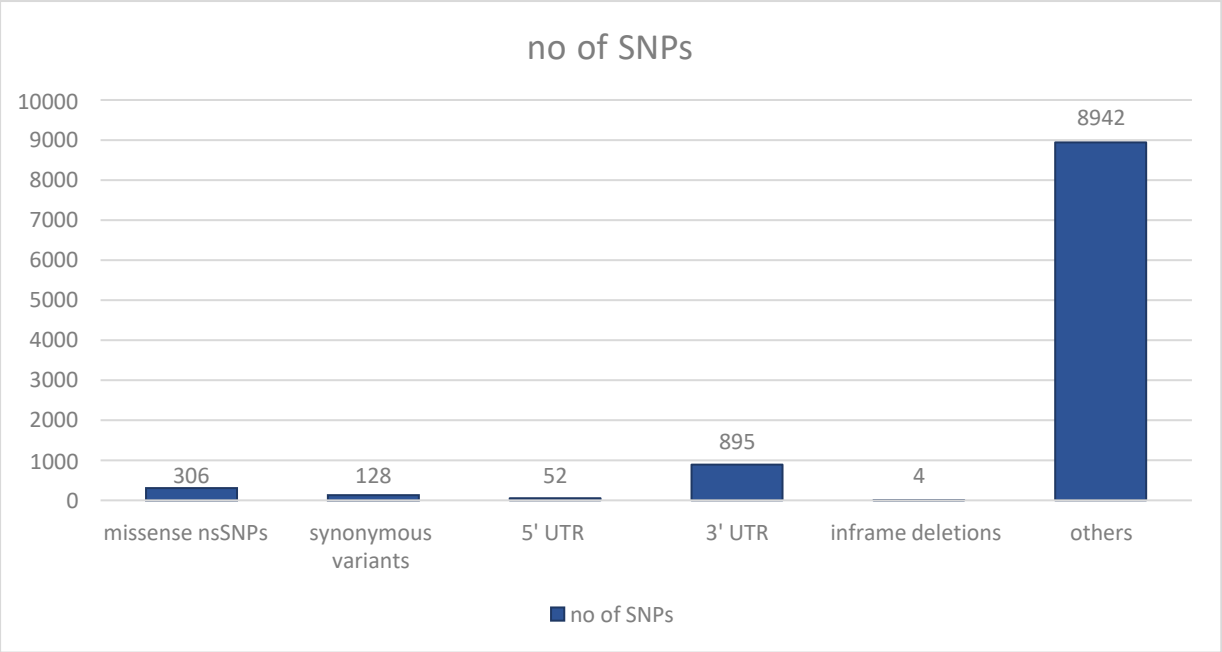


Figure 15 *Distribution of SNPs in PON1*

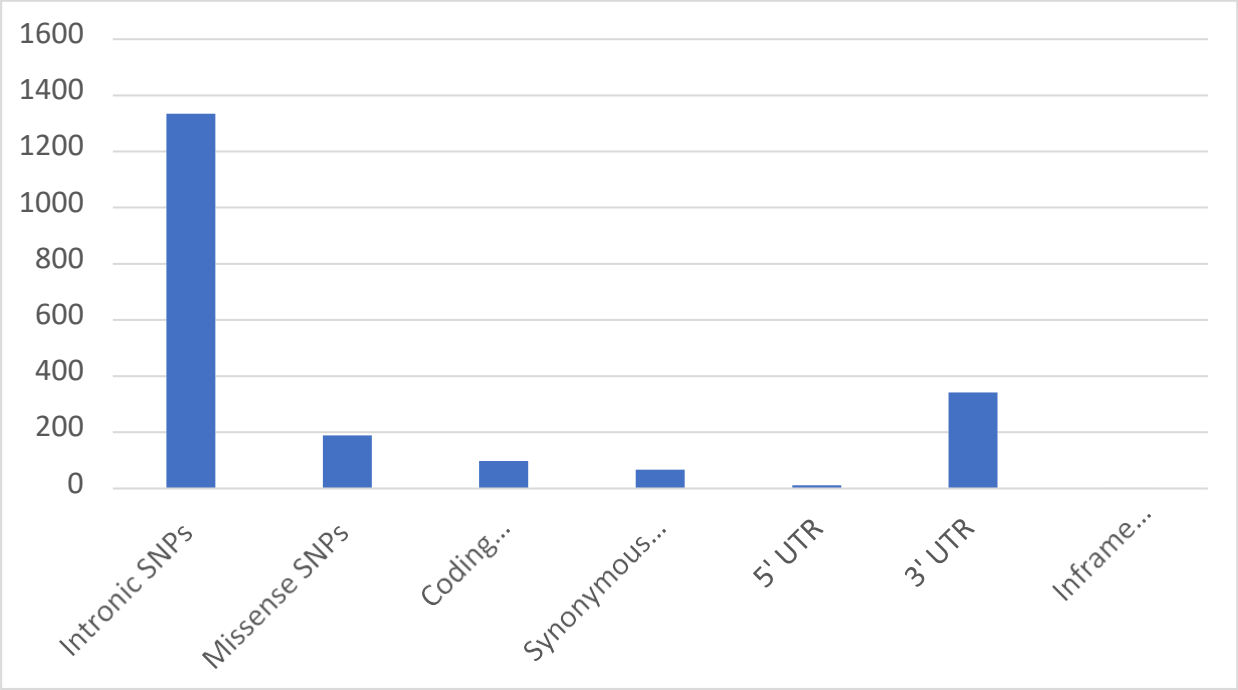


Figure 16 *Distribution of SNPs in IL-1RN*

Deleterious SNPs in paraoxonase-1 (PON1) and interleukin 1 receptor antagonist (IL1RN)

All 306 for PON1 and 189 for IL1RN missense nonsynonymous SNPs were submitted to 6 different in-silico prediction software to predict whether the SNPs are tolerated or in-tolerated. The software used include SIFT, PolyPhen 2, PhD-SNP, SNAP 2, PANTHER, SNP&GO.

According to SIFT, the non-synonymous SNPs which scored less than or equal to 0.05 are intolerant or deleterious. And those nsSNPs which scored greater than 0.05 are tolerant and have no effect on the structure and function of proteins. So PON1 showed 14 nsSNPs which are intolerant and hence affects the function of protein and IL1RN showed 7 nsSNPs which comes out to be intolerant.

According to PolyPhen 2, the score equals to 1 means probably damaging and the score equals to 0 means benign having no effect and from 0.15 to 0.85 depicts possibly damaging, probably damaging being the most confident. PolyPhen 2 outcomes for PON1 showed 9 probably damaging, 6 possibly damaging and 23 benign. For IL1RN, outcomes include 7 probably damaging, 1 possibly damaging and 15 benign nsSNPs.

According to PhD-SNP, 14 nsSNPs of PON1 comes out to be diseased and 24 comes to be neutral. And for IL1RN, 5 nsSNPs were found to be diseased and 18 were neutral. SNP&GO showed 7 nsSNPs of PON1 as diseased and 31 as neutral. And for IL1RN only 4 nsSNPs were diseased and 19 were neutral. According to PANTHER, for PON1 16 nsSNPs were come out to be probably damaging, 5 were possibly damaging and 17 were probably benign. And nsSNPs of IL1RN, 4 were probably damaging4 were possibly damaging and 15 were probably benign. While SNAP2 revealed 12 nsSNPs of PON1 as effective and 26 as neutral and 12 nsSNPs of IL1RN as effective and 11 as neutral.

The SNPs which were found deleterious, intolerated or probably damaging from all these six software, were shortlisted and considered for further analysis. Interestingly 5 SNPs from PON1 and 1 from IL1RN are shortlisted according to the above-mentioned criteria so the further analysis was performed on them.

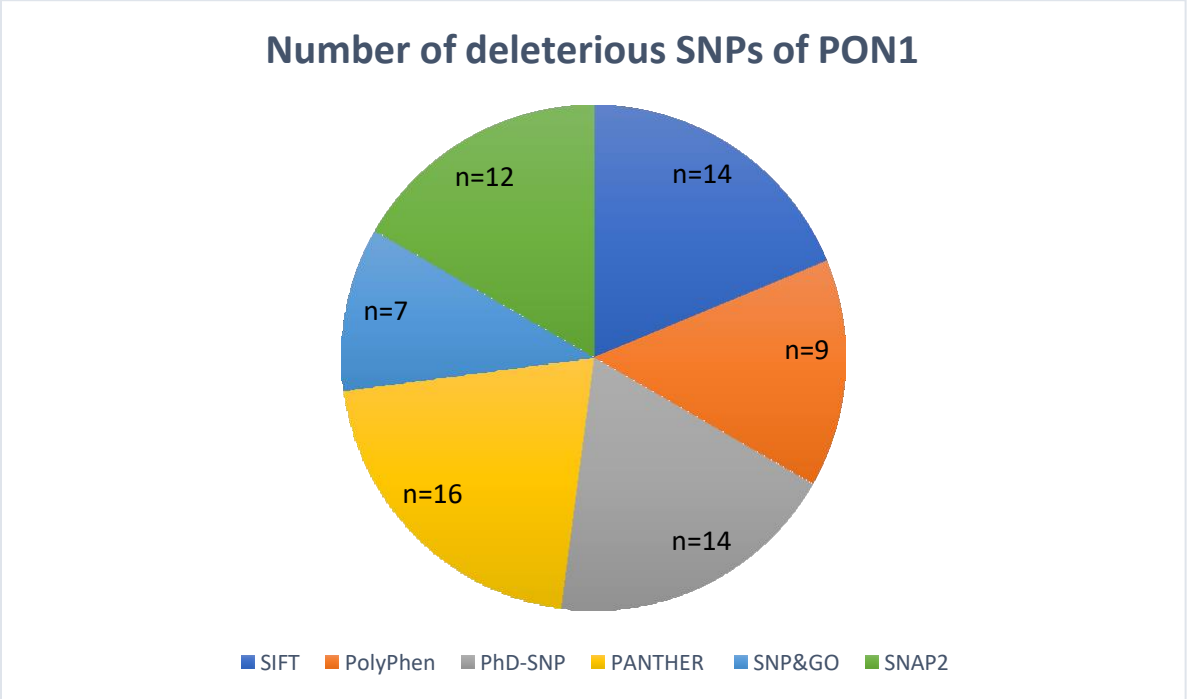


Figure 17 *Pie chart showing number of deleterious SNPs (n) of PON1 by six Insilco tools; SIFT, PolyPhen2, PhD-SNP, PANTHER, SNP&GO, SNAP2*

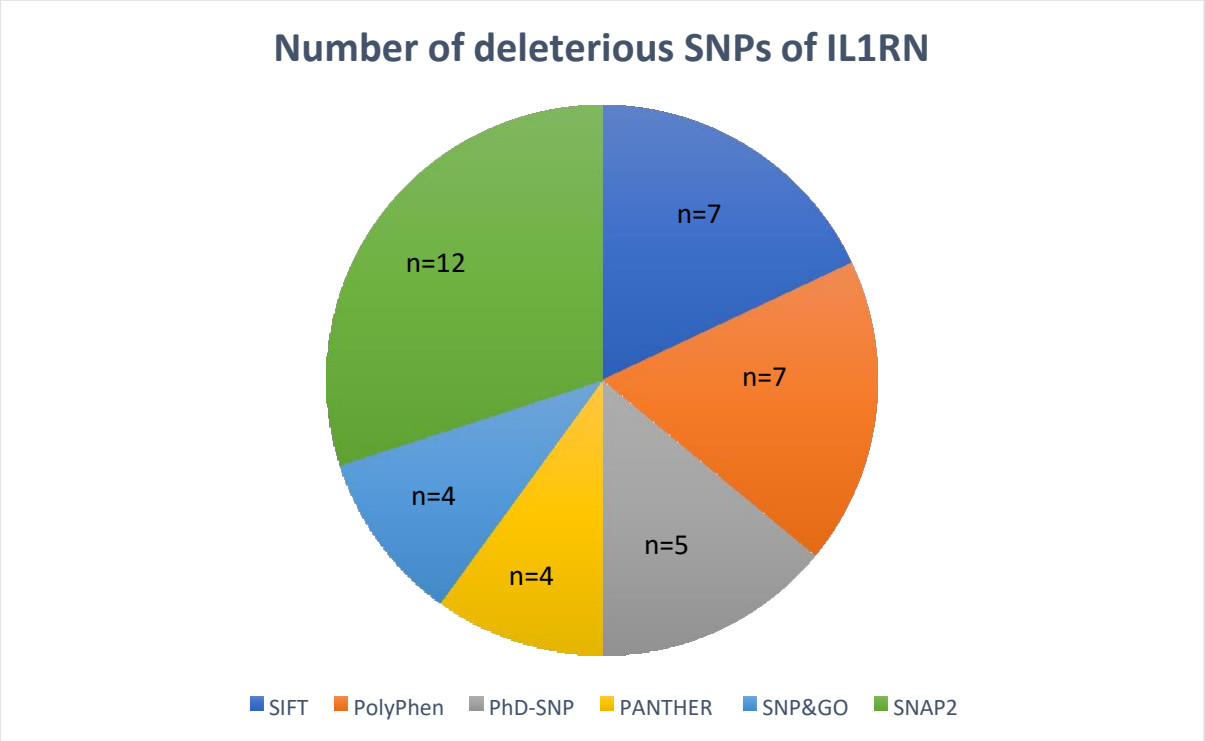


Figure 18 *Pie chart showing number of deleterious SNPs (n) of IL1RN by six Insilco tools; SIFT, PolyPhen2, PhD-SNP, PANTHER, SNP&GO, SNAP2.*

Structural and functional modifications of PON1 and IL1RN predicted by MutPred

All the shortlisted nsSNPs of PON1 and IL1RN were submitted to MutPred web server and the alterations in structure and function of proteins were predicted. For the SNPs of PON1 i.e., for L90P, gain of intrinsic disorder (p=0.04), gain of B-factor (p=0.0069), altered stability (p=0.0066) and loss of strand (p=0.04) were predicted. For C42R loss of disulfide linkage at C42 (p=0.00014), altered transmembrane proteins (p=0.00084), altered metal binding (p=0.03) and gain of ADP-ribosylation (p=0.03) were predicted. For variation S302L, altered transmembrane protein (p=0.00077) and loss of B-factor (p=0.05) were predicted. For substitution G344C, loss of relative solvent accessibility (p=0.009), altered ordered interface (p=0.03), loss of strand (p=0.0077), altered transmembrane protein (p=0.00057) and altered metal binding (p=0.03) were predicted. For substitution W281C, altered metal binding (p=0.01), altered ordered interface (p=0.04), loss of strand (p=0.02), loss of relative solvent accessibility (p=0.04), altered transmembrane protein (p=0.01) and gain of catalytic site at H285 (p=0.02) were predicted. While in case of IL1RN, altered metal binding (p=0.0066) were predicted for substitution C94F.

Stability modification prediction

The stability of the variations occurred was predicted by I-MUTANT. The 5 shortlisted high-risk SNPs of PON1 and 1 high-risk SNP of IL1RN were submitted to I-Mutant to calculate the reliability index and Gibbs free energy value. Results revealed that after mutation all the SNPs of both PON1 and IL1RN have decreased their stability hence cause extreme damage to the protein. The details are given in the table below.

Table 1 I-Mutant results for high-risk SNPs of PON1

I-MUTANT						
SNP IDs	Mutations	pH	temp	SVM2 prediction effect (stability)	DDC value prediction Kcal/mol	RI
rs72552788	L90P	7	25	decrease	-1.66	4
rs138512790	C42R	7	25	decrease	-0.31	4
rs185623242	S302L	7	25	decrease	-0.03	1
rs368206333	G344C	7	25	decrease	-1.22	7
rs369422555	W281C	7	25	decrease	-1.57	7

Table 2 I-Mutant results for high-risk SNPs of IL-1RN

I-MUTANT						
SNP	Mutations	pH	Temp	SVM2 Prediction effect	DDG value prediction kcal/mol	RI
rs201638660	C94F	7	25	decrease	-0.02	2

Conservation profiling of the deleterious non-synonymous SNPs in PON1 and IL1RN

The web server ConSurf was performed to calculate the conservation score of amino acid residues of the protein PON1 and IL1RN, to explore and analyse the effect of their high-risk non-synonymous SNPs. Outcomes were represented in pictorial form shown in figure 19 & 20. It tells us about which residue is important for its structure or function, based on the surface accessibility. Results revealed structural and functional importance and conservation levels of all the amino acid residues of PON1 and IL1RN but here only high-risk nsSNPs were focused on. The results revealed that for PON1, L90, S302, G344, W281 all are buried while C42 is exposed and highly conserved. G344 and S302 are structural residues and C42 is a functional residue of PON1. In case of IL1RN, C94 is buried in its whole structure and its highly conserved.

Table 3 conservation profiling of substitutions of PON1

ConSurf results for PON1			
SNP IDs	Residue & position	Conservation score	Prediction
rs72552788	L90P	6	Buried residue
rs138512790	C42R	9	Highly conserved and exposed residue (f)
rs185623242	S302L	9	Highly conserved and buried residue (s)
rs368206333	G344C	9	Highly conserved and buried residue (s)
rs369422555	W281C	7	Buried residue

Table 4 Conservation profiling of substitutions of IL1RN

Consurf results for IL-1RN			
SNP ID	Residue & position	Conservation score	Prediction
rs201638660	C94F	8	Buried residue

Where (f) is functional residue, (s) is structural residue.

HOPE (have your protein explained)

The outcomes of HOPE revealed that for L90P substitution of PON1 protein, in which leucine at position 90 substituted into proline. As every amino acid or nucleotide has its own nature, size, charge, and hydrophobicity. Wild type and mutant vary in these properties. Wild type residue leucine is bigger in size than mutant type residue proline. Wild type amino acid is very conserved, but mutant is not included in this. The figure shows leucin- the wild type residue is mutated in proline. As the wild type and mutant residues vary in size and mutant is smaller in size than wild type residue leucine so it causes an empty space in the core of the protein.

The structure of protein PON1 showing green part which is the wild type of residue i.e., leucin and red portion is the mutated part that is proline, and the protein is coloured grey.

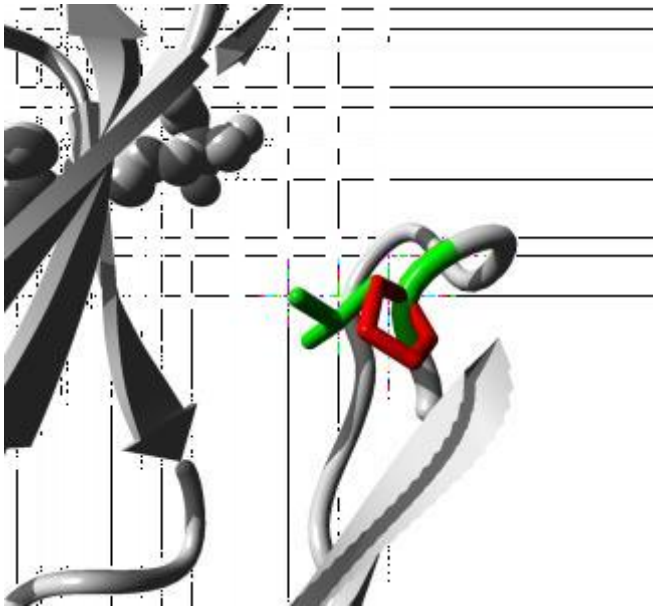


Figure 21a. *Structure of substitutions L90P of PON1 by HOPE*

For C42R substitution of PON1, arginine replaced cysteine at position 42. Cysteine is neutral while the mutant arginine has positive charge and the wild type of residue cysteine is smaller in size than the mutant type i.e., arginine as well as also differ in their hydrophobicity. Cysteine is more hydrophobic than arginine. The figure shows the structure how cysteine is substituting

into arginine. The residue is highly conserved, and it have maximum chances to cause the damage to protein. Cysteine is involved in cysteine bridge which plays part in protein stability. As only cysteine can make these bonds, so the substitution in this region causes the loss of interactions with ligands and due to this destabilization, they also affect protein structure. The grey coloured portion shows the protein structure and green and red portion shows the wild type of residue cysteine and mutant type arginine respectively.

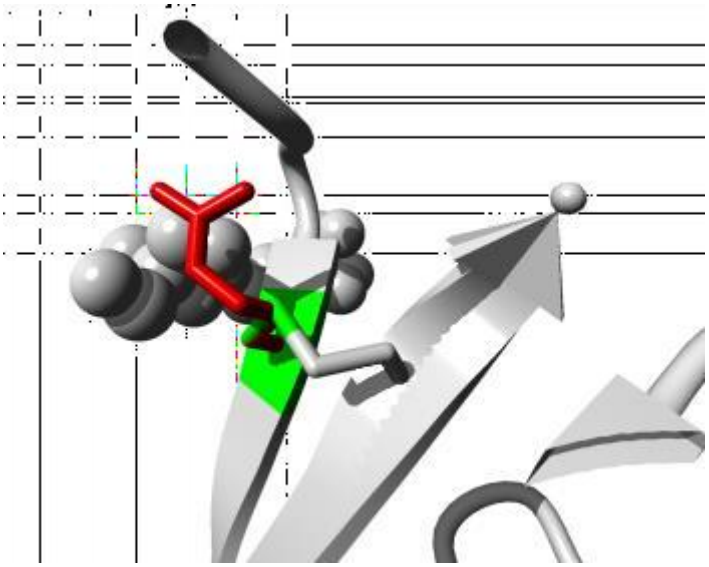


***Figure 21b**. Structure of substitutions C42R of PON1 by HOPE*

For G344C substitution of PON1 protein, where glycine is mutated into cysteine at position 344. Results revealed that the wild type of residue is smaller in size as compared to the mutant. And the mutant comes out to be more hydrophobic than wild type. The mutant is larger than the wild type and buried in the core of protein so mutant obviously cannot fit the area.

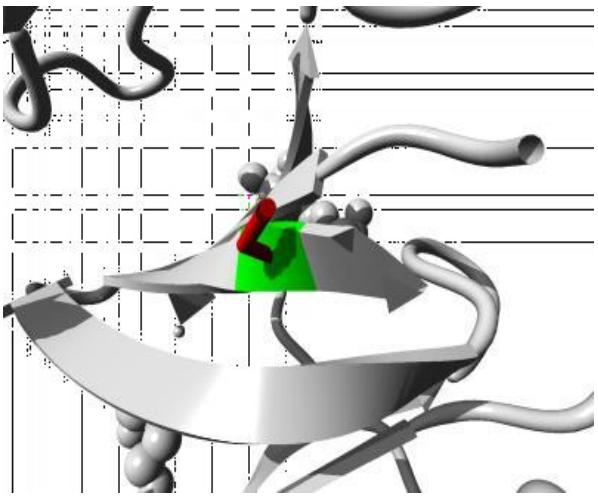


***Figure 21c.** Structure of substitutions G344C of PON1 by HOPE*

For S302L substitution of PON1, where serine changes into leucine at position 302. Results of HOPE revealed that mutant residue is bigger than wild type and more hydrophobic as well. the black part in the structural formula shows the side chain and red part shows backbone that is same for both amino acids. Serine makes hydrogen bond with leucin at 302 position and as the wild type of residue is smaller and the mutant residue leucin is smaller so the mutant cannot fit properly in the space and hence unable to make hydrogen bond. The difference in hydrophobicity also affects formation of hydrogen bond. Both wild and mutant are not similar as serine is more conserved, so mutation causes damage to protein. Due to loss of hydrogen bond in the core of the protein, it resists correct folding.

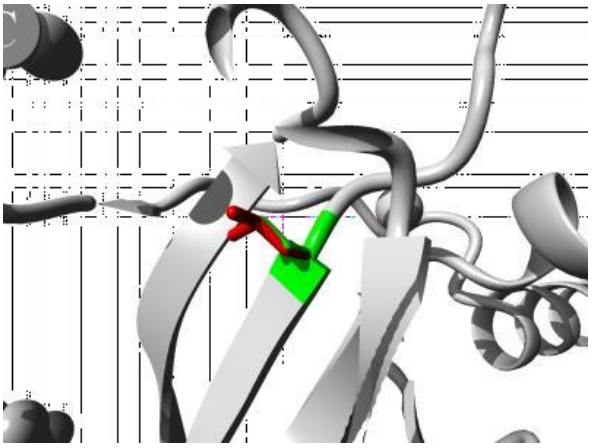


Figure 21d. *Structure of substitutions S302L of PON1 by HOPE*

For W281C substitution of PON1 where tryptophan at position 281 changes to cysteine. Results revealed that wild type and mutant vary in size. Wild type is larger than mutant residue. The red coloured structure represents backbone and black portion is the side chain which differ in both amino acid residues.

As the mutant is smaller than wild type so it will cause empty space in the core of the protein. And the mutant residue lies at the location which is important for the activity of the protein.so the interactions between protein and ligand will be disturbed and hence protein function will be affected.

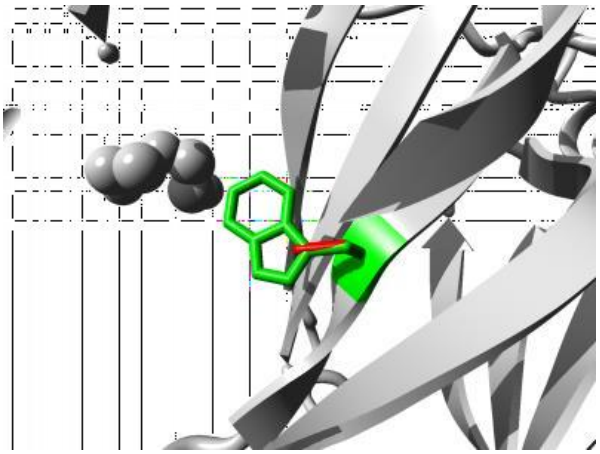


Figure 21e. *Structure of substitutions W281C of PON1 by HOPE*

For the substitution C94F of IL1RN protein, where cysteine is replaced with phenylalanine at position 94. The figure below shows the original versus mutated structure. Red portion represents the backbone and black portion shows the side chain that is different in both amino acids. HOPE results revealed that mutant residue is bigger in size than wild type residue. Wild type residue is buried in the protein, but the mutant is bigger enough that it will not fit in the place properly. Secondly cysteine is involved in making bridge, that is important in protein stability. Due to the mutation no proper interaction will occur and affect the protein structure. Also, the loss of interactions causes protein destabilization. And the activity of the protein gets affected by this.

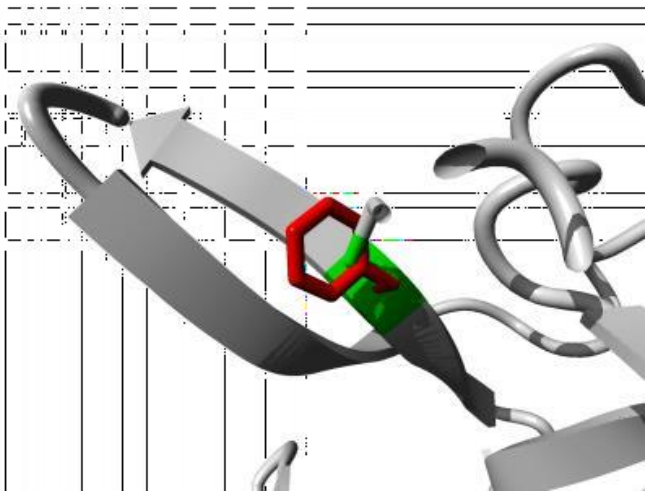


Figure 21f*. Structure of substitutions C94F of IL-1RN by HOPE*

Comparative modelling of PON1 and IL1RN and their mutants

The short-listed high-risk SNP of PON1 and IL1RN were modelled using tools including Phyre2, I-TASSER, trRosetta along with wild type 3D structure. Later PyMOL was used to visualise them and to superimpose the wild and mutant structures. The wild type of structure of protein was generated from Phyre2 and I-TASSER. And the mutant protein structures of both PON1 and IL1RN were generated by using trRosetta. After visualization by PyMOL, further analysis was performed and Tm score and RMSD values were calculated by TM-align. TM-score tells us the topological similarity of wild and mutant type structure while RMSD (root mean square deviation) tells us the average distance of alpha carbon backbone between wild type and mutant type. Greater RMSD value corresponds to greater deviation of mutant type structure from wild type structure of protein. The structure of substitution S302L of PON1 comes out with maximum RMSD value i.e., 1.00 followed by L90P and G344C, which showed RMSD value equals to 0.68 and 0.64 respectively. W281C showed RMSD equals to 0.60 and C42R showed slight variation from wild type structure of PON1 with RMSD value equals to 0.59. The substitution C94F of IL1RN shows TM-score equals to 0.94133 and RMSD value equals to 1.32. he generated structure was validated by ERRAT, PROCHECK and Molprobity.

ERRAT value of C94F substitution of IL1RN comes out to be 94.545. PROCHECK verified that 90.4% residues were present in core- the most favoured region, 8.3% residues were present in additional allowed region, 1.3% residues were present in generally allowed region with 0% residues present in disallowed region. MolProbity gives 98th percentile along with 1.30 MolProbity score predicting the structure good enough.

ERRAT value of C42R substitution of PON1 comes out to be 91.496. PROCHECK verified that 86.7% residues were present in core- the most favoured region, 12.3% residues were present in additional allowed region, 0% residues were present in generally allowed region with 1.0% residues present in disallowed region. MolProbity gives 99th percentile along with 1.13 MolProbity score predicting the structure good enough.

ERRAT value of G344C substitution of PON1 comes out to be 90.588. PROCHECK verified that 87.4% residues were present in core- the most favoured region, 12.3% residues were present in additional allowed region, 0% residues were present in generally allowed region with 0.3% residues present in disallowed region. MolProbity gives 99th percentile along with 1.26 MolProbity score predicting the structure good enough.

ERRAT value of L90P substitution of PON1 comes out to be 88.953. PROCHECK verified that 87.0% residues were present in core- the most favoured region, 12% residues were present in additional allowed region, 0.6% residues were present in generally allowed region with 0.3% residues present in disallowed region. MolProbity gives 99th percentile along with 1.21 MolProbity score predicting the structure good enough.

ERRAT value of S302L substitution of PON1 comes out to be 93.210. PROCHECK verified that 85.8% residues were present in core- the most favoured region, 12.6% residues were present in additional allowed region, 0.3% residues were present in generally allowed region with 1.3% residues present in disallowed region. MolProbity gives 99th percentile along with 1.21 MolProbity score predicting the structure good enough.

ERRAT value of W281C substitution of PON1 comes out to be 92.477. PROCHECK verified that 86.7% residues were present in core- the most favoured region, 11.7% residues were present in additional allowed region, 1% residues were present in generally allowed region with 0.6% residues present in disallowed region. MolProbity gives 98th percentile along with 1.45 MolProbity score predicting the structure good enough. The Ramachandran plots of all these mutated structures are given below.

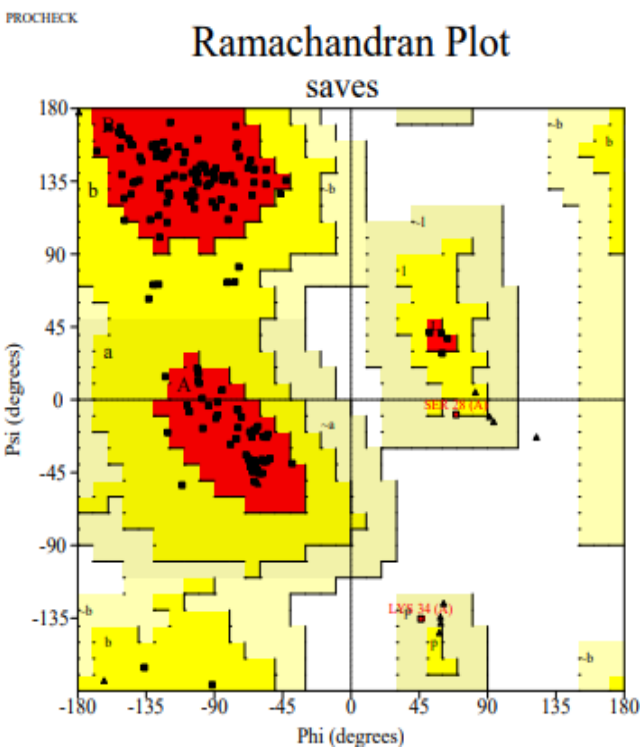


***Figure 22a**. Ramachandran plot of substitutions C94F of IL-1RN*

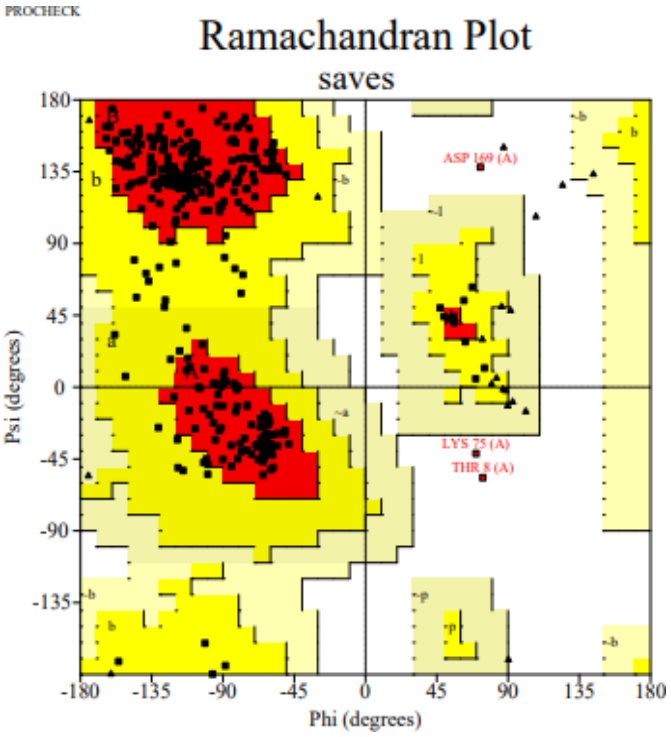


***Figure 22b.** Ramachandran plot of substitutions C42R of PON1*

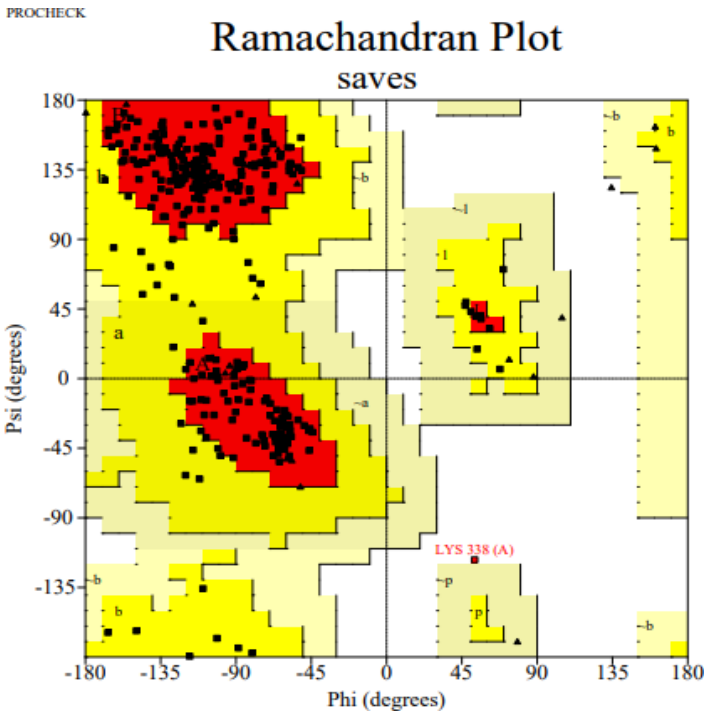


Figure 22c. *Ramachandran plot of substitutions G344C of PON1*

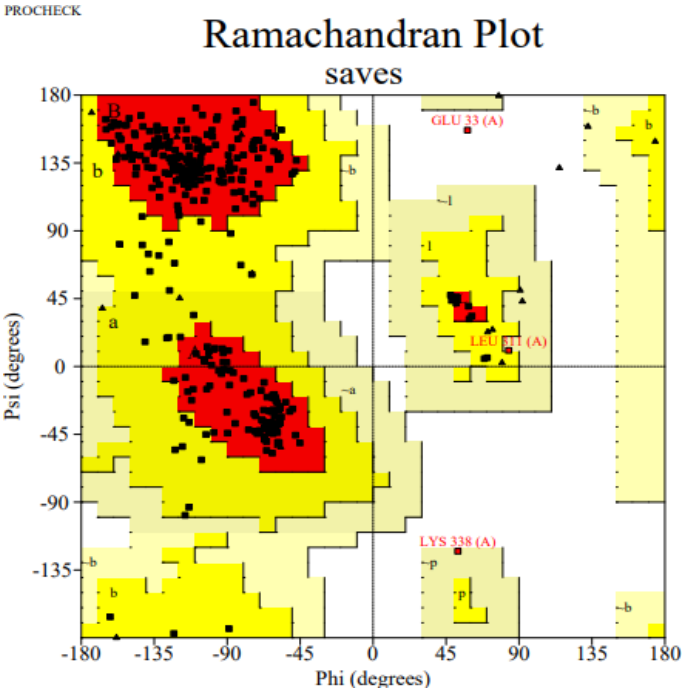


Figure 22d. *Ramachandran plot of substitutions L90P of PON1*

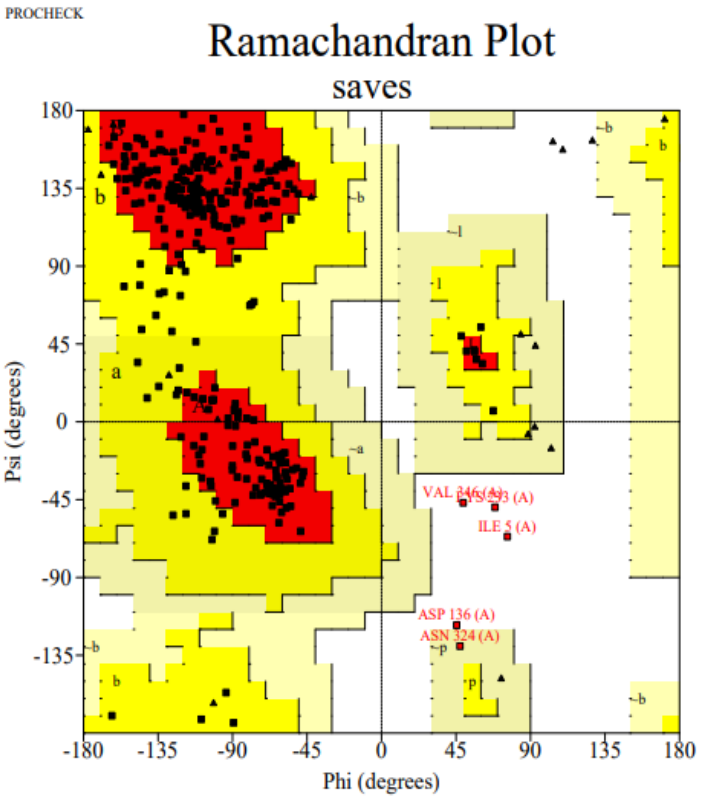


Figure 22e. *Ramachandran plot of substitutions S302L of PON1*

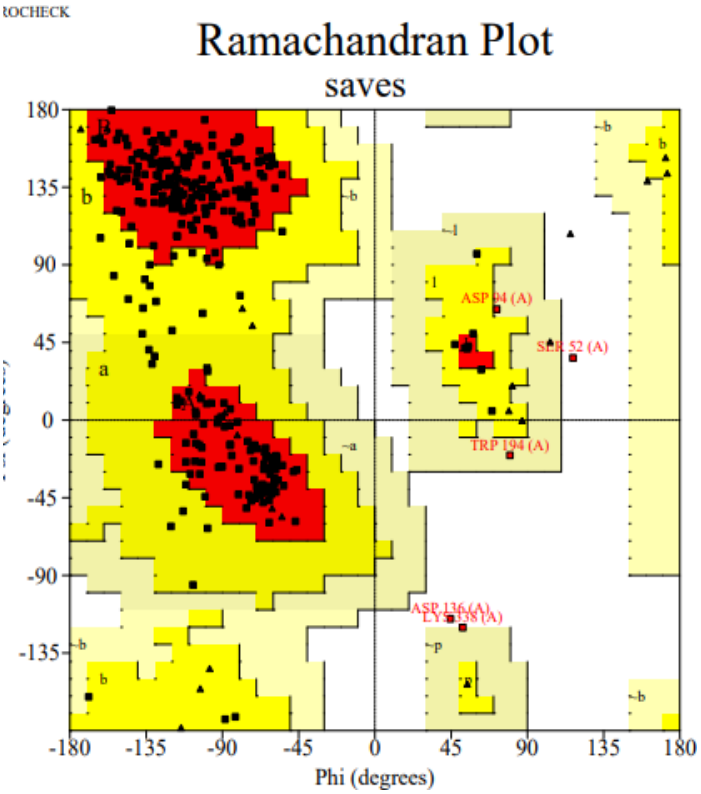


Figure 22f. *Ramachandran plot of substitutions W281C of PON1*

The generated wild type and mutant structure of IL1RN and PON1 were visualized using PyMOL and the mutant and native amino acid residues are highlighted in red and blue respectively.

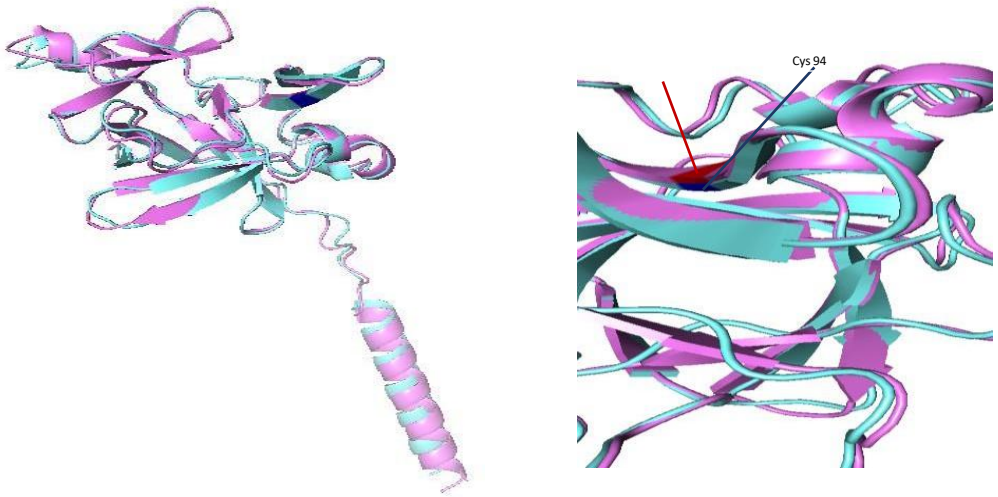


Figure 23a. *Comparative modelling of the substitutions C94F of IL-1RN*

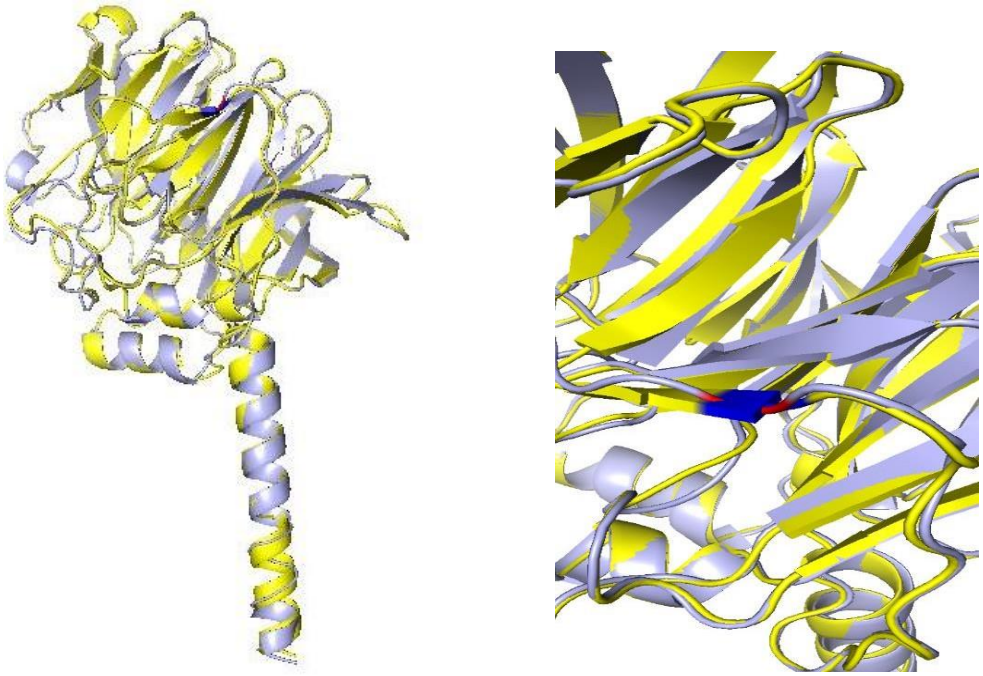


Figure 23b. *Comparative modelling of the substitutions C42R of PON1*

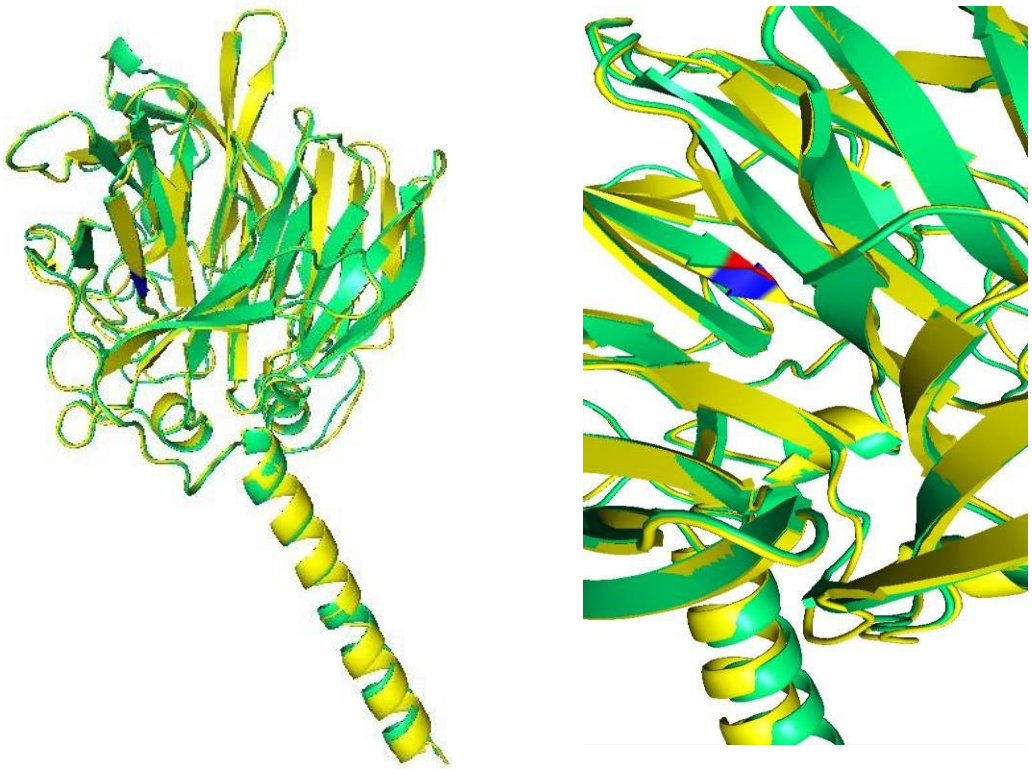


Figure 23c.*Comparative modelling of the substitutions G344C of PON1*

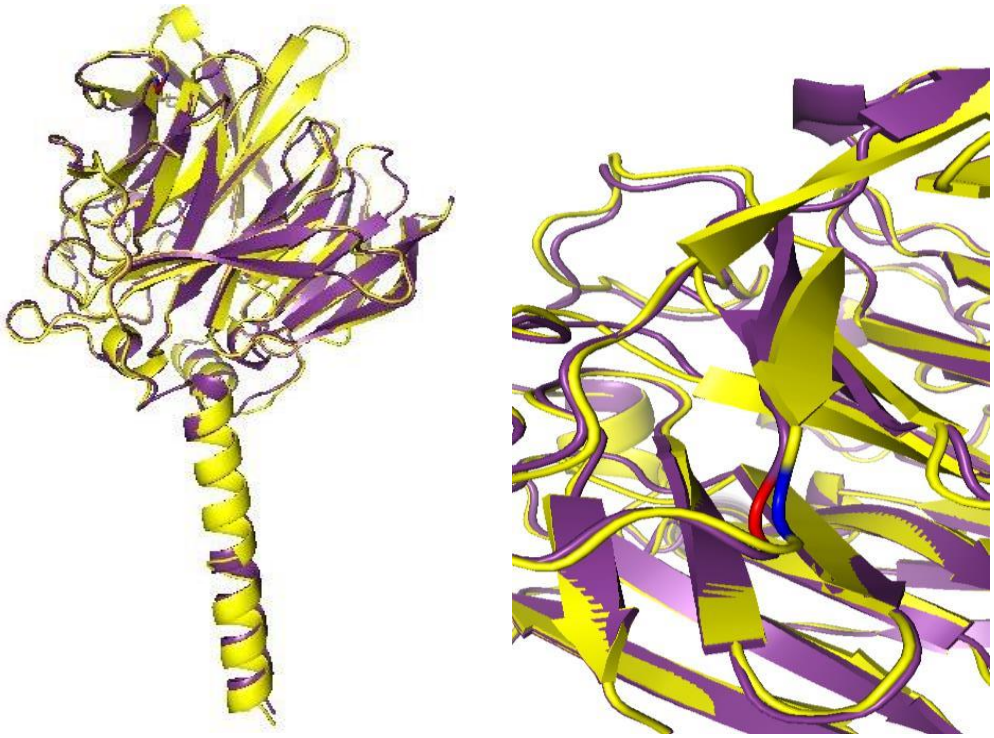


Figure 23d.*Comparative modelling of the substitutions L90P of PON1*

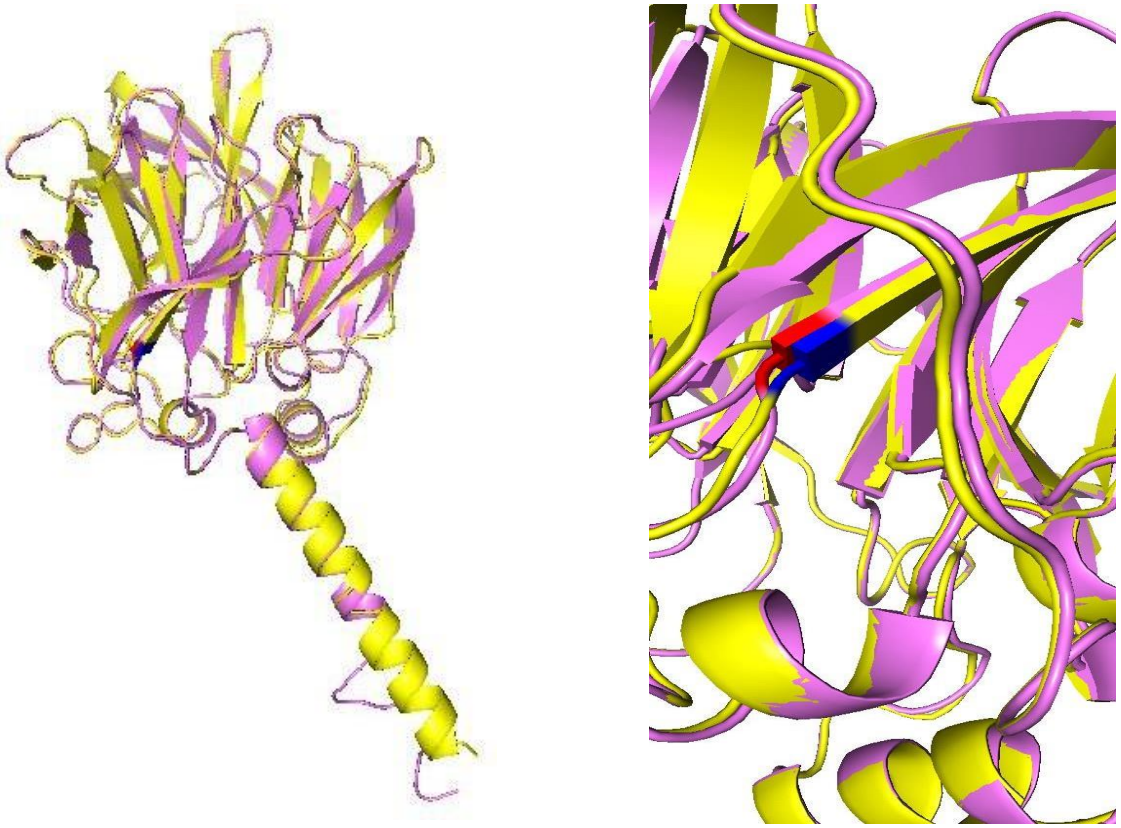


Figure 23e.*Comparative modelling of the substitutions S302L of PON1*

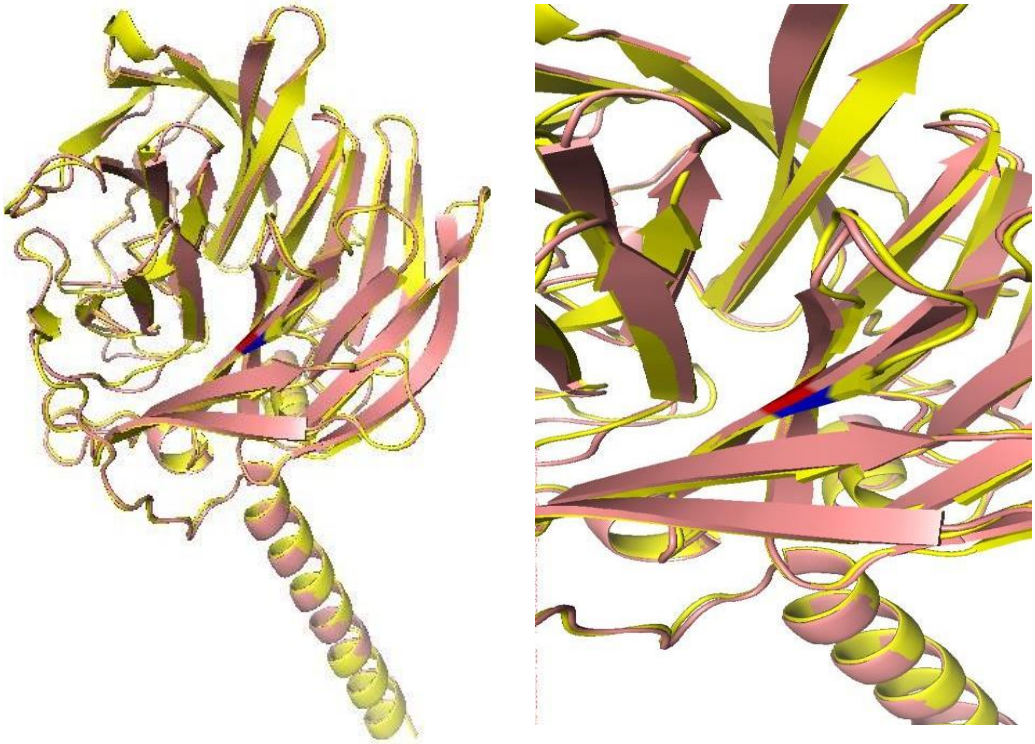


Figure 23f.*Comparative modelling of the substitutions W281C of PON1*

Post translational modifications of IL1RN & PON1

Post Translational Modifications play an essential role in the regulation of structure and function, cell signalling and interactions of proteins. To determine whether high risk SNP of IL1RN i.e., C94F and PON1 i.e., C42R, L90P, S302L, G344C, W281C have any impact on the post translational modification in IL1RN and PON1 respectively.

Phosphorylation sites

Phosphorylation plays an important regulatory role in protein's function like confirmational changes in structure of protein, in activation and deactivation, and cell signalling. For IL1RN and PON1, phosphorylation sites were predicted by NetPhos 3.0 and GPS 3.0. Former predicted for 17 kinases and GPS 3.0 predicted for mainly 3 kinases. According to NetPhos 3.0, there are a total of 17 residues of IL1RN (ser:11, thr:5, tyr:1) that are prone to phosphorylation. According to GPS 3.0, 15 residues have the potential of being phosphorylated. These residues include 8 serine, 5 threonine and 2 tyrosine. The common sites that were predicted by both web servers include 6 serine residues, 3 threonine residues and no tyrosine residue. The serine residue at position 97 is a potential phosphorylation site and is present in a close vicinity to our mutation C94F. Due to its close proximity, phosphorylation at position 97 will likely alter the tertiary protein structure and will disturb the function of protein as well. As cysteine is involved in disulfide linkages, the mutation will cause loss of interactions as the protein will be unable to make disulfide bridges.

According to NetPhos 3.0, there are a total of 27 residues of PON1(ser:12, thr:9, tyr:6) that are prone to phosphorylation. According to GPS 3.0, 11 residues have the potential of being phosphorylated. These residues include 2 serine, 2 threonine and 7 tyrosine. The common sites that were predicted by both web servers include 1 serine residue at position 139, 2 threonine residues at position 103 & 255 and 1 tyrosine residue at position 321.

Methylation sites

The putative methylation sites in IL1RN and PON1 were predicted by GPS-MSP-group based prediction system- methyl specific predictor. The outcomes of GPS-MPS revealed there is no methylation sites exists in IL1RN and PON1.

Glycosylation sites

For the prediction of putative site of glycosylation, NetNGlyc, NetOGlyc, NetCGlyc were used specifically for N-glycosylation, O-glycosylation, and C-glycosylation respectively. If the score is greater than 0.5 it will correspond to site having potential to be glycosylated. These web server mention ‘’POSITIVE’’ along with the score that means site with potential of being glycosylated. NetNGlyc predicted only 1 site in IL1RN with a score 0.6708 that have potential of getting N-glycosylated at position 109 and 3 sites in PON1 with a score 0.6607, 0.6737 and 0.5470 at position 253, 270, 324 respectively. And no C-glycosylated and O-glycosylated sites were predicted in both IL1RN and PON1.

Wet Lab Results

Research was conducted to ascertain the association analysis of genetic polymorphisms in IL1RN and PON1 gene and for this samples were collected from patients of type 2 diabetes mellitus and healthy controls from Ali Hospital, Islamabad. Total 180 samples were collected out which 130 were the type 2 diabetic patients and 5 were healthy controls.

Genomic DNA extraction

Blood samples were collected, and DNA was extracted from these samples by using phenol, chloroform method. For quantification of DNA, nanodrop was used. 260/280 ratio was penned down to ascertain purification of genomic DNA.

Genotyping of IL1RN rs380092 and PON1 rs854560

High resolution melting polymerase chain reaction has been performed on rs380092 of IL1RN and rs854560 of PON1. Screening has been done on all the samples to figure out the present of absence of single nucleotide polymorphism. After PCR, PCR product is visualized through gel electrophoresis.

High resolution melting polymerase chain reaction mainly involves amplification as per normal PCR, then perform melt curve analysis. In this step temperature increases gently and the DNA melts down, causing change in fluorescence due to different DNA sequence. This change in fluorescence decreases as the temperature increases because the strands of DNA get separated.

Then genotyping differentiation occurs in which melt curves were plotted between fluorescence and temperature. Different curves show different shapes depending on the DNA sequence. If there is a peak at a particular temperature, it depicts homozygous wild type. If

single peak appears but a temperature different from the wild one, it depicts homozygous mutant. And if there appear two peaks, it depicts heterozygous mutant (one peak for mutant allele and one for wild allele). If no peak appears, it means no amplification occurred.

HRM PCR on 20 T2DM samples and 10 healthy controls were performed along with 1 negative and 1 positive control. The results of these polymorphism were analysed by uAnalyze v2.0 and the melt curves were plotted. This software works when data input file is uploaded in excel format. Then it performs pre-processing link normalizing, remove noise and background subtractions. Then it performs curve analysis, by analysing melting curve data obtained by HRM PCR and melting temperatures and the shapes of the curve. It has inbuilt curve fitting algorithm through which it analyses the height and position of the peaks formed. After this it performs genotype calling step in which it determines genotype based on the melting curves. It compares the curves of the samples with that of controls whose genotype is known. Quality control is one of the important steps of this software. Results are visualized via curves and peaks and can be exported to the excel file.

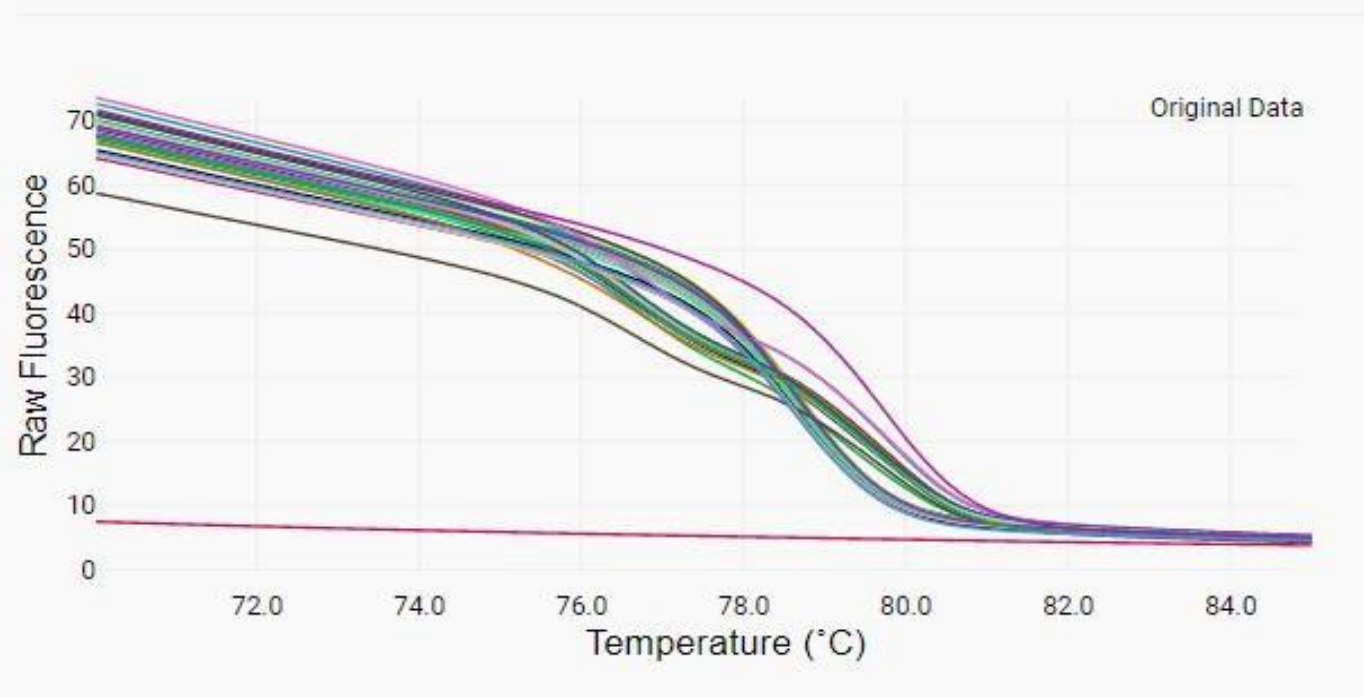


Figure 24 standard curve between raw data of fluorescence and temperature of rs380092 of IL1RN

For the detection of polymorphism of rs380092 of IL1RN which is present in the intron 2, this is the graph plotted between the raw data the fluorescence and the temperature after performing HRM PCR. Normalization of this data is performed.

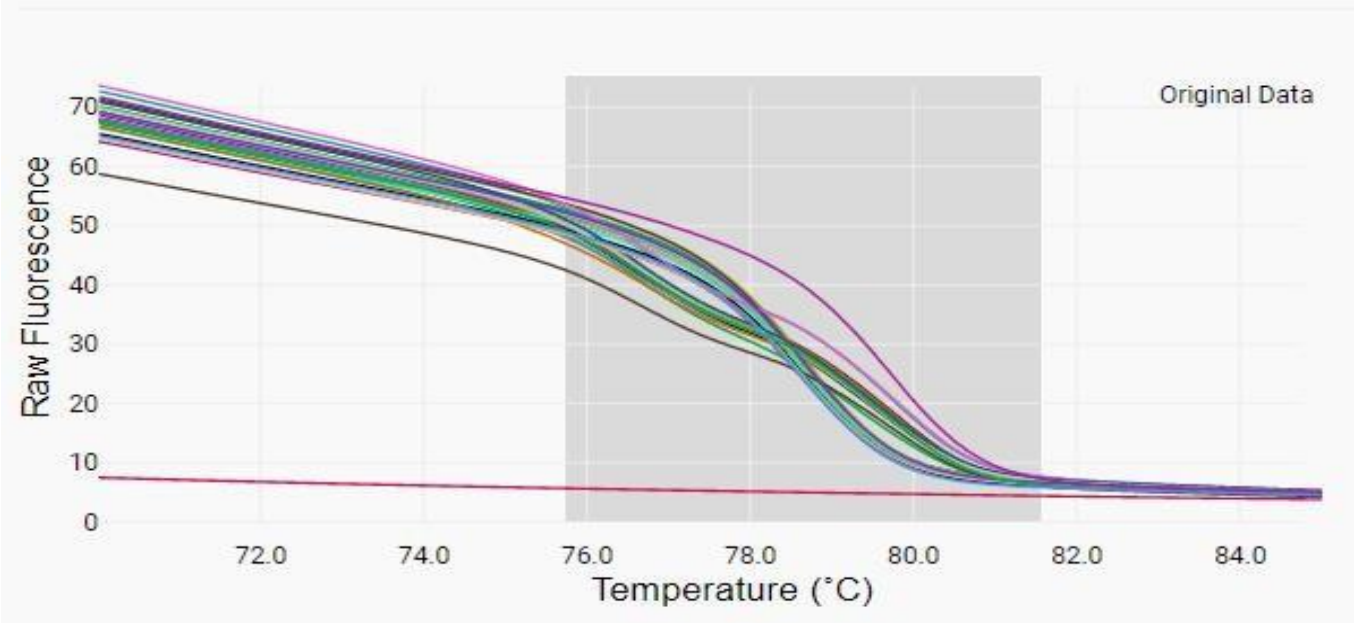


Figure 25a. Normalization of the raw data of fluorescence against temperature

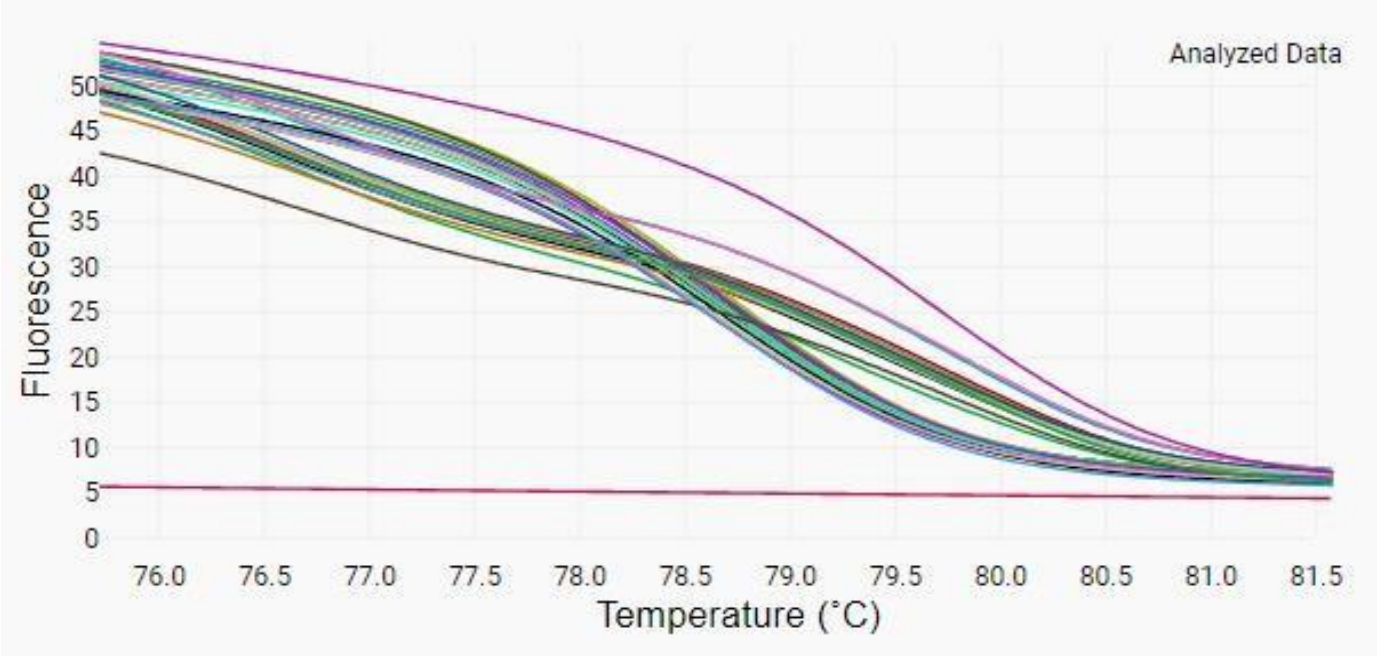


Figure 25b. Normalized melt curves of the samples and controls.

Normalized graphs showing cleaned data after removing noise and background subtraction. This data is further used for melt curve analysis.

The derivative melt curve which is a sensitive and more specific way to detect alterations in fluorescence, has been plotted. It measures that how fast fluorescence changes. It will give peak

for the allele at a specific melting temperature easily depicting the SNPs, where the peak of heterozygous is lower than the peak of homozygous. The reason behind this is because heterozygotes cause the DNA unstable. The peak of heterozygote in a derivative melt curve appears between twos different temperatures of the genotypes. Here samples that are showing single peak are homozygous and with two peaks at twin different temperatures are showing heterozygotes.

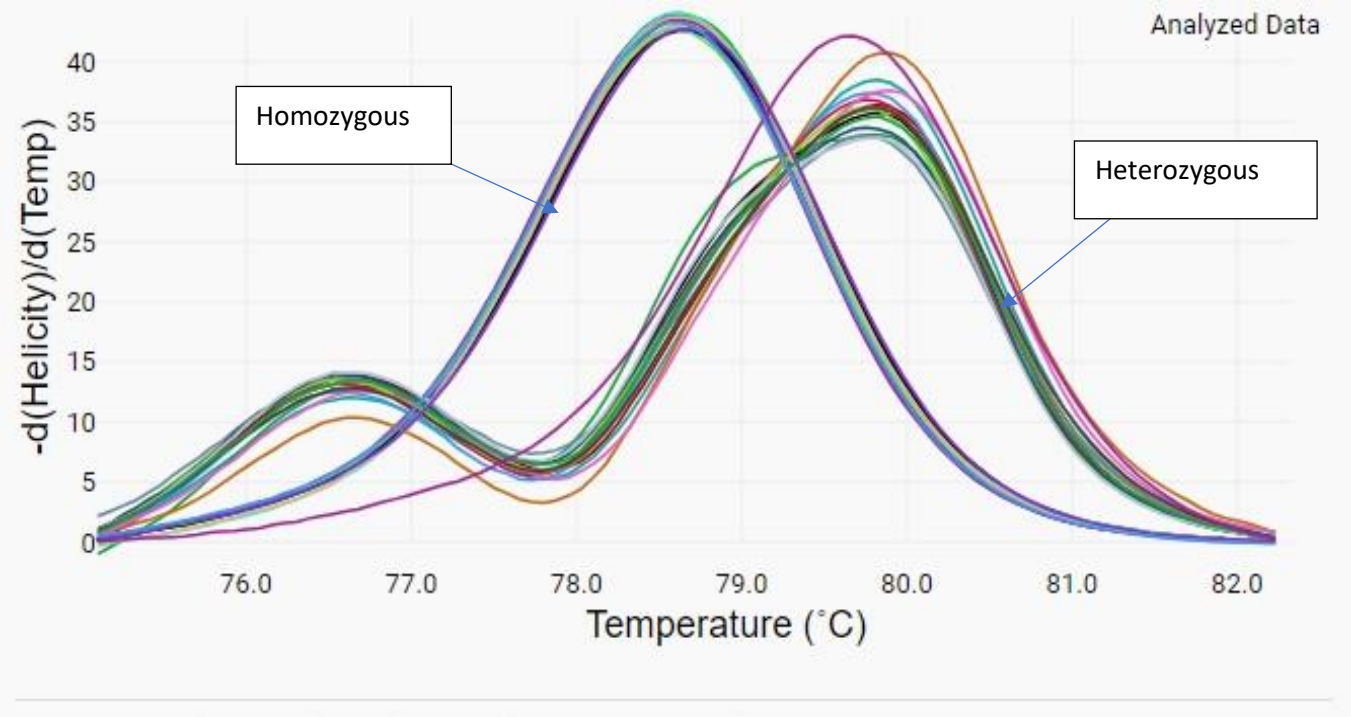


Figure 26 Derivative melt curve of the samples and controls for the detection of rs380092 of IL1RN gene

Difference graph has also been plotted that showed the difference between the melting temperatures of two genotypes for specific polymorphism or SNP. It compares the melting temperatures of different samples. If the temperature difference is equal or greater than 5˚C, it depicts two samples have different genotypes.

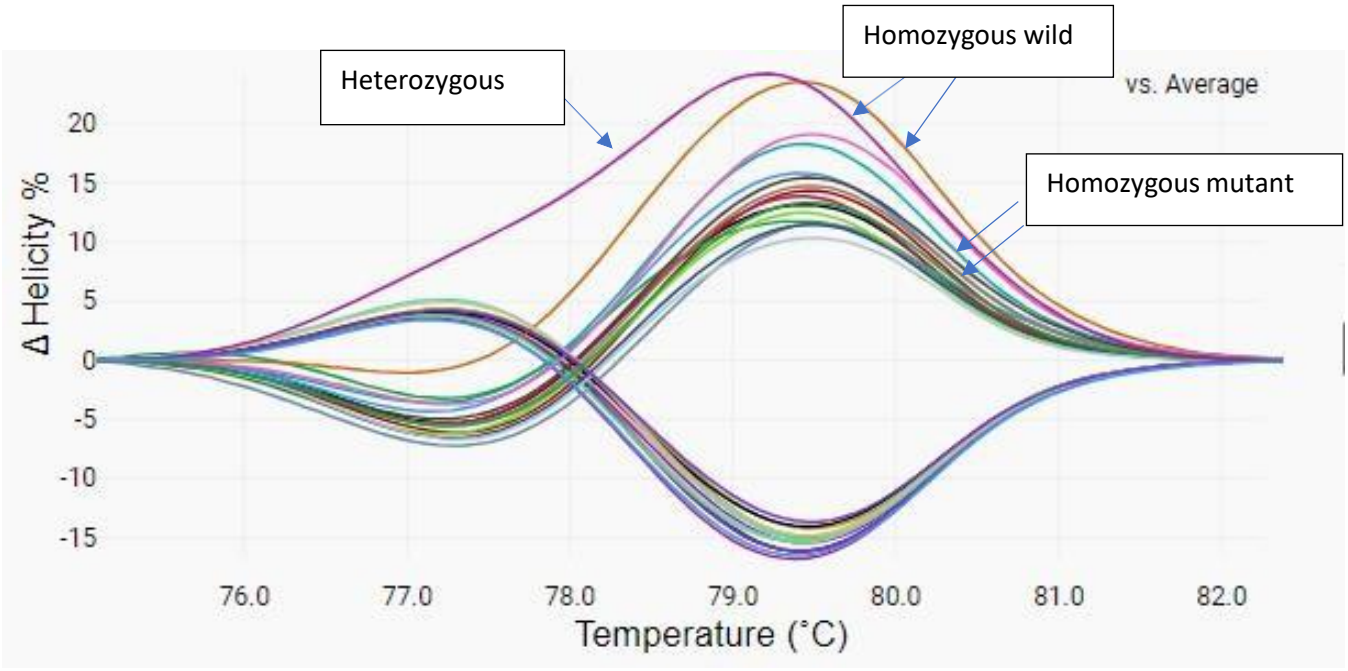


Figure 27 Difference graph of the samples and controls for detection of rs380092 of IL1RN gene

The graphs of IL1RN showed that controls contain 7 homozygotes TT and 3 heterozygotes TA, and 8 samples showed genotype AA, TT (homozygotes) and 12 samples showed genotype TA (heterozygotes).

High resolution melting polymerase chain reaction has been performed on rs854560 of PON1. Screening has been done on all the samples to figure out the present of absence of single nucleotide polymorphism. After PCR, PCR product is visualized through gel electrophoresis.

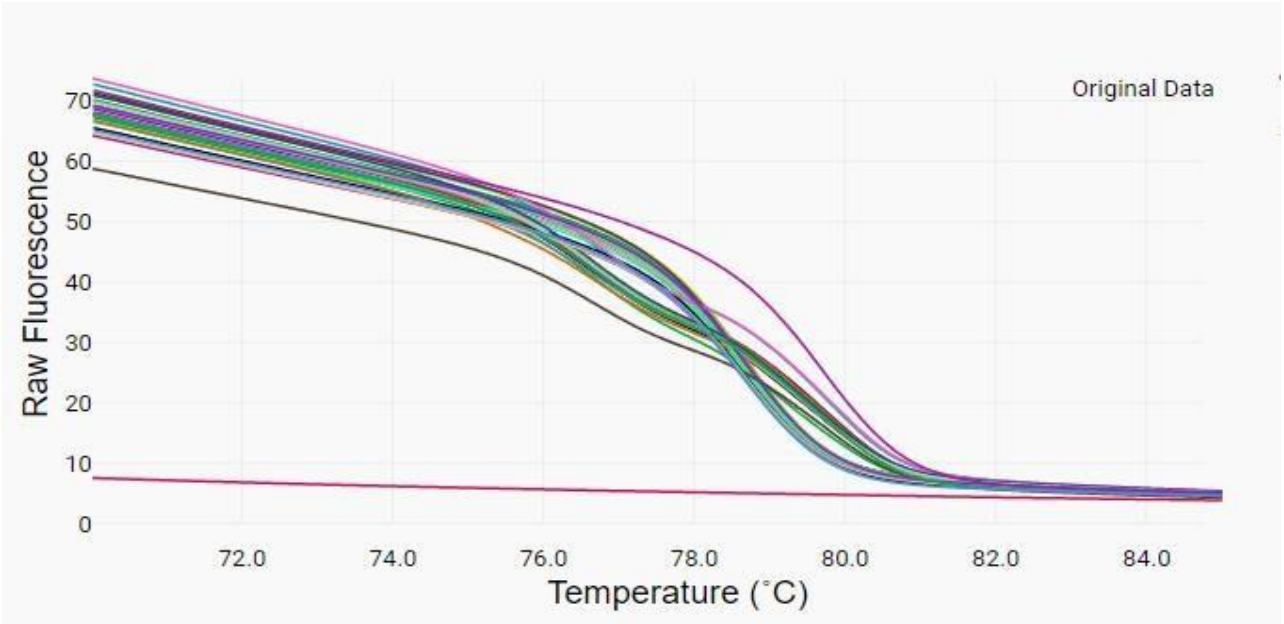


Figure 28 standard curve between raw data of fluorescence and temperature of rs854560 of PON1

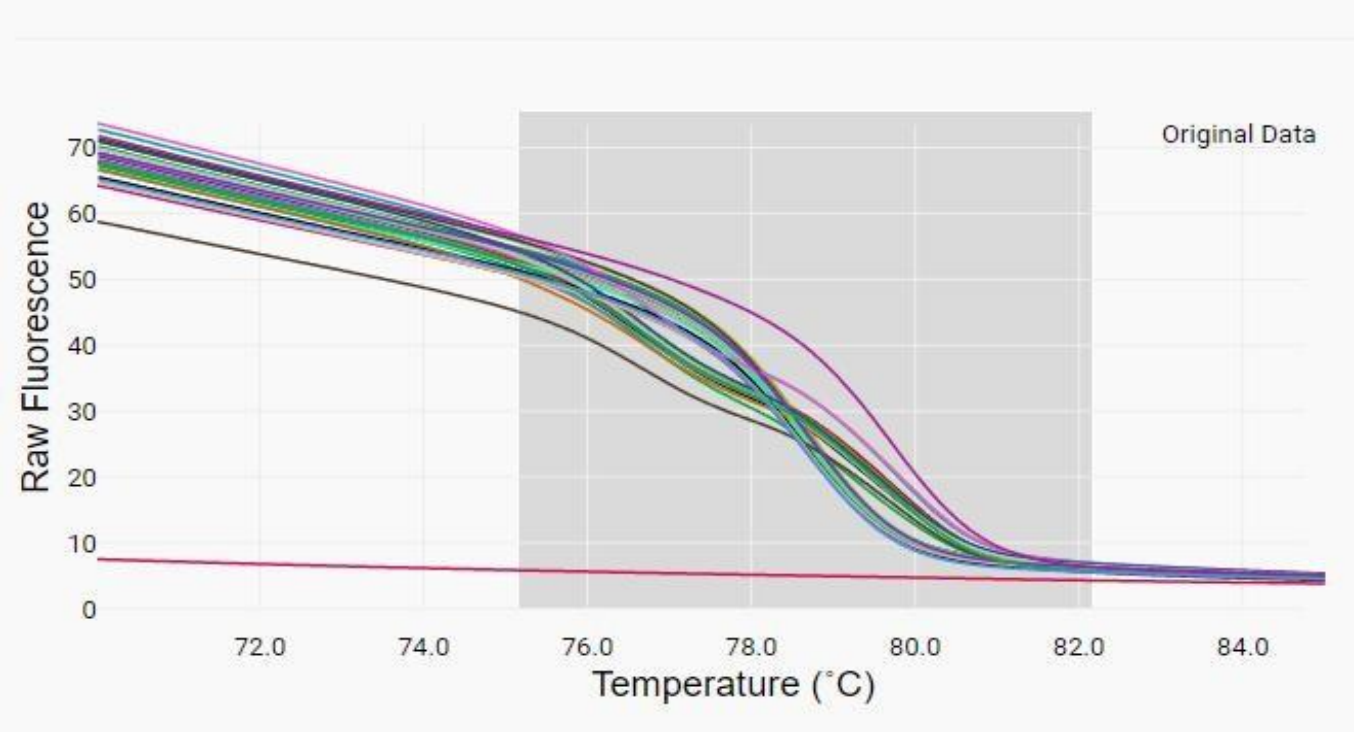


Figure 29a. Normalization of the raw data of fluorescence against temperature of rs854560 of PON1

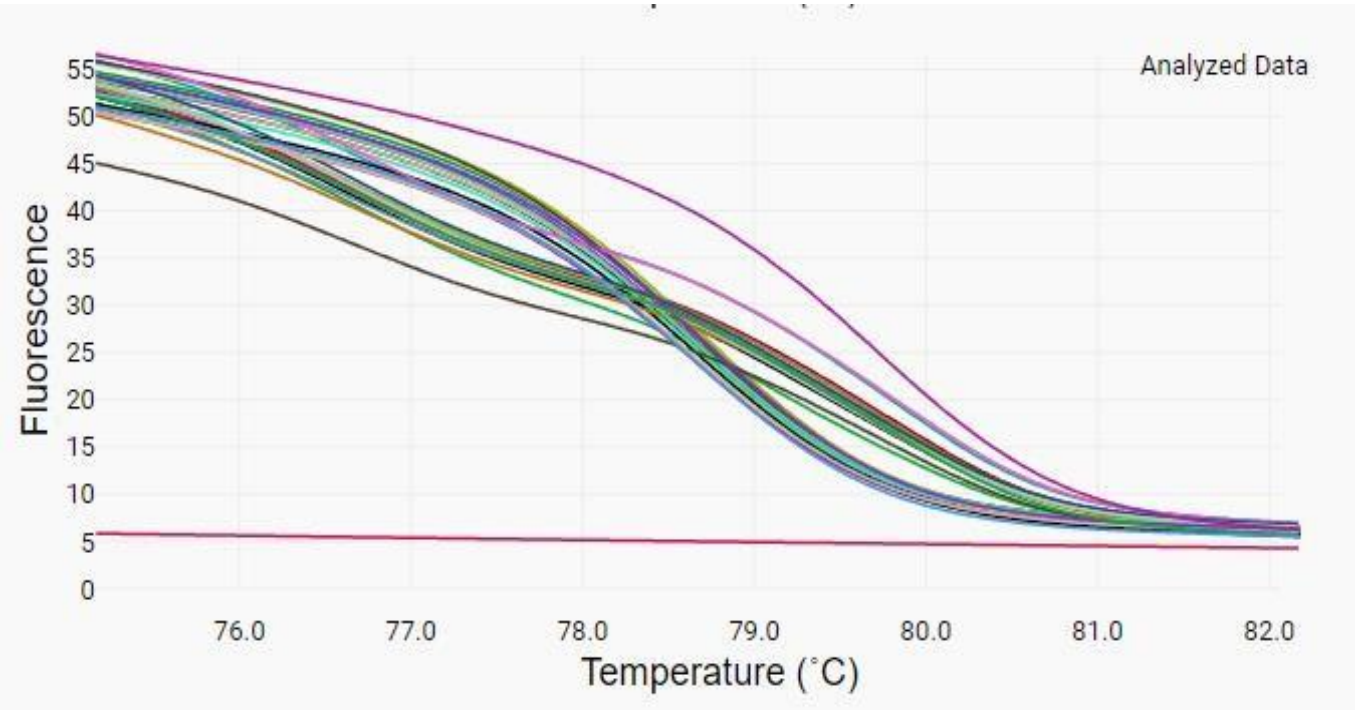


Figure 29b. Normalized of the raw data of fluorescence against temperature of PON1

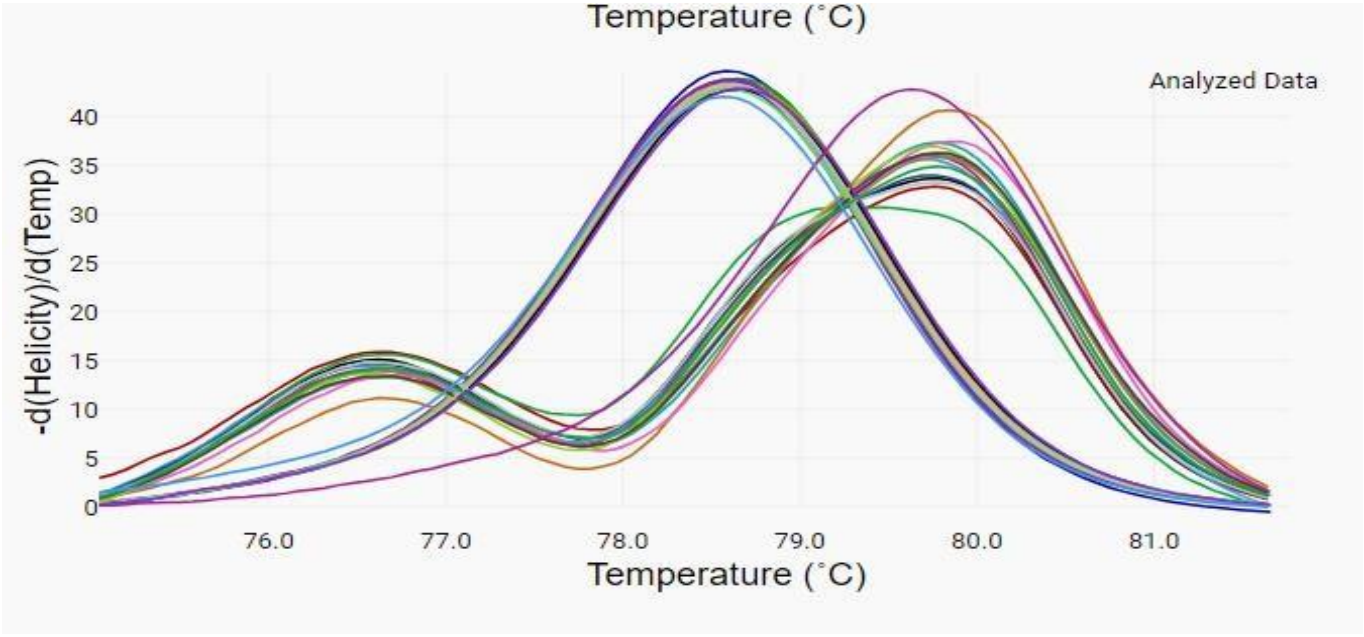


Figure 30 Derivative melt curve of the samples and controls for the detection of rs854560 of PON1 gene

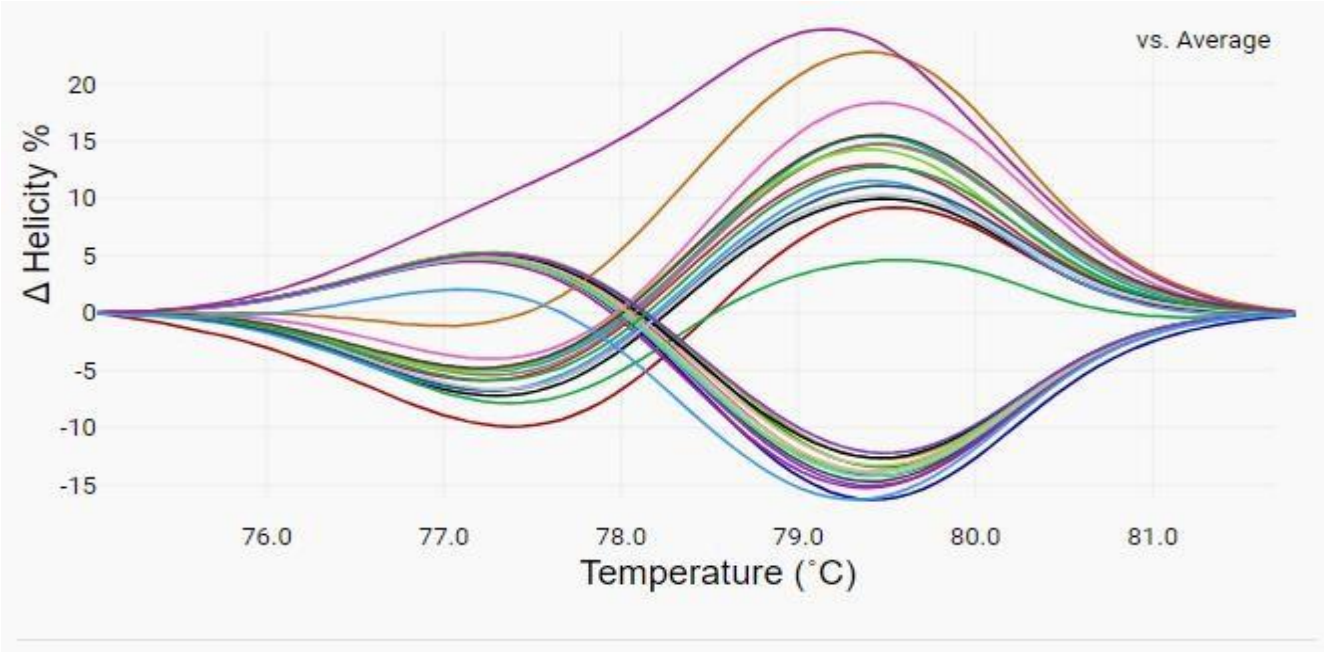


Figure 31 Differential melt curve of the samples and controls for the detection of rs854560 of PON1 gene

The graphs of rs854560 f PON1 showed that it contains 10 controls out of which 7 are homozygotes AA and 3 heterozygotes. And out of 20 samples, 8 showed genotype AA homozygotes, and 12 showed heterozygous genotype.

DISCUSSION

Type 2 diabetes mellitus is a chronic multifactorial disorder attributed to hyperglycaemia, insulin resistance, defective insulin secretion, oxidative stress, adiposity, increased glucagon secretion along with dyslipidaemia (Block & Baily, 2023) . There exists a lot of risk factors including genetic and environmental factors such as obesity, sedentary lifestyle, hypertension, dietary pattern etc. (Harrigan, 2007), (Wild & Byrne, 2006). Interleukin 1 receptor antagonist gene which is located at chromosome 2 comprises of 13 exons. The IL1RN gene encodes for the protein interleukin 1 receptor antagonists that comprises 177 amino acid residues and having weight of 20.055 kDa. The cells of the immune system produce this protein. At the time of need, IL1RN binds to receptors and prevents IL-1 activation and hence prevents uncontrolled inflammation and tissue damage (Nowak *et al*., 2013, Jesus *et al*., 2011). Insulin resistance may be caused by low-grade inflammation, which is a contributing factor of type 2 diabetes and related complications (Kim *et al*., 2012) . When the body shows less response to insulin, blood glucose level rises and causes damage to body. More specifically, low levels of IL1RN correspond to increased risk of developing the disease i.e., Type 2 Diabetes Mellitus as IL1RN has anti-inflammatory properties (Marculescu *et al*., 2002) and help improving insulin sensitivity (Jiao *et al*., 2021). Serum paraoxonase/ arylesterase 1 is a protein (enzyme) that is encoded by PON1 gene. In consonance with genome assembly GRCh38.p14, Serum Paraoxonase 1 gene (IL1RN) is located at chromosome 7 corresponds to position 7q21.3 with genome coordinates 7: 95,297,676- 95,324,532 comprises of 9 exons. The *PON1* gene encodes for the protein serum paraoxonase 1 that comprises of 355 amino acids (accession # P27169) and possesses a molecular mass of 39,731 Da (from UniProt). PON1 is involved in the metabolism of lipids and show antioxidant properties. Low levels of PON1 contributes to type 2 Diabetes Mellitus. A comprehensive and in-depth analysis of alleged missense SNP of IL1RN and PON were performed. Initially 189 SNPs were shortlisted as missense SNPs of IL1RN and 306 missense SNPs of PON1, out of which only 1 nsSNP of IL1RN and 5 SNPs of PON1 come out to be deleterious or pathogenic by all 6 in-silico tools used. Single nucleotide polymorphism is the simplest type of polymorphism, occurs every 200-300 base pairs and comprises 90% of polymorphisms. It may be coding (missense) or non-coding. Coding or missense results in amino acid change in protein hence affecting structure and function (Chen *et al*., 2010). After shortlisting high-risk nsSNP of IL1RN and PON1, further analysis was conducted. I-MUTANT and MUpro predicted that the high risk nsSNP C94F of IL1RN decreases the stability of protein. Besides this, it has a certain impact on the

molecular nature of protein encoded by IL1RN. The most common impact predicted by MutPred2 includes altered metal binding. I-MUTANT and MUpro predicted that the high risk nsSNP L90P, C42R, S302L, G344C, W281C of PON1 decreases the stability of protein and have a certain impact on the molecular nature of protein encoded by IL1RN. ConSurf showed the conservation profiling of nsSNP of IL1RN and PON1, according to which C94F substitution (rs201638660) of IL1RN is highly conserved and buried in the structure and nsSNPs (rs72552788) L90P is buried, (rs138512790) C42R is exposed and important for function, (rs185623242) S302L and (rs368206333) G344C are buried and important structurally , and (rs369422555) W281C is buried. Spatiotemporal dynamics of the protein interactions with others are affected by charge, a protein have. Using HOPE, it is investigated that Cysteine replaced Phenylalanine at 94th position, a bigger residue in size so can't fit in the space properly and bridge formation, due to mutation in cysteine, is affected so, protein stability and interactions were lost, and its activity gets affected (Islam *et al*.,2019). In case substitutions of PON1, bigger leucin is replaced by smaller proline at 90th position, creating an empty space in the core of the protein. For C42R, cysteine is neutral which is replaced by arginine that has positive charge and larger in size, and it will cause damage to protein and stability as cysteine is involved in interaction by bridge formation. For G344C, glycine replaces cysteine which is larger in size and will not fill in space properly and affect the function of protein. For S302L, serine is replaced by leucin, which is larger in size and hydrophobic in nature. It will be unable to fit in the space and hydrogen bonds will be lost. Hence structure and function of protein gets affected. For W281C, tryptophan is replaced by cysteine which is smaller in size hence creates empty space in the core of the protein. Also affects the activity of the protein.

The structure of IL1RN and PON1 were obtained from Phyre2 and I-TASSER and modelled also by using trRosetta. The structure of sequence containing high-risk SNPs were also modelled by trRosetta and then these structures were validated by ERRAT, PROCHECK and MolProbity. Using PyMOL, structure of wild type of IL1RN and mutant type structures were superimposed and RMSD value calculated i.e., 1.32 and tm score 0.94133. Structure of wild type of PON1 and mutant type structures were superimposed and RMSD value and tm score were calculated . The structure of substitution S302L of PON1 comes out with maximum RMSD value i.e., 1.00 followed by L90P and G344C, which showed RMSD value equals to 0.68 and 0.64 respectively. W281C showed RMSD equals to 0.60 and C42R showed slight variation from wild type structure of PON1 with RMSD value equals to 0.59. Then post translational modifications were performed for both IL1RN and PON1. For IL1RN, NetPhos

3.0 predicted 17 sites (2 serine, 2 threonine, 7 tyrosine) and GPS 3.0 predicted 15 sites (6 serine residues and 3 threonine residues and no tyrosine residue) having potential of get phosphorylated. GPS-MSP predicted no methylation site exists and NetNGlyc, NetOGlyc, NetCGlyc (specifically for N-glycosylation, O-glycosylation, and C- glycosylation respectively) were used for glycosylation. NetNGlyc predicted only 1 site having potential of getting N-glycosylated at position 109. And no C-glycosylated and O- glycosylated sites were predicted. For, PON1, NetPhos 3.0 predicted 27 sites (12 serine, 9 threonine, tyrosine) and GPS 3.0 predicted 11 sites (2 serine residues and 2 threonine residues and 7 tyrosine residue) having potential of get phosphorylated. GPS-MSP predicted no methylation site exists and NetNGlyc, NetOGlyc, NetCGlyc (specifically for N-glycosylation, O-glycosylation, and C-glycosylation respectively) were used for glycosylation. NetNGlyc predicted 3 sites at position 253, 270, 324 with a score 0.6607, 0.6737, 0.5470 respectively, having potential of getting N-glycosylated at position 109. And no C-glycosylated and O- glycosylated sites were predicted for both PON1and IL1RN. High melting Resolution analysis was performed for rs854560 of PON1 and rs380092 of IL1RN and it showed that fluorescence decreases with gradual increase in temperature and derivative melt curves were obtained that showed single and double peak graphs depicting homozygotes and heterozygotes in samples. Graph for IL1RN showed that controls have 14 homozygotes and 6 heterozygotes while samples have 24 homozygotes and 16 heterozygotes. Fisher's test showed a p-value less than 0.05 depicting significant association of rs380092 of IL1RN with T2DM. Graph for rs854560 of PON1 showed that controls have 16 homozygotes and 4 heterozygotes while samples have 26 homozygotes and 14 heterozygotes. Fisher's test showed p-value 0.0539, depicting significant association of rs854560 of s PON1 with T2DM.

Conclusion

It is concluded that computational analysis of IL1RN showed that Cys94phen is the deleterious nsSNPs which alter the structure and function of protein due to loss of cysteine disulfide linkage and make protein unstable. Computational analysis of PON1 showed that Leu90Pro, Ser302Leu, Tryp281Cys, Gly344Cys and Cys42Arg are the most deleterious SNPs which cause damage to protein structure and hence disable the protein to perform proper function and decreases protein stability. Invitro studies of rs380093 of IL1RN and rs854560 of PON1 by HRM analysis which is an advanced technique that doesn't require any separate machine for analysis and highly specific, confirmed the association of these polymorphisms with Type 2 diabetes mellitus and their microvascular complications. This will help us in early screening of the patients with these SNPs and will help us to design personalized medicine.

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