Relationship of the glycation gap to diabetes and its complications, and the potential role of adipokines

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A thesis submitted in partial fulfilment of the requirements of the University of Wolverhampton for the degree of Doctor of Philosophy

DECLARATION

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ABSTRACT

Background: Diabetes mellitus has become a global health menace and the management cost to both developed and developing countries is biting hard on the economy. Diabetes mellitus is primarily caused by hyperglycaemia and research has confirmed the strong link of obesity as a precursor of type 2 diabetes. Hyperglycaemia is a major and an independent risk factor of cardiovascular disease (CVD) and atherosclerosis in diabetes. Obesity is also associated with cardiovascular disease which is one of the diabetic complications. Stress which is one the predisposing factor of obesity generates a vicious cycle leading to the release of high level of inflammatory adipokines and this is the link between obesity and CVD. Adipokines are believed to have a role in diabetic complications. This research intends to understand the role some specified adipokines plays in insulin secretions and beta cell failure.

Glycation is a common and spontaneous reaction of proteins or lipids becoming glycated after exposure to sugars, occurring *in vivo* without the controlling action of an enzyme. Deglycation is an enzyme-mediated pathway and fructoseamine-3-kinase (FN3K) is believed to be one of the major enzymes. FN3K is known to play a protective role in the development of vascular complications in diabetes patients. In the absence of deglycation or deglycating enzymes, advanced glycation endproducts (AGEs) are formed. This research work employed 1-deoxy-1-morpholino fructose (DMF) a major enzyme which can prevent deglycation to show the importance of deglycation in beta cell and FN3K role in insulin secretion.

Method: This research work analysed glycoprotein acetylation (GlycA) a known inflammatory marker that tracks systemic inflammation and cardiovascular risk. The investigation of the potential role of inflammation in the GGap using a novel (and putatively better than existing measures such as CRP) marker of inflammation, GlycA was carried out. A total of 54 diabetic

patients were used for this research work and divided into 2 groups. GGap negative (G0) = 34 and GGap positive (G1) = 20. 1H- Nuclear Magnetic Resonance (NMR) was used to analyse the samples and measuring the different peaks. Glycoscale was used for glycoproteins while liposcale was used for lipoproteins.

Laboratory analyses were carried out to ascertain the pathophysiological role of adipokines in inducing insulin secretion. The laboratory analysis includes assessment of insulin secretion from MIN6 and BRIN-BD11 cells, effects of WISP1 on beta cells viability**,** effects of some adipokines (WISP1, eNAMPT/Visfatin, sFRP4) on insulin secretions/release from pancreatic beta cell. To this end, MIN6 cells were cultured in low and high glucose media, treated with different concentrations of adipokines, and tested for insulin secretion, beta cell failure and cell viability. Using insulin ELISA assay, the concentrations of insulin release/secretions was measured while cell viability was determined by using prestoleblue.

Results: Visfatin/eNAMPT exhibited a dose dependant insulin response at high concentrations. WISP1 acute effects (incubating cells for 48hours) shows a dose-dependent outcome on insulin secretions and a reduced effects at high concentrations. Chronic effects of WISP1 (incubation of cells for over 72 hours) shows increase acute GSIS over 72hr period independent of glucose or WISP1 concentrations (*P*-value = 0.0025).

With low glucose, MIN6 cell viability decreases over 72 hours while at high glucose, cells didn't appear to have proliferated much over 72 hours. sFRP4 had an increased effect at higher glucose levels.

The introduction of FN3K inhibitor in the presence of high glucose led to a drastic fall in insulin release with *P value* = 0.005.

GlycA and GlycB but not GlycF concentrations were elevated in the Positive GGap group ($p<0.001$). BMI was higher in positive GGap indicating its link to diabetes and its complications. VLDL was higher in cholesterol and triglyceride in positive GGap patients while HDL was lower in cholesterol and triglyceride in positive GGap patients ($p<0.001$).

Conclusion: This research has been able to show that the selected adipokines are able to induce insulin secretion. GGap positive patients are more susceptible to diabetes complications. GlycA and GlycB but not GlycF shows to be potent biomarker of inflammation. Lipoproteins particles of GGap positive patients are more exposed to diabetes complications. Lipoprotein particle measurement may be useful in patients at risk of CVD.

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ABBREVIATIONS

AP-1	Activator protein 1
AGEs	Advance Glycation Endproducts
BFGF	Basic Fibroblast Growth Factor
BMI	Body Mass Index
BSA	Bovine Serum Albumin
CRD	Cysteine Rich Domain
CVD	Cardiovascular Disease
DCCT	Diabetes Control and Complications Trial
Dlo	Disposition index
DM	Diabetes Mellitus
DN	Diabetic Nephropathy
EC	Endothelial Cell
EDIC	European Diploma in Intensive Care Medicine
EPCs	Endothelial Progenitor Cells
FBG	Fasting Blood Glucose
FPG	Fasting Plasma Glucose
FN3K	Fructosamine 3 kinase

GA	Glycated Albumin
GGap	Glycation Gap
GlycA	Glycoprotein Acetylation
GSIS	Glucose-Stimulation Insulin Secretion
G0	Negative GGap
G1	Positive GGap
HB	Haemoglobin
HGI	Haemoglobin Glycation Index
IDF	International Diabetes Federation
IDL	Intermediate-Density LIpoprotein
IR	Insulin Resistance
LDL	Low-Density Lipoprotein
MAP	Mitogen Activated Protein
MAPK	Mitogen Activated Protein Kinase
MBG	Mean Blood Glucose
MNC	Mononuclear Cell
MODY	Maturity-onset Diabetes of the Young

MTP	Microsomal Triglyceride transfer Protein
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	Non Alcoholic Fatty Liver Disease
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
NOS	Nitric oxide synthase
OS	Oxidative Stress
PAI-1	Plasminogen Activator Inhibitor-1
PKC	Protein Kinase C
RAGEs	Receptors for Advanced Glycation Endproducts
ROS	Reactive Oxygen Species
SDH	Sorbitol dehydrogenase
TGF-β	Transforming Growth Factor-β
TIDM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TGRL	Triglyceride Rich Lipoprotein
UKPDS	UK Prospective Diabetes Study

VEC	Vascular Endothelial Cell
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low-Density Lipoprotein
WHO	World Health Organisation
WISP1	Wingless-type inducible signaling pathway protein-1
WNT	Wingless type

CHAPTER ONE

INTRODUCTION/LITERATURE REVIEW

1.0 DIABETES MELLITUS: AETIOLOGY AND EPIDEMIOLOGY

Diabetes mellitus (DM) is a metabolic disease characterised by insufficient production of insulin or inability to make use of the insulin produced by the body, resulting in problems regulating blood sugar. It was estimated that 422 million people had diabetes in 2014, representing 8.5% of adult worldwide according to World Health Organisation (WHO), (Danna *et al,* 2016). Diabetes is one of the biggest global health problems of the 21st century, International Diabetes Federation (IDF) (Teo *et al*, 2021). The prevalence of diabetes globally is of 1 in 11 adults as of 2015 and these numbers are estimated to further increase, especially in the urban population. This result in more medical and economic challenges, added on top of the 12% global health expenditure presently spent on diabetes (Badescu *et al,* 2016). The global health expenditure for diabetes cost about $966 billion dollars which is a 316% increase from the last 15 years (IDF, 2021).

Diabetes has become one of the most severe and common prolonged diseases of present times, producing life threatening, disabling, and costly complications thereby, reducing life expectancy (Xu *et al,* 2022). The global prevalence of diabetes had reached pandemic proportions with the 9th edition of the IDF reporting a prevalence of 9% (463 million adults) in 2019. The rising prevalence of diabetes has been attributed principally to the ageing of populations. However, decreasing mortality among those with diabetes due to improving medical care as well as increases in diabetes incidence in some countries resulting from increasing prevalence of diabetes risk factors, especially obesity, are also important drivers of higher prevalence (Sun *et al,* 2021). The 10th edition IDF diabetes Atlas estimates that in 2021 there are 537 million people living with diabetes

worldwide. Overall, the global prevalence of diabetes is now estimated to be over 10% of the world population. Among income groups, the highest prevalence was observed in the middle-income countries compared to the previous 9th IDF. Diabetes Atlas edition, we report an overall 12.9% increase in diabetes prevalence, with prevalence increasing in many world regions (Sun *et al,* 2021). Future projections suggest that by 2045 that the number of people with diabetes would have increased by 46% (700 million), and majority of the cases will be in middle-income countries (Sun *et al*, 2021).

Unsurprisingly, countries with large populations such as China, India, Pakistan, and the USA contribute the most to the total number of people with diabetes (Teo, *et al*, 2022). About 60% of Asian people (139.9 million) in China lives with diabetes whilst about 65 million people with diabetes lives in India. These finding has earned Asia as the epicentre of diabetes (Yusufi *et al,* 2023). IDF suggests that in 2021 more than 1 in 10 adults (20 – 79 years) lived with diabetes globally. According to Diabetes.co.uk, 2022, above 4.8 million people have diabetes in the UK while over 130 million adults have diabetes/prediabetes in the United State (Centre for disease control, 2022). With aging and better diabetes treatment paradoxically increasing prevalence through decreased mortality, a strong need exists for the implementation of effective intervention strategies and policies that aim to stall the increase in the number of people developing diabetes and its complications (Sun *et al*, 2021).

Comparing countries by population size, the prevalence of diabetes in China has increased from 0.9% in 1980 to 11.6% in 2010, i.e., more than 1in 10 people in China have diabetes, with over 100 million adults having the disease, a figure higher than in any other country according to a national survey (Sun *et al*, 2016; Wan *et al*, 2016). According to the research carried out by Su *et al,* 2022, looking at the trends in diabetes mortality in China between 1987-2019, the incidence

and prevalence of diabetes in China is believed to have changed extremely. There is a rapid growth of mortality rate of diabetes in rural settlements which has bridged the urban-rural gap. The burden of death and disability associated to diabetes and its complications will continue to rise just as China's population ages (Su *et al*, 2022). The trends in the diabetes incidence and mortality in India from 1990-2019 shows a decreased in younger age but increased in older age group (Jha *et al,* 2021). In 2019, with 77 million of diabetic adults individual and with the effect still rising rapidly over the years, India is termed as the diabetes capital of the world (Jha *et al,* 2021). Diabetes prevalence in Romania is of 11.6% while prediabetes one is of 16.5% (Badescu *et al,* 2016) and 35.9% of adult population has diabetes in Korea according to a 2015 survey (Yang *et al*, 2016).

There are two main types of diabetes namely type 1 and type 2 diabetes mellitus (Siddiqui *et al,* 2013). There are other forms of diabetes such as gestational diabetes, maturity onset diabetes of the young (MODY), neonatal diabetes, latent autoimmune diabetes in adults (LADA), type 3c diabetes, Steroid-induced diabetes, and cystic fibrosis diabetes (Diabetes UK). Diabetes is a major source of morbidity, mortality, and economic cost to society (Singh *et al*, 2019). People with diabetes are at risk of developing acute metabolic complications such as diabetic complications such as diabetic ketoacidosis, non-hyperglycaemic hyperosmolar coma, and hypoglycaemia (Lotfy *et al,* 2017). Diabetics are also at increased risk for chronic complications such as coronary heart disease, retinopathy, kidney and neurological disease, and foot ulcers (Mezil and Abed, 2021). Various factors influence the development of diabetes (Rhee *et al,* 2017). Insulin resistance develops as a result of obesity and physical inactivity that serve as substrates for genetic susceptibility. Because diet affects the amount of insulin needed to reach target glycaemic goals, diet, especially carbohydrate intake, can contribute to the pathology of diabetes (Chaudhury *et al,* 2017). Dietary carbohydrates have the most influence on postprandial blood glucose and are a

major determinant of meal-related insulin requirements (Tiwari *et al*, 2013). One Diabetes Control and Complications Trial (DCCT) follow-up study found that when closely controlled for 6.5 years of blood sugar regulation in the early stages of type 1 diabetes (T1D), the effect persisted to the point that progression and the development of microvascular complications is delayed for at least 8 years after the end of the original study and the occurrence of cardiovascular events was delayed by 11 years (Rhee *et al,* 2017).

Currently, diabetes is the 7th leading cause of disability worldwide making its management and complications a high priority for healthcare systems worldwide (Sartorius, 2022). A preventive public health effort at societal and global scales is the best way to address the epidemic levels of diabetes and its complications (*Danna et al,* 2016). DM also increases the risk of future cardiovascular disease accounting for the leading cause of death in most high-income and developing countries, with significant evidence that it is changing into an epidemic in many low and middle-income countries (Bener *et al,* 2016).

Diabetes has been regarded as a risk factor for periodontitis traditionally, but this is not the case recently as it has become evident that it is not merely the diagnosis diabetes that is important, but rather the elevated blood glucose levels (hyperglycaemia) that is pivotal (Monje *et al*, 2017). Diabetes is a well-established independent risk factor for cardiovascular diseases (CVD). Compared with non-diabetic individuals, diabetic patients have 2 to 4 times increased risk for stroke and death from heart disease. Hyperglycaemia cannot entirely account for the high cardiovascular risk in diabetic patients (Wu *et al,* 2014).

1.1 Types of Diabetes

Type 1 Diabetes Mellitus (T1DM) is an autoimmune diabetes which is a chronic disease characterised by insulin deficiency due to pancreatic beta-cell destruction leading to hyperglycaemia (Ilonen *et al,* 2019). Although symptoms usually occur during childhood or adolescence but sometimes can be seen much later. Amongst factors responsible in the development of T1DM are multiple genetic and environmental risk factors (Lindbladh *et al,* 2020). The environmental trigger and genetic factors also determine the type of autoantibody that is seen first. No cure is known at present, so patients depend on lifelong insulin treatment (Vallianou *et al,* 2021).

Type 2 diabetes mellitus (T2DM) is a group of disorders of carbohydrate metabolism characterised by hyperglycaemia, which is primarily caused by an inadequate response to insulin (Ozougwu *et al,* 2013). The feature of Type 2 diabetes mellitus (T2DM), which is one of the most common complex metabolic disorders, are insulin resistance in the peripheral tissues, including liver, adipose, and muscle, along with progressive β-cell dysfunction, which results in hyperglycaemia (Xiao *et al*, 2019). A direct correlation between blood D-glucose and HbA1c has been known for a long time, and both are known predictors of diabetes complications. On the other hand, D-ribose has so far not been considered a potential risk factor in the development of T2DM (Chen *et al,* 2017).

One in 11 adults has diabetes worldwide (90% of them have type 2 diabetes mellitus (T2DM)) and Asia is the epicentre of this global T2DM epidemic (Zheng *et al,* 2018). The global pandemic of T2DM is mainly caused by overweight and obesity, a sedentary lifestyle and increased consumption of unhealthy diets high in red and processed meats, refined grains, and beverages

(Cheng *et al,* 2016). It is very important to break the cross-generational diabetes cycle of diabetes by implementing effective strategies to prevent gestational diabetes from having a global impact (McCance, 2015). Cardiovascular complications are the leading cause of morbidity and mortality among patients with T2DM and patients in Asia with diabetes mellitus are highly prevalent kidney diseases (Dal Canto *et al,* 2019). Diet and lifestyle modifications are effective in preventing T2DM in high-risk individuals as demonstrated by major clinal trials. T2DM management strategies including lifestyle modifications, social support and ensuring medication adherence are key to reducing the incidence of diabetes mellitus complications (Zheng *et al,* 2018). T2DM patients mostly have some level of overweight or obesity which has also been connected to insulin resistance and defects in insulin secretion. These modifications facilitate the appearance and worsening of diabetes so reducing the caloric intake is a must and a major course to take in other to achieve weight reduction in addition to a proper distribution of macro and micronutrients (Marin-Penalver *et al,* 2016).

Gestational diabetes Mellitus (GDM) is a condition in which instinctive hyperglycaemia occurs during pregnancy and if often common (Johns *et al,* 2018). There is about 60% reduction in insulin sensitivity during gestation which is a predisposing baseline insulin resistance. When this is further exacerbated and combine with beta cell dysfunction, leads to hyperglycaemia and it is therefore referred to as gestational diabetes mellitus (Catalano, 2014). In 2017 the International Diabetes Federation (IDF) estimates 14% of pregnancies globally are affected by GDM which represent 18 million births annually (Plows *et al,* 2018). Although GDM normally reverses back to normal after delivery, it could have long-lasting health consequences in the mother and child. Overweight or obesity, micronutrient deficiencies, advanced maternal age, western diet, and family history of insulin resistance or diabetes are the risk factors associated with GDM (Johns *et al,* 2018). There

are many management strategies for example, insulin and lifestyle interventions but there is no known cure yet and no effective prevention approach reason not far from poorly defined molecular mechanisms underlying GDM (Plows *et al,* 2018).

Maturity-onset diabetes of the young (MODY) is a group of inherited disorders of non-autoimmune diabetes mellitus which is usually present in adolescence or young adulthood (McDonald and Ellard, 2013). This type of diabetes was devised to show the mixed clinical presentation of the juvenile-onset and maturity onset diabetes (Naylor and del Gaudio, 2018). Its characteristics include early onset, autosomal dominant mode of inheritance and a primary defect in pancreatic β-cell function (Kim, 2015). Prevalence of MODY is believed to account for at least 1-3% of all diabetes (Naylor and del Gaudio, 2018). Diagnosis of MODY can be by direct sequencing with up to 100% sensitivity. Due to overlapping clinical features of MODY with both types of diabetes, about 80% of patients in the UK are wrongly diagnosed with T1DM and T2DM (Kim, 2015). Rapid MODY diagnosis is vital for patients and their family members since it can provide individualised treatment and prognosis predictions (Kim, 2015).

According to Chen *et al*, 2017, D-ribose may play a role in diabetes due to the following reasons: the ribosylation of proteins such as alpha-synuclein, Tau protein, and bovine serum albumin (BSA) occurs much more rapidly than glycation with D-glucose, due to a D-ribose glycation. The formation of AGEs in the reaction of proteins with D-ribose is also much quicker than the cytotoxicity of the ribosylation products is higher than that of the glycated products (Chen *et al,* 2019) a high level of D-ribose may be one of the important risk factors for the formation of HbA1c in the development of diabetes; and decreasing the concentration of D-ribose with benfotiamine results in a decrease in glycated haemoglobin (Javed *et al,* 2020).

Consequently, the role of an increase in D-ribose and the subsequent ribosylation of proteins in the progression of T2DM should not be neglected (Chen *et al*, 2017).

1.2 DIABETES AND HYPERGLYAEMIA

Hyperglycaemia (dysglycaemia) refers to the elevation in blood glucose levels regardless of the classification and categorisation such as pre-diabetes, gestational diabetes, type 1 diabetes, type 2 diabetes, and maturity-onset diabetes of the young (MODY) or surgically provoked diabetes (Monje *et al*, 2017). This is usually when the blood sugar level is above 7 mmol/L before meal and 8.5mmol/L 2 hours after a meal (Mouri and Badireddy, 2022). Whether or not it results in diabetes mellitus, it leads to high morbidity and mortality rate and high cost of management (Pasquel *et al*, 2021). Despite every cell in the body of people with diabetes is exposed to abnormally high glucose concentrations, hyperglycaemia selectively damage some cell types and not others (B Arden and Sivaprasad, 2011). The targeting of specific cell types by generalised hyperglycaemia reflects the failure of those cells to downregulate their uptake of glucose when extracellular glucose concentrations are elevated (Röder *et al,* 2016). Cells that are not directly susceptible to direct hyperglycaemic damage show an inverse relationship between extracellular glucose concentrations and glucose transport (Giacco *et al*, 2011). In contrast, vascular endothelial cells, major targets of hyperglycaemic damage, show no significant change in glucose transport rate when glucose concentration is elevated, resulting in intracellular hyperglycaemia (Giacco *et al*, 2010).

This role of hyperglycaemia has been established in 2016 by large-scale prospective studies for both type 1 and 2 diabetes, the Diabetes Control and Complications Trial/European Diploma in Intensive Care Medicine (DCCT/EDIC), and the UK Prospective Diabetes Study (UKPDS) (Eckel

et al, 2021). However, further analysis of the DCCT data shows that although intensive therapy reduced the risk of sustained retinopathy progression by 73% compared to standard treatment, haemoglobin (Hb)A1c and duration of diabetes (glycaemic exposure) explained only ≈11% of the variation in retinopathy risk for the entire study population (Neumiller *et al,* 2017). This suggest that the remaining 89% of the variation in risk is presumably explained by aspects of glycaemia not captured by HbA1c (William *et al*, 2009). Similar data have been reported by the Steno-2 study (Gaede *et al*, 2008).

Acute hyperglycaemia has been shown to reduce endothelium-dependent vasodilation, insulin secretion in addition to promoting the release of inflammatory proteins (Fain, 2016). The contribution of hyperglycaemia alone confers the risk of developing endothelial dysfunction and insulin resistance (Laakso and Kuusisto, 2014). Insulin resistance can be a pathophysiological phenomenon as transient adaptation to puberty, dehydration, infections, several drugs, and smoking (Hanson and Gluckman, 2014). Meanwhile, insulin resistance as observed in obesity and type 2 diabetes mellitus results from a complex interaction of environmental and inherited factors and progresses chronically (Kaul *et al*, 2015). Insulin resistance is when your body's cells do not respond properly to the insulin that your body makes or the insulin you inject as a medication (Diabetes UK, 2022).

Chronic hyperglycaemia is a manifestation of all types of diabetes as well as the development of diabetes-specific microangiopathy in the retina, glomeruli, and peripheral nerves (Davey *et al,* 2014). Due to microvascular disease, diabetes is a major cause of blindness, end-stage renal disease, and various debilitating neurological diseases (Ighodaro and Adeosun, 2018). Chronic hyperglycaemia is associated with long-term damage, dysfunction, and impairment of the normal functioning of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels.

(Tiwari *et al*, 2013). Diabetes is also associated with macrovascular disease due to rapid atherosclerosis affecting the arteries that supply blood to the heart, brain, and lower extremities (Nathan *et al*, 2009).

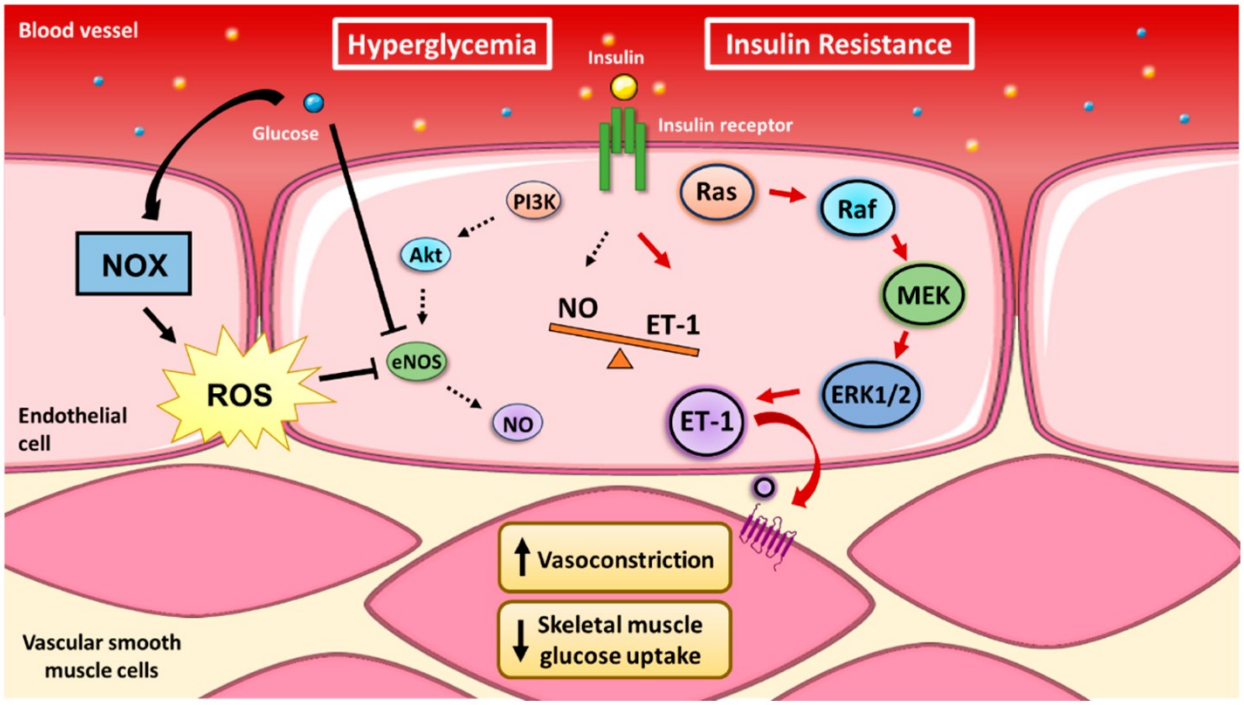


Figure 1: Diagram representing endothelial dysfunction due to hyperglycaemia as explained above (Meza *et al,* 2019).

1.2.1 *Reactive Oxygen Species (ROS) and Oxidative Stress*

Reactive oxygen species (ROS) are reactive chemicals that result from oxidative metabolism and the cellular response to foreign invasion (Bardaweel, *et al*, 2018). ROS can act by targeting molecules that modulate transcription factors and epigenetic signalling pathways, thereby regulating cell survival and death (Mittler, 2017). Cellular ROS levels are always unstable within the physiological redox range. The redox state of the cell, which regulates basic biological processes such as cell proliferation/differentiation, metabolic homeostasis, and immune responses,

is very important (Bellanti *et al,* 2020). Oxidative stress arises as a result of ROS generation exceeding ROS clearance, thereby placing the redox state outside the physiological redox range (Tiwari *et al,* 2013). Oxidative stress, when sustained, leads to cell, tissue, and organ damage, and this is associated with the pathogenesis of cancer, diabetes, cardiovascular disease, neurodegenerative diseases, diseases of the reproductive system and the aging process (Rahal *et al,* 2014; Wei *et al,* 2019). Oxidative stress in T2DM is an important pathological state caused by hyperglycaemia and generation of reactive oxygen species (ROS). In an insulin-sensitive tissue like liver, a rise in ROS elicits a chain reaction between free-radicals and membrane-bound free-fatty acids (FFAs). This in turn, results in the formation of noxious lipid peroxidation products such as hydroxynonenal (HNE) and MDA, which provoke the release of pro-inflammatory cytokines. A reduced integral antioxidant defence system in the liver is seen when there is an increase in hepatic lipid peroxidation. Oxidative stress plays a role in apoptosis and tissue degeneration (Dong *et al,* 2019). ROS types can be divided into free radicals and non-radicals. Examples of free radicals are nitric oxide (NO), the superoxide radical anion (O_2-), the hydroxyl radical (OH) (Ozougwu, 2016). Protein, lipid, and DNA oxidation, which can lead to cytotoxicity and/or cell death, can be caused by oxidative stress, but can also lead to modification of protein cysteine residues, impairing redox signalling within different cellular Signal transduction pathways enabled (Zhang *et al*, 2022).

1.3 THE ROLE OF HYPERGLYCAEMIA IN THE DEVELOPMENT OF DIABETIC COMPLICATIONS.

In general, the negative effects of hyperglycaemia are separated into two groups namely macrovascular complications (coronary artery disease, peripheral arterial disease, and stroke) and microvascular complications (diabetic nephropathy, neuropathy, and retinopathy) (Fowler, 2008).

All forms of diabetes are characterized by hyperglycaemia, a relative or absolute lack of insulin action, and the development of diabetes-specific pathology in the retina, renal glomerulus, and peripheral nerve (Blair, 2016). Diabetes is also associated with accelerated atherosclerotic disease affecting arteries that supply the heart, brain, and lower extremities. In addition, diabetic cardiomyopathy is a major diabetic complication (Giacco *et al*, 2010, Inzucchi *et al*, 2012).

Diabetic accelerated lower extremity arterial disease in conjunction with neuropathy accounts for 60% of all nontraumatic amputations in the United States (Long and Dagogo-Jack, 2011). Diabetes and impaired glucose tolerance increase cardiovascular disease (CVD) risk 3 to 8-fold. Thus, more than 30% of patients hospitalized with acute myocardial infarction have diabetes, and 35% have impaired glucose tolerance (Bartnik *et al,* 2004). Finally, new blood vessel growth in response to ischemia is impaired in diabetes, resulting in decreased collateral vessel formation in ischemic hearts and in non-healing foot ulcers (Qadura *et al,* 2018). Hyperglycaemia causes tissue damage through 5 major mechanisms: (1) increased flux of glucose and other sugars through the polyol pathway; (2) increased intracellular formation of AGEs (advanced glycation end products); (3) increased expression of the receptor for AGEs and its activating ligands; (4) activation of protein kinase (PK) C isoforms; and (5) over activity of the hexosamine pathway (Laakso 1999, Giacco *et al*, 2010).

Several lines of evidence indicate that all five mechanisms are activated by a single upstream event: mitochondrial overproduction of reactive oxygen species (ROS) (Giacco *et al*, 2010). In the diabetic microvasculature, this is a consequence of intracellular hyperglycaemia. In the diabetic macrovasculature and in the heart, in contrast, this appears to be a consequence of increased oxidation of fatty acids, resulting in part from pathway-specific insulin resistance (Bensellam *et al*, 2012).

Generally, diabetic microvascular complications are caused by prolonged exposure to high glucose levels. The extent of diabetic tissue damage is also determined by genetic determinants of individual susceptibility and, as with atherosclerosis, by the presence of such independent accelerating factors as hypertension and dyslipidaemia (Inzucchi *et al*, 2015). Diabetes-specific microvascular disease is a leading cause of blindness, kidney failure, and nerve damage (Tiwari *et al*, 2013). Understanding the mechanism that combines endothelial function with metabolic function may improve the treatment of various vascular complications associated with type 2 diabetes mellitus (T2DM) (Barnes, 2010). T2DM damages blood vessels throughout the body, putting patients at increased risk for other chronic conditions such as diabetic kidney disease and diabetic retinopathy. Aberrations in endothelial function often precede many of the abnormalities seen in T2DM and cardiovascular disease (CVD). Diabetes is associated with endothelial cell (EC) dysfunction and reduced neovascularisation in response to tissue ischemia, processes that are essential for wound healing and prevention of cardiovascular ischemia (Loomans *et al,* 2004). A growing body of evidence indicates that neovascularisation does not exclusively rely on proliferation of local ECs but also involves bone marrow–derived circulating stem cells (Monnier *et al,* 2006). Vascular endothelium is an active endocrine organ involved in the regulation of vascular tone and maintenance of vascular homeostasis. This monolayer of tissue lines the inner lining of blood vessels, thereby positioning endothelial cells in direct contact with the flowing blood and resulting in shear stress (Krüger-Genge *et al,* 2019). Exposure of endothelial cells to shear stress results in mechanical signal transduction for tight control of lumen size. The delivery of circulating molecules such as insulin and glucose to skeletal muscle fibres is regulated by endothelial cells (Li *et al,* 2005). Skeletal muscle accounts for about 80% of insulin-stimulated

glucose, and the haemodynamic forces resulting from shear stress increase the vasodilator action of insulin (Meza *et al,* 2019).

The endothelium is an important factor in mediating the links between vascular function and metabolic requirements. Endothelial injury is characterised by phenotypic changes, inflammation, altered permeability as well as decreased endothelium-dependent dilation. Endothelial dysfunction is a predictor of future cardiovascular events and insulin resistance (Meza *et al*, 2019). These cells can be cultured from the circulating mononuclear cell (MNC) fraction and are commonly referred to as endothelial progenitor cells (EPCs) because they exhibit characteristic endothelial surface markers and properties (Loomans *et al,* 2004). Moreover, a number of studies have shown that injected EPCs home to sites of ischemia, incorporate into the newly formed capillaries, and augment neovascularisation (Balaji *et al,* 2013). Consequently, if EPCs are critical to endothelial maintenance and repair, EPC dysfunction could contribute to the pathogenesis of ischemic vascular disease (Li *et al,* 2010). Indeed, studies have demonstrated that, in patients with cardiovascular risk factors, the number of EPCs that can be isolated from peripheral blood is reduced and EPC function is impaired (Wils *et al,* 2017). It was recently reported that a strong inverse correlation exists between the number of EPCs and the subjects' combined Framingham risk factor score (Aragona *et al,* 2016). In addition, measurements of flow-mediated brachial-artery reactivity also revealed a significant relation between endothelial function and the number of EPCs, supporting a role for EPCs in the maintenance of endothelial integrity (Loomans *et al*, 2004).

The major cause of death and complications in patients with type 2 diabetes is cardiovascular disease. More than 60% of all patients with type 2 diabetes die of cardiovascular disease, and an even greater percentage have serious complications (Hirshberg *et al,* 2011). The prevalence of

vascular disease, hypertension, dyslipidaemia, and other abnormalities is very high, and the consequences of these abnormalities are burdensome to patients, their families, and society (Evarson-Rose, 2011). Hyperglycaemia of diabetes is associated with increased glycation of free amino groups in proteins. Approximately 50% of plasma protein is the protein albumin, which is a major contributor to plasma osmolality and aids in the transport of lipids and steroid hormones (Ростока *et al,* 2021). Globulin makes up 35% of plasma proteins and is used in the transport of ions, hormones and lipids that contribute to immune function (Xiao *et al,* 2011). Fibrinogen is required for blood clotting and can be converted to insoluble fibrin accounting for 4%. Regulatory proteins such as enzymes, proenzymes and hormones make up less than 1% of plasma proteins (Singh *et al,* 2014).

Interventions such as lifestyle changes, control of blood pressure and lipids, and antiplatelet therapy can reduce the development, progression, and complications associated with T2DM (de Boer *et al,* 2020). Blood glucose control may reduce microvascular complications, but not cardiovascular complications (Valencia and Florez, 2017). Even with microvascular complications, blood-pressure control has a greater effect than glucose control. In patients with advanced type 2 diabetes, the unanswered question is whether glucose control independently reduces cardiovascular complications (Duckworth *et al*, 2009).

Figure 2 shows how genetic individual susceptibility like hypertension, hyperlipaemia etc and genetic determinants of individual susceptibility resulting from hyperglycaemia result in diabetic tissue damage.

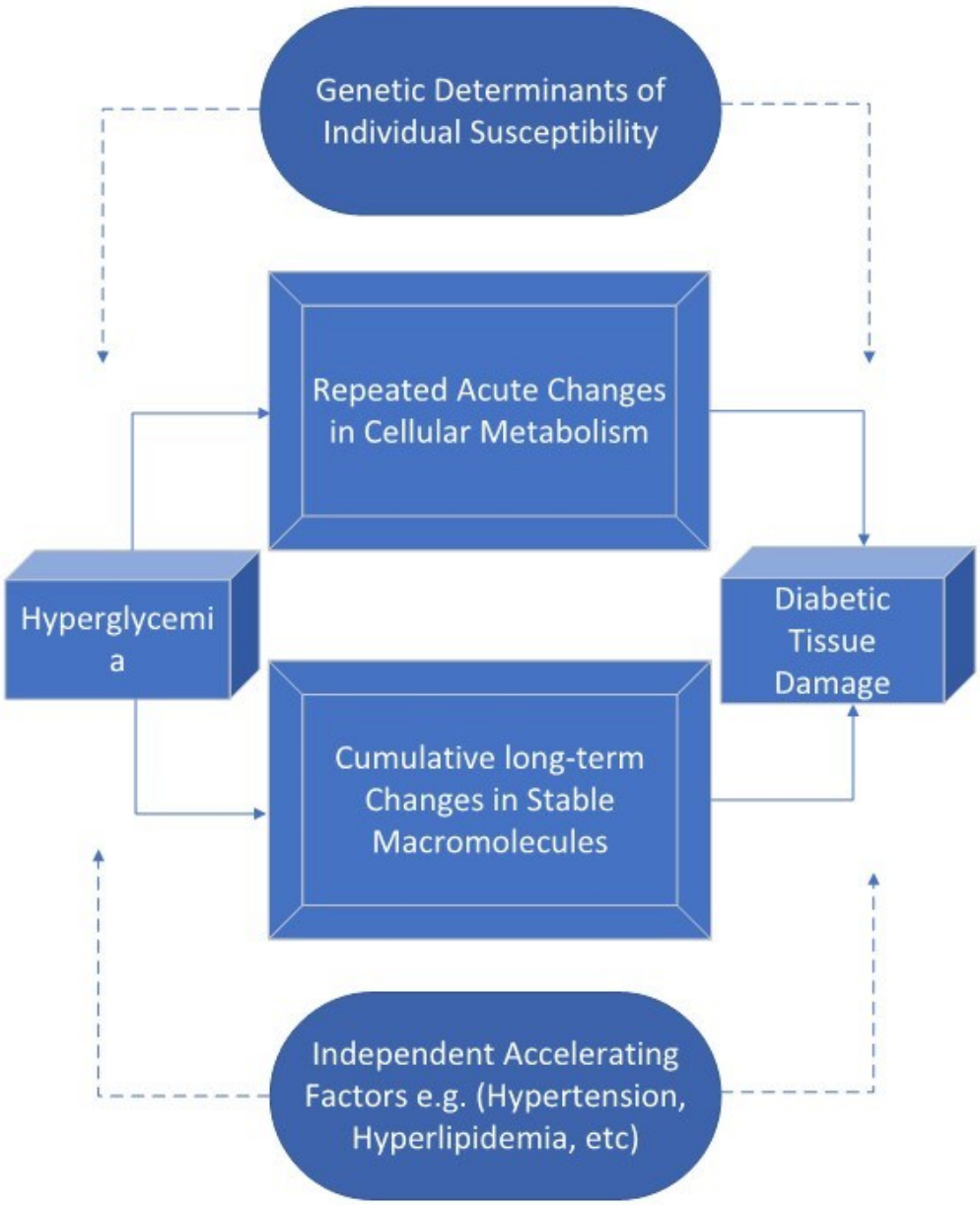


Figure 2: General features of hyperglycaemia-induced tissue damage, adapted from Giacco and Brownlee, 2010.

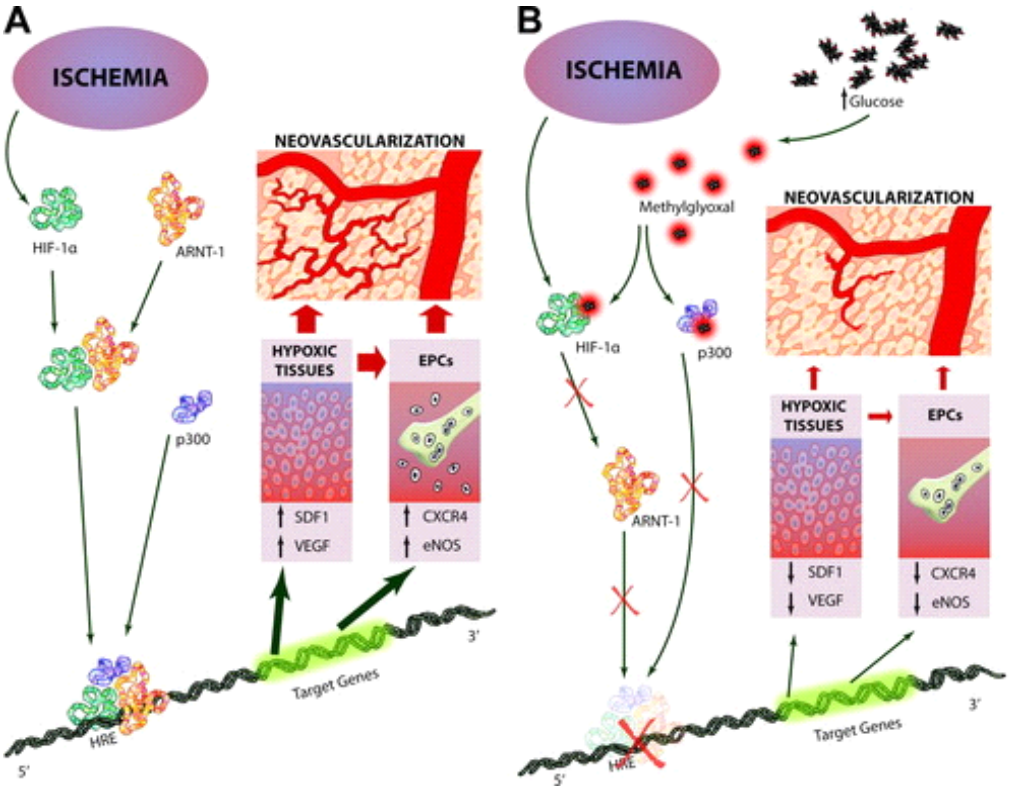


Figure 3: Model of ischemia-induced neovascularisation in normal and high glucose (Giacco and Brownlee, 2010).

Figure 3 shows how reduced blood flow in normal glucose and high glucose leads to neovascularisation.

All types of diabetes are represented by chronic hyperglycaemia and the development of diabetes-specific microangiopathy in the retina, glomeruli, and peripheral nerves (Mauricio *et al,* 2020). Due to microvascular disease, diabetes is a major cause of blindness, end-stage renal disease, and various debilitating neurological diseases (Faselis *et al,* 2020). Diabetes is also associated with macrovascular disease due to rapid atherosclerosis affecting the arteries that supply blood to the heart, brain, and lower extremities (Nathan *et al,* 2009). As a result, patients with diabetes have a much higher risk of heart attack, stroke, and amputation. Large prospective clinical studies show a strong relationship between blood glucose and diabetic microvascular complications in type 1 and 2 diabetes (Zoungas *et al,* 2017). Both hyperglycaemia and insulin resistance appear to play

an important role in the pathogenesis of macrovascular complications as seen in figure 3 (Brownlee, 2010).

Hyperglycaemia is associated with an increase in cytokine production mediated by oxidative mechanisms (Luc *et al*, 2019). Thus, the excess production of reactive oxygen species (ROS), together with the interaction of advanced glycation end-products (AGEs) with their receptors (RAGEs) on endothelium, leads to cytokine production by endothelial cells (Yan *et al,* 2008). Overproduction of cytokines is also associated with endothelial dysfunction, a diabetes-specific microvascular disease of the retina, glomeruli, and blood vessels with similar pathophysiological features (Akhil, 2010). In the early stages of diabetes, increased intracellular glucose leads to blood flow abnormalities and increased vascular permeability. This reflects the decreased activity of vasodilators such as nitric oxide, increased activity of vasoconstrictors such as angiotensin II and endothelin 1, and the expansion of permeability factors such as vascular endothelial growth factor (VEGF). (Bensellam *et al,* 2012). Quantitative and qualitative abnormalities of the extracellular matrix contribute to irreversible increased vascular permeability (Chronopoulos *et al,* 2010). Over time, loss of microvascular cells occurs, partly due to apoptotic cell death, and there is progressive capillary occlusion due to both extracellular matrix overproduction (Al-Kharashi, 2018). This is caused by growth factors such as transforming growth factor-β (TGF-β) and deposition of extravascular circulating acid-Schiff plasma proteins (Fournet *et al,* 2018). Hyperglycaemia can also reduce the production of nutritional factors for endothelial cells and neurons (Hammes, 2018). Together, these changes lead to hypoxic-induced oedema, ischemia, and neovascularization in the retina, proteinuria, mesenchymal matrix enlargement, and glomerular fibrosis in the kidney, and axonal degeneration. multifocal peripheral nerves (Mrugacz *et al,* 2021). The pathogenesis of atherosclerosis in nondiabetic subjects has been extensively described in recent reviews and begins

with endothelial dysfunction (Al-Hamodi *et al*, 2014). In diabetic arteries, endothelial dysfunction appears to be associated with both insulin resistance specific to the phosphatidylinositol-3-OH kinase pathway and hyperglycaemia (Van *et al,* 2006). Pathway-selective insulin resistance leads to decreased endothelial production of the anti-atherogenic molecule nitric oxide and increased vascular smooth muscle cell proliferation and plasminogen activator inhibitor-1 (PAI-1) production via the Ras—›Raf—› MEK kinase—›mitogen-activated protein kinase (MAP) pathway (Muniyappa and Sowers, 2013). Hyperglycaemia itself inhibits nitric oxide production in arterial endothelial cells and stimulates PAI-1 production (Brownlee, 2010). There is a ≥10% risk reduction in major macrovascular and microvascular events resulting from intensive T2DM treatment (Henning, 2018).

1.3.1 Mechanisms of Damage from Hyperglycaemia.

The DCCT study conclusively demonstrated that the relative risks of developing diabetic complications increased with increasing haemoglobin (Hb) mean A1c levels (DCCT/EDIC research group, 2014). A relationship between chronic hyperglycaemia levels and diabetic microvascular and macrovascular disease in patients with type 2 diabetes has also been established in several studies (van Wijngaarden *et al,* 2017). Hyperglycaemia is the most important precipitating factor in the pathogenesis of diabetic complications (Nishikawa *et al*, 2000). Among the wide range of molecules produced by endothelial cells, nitric oxide (NO) can be considered essential to mediate the various actions of the endothelium (*Meza et al,* 2019). The effect of hyperglycaemia on endothelial cells is very similar to that of inflammatory initiators (Funk *et al*, 2012). Endothelial cells exposed to hyperglycaemic cell culture media show decreased NO production with enhanced NF-kB activation, inflammatory gene expression, and leukocyte recruitment (Funk *et al*, 2011). Reactive oxygen species (ROS) generated by hyperglycaemia

contribute to the development and progression of diabetic vascular complications and are associated with endothelial dysfunction (Sun *et al,* 2013). Intracellular hyperglycaemia causes tissue damage through mechanisms that can be divided into two categories (Giri *et al*, 2018). One category of mechanisms involves repeated acute changes in cellular metabolism that are reversible when euglycemia is restored (Taylor, 2013). Another category of mechanisms involves cumulative changes in long-lived macromolecules that persist despite recovery of euglycemia (Grohová *et al*, 2019). These mechanisms are influenced by genetic determinants of susceptibility or resistance to hyperglycaemic damage (Nishikawa *et al,* 2000). A healthy endothelium modulates vasculature homeostasis in response to physical and chemical stimuli through the secretion of vasoactive molecules that act in an autocrine, paracrine, or endocrine manner (*Meza et al,* 2019). These endothelial-derived factors control a number of physiological outcomes, including vascular tone, diameter, vascular smooth muscle cell (VSMC) proliferation, platelet activation, and leukocyte adhesion (Méndez-Barbero *et al,* 2021). The endothelium is considered a powerful predictor of cardiovascular health and elucidation of healthy physiological mechanisms, and therefore provides a basis for clinical strategies to treat cardiometabolic diseases (Meza *et al*, 2019). Four main hypotheses about how hyperglycaemia causes diabetic complications to have generated a wealth of data as well as several clinical studies based on specific inhibitors of these mechanisms. The four hypotheses are: increased flux from the polyol pathway; increased formation of advanced glycation end products (AGEs); activation of protein kinase C (PKC) isoforms; and increased flux from the hexosamine pathway. Until recently, there was no unified hypothesis linking these four mechanisms (Brownlee, 2001)

1.3.2 Four ways Hyperglycaemia Causes Diabetic Complications

1.3.2.1 Activation of protein kinase c (PKC)

PKC involves 12 isoforms and belongs to a family of serine or threonine kinases. Hyperglycaemia increases intracellular reactive oxygen species (ROS) and lipid peroxidation (Noh and King, 2007). In a hyperglycaemic environment, oxidative stress (OS) is increased, and reactive aldehydes are subsequently detoxified. However, in contrast to the normal state, increased intracellular glucose levels lead to increased metabolism of glucose to polyalcohol sorbitol, while reducing nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione (Dymkowska *et al*, 2014). In cells in which aldose reductase activity is sufficient to deplete glutathione, the oxidative stress induced by hyperglycaemia is increased. Sorbitol is oxidized to fructose by the enzyme sorbitol dehydrogenase (SDH). In cells with elevated sorbitol dehydrogenase activity, can lead to an increase in NADH (Ramana *et al,* 2005).

NAD^+ in its normal state, may increase de novo diacylglycerol (DAG) synthesis by inhibiting the oxidation of triose phosphate, thereby activating PKC (Nishikawa *et al,* 2000). The PKC family includes at least eleven isoforms, nine of which are activated by the lipid second messenger DAG. Intracellular hyperglycaemia increases the amount of DAG in cultured microvascular cells and in the retina and glomeruli of diabetic animals (Madonna and Caterina, 2011). Hyperglycaemia may also indirectly activate PKC isoforms through ligation of AGE receptors and through increased activity of the polyol pathway, possibly by increasing reactive oxygen species. Abnormal PKC activation is associated with decreased glomerular nitric oxide production due to experimental diabetes and decreased nitric oxide production in smooth muscle cells due to hyperglycaemia (Kizub *et al,* 2014). PKC activation also inhibits insulin-stimulated expression of endothelial nitric oxide synthase (eNOS) (an enzyme responsible for the synthesis of NO) and messenger RNA in cultured endothelial cells (Brownlee, 2001).

1.3.2.2 Increased polyol pathway flux

Aldose reductase (alditol:NAD(P)$^+$ 1-oxidoreductase, EC 1.1.1.21) is the number one enzyme in the polyol pathway. The polyol pathway comprises of two reactions catalysed by two different enzymes (Şengül *et al,* 2018). It is a cytosolic substance, monomeric oxidoreductase that catalyses the NADPH-dependent reduction of a variety of carbonyl compounds, including glucose (Dunlop, 2000; Brownlee, 2001). Aldose reductase which is the rate-limiting reaction has a low affinity (high K_m) for glucose and at the normal glucose concentrations found in non-diabetic individuals, glucose metabolism by this pathway accounts for a very small proportion of the total amount of glucose used (Elimam *et al,* 2017). But in a hyperglycaemic environment, an increase in intracellular glucose leads to an increase in its enzymatic conversion to polyalcohol sorbitol while also reducing NADPH (Rabbani and Thornalley, 2018). In the polyol pathway, sorbitol is oxidized to fructose by the enzyme sorbitol dehydrogenase, NAD+ is reduced to NADH (Brownlee, 2001).

Several mechanisms have been proposed to explain the potential adverse effects of hyperglycaemia-induced hyperglycaemia in polyol pathway flux (Javed *et al,* 2015). These include sorbitol-induced osmotic stress, decreased (Na+K+) ATPase activity, increased intracellular NADH/NAD+, and decreased intracellular NADPH. Sorbitol does not readily diffuse across cell membranes, and this was originally thought to lead to osmotic damage to microvascular cells (Rabbani and Thornalley, 2018). Another early suggestion is that increasing flux via the polyol pathway reduces ATPase (Na+K+) activity (Garg and Gupta, 2022). Although this depletion was initially attributed to a polyol pathway-related impairment in phosphatidylinositol synthesis, it has recently been shown to be due to PKC activation (Safi *et al,* 2014). Activation of PKC by hyperglycaemia increases the activity of cellular phospholipase A2, which increases the production of two inhibitors (Na+K+) ATPase- arachidonate and prostaglandin E (PGE). (Giacco

et al, 2010). In vivo polyol pathway inhibition studies have yielded inconsistent results (Dodda and Ciddi, 2014). In a 5-year study in dogs, aldose reductase inhibition prevented diabetic neuropathy, but did not prevent retinopathy or thickening of capillary basement membranes in the retina, kidneys, and muscles (Setter *et al,* 2003). Several negative clinical trials have questioned the relevance of this mechanism in humans (Lorenzi, 2007). The positive effect of aldose reductase inhibition on diabetic neuropathy has, however, been confirmed in humans in a rigorous multi-dose, placebo-controlled trial with the potent aldose reductase inhibitor zenarestat (Brownlee, 2001).

1.3.2.3 Increased intracellular formation of advance glycation end-products.

AGEs can arise from autooxidation of glucose to glyoxal, decomposition of the Amadori products to 3-deoxyglucosone, and fragmentation of glyceraldehyde-3-phosphate to methylglyoxal (Vistoli *et al,* 2013). These reactive dicarbonyls react with amino groups of protein to form AGEs. Methylglyoxal and glyoxal are detoxified by the glyoxalase system (Nishikawa *et al,* 2000). AGEs are produced by the Maillard reaction and are found in increased amounts in diabetic retinal vessels and renal glomeruli (Brownlee, 2001). Reducing sugars, such as glucose, react non-enzymatically with protein amino groups to produce Schiff bases, which rearrange to Amadori compounds (Liu, 2013). These Amadori adducts undergo very slow, irreversible dehydration and condensation reactions, ultimately producing AGEs, which are yellow-brown, fluorescent substances (Kuerban, 2018). AGEs are produced not only from glucose, but also from glucose degradation products such as glyoxal, methylglyoxal and 3-deoxyglucosone, and from dicardonyl compounds produced by autoxidation of α-hydroxyaldehydes such as glyceraldehyde and glycolaldehyde is generated (Papachristoforou *et al,* 2020). In chronic hyperglycaemia, AGEs are actively produced and accumulate in the circulating blood and various tissues, leading to vascular complications of

diabetes (Chawla and Tripathi, 2019). AGEs are endogenously produced by oxidative stress and hyperglycaemia, but they can also be ingested exogenously from the diet. About 6% to 7% of his AGEs from diet are known to reside in the body over time which accelerate the expression of receptors for advanced glycation end products (RANGEs) (Zhang *et al,* 2020). Persistent activation of the AGE-RAGE system is thought to produce long-term metabolic memory or legacy effects (Reddy *et al,* 2015). In endothelial cells, recognition of AGEs by RAGE promotes the production of intracellular oxidative stress and activation of factor NF-kB induces the secretion of various cytokines and growth factors, induces the expression of adhesion factors, and triggers an inflammatory response (Tobon-Velasco *et al,* 2014). In diabetic patients, RAGE expression is accelerated in atherosclerotic lesions in proportion to the deterioration of glycaemic control (Rhee *et al,* 2018). AGEs may promote oxidative stress, thereby increasing the expression of transcription factors, pro-inflammatory cytokines, inflammatory cytokines, and acute-phase proteins (Xu *et al,* 2018). Furthermore, accumulation of AGEs and binding to RAGEs can lead to metabolic disturbances, inflammation, and oxidative stress (Beereka *et al,* 2021).

1.3.2.4 Hexosamine pathway

Through the hexosamine pathway, only a little portion (about 1-3%) of glucose is metabolised in a normal glucose condition. Intracellular glucose levels elevation can result in an increased flux through the hexosamine pathway (Kitada *et al,* 2010). The rate limiting enzyme, glutamine-fructose-6-phosphate amidotransferase (GFAT), converts fructose-6-phosphate which is a glycolysis intermediate to glucosamine-6-phosphate (Walter, 2013). Uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) is the major end product and is also a substrate for the subsequent O-linked GlcNAc modification of target proteins at serine and threonine residues (Chatham *et al,* 2021). The O-GlcNAc transferase (OGT) vigorously add O-GlcNAc but it is

removed by O-GlcNAcase (OGA) to serine and threonine residues of nucleocytoplasmic proteins. Through the O-GlcNAc modification at serine 1177 in endothelial cells, hyperglycaemia could inhibit endothelial nitric oxide synthase (eNOS) activity (Qin *et al,* 2017). Hyperglycaemia is known to cause a 4-fold increase in O-GlcNAcylation of the transcription factor Sp1 which mediates hyperglycaemia-induced activation of the PAI-1 promoter in vascular smooth muscle cells and TGF-β1 and PAI-1 in arterial endothelial cells (Thomas, 2018). GFAT activity in aortic smooth muscle cells is increased by hyperglycaemia and it also increases O-GlcNAc modification of several proteins in the cell (Giacco and Brownlee, 2010).

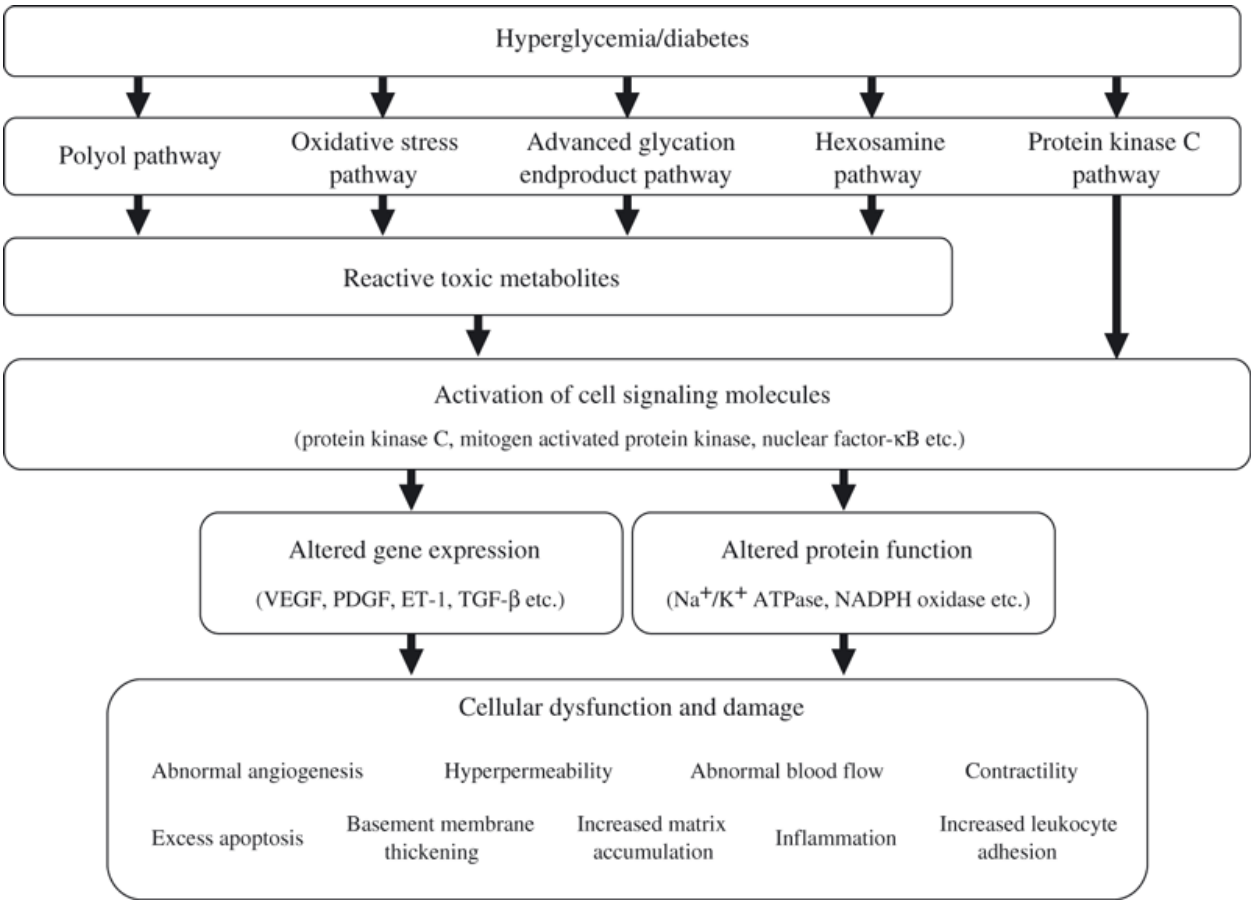


Figure 4: The diagram above illustrates the different mechanism through which hyperglycaemia leads to diabetes complications as discussed in the four pathways above (Kitada *et al*, 2010).

1.4 GLYCATION

Glycation is a non-enzymatic process mainly dictated by the glucose concentration and has long been considered as irreversible, although some findings were suggesting the existence of intracellular deglycation mechanisms. Kinases are used to deglycate proteins under physiological conditions (Dunmore *et al,* 2017 and Avemaria *et al*, 2015). Glycation is a common and spontaneous reaction which occurs *in vivo*. About 5% of total haemoglobin in normo-glycaemic subjects has a fructosamine bound to the amino terminus of its β-chains (HbA1c) as seen in figure 5 (Kohzuma *et al,* 2021; Van Schaftingen *et al,* 2012). It is well known that HbA1c represents an important tool for the clinical management of diabetes and evidence confirms its important role as a retrospective indication of the quality of glycaemic control (Avemaria *et al*, 2015). Glycation occurs as a result of the well-known reaction of carbohydrate moieties with amino groups of proteins known since 1910 as the Maillard, or more specifically when involving glucose, as the Schiff reaction. The almadori product of the Schiff reaction undergoes slow but reversible rearrangement to form the Amadori ketoamine product as shown in figure 9 and figure 10 (Sharma *et al*, 2020). The ketoamine is then slowly converted to advance glycation end-products which comprise a wide range of chemical moieties which contribute to the development of diabetes complications (Nayak *et al*, 2019).

Human serum albumin is a single-chain polypeptide consisting of 585 amino acid residues with a molecular weight of 66,460kDa (Yu *et al,* 2021). In the progression of T2DM, glyco-oxidation of proteins has been implicated and human serum albumin is the protein prone to glyco-oxidation (Luna *et al,* 2021). It is attacked by sugars and methylglyoxal which is a strong glycating enzyme and may have serious effect on its structure and successive role in diabetes (Mir *et al*, 2016). Proteins appears to be the first seen target by the glucose molecules which is circulating at increased level in diabetic individual (Pessin *et al,* 2000). The nonenzymatic reaction involving

proteins (fructose) and sugars (glucose) results in glycated proteins, depending on the amount or quantity of glucose molecule condensed on the protein, would display, or produce different functionality (Lapolla *et al,* 2013).

Glycation of protein is known to be involved in a variety of biological processes example of which are receptor interaction, immune response, protein secretion and transport (Fournet *et al*, 2018). This involvement affects protein properties such as solubility, stability, and folding which differ between individuals, but remarkably stable per person except in disease state or change in lifestyle (Clerc *et al*, 2016).

One particular protein may have multiple sites of glycation, of which its glycoforms may vary by site occupancy and occupying glycan structure (Woods, 2018). Multiple parameters determine the biosynthetic pathways leading up to the variety of glycans and may also be influenced by other factors including genetic regulation, the availability of nucleotide sugars, the time spent in the endoplasmic reticulum and Golgi apparatus, and also the accessibility of a specific glycation site (Clerc *et al,* 2016).

The major plasma glycoproteins are as follows; Alpha-1-acid glycoprotein (P02763; P19652), Alpha-1-antitrypsin (P01009), Alpha-1B-glycoprotein (P04217), Alpha-2-HS-glycoprotein (P02765), Alpha-2-macroglobulin (P01023), Antithrombin-111 (P01008), Apolipoprotein B-100 (P04114), Apolipoprotein D (P05090), Apolipoprotein F (Q13790), Beta-2-glycoprotein 1 (P02749), Ceruloplasmin (P00450), Fibrinogen (P02671; P02675; P02679), Haptoglobin (P00738), Hemopexin (P02790), Histidine-rich glycoprotein (P04196), Kininogen-1 (P01042), Serotransferrin (P02787), Vitronectin (P04004), Zinc-alpha-2-glycoprotein (P25311), Immunoglobulin A (P01876; P01877), Immunoglobulin D (P01880), Immunoglobulin E

(P01854), Immunoglobulin G (P01857; P01859; P01860) and Immunoglobulin M (P01871) (Clerc *et al*, 2016).

Miyazawa et al, 2010 found out that Pyridoxal 5′-phosphate and Pyridoxal are the most effective inhibitors of lipid glycation of which phosphatidylethanolamine-pyridoxal 5′-phosphate adduct is measurable in human red blood cells. This suggest that a potential clinical marker can be developed for glycaemic control and also to avoid the pathogenesis of diabetic complications. Protein glycation level can give account for the long-term diabetic complications which also can be used as biomarkers, prognostic instruments, and as secure points for treatment targeting (Lapolla *et al,* 2013; Clerc *et al*, 2016).

AGE which stands for advance glycation end-products are products of nonenzymatic reactions that occur between reducing sugars and proteins (Seigi *et al*, 2021). RAGE (stands for receptor for advanced glycation end-products) belongs to the immunoglobulin superfamily of cell surface proteins encoded in the Class 111 region of the major histocompatibility complex. AGE-RAGE interactions may promote abnormal signaling pathways and genes overexpression (Dariya *et al,* 2020). Oxidative stress accelerated by AGE-RAGE inactivates nitric oxide and subsequently induces an inflammatory response or exacerbates the propensity to thrombosis leading to the progression of arteriosclerosis. In endothelial cells, the AGE-RAGE system promotes the expression of genes that are components of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and accelerates the generation of oxidative stress by activating oxidative stress (Chen *et al,* 2022). Membrane transport of small GTPase 1 of the Rac family (Rac1) causes endothelial cell dysfunction (Rhee *et al*, 2018). AGEs promote the production of vascular endothelial growth factor (VEGF) in endothelial cells, which subsequently induce pathological angiogenesis. VEGF is known to be involved in increasing atheroma in

atherosclerotic lesions. This suggests that the AGE-RAGE system, through the production of VEGF, activates angiogenesis in atheroma and exacerbates inflammation in plague, possibly through haemorrhage or increased atherosclerotic plaque (Finch, 2007). In addition, the AGE-RAGE system inhibits prostacyclin (PG12) production in endothelial cells and promotes de novo synthesis of the plasminogen activator inhibitor-1, thereby inhibiting fibrinolytic activity and thus contribute to the stabilization of thrombosis (Yamagishi and Matsui, 2011). The reduction of circulating adenosine monophosphate production by the AGE-RAGE system is associated with a propensity to thrombosis (Robson *et al,* 2018). AGEs are known to not only promote platelet aggregation but also promote blood clotting through the production of tissue factors (Kaur *et al,* 2018). Propensity to thrombosis induced by AGEs is implicated as a cause of acute coronary syndromes, such as unstable angina or acute myocardial infarction due to atherosclerotic plaque rupture and subsequent thrombus formation. in coronary arteries (Rhee *et al*, 2018).

Glycation gap (GGap) is the difference between HbA1c, and other measures of glycaemia obtained from fasting plasma glucose (FPG) and fructosamine or glycated albumin as predicted (Wang *et al,* 2022). Negative Glycation Gap represent a situation where the HbA1c is lesser than the value estimated from fructosamine whilst Positive Glycation Gap is when the HbA1c is more than the value predicted by fructosamine (Nayak *et al,* 2013). It can be a useful tool to alert clinicians to patients with unstable glycaemic control or when different pre-analytical conditions may affect measurement consistency. glycated albumin (GA) or glycated haemoglobin (HbA1c) (Renata *et al,* 2016). Inter-individual differences in protein turnover may contribute, and it is more likely that the amount of glucose the protein is exposed to and the rate of glycation/deglycation may explain the differences in Glycation Gap (GGap) (Wang *et al,* 2022).

Glycoprotein acetylation (GlycA) is a composite and accurate measure of system inflammation and is reduced in individuals at risk for the development of T2DM. Increased GlycA is associated with incidence of T2DM and CVD and with vascular disease in patients with rheumatoid arthritis (RA) and psoriasis. Diet, and/or exercise-induced GlycA reductions may reflect a reduced risk of developing T2DM and diabetes-associated cardiometabolic complications for a number of inflammatory conditions (Bartlett *et al*, 20017). GlycA, a marker of systemic inflammation, is associated with incident T2DM in a general population of both men and women. GlycA may provide complementary and possibly superior information to high sensitive C-reactive protein (hsCRP) for the prediction of future T2DM (Bartlett *et al,* 2017). GlycA is unique in that it is an NMR signal derived from residues within the carbohydrate sidechains of multiple acute phase reactions. Not only are the synthesis and secretions of these proteins increased during inflammation, but their glycans structures are dynamically altered (Fuertes-Martin *et al,* 2020). For example, the carbohydrate side-chains of α 1-acid glycoprotein, one of the acute phase glycoproteins captured in the GlycA signal, become highly branched and contain additional GLCNAc residues in the context of chronic inflammation and cancer (Connelly *et al,* 2016).

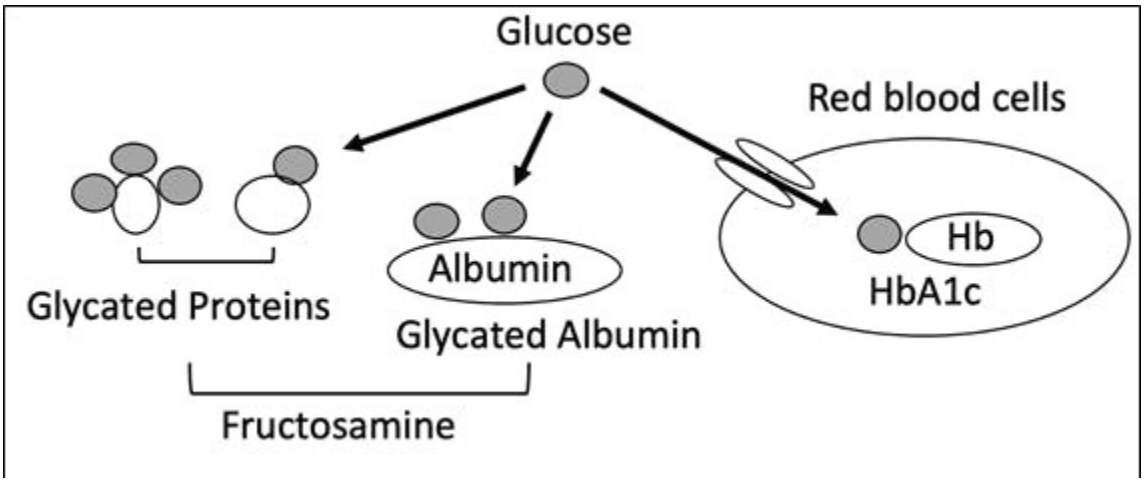


Figure 5: above shows the attachment of fructosamine to glucose in red blood cells (Kohzuma *et al,* 2021).

1.4.1 Glycation and Diabetic Complications

Serum AGE levels are known to be an independent risk factor for endothelial dysfunction in patients with T2D (Ding *et al*, 2021). Serum AGE levels are also known to be associated with left diastolic dysfunction and vascular hardening in T1D patients (Salazar *et al,* 2021). RAGE has three splice variants namely full-length RAGE, N-terminal variant containing no AGE binding domain, soluble receptor for advanced glycation end product (sRAGE), and C-terminal slice variant which does not contain transmembrane and effector domains (Zeni *et al,* 2017). The physiological function of sRAGE has not been fully described, but it is known to be induced by cleavage of RAGE on the cell surface and is known to reflect injury to endothelial cells. However, sRAGE is known to modulate the AGE-RAGE system as a full-time RAGE antagonist (Rhee *et al,* 2018).

The AGE reaction may also be called the browning reaction, a change in the browning of foods that can be a measure of their AGE content (Lund *et al,* 2017). In general, it is known that large amounts of AGEs are produced when meat or fatty foods are heated or cooked to high temperatures. On the other hand, AGEs are not produced in significant quantities when foods are slowly steamed or heated with a lot of water for a long time (Rhee *et al,* 2018). Foods high in protein and fat, mainly of animal origin, cooked at high heat and dry, such as baking, broiling, frying and roasting, tend to be the highest source of AGEs, while Low-fat foods and high-carbohydrate foods tend to be relatively low in AGEs (Sharma *et al*, 2015). AGEs in foods are generated not only by the Maillard reaction but also by the interaction between lipids and oxidized proteins, reactions that can generate AGEs such as carboxymethyllysine (Uribarri *et al,* 2015).

Food cooking, processing and preservation is the result of the Maillard reaction, non-enzymatic browning, between the carbonyl group of the reducing sugar and the primary amine group, forming food AGEs (Xiang *et al,* 2021). With dietary and circulating free AGE concentrations being strongly correlated with dietary AGEs absorbed from the gastrointestinal tract (Šebeková *et al*, 2019). This have effects on their health, rendering naturally occurring AGEs to be a good marker for dietary AGEs while plasma protein-bound AGEs better represent endogenously produced AGEs (Bettiga *et al,* 2019). Low molecular weight AGEs are absorbed more efficiently than protein bound AGEs because modification of AGE inhibits proteolysis (Sergi *et al*, 2021).

Consuming AGEs in the diet is likely to increase the risk of pancreatic cancer in men. However, women who consumed a diet rich in CML AGEs (N-(carboxymethyllysine) had no increased risk of pancreatic cancer (Wada *et al*, 2022).

Besides endogenous AGEs, dietary AGEs contribute significantly to the body's AGE pool (Gary-Sevilla *et al,* 2021). It assumes that if the amount of AGEs in the diet is reduced, any chronic diseases, such as diabetes and cardiovascular disease, can be improved or even cured (Zhu *et al,* 2018). For this reason, much attention has recently been paid to the identification and quantification of AGE products in foods consumed. Some of the knowledge found in the literature regarding the formation of AGEs in food, their gastrointestinal absorption, and their toxic effects was reviewed (Abate *et al,* 2015). Since modern diets are largely processed by heat, they are more likely to contain high levels of glycation end products (AGEs). AGEs are a complex and heterogeneous group of compounds known as brown, fluorescent crosslinkers such as pentosidine, non-fluorescent crosslinking products such as methylglyoxal-lysine (MOLD) or non-fluorescent dimer, no crosslinking. narcotics such as carboxymethyllysine (CML) and pyraline (a pyrrole aldehyde) (Sharma *et al,* 2015). Activation of RAGE is not only implicated in the promotion of

oxidative stress by activating NAPDH oxidase which enhances ROS production with ROS promoting AGE formation but also activation of inflammatory pathways (Tarafdar and Pula, 2018). In addition, ROS enhances the inflammatory response by activating NF-kB, which upon activation migrates to the nucleus and induces both the expression of inflammatory mediators and up-regulates RAGE, thereby establishing positive feedback (Sergi *et al,* 2021).

In both health and disease state, modern diet is an important contributor to the circulating AGE pool (Gill *et al*, 2019). It is further postulated that continuous exposure to these exogenous pro-oxidant substances gradually wear down native defences, preparing the stage for abnormally high OS and inflammation, which are the precursors of disease (Prabha, 2020). This scenario seems to be reversible simply by following a very simple dietary intervention, the low-AGE diet, which has been shown to be a feasible, effective, and safe intervention in different populations and countries (Uribarri *et al*, 2015).

AGEs accumulate at a much higher rate in individuals with diabetes than in those without diabetes (Peppa *et al*, 2003). In a study involving tissue AGE levels, Makita *et al*, (1991) reported significantly higher AGE levels in arterial wall collagen from diabetics compared with samples from those without. diabetes. In addition to this study, another group reported that the AGE levels found in the renal tissue of diabetic patients with end-stage renal disease were approximately twice the levels of AGEs from diabetic patients without renal disease (Yamagishi and Matsul, 2010).

Pentosidine is a biomarker of AGEs that form continuously under normal circumstances and more rapidly under various stressful situations, for example oxidative stress and hyperglycaemia (Dyer *et al*, 1991). Yoshida *et al,* (2005) observed that serum pentosidine concentrations were significantly higher in diabetic patients than in non-diabetic subjects. Furthermore, in diabetic

patients with cardiovascular disease, serum pentosidine concentrations were significantly higher than in those without cardiovascular disease (Kerkeni *et al*, 2014). This serum pentosidine concentration correlates with arterial wall stiffness in diabetic patients (Kerkeni *et al*, 2014).

The findings of Koyama *et al*. (2007) studied 141 patients with heart failure and 18 controls. This study reported that serum pentosidine concentrations were higher in patients with heart failure compared with those without ($p < 0.001$). This suggests that serum pentosidine could be considered an important risk factor in predicting heart failure in relation to heart failure severity. Similarly, Lapolla *et al.* (2007) examined the association between AGEs, malondialdehyde, potential total reactive antioxidants (TRAP) and vitamin E in type 2 diabetic patients with and without the disease. peripheral arteries. They found that levels of AGEs, pentosidine, and malondialdehyde were significantly higher in patients with peripheral artery disease than in patients without the disease and compared with control subjects.

As previously mentioned, many studies have examined the association between AGEs and diabetes-related macrovascular and microvascular complications in order to understand how AGE levels correlate with the development of diabetes-related cardiovascular complications (Huang *et al,* 2017). Obayashi *et al*. (1996) reported the presence of AGEs in atheromatous lesions located in the coronary arteries of patients with diabetes. They suggested that AGEs might accelerate the development of atherosclerosis, as reported in patients diagnosed with diabetes. In support of this result, Yeboah *et al*. (2004) showed that serum AGE levels in patients with type 2 diabetes can be considered as a biomarker to detect the severity of coronary artery atherosclerosis. In addition, Kiuchi *et al.* (2001) on the demonstration of higher levels of AGE in type 2 diabetics with obstructive coronary artery disease compared to diabetics without obstructive coronary artery disease. A correlation between AGE levels and the degree of coronary arteriosclerosis has also

been observed in patients with obstructive coronary disease (Kosmopoulos *et al,* 2019). In addition, it has been reported that elevated serum AGE levels in patients undergoing percutaneous coronary intervention served as an independent risk marker for restenosis in patients with diabetes (Choi *et al*, 2005).

Figure 6 below describes the physiological mechanisms of hyperglycaemia leading to diabetic complications. Here we see hyperglycaemia (high blood sugar) the driving force triggering glycation, oxidative stress, and endothelial dysfunction. This leads to increase arterial stiffness (which can also result from age, smoking, and atherosclerosis) bringing about increase in central blood pressure (this can also result from endothelial dysfunction) and thereby leading to increased chances of various diabetic complications such as CVD, CHD, TOD and increase mortality rate.

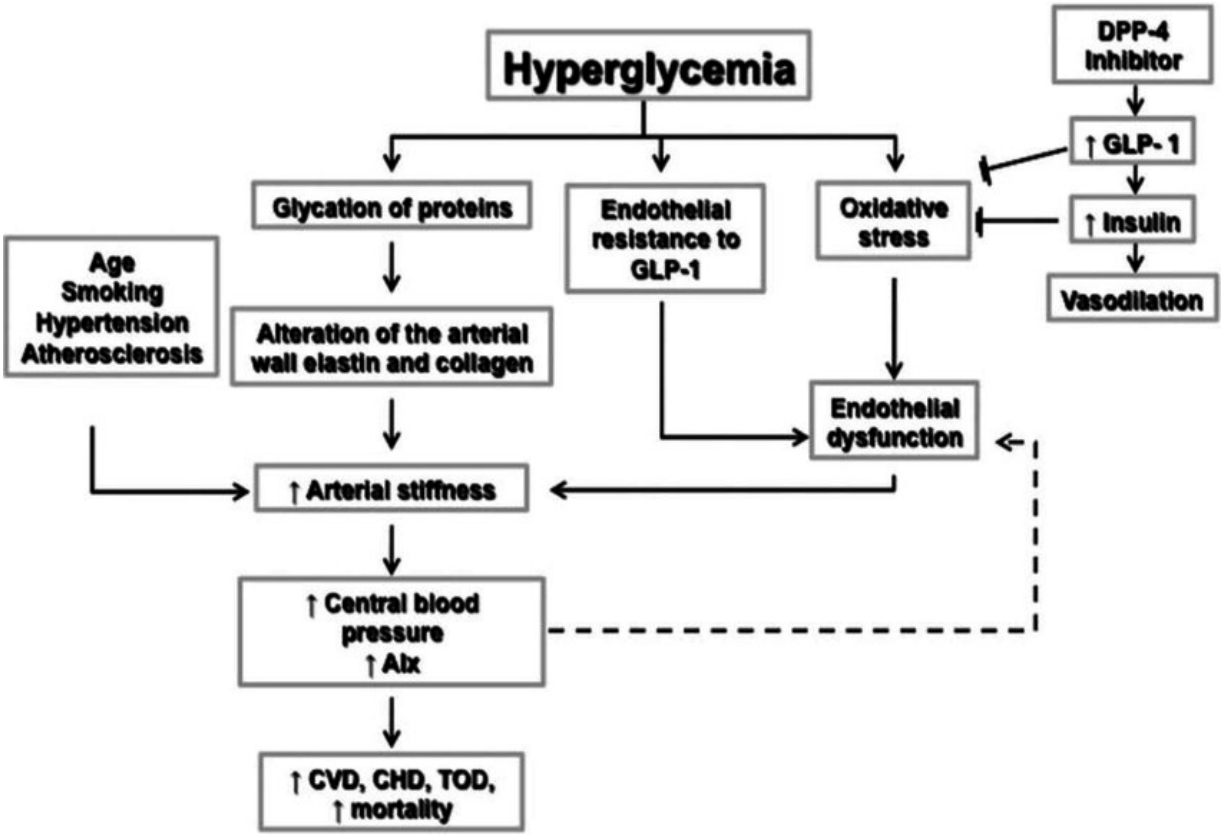


Figure 6: Pathophysiological Mechanisms that Associate Hyperglycaemia, Endothelial Dysfunction, Arterial Stiffness and Cardiovascular Diseases (Kaur *et al*, 2018).

Peripheral vascular disease (PVD) is a common and severe complication of diabetes mellitus (DM) and is characterised by a high prevalence, early development, and rapid progression (Punia *et al,* 2022). In diabetes, there is also an impaired collateralisation of vascular ischemic beds but mechanisms that hinder ischemia induced neovascularisation in diabetes remain ambiguous (Avogaro *et al*, 2008). Emerging evidence indicates that bone marrow-derived endothelial progenitor cells (EPCs) take part in postnatal neovascularization (Rana *et al,* 2018). The EPCs co-express surface markers of both hematopoietic stem cells (CD34 and CD133) and endothelial cells [VEGF-R2, also known as Kinase insert Domain Receptor (KDR)] (Lin *et al,* 2014). The EPCs express endothelial phenotype in culture, promote in vivo re-endothelisation, and can be incorporated into new vessels in animal models of hind limb ischemia (Shen *et al,* 2015). The EPCs are reduced in the presence of risk factors for coronary artery disease (CAD), endothelial

dysfunction, hypercholesterolemia, smoking, and chronic renal failure (Chopra *et al,* 2018). Lambiase *et al.* (2004), have shown that poor coronary collateral development is associated with reduced numbers of circulating EPCs. Thus, depletion of circulating EPCs may contribute to both endothelial dysfunctions, as an early event in the atherogenic process, and poor collateralization, as a late event leading to clinical manifestations of atherosclerosis and cardiovascular disease progression (Leal *et al,* 2019). Recently, circulating EPC reduction and dysfunction have been reported in both type 1 and type 2 diabetic patients: these alterations are likely to be involved in the pathogenesis of vascular complications of DM, but there are no data on this topic currently available in the literature (Nandula *et al,* 2021).

1.4.1.1 AGEs and Diabetic Retinopathy

The leading cause of blindness in individuals between the ages of 30 and 70 years is retinopathy which is a serious microvascular problem of diabetes (Sasongko *et al*, 2017). It is characterised by increased proliferation of blood vessels, vascular occlusion, angiogenesis, loss of pericytes from retinal capillaries, microaneurysms, haemorrhages increased retinal capillary permeability, thickening of the capillary basement membrane and infarction affecting the retina of the eye (Singh *et al*, 2014). Diabetic retinopathy could be due to the death of various lens proteins and retinal cells of the eyes due to AGEs accumulation. The RAGE receptors in the lens are bind to by AGEs and this results to the activation of the signalling pathway, causing oxidative stress and the release of some hormones, cytokines, and adhesive molecules (Vadakedath and Kandi, 2018). RAGE blockade, a potential clinical application includes the decreased progression of diabetic retinopathy as upregulation of RAGE leads to pro-inflammatory responses by retinal Muller glia cells. The role of AGEs in the progression of diabetic retinopathy, which leads to dysfunction and death of various retinal cells, cannot be overstated (Xu *et al*, 2018). Many components of the AGE-RAGE

axis including signal transduction, ligand formation, and terminators may represent promising targets for treatments for diabetic retinopathy (Jangde *et al,* 2020). Preclinical and clinical studies suggest that AGEs and MGOs affect various aspects of diabetic retinopathy. MGO detoxification has recently been evaluated to reduce AGE accumulation, which in turn may prevent the formation of important retinal nerve and vascular lesions. The AGE-RAGE interaction appears to play a key role in the persistent inflammation, neurodegeneration, and retinal microvascular dysfunction that occur in diabetic retinopathy (Shen *et al*, 2020). Several reports have shown that increased formation of AGEs in the vitreous may be involved in the development of diabetic retinopathy by inducing the production of BFGF (basal fibroblast growth factor) by the Müller cells of the retina (Singh *et al,* 2014).

1.4.1.2 AGEs in Diabetic cataract

The loss of the normal transparency of the crystalline lens as a result of an opacity (lens opacity or crystalline opacity) is called cataract (Gupta *et al*, 2014). AGEs play a central role in the loss of vitreous transparency, i.e., the development of cataracts. Cataracts are the main cause of blindness in developed and developing countries and cataract progression is increased in patients with diabetes mellitus (Cicinelli *et al,* 2023). The ocular lens protein glycation is considered as one of the mechanisms of diabetic cataract formation, which is the main cause of blindness, so the identification of risk factors for the prevention and treatment of blindness is important (Li *et al*, 2014; Vadakedath and Kandi, 2018). Recent findings have shown that AGEs play an important role in degenerative changes in the vitreous and that an approach to consuming foods low in AGEs may be beneficial in delaying cataract formation. Crystals (Lim *et al,* 2020). Several reports have revealed that AGEs accumulate in the vitreous and cause visual impairment and cataracts (Bejarano and Taylor, 2019). In the lens, AGE causes irreversible changes in structural proteins,

leading to the synthesis of vitreous proteins and the formation of high-molecular-weight aggregates that scatter light and interfere. Vision (Singh *et al,* 2014). It has been shown that AGEs, by altering the surface charge of proteins, lead to conformational changes which, in turn, can influence protein-protein and protein-water interactions and ultimately lead to a decrease in the degree of solubility. transparency of the vitreous (Kumar *et al,* 2007). The rate of AGEs accumulation is related to the severity of diabetic cataract. Increased glucose levels in the aqueous humour may induce glycation of lens proteins, a process resulting in the generation of superoxide radicals and in the formation of AGEs (López-Contreras *et al*, 2020). Interaction of AGE with RAGE in the epithelium of the lens further increased the O_2- and H_2O_2 generation (Perez-Garmendia *et al,* 2020). In addition to increased levels of free radicals, diabetic lenses show an impaired antioxidant capacity, increasing their susceptibility to oxidative stress (Singh *et al*, 2014). Liu *et al.* (2017) suggested that nuclear factor (erythroid-derived 2)-like 2 (Nrf2) inducers may reduce the oxidative stress of the lens and therefore could be a hopeful therapeutic target for cataract treatment.

1.4.1.3. AGEs in Diabetic Nephropathy

Diabetic nephropathy (DN) is defined as a progressive decline in glomerular filtration rate, accompanied by proteinuria and other end-organ complications such as retinopathy (Sagoo and Gnudi, 2018). Diabetic nephropathy progresses to end-stage renal disease via a number of stages including normal albuminuria, incipient diabetic nephropathy, micro albuminuria and finally end-stage renal disease (Singh *et al*, 2014). DN also referred to as diabetic kidney disease is known by the presence of urine albumin excretion of pathological amounts, diabetic glomerular lesions, and absence of glomerular filtration rate (GFR) in diabetics. DN becoming the most common cause of end-stage renal disease (ESRD) in most countries is as a result of the prevalence of diabetes (Lim,

2014). The formation of AGEs has been attributed to the pathological link between hyperglycaemia and development of diabetic nephropathy. The formation of AGEs also triggers the production of inflammatory cytokines, chemokines, adhesive molecules, and growth factors which may be involved in the pathogenesis of diabetic nephropathy (Vadakedath and Kandi, 2018). Progression to end stage renal disease is enhanced by hyperglycaemia, hypertension, and proteinuria, which are all common in diabetes (Maclsaac *et al,* 2014). Renal disease in diabetic patients is characterized by haemodynamic (hyperfiltration and hyperperfusion) as well as structural abnormalities (glomerulosclerosis, alterations in tubulointerstitium including interstitial fibrosis) and metabolic changes (Liu *et al*, 2017). Within glomeruli, there is thickening of basement membranes, mesangial expansion and hypertrophy and glomerular epithelial cell (podocyte) loss (Anil Kumar *et al,* 2014). Disease progression is also seen in the tubulointerstitial compartment causing expansion of tubular basement membranes, tubular atrophy, interstitial fibrosis, and arteriosclerosis (Agarwal and Nath, 2020). It has been reported that AGE-RAGE axis plays an important role in diabetic nephropathy (Wu *et al*, 2021). Pravastatin has been shown to exert beneficial effects on tubular damage in diabetic nephropathy by inhibiting the AGEs-induced apoptosis and asymmetric dimethylarginine (ADMA) generation tubular cells via suppression of RAGE expression (Ishibashi *et al*, 2012). It is suggested that AGEs play an important role in the pathogenesis of diabetic nephropathy through interacting with RAGE, which activate a series of intracellular signalling pathways (Singh *et al*, 2014). Treatment of DN involves control of metabolic and haemolytic abnormalities adequately. This involves proper blood glucose lowering and control of hypertension (antihypertensives) which helps in the reductions of proteinuria or preservation of GFR, or both (Lim, 2014).

1.4.1.4 AGEs in Diabetic Neuropathy

Diabetic neuropathy is a serious complication, affecting both autonomic and peripheral nerves (Deli *et al,* 2014). Diabetic patients with neuropathy present variable symptoms and physical findings, ranging from asymptomatic loss of tendon reflex to severe painful neuropathy (Smith and Singleton, 2012). Diabetic neuropathy also causes urinary incontinence, diarrhoea, and constipation, impairing the quality of life of diabetic patients (Kurniawan *et al*, 2019). Damage of the peripheral nerves (polyneuropathy) or damage of a nerve due to injury (mononeuropathy) is a common diabetic neuropathic complication. Diabetic neuropathy results from the glycation of cytoskeletal proteins, structural or functional changes to nerve fibres (Vadakedath and Kandi, 2018). Habib and Brannagan in 2010 stated that tight glycaemic control is one of the treatments to manage diabetic neuropathy. Studies have revealed that the AGE-RAGE axis plays an important role in the pathogenesis of diabetic foot involvement in diabetic neuropathy (Juranek *et al,* 2022). The formation of reactive dicarbonyl-induced AGEs has been recognized to play an important role in the pathogenesis of sensory neuronal injury (Singh *et al,* 2014). Nitric oxide (NO), a vasodilator mediator, is reported to be deactivated by AGE (Chen *et al,* 2018). In addition, AGEs affect the expression level of NO synthase, thereby reducing nerve blood flow and causing hypoxia in peripheral nerves (Liu *et al,* 2015). Since RAGE is expressed in endothelial cells of perivascular and endothelial vessels, the interaction between AGEs and RAGE on endothelial cells is thought to play a role in the development of peripheral neuropathy (Egaña-Gorroño *et al*, 2020). Binding of AGEs to RAGE in endothelial cells has been shown to activate NF-B and activate transcription factors protein-1 (AP-1) to increase expression of the vascular cell adhesion molecule-1. and cytokines such as tumour necrosis factor and interleukin 6 (Fukami *et al,* 2014). It was concluded that the formation and accumulation of AGEs in the peripheral nerves suggests the development

of diabetic neuropathy, directly by affecting structural and functional proteins and indirectly by activates AGE receptors (Singh *et al*, 2014).

1.4.1.5 AGEs in Diabetic Cardiomyopathy

Diabetic cardiomyopathy is characterised by myocellular hypertrophy and myocardial fibrosis, resulting in diastolic dysfunction, and has a high incidence of heart failure in diabetes (Bodiga *et al*, 2014). Diastolic dysfunction is present in 50~60% of type 2 diabetics and is almost present in diabetics with microalbuminuria who later progress to systolic dysfunction. Diastolic dysfunction is related to HbA1c levels and the most likely reason for this is accumulation of AGE in the myocardium (Chen *et al*, 2023). AGEs can contribute to the development of heart failure in two ways: AGEs can influence the physiological properties of proteins in the ECM and by inducing cross-linking (Deluyker *et al*, 2016). AGEs can also cause intracellular changes in vascular and myocardial tissue through interaction with AGE receptors (Neviere *et al*, 2016). The diabetic myocyte is subject to a number of metabolic changes that may contribute to contractile dysfunction. Glucose uptake is limited by depletion of glucose transporters, and glucose oxidation is inhibited by high free fatty acid circulation, potentially reducing ATP availability and thus contractile function (Singh *et al,* 2014).

1.4.1.6 Diabetic hepatopathy

The liver plays an important role in regulating carbohydrate metabolism under both physiological and pathological conditions (Bechmann *et al,* 2012). In type 2 diabetes mellitus, insulin resistance causes hyperglycaemia in the liver and in turn further affects glucose uptake or utilization (Mu *et al,* 2019. Diabetes mellitus and its complications, especially complications related to the liver have been linked to a low-grade chronic inflammatory response according to recent research (Pereira

and Alvarez, 2014). Diabetes mellitus is associated also to a variety of liver alterations such as abnormal glycogen synthesis, fibrosis, cirrhosis, acute liver disease and liver hepatitis (Tilg *et al*, 2017). Liver integrity and function can be negatively affected by the over production of fatty acids in the liver and hyperglycaemia thereby contributing to morbidity and mortality in people with diabetes mellitus (Oguntibeju, 2019). Diabetes mellitus (DM) and liver disease have a direct relationship and the joint prevalence of co-morbidity is higher than that associated with an individual condition (Liu and Lu, 2014). Hepatic complications of DM are not easily noticeable. A clinical link between DM and liver diseases such as non-alcoholic steatohepatitis (NASH), non-alcoholic fatty liver disease (NAFLD), liver cirrhosis and metastatic hepatocellular carcinoma have been made known by pre-clinical and epidemiological evidence (Tuttolomondo *et al*, 2015). Non-alcoholic fatty liver disease is a common co-morbidity in approximately 70% of patients with T2DM. This explains the persistent occurrence of obesity and insulin resistance in diabetes (Dong *et al*, 2019). The liver is known to be one of the most susceptible organs to oxidative stress due to hyperglycaemia leading to liver damage (Mohamed *et al,* 2016). Oxidative stress and inflammatory cytokines that contribute to the pathogenesis and progression of diabetes affect the liver (Li *et al*, 2016). According to Oguntibeju 2019, the most common cause of liver disease in the United States today is diabetes, and various types of liver disease such as acute liver failure, cirrhosis, and hepatocellular carcinoma can be seen in patients with type 2 diabetes mellitus and type 1 diabetes (T1DM)

1.4.1.7 Diabetic Autonomic Neuropathy

Autonomic neuropathy, which was once considered to be the Cinderella of diabetes complications, is now of age (Vinik and Erbas*,* 2013). The entire human body is supplied by the autonomic nervous system which is also involved in the regulation of every single organ in the body (Park,

2023). Everything from abnormalities in pupillary function to gastroparesis, intestinal dysmotility, diabetic diarrhoea, genitourinary dysfunction, amongst others are as a result of anxiety from autonomic function (Bell, 2023). Becoming very clear now is that before the advent of severe pathological damage to the autonomic nervous system, there may be an imbalance between the two major arms, namely the sympathetic and parasympathetic nerve fibers (Vinik *et al,* 2013). These supplies the heart and blood vessels, resulting in abnormalities in heart rate control and vascular dynamics. Daily activities of individuals with diabetes can be affected by dysfunction which can lead to potentially life-threatening outcomes (Balcıoğlu and Müderrisoğlu, 2015). Glycaemic control intensification in the presence of autonomic dysfunction (especially if it is combined with peripheral neuropathy) increases the possibility of sudden death and is a warning for aggressive glycaemic control (Vinik *et al*, 2013). Moreover, in patients with diabetes, a decrease in the bioavailability of NO, which acts as a potent vasodilator, was seen, in addition to an increase in the secretion of vasoconstrictor endothelin-1 (Koh *et al,* 2005). This decrease in the bioavailability of NO in diabetic patients might be due to insulin deficiency or resistance in endothelial cells, plus hyperglycaemia was proven to inhibit NO production in arterial endothelial cells (Williams *et al,* 1998). The commonest cause of an autonomic neuropathy in the developed world is diabetes mellitus (Freeman, 2014). These group of symptoms and signs affecting cardiovascular, urogenital, gastrointestinal, pupillomotor, thermoregulatory, and sudomotor systems are caused by diabetic autonomic neuropathy (Tentolouris and Tentolouris, 2021). Several distinct or separate syndromes associated with diabetes cause autonomic dysfunction (Spallone, 2019). Generalised diabetic autonomic neuropathy, autonomic neuropathy associated with the prediabetic state, treatment-induced painful and autonomic neuropathy, and transient hypoglycaemia-associated autonomic neuropathy are the

most prevalent of autonomic dysfunction (Hohler *et al,* 2023). The most problematic and disabling features of diabetic peripheral neuropathy which result in a significant proportion of the mortality and morbidity associated with the disease are products from these autonomic manifestations of diabetes (Freeman, 2014).

1.4.2 Haemoglobin Glycation Index (HGI) And Glycation Gap (GGap)

Diagnosis of diabetes has traditionally relied on blood glucose levels, but now, glycated haemoglobin A1c (HbA1c) as a long-term (approximately 3 months) glycaemic indicator as an essential requirement for the diagnosis and management of diabetes (Parrinello and Selvin, 2014). However, some conditions such as haemolysis and other blood disorders, kidney disease, and iron deficiency affect HbA1c independently of blood sugar levels (Copur *et al*, 2020). Studies have confirmed the difference between HbA1c, and other measures of blood glucose found in a significant proportion (nearly 40%) of people with diabetes (Wang *et al*, 2022). HbA1c is not suitable for assessing a person's glycaemic status in all situations, therefore, the overt use of HbA1c as a diagnostic and therapeutic guide may lead to potential clinical errors (Nayak *et al,* 2019). Recently, the haemoglobin glycation index (HGI) and glycation gap (GGap) were introduced to provide an individual link between HbA1c, and other measures of blood glucose derived from fasting plasma glucose (FPG) and fructosamine/glycated albumin (GA) (Zhang *et al*, 2020). Among them, GA, which is not affected by changes in erythrocyte lifespan, is considered a good surrogate indicator of glycaemic control (Tseng, 2023). Several studies have shown that a higher HGI in a diabetic patient is associated with a higher risk of macrovascular and microvascular complications (Chen and Luo, 2023). The higher the HGI, the higher the risk of developing coronary artery calcification (Klein *et al,* 2021). Few studies have shown a positive association of GGap with nephropathy and retinopathy in patients with type 2 diabetes (Wu *et al*, 2022).

Measurement of HGI and GGap is therefore important for clinicians to enable individualised treatment and to avoid harm from blunt application of HbA1c (Nayak *et al,* 2019) So far, studies on HGI and GGap have been conducted mainly in diabetic patients, especially with type 2 diabetic patients, and there are few clinical or epidemiological studies on GGap from GA. The association of HGI and GGap with CVD in the general population remains unclear (Wang *et al,* 2022).

1.4.2.1 Derivation/calculation of haemoglobin glycation index (HGI) and glycation Gap (GGap)

Some researchers used fructosamine in estimating or calculating GGap while others use glycated albumin and, in both ways, GGap was significantly associated with BMI, diabetes and its complications.

To estimate the discrepancy between HbA1c and other blood glucose measurements, HbA1c and FBG values were used to define HGI, and HbA1c and GA values were used to define GGap (Joung *et al,* 2020). In recent research where researchers analysed the association of FPG-derived HGI and GA-derived GGap with CVD. GGap was found to be significantly and positively associated with the CVD prevalence in adults in general. Furthermore, the high GGap/low HbA1c group was associated with increased CVD compared to the low GGap/high HbA1c group (Wang *et al,* 2022). GGap was generally positively associated with the CVD in adults, and this association was independent of many conventional risk factors, including the HbA1c. HGI, which reflects the difference between observed HbA1c and FPG-predicted HbA1c, was first proposed by Hempe and colleagues (Wang *et al,* 2022). A study showed that high HGI was associated with the risk of complications in his type 2 diabetic, and this association may be mediated by his HbA1c (Li *et al*, 2021). The DEVOTE study found that HGI failed to predict adverse cardiovascular events in his type 2 diabetic patient when HbA1c was included in the statistical model (Klein *et al*, 2021). GGap is primarily attributed to the gradient of nonenzymatic glycation of proteins between the plasma

and intracellular compartments, with several unrecognized factors differentially altering the forward or reverse rate of nonenzymatic glycation (Wang *et al,* 2022). AGEs are known to activate inflammatory signals, increase oxidative stress, ultimately damage arterial endothelial cells, and promote atherosclerosis by promoting lipid oxidation (Hu *et al*, 2020).

GGap is the difference between the HbA1c and Fructosamine predicted FHbA1c. FHbA1c was calculated from the simultaneously measured Fructosamine standardized to the HbA1c distribution as follows: FHbA1c = ((Fructosamine – mean Fructosamine)/SD Fructosamine) x SD HbA1c) + mean HbA1c (Nayak *et al,* 2019).

1.4.2.2 Glycation and the maillard reaction

L.C. Maillard in the early 1900s was the first to study the non-enzymatic reaction of the amino groups of biological amines with reducing sugars, leading to the formation of protein-protein cross-links (Popova *et al,* 2014). Glycation is the result of the covalent bonding of a sugar molecule, e.g., glucose or fructose, to a protein or lipid molecule, without the controlling action of an enzyme (Ahmed *et al,* 2014). Glycosylation on the other hand is the enzyme-mediated ATP-dependent bonding of sugars to protein or lipid molecules at specified site on the target molecule (Liddy *et al*, 2013). Deglycation is an enzyme-mediated pathway and fructoseamine-3-kinase (FN3K) is believed to be one of the major deglycation enzymes (Minoru *et al*, 2005).

Factors that influence erythrocyte survival and regulate intracellular glucose concentrations have been shown. Glucose is the most important free sugar in the body and the least reactive of the other reducing sugars (Tessier, 2010). Nevertheless, glucose reacts with free α- or ε-amino groups of amino acids, peptides, or proteins that form a Schiff base (Djellouli *et al*, 2020). This is unstable

and undergoes a rearrangement process to form a ketoamine product called the Amadori product (Popova *et al,* 2014). This process is described in three phases below:

Level 1): It participates in the formation of a glycoprotein complex called the Amadori rearrangement. This is considered as the precursor of all later compounds.

Level 2): This stage involves the formation of several intermediate compounds. Some of them are very reactive whilst some are not. Reactive compounds continue to participate in the reaction and proceed to the third and final stage.

Level 3): Reaction products formed in the previous step polymerise to form AGEs (Alderawi, 2018, Popova *et al,* 2014).

1.4.2.3 Picture/structures showing glycation and Millard reaction.

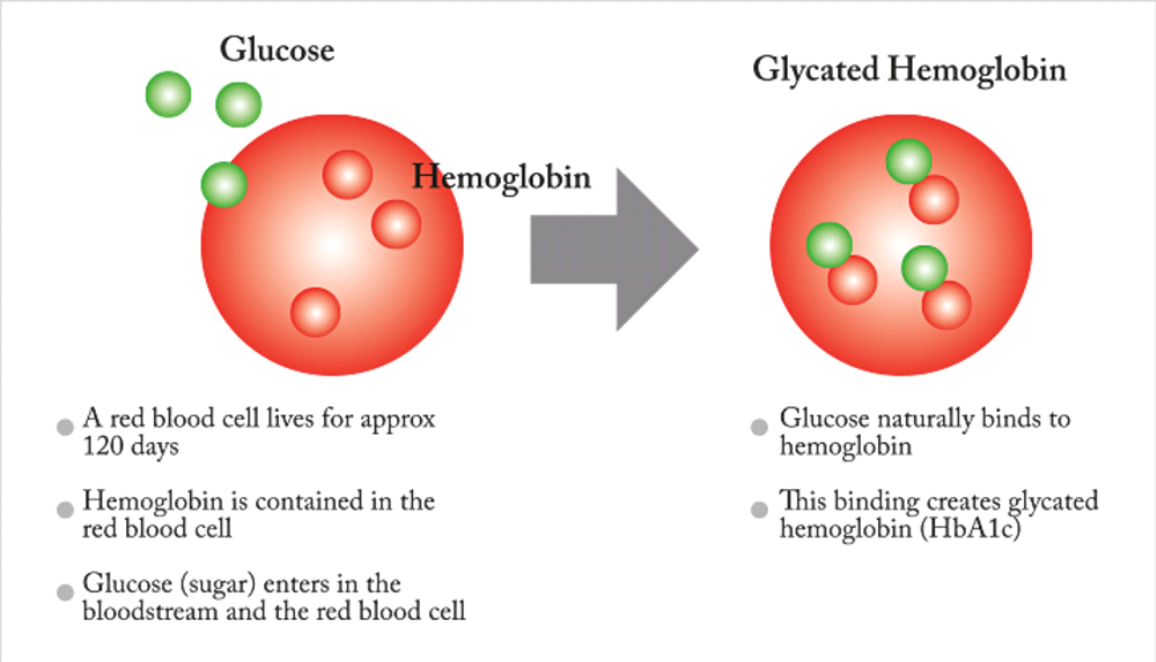


Figure 7: Formation of Glycated haemoglobin (Timothy Warlow Jr, 2015)

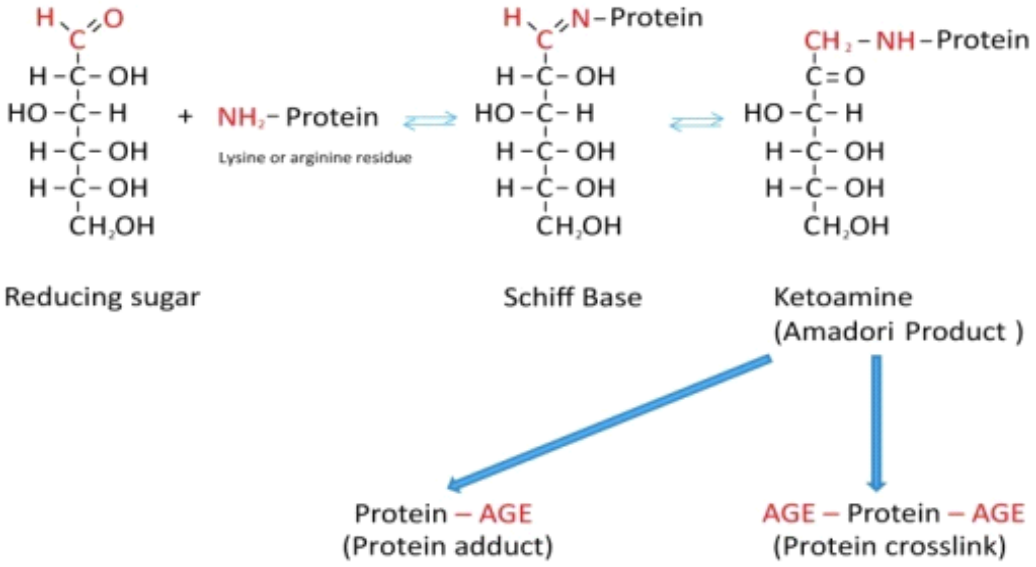


Figure 8: The initial steps of glycated haemoglobin formation from D glucose (Paul *et al,* 2021)

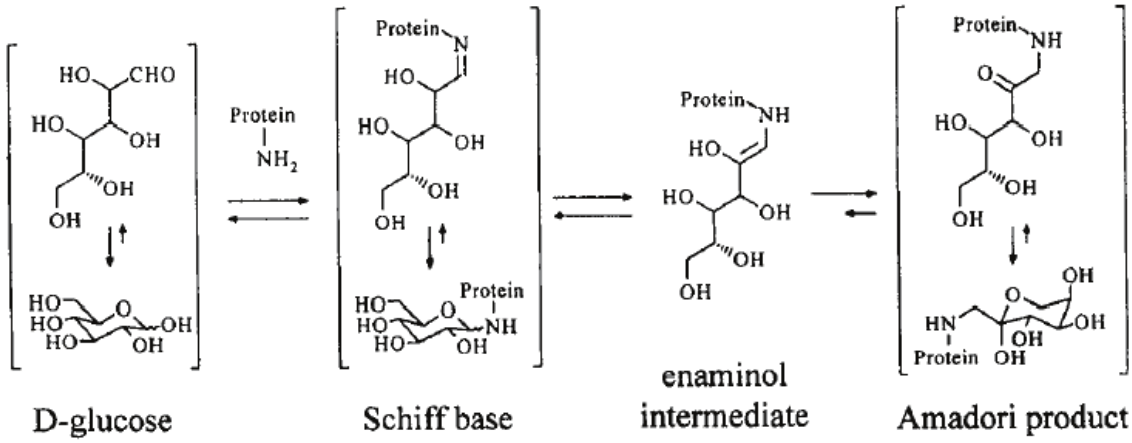


Figure 9: Formation of Amadori product (Sharma *et al*, 2020)

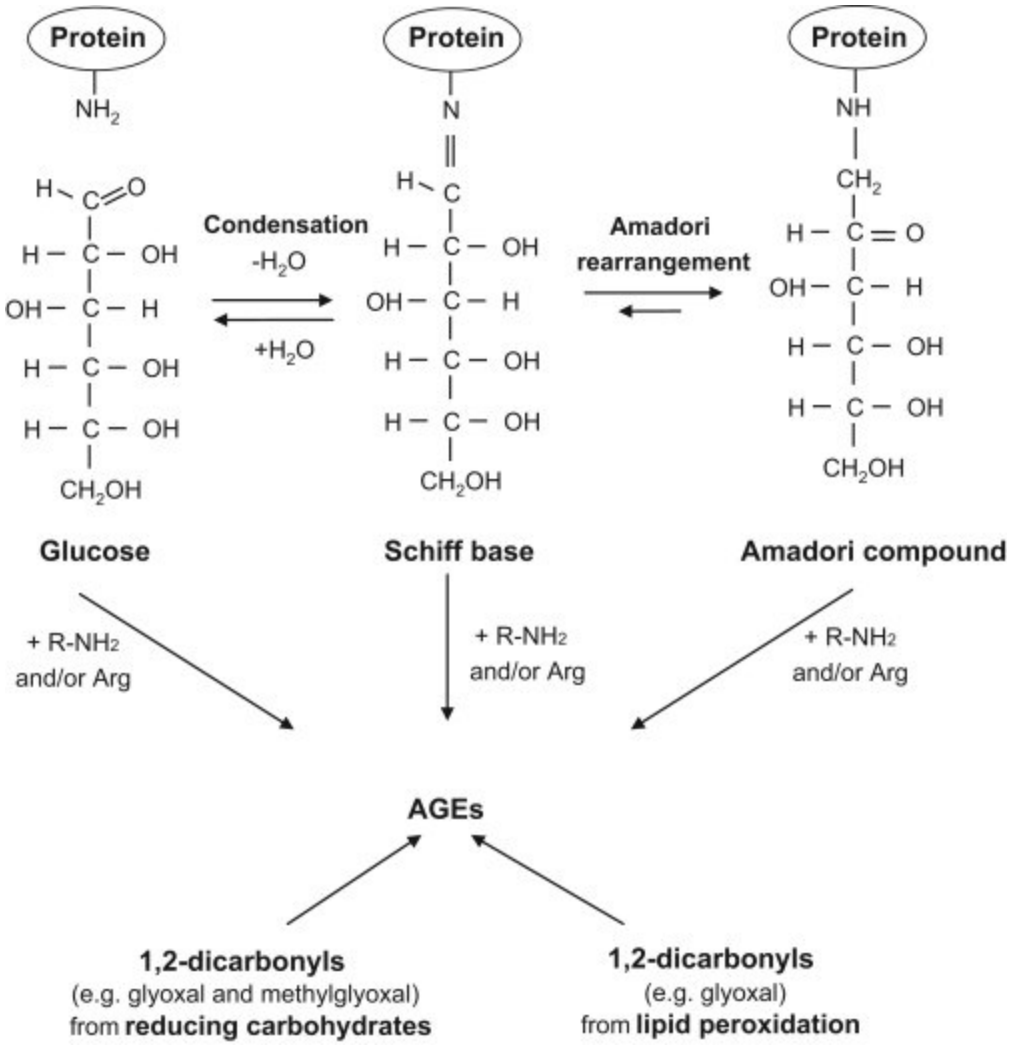


Figure 10: above shows the formation of glycated haemoglobin, the rearrangement of amadori products and the formation of AGEs (Chen *et al,* 2022; Stirban *et al*, 2014).

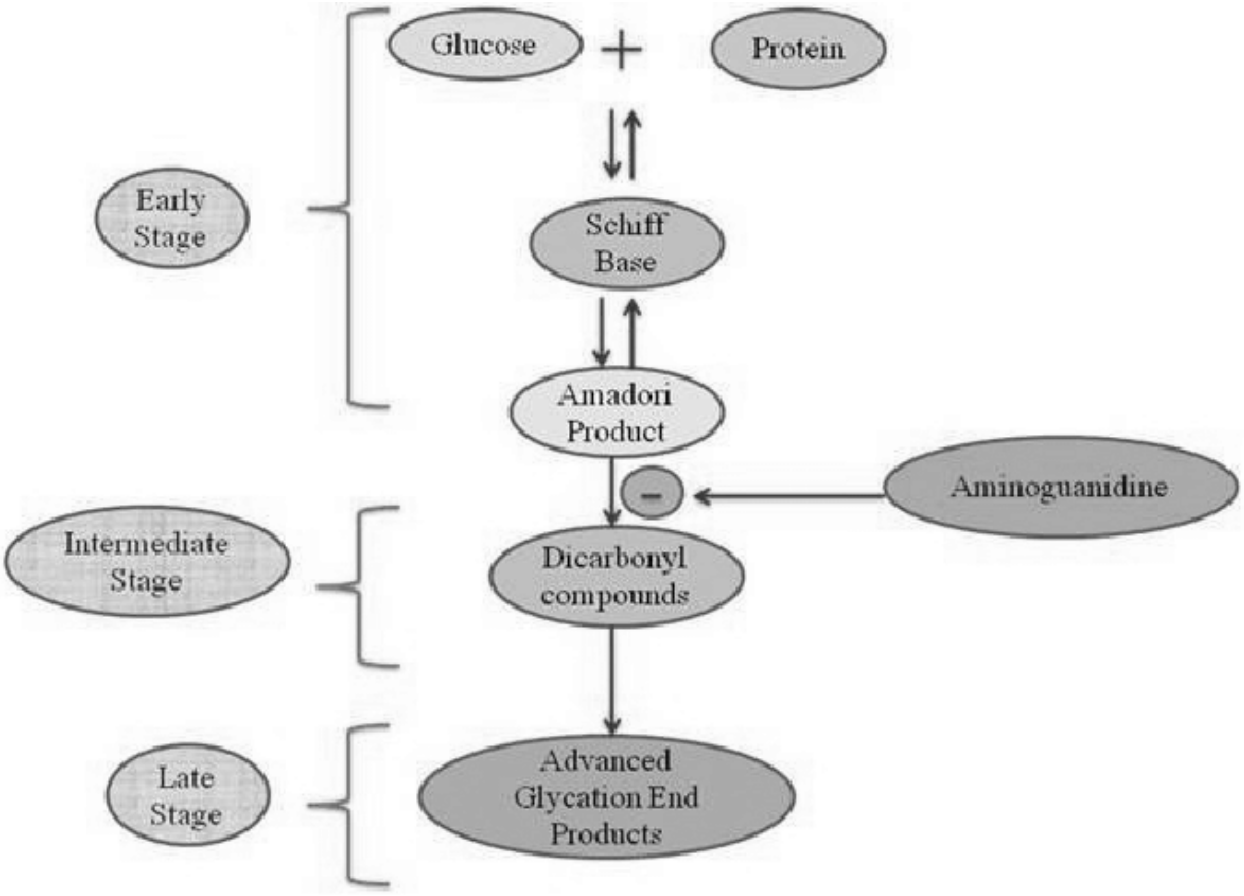


Figure 11 Early stage (Reversible) and AGE formation (Irreversible) (Singh *et al,* 2014)

1.4.2.4 Deglycating enzymes

Deglycation is the removal of sugars from proteins by FN3K, a known kinase that has been reported to be directly involved in phosphorylation of bound sugars and protein destabilisation (Beeraka *et al*, 2021).

The discovery of fructosamine-3-kinase (FN3K), a protein distantly related to aminoglycoside kinases and distantly related to protein kinases, began with the identification of fructose-3-phosphate in human and animal tissues (Motshwari, 2018). Fructose-3-kinase phosphorylates fructosamine with a Km in the micromolar range (Alderawi, 2017). The enzyme has been purified

from human erythrocytes and its cDNA cloned (Avemaria *et al*, 2015). `Basic amino acids such as lysine and arginine are phosphorylated by FN3K to mediate deglycation (Beeraka *et al,* 2021). FN3K mediates the phosphorylation of fructoseridine residues on glycated proteins, resulting in the formation of protein-bound fructolysine-3-phosphate in human tissues (Schhaftingen *et al*, 2012). FN3Ks are actively involved in promoting cell signalling to mitigate oxidative stress, promote cellular repair, and exhibit antitoxin defence mechanisms with pronounced transcriptional regulation (Beeraka *et al,* 2021). In human erythrocytes, FN3K mediates the phosphorylation of sorbitol or fructose to produce sorbitol-3-phosphate and fructose-3-phosphate, respectively (Alderawi, 2017). Therefore, both FN3K and FN3K-RP have unique substrate specificities to phosphorylate protein-bound ketosamines (Avemaria *et al*, 2015). FN3K activity can be recycled in the deglycation pathway to mitigate AGE formation during metabolic diseases (Beeraka *et al,* 2021). FN3K is abundant in both eukaryotes and prokaryotes as it contains one copy of the FN3K gene. However, mammalian cells are composed of her two copies of the gene encoding FN3K and FN3K-related protein (FN3K-RP) (Naseem, 2019). FN3K is highly conserved in various organisms and maintains cellular homeostasis by mitigating AGE-mediated oxidative stress (Chaudhuri *et al*, 2018). Prospective studies should focus on elucidating the fundamental role and regulation of FN3K in multiple cellular functions and disease models (Schhaftingen *et al*, 2012). FN3K is found more frequently in liver, heart, kidney, brain, skeletal muscle and relatively frequently in erythrocyte (Alderawi, 2017). FN3K consists of 309 amino acids and is encoded by chromosome 17q25.3 of the human genome (Motshwari, 2018). FN3K and fructosamine-3-kinase-related protein (FN3KRP) isoforms are found in eukaryotes such as mammals, birds, amphibians, fish, and nematodes (Beisswenger, 2009). Several studies have shown an important role for FN3K

in protein glycation and the accumulation of AGEs in several types of cancer. (Beeraka *et al,* 2021).

Three group of enzymes which are capable to reverse the Amadori formation have been discovered to date.

First includes oxidases, which were first reported in Cornebacterium sp. and Aspergillus sp. These flavin adenine dinucleotide (FAD)-containing enzymes were found to deglycate fructosyl amino acids into glucosone and the primary amine under formation of H_2O_2 (Popova *et al,* 2014).

A second group was discovered by Van Shaftingen and co-workers who cloned and fully characterised the enzyme fructosamine 3-kinase. This enzyme phosphorylates with high affirnity both low molecular mass and protein bound fructosamines, leading to the formation of fructosamine 3-phosphates which are unstable and spontaneously decompose into inorganic phosphate and 3-deoxyglucosone (Collard *et al,* 2004, Popova *et al*, 2014).

FN3K is present in many tissues including skeletal muscle and heart, where the intracellular glucose concentration is more than 10-fold lower than that in plasma. Fructosamine-6-phosphates, which are more powerful glycating agents than glucose result from the reaction of amines with Glu-6-P, but they are not substrates for FN3K (Van Schaftingen *et al*, 2012). Magnesium-dependent phosphatase (MDP-1) converts fructosamine-6-phosphates to fructosamines (Popova *et al,* 2014).

A third group was reported by Van Shaftingen and co-workers who found that fructoselysine can maintain the growth of Escherichia coli. These findings proposed the presence of a whole set of genes (a bacterial operon) which controls the uptake and metabolism of fructoselysine (Popova *et al,* 2014).

1.4.2.5 FN3K Inhibitors

DMF (1-deoxy-1-morpholinofructosamine), a cell permeable substrate and competitive inhibitor of FN3K has provided evidence that this enzyme remove fructosamine residues from haemoglobin in erythrocytes (Collard *et al*, 2004).

Collard *et al*, 2004 states that NMR analysis indicated that 1-deoxy-1-morpholinopsicose (DMP, a substrate for FN3K and FN3K-RP) also penetrate erythrocytes and converted into the corresponding 3-phospho-derivative. FN3K-RP phosphorylate intracellular, protein-bound psicosamines and ribulosamines, thus leading to deglycation (Conner *et al,* 2004 and 2005, Collard *et al*, 2004).

1.4.2.6 Clinical Importance of Glycoproteins

N-glycans and O-glycans play an important role in glycoprotein functions involved in cell recognition signals and various pathological situations, thus they are powerful and reliable biomarkers. reliability of various diseases (Guo *et al,* 2022). Given the large number of biological processes in which glycans are involved, it is not surprising that defects in glycan synthesis can be a direct cause of many diseases and thus a hallmark of disease (Smith and Bertozzi, 2021). It was not until the 1980s that NMR began to attract great interest in the search for clinically relevant signs, including APP (Wishart, 2019). It is important to note that most serum glycoproteins have N-linked sugars and, less often, O-linked sugars in their structure (Yang *et al,* 2017). As more and more information about protein glycation emerges, it becomes increasingly clear that glycation is tightly regulated and that protein-binding glycans are of paramount physiological importance (Fuertes-Martin *et al*, 2018).

The most clinically important and most studied glycoproteins are the membrane glycoproteins, whose glycans, called glycocalyxes, play an important role in the immune response (Štambuk *et*

al, 2021). Human plasma glycoproteins belong to the large APP family, which is characterised by an increase or decrease in their concentration (positive or negative acute phase proteins, respectively) by up to 25% in inflammatory disorders (Roy *et al,* 2017). Most of these APPs are glycosylated proteins secreted by hepatocytes (Kuscuoglu *et al*, 2018). Changes in the glycosylation of these proteins indicate cellular changes in a large number of diseases, which is why they can be considered diagnostic markers of disease (Silsirivanit, 2019). Multiple alterations in serum protein glycosylation have been reported for inflammatory diseases (Fuertes-Martin *et al*, 2020).

1.4.3 Glycoprotein Acetylation (GlycA)

GlycA, a marker of systemic inflammation, was found to be connected with incident T2DM in a general population of both men and women (Connelly *et al*, 2016). GlycA may also give corresponding and maybe interesting information to hsCRP (C-reactive protein) for the likelihood of future T2DM (Brunoni *et al*, 2020). Not only are the synthesis and secretions of these proteins increased during inflammation, but their glycans structures are dynamically altered (Groux-Degroote *et al,* 2020). For example, the carbohydrate side-chains of α 1-acid glycoprotein, one of the acute phase glycoproteins captured in the GlycA signal, become highly branched and contain additional GLCNAc residues in the context of chronic inflammation and cancer (Margery *et al*, 2015).

GlycA is a new composite measure of systemic inflammation and a forecaster of many inflammatory diseases, measured through nuclear magnetic resonance (NMR), whose signal arises from N-acetyl glucosamine residues on the glycans of acute-phase proteins (APPs) (Bartlett *et al*, 20017). GlycA receives contributions from glycoproteins which are involved in the so-purported ‘acute-phase response’ to exogenous insults, including infections and physical injury (Ritchie *et*

al, 2015). This is a broad set of systemic biochemical and physiological changes, primarily driven by cytokine production of inflammatory cells, which occurs immediately after the insult (Turner *et al,* 2014). With the exception of transferrin, the circulating concentrations of the proteins that constitute the GlycA signal are known to increase during the acute-phase response (Ritchie *et al,* 2015).

Proton nuclear magnetic resonance (NMR) spectroscopy has the ability to detect signals from circulating glycoproteins (Unione *et al*, 2021). NMR-based glycoprotein biomarker, labelled GlycA, which shows both the protein levels and enhanced glycosylation states of the most abundantly expressed acute-phase proteins, has been shown to predict the development of CVD as well as the progression of type 2 diabetes mellitus (T2DM) in women (Dullaart *et al*, 2015).

Specific glycan structures of acute-phase proteins are enzymatically modified under chronic inflammatory conditions suggesting that GlycA, which represent an integrated plasma glycoprotein biomarker, reflects a pro-inflammation state beyond associations with the individually captured protein (Gruppen *et al*, 2015). NMR-based risk score, and α-1-acid glycoprotein (GlycA NMR signal), may predict all cause and CVD mortality. The relationships of GlycA and hs-CRP with the leptin/adiponectin ratio are independent from BMI which agrees with the hypothesis that abnormalities in adipose tissue function could contribute to a pro-inflammatory state (Dullaart *et al*, 2015).

Although GlycA is understood to mark the acute-phase response, it has also been shown to be elevated in patients with a diverse range of inflammation-linked chronic conditions, including rheumatoid arthritis, hypertension, obesity, and metabolic disorders. Evidence is now evolving to suggest that elevated CRP is not a universal feature of chronic inflammation. Importantly, GlycA,

a composite marker of multiple molecular species, has been shown to predict risk of all-cause mortality and cardiovascular disease (CVD), even when adjusted for CRP (Ritchie *et al,* 2015).

GlycA is a biomarker for cardiometabolic disease risk which is also associated with CVD (Connelly *et al*, 2017). It measures CVD risk independently of CRP and may be a marker of a different inflammatory process. GlycA concentrations are higher in patients with inflammatory autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus (SLE), and psoriasis. It is also associated with disease activity and coronary atherosclerosis in RA as well as active vascular inflammation in psoriasis (Bartlett *et al*, 20017).

Inflammatory processes are characterised by enhanced acute phase protein secretion and glycosylation. There is a positive relationship of GlycA with high-sensitive C-reactive protein (hs-CRP) but with cardiometabolic risk factors are still limited (Dullaart *et al,* 2015). GlycA is elevated in several chronic inflammatory diseases and associated with disease incidence. It is elevated in patients with various autoimmune diseases and is associated with disease activity and coronary atherosclerosis in rheumatoid arthritis patients (Connelly *et al*, 2016). In the Women's Health Study (WHS), PREVEND study and the JUPITER trial, GlycA was associated with incident CVD events, independent of traditional risk factors. GlycA was also found to predict incident T2DM in a large population of healthy women. These data raise the possibility that GlycA provides added value for the evaluation of CVD and diabetes risk (Connelly *et al*, 2016; Gruppen *et al*, 2019).

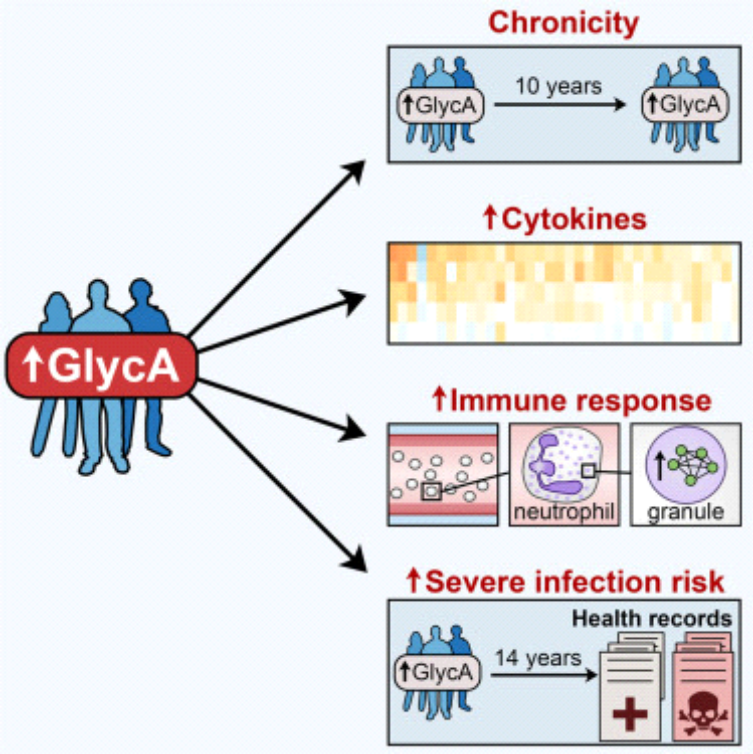


Figure 12: GlycA association with chronic inflammation (Ritchie *et al*, 2015)

Low grade inflammation is known to trigger the development of IR and loss of β-cell function, both of which are proposed to be implicated in the pathogenesis of T2DM (Zetterale *et al,* 2020). Accumulating evidence suggests that carbohydrate metabolism and the immune system are intricately linked (Delmastro-Greenwood and Piganelli, 2013). Nutritional excess leads to enhanced systemic inflammation via multiple mechanisms including obesity and ensuing adipose tissue inflammation and alterations in the intestinal microbiome (Reilly and Saltiel, 2017). In turn, tissue and circulating inflammatory mediators may be casually implicated in the development of IR and β-cell dysfunction, factors that are key for the progression to T2DM (Margery *et al*, 2015).

Insulin-secreting pancreatic beta-cells may likely undergo strong intracellular protein glycation at elevated glucose concentrations because of high glucose permeability (Maulucci *et al*, 2016). They may, however, be partly protected from the glucotoxic alterations of their survival and function by

fructosamine-3-kinase (FN3K), a ubiquitous enzyme that initiates deglycation of intracellular proteins (Pascal *et al*, 2010).

NMR can also provide quantifications of serum glycoprotein acetylation as well as lipoprotein parameters, which are a synthetic inflammatory biomarker that predicts CV events in other patient populations (Ritchie *et al*, 2015). It has been hinted that neutrophils are an important source of two major proteins that contribute to GlycA signaling, the acid glycoprotein α1 and haptoglobin, although GlycA represents the aggregate NMR signaling of several acetylated glycoproteins (Scott, 2019). chemical. High GlycA has also been shown to be associated with neutrophil activity. Inflammatory pathways produce acetylated glycoproteins that may play a role in promoting atherogenesis, a marker of GlycA with markers of inflammation and plague (Purmalek *et al*, 2019).

Glycans are complex oligosaccharides which does not only play a fundamental role in human health but also contribute to the development of many complex inflammatory diseases (Singh, 2019). Glycoprotein acetylation which is a novel nuclear NMR biomarker measured in blood serum or plasma, summarises the NMR signal coming from the glycan groups of certain acute-phase glycoproteins (mainly α-1-acid glycoprotein, haptoglobin, α-1-antichymotrypsin, and transferrin). GlycA has been intensively researched in CVD and was found to be wholesomely connected with atherosclerosis and risk of future CVD (Mostashari *et al,* 2023). Wholesome links with both CVD and diabetes in large cohorts have resulted in the sum up that GlycA summarises the summative risk resulting from multiple inflammatory pathways (Otvos *et al,* 2015).

The GlycA advantage over CRP likely comes from its greater stability, due to its composite nature as a summary measure of glycan signal coming from several acute-phase proteins (Menzel *et al*, 2021). Circulating the true set point for biological homeostasis of CPR requires 33 concurrent

measurements, whereas for the GlycA only a single measurement is required as showed by various research (Rodriguez-Carrio *et al,* 2020). GlycA has proved to be a stable and sensitive marker for chronic inflammatory rheumatoid arthritis in contrast to CRP (Mehta *et al*, 2020). Seeing that patients prefer regular blood instead of regular stool sampling, replacing CRP with another serological biomarker in patients who cannot be biochemically monitored using CRP might be a better alternative than a faecal biomarker (Singh *et al*, 2023). Several metabolic biomarkers such as amino acid levels, glycolysis and fatty acid metabolites, and an extensive lipoprotein profile are quantified by GlycA also (Dierckx *et al,* 2019).

Although the role of glycans in the pathogenic mechanism is still not fully understood, plasma levels of glycosylation have been implicated in various diseases, most of which have a marked inflammatory component (Theodoratou *et al*, 2014). Plasma glycoproteins belong to the APP family, the concentration of which is increased in the presence of an inflammatory process (Dungan *et al*, 2015). To a large extent, they can be considered diagnostic markers of disease. It is important to note that the 1H-NMR technique is a quantitative technique for measuring the overall state of glycation, but it does not identify exactly which proteins are involved (Silva *et al*, 2020). What all results had in common was an increase in GlycA levels or circulating levels of N-acetyl glycoprotein compared with the control group (Fuertes-Martin *et al*, 2020). The GlycB variable was found to be associated with these parameters but less strongly. Although CRP has been the most studied inflammatory biomarker since ancient times, it has been shown to fluctuate for a variety of reasons (Dungan *et al,* 2015). These associations give room for the following questions. The first is that the two markers may follow separate inflammatory pathways as several studies have reported different behaviour between GlycA and hsCRP. The second is that GlycA can integrate more inflammatory pathways by global signalling of certain proteins and thus, better

capture systemic inflammation levels. However, regardless of their possibly distinct inflammatory origin, GlycA has clear advantages over CRP (Fuertes-Martin *et al*, 2020).

While hsCRP needs to be measured multiple times on consecutive days to determine cardiovascular disease risk, GlycA only needs one measurement (Brunoni *et al*, 2020). On the other hand, in previous studies ensures high reliability of GlycA because its measurements are similar in serum and plasma samples, during fasting and non-fasting, as well as after short-term or long-term storage for example, there is a lower intra-individual level of GlycA variability than hsCRP (Otvos *et al*, 2015).

1.5 INFLAMMATION AND DIABETIC COMPLICATIONS

Inflammation is a section of the body's defence mechanism through which the innate immune system recognises and responds to harmful and foreign stimuli and begins the healing process (Antonelli and Kushner, 2017). Inflammation can be classified into two main groups, which is acute and chronic. There is also subacute inflammation which is in between acute and chronic inflammation and can persist for 2 to 6 weeks. Acute inflammation refers to a situation where the tissue damage is due to trauma, microbial invasion, or noxious compounds. It begins rapidly and becomes severe in a short time; however, symptoms may persist for a few days. Chronic inflammation on the other hand is referred to as slow, but long-term inflammation lasting for prolonged periods ranging from several months to years. The extent and effects of chronic inflammation depends on the cause of the injury and the ability of the body to repair and overcome the damage (Pahwa *et al*, 2021).

Inflammatory stimulation of cytokines can also lead to inducible nitric oxide (iNOS) (one of the reactive oxygen and nitrogen metabolite responsible for metabolising enzymes) induction (Zamora

et al, 2000). Overproduction of nitric oxide also seems to contribute to impaired insulin action in muscle cells and β-cell function in obesity (Bahadoran *et al,* 2020). Inhibition of iNOS prevents the attenuation of insulin signalling in muscle caused by a high-fat diet. Thus, induction of suppressor of cytokine signalling (SOCS) and iNOS proteins represent complementary and potentially important mechanisms that contribute to cytokine-mediated insulin resistance (Dragone *et al,* 2014). It is likely that additional mechanisms linking inflammation to insulin resistance remain unexplored (Gacia *et al,* 2010).

1.5.1 Inflammatory Marker

Endothelial function does more than just provide a smooth, non-sticky lining for blood vessels (Tariq *et al,* 2020). The properties of the endothelium, especially its secretion, not only keep the endothelium itself healthy, lubricious and "free of stickiness", but also regulate the function of smooth muscle in the vessel wall, platelet activity, and leukocytes within the vessel (Krüger-Genge *et al*, 2019). The endothelium plays a role in maintaining vascular tone while regulating vascular responsiveness to various challenges (stresses), 2, modulating platelet aggregation and plasma coagulability, thereby regulating the thrombotic process; 3, regulation of fibrinolysis and dissolution of formed intravascular thrombin; 4, regulation of leukocyte adhesion, activation and chemokines; hence regulation of inflammation (Kim *et al*, 2017).

Inflammation as a major mediator of atherogenesis may also adversely affect other endothelial functions (Tousoulis *et al,* 2008). Studies have shown that endothelial inflammation and a wide range of other functions are in turn regulated by ambient glucose levels, insulin, insulin-resistant states, and diabetes (Bakker *et al,* 2009). Inflammation is thought to play a role in the pathogenesis of T2DM, also increased concentrations of interleukin 6 (IL-6) and high-sensitive C-reactive protein (hs-CRP) have been associated with an increased risk of T2DM (Nkinda *et al*, 2019).

Elevated plasminogen activator inhibitor type 1 (PAI-1) has also been found to be a predictor of the development of T2DM (Kim *et al*, 2017).

1.5.2 The role of adipokines in diabetes-induced inflammation

Adipokines, secreted by adipose tissue, are required for a number of physiological and metabolic processes (Balistreri *et al,* 2010). Despite the potential importance of these agents as putative mediators of metabolic disorders, little is known about their implications in gestational diabetes mellitus (GDM) and macrosomia (Caputo *et al,* 2020). It has been shown that adiponectin and TNF-α produce opposite effects on insulin signaling, with TNF-α inhibiting and adiponectin increasing tyrosine phosphorylation of the insulin receptor (Barchetta *et al,* 2017).

Adipocytokines are a group of biologically active molecules derived from the adipose tissue, with an increasingly recognised role in glucose metabolism and inflammatory disease states (Karampela *et al,* 2019). Adipocytokines have been implicated in various glucoregulatory processes such as insulin sensitivity, insulin secretion, and inflammation. The role of adipocytokines in glucose metabolism has been abundantly explored in the context of type 1 and type 2 diabetes mellitus. Studies to date have typically investigated adipocytokines as predictors of acute pancreatitis severity and a recent study highlighted a significant association between leptin and persistent hyperglycaemia in acute pancreatitis (Gillies *et al,* 2016).

Interleukin-6 (IL-6) is a cytokine with diverse physiological and pathological functions, including an established role in glucose metabolism (Pedersen *et al*, 2007). Extensive research has been undertaken to determine how adipocytokines such as IL-6 affect glucose metabolism in type 1 and type 2 diabetes mellitus (Libby *et al,* 2012). Findings however, evolved as controversial, with IL-6 shown to have beneficial effects in skeletal muscle through enhanced glucose metabolism and

clearance, yet appearing to have a proinflammatory action in adipose and hepatic tissue. Research surrounding the role of IL-6 in glucose metabolism maintains that diabetic patients present with elevated serum IL-6 concentrations compared to non-diabetic patients (Murahorschi *et al,* 2014). Several literatures support an increased risk of developing type 2 diabetes mellitus with raised IL-6 concentrations and a positive correlation between IL-6 and HbA1c, a known marker of chronic hyperglycaemia and the development of diabetes mellitus, with further long-term inflammatory and clinical implications also associated with IL-6 (Gillies *et al*, 2016).

Omentin is a novel adipokine known to prevents TNFα-induced endothelial cell COX2 expression and also causes endothelial nitric oxide synthase. It is mostly found in visceral fat tissues and places like the heart, lungs, ovary, and placenta (Shibata *et al,* 2012).

Secreted frizzled-related protein 5 (SFRP5) is known to have anti-inflammatory effects in adipose tissue and in macrophages which are mediated through the inhibition of Wnt5a/JNK signaling, which eventually inhibits macrophage TNFα, IL-1β, and CCL2–MCP1 synthesis (Cho *et al,* 2018). It belongs to the SFRP family, and it's known to have insulin-sensitizing effects. It is a secreted protein produced by many tissues in the heart, liver, pancreatic islets, and mononuclear blood cells (Carstensen-Kirberg *et al*, 2017).

Apelin is an endogenous peptide, commonly known as a ligand of the orphan G protein-coupled receptor APJ (De Falco *et al,* 2002). It is mostly found in the lung, testis, and uterus. Apelinergic system might play a role in hypertension, cardiac contraction, heart failure, T2DM, and obesity. Signal transduction pathways-PI3K/Akt, extracellular signal-regulated kinase (ERK), MAPK, and endothelial nitric oxide synthase (eNOS) might be anticipated as mechanisms underlying the preventive effects of apelinergic system in CVDs (Castan-Laurell *et al,* 2011).

Table 1.1 shows a summary of different adipokines and their functions.

Adipokines	**Functions**
Anti-inflammatory Adipokines	These are adipokines with substance or properties that fight against inflammation.
Adiponectin	It has anti-inflammatory properties proven to inhibit inflammation by way of blocking NF-κB activation and reducing cytokines such as TNFα, IL-6, and IL-18.
C1q/TNF-related protein (CTRPs)	It inhibits TLR4 activation. Also inhibits inflammation in lipid-loaded hepatocytes and increases insulin sensitivity.
Omentin	It prevents TNFα-induced endothelial cell COX2 expression and causes endothelial nitric oxide synthase (Shibata *et al,* 2012).
Secreted frizzled-related protein 5 (SFRP5)	SFRP5 have anti-inflammatory effects in adipose tissue and in macrophages. It's known to have insulin-sensitizing effects.
Apelin	It might play a role in hypertension, cardiac contraction, heart failure, T2DM, and obesity.
sFRP4	Interact with FZDs and Wnts, act as soluble modulators, change Wnt signals by contending with FZDs to bind Wnts. associates with the development of beta cell dysfunction. It contributes to lipid accumulation in the liver, sFRP4 selectively inhibits the insulin signalling pathway.
Pro-inflammatory Adipokines	These adipokines can cause inflammation and they play a crucial role in immune system modulation. The production of most adipokines is upregulated in the obese state, and these pro-inflammatory proteins typically function to promote obesity-linked metabolic diseases (Ouchi *et al,* 2011).
Leptin	It initiates intracellular signaling, activates the tyrosine kinase, the latent transcription factor and PI3K pathways, then activate the innate immune response. Leptin may improve the production of several proinflammatory cytokines, chemokines and CCL2/MCP-1, and the lipid mediators and leukotriene in peripheral blood monocytes and resident tissue macrophages. Leptin induces the production of reactive oxygen intermediates in macrophages, neutrophils, and endothelial cells and potentiate IFNγ-induced expression of nitric oxide synthase. It enhances platelet aggregation and promotes leukocyte–endothelial cell interactions. It enhances T_H1 and T_H17 responses, prevent T-cell apoptosis.
Resistin	Resistin influences insulin homeostasis.
Chemerin	Chemerin functions as chemo-attractant for cells of the innate immune system. It prevents macrophages from polarizing into an anti-inflammatory phenotype.
Retinol binding protein 4 (RBP4)	It transports retinol from the liver to the peripheral tissues, linked in the development of insulin resistance, contribute to inflammation. It inhibits insulin in skeletal muscle cells leading to insulin resistance.
Lipocalin 2 (LCN2)	LCN2 binds and transports hydrophobic molecules. Plays an essential role in host defence against bacterial infections by sequestering iron. It serves as protective factor in the CNS in response to systemic inflammation.
Adipsin	Adipsin plays pivotal roles in models of ischemia reperfusion and sepsis. It is a key link between fat cells, obesity and beta cell function.
WISP1 (Wnt1-inducible signalling pathway protein-1)	Regulates adipogenesis and-low grade inflammation in obesity. It plays a vital modulatory role in cell growth. Play a key role in the pathogenesis of polycystic ovarian syndrome (PCOS). It impairs insulin signalling in muscle and liver cells.
Visfatin	Function in the immune system, a growth factor for early B cells, binds and activates insulin receptor. It has an enzymatic nicotinamide phosphoribosyltransferase (Nampt) function.

Resistin is a polypeptide produced and released by adipocytes with a molecular weight of 11.3 kDa and 108 amino acids, including 11 cysteines, in rodents and mainly by macrophages, monocytes, and pre-adipocytes in humans (Farkhondeh *et al,* 2020). Resistin in mouse and human share 46.7% genomic DNA homology, 64.4% mRNA sequence resemblance, and 59% amino acid's identity (Li *et al,* 2021). Resistin is known to influence insulin homeostasis, but the association between its serum levels and T2DM, insulin resistance, or obesity is unclear. An increase in resistin levels correlating with these pathologies is still questionable (Acquarone *et al,* 2019).

Chemerin is found in a number of tissues like liver, pancreas, and lung and has been demonstrated to function as chemo-attractant for cells of the innate immune system. The presence of chemerin was found to prevent macrophages from polarizing into an anti-inflammatory phenotype resulting in impaired expression of Arginase-1 and IL-10 (Bozaoglu *et al,* 2007). The chemokine-like receptor 1 (CMKLR1) is a receptor for chemerin and highly expressed in adipose tissue. Mice deficient in CMKLR1 was found to develope DSS-induced colitis in a delayed time course, although presented with a related disease activity (Weidinger *et al,* 2018).

Retinol binding protein 4 (RBP4) is a member of the lipocalin family of proteins that transports retinol from the liver to the peripheral tissues. RBP4 is produced by the liver, adipose tissue, and macrophages known to be linked in the development of insulin resistance (Broch *et al,* 2010). RBP4 may contribute to inflammation by activating adipose tissue antigen-presenting cells that promote T_H1-cell polarization. The signal pathways stimulated by insulin in skeletal muscle cells is inhibited by circulating RBP4, which leads to the development of insulin resistance (Majerczyk *et al,* 2016).

Lipocalin 2 (LCN2) is also referred to as neutrophil gelatinase-associated lipocalin, which binds and transports hydrophobic molecules, such as retinoids, arachidonic acid, LTB_4, platelet-activating factor, and steroids (Abella *et al*, 2015). LCN2 also plays an essential role in host defence against bacterial infections by sequestering iron (Asimakopoulou *et al,* 2016). It is believed to serve as a effective protective factor in the CNS in response to systemic inflammation (Kang *et al,* 2018).

In 1987 Adipsin also known as complment factor D was identify as an adipokine. It is one of the major proteins of adipose cells, but in contradiction decline in many animal models of obesity and diabetes (Lo et al, 2014). Adipsin has been shown to play pivotal roles in models of ischemia reperfusion and sepsis. Adipsin is a major link between fat cells, obesity and beta cell function (Ouchi *et al*, 2011).

Adiponectin was one of the earlier adipokines identified and, unlike many others, has beneficial effects improving insulin sensitivity and vascular function, thus being both anti-diabetic and anti-atherogenic (Chehab, 2014). Increased adiposity is associated with decreased adiponectin secretion, apparently because hypertrophic adipocytes release less of the adipokine (Maury and Brichard, 2010). Adiponectin has been suggested to circulate primarily as a multimeric (trimeric, hexameric and high molecular weight) associated of full-length adiponectin and is locally proteolytically cleaved to a globular (trimeric) form in which the collagen-like amino-terminal domain is released (Watt, 2010). Adiponectin receptors (AdipoRs) are widely expressed, and two forms have been cloned that exhibit 67% homology (Simon and James, 2013). Adiponectin has been shown to enhance insulin sensitivity, although its mechanism of action remains unclear (Ategbo *et al,* 2006).

Adiponectin is the foremost and most abundant adipokine seen in human serum, with concentrations normally in the µg/mL range (Boutari *et al,* 2016). It is predominantly produced by bone marrow adipose tissue (BMAT), unlike other adipokines, which are found in greatest quantities in visceral and subcutaneous adipose tissue (SCAT). The serum level of adiponectin increases during calorie restriction, aging, estrogen deficiency, T1DM, and treatment with thiazolidinediones, while obesity, T2DM, oxidative stress, and cigarette-smoke exposure decrease serum adiponectin levels (Castela *et al*, 2023). Specifically, the high-molecular-weight complex has anti-inflammatory properties proven to inhibit inflammation by way of blocking NF-κB activation and reducing cytokines such as TNFα, IL-6, and IL-18. (Mancuso, 2016). Adiponectin is a protein which is encoded by the ADIPOQ gene in humans. It is implicated in regulating glucose level as well as fatty acid breakdown. The level of adiponectin decreases as the BMI (body mass index) increases which correlate negatively with insulin resistance (IR) high in obese people (Minoru *et al,* 2005).

C1q/TNF-related protein (CTRPs) are structurally comparable to adiponectin, and a minimum of 15 isoforms have been described. CTRPs is known to reduce cytokine production in human monocytes and adipocytes which is stimulated with lipopolysaccharide and free fatty acids by inhibiting TLR4 activation. Additionally, CTRPs inhibits inflammation in lipid-loaded hepatocytes and increases insulin sensitivity (Li *et al,* 2017). Clinical observations also support a relationship between adiponectin levels and obesity-linked metabolic dysfunction (Engin, 2017). Adiponectin uniqueness is expressed at the highest levels by the functional adipocytes that are found in lean organisms, but its expression is down regulated in dysfunctional adipocytes that are associated with obesity (Ouchi *et al*, 2011).

Tumour necrosis factor α (TNF-α) is a pro-inflammatory cytokine with a well-established role in the immune system-mediated induction of β-cell death in type 1 diabetes (Jose, 2014). It is an adipokine, whose high circulating levels in obesity have been implicated in the induction of insulin resistance (Balsan *et al,* 2015). There is also evidence of TNF-α effects on the β-cell, which may further contribute to type 2 diabetes (although one review asserts that the elevation in TNF-α in obese humans and animals does not reach levels concomitant with those known to deleteriously affect β-cell function or survival (Simon and James, 2013).

Leptin is a protein whose genes are precisely expressed in adipose cells. It can be used as a sensitive chemical marker for the diagnosis of obesity and obesity-related diseases which is usually significantly raised. Leptin was primarily identified as a satiety factor and the best known proinflammatory adipokine. It increases in proportion to white adipose tissue mass and was first called a satiety hormone (Friedman, 2014). Leptin is principally produced by adipocytes and secreted into the blood stream. It is an appetite suppressant agent, and it exerts its effects by interacting with neuropeptide Y, MSH, and the melanocortin-4 receptor in the hypothalamus (Goit *et al*, 2022).

Leptin production from the obese gene which was identified in ob/ob mice by positional cloning was found to regulates feeding behaviour through the CNS (central nervous system) (Obradovic *et al,* 2021). It has also shown to be effective at improving metabolic dysfunction in patients with lipodystrophy or congenital leptin deficiency (Araújo-Vilar *et al,* 2019)). However, leptin levels in the blood positively correlate with adipose mass, indicating the occurrence of leptin resistance, and obese individuals have high levels of leptin without the expected anorexic response (Ouchi *et al*, 2011). Leptin upregulates proinflammatory cytokines such as TNF-α and IL-6 which are associated with IR and T2DM. On the other hand, adiponectin has anti-inflammatory properties

and downregulates the expression and release of a number of proinflammatory immune mediators (Patricio *et al*, 2014).

Leptin and adiponectin play key roles in adipocyte differentiation and inflammation. The leptin/adiponectin ratio is a determinant of insulin resistance (IR) in non-diabetic individuals, highlighting the contribution of adipose tissue dysfunction to the pathogenesis of diminished insulin action (Castela *et al*, 2023). A high leptin/adiponectin ratio may confer increased intima media thickness and predict CVD (Dullaart *et al,* 2015). Elevated leptin concentrations during diabetic pregnancy may be a result of its secretion by the adipocytes in the presence of elevated estrogen and placenta (Wójcik *et al*, 2014). Leptin acting as a signal for sufficient energy supply is persistently increased in women with GDM after delivery and associated with hyperglycaemia and insulin resistance (Ategbo *et al,* 2006).

The long form of the leptin receptor is expressed by almost all immune cells. It initiates intracellular signaling which activates the tyrosine kinase JAK2, the latent transcription factor STAT3, MAPK, ERK1/2, and PI3K pathways which then activate the innate immune response (Yuan *et al,* 2022). Leptin believed to directly improve the production of several proinflammatory cytokines, such as IL-6, IL-12, IL-18, and TNFα, the chemokines IL-8 and CCL2/MCP-1, and the lipid mediators PGE_2, cysteinyl leukotrienes (cysLTs), and leukotriene B_4 (LTB_4) in peripheral blood monocytes and resident tissue macrophages in mice and humans (Mancuso, 2016). Leptin can also induce the production of reactive oxygen intermediates in macrophages, neutrophils, and endothelial cells and potentiate IFNγ-induced expression of nitric oxide synthase (Procaccini *et al*, 2012). Amongst things enhanced by leptin are platelet aggregation and promotes leukocyte–endothelial cell interactions by increasing the expression of adhesion molecules on myeloid cells

and vascular endothelial cells. Leptin also enhances T_H1 and T_H17 responses, which can also prevent T-cell apoptosis (Mancuso, 2016).

WISP1 (Wnt1-inducible signaling pathway protein-1, also known as CCN4) is a member of the secreted extracellular matrix-associated proteins of the CCN family and a target gene of the Wingless-type (WNT) signaling pathway (Klimontov *et al*, 2020). Growing evidence links the WNT signalling pathway to the regulation of adipogenesis and-low grade inflammation in obesity (Veronica *et al,* 2015). WISP1 is said to be positively associated with blood glucose in the OGTT and circulating haem oxygenase-1 but negatively associated with adiponectin levels. Also, it has a compound relationship with several of proliferative and protective pathways (Kenneth Maiese 2016).

WISP1 also is intricated in a broad spectrum of biological functions and pathological processes. During organ development and under diseased conditions, such as fibrosis, cancer, and inflammatory diseases, WISP1 is usually expressed and recently, it has been proposed as a novel adipokine (Murahovschi *et al*, 2015). It can be found in several normal tissues including heart, kidney, lung, pancreas, placenta, ovary, small intestine, and spleen (Ferrand *et al*, 2017).

It is also highly expressed in both visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) which is released by fully differentiated human adipocytes and stimulates cytokine responses in AT associated macrophages (Barchetta *et al,* 2017). Quite a number of tissues including osteoblasts, cardiomyocytes, hepatocytes, neuronal cells, colon, lungs, myocytes, as well as by adipocytes produces WISP1. It plays a vital modulatory role in cell growth rather than a structural role. Levels of WISP1in the circulation are a marker of systemic and tissue inflammation that are raised with other inflammatory response markers (Barchetta *et al*, 2017).

Under conditions of excessive caloric intake, adipose tissue (AT) undergoes structural and functional rearrangement characterised by hypertrophy and hyperplasia of adipocytes; insufficient neovascularisation; aberrant fibrogenesis; and migration and activation of macrophages, natural killers, and lymphocytes (Barchetta *et al,* 2017). Thus, the over-production of pro-inflammatory adipokines, such as tumour necrosis factor (TNF)-α, IL-1β, IL-6, and IL-8, along with reduced AT capability of storing free fatty acids and the resultant aberrant efflux of free fatty acids into the circulation, lead to insulin resistance and its related diseases (Ilaria *et al,* 2017).

Wnt1-inducible signalling pathway protein1 (WISP1 or Cyr61/CTGF/NOV) is an extracellular matrix-associated protein belonging to the Cyr61/CTGF/NOV family, which includes matricellular proteins operating at the border between cells and extracellular matrix and exerts regulatory actions on several cellular responses (Barchetta *et al,* 2017). Thus, WISP1 is involved in a broad spectrum of biological functions and pathological processes. It is mainly expressed during organ development and under diseased conditions, such as fibrosis, cancer, and inflammatory diseases; and has recently been proposed as a novel adipokine (Maiese, 2016).

WISP1 is widely expressed in visceral (VAT) and subcutaneous (SAT) human AT (Hörbelt,*et al,* 2017). It is released by fully differentiated human adipocytes and stimulates cytokine responses in AT-associated macrophages. Circulating WISP1 concentration correlates with its expression in AT, which therefore represents a major source of this adipokine in humans (Murahovschi *et al*, 2015). Among the many cell types on which it exerts proliferative effects, WISP1 has induced proliferation of mesenchymal stem cells and, thus, AT expansion, in experimental models of visceral obesity (Ilaria *et al*, 2017). Gulcin *et al,* 2017 demonstrated that there is a relationship between WISP1 and metabolic parameters of gestational diabetes mellitus (GDM). WISP1 and betatrophin may play a key role in the pathogenesis of polycystic ovarian syndrome (PCOS).

Circulating WISP1 levels and WISP1 expression in VAT are increased in obesity independent of glycaemic status. WISP1 impaired insulin signaling in muscle and liver cells and serum WISP1/CCN4 levels may be used as a suitable biomarker of obesity (Horbit *et al*, 2018; Christoper *et al,* 2018).

WISP1 (Wnt1-inducible signaling pathway protein-1, also known as CCN4) is a member of the connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpression (CCN) family (Tanaka *et al,* 2001). The CCN family consists of six members, Cyr61/CCN1, CTGF/CCN2, NOV/CCN3, WISP1/CCN4, WISP2/CCN5 and WISP3/CCN6, named CCN1-6 based on the unified nomenclature (Horbit *et al,* 2018). Three of these proteins, WISP1-3, were first identified in C57MG cells, a mouse mammary epithelial cell line that expresses Wnt-1, and subsequently identified as Wnt-1-inducible genes (Ferrand *et al*, 2017). WISP1/CCN4 (hereafter referred to as WISP1) is both an intracellular and a secreted protein found in the extracellular matrix (ECM) and, like many other matrix cellular proteins, is a cell-mediated protein that is involved in cell proliferation, differentiation, etc. (Christoper *et al,* 2018). Response can be adjusted and survival of WISP1 is expressed in a variety of normal tissues including heart, kidney, lung, pancreas, placenta, ovary, small intestine, and spleen (Tsai *et al*, 2017). Interestingly, increased WISP1 expression has also been observed in various cancers such as hepatocellular carcinoma, colon adenocarcinoma, lung cancer, breast cancer and cholangiocarcinoma (Feng and Jia, 2016). The Wnt signalling pathway plays an important role in keeping cells in an undifferentiated state. Wnt proteins are secreted signalling factors that influence cell fate and differentiation, including adipogenesis, myogenesis, and breast development (Nathalie *et al,* 2016). Upon activation of Wnt signalling, GSK-3β is inhibited and β-catenin accumulates in the nucleus, where it binds to the TCF-LEF transcription factor and then activates Wnt target genes (Liu *et al*, 2022). Wnt-1 protein

has been described as an inhibitor of adipocyte differentiation. Interestingly, ectopic expression of Wnt-1 in mouse 3T3-L1 preadipocytes induces the expression of multiple downstream genes, including WISP1, suggesting a potential role of WISP1 in adipocyte differentiation (Maiese, 2016). Adipogenesis is characterized by dynamic changes in gene expression. It is well established that both peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding protein (C/EBP) function as key regulators of adipogenesis PPARs are members of a nuclear receptor family that includes a subset of three homologous genes (Smith *et al,* 2018) Of these, PPARγ function primarily as a key regulator of adipocyte differentiation and metabolism (Yuan *et al,* 2016). After ligand-mediated activation, PPARγ receptors bind to PPARγ-responsive elements (PPREs), thereby promoting target gene expression (Ferrand *et al,* 2017). Forced expression of PPARγ can convert fibroblasts into adipocytes, whereas a dominant-negative PPARγ mutant in cultured preadipocytes inhibits adipogenesis PPARγ is a pharmacological target of thiazolidinedione (TZD) drugs that act as potent insulin sensitisers (Nathalie *et al*, 2016).

Visfatin, previously known as a pre-B cell colony-enhancing factor (PBEF), also has a function in the immune system, in which it was described as a growth factor for early B cells. Visfatin/PBEF binds and activates the insulin receptor in different insulin-sensitive cells *in vitro* and treating mice with recombinant visfatin/PBEF elicited insulin-like effects also *in vivo* (Mayi *et al*, 2010). Visfatin/PBEF was recently identified as a protein highly expressed in VAT, compared with SC adipose tissue (SAT) and hepatocytes could also be a fundamental source (Garten *et al,* 2010). Visfatin, is defined by 3 different properties it possesses, which are as follows; it was first referred to as an adipokine secreted by visceral fat which impersonate the effects of insulin (Moreno-Aliaga *et al,* 2010). It was initially identified as a secreted growth factor that improves B-cell precursor maturation, called pre-B-cell colony-enhancing factor (PBEF) (Lu *et al*, 2009). It was later shown

that visfatin also has an enzymatic nicotinamide phosphoribosyltransferase (Nampt) function, which is required for the synthesis of NAD, an important cofactor necessary for cell metabolism (Jacques *et al*, 2012).

The names visfatin, PBEF, and eNAMPT are used to refer to the same protein. Visfatin which is the most common name was designated in 2005, meaning visceral fat-enriched adipocytokine. Visfatin also has features of cytokines and is universally formed in several tissues and various cells including synovial fibroblasts, articular chondrocytes, or monocyte. This reinforcing the assumption that visfatin plays vital roles in various pathophysiological processes (Wang *et al,* 2021). Visfatin was found to be overexpressed in several inflammatory diseases, example include atherosclerosis, rheumatoid arthritis, or osteoarthritis (OA). This indicate that it could be a viable target for treating OA. It is also believed to be involved in obesity associated low-grade inflammation state and metabolic syndrome (Jacques *et al*, 2012). visfatin is capable of exerting proinflammatory action through regulating IR pathway activity and by initiating Nampt enzymatic activity. Visfatin could play a functional interplay between these obesity-related factors insulin and leptin, which promotes it expression in primary human adipocytes or adipose tissue culture through a negative feedback mechanism (Wang *et al,* 2021). Visfatin also has the ability to stimulates insulin secretion, increases insulin sensitivity as well as glucose uptake by muscle cells and adipocytes. Among its capabilities is the robust proinflammatory function, activates leukocytes and cytokines, and increases adiposity (Kuryszko *et al,* 2016).

Plasma glucose decreased by visfatin/PBEF treatment, while heterozygous visfatin/PBEF knockout mice had higher plasma glucose than wild-type littermates. In 3T3-L1 adipocytes, visfatin/PBEF expression is up-regulated by differentiation and dexamethasone and down-regulated by GH, isoproterenol, forskolin, and cholera toxin. Insulin has no effect on visfatin/PBEF

mRNA. Furthermore, visfatin/PBEF is upregulated by peroxisomal proliferative-activated receptor agonist (PPAR) and PPAR in obese mice along with improved glycaemic control and lipid profile, due to which suggests that PPAR and PPAR agonists may act, at least in part, by upregulating visfatin/PBEF expression (Mayi *et al,* 2010). A recent human study in a large population reported that plasma visfatin/PBEF was directly correlated with body mass index (BMI) and body fat content only in men and did not find found different expression between visceral fat and sc (Olszanecka-Glinianowicz *et al,* 2011). Visfatin/PBEF is a candidate for establishing the link between excess visceral fat, insulin resistance, and metabolic syndrome. However, it is not known whether lipid substrate transfection and induction of insulin resistance can affect circulating levels of visfatin/PBEF and its expression in adipose tissue (Mayi *et al,* 2010). The association between visceral adipose tissue (VAT) accumulation and insulin resistance is well established in obesity and type 2 diabetes mellitus(T2DM), and visceral fat and insulin resistance are closely associated with increased cardiovascular risk. (Olszanecka-Glinianowicz *et al,* 2011; Mayi *et al*, 2010).

Secreted Frizzed Related Protein **(**sFRP4) is a member of the active regulator of the wingless-like (Wnt) signalling pathway, and it is also one of the adipokines that show impaired visceral adipose tissue (VAT) expression and secretion changes in obese subjects and in patients with nonalcoholic liver disease. (NALD) (Baldane *et al*, 2018). In vitro studies suggest a direct effect of sFRP4 on insulin secretion by beta cells, whereas little is known about the metabolic effects of sFRP4 in target tissues of insulin action, such as liver. sFRP4 expression is increased in VAT of obese men and is associated with insulin resistance and triglycerides (Anand *et al*, 2016). Wnt pathways are generally classified as canonical β-catenin-dependent pathways and non-canonical β-catenin-

dependent pathways (Bukhari *et al*, 2019). sFRP4 is an activity modulator of the canonical Wnt signalling pathway, which promotes beta-catenin stability and stimulates gene expression through interaction with T-cell factor transcription factors. (Hoffman *et al,* 2014). Activation of Wnt signalling is initiated by binding of Wnt ligands to disturbed receptors, while inactivation is regulated by binding of sFRPs, including sFRP4, to Wnt ligands. Wnt ligands secrete cysteine-rich glycoproteins of approximately 340–400 amino acids with signal peptides at their N-terminus (Mahdi *et al*, 2012).

As many as 19 different Wnt ligands have been identified that can bind to at least 10 different Frizzled (FZD) receptors. It can be assumed that the difference in tissue distribution of the Wnt ligand and the Frizzled receptor causes the differences in sFRP4 signalling between hepatocytes and muscle cells (Baldane *et al*, 2018). Wnt-mediated signalling is activated by the binding of these ligands to FZD receptors. Wnt ligands and receptors play an important role in development, differentiation, determination of tissue polarity, control of cell movement, and patterning of the central nervous system (Bukhari *et al*, 2019). Brain/Wnt inhibitory factors (WIF) and secretory dysregulation-associated proteins (SFRP) bind directly to Wnt proteins as well as to FDZ receptors and block canonical and non-canonical pathways. Bukhari *et al,* 2019). In addition to the effect of sFRP4 on adipogenesis found in hepatocytes from healthy transfected C57Bl6 mouse hepatocytes, indirect effects of sFRP4 may also contribute to lipid accumulation in the liver. Other tissues, such as liver, pancreas, and skeletal muscle, also secrete and/or express sFRP4. According to an in vitro hepatocyte study, it was shown that sFRP4 selectively inhibits the insulin signalling pathway that regulates glucose metabolism but enhances DNL stimulation. DNL could be further enhanced by increased glycolysis in sFRP4-treated myotubes. During the development of type 2 diabetes and fatty liver disease, sFRP4 may be an attractive target for attenuating the pathophysiological

changes that occur during the development of type 2 diabetes and fatty liver disease. (Horbelt *et al*, 2019).

Five members of SFRP were identified for their antagonistic effects on mammalian Wnt signalling cascades and named SFRP1-5. Each SFRP has different binding specificity for different Wnt ligands. Another study described different functions of SFRP protein in Wnt ubiquitination, Wnt signalling enhancement and Wnt independence (Bukhari *et al*, 2019). During embryonic development, such as growth, proliferation, and cell differentiation, wing-uncoupled integrated site (Wnt) signalling is one of the evolutionarily conserved pathways for the regulation of function. Difference. Modulation of SFRPs in various diseases, including cancer and diabetes, has been reported by various studies. Wnt diffusion is enhanced by SFRP molecules, thus, SFRP may act as a positive modulator of Wnt signalling. Wnt signalling can also be activated by diffusion of Wnt8 and Wnt11, which is supported by sFRP molecules (Bukhari *et al,* 2014).

sFRP4 is the largest member of sFRP, which is quite distinct from other members of the family due to the presence of six conserved cysteine residues linked together by disulfide bridges. The sFRP4 protein contains 346 amino acids with an expected molecular weight of about 39.9 kDa and an actual weight of 50-55 kDa. sFRP4 is secreted by the cell into the extracellular matrix where it is bound to the plasma membrane (Brix *et al,* 2016). sFRP4 is produced in the nucleus, accumulates and transforms in the endoplasmic reticulum in the perinuclear region, and migrates to the cell membrane via vesicles for secretion. The perinuclear region also contains oligomeric aggregates of sFRP4. Intracellular localization and secretion of sFRP4 is modulated by Wnt3a. Wnt signaling is strongly regulated by a feedback mechanism through the expression of various components of the Wnt pathway (Anand *et al*, 2016).

sFRP4 are secreted glycoproteins, which contain a characteristic cysteine rich domain (CRD) that has properties to potentially interact with FZDs and Wnts. Signal sequence and CRD constitute the N-terminus while the C-terminal consists of a hydrophilic heparin binding domain. The sFRPs act as soluble modulators and are thought to change Wnt signals by contending with FZDs to bind Wnts (Bukhari *et al,* 2019). In obesity and type 2 diabetes, circulating levels of sFRP4 are raised and are associated with the development of beta cell dysfunction. Hormones called adipokines, from adipose tissue and alterations in adipokine secretion are altered by obesity and have been linked to the inhibition of insulin action in peripheral tissues like the liver and skeletal muscle, but also to the development of diabetes associated complications like non-alcoholic fatty liver disease (NAFLD) (Bukhari *et al*, 2014).

After eating, blood glucose levels rise, triggering insulin secretion by pancreatic β-cells in the islets of Langerhans, which stimulates glucose uptake by skeletal muscle and adipose tissue (Bergmann *et al*, 2014). Chronic, low-grade islet inflammation in response to metabolic stress leads to T2DM. Patients with T2DM are also commonly found to have elevated levels of inflammatory cytokines, including C-reactive protein (CRP), interleukin 6 (IL-6), and IL-1β, which can trigger an inflammatory innate immune response that leads to its inflammation of islets mechanism. In addition, several inflammatory mediators, including IL-6, tumour necrosis factor-α (TNF-α), IL-1β, and IL-1-dependent cytokines, are released from pancreatic islets in response to hyperglycaemia (Hoffman *et al*, 2014). Similarly, many components of the Wnt signaling pathway are involved in glucose and lipid metabolism and play critical roles in the development of metabolic disorders, including diabetes. T2DM is involved in adipogenesis and insulin signaling through the dysregulation of Wnt signaling. Wnts inhibit preadipocytes and therefore preadipocytes are maintained in an undifferentiated state (Baldane *et al*, 2018). Wnt ligands,

including sFRP4, are produced by mature adipocytes with anti-inflammatory and insulin-sensitising properties. Approximately 300 adipokines are secreted by human white adipose tissue (WAT), and most of them show altered expression in obesity and induce low-grade chronic inflammation and impaired insulin sensitivity (Bukhari *et al,* 2019). Wnt proteins are involved in cell differentiation and function in various tissues, including WAT (Mahdi *et al,* 2012). People with high blood levels of sFRP -4 are five times more likely to develop diabetes in the next few years. sFRP-4 is a regulator of insulin exocytosis in mouse and human islet cells. sFRP-4 and DPP-4 levels may be predictive markers of atherosclerosis in patients with diabetes (Senigigit *et al*, 2019). Table 1.1 provides a summary of the functions of key adipokines that are involved in hyperglycaemia-induced inflammation.

1.5.3 Inflammation, Obesity and Diabetes

Diabesity is referred to the collective adverse health problems of obesity and diabetes mellitus (Zhang *et al,* 2023). In other words, it is the pathological interlink between T2DM and obesity/overweigh (Pappachan *et al,* 2018). The dual epidemic of obesity and T2DM globally is an important public health issue. A sixfold increase in the number of adults with obesity in 40 years has been projected (Patil *et al,* 2023). Obesity increases morbidity and mortality with a negative impact on the quality of life. This impact poses a global health threat to health systems in all economies (Mohajan *et al,* 2023). The strongest risk factor for developing diabetes is known to be increased in adiposity (Eibl *et al,* 2018). The optimal implementation of effective therapies that prevent atherosclerosis progression, cardiac remodelling and the resulting ischaemic heart disease, and heart failure is the early detection of the effects of diabesity on the cardiovascular system (Ng *et al*, 2021).

It is confirmed from numerous numbers of clinical trials that there is a link between being overweight and the higher risk for the development of T2DM (Wimalawansa, 2018). Abdominal fat accumulation singularly, deteriorates insulin resistance, therefore act as a strong, independent risk factor of developing T2DM (Paniagua). Free fatty acids (FFA), which are often elevated in obese individuals, are implicated as an important causal link between obesity, insulin resistance, and T2D (Rachek, 2014). Infusion of AGF in humans and rodents rapidly induces hyperinsulinemia and insulin resistance in both skeletal muscle and liver. This is thought to be due to multiple mechanisms, including competition for glucose utilisation, hepatic gluconeogenesis, and glucose production (Abdul-Waheb *et al,* 2014). Obesity is at epidemic levels throughout the world, which is now treated as one of the main lifestyle diseases (Saad *et al*, 2022).

Just over a decade ago, the first molecular link between inflammation and obesity - TNF-α - was identified when it was discovered that this inflammatory cytokine was overexpressed in adipose tissue from adipose tissue obesity in rodents (Kahles *et al,* 2014). As in mice, TNF-α is overproduced in adipose and muscle tissue of obese humans (Tzanavari *et al,* 2010). Application of recombinant TNF-α to cultured cells or whole animals mitigated insulin action, and obese mice lacking functional TNF-α or TNF receptors improved insulin sensitivity compared with their wild-type counterparts (Wellen and Hotamisligil, 2005). Therefore, especially in experimental models, it is clear that overproduction of TNF-α in adipose tissue is an important feature of obesity and it contributes significantly to insulin resistance (Biondi- Zoccai *et al*, 2003).

However, it is clear that obesity is characterised by a broad inflammatory response and that many inflammatory mediators exhibit expression patterns and/or impact insulin action in a manner similar to TNF-α during obesity, in animals ranging from mice and cats to humans (Wellen and Hotamisligil, 2005). Transcriptional profiling studies suggest that inflammation and stress

response genes are among the most upregulated in the adipose tissue of obese animals (Gustafson *et al*, 2010).

In addition to inflammatory cytokines that regulate metabolic homeostasis, molecules typical of adipocytes, with well-established metabolic functions, can modulate the immune response (Kawai *et al*, 2021). Leptin is one of the hormones that play an important role in adaptive and innate immunity, and mice and humans lacking leptin function show impaired immunity (Pérez-Pérez *et al*, 2020). Decreased leptin levels may be responsible, at least in part, for hunger-related immunosuppression, as administration of leptin has been shown to reverse immunosuppression in rats starved for 48 hours (Hurley, 2018). Adiponectin, resistin and visfatin are also examples of immunoactivity molecules induced in adipocytes (Ouchi *et al*, 2011). Finally, lipids themselves are also involved in the coordinated regulation of inflammation and metabolism. High plasma lipid levels are characteristic of obesity, infections, and other inflammatory conditions (Jung and Choi, 2014). Hyperlipidaemia in obese individuals is partly responsible for the induction of insulin resistance in peripheral tissues and dyslipidaemia, and also contributes to the development of atherosclerosis (Libby *et al,* 2013). Macrophages are the link between inflammation and metabolism. The high degree of coordination of inflammatory and metabolic processes is evidenced by the overlap in biology and function of macrophages and adipocytes in obesity (Engin, 2017). Macrophages express many, if not the majority, of "adipocyte" gene products such as adipocytes/macrophages FABP aP2 (also known as FABP4) and PPARγ, whereas adipocytes have can express many "macrophage" proteins such as TNF-α, IL-6 and MMP (Garcia *et al*, 2010). The functional abilities of these two cell types also overlap and macrophages can take up or/and store lipids to become atherosclerotic foam cells (Volobueva *et al*, 2018). Preadipocytes can under certain conditions exhibit phagocytic and antimicrobial properties and even appear to have the

ability to differentiate into macrophages in the appropriate environment, suggesting a potential immunological role for preadipocytes (Mraz and Haluzik, 2014). In addition, macrophages and adipocytes colocalize in obese adipose tissue (Wellen and Hotamisligil, 2005). The recent finding that obesity is characterised by the accumulation of macrophages in white adipose tissue adds another dimension to our understanding of the development of adipose tissue inflammation in obesity (Herrada *et al*, 2021). It is likely that macrophages in adipose tissue contribute to the production of inflammatory mediators, either alone or in conjunction with adipocytes, suggesting a potentially important role of macrophages in promoting insulin resistance in adipose tissue (Barchetta *et al*, 2017). However, no direct evidence has been offered to establish this connection thus far. In terms of the immune response, integration between macrophages and adipocytes makes sense, given that both cell types participate in the innate immune response (Michailidou *et al,* 2022). Macrophages in their role as immune cells by killing pathogens and secreting inflammatory cytokines and chemokines; and adipocytes by releasing lipids that may modulate the inflammatory state or participate in neutralization of pathogens (Gilles *et al*, 2016). While it is not yet known whether macrophages are drawn to adipose tissue in other inflammatory conditions, it is conceivable that macrophage accumulation in adipose tissue is a feature not only of obesity, but of other inflammatory states as well (Dandona P, 2002).

It is apparent that obesity is associated with a state of chronic, low-grade inflammation, particularly in white adipose tissue (Castro *et al*, 2017). In recent years, much has been learned about the intracellular signalling pathways activated by inflammatory and stress responses and how these pathways intersect and inhibit insulin signalling (Verdile *et al*, 2015). Insulin affects cells through binding to its receptor on the surface of insulin-responsive cells (Danna *et al*, 2016). The stimulated insulin receptor phosphorylates itself and several substrates, including members of the insulin

receptor substrate (IRS) family, thus initiating downstream signalling events (Boucher *et al,* 2014). The inhibition of signalling downstream of the insulin receptor is a primary mechanism through which inflammatory signalling leads to insulin resistance (Libby *et al*, 2012). Exposure of cells to TNF-α or elevated levels of free fatty acids stimulates inhibitory phosphorylation of serine residues of IRS-1 (Copps and White, 2012). This phosphorylation reduces both tyrosine phosphorylation of IRS-1 in response to insulin and the ability of IRS-1 to associate with the insulin receptor and thereby inhibits downstream signalling and insulin action (Wellen and Hotamisligil, 2005).

Recently it has become clear that inflammatory signalling pathways can also become activated by metabolic stresses originating from inside the cell as well as by extracellular signalling molecules (Forrester *et al*, 2018). It has been demonstrated that obesity overloads the functional capacity of the endothelial reticulum (ER), and that this ER stress leads to the activation of inflammatory signalling pathways and thus contributes to insulin resistance (Barchetta *et al,* 2017). Additionally, increased glucose metabolism can lead to a rise in mitochondrial production of ROS. ROS production is elevated in obesity, which causes enhanced activation of inflammatory pathways (Murahorschi *et al*, 2014).

Several serine/threonine kinases are activated by inflammatory or stressful stimuli and contribute to inhibition of insulin signalling, including JNK (c-Jun N terminal), inhibitor of NF-κB kinase (IKK), and protein kinase C (PKC) (Yung and Giacca, 2020). Again, the activation of these kinases in obesity highlights the overlap of metabolic and immune pathways (Tanti *et al,* 2013). These same kinases, particularly IKK and JNK, that are activated in the innate immune response by Toll-like receptor (TLR) signalling in response to LPS, peptidoglycan, double-stranded RNA, and other microbial products (Verhelst *et al,* 2013). Hence it is likely that components of TLR signalling pathways will also exhibit strong metabolic activities (Yan *et al*, 2008).

The 3 members of the JNK group of serine/threonine kinases, JNK1, 2, and 3, belong to the MAPK family and regulate multiple activities in development and cell function (Chen *et al,* 2021). They act largely through their ability to control transcription by phosphorylating activator protein–1 (AP-1) proteins, including c-Jun and JunB (Kappelmann *et al,* 2014). JNK has recently emerged as a central metabolic regulator, playing an important role in the development of insulin resistance in obesity (Murahorschi *et al*, 2015). In response to stimuli such as ER stress, cytokines, and fatty acids, JNK is activated, which then binds to and phosphorylates IRS-1 on Ser307, impairing insulin action. In obesity, JNK activity is increased in liver, muscle, and adipose tissue, while loss of JNK1 prevents the development of insulin resistance and diabetes in genetic and dietary models of obesity diet in rats (Wellen and Hotamisligil, 2005). Modulation of JNK1 in the liver in adult animals also confers systemic effects on glucose metabolism, highlighting the importance of this pathway in the liver. The contribution of the JNK pathway in adipose, muscle, or other tissues to systemic insulin resistance is currently unclear. In addition, a mutation in the JNK-interacting protein-1 (JIP1), a protein that binds to JNK and regulates its activity, has been identified in humans with diabetes (Danna *et al*, 2016). The phenotype of the JIP1 loss-of-function model is very similar to the murine JNK1-deficiency phenotype, with decreased JNK activity and increased insulin sensitivity. Interestingly, the JNK2 isoform plays an important non-necessary role in atherosclerosis, but apparently not in type 2 diabetes. Recent studies in mice demonstrate that JNK inhibition in diabetes or established atherosclerosis may be a possible treatment for these diseases in humans (Barchetta *et al,* 2017).

PKC and IKK are two other inflammatory kinases that play an important role in counteracting insulin action, especially in response to lipid metabolites, are IKK and PKC-θ (Hotamisligil, 2017). Lipid infusion has been shown to increase levels of intracellular fatty acid metabolism, such as

diacylglycerol (DAG) and fatty acyl CoAs (Consitt *et al,* 2009) This increase correlates with activation of PKC-θ and increased phosphorylation of Ser307 by IRS-1. PKC-θ can alter insulin activity by activating another serine/threonine kinase, IKKβ or JNK (Nandipati *et al*, 2017). Other PKC isoforms have also been reported to activate lipids and may also be involved in the inhibition of insulin signaling (Kathryn *et al,* 2005).

IKKβ can affect insulin signaling through at least two pathways. First, it can directly phosphorylate IRS-1 on serine residues (Perkins, 2007). Second, it can inhibit NF-κB (IκB) phosphorylation, thereby activating NF-κB, a transcription factor that, among other targets, stimulates the production of many inflammatory mediators, including TNF-α and IL-6 (Liu *et al,* 2017). Mice heterozygous for IKKβ are partially protected against insulin resistance induced by lipid infusion, a high-fat diet, or genetic obesity (Garcia *et al*, 2010). In addition, inhibition of IKKβ in human diabetics by high-dose aspirin treatment also enhances insulin signaling, although at this dose it is unclear whether other kinases involved are also affected. Enjoy (Wieser *et al,* 2013). Recent studies have also begun to elucidate the importance of IKKs in individual tissues or cell types in the development of insulin resistance (Sethi and Hotamisligil, 2021). Activation of IKK in hepatocytes and marrow appears to contribute to obesity-induced insulin resistance, although this pathway may not be as important as muscle (Duncan *et al,* 2003). In addition to the serine/threonine kinase cascades, other pathways contribute to inflammatory insulin resistance. For example, at least 3 SOCS (suppressors of cytokine signaling) family members, SOCS1-3 and 6, have been implicated in cytokine-mediated inhibition of insulin signaling (Suchy *et al,* 2013). These molecules appear to inhibit insulin signaling by interfering with IRS-1 and IRS-2 tyrosine phosphorylation or by targeting IRS-1 and IRS-2 for proteosomal degradation (Fritsche *et al,* 2011). SOCS3 has also been shown to modulate central leptin activity and both whole-body

reduction of SOCS3 and neural disruption of SOCS3 resulting in resistance to regimen-induced obesity, high fat intake and insulin resistance (Ouchi *et al,* 2011).

The role of lipids in metabolic diseases is complex. As discussed above, hyperlipidemia leads to increased uptake of fatty acids by muscle cells and the production of fatty acid metabolites that stimulate inflammatory episodes and inhibit insulin signaling (Chen *et al,* 2021). On the other hand, intracellular lipids may also be anti-inflammatory (Teng *et al,* 2020). The ligands for the hepatic X receptor (LXR) and the PPAR family of nuclear hormone receptors are oxysterols and fatty acids, respectively, and activation of these transcription factors inhibits the expression of inflammatory genes in macrophages and adipocytes, largely due to inhibition of NF-κB (Gustafson *et al*, 2010). LXR function is also regulated by the innate immune pathway. TLR signaling inhibits LXR activity in macrophages, increases cholesterol accumulation, and explains, at least in part, the precancerous effect of infection (Kolliniati *et al,* 2022). Interestingly, despite the inhibitory effect of TLR signaling on LXR cholesterol metabolism, LXR appears to be required for the full response of macrophages to infection. In the absence of LXR, macrophages undergo accelerated apoptosis and are therefore unable to respond appropriately to infection (Noelia and Castrillo, 2011).

The ligand-free PPARδ also appears to have an inflammatory function, mediated at least in part by its association with transcriptional repressor 6 (BCL-6) B-cell lymphoma (Wellen and Hotamisligil, 2005). The activity of these lipid ligands is affected by cytosolic FABP. Animals lacking ap2 and mal1 adipocyte/macrophage FABP were strongly protected against type 2 diabetes and atherosclerosis, a phenotype reminiscent of thiazolidinedione-treated rats and humans (Babaev *et al,* 2011). One mechanism for this phenotype is likely related to the availability of endogenous ligands for receptors that stimulate lipid storage in adipocytes and the blocking of inflammatory

pathways in macrophages (Gustafson *et al,* 2016). Collectively, it appears that the composition of the cellular environment, and association with target signaling pathways are important in determining whether lipids promote or prevent the condition of inflammation and insulin resistance (Khodabandehloo *et al,* 2016). Accumulation of cholesterol in macrophages promotes atherosclerosis and lipids in muscle and liver promote insulin resistance (Prieur *et al*, 2010). Meanwhile, as seen in FABP-deficient mice treated with TZDs, if lipids are forced to stay in adipose tissue, insulin resistance against obesity may be reduced (Lee *et al,* 2018). Thus, lipids and their targets clearly play both metabolic and inflammatory roles; however, the functions they perform depend on several factors (Duncan *et al*, 2003). To corroborate genetic evidence in mice that lose inflammatory mediators or signaling molecules that would prevent insulin resistance, pharmacological targeting of inflammatory pathways also enhances insulin action (Zatterale *et al,* 2020). Effective treatment has been demonstrated with both inflammatory kinase inhibitors and related transcription factor agonists (Patterson *et al,* 2014). As discussed above, salicylates promote insulin signaling by inhibiting the influx of inflammatory kinases in cells. Through inhibition of IKK and possibly other kinases, salicylates can enhance glucose metabolism in obese rats and diabetic humans (Luo *et al,* 2015). Targeting JNK using a synthesis inhibitor and/or peptide inhibitor has been shown to enhance insulin action in obese mice and reduce atherosclerosis in a deficient rodent model. insulin. apoE. These results directly demonstrate the therapeutic potential of JNK inhibitors for diabetes (Kathryn *et al*, 2005).

Synthetic ligands have been produced to all 3 PPAR isoforms as well as LXR-α, though only PPARγ and PPARα ligands have been approved for clinical treatment (Tanaka *et al*, 2017). TZDs, high-affinity ligands of PPARγ, which are given clinically as insulin-sensitizing agents, likely improve insulin action through multiple mechanisms, including both activating lipid metabolism

and reducing production of inflammatory mediators such as TNF-α (Ahmadian *et al,* 2013). Synthetic PPARα ligands, fibrates, are used to treat hyperlipidaemia (Choi *et al,* 2014). These drugs appear to work predominantly through stimulation of fatty acid oxidation, though they also have anti-inflammatory actions that contribute to their effects (van Diepen *et al,* 2013). LXR ligands have been demonstrated to improve glucose metabolism in experimental animals, and it remains to be seen whether suppression of inflammation contributes to this action (Wellen and Hotamisligil, 2005).

In targeting inflammation for the treatment of insulin resistance and diabetes, finding inhibitors for each of the inflammatory mediators may not be a maximally effective strategy, because of the other redundant components. may be sufficient to further spread inflammatory pathways (Esser *et al,* 2015). For example, targeting individual inflammatory cytokines may not be very effective, whereas targeting the inflammatory kinases JNK and IKK produces potent antidiabetic effects because these factors integrate signaling from many inflammatory mediators (Sethi and Hotamisligil, 2021). On the other hand, if a more central process or mediator can be identified, this could provide an even more compelling goal (Libby *et al,* 2012). The ER stress pathway is likely one of these central processes, in which this pathway is likely to activate both JNK and IKK; therefore, inhibition of the ER stress response through chaperone addition or other mechanisms could potentially disable these two branches of the inflammatory response and rescue insulin action (Reverendo *et al,* 2019). A decrease in Expression of this molecule in the liver leads to increased ER stress and insulin resistance (Duncan *et al*, 2003). Looking at the facts, it's pretty clear that obesity promotes both low-grade chronic inflammation and insulin resistance. However, even without obesity, infusing animals with inflammatory cytokines or lipids can induce insulin resistance (Kojta *et al,* 2020). In addition, people with other chronic inflammatory diseases have a

higher risk of diabetes; for example, about one-third of patients with chronic hepatitis C develop type 2 diabetes, and elevated TNF-α levels are associated with this association (Danna *et al,* 2016). Rheumatoid arthritis also predisposes patients to diabetes and cardiovascular disease in particular, and some evidence points to a link between pneumonia and the risk of cardiovascular disease and diabetes (Murahorschi *et al,* 2014). Finally, knockdown of inflammatory mediators or components of the pathway, such as TNF-α, JNK and IKK, protects against insulin resistance in a mouse model of obesity and human treatment. with drugs that target these pathways, such as salicylates, improve insulin sensitivity (Zatterale *et al,* 2020). Therefore, the available evidence strongly suggests that type 2 diabetes is an inflammatory disease, and that inflammation is a major cause of insulin resistance, hyperglycaemia, and obesity-associated hyperlipidaemia. is not a simple consequence (Dandona P, 2002).

One mechanism, based on emerging data, that appears to be of particular importance is the activation of inflammatory pathways by ER stress (Almanza *et al,* 2019). Obesity creates conditions that increase the need for the ER. This is particularly the case for adipose tissue, which undergoes severe changes in tissue structure, increases protein and lipid synthesis, and disrupts the intracellular flow of energy and nutrients (Kahn *et al,* 2019). In cultured cells and whole animals, ER stress leads to JNK activation and thus contributes to insulin resistance. Interestingly, ER stress also activates IKK and thus may represent a common mechanism for the activation of these two important signaling pathways (Kathryn *et al* 2005).

A second mechanism that may be involved in the initiation of inflammation in obesity is oxidative stress (Colak and Pap, 2021). Due to increased glucose transport to adipose tissue, endothelial cells are able to absorb increasing amounts of glucose through their constitutive glucose transporters (Duncan *et al,* 2003). Increased glucose uptake by endothelial cells under hyperglycaemic

conditions leads to overproduction of ROS in mitochondria, causing oxidative damage and activation of inflammatory signaling cascades within endothelial cells (Teodoro *et al,* 2019). Endothelial damage in adipose tissue can attract inflammatory cells such as macrophages to this site and exacerbate local inflammation (Chen *et al*, 2021). Hyperglycaemia also stimulates ROS production in adipocytes, leading to increased production of proinflammatory cytokines (Bondi-Zoccai *et al*, 2003). Perhaps the stresses of obesity are similar to those caused by infections that cause the body to respond to obesity in the same way as it does to an infection. For example, in infection and obesity, intracellular stress pathways such as the JNK and IKK–NF-κB pathways are activated.

On the other hand, the inflammatory response to obesity is not just an unwanted by-product, but rather a homeostatic mechanism to prevent the body from reaching a point where excessive fat accumulation impairs its ability to function or reduced fitness (Libby *et al*, 2012). Lipid storage and fat accumulation require anabolic processes, exemplified by insulin action, while inflammation stimulates catabolism, including lipolysis of adipocytes (Schwartsburd, 2019). It is conceivable that mechanisms such as activation of catabolism through inflammation (and thus resistance to anabolic signals) could be an attempt to maintain body weight within acceptable limits (Murahorschi *et al*, 2015).

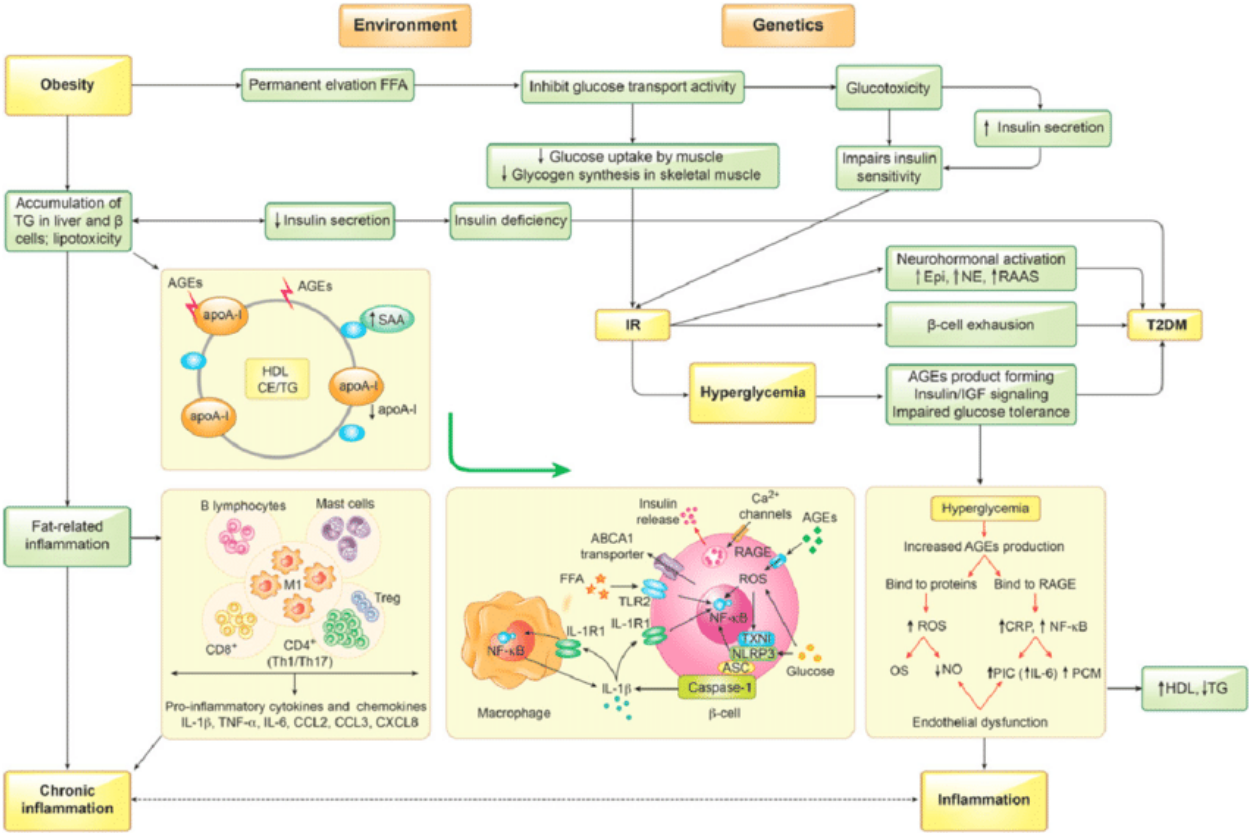


Figure 13: Relationship between Inflammation, obesity, and diabetes (Zhao *et al*, 2019)

1.6 DIABETES AND LIPID PROFILE

Lipid profile consist of five different types of lipids which are total cholesterol, low-density lipoprotein cholesterol (LDL-C), very low-density lipoprotein (VLDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides (Amigó *et al*, 2021). The main risk factors for cardiovascular disease are diabetes mellitus, race, family history of heart disease, high blood pressure, high blood cholesterol, lack of exercise, smoking, and obesity/overweight (Grundy *et al,* 1999). According to the American Heart Association (2016), people with diabetes are at high risk of CVD because diabetics, particularly those with type 2 diabetes, may also have other CVD risk factors. These include high blood pressure, abnormal cholesterol and triglyceride levels, obesity,

and lack of exercise (Gallagher *et al*, 2010). Nathan *et al,* (2005) found that hyperglycaemia increases the risk of heart attack, stroke, angina, and coronary artery disease in adult diabetics. Furthermore, Betsy, (2008) mentioned that hyperglycaemia as a symptom of diabetes is a contributing factor to myocardial damage after ischemic events. Recently, strategies for the management of vascular complications associated with diabetes have moved away from a glucocentric approach to address additional risk factors that contribute to the development and progression of atherosclerosis (Yun and Ko, 2021). A common pattern of lipid abnormalities, known as diabetic dyslipidaemia, includes hypertriglyceridemia, reduced high-density lipoprotein (HDL) cholesterol, and a shift toward small, dense, low-density lipoprotein (LDL) cholesterol (Wu *et al*, 2014).

Hypertriglyceridaemia is considered the dominant lipid abnormality in insulin resistance and plays a pivotal role in determining the characteristic lipid profile of diabetic dyslipidaemia (Thambiah and Lai, 2021). Elevated triglyceride levels are the result of increased production and decreased clearance of triglyceride-rich lipoproteins in both fasting and non-fasting states (Keirns *et al,* 2021). Increased production of very low-density lipoprotein (VLDL), the main transporter of fasting triglycerides, is a prominent feature of insulin resistance (Wu *et al*, 2014). Insulin is involved in all stages of VLDL production and secretion (Sparks *et al,* 2012). In adipose tissues, insulin suppresses lipolysis by inhibiting the activity of hormone sensitivity lipase, which catalyses the mobilisation of free fatty acids from stored triglycerides (Carpentier, 2021). Insulin regulates the amount of circulating free fatty acids, which acts as substrates and regulatory factors for VLDL and assembly and secretion (Wu *et al,* 2014). Increased oxidative stress, disruption of protein kinase C signalling and increase advanced glycation end-products that result in vascular

inflammation, vasoconstriction, thrombosis and atherogenesis are caused by hyperglycaemia, insulin resistance and excess fatty acids (Henning, 2018).

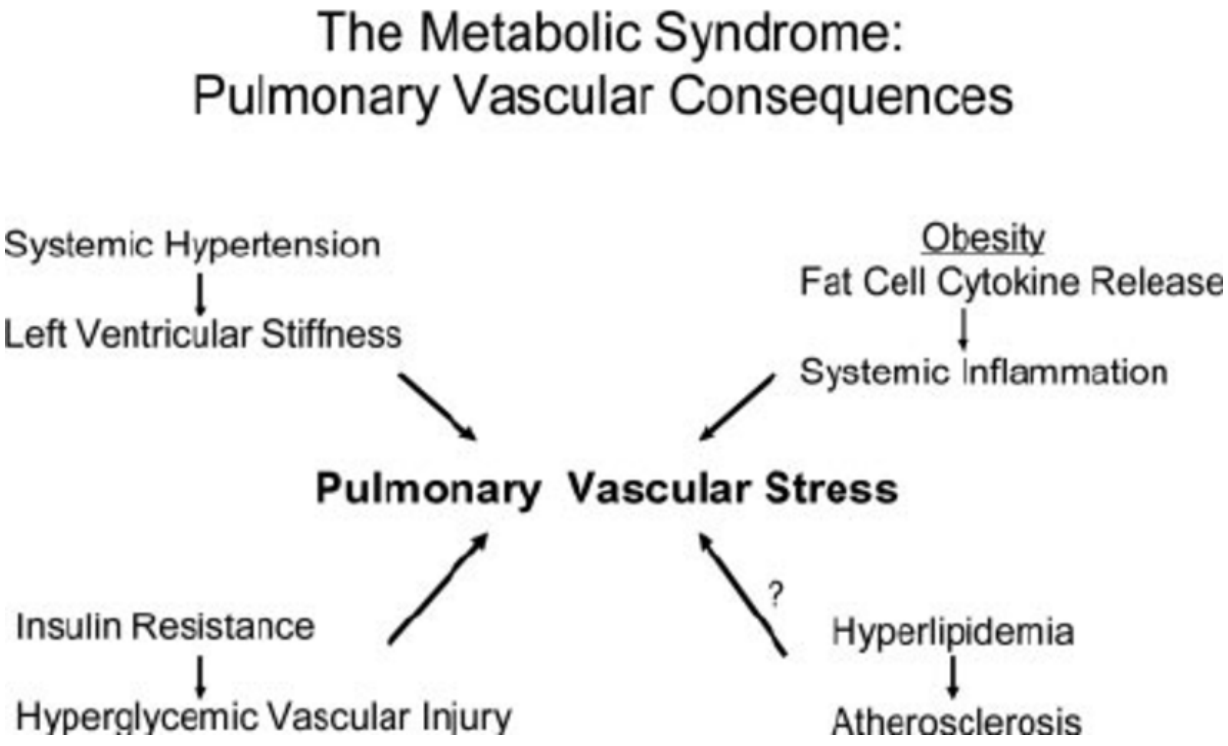


Figure 14: Pulmonary vascular stress the centre of metabolic syndrome (Robbins *et al*, 2009).

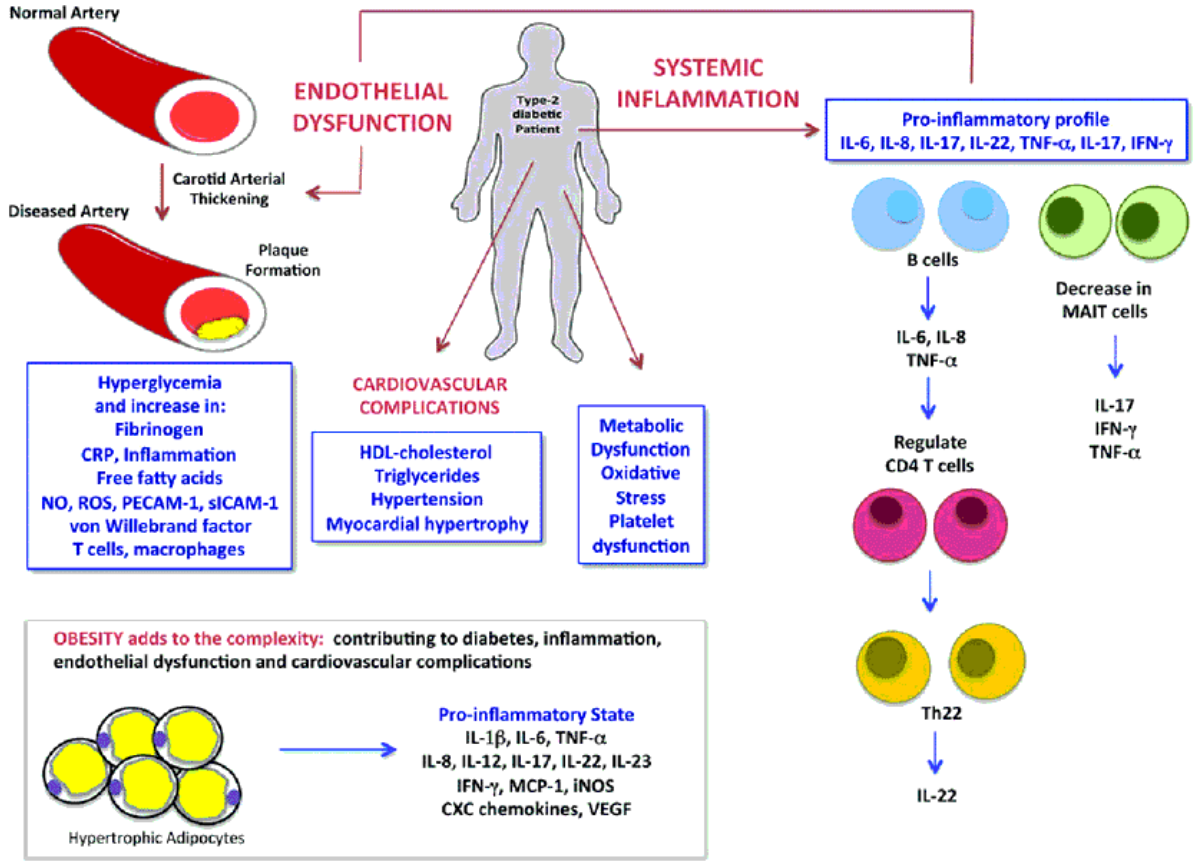


Figure 15: Endothelial Dysfuntion and Cardiovascular complications (Bosevski *et al*, 2015). This figure shows the link between endothelial dysfunction and systemic inflammation, and cardiovascular complications.

1.6.1 Diabetic Dyslipidemia

A very common metabolic abnormality associated with diabetes is dyslipidaemia, characterized by a spectrum of quantitative and qualitative changes in lipids and lipoproteins (Mallol *et al*, 2013). Both insulin resistance and hyperglycaemia have also been implicated in the pathogenesis of diabetic dyslipidaemia (Beverly and Budoff, 2020). Insulin resistance as well as the resultant fatty acids increase are understood to be responsible for the abnormal TRL metabolism in T2DM (Perona, 2017). For example, inhibition of the transcription factor FoxO1 by insulin induced Akt activation represses expression of microsomal triglyceride transfer protein (MTP), thereby inhibiting lipidation of APOB and secretion of VLDL (Henkel *et al,* 2012). In parallel, insulin

promotes hepatic clearance of circulating APOB particles via the LDL receptor (LDLR), LDLR-related protein 1 (LRP1), and syndecan, in part by suppressing APOC3, an apolipoprotein that prevents clearance of TRLs (Kothari and Bornfeldt, 2020). Hyperglycaemia appears to cause increased levels of atherogenic cholesterol-enriched apolipoprotein B-containing residual particles by decreasing expression of the heparan sulfate proteoglycan perlecan in hepatocytes (Takechi, 2010). Associations of atherosclerosis and atherosclerosis risk factors with glycemia have been demonstrated across a wide range of glucose tolerance, from normal to diabetic. Postprandial hyperglycaemia may be a better predictor of atherosclerosis than fasting plasma glucose or haemoglobin A1c (Monnier *et al,* 2006).

Serum lipids are also strongly affected by insulin and hyperglycaemia due to deficiency in insulin actions leading to diabetes (Tangvarasittichai, 2015). Irrespective of insulin deficiency or insulin resistance, serum lipid abnormalities (dyslipidaemia) are commonly seen in diabetic populations (Fatima *et al,* 2020). The underlying pathophysiology of diabetic dyslipidaemia is complex and still not well understood (Martín-Timón *et al,* 2018). Hypertriglyceridemia, low HDL-cholesterol, and a predominance of small dense LDL can be detected years before the clinical diagnosis of T2DM in insulin-resistant, prediabetic individuals with normal glucose concentrations (Wu and Parhofer, 2014). Hyperglycaemia alone cannot fully explain the lipid changes and insulin resistance is believed to be the main trigger for diabetic dyslipidaemia (Jung and Choi, 2014). Research findings have it on a good note that low-density lipoprotein (LDL)-cholesterol (C) is the most important risk factor for atherosclerotic cardiovascular disease (CVD) such as coronary arterial disease (Hirano, 2018).

The risk of cardiovascular disease**,** a leading cause of death in industrialised countries, is increased by abnormal levels of blood lipids such as: High levels of LDL cholesterol (LDL-C) and low levels

of HDL cholesterol (HDL-C) concentration (Nunes *et al,* 2011). The European Atherosclerosis and Cardiology Societies and the National Cholesterol Education Program, through their 3rd report of the Adult Treatment Panel, identified LDL-C and non-HDL-C as the primary and secondary endpoints, respectively, of cholesterol-lowering therapies to reduce cardiovascular risk (Catapano *et al*, 2011). LDL-C lowering therapy has been shown to reduce the rate of cardiovascular events in patients with and without cardiometabolic risk (CMR) (Rosenson *et al,* 2010). As a result, a large number of patients on treatment, as well as those who are undiagnosed, may experience cardiovascular events whether or not they have normal LDL-C levels (Mortensen *et al,* 2022). Patients with diabetes and metabolic syndrome are more likely to suffer from cardiovascular events. These pathologies share a common phenotype characterised by high triglyceride levels, predominance of dense small LDL particles, and low HDL levels (Vekic *et al*, 2022). In individuals with this phenotype, LDL-C has been shown to be a poor predictor of cardiovascular risk, so standard lipid panels measuring cholesterol or lipoprotein triglyceride levels are unlikely to be sufficient to predict CVD risk (Ramasamy, 2018). The risk associated with Type 2 diabetes (T2D) leading to increased cardiovascular disease (CVD) risk is maybe partly due to Diabetic dyslipidaemia – a condition classified by increased plasma triglyceride-rich lipoprotein (TRL) levels, smaller and denser LDL particles, and decreased HDL cholesterol (Huh *et al,* 2022).

To fill this gap, advanced lipoprotein tests (ALT) that allow in-depth characterization of lipoprotein particles across a range of additional parameters, such as particle size and number, have been proposed to improve CVD risk assessment and to guide lipid reduction therapy (Mollol *et al,* 2015).

NMR spectroscopy is a technique that allows the analysis of lipoprotein particles. In short, depending on particle size, the methyl radicals of lipids in lipoprotein particles resonate at slightly

different frequencies, with smaller particles resonating at lower frequencies (Ma and Gao, 2021). Thus, lipoproteins can be quantified by dissociating the core lipid methyl signal into individual signals or by using methyl shell-wide statistical methods to estimate lipid concentrations (Mollol *et al,* 2015).

1.7 JUSTIFICATION FOR THE STUDY

As previously discussed, many adipokines are thought to have a role in the pathophysiology of obesity-associated type 2 diabetes by inducing insulin resistance and more particularly, in many instances, by negative effects on pancreatic beta-cells. Adipokines are also thought to play a significant role in diabetic complications, particularly in light of the pro-inflammatory actions of the majority. This is significant since previous research by the Wolverhampton Diabetes Research Group has demonstrated an increase in the plasma concentration ratio of the pro-inflammatory adipokine leptin relative to the anti-inflammatory adipokine adiponectin (L/A ratio) in diabetic patients with a positive GGap compared with negative GGap, and consistent with the higher incidence of diabetic complications in the former group (Majebi, 2015). Naseem, 2019 looked into defining the molecular, genetic and transcriptomic mechanisms underlying the variation in glycation gap between individuals The selected adipokines in this research are already known for their various roles in beta cell function and failure. Nevertheless, there is a need to further investigate their role in insulin secretion/release.

1.8 AIMS AND OBJECTIVES

The major aim of this research is therefore to:

- Further investigate the role of adipokines in diabetes and diabetic complications with particular emphasis on the part played by novel and established adipokines in beta-cell failure.
- To investigate the role of FN3K inhibitor in insulin secretion in beta cell.
- To investigate the role of inflammation in the GGap in type 2 diabetes.

The objectives of the project were focused on.

- establishing the effect of specific novel and existing adipokines (WISP-1, Visfatin, and sFRP4) on beta cell function and insulin release.
- the effect of inhibition of FN3K {previously shown by us (Wolverhampton Diabetes Research Group) to play a role in the GGap and which is expressed in the beta-cell} on insulin release.
- Further set out was to investigate the potential role of inflammation in the GGap using a novel (and putatively better than existing measures such as CRP) marker of inflammation, GlycA.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Procedures and equipment (In vitro Studies)

2.1.1 Cell lines and cell culture

MIN-6 cells, obtained from the stock at the Diabetic Research Group stored in the liquid nitrogen room at the University of Wolverhampton, were used for all in vitro tests. The passage number for MIN6 cell line was 4 and that of BRIN-BD 11 was 14. MIN6 cell line was obtain from mouse pancreatic beta cells whilst BRIN-BD 11 was obtained from rat pancreatic beta cell. Both cell lines were chosen to be used for this research because they exhibit morphological characteristics of pancreatic beta cells. Cells were cultured in Dulbecco's Modified Eagle's Medium which was supplied by Sigma supplemented with 4500mg/L glucose, L-glutamine, sodium pyruvate, and sodium bicarbonate, liquid, sterile-filtered, cell culture tested, Penicillin/Streptomycin, foetal bovine serum and 2- mercaptoethanol (a reducing agent which prevent toxic levels of oxygen radicals). The culture medium was made up of the following concentrations, DMEM containing 25mM glucose (500ml), 15% foetal bovine serum (75ml), 75mg/l penicillin/ 50mg/l streptomycin (5ml), 5µl/l β-mercaptoethanol (2.5µl stock). BRIN-BD 11 cells were also used for the assessment of insulin secretion. BRIN-BD11 cells were cultured using RPMI1640 made with 11.1mM glucose and supplemented with 10% foetal bovine serum and 1% of 100 U/ml penicillin and 0.1 mg/ml streptomycin. All cells were maintained in an incubator conditioned with 5% CO_2 and 95% air. Cells were sub-cultured every 3 to 4 days or retrieved and seeded in 24-well plates for experiments. Whilst MIN6 has 48 hours doubling time, BRIN-BD 11 has 20 hours doubling time and both cell lines were checked regularly for mycoplasma testing.

2.1.2 Assessment of insulin secretion from MIN6 cells

For insulin secretion assays, cells were seeded at a density of 100,000 cells per well and allowed to attach overnight in DMEM media. Incubation of cells for insulin secretion was carried out using Krebes-Hepes buffer (KHB, pH 7.40), which was made up 119mM sodium chloride (NaCl), 4.6 mM potassium chloride (KCl), 2mM calcium chloride ($CaCl_2$), 1mM magnesium sulphate ($MgSO_4$), 0.4mM monobasic potassium phosphate (KH_2PO_4), 25mM sodium hydrogen carbonate ($NaHCO_3$), 0.15mM sodium hydrogen phosphate (Na_2HPO_4), 20mM Hepes, and 0.05% bovine serum albumin (BSA).

On the day of experiment, confluent cells (80-90%) were initially pre-incubated with KHB containing 0.2 mM glucose for 2 hours for insulin secretion. This is subsequently followed by incubation of cells for 1 hour with graded concentrations of FN3K inhibitor called 1-deoxy-1-morpholinofructose (DMF) (0.2mM to 30mM), novel adipokine (WISP1, 0.1 to 1.0ng/ml), sFRP4 (0 to 100ng/ml), and eNAMPT/Visfatin (0.1 to 10ng/ml). Incubation was carried out at low (2mM) glucose, and high (20mM) glucose. To assess chronic effects of adipokines (WISP1), media were removed from wells of attached cells and replaced with high glucose media in the presence of WISP1 (0.1ng/ml) and further incubated for 48 hours. Treated and untreated cells were then incubated with KHB buffer containing low (2mM) or high (20mM) glucose as previously described to assess the impact of pre-treatment on insulin secretion. Cell supernatants were retrieved from all incubations and stored at -20^{o}C until used for the measurement of insulin concentrations (ELISA ASSAY). During the treatment of cells with different adipokines, 0 glucose was used as negative control whilst high glucose was used as positive control. Each sample was replicated, and the experiment carried out 3 different times.

2.1.3 Assessment of insulin secretion from BRIN-BD11 cells

Effects of adipokines were also assessed using BRIN-BD11 clonal pancreatic cells. Incubation and treatment of cells with different agents were carried out as described for MIN6. However, pre-incubation of BRIN-BD11 cells were for minutes while acute incubation with test agents was for 20 minutes. This is consistent with previous protocols for the use of BRIN-BD11 cells for insulin secretion studies (Davies *et al,* 2001).

2.1.4 Insulin ELISA

ELISA (which stands for enzyme-linked immunosorbent assay) is a technique to detect the presence of antigens in biological samples. An ELISA, like other types of immunoassays, relies on antibodies to detect a target antigen using highly specific antibody-antigen interactions.

The measurement of insulin concentration in cell supernatants was carried out using commercially available ultrasensitive insulin ELISA kits from Mercodia (Sylveniusgatan 8A, Uppsala, Uppland, 75322, Sweden). All assays were carried out using the procedure recommended by the manufacturer. Prior to assays and as recommended by the manufacturer, all reagents and samples were brought to room temperature. 10µl of calibrators, controls, and samples were added, 100µl of enzyme conjugate was added to each well, then incubated for 2 hours on a plate shaker. After which, plate was washed with buffer, substrate added and incubated for 15 minutes. Then stop solution was added and measurement taken. A calibration curve was also constructed using calibrators with known insulin concentrations supplied by the manufacturer. All measurements were carried out manually and prolong soaking was avoided as recommended. Optical density was read at 450 nm and results calculated. Readings were taken within 30 minutes of carrying out assay.

2.1.5 Effects of WISP1 on beta cells viability

Prestoblue was used to assess beta cell viability in this study. Prestorblue indicates cell viability by using the reducing power of living cells to quantitatively measure the proliferation of cells. When added to cells, the PrestoBlue® reagent is modified by the reducing environment of the viable cell and turns red in colour, becoming highly fluorescent. In order to determine the effects of WISP1 on beta cells viability, MIN6 cells were prepared as already described above and incubated for 2 days. Using a 96 well plate, 100µl of cell suspension from a trypsinised confluent flask was added to each well. WISP1 with varying concentrations (0.1 to 10ng/ml) were also treated as described above. 10µl of prestoblue was added to each well and incubated for 10 minutes at 37 ^{O}C to measure cell viability and media was changed to WISP1 containing media. In different dilutions range, WISP1 concentrations were incubated for 72 hours, and aliquot samples were taken and frozen at -20^{O}C for insulin assay. Cell viability was measured again with prestoblue to examine the difference between measuring without WISP1 and with WISP1. Cell viability was measured using fluorescence (540-570nm) excitation or (580-610nm) emission as a more sensitive than absorbance and a preferred method of detection. Cell viability was not done for other adipokines because of time constraint and cytotoxicity tests were not carried out as well.

2.2 Human Sample Studies

2.2.1 Recruitment of Participants

Participants were diabetic patients attending clinic at New Cross Hospital, Wolverhampton. Their serum blood samples have been collected and used by my predecessor who worked on Genetics and transcriptomic (GGap) studies. These serum samples were stored in freezer at -80 degrees until ready to be used. Out of the 184 selected patients with positive and negative GGap, used for

the previous study, I selected 54 samples, both positive (n=20) and negative (n=34) GGap for my research. The clinical data (type of diabetes, age, BMI, duration of diabetes, ethnicity, blood pressure, smoking, CVD risk, gender, etc) was accessed from records from my predecessor. The GGap, HbA1c and fructosamine of the samples were already calculated and determined as well as the demographic and other baseline scale by my predecessor. The GGap was calculated as the difference between the true HbA1c and the fructosamine derived standardised predicted F_HbA1c (GGap = HbA1c – F_HbA1c) (Nayak *et al,* 2019).

2.2.2 Ethical Approval

This was obtained by my predecessor since this research is analysing the samples already collected. The reference is quoted below adapted from previous work.

The UK National Health Service Research Ethical Committee (ref. no. 11/WM/0224) approved the genetic and transcriptomic studies. The study was also approved by the University Life Sciences Ethics Committee (Naseem, 2019).

2.2.3 Sample handling and preparation

Serum and plasma fractions were collected from blood samples that had undergone different biochemical procedures after collection. In the case of serum, clotting factors (i.e., fibrinogen) and blood cells are removed by centrifugation, while plasma is usually obtained from blood samples with the addition of an anticoagulant (i.e., heparin). or EDTA). These agents produce intense peaks (EDTA) or overlapping signals (heparin) in the NMR spectrum, so for some NMR applications, serum may be preferred over plasma. However, the metabolic profiles detected in plasma and serum by NMR were comparable, although the EDTA signal complicates the plasma spectral profile. A major advantage of NMR in plasma and/or serum studies is that measurements can often

be performed with minimal sample preparation. Typically, in biological fluid NMR experiments, samples require only the addition of phosphate buffer in a small amount of decolorized solvent and an internal standard for stoichiometric reference and standardization. quantity (Fuertes-Martin *et al*, 2020).

2.2.4 Sample storage

For the analysis of lipoproteins and other plasma/serum metabolites, certain preservation issues must be considered. Samples can be stored under good refrigeration for several days at 2–4 ◦C and up to 7 days at below 4 ◦C. They can be successfully stored at -20◦C for a moderate amount of time (up to 1-2 months), but some enzymes, such as plasma esterases, can still be active at this temperature. Therefore, for longer storage times, −70 or −80 ◦C is required. For the purpose of this research, the samples were stored at−80 ◦C. Regarding stability, several studies reported high levels of stability of glycoproteins measured by NMR in samples frozen and stored for more than 10 years (Fuertes-Martin *et al*, 2020).

2.2.5 Profile of glycoproteins using 1H NMR spectroscopy

Blood samples were shipped on dry ice to the Biosfer Teslab facilities (Reus, Spain) for glycoproteins and lipoprotein testing using the glycoscale and liposcale test respectively.

GlycA was analysed using a software called Glycoscale while lipoproteins were analysed using liposcale. After the preparation of samples, it was placed in the automatic sampler and the exact quantity of sample taken into the machine which is then processed as programmed. Before 1H-NMR analysis, 200 µl of serum was diluted with 50 µl of deuterated water and 300 µl of 50 mM phosphate buffer (PBS) at pH 7.4. 1H-NMR spectra were recorded at 310 K on a Bruker Avance

III 600 spectrometer (Bruker BioSciences Española S.A., Rivas Vaciamadrid, Madrid, Spain) operating at a proton frequency of 600.20 MHz (14.1 T). The Bruker Avance 111 600 (magnetic strength) spectrometer operating at a proton frequency of 600.20 MHz (14.1 T) was used because it was bigger and can accommodate more samples.

The region of the 1H-NMR spectrum where the glycoproteins resonate (2.15-1.90 ppm) were analysed using several analytical functions according to a previously published procedure (Fuertes-Marti *et al*, 2018). For each function, we determined the total area (proportional to concentration), height, position, and bandwidth. The area of GlycA provided the concentration of acetyl groups of protein-bond N-acetylglucosamine and N-acetylgalactosamine, and the area of GlycB those of N- acetylneuraminic acid. GlycF area arises from the concentration of the acetyl groups of N-acetylglucosamine, N-acetylgalactosamine and N-acetylneuraminic acid unbound to proteins (free fraction). H/W ratios of GlycA and GlycB were also reported, being a parameter associated to the aggregation state of the sugar-protein bonds, height was calculated as the difference from baseline to maximum of the corresponding NMR peaks and the width value corresponds to the peak width at half height (Fuertes-Martin *et al*, 2018).

2.2.6 Lipoprotein analysis

250-µl aliquot of plasma were shipped on dry ice to Biosfer Teslab (Reus, Spain) for lipoprotein analysis by using the Liposcale Test. This is an advanced lipoprotein test based on 2D NMR diffusion-ordered spectroscopy which enables exhaustive analysis of lipoprotein particles. The methyl signal was deconvoluted by using 9 lorentzian functions to determine the lipid concentration of the large, medium, and small subclasses of the main lipoprotein classes (VLDL, LDL and HDL), and their size associated diffusion coefficients. Then combined the lipid

concentration with their associated particle volume in order to quantify the number of particles required to transport the measured lipid concentration of each lipoprotein subclass. The lipid composition and the mean size for each particle class was determined and the particle concentration of the 9 subclasses (large, medium, and small VLDL, LDL, and HDL-P).

Finally, weighted average VLDL, LDL and HDL particle sizes were calculated from various subclass concentrations by summing the known diameter of each subclass multiplied by its relative percentage of subclass particle number. The variation coefficients for particle number were between 2% and 4%, and for the particle sizes were lower than 0.3% (Fuertes-Martin *et al*, 2020)

2.3 STATISTICAL ANALYSIS

For in vitro studies, all experiments were carried out with n=3. Therefore, results were expressed as mean ± Standard Error of Mean. To assess statistical significance, data were compared using One Way ANOVA followed by Dunnett's post hoc test and normality of data checked. Statistical significance was established at $P<0.05$.

For the human studies, the total number of samples were 54 divided into 2 groups of negative and positive GGap. Using SPSS 26, sample (clinical data) were analysed based on age, BMI, type of diabetes, duration of diabetes, gender, ethnicity, smoking status, systolic blood pressure, total cholesterol, HDL cholesterol, cholesterol/HDL cholesterol ratio and results were expressed as mean ± Standard Error of Mean. Statistical significance was established at $P<0.05$.

GGap data were also analysed using SPSS 26 based on GlycA, GlycB, GlycF, H/W GlycA, H/W GlycB and results were expressed as mean ± Standard Error of Mean. Statistical significance was established at $P<0.05$.

CHAPTER THREE

3.0 EFFECT OF SELECTED ADIPOKINES ON INSULIN SECRETION/RELEASE AND THE EFFECT OF WISP1 ON CELL VIABILITY

The importance and relevance of adipokines in diabetes pathogenesis and treatment has been over emphasised for over two decades by so many researchers (Liu *et al*, 2014). To date more research has still been carried out to uncover more adipokines that are clinically useful and elucidate more involvement of the already existing adipokines (Schreiner and Genes, 2021). It is of good note that unravelling the clinical importance of adipokines to diabetes pathogenesis will bring a lasting solution to some diseases and thereby cutting clinical and economic cost and improving life (Kim *et al,* 2019). Adipokines could reduce the insulin sensitivity of tissues thereby inducing inflammation and the development of atherosclerosis, diabetes, diabetic foot, and psoriasis (Zorena *et al,* 2020). Adipose tissue, skeletal muscle, and heart are the endocrine organs mainly secrets adipokines both in normal and pathological conditions (Sabbatini *et al,* 2014)

Adipokines have been strongly linked to diabetes and its related complications and known to play a key role as biomarkers for diagnosis and predictions, and also as therapeutic targets (Kim *et al*, 2019). The organs mainly affected by diabetes pathogenesis connect and exert corresponding effects on each other to be able to sustain energy balance. This connection is made possible by secretory peptides or proteins from metabolic organs of which that from adipose tissues are referred to as adipokines (Kim *et al,* 2019).

Adipokines such as adiponectin, leptin, resistin, ghrelin, and visfatin have been implicated in the pathogenesis of Nonalcoholic fatty liver disease (NAFLD) (Qiu et al, 2019). NAFLD and T2DM are shown to regularly collaborate and act in synergy to raise the risk adverse clinical outcomes. The risk of T2DM is approximately double with the association of NAFLD irrespective of obesity

and other metabolic factors (Targher *et al,* 2021). NAFLD aggravates hepatic and peripheral insulin resistance, influences to atherogenic dyslipidaemia, and causes the systemic release of pro-inflammatory cytokines and hepatokines which can advance the development of T2DM (Ferguson and Finck, 2021). Adiponectin and leptin have also been implicated in obesity. A reduction in circulating levels of adiponectin leads to a reduced insulin sensitivity effects in the liver and muscle, thereby resulting in insulin resistance (IR) and hyperglycaemia (Stumvoll *et al,* 2005). Overweight is a predisposing factor to CVD, T2DM, and some common cancers e.g., colon cancer, postmenopausal breast cancer, renal cancer, oesophageal adenocarcinoma, and endometrial cancer (Scully *et al,* 2021). In obesity, adipose tissue expands and releases anti-inflammatory adipokines like adiponectin and pro-inflammatory adipokines like leptin, resistin, visfatin, and chemerin (Zelechowska *et al,* 2020). Chronic low-grade inflammation driven by obesity, characterised by IR and hyperinsulinemia leads to increase levels of insulin-like growth factor-1 (IGF-1) and initiation of transcriptions factors like nuclear factor kappa-B (NF-Kb), a promoter of carcinogenesis (Dalamaga, 2013).

So much effort has been made to develop strategies against new therapeutic targets such as adipokines, multiligand receptor, and inflammatory mediators (Francisco *et al,* 2018). The control of bioactive levels of leptin by high-affinity leptin-binding molecules, monoclonal humanised antibodies blocking leptin receptors, administration of leptin receptor antagonists, or miRNAs targeting leptin, are possible feasible therapeutic options (González-Rodríguez *et al*, 2022). Adipokines and their signalling pathways has been proposed to represent innovative therapeutic strategies for inflammatory disorders (Wang *et al*, 2017). However, more awareness into the pathophysiological role of adipokines in the immune system and other metabolic disorders will be important for the development of novel therapeutic approach (Francisco *et al,* 2018). There are

developing and hopeful strategies that could influence endocrinological function of adipose tissue, skeletal muscle, and heart. These include caloric restriction, prebiotic supplementation, omega-3 polyunsaturated fatty acid (3-n PUFA), and polyphenols (Senesi *et al,* 2020). Further insights suggest that some proteins (C-C chemokine motif ligand, Growth differentiation factor-15, and Insulin-like growth-factor binding protein-6) could serve as biomarkers for the therapeutic success of conventional and surgical strategies, targeting morbid obesity, and or metabolic syndrome (Schmid *et al,* 2022).

WISP1 recently recognised as a novel adipokine is mainly expressed when organs are developing and during disease circumstances (Ferrand *et al,* 2017). It has been implicated in many diseases like visceral obesity, polycystic ovarian syndrome (PCOS), fibrosis, cancer, inflammatory diseases. It can be found in the liver, heart, spleen, colon, lungs, muscles etc (Barchetta *et al*, 2017). The effect of WISP1 on insulin secretion/release was tested to justify its role in beta-cell failure. Because insulin secretion is very vital to cells in order to drive normal body metabolic functions and prevent hyperglycaemia.

Visfatin/eNAMPT: Visfatin exhibits a proinflammatory properties and it is raised in patients with insulin resistance. The level of visfatin also correlate with the severity of hepatic steatosis and fibrosis (Boutari *et al*, 2016). This adipokine was tested since it has been implicated in many diseases state as well as diabetes. Since this research was centred around diabetes and its complications (possible effect of adipokines), visfatin was tested to see the role it plays in insulin release or secretion as a proinflammatory adipokine.

sFRP4 is an adipokine that is also expressed in visceral adipose tissue thereby exert changes in obese and nonalcoholic liver disease (NALD) patients (Su and Peng, 2020). It is found in the liver,

pancreas, skeletal muscle etc. Apart from its role in adipogenesis, it is also implicated in lipid accumulation in the liver (Horbelt *et al,* 2019). This adipokine was tested because of its implications in selectively inhibiting the insulin signalling pathway which is known to regulate glucose metabolism.

3.1 Summary of Methods

In this research, MIN6 cell and BRIN-BD11 cell lines were used for all cell based invitro experiments. MIN6 cells were chosen because it is previously established and confirmed to be a source of pancreatic beta cells (Davies *et al,* 2001). It is from mouse, and it is observed to stably maintains its GSIS (Miyazaki *et al,* 2021). It was readily available and easy to obtain. BRIN-BD11 cells was chosen because it is confirmed as a good source of pancreatic beta cells. It is a clonal glucose-responsive insulin-secreting cell line that is responsive to an array of pharmacological modulators of insulin secretion (Mel *et al,* 2010). It was also readily available and accessible. Preparation and treatment of MIN6 and BRIN-BD11 cell lines are similar and straight forward (Thompson *et al,* 2014).

During the experiments, various concentrations of the adipokines were tested in other to known at which concentration they perform best. If adipokines properties are affected by varying concentrations. In this research, concentrations ranging from 0.1 to 1.0ng/ml was used because the original/stock supply was 20µg powder which was dissolved in PBS or KHB-Low glucose to make a volume of 2.0ml. (Deng et al, 2019; Chaing *et al,* 2015). The experiments were carried out at both low and high glucose concentrations in other to confirm how adipokines perform at low and high glucose concentrations. Low glucose concentration used in this research was between 0.1 - 2mM whilst 20mM was considered high glucose. We also tested acute and chronic (after 48 hours)

effects of adipokines on beta cell secretions. Details of the preparation and treatment of cells for the experiments can be seen in the methodology section.

3.2 RESULTS FROM CELL CULTURE

3.2.1 Effects of FN3K inhibition on insulin secretion from MIN6 Cells

From the experiment it is observed that at the lowest glucose concentration 0.2mM, the effect of FN3K no inhibitor was noticed and basal insulin release from MIN6 cells was 8µg/ml as shown in Figure 16. The introduction of FN3K inhibitor (1-deoxy-1-morpholino-fructose) was evident in the presence of glucose concentration 5mM up to 15mM led to a drastic fall in insulin release, but this effect started to decline as glucose concentration increases to 20mM as seen in the figure below. This suggest that FN3K triggers insulin secretion across different glucose concentrations.

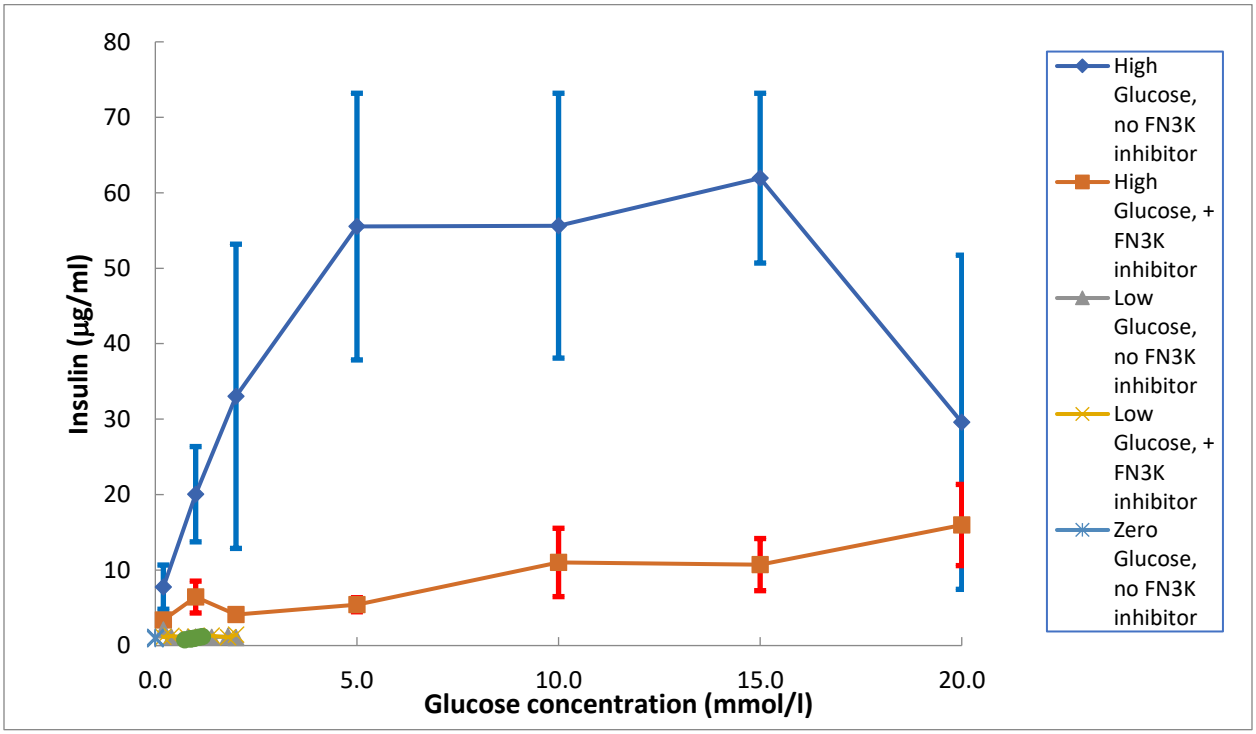


Figure 16 *shows the effects of inhibition of FN3K on insulin secretion with high and low glucose.*

3.2.2 Effects of Visfatin/eNAMPT on insulin release from MIN6 cells

In the figure below, it was observed that visfatin concentration at 0ng/ml had no effect on insulin release. When the concentration was increased from 0.1ng/ml to 1.0ng/ml, insulin secretion effect was observed in a U shape pattern, meaning insulin secretion decreased initially at decreased visfatin concentrations reaching a minimum before increasing as visfatin concentrations increases. Though it is statistically not significant *(P=0.77, 0.22),* it could mean that visfatin is more likely to behave like a monomer (binding with other molecules) in high concentration that dimer.

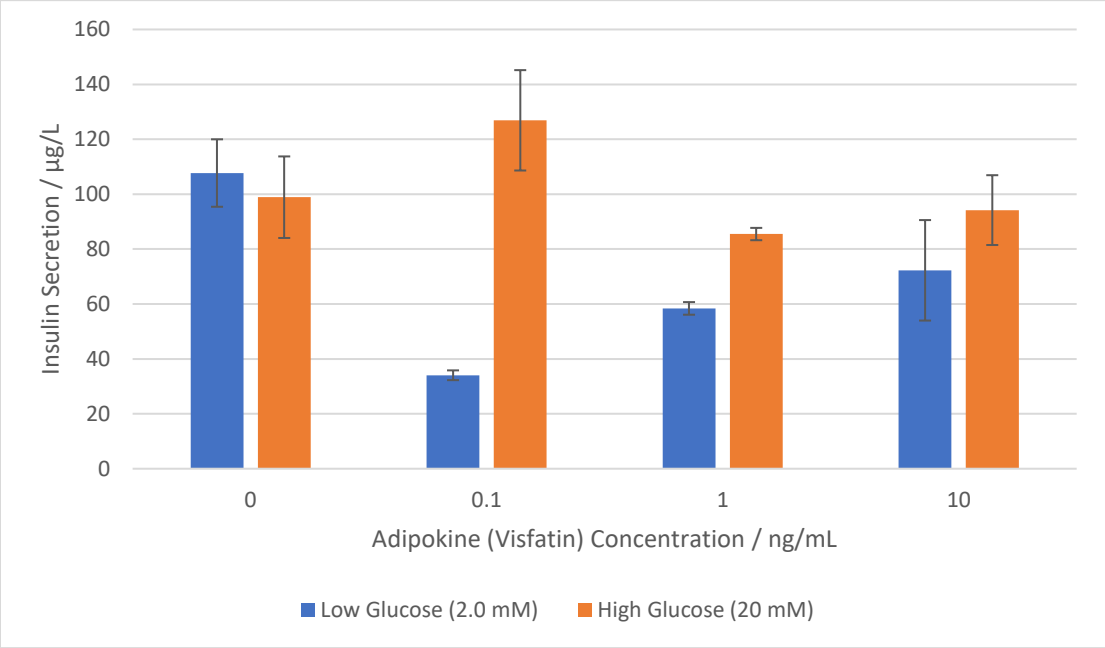


Figure 17 *shows U shape effect of visfatin on insulin release with low and high glucose. Values are mean ± SEM with n=3*
*eNAMPT likely to be more monomer than dimer at high concs. P=*0.77, 0.22

3.2.3 Effects of eNAMPT on insulin release from BRIN-BD11 cells

In the figure below, there appear not to be any significant effect of visfatin irrespective of the concentrations (0 – 1.0ng/ml) in the presence of low glucose on insulin release using BRIN-BD11 cells. This was proven using ANOVA Statistical test (*P* value= 0.32). On the other hand, there is a possible effect of visfatin on insulin secretion at high glucose concentrations (*P* value = 0.033).

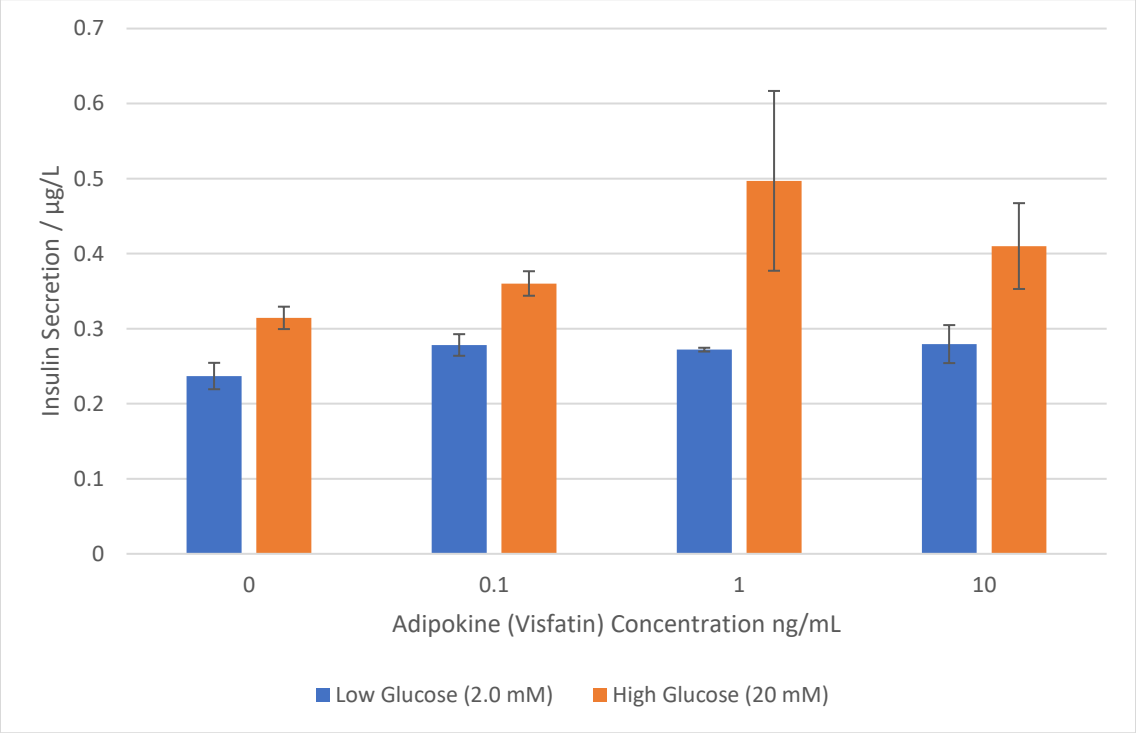


Figure 18 *shows no significant effect eNAMPT/Visfatin on insulin release using ANOVA (Statistical test). Values are mean ± SEM with n=3.*

3.2.4 Acute effects of WISP1 on insulin secretion from MIN6 cells

WISP1, dose-dependently stimulated insulin release from MIN6 cells. This was observed predominantly when cells were treated acutely for 1 hour at low glucose (2 mM) and 0.1 ng/mL as shown below. WISP1 with a smaller effect at high concentrations of the adipokine.

Nevertheless, when MIN6 cells were incubated acutely with WISP1 for 1 hour at high (20 mM) glucose, inhibition of insulin secretion was observed at 0.1 ng/ml WISP1 with reduced inhibition at higher WISP1. This appears to be another acute, dose-dependent effect as seen in figure 4.4.

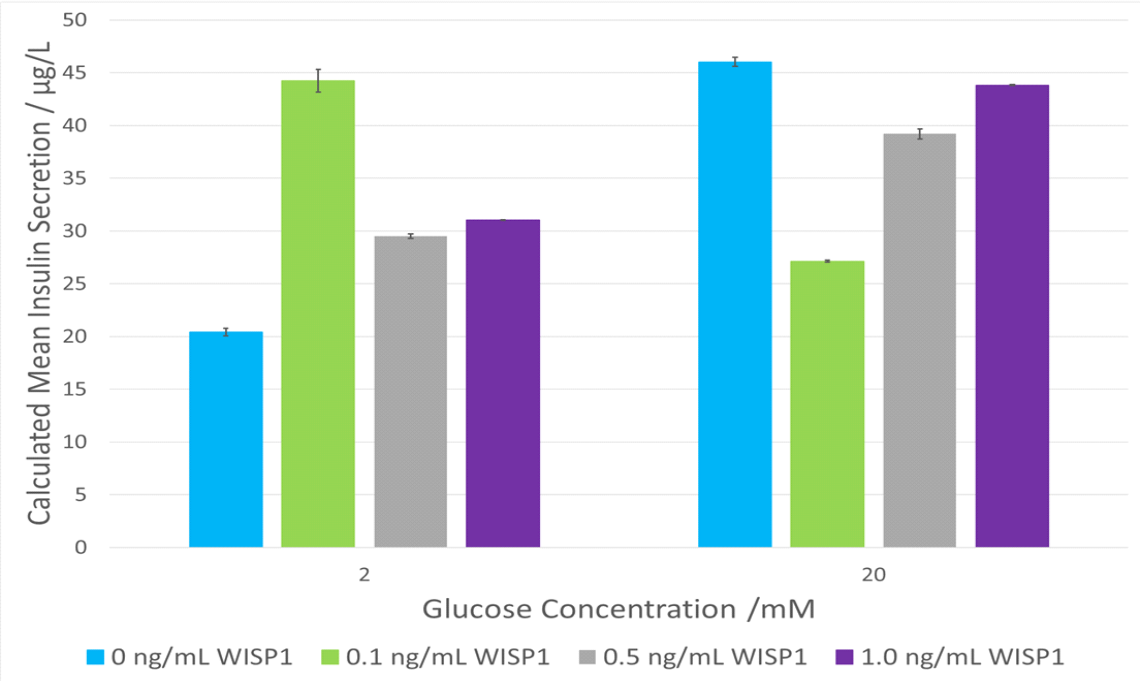


Figure 19 *Acute effect of WISP1 on insulin release from MIN6 cells at low (2 mmol/L and high (20 mmol/L) glucose concentrations (shown as mean+SE, n=3).*

3.2.5 Chronic effects of WISP1 on insulin secretion from MIN6 cells

When MIN6 cells were incubated chronically with WISP1 for 48 hours, stimulation of insulin release occurred at low and high WISP1 concentrations, even when the glucose concentration was only 2mM as seen in the figure below. At 0.1ng/ml WISP1 concentration, the secretion of insulin was at its peak with increase in glucose concentration of 20mM.

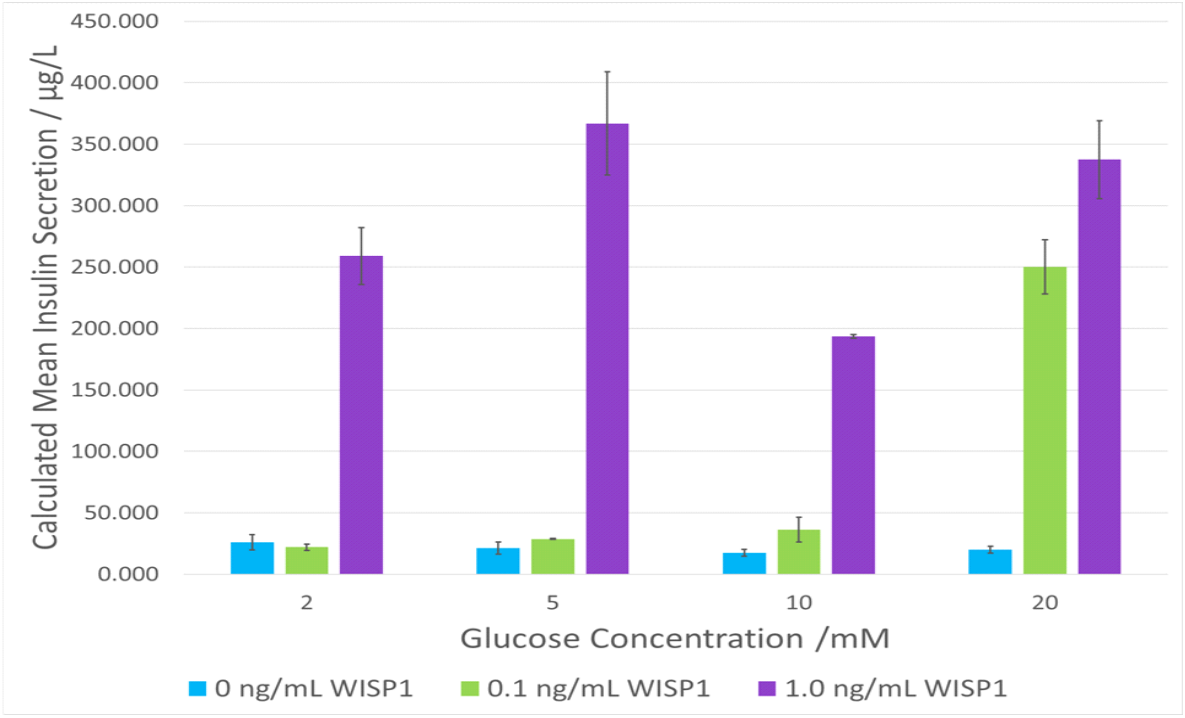


Figure 20 *This graph shows the chronic effects of WISP1 on acute GSIS- so high chronic concs of WISP1 increase acute GSIS over 48hr period (shown as mean+SE, n=3).*

3.2.6 Effects of WISP1 on insulin release from BRIN-BD11 cells

The figure below shows that there was no secretion of insulin from BRIN-BD11 at low or high glucose concentrations. As observed in the graph below, increase in the concentrations of glucose from 2 – 20mM and WISP1 from 0.1ng/ml to 1ng/ml showed a significant difference or effect statistically with *P*-value = 0.0025, 0.005 respectively.

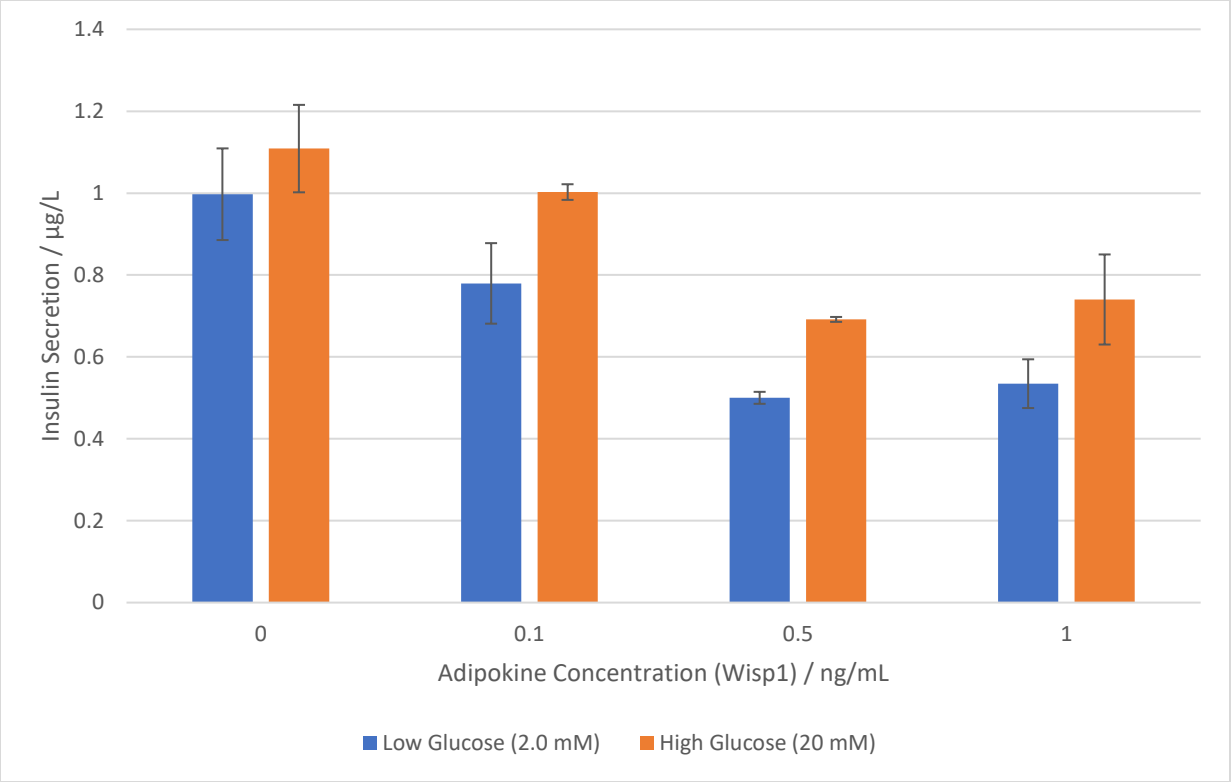


Figure 21: *Effect of WISP1 on insulin release from BRIN-BD11 cells at low and high glucose concentration. Values are mean ± SEM with n=3. P*-value = 0.0025, 0.005

3.2.7 Effect of WISP1 on cell viability in MIN6 cells

The figure below shows how cell viability responded in the presence of low and high glucose concentrations. Cells appears to have started proliferating at increased glucose concentrations 1ng/ml, but this proliferation was not proportional to the period of incubation (72 hours). A decrease in insulin secretion or proliferation was noted after 72 hours of incubation.

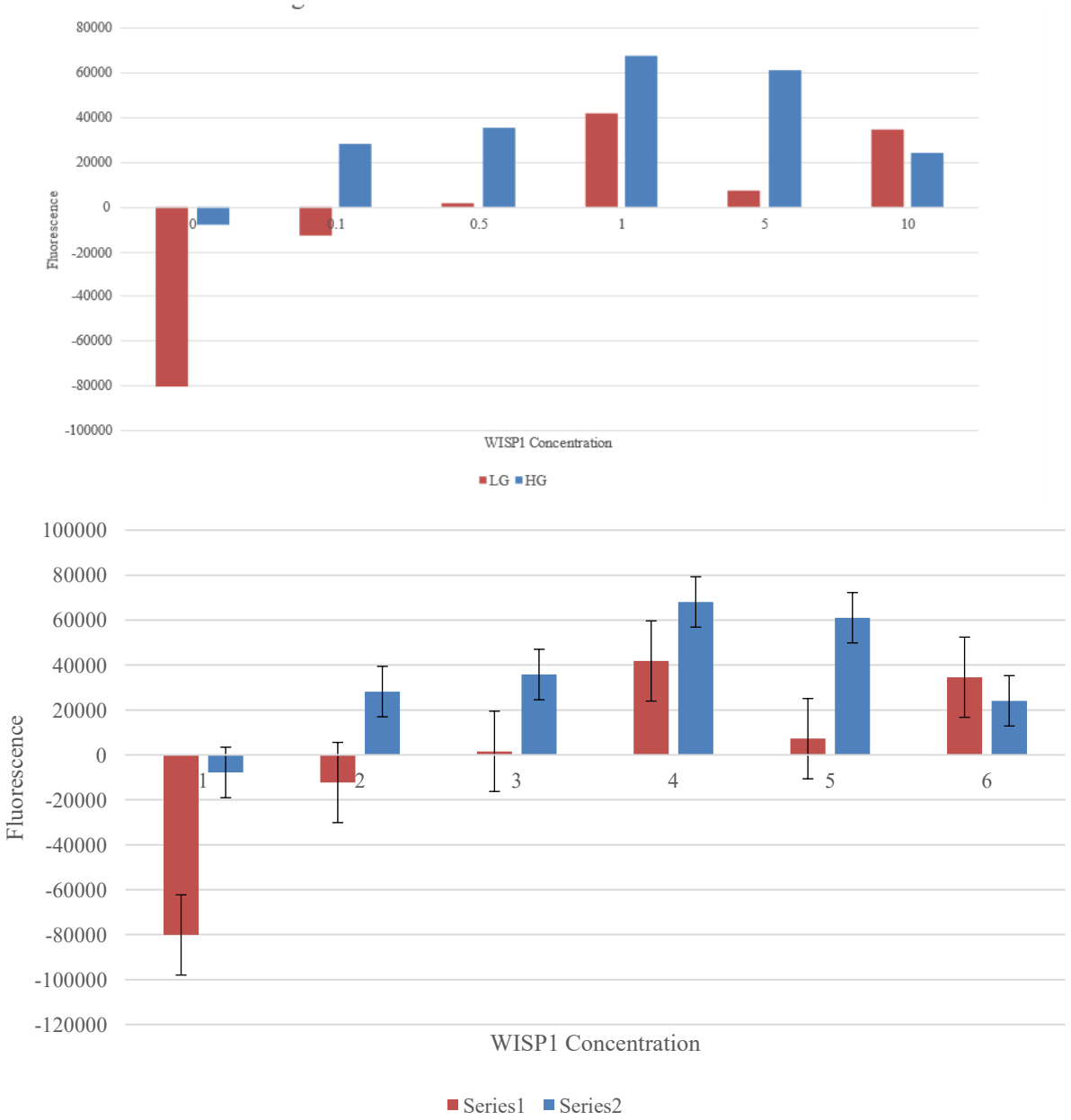


Figure 22: *Effects of WISP1 on cell viability in MIN6 cells. Cells were treated for a period of 72 hours. Values are mean ±SEM with n = 3.*

3.2.8 Effects of sFRP4 on insulin release from MIN6 cells

Inferences from the graph below, sFRP4 has a dose dependent effect, in both low and high glucose samples. Glucose concentration seems at first place to be negligible in effect (could see whether the change in insulin secretion is greater or smaller between concentrations of sFRP4). Generally,

seems to be that the more sFRP4 the MIN6 cells are incubated with, the more insulin that the cells release. This effect is more apparent when comparing HG AK0 and HG AK1, compared to LG AK0 and LG AK1, so potentially sFRP4 has an increased effect at higher glucose levels. However, between HG AK1, HG AK2, HG AK3, the differences are very smell. Between LG AK1, LG AK2, LG AK3, there is a more apparent difference between LG AK2 and LG AK3. Where LG = Low glucose, HG = High glucose, and AK = Adipokine.

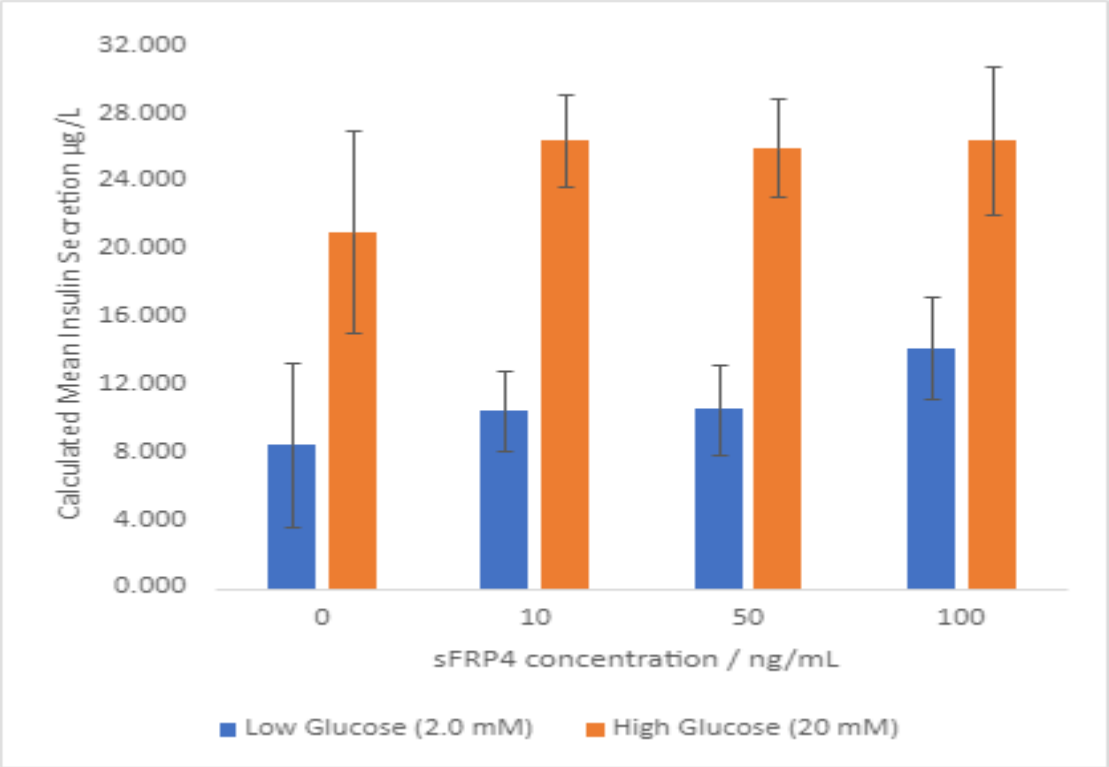


Figure 23 *Acute effect of sFRP4 on insulin release at low (2mmol/l) and high (20mmol/l) glucose concentration (shown as mean +SE, n=3) on MIN6 after incubation with sFRP4 for 24 hours.*

3.3 Discussion

In this research, some cell experiments was carried out which include; effects of FN3K inhibitor on insulin secretion from MIN6 cells, effects of eNAMPT on insulin release from MIN6 cells, effects of eNAMPT on insulin release from BRIN-BD11 cells, acute effects of WISP1 on insulin

secretion from MIN6 cells, chronic effects of WISP1 on insulin secretion from MIN6 cells, effects of WISP1 on insulin release from BRIN-BD11 cells, effects of sFRP4 on insulin release from MIN6 cells, and effect of WISP1 on cell viability in MIN6 cells.

This study found out that FN3K inhibitor when introduced in the presence of low glucose, had no significant effects on insulin release as well as when FN3K inhibitor is not introduced (low glucose). When cells were incubated in the presence of high glucose, there was a significant release of insulin but when FN3K inhibitor was introduced, there was a significant reduction in the release of insulin. From the experiment, FN3K triggers insulin secretion across different glucose concentrations. It was observed that the introduction of the inhibitor (DMF) resulted to a drastic fall in the secretion or release of insulin. This inhibitor has been able to alter or stop the activities of FN3K which is a deglycating enzyme, giving room for more glycation to occur. This observation support already existing claim that FN3K protects against glycation damage by high glucose. Fructosamine 3-kinase (FN3K) is an enzyme involved in the protein deglycation process by fructosyl phosphorylation, returning amino acid residues to their pristine state with 3-deoxyglucosone (3-DG) and inorganic phosphate as by-products (Sartore *et al,* 2020). 3-DG is a very reactive electrophile that is even more potent at generating AGEs than glucose; therefore, inhibitors specific to FN3K have been suggested as a means to suppress 3-DG formation (Tsai *et al*, 2006). The ability of FN3K to convert protein-bound fructosamines into unstable 3-phospho derivatives suggested that it could act as a protein repair enzyme, responsible for deglycation of intracellular proteins (Da-Cunha *et al,* 2006). As the role of FN3K is inhibited, glycation occurs and when sustained result in advanced glycation end-products (AGE) which increases the risk of CVD comorbidity and mortality (Alderawi *et al,* 2020). FN3K functions intracellularly by catalysing ATP-dependent removal of D-fructoselysines from proteins, resulting in the Maillard

reaction flux from glucose to AGE's (Szwergold, 2021). In attempt to knockout or isolate FN3K, a closely related protein called fructoseamine-3-kinase related protein (FN3KRP) copurified with it which does not phosphorylate D-fructoselysines but several others (non-physiological) substrates (Beeraka *et al,* 2021). FN3K activity is attributed to its ability to deglycate a principal regulator of oxidative stress called Nrf2 in cells (Beeraka *et al*, 2021). Sartore *et al*, 2020, show that patients carrying the beneficial allele for the polymorphisms of the FN3K gene have less serious microangiopathy and macroangiopathy indicating a protective role of this genotype against the onset of diabetic complications. FN3K genetic variability have been linked to its enzymatic activity and glycated haemoglobin (HbA1c) levels by many researchers (Sartore *et al,* 2020). AGE accumulation and binding to RAGE can lead to metabolic disturbances, inflammation, and oxidative stress. The excess production of reactive oxygen species (ROS), together with the interaction of advanced glycation end-products (AGEs) with their receptors (RAGEs) on endothelium, leads to cytokine production by endothelial cells (Chen *et al*, 2016). As the endothelium become affected by the accumulation of AGEs, it alters adipokines activities and subsequent cytokine release. Hence, the interest in looking at the selected adipokines. The endothelial layer mediates several vascular protective effects such as vasodilation and inhibition of smooth muscle cell growth and suppression of inflammation through nitric oxide (NO) (Vanhoutte *et al*, 2017). Any defect in NO production can lead to endothelial dysfunction and atherosclerosis (Feldman *et al*, 2000).

From the visfatin experiment using MIN6 cells, a U shape insulin response was observed at low and high glucose concentrations. This implies that visfatin could be more monomer than dimer in high concentrations. Being monomer means it can bind with other similar molecules to form a larger one, example of such is glucose. Visfatin exists in two forms, intracellular (iNAMPT) and extracellular (eNAMPT) forms. The former plays a regulatory role in NAD^+ biosynthesis whilst the latter is associated with many hormone-like signaling pathways and intracellular signaling cascades (Dakroub *et al,* 2021). When visfatin was originally discorvered, it shown to act as an immune modulating cytokine stimulating the release of many inflammatory mediators (Wu *et al*, 2020). It appears to induce MCP-1 production and matrix metalloproteinases expression. It elicits the activation of many inflammatory signaling pathways including NF-kB, mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3 kinase (PI3K) (Behranvand *et al*, 2022). NF-kB regulates the synthesis of key receptors in immune recognition, like major histocompatibility complex (MHC), and cytokines essential to provoke immune reactions, proliferation, and differentiation of lymphocytes. NF-κB is a major regulator of cell adhesion, migration, invasion, metastasis, angiogenesis, and many other processes (Panahi *et al*, 2016). The dysregulation of NF-κB signaling cascade is able to modify the regulation of these processes and may likely affect a large number of genes. Thereby altering several inflammatory molecules promoting atherosclerosis vasculo-pathology, cardiovasculo-metabolic disorders, β-cell destruction in type 1 diabetes (Szekanecz *et al*, 2016). eNAMPT does not exert insulin-mimetic effects in vitro or in vivo but rather exhibits strong NAD biosynthetic activity. NAMPT-mediated systemic NAD biosynthesis is crucial for β cell function, suggesting a vital structure for the regulation of glucose homeostasis (Pavlová *et al*, 2015). Visfatin is believed to employ insulin-mimetic effects in cultured cells and bring down plasma glucose levels in mice by binding to and activating the insulin

receptor. Nevertheless, the physiological relevance of visfatin remains contentious (Revollo *et al,* 2007). There is a need to further investigate the precise and exact mechanisms underlying visfatin's biological effects (Dakroub *et al,* 2021).

Using BRIN-BD11 cells was able to show a possible effect of visfatin on insulin release at high glucose concentrations. Kuryszko *et al,* 2016 believes that visfatin is capable of stimulating insulin release, increase it activities, and increase glucose uptake. Just like visfatin, WISP1 is also implicated in cell adhesion, survival, proliferation, differentiation, and migration (Tao *et al*, 2020).

Acute effect of WISP1 on insulin secretion using MIN6 cells shows that the adipokine dose dependently stimulates insulin secretion even at low glucose concentrations but reduced effect at higher adipokine concentrations. It further showed insulin secretion inhibition at higher glucose concentrations but reduces when the concentration of WISP1 is increased. On the other hand, chronic effect shows the insulin secreting ability of WISP1 regardless of the concentrations of glucose or adipokines. Many researchers have shown that WISP1 impairs insulin sensitivity, interfere with insulin signalling, and promote inflammation (Mirr *et al*, 2021). In subcutaneous and visceral adipose tissue WISP1 mRNA expression, correlate with macrophage infiltration and also associated with IL-18 which is a strong proinflammatory cytokine (Mirr and Owecki, 2021). Evidence shows that WIPS1 adipokine impairs glucose homeostasis and induces diabetes mellitus. In obesity, circulating WISP1 levels and WISP1 expression in VAT are raised irrespective of glycaemic or diabetes status. Additionally, WISP1 impaired insulin signalling in muscle and liver cells (Yaribeygi *et al,* 2019). WISP1 may play a role in linking obesity to inflammation and insulin resistance and could be a novel therapeutic target for obesity. The visceral fat from obese subjects overexpressed WISP1 a novel adipokine considerably and speculate insulin resistance and adipose tissue inflammation (Murahovschi *et al,* 2015). The mechanisms of WISP1 action seems impairing

insulin sensitivity which involves inhibition of glycogen synthesis, interfering with insulin signalling, and promotion of the inflammatory state. WISP1 ability to promote human beta cell proliferation makes it a potential candidate for therapeutic use (Mirr and Owecki, 2021).

The adipokine sFRP4 in this research also shows a dose dependent effect whether in low or high glucose concentrations. However, sFRP4 has an increased effect at higher glucose levels or concentrations which shows its ability to stimulate insulin secretion in hyperglycaemic state. Although, this has some similarities and in agreement with some previous research. Previous research showed that sFRP4 enhances the effect of insulin and its levels increased before the onset of T2DM (Ullah *et al*, 2020). Analysis of sFRP4 in embryonic pancreas of mice using polymerase chain reaction (PCR) shows that throughout development, sFRP4 expression was extremely low but at postnatal 7^{th} day peak of expression was observed (Bukhari *et al,* 2019). The association of sFRP4 with β cell dysfunction in diabetes is strongly associated to expression in pancreatic islets (Shrewastwa *et al*, 2023). Unlike WISP1, it is a Wnt antagonist which inhibits Wnt signaling pathway and may therefore leads to its raised expression in β cells of diabetic individuals. Just like visfatin and WISP1, sFRP4 is associated with obesity and T2DM. Mature adipocytes produces Wnt ligands and sFRP4 with insulin sensitising and anti-inflammatory properties (Liu *et al,* 2018). The overexpression of sFRP4 leads to the elevation in lipid accumulation and its expression in adipose tissue of obese people is increased which is associated to insulin resistance (Bukhari *et al,* 2019). sFRP4 was investigated as a biomarker of pancreatic islet dysfunction in T2DM and was suggested to be useful in predicting the early risk especially in people who are apparently healthy (Shrewastwa *et al*, 2022). It may be interesting to target sFRP4 to mitigate pathophysiological changes that occur during diabetes mellitus development of type 2 diabetes and fatty liver disease (Hörbelt *et al*, 2019). The levels of sFRP4 are seen to be high in diseases which results in less

insulin production, thereby leading to reduced carbohydrate metabolism. The *in-silico* approach was used to discover some potential inhibitors against sFRP4. This could pave a way for diabetes mellitus cures in the future by reducing the expression of sFRP4 and increasing insulin production. Using the inhibitors cyclothiazide, clopamide and perindopril to inhibit sFRP4, it could lead to possible treatment of T2D (Bukhari *et al,* 2014). sFRP4 an inflammatory mediator which causes dysfunction of β-cells and reduces secretion of insulin in humans but do not have any effect on β-cells viability or insulin content (Bukhari *et al*, 2019).

The cell viability test shows that more viable cells proliferate at increase in glucose concentration but not with extended period of incubation. At prolong incubation period, the viable cells proliferation decline. This could be attributed to cell death (apoptosis) due to prolong incubation. The control (low glucose) sample after 72 hours of incubation has the least viable cells. This could be because of the prolong incubation in which the cells would have been depleted of glucose, an essential nutrient for cell growth. Deficiency of WISP1 has been shown to provoke lipopolysaccharide-induced adipocyte apoptosis (Liu *et al,* 2022). It is also believed that the adipokine (WISP1) cytotoxicity may also have contributed to the cell death leading to the reduction in cell viability at prolong incubation. The cell viability of other adipokines (sFRP4 and Visfatin) were not tested due to time contrail.

3.4 Conclusion

This research shows that FN3K (a vital protein which helps prevent glycation and diabetes complications) is able to induce insulin secretion in the presence of glucose irrespective of the concentration. This research indicates that FN3K inhibitor (DMF) is capable of preventing the glycation of proteins (supporting already existing record) or action of FN3K thereby allowing

protein glycation. This will thereafter lead to insulin resistance, hyperglycaemia and finally T2DM. This research also shows that adipokines like visfatin, WISPI, sFRP4 are all important and are capable of participating in insulin secretion. The ability to secrete insulin is very important in diabetes treatment and management. It could also help as therapeutic target and avoid diabetes complications. Amongst the findings is that with increase in glucose concentration, more viable cell proliferates. This is very important because viable cells are required for insulin secretion or release and also to maintained healthy cells, organs, and tissues.

CHAPTER FOUR

4.0 ANALYSIS OF GLYCOPROTEIN ACETYLATION (GlycA) IN PATIENTS BLOOD SAMPLE

4.1 Introduction

The diagnostic utility of glycoprotein acetylation (GlycA) as a biomarker of many diseases, including type 2 diabetes, is emerging (Amigó *et al*, 2021). Consistent with this observation is the report that GlycA is also a strong indicator of severe and prolonged infection and associated mortality (Shah *et al*, 2023)). GlycA is a protein and inflammatory biomarker that has been linked with T2DM irrespective of gender orientation. GlycA as a biomarker of system inflammation, quantifies both the protein concentrations and glycosylation states of various acute phase proteins (Mehta *et al,* 2020). The synthesis and secretions of GlycA are raised during inflammation or physical injury and their glycan are altered vigorously (Amigó *et al*, 2021). Both subclinical atherosclerosis and CVD have been linked to GlycA and high levels are found in individuals with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and psoriasis (Tibuakuu *et al*, 2019). GlycA levels are higher in acute and chronic phase inflammation where reactants and proteins experience circulating concentration and glycosylation pattern changes, and these changes are displayed in the GlycA NMR signal (Mehta *et al*, 2020).

Other than the NMR, there are other techniques used to measure or determine glycoproteins. Amongst these techniques are lectin analysis, DNA sequencing equipment-fluorophore assisted carbohydrate electrophoresis (DSA-FACE), chromatographic methods [high performance liquid chromatography (HPLC) , or hydrophilic interaction liquid chromatography (HILIC), MALDI-TOF analysis of tryptic glycopeptides, electrophoresis, mass spectrometry (MS), glycoblotting method (combination of BlotGlycoABC bead and MALDI-TOF MS) to detect abnormal

glycosylation patterns in the whole serum glycoproteins. Recently enzyme-linked immunosorbent assays (ELISAs), electrochemiluminescence immunoassay (ECLIA), Luminnex-based assays, radioimmunoassay (RIA), and nephelometric assays, was used (Fuertes-Martín *et al,* 2020). In recent years, nuclear magnetic resonance (NMR) has played a major role as an analytical tool for biological fluid metabolism studies, especially for serum and plasma samples. A clear example of this is its application to lipoprotein determination. Unlike other techniques, NMR can reproducibly and efficiently quantify metabolites, so it is widely used in large epidemiological studies and has begun to be put into practice. routine clinical practice (Fuertes-Martin *et al*, 2020).

Cholesterol and triglycerides are the lipids measured as total plasma values in lipid profiles, are significant for most cells. Cholesterol is a fundamental component of cell membranes, which also acts as precursor for bile acids and steroid hormones (Nordestaard and Varbo, 2014). They are taken up via the intestine when newly synthesised and will remain in the body unless it is converted to steroid hormones, lost through skin cell detachment, or secreted via bile because it cannot be broken down by human cells (Nordestgaard, 2017).

Triglycerides are significant as source of energy and act as an energy reserve after storage, as well as for insulation against cold weather (Nordestgaard, 2017). The enzyme lipoprotein lipase helps to degrade triglycerides in very low density lipoprotein (VLDL) in fat and muscle tissue, and the cholesterol-rich intermediate density lipoprotein (IDL) particle is formed (Shabana *et al*, 2020). With the help of the triglyceride-degrading enzyme hepatic lipase, some IDL particles are cleared by liver cells, while others are converted to low density lipoprotein (LDL) particles. LDL particles are taken up via the LDL receptor in the liver and other tissues (Nordestaard and Varbo, 2014). There is weaker and weaker evidence to support the thought that High density lipoprotein (HDL) particles are significant in atherosclerosis and cardiovascular disease (Shabana *et al*, 2020).

Lipoprotein abnormalities are noted to be prevalent in obesity and heart diseases. Due to the increasing incidences of obesity, insulin resistance, and type 2 diabetes, knowing/lowering triglyceride levels is very important (Scordo and Pickett, 2017).

It was necessary to conduct a human study in this research work because it will help in finding out the various glycated proteins, the extent of glycation, and the potential damage. It is also important in that it could reveal novel inflammatory marker that could be useful therapeutic targets in the treatment and management of diabetes as well as its complications. The in vitro cell culture work which looked into different adipokines roles in insulin secretion or inhibition that could help in insulin resistance which is the foundational problem in T2DM and metabolic disorders. Both in vitro and human study were carried out to identify any potential therapeutic target for the proper management and prevention of diabetes and its complications. The relationship of the cell culture work to human cell work is that adipokines and GlycA are both implicated in inflammation which is implicated in the development of insulin resistance and beta cell failure, a strong link to T2DM and other metabolic complications.

Based on these, investigations conducted in this study was aimed at determining the level of glycoprotein acetylation in patients with type 2 diabetes. Moreover, the impact of glycation gap on lipid profiles of type 2 patients recruited for this study will be assessed.

GlycA measurement is very important to this research work to achieve one of the major aims and objectives. To measure GlycA in order to elucidate the role of inflammation in the GGap in T2DM patients. As an inflammatory marker, that could detect and measure the circulating glycoproteins early in the GGap samples (positive and negative) which will then give a direction on the implication in diabetes and its complications.

To measure lipoproteins of GGap of type 2 diabetes patients. Lipoproteins were measured to ascertain the potential risk of GGap samples with type 2 diabetes patients to its complications.

4.2 SUMMARY OF METHODS

4.2.1 Participants selection and sample collection

Selection of suitable patients for the study was critical after ethical approval. All simultaneous HbA1c and fructosamine values measured over 10 years were identified from laboratory records at New Cross Hospital Wolverhampton people with diabetes. From amongst those with at least 3 estimations, whose average G-gap was either <= -0.5 or >= +0.5, that were confirmed as having a long-term over 10 years consistent negative or positive G-gap status and who were alive (n=1663), 150 each of G-gap negative or positive subjects were invited by post to participate according to the ethical committee approved research protocol of whom in total 54 patients were collected from the attendees with complete data. Pregnant women, participants with creatinine>200 μmol/L, with known haemoglobinopathy, or those with an unusual electrophoretic pattern on HbA1c testing were excluded from this study. No other demographic or clinical selection criteria were used. The 54 selected patients had 2 pairs of simultaneous estimations of HbA1c and fructosamine and 3 calculated G-gaps that were consistently greater than +0.5% or lower than -0.5% in a positive or negative direction respectively.

4.2.2 Blood collection and storage

Venous blood was collected from all volunteers (refer to section 2.1.2) by venepuncture into heparin-based S-Monovette system (9 ml, Sarstedt), EDTA coated blood collection tubes (sterile 9 ml, Vacutainer, Becton Dickinson, UK) and Tempus Blood RNA tubes (Thermo Fisher

Scientific). Plasma was separated by centrifugation (2000g for 10 minutes at 4°C), aliquoted into 200µl aliquots and stored at -80°C until needed.

4.2.3 Sample analysis procedure

GlycA and lipoproteins were analysed at Biosfer Teslab, Reus Spain. Specifically, GlycA was analysed using a software called Glycoscale while lipoproteins were analysed using liposcale. After the preparation of samples, it was placed in the automatic sampler and the exact quantity of sample taken into the machine which is then processed as programmed. The Bruker Avance 111 600 (magnetic strength) spectrometer operating at a proton frequency of 600.20 MHz (14.1 T) was used because it was bigger and can accommodate more samples. The area, height, position, and bandwidth are determined from the peaks generated by the NMR machine for GlycA (Fuertes-Martin, et al). For lipoproteins, the lipid content, size, and particle number are analysed to determine the large, medium, and small subclasses of the main lipoprotein classes (Mallol *et al*, 2015). 1H-NMR measures GlycA by picking up the signals from glycan groups which is usually α-1-acid glycoprotein, haptoglobin, α-1-antichymotrypsin, and transferrin (Dierckx *et al,* 2019). There are 3 principal parameters in an NMR spectrum: the chemical shift, the coupling, and the relaxation. A proton has a positive charge and a property called spin. A moving charge create a magnetic field meaning that protons can generate their own magnetic fields and have a random orientation (Derome, 2013). Protons in samples applied to a magnetic field may go the opposite way (beta spin), they may align with external magnetic field also. Anytime the nuclei align with magnetic field, it is called alpha spin and majority of the nuclei with high energy state are in alpha state because it is more stable and have lower energy. It requires more energy to move against the applied magnetic field and the difference in energy represent the difference in alpha and beta spin states. When the right energy is applied, alpha spin state can flip to beta spin state and when it flips

back to alpha spin state, it emits radio frequency energy. When it continuous flipping back and forth, it's in resonance, bringing about NMR (Akitt and Mann, 2017). This is the summary of the chemistry behind 1H-NMR spectroscopy which was used to measure GlycA and lipoproteins. This was how we were able to ascertain the role of inflammation in the GGap of diabetic patients. A summary of the procedure followed for the entire analysis is presented in Figure 24

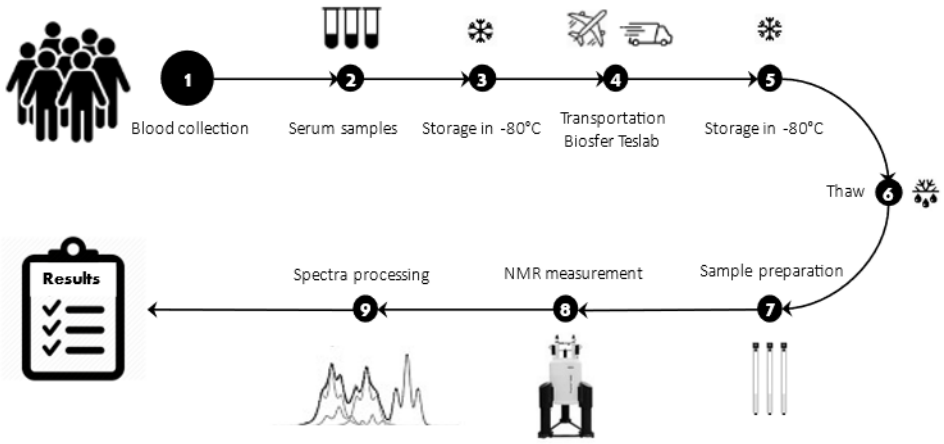


Figure 24*: Summary of the analysis of GlycA and lipoproteins* (Amigó *et al*, 2021)

4.2.4: Statistical analysis

All data analysis were conducted using Microsoft Excel and GraphPad Prism version 11.0. Briefly, values obtained were expressed as mean ± SEM. Groups of data were compared by One Way ANOVA followed by Bonferroni post hoc test. Statistical significance was taken at $P<0.05$.

4.3 Results

4.3.1 Participants' Anthropometric Profile

A summary of participants' profile is presented in Table 4.1. Data presented in the table indicated that majority of participants recruited were male (72.2%, n = 39) and the mean age of participants was (61.95 ± 2.2). The analysis also indicated that 62.9% (n = 34) of participants live with type 2 diabetes while 72.2% (n = 39), 72.2% (n = 39), and 43% (n = 79.63) are from white ethnic origin, non-smokers and have significant cardiovascular risk. Mean values for Body Mass Index (31.2 ± 1.7), systolic blood pressure (134.5 ± 3), Framingham 10-year CHD risk (22.4 ± 4), Total cholesterol (4.16 ± 1), HDL cholesterol (3.5 ± 0.2), Creatinine (88 ± 37), eGFR (77.5 ± 6), and urinary ACR (9.7 ± 11) were also computed for the cohort.

Table 4.1: Summary of anthropometric and clinical data of selected participants

Parameter	Frequency (n, %)
Gender (n, % male)	n = 39, 68.5%
Age (years)	59 – 64
Ethnicity (n, % white)	n = 39, 68.5%
Body mass index (kg/m2)	31.2 kg/m2
Type diabetes (n, % type 2)	n = 34, 62.9%
Duration of diabetes (years)	16.2yrs
Systolic blood pressure (mmHg)	134.5
Smoking status (n, % never)	n = 39, 79.6%
Cardiovascular risk status (n, % primary)	n = 43, 20.4%
Framingham 10 year CHD risk (%) (primary group only)	41.5%
Total cholesterol (mmol / l)	4.15 (mmol/l)
HDL cholesterol (mmol / l)	1.2 (mmol/l)
Creatinine (umol /l)	88 (umol /l)
eGFR (ml / min)	77.5 (ml / min)
Urinary ACR (mg / mmol)	9.7 (mg /mmol)
Retinopathy status (n, % none)	n = 21, 61.1%
Any micro or macrovascular complication	39
Cholesterol / HDL cholesterol ratio	3.5:1

4.3.2 Prevalence of GGAP and the association of GGAP with anthropometric parameters

The analysis conducted in this study indicated that the majority of participants selected for this study are GGAP negative (63%, n = 34). Data indicating the association between GGap and clinical as well as anthropometric data are presented in Table 4.2. The analysis of the data indicated that the prevalence of type 2 diabetes is significantly higher (P<0.001) among GGap positive (90%)

compared to GGap negative patients. However, no significant association was observed between GGap and many other parameters presented in Table 4.2 except BMI (higher in GGap positive, p <0.001), HDL cholesterol (higher in GGap negative, p < 0.001), and total cholesterol/HDL ratio (higher in GGap positive, p < 0.01) (Table 4.2).

Table 4.2: Association between GGap and clinical data

	GGap		
Clinical data	**Negative**	**Positive**	**Statistical significance**
GGap category (n, %)	34 (63%)	20 (37%)	
Type diabetes (n, % type 2)	16 (47%)	18 (90%)	p<0.01
Duration of diabetes (years)	24.1± 12.0	19.1± 9.7	ns
Age (years)	59.0 ± 14.4	64.9 ± 12.2	ns
Gender (n, % male)	27 (79%)	12 (60%)	ns
Ethnicity (n, % white)	25 (74%)	14 (70%)	ns
Body mass index (kg/m2)	27.6 ± 4.3	34.8 ± 6.0	p<0.001
Systolic blood pressure (mmHg)	137 ± 16	132 ± 13	ns
Smoking status (n, % never)	26 (77%)	13 (65%)	ns
Cardiovascular risk status (n, % primary)	27 (79%)	16 (80%)	ns
Framingham 10 year CHD risk (%) (primary group only)	10.4 ± 8.3	12.0 ± 4.3	ns
Total cholesterol (mmol / l)	4.42± 0.8	3.9 ± 0.9	ns
F=4.04, p=0.05) HDL cholesterol (mmol / l)	1.4 ± 0.4	1.0 ± 0.2	p<0.001
Cholesterol / HDL cholesterol ratio	3.1 ± 0.9	3.9 ± 1.1	p<0.01
Creatinine (umol /l)	91 ± 68	85 ± 31	ns
eGFR (ml / min)	76 ± 32	79 ± 26	ns
urinary ACR (mg / mmol)	11.2 ± 34.1	8.2 ± 23.1	ns
Retinopathy status (n, % none)	10 (29%)	11 (55%)	ns
Any micro or macrovascular complication	26 (77%)	13 (65%)	ns

4.3.3 Analysis of GGAP and haemoglobin glycation

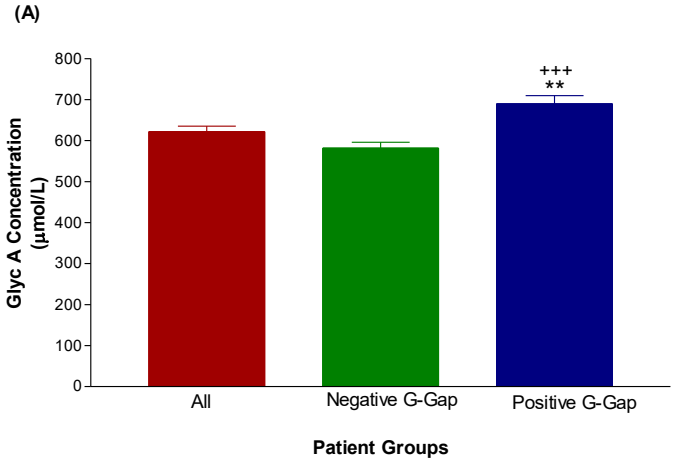
The analysis of the role of GGap on indicators of glycation is presented in Table 4.3. Results presented in the table shows that the number of HbA1c/Fructosamine estimations was not significant different in both GGap negative and positive groups. Time span (period in which HbA1c/Fructosamine was measured) was not significant in both groups, GGap negative and positive groups. However, the mean HbA1c DCCT (%) in GGap negative group (7.7 ± 1.0) was observed to be significantly lower ($p < 0.001$) in negative GGap group compared with positive GGap group (9.4 ± 1.2). However, higher mean fructosamine levels (1.2-fold, $p < 0.001$) and fructosamine: HbA1c ratio (1.2-fold, $p < 0.001$) were observed in the negative GGap group. GGap (negative 3.1 - negative 1.0 and (positive 1.1 – positive 2.3) were all significant $p<0.001$.

Table 4.3: Effects of GGap on indicators of glycation

Indicators	**GGap Groups**		
	Negative	**Positive**	**Statistical Significance**
Number of HbA1c / Fructosamine estimations	5.4 ± 2.5 (3 -13)	5.4 ± 2.5 (3 -10)	ns
Time span (months)	72 ± 36	65 ± 35	ns
Mean HbA1c DCCT %	7.7 ± 1.0	9.4 ± 1.2	p<0.001
Mean Fructosamine	357 ± 50	298 ± 43	p<0.001
Mean F_HbA1c	9.3 ± 1.2	8.0 ± 1.0	p<0.001
Mean GGap (range)	-1.6± 0.5 (negative 3.1 - negative 1.0)	1.5 ± 0.3 (positive 1.1 – positive 2.3)	p<0.001

4.3.4 Analysis of glycoprotein levels in GGap negative and GGap positive participants

Results of the analysis of the levels of GlycA, Glyc B and Glyc F in selected participants is presented in Figure 25. The analysis revealed that the average mean GlycA level across all participants (621.8 ± 13.6 µmol/L) is similar to the level observed for participants in the GGap negative (581.7 ± 14.39 µmol/L). However, the value observed for the GGap positive group is 10.9% ($p < 0.01$) and 18.6% ($p < 0.001$) higher compared to the combined and GGap negative groups (Figure 25). For GlycB, the value obtained for the negative GGap group (330.5 ± 7.2 µmol/L) is lower by 7.8% ($p < 0.05$) compared to the group and by 21.0% ($p < 0.001$) compared with the GGap positive group. However, for GlycF, values obtained across all the groups are similar. Average mean GlycF level across all participants was (216.4 ± 6.6) which is similar to participants in GGap negative (208.6 ± 7.0) and GGap positive (229.7 ± 12.9) groups. (Figure 25).



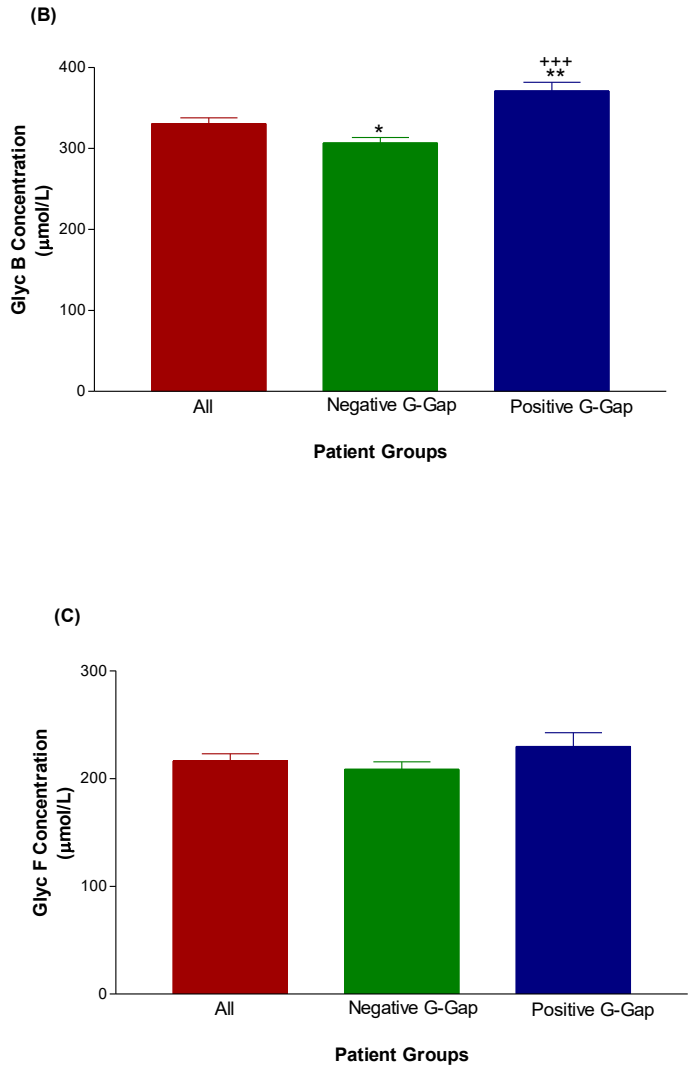


Figure 25: Serum levels of the height:width ratio for Glyc A (A) and Glyc B (B) peaks.

Values are presented as mean ± SEM. p <0.05, 10.9% ($p < 0.01$) compared to combined group 18.6% ($p < 0.001$) compared to the GGap negative group.

The analysis of the height: width ratio of peaks observed for GlycA and GlycB (Figure 26) also indicated that the value for GGap positive groups is significantly higher than the value for the combined group ($p < 0.05$) and the GGap negative group ($p < 0.001$).

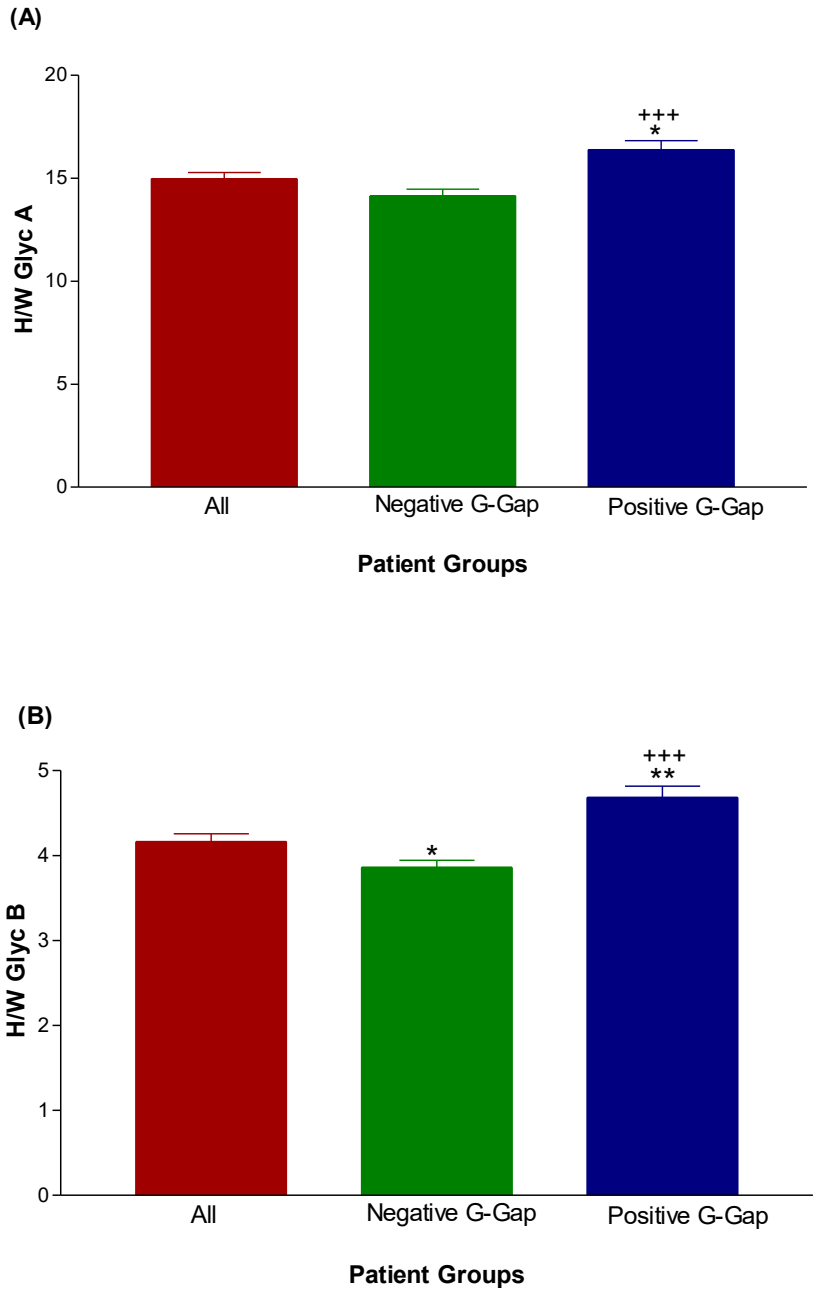


Figure 26: Serum levels of the height:width ratio for Glyc A (A) and Glyc B (B) peaks. Values are presented as mean ± SEM. $p < 0.05$, (7.8%) $p < 0.01$ compared to combined group (21.0%) $p < 0.001$ compared to the GGap negative group.

4.3.5 Analysis of lipoprotein levels in GGap negative and GGap positive participants

The analysis of the impact of GGap on levels of cholesterol and triglycerides selected patients were also analysed in this study. For cholesterol, the distribution of participants based on whether their cholesterol level is within or above the normal range was initially computed. Results of the analysis is presented in Table 4.4.

Table 4.4: Distribution of participants based on their cholesterol levels.

Type of cholesterol	Level category	All (n = 54) Frequency (%)	GGap Negative (n = 34) Frequency (%)	GGap Positive (n = 20) Frequency (%)
VLDL	Normal	49 (90.7)	33 (97.1)	16 (80.0)
	High	5 (9.3)	1 (2.9)	4 (20.0)
IDL	Normal	50 (92.6)	33 (97.1)	17 (85.0)
	High	4 (7.4)	1 (2.9)	3 (15.0)
LDL	Normal	53 (98.1)	34 (100)	19 (95.0)
	High	1 (1.9)	0 (0.0)	1 (5.0)
HDL	Normal	44 (81.5)	24 (70.6)	20 (100.0)
	High	10 (18.5)	10 (29.4)	0 (0.0)

Key: Normal Range: VLDL = 2-30 mg/dL, LDL = <130 mg/dL, HDL = 35-65 mg/dL, IDL = <15 mg/dL

Data presented in Table 4.4 indicate that majority of combined participants (90.7%), GGap negative (97.1%) and GGap positive (80.0%) had normal VLDL cholestoreol. A similar trend was observed for all types of cholesterol. However, a higher percentage of GGap positive participants had higher VLDL (20%) and IDL (15%). Only 1 GGap positive participant had high LDL while none was observed in the negative GGap group. However, significantly higher number of negative GGap participants (29.4) had HDL cholesterol.

The size of cholesterol molecules was also analysed in this study. The measurement strategy employed in this study is shown in Figure 27. The analysis revealed that majority of the VLDL in across the combined group, the GGap negative and GGap positive are small particles (Figure 27A).

In the combined group, the concentration of medium sized particles was 3.7-fold ($p < 0.001$) compared to large sized particles, and the concentration of small sized molecules was 8.4-fold ($p < 0.001$) and 30.9-fold ($p < 0.001$) higher compared to medium-sized and large-sized particles. The trend observed for negative and positive GGap groups are similar (Figure 27A).

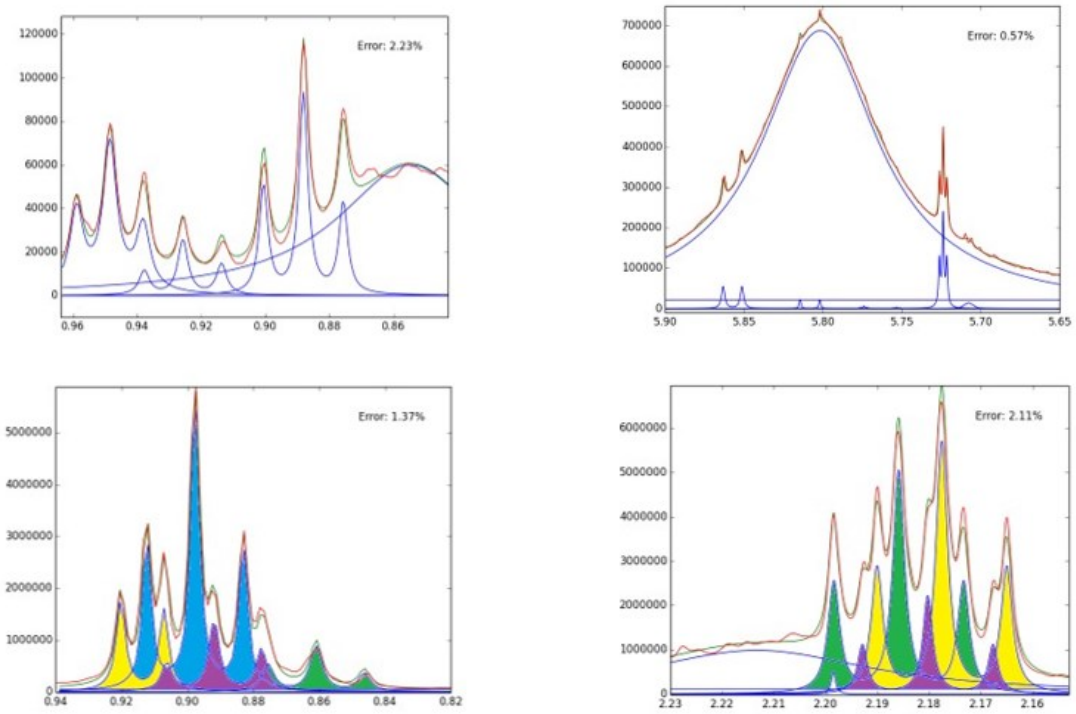


Figure 27: Measurement strategy for cholesterol particle size.

The majority of LDL in across the combined group, the GGap negative and GGap positive are small particles (Figure 4.4B). In the combined group, the concentration of medium sized particles was 1.6-fold ($p < 0.001$) compared to large sized particles, and the concentration of small sized molecules was 2.1-fold ($p < 0.001$) and 3.3-fold ($p < 0.001$) higher compared to medium-sized and large-sized particles. The trend observed for negative and positive GGap groups are similar (Figure 27B). Also, the majority of HDL in across the combined group, the GGap negative and GGap

positive are small particles (Figure 27C). In the combined group, the concentration of medium sized particles was 38.2-fold ($p < 0.001$) compared to large sized particles, and the concentration of small sized molecules was 1.7-fold ($p < 0.001$) and 63.8-fold ($p < 0.001$) higher compared to medium-sized and large-sized particles. The trend observed for negative and positive GGap groups are similar (Figure 27C).

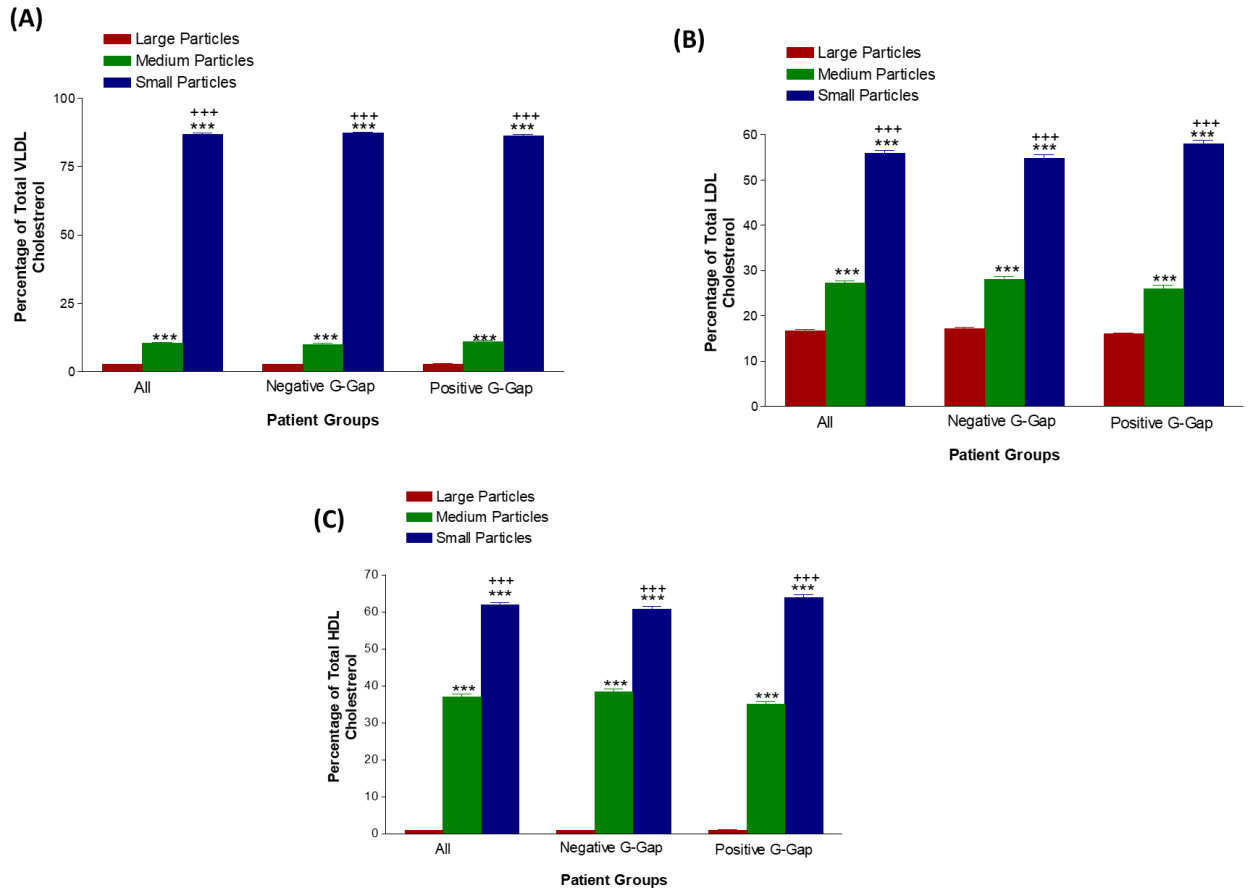


Figure 28: Analysis of the particle size of VLDL, LDL and HDL cholesterol. (3.3-fold) $p < 0.001$compared to large particle and (2.1-fold) $p < 0.001$ compared to the medium particles.

For triglycerides, the distribution of participants based on whether their triglyceride level is within or above the normal range was initially computed. Results of the analysis is presented in Table 4.5

Table 4.5: Distribution of participants based on their triglyceride levels.

Type of cholesterol	Level category	All (n = 54) Frequency (%)	GGap Negative (n = 34) Frequency (%)	GGap Positive (n = 20) Frequency (%)
VLDL	Normal High	43 (79.6) 11 (20.4)	29 (85.3) 5 (14.7)	14 (50.0) 6 (30.0)
IDL	Normal High	36 (66.7) 18 (33.3)	26 (74.5) 8 (23.5)	10 (50.0) 10 (50.0)
LDL	Normal High	53 (98.1) 1 (1.9)	34 (100) 0 (0.0)	19 (95.0) 1 (5.0)
HDL	Normal High	4 (7.4) 50 (92.6)	3 (8.8) 31 (91.2)	1 (5.0) 19 (95.0)

Key: Normal Range: VLDL = <98 mg/dL, LDL = <19 mg/dL, HDL = <12 mg/dL, IDL = <12 mg/dL

Data from the above table shows that a high percentage of the patients have normal VLDL cholesterol (79.6%). There is not much difference in their GGap (negative - n=5, positive - n=6). A very high percentage of the patient LDL is normal (98.1%) and 95 % of GGap positive patients have normal LDL. The percentage of patients with normal HDL is very low (7.4%) while 92.6% was high. There was not much percentage difference between GGap negative (91.2) and positive (95.0) patient. Traditionally, it is believed that LDL-C being bad cholesterol should be high and HDL should be normal/close to normal to show proper management. Higher HDL (which can be harmful rather than protective against CVD) could be from certain medications used by patients or genetic mutation (Franczyk *et al,* 2021). Judging from table 4.5, the patients are not at risk of CVD or metabolic syndrome due to normal LDL-C whether GGap negative (100%) or GGap positive (95%). But recent research has shown that smaller and denser LDL particles are self-determining indicator of

patients at risk of CVD as a result of their higher susceptibility to oxidation, and permeability in the endothelium of arterial walls (Talebi *et al,* 2020).

4.4 Discussion

From the results above it was found out that the positive and negative GGap groups differ significantly in their type of diabetes, especially type 2 diabetes which was significantly excess in the positive GGap group as well as their BMI which was higher. Though the relationship between GGap and BMI Has been established but this research has shown more about the class of GGap (positive). This suggests that the GGap positive diabetic patients have less deglycation and the corresponding BMI is a further agreement and confirmation of its link to diabetes and its complications. As shown in the table 1 above, other clinical variables were not significant, e.g., age, duration of diabetes, gender, ethnicity, systolic blood pressure, smoking, retinopathy status, total cholesterol, any micro or macrovascular complication, cardiovascular risk status, creatinine, eGFR, urinary ACR. This suggests that irrespective of the GGap group, the mentioned clinical variables were not statistically significant. Observation from the results shows a lower HDL cholesterol which is known as the good cholesterol, and a higher cholesterol/HDL cholesterol ratio in the Positive GGap group. Despite the good cholesterol was low in the positive GGap group, but in combination with the total cholesterol, it became high. Statistically, there was no significant difference in macrovascular complications prevalence, or in primary macrovascular risk, and microvascular retinopathy or renal status. But this could be an indication that, the GGap positive group are susceptible to dyslipidaemia or other lipids complications. Puig-Juve *et al*, 2022 believes GlycA is highly associated with myocardial dysfunction in T1DM and suggest that diabetic dyslipidaemia could also be a contributor. Nayak *et al*. (2019) proved the relationship of GGap

with retinopathy and nephropathy. Memarian *et al*, 2021 showed the association of nephropathy and retinopathy to T2DM.

GlycA and GlycB concentrations are seen to be elevated but GlycF was not, in the positive GGap group. This suggest that GlycF which is the unbound portion of the glycans may be less relevant as an inflammatory marker compared to GlycA and GlycB. 1H-NMR detect signals from glycoproteins circulating in the blood indicating that GlycA is more important follow by GlycB. This finding is in alliance with existing data like, Seres-Noriega *et al*, 2020 Carmona-Maurici *et al,* 2020 Fuertes-Martin *et al*, 2020 and Amigo *et al*, 2021. Connelly *et al*. (2017) looking at a population of men and women, found out that GlycA is significantly correlated with T2DM and also independent of hsCRP. GlycA independently predict T2DM even after adjusting for conventional diabetes risk factors. Akinkuolie *et al,* 2013 also agree that GlycA is strongly associated with diabetes (though in women), suggesting it to play a novel part of protein glycans in diabetes development. Dugan *et al*, 2015 did not find GlycA a significant predictor of glucose control but found that GlycA was associated with increased IL-6 and CRP, lower haemoglobin and GFR.

This research has been able to show that GlycA is elevated in GGap positive diabetic patients as well as the BMI of GGap positive patients were higher compared to GGap negative diabetic patients. GGap positive group was significantly correlated with T2DM. This agrees with the research carried out by Nayak *et al,* 2019 and Wang *et al,* 2022 were they found out that GGap was positively associated with T2DM patients. Both positive and negative GGap group were significantly associated with the type of diabetes. Our findings that GGap varies significantly with demographic characteristics and important clinical outcomes agrees with Nayak *et al*, 2019. GlycA was significantly associated with mean HbA1c, fructosamine, FHbA1c, and GGap individually

but not combined HbA1c/fructosamine estimations. GlycA and GlycB were found to be significantly correlated with GGap and types of diabetes confirms it as an inflammatory marker and a potential or possibly better marker. Some researchers have carried out some work on GGap in relation to diabetes, but this is the first time of looking at GlycA as a marker in the GGap of diabetes patients. Our findings are very interesting and intriguing as we were the first to link GlycA and GlycB (glycoproteins) as a better biomarker in the GGap of diabetic patients.

Lipoprotein analysis shows that cholesterol, very low-density lipoprotein-C (VLDL-C), intermediate density lipoprotein-C (IDL-C), low density lipoprotein-c (LDL-C), and high-density lipoprotein-C (HDL-C) all together did not significantly corelate with the GGap. VLDL-C and HDL-C were significantly corelated with GGap. VLDL-Particles (large, medium, and small) were significantly corrected with both negative and positive GGap. Large LDL-Particles and medium HDL-Particles were significantly correlated with both positive and negative GGap. LDL-Z and HDL-Z were also significantly correlated with both negative and positive GGap. Despite administering LDL-C lowering therapy which has shown to reduce the rate of CVD events, CVD events remain prevalent among individuals with low or normal LDL-C (Amigó *et al,* 2021). CVD risk which may not be apparent from LDL-C concentrations could be associated with higher concentrations of atherogenic lipoproteins (Puig-Jove *et al,* 2022). According to Subramanian and Gundry, 2022, apolipoprotein that are glycosylated have been firmly linked with lipoprotein-associated diseases like dyslipidaemia, CVD, stroke, obesity, cancer, T2DM, and neurological disorders. Puig-Juve *et al*, 2022 suggested that triglyceride-rich lipoproteins (TGRLs) perform a key role in the subclinical myocardial dysfunction in T1DM development. Decreased levels of LDL-C are associated with congenital defects of glycosylation. A general characteristic of rheumatoid arthritis (RA) is an altered lipoprotein function (Rodríguez-Carrio *et al*, 2020).

In as much as measuring GlycA was important to this research work, it came with a lot of challenges which include, NMR machine not readily available in the place where this research was carried out, the logistics of carrying or transporting the samples to the place of measurement, the legal requirements, and the financial involvement.

4.5 Conclusion

GlycA is correlated with BMI and type 2 diabetic patients with positive GGap. Both negative and positive GGap are associated with diabetes irrespective of the type. GlycA appears to be a better inflammatory marker than GlycB and the traditional HbA1c.

Although total cholesterol measurement did not correlate with GGap, VLDL-C and HDL-C did which may be indicative of CVD. Measurement of the particles size indicate that VLDL-P (large, medium and small) and LDL-P significantly correlated with both positive and negative GGap. Measurement of particle diameter, LDL-Z and HDL-Z correlated significantly with both positive and negative GGap. This is an indication that measuring lipoprotein particles (size and diameter) could give a more insight in patient at risk of CVD and general health condition. There is also a strong indication that measuring GGap in patients with lipid abnormalities could be very helpful as a guide for clinicians and other healthcare scientist/practitioners.

CHAPTER FIVE

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Introduction

Diabetes being the pivotal/centre point of this research work, its link with adipokines, and thereafter its complications will be summarised here. Other preceding chapters will be summarised, then conclusion and recommendations will follow. This research work was carried out to uncover the relationship of the GGap to diabetes and its complications, and the possible role or involvement of adipokines. This research work has been able to establish the relationship or connection between GGap and diabetes and its complications with the help of an inflammatory biomarker called GlycA.

5.2 Discussion

Before now, in the recent past, hsCRP was used to determine the inflammatory state and the risk of CVD. Here are some of the reasons GlycA is preferrable to hsCRP; GlycA shows both the protein level and the glycosylation states. It does not only mark acute phase response, but it is also linked with chronic diseases e.g., in RA it measures CVD risk independent of hsCRP. It is possible to measure serum glycoproteins acetylation quantifications as well as lipoprotein indices using 1H-NMR. It is more stable, consistent, sensitive, powerful, and reliable. It requires just a single measurement compared to hsCRP which requires many measurements. Patients prefer regular blood to regular stool sampling making serological marker replacing faecal biomarker. It is preferred in patients who cannot be monitored biochemically using faecal samples. hsCRP is known to fluctuate due to unstable parameters involve. GlycA can integrate more inflammatory pathways making it more accountable. The reasons listed above have been confirmed by some other researchers amongst whom are Connelly *et al,* 2017; Fuertes-Martin

et al, 2020; Gruppen *et al*, 2015.The use of GlycA to access GGap in this research work confirmed what have been said by others and also shows new findings which open doors for further research.

With the knowledge that glycation leads to AGEs and thereby cause distortion in adipose tissue resulting in IR (hyperglycaemia) which is the root cause of diabetes and its complications. There was a need to use an inhibitor of glycation enzyme called 1-DMF to confirm the secretion or release of insulin. The application of the inhibiting enzyme produced a crash in insulin secretions or release. This research has been able to establish the fact without the presence of the FN3K (a deglycation enzyme), glycation of proteins will accumulate to form AGEs and thereby leads to diabetes and its complications. Protein deglycation catalysed by FN3K is a process of restoring proteins to their original structure and function prior to the Maillard modification. Once glycated, some protein residues remain glycated until the protein is turned over, because of inaccessibility or low affinity to FN3K. In this research, we were able to show the importance and potency of FN3K inhibitor in protein deglycation. Our research confirmed that FN3K inhibitor 1-deoxy-1-morpholinofructosamine (DMF) is capable of stopping the deglycation proteins thereby, allowing the reaction to proceed to AGE formation. From our result, we noticed that FN3K acts in its maximum capacity in the presence of high glucose. This is a confirmation that there is more glycation in the presence of more glucose. FN3K protects beta-cells against glycation damage and when this role is defeated, the progression of AGE continues which then leads to IR, the release of adipokines and cytokines from adipose tissues. This suggest that FN3K is capable of protecting beta-cells against glycation damage thereby adverting diabetes complications. This is also in agreement with Collard *et al*, 2004 who showed evidence that DMF removes fructosamine residues from haemoglobin in erythrocytes. It is also observed that at higher concentration of glucose, insulin secretion begins to diminish. According to Pascal *et al*, (2010), FN3K is neither needed for the maintenance of β-cell survival and function when the condition is under control nor involved in protection against β-cell glucotoxicity notwithstanding its ability to reduce

the glycation of intracellular islet proteins. FN3K and FN3K-RP (related protein) is believed to have effect on the glycation cascade in vivo with potential positive and negative effects (Hellwig *et al*, 2013). AGEs may be a key factor in the development of metabolic memory in diabetic complications because AGEs are irreversibly produced and accumulated in the body, depending on the extent and timing of their adjustment in blood sugar (Rhee *et al,* 2017).

Although Ross *et al.* (2018) proposed that atherosclerosis is an inflammatory response of the arterial wall to injury more than four decades ago, it is only recently that inflammation has become widely accepted as a mechanism (central mechanism), perhaps the ultimate common pathway leading to the atherosclerotic process. Therefore, high cholesterol, hypertension, high blood sugar, smoking, lack of estrogen, and obesity can all be triggers for inflammation (Ruiz-Nunez *et al,* 2013). These metabolic disorders induce chronic inflammation unlike endotoxins and bacterial infections, which cause acute inflammation (Lassenius *et al*, 2011). Endotoxins and inflammatory cytokines induce a cascade of events that ultimately lead to increased expression of adhesion molecules on the endothelial layer and their respective ligands on leukocytes (Paresh, 2002). During acute inflammation, polymorphonuclear leukocytes adhere to the endothelium, whereas in chronic inflammation, monocytes are predominant (Ward, 2010). This chronic inflammatory process is initiated by adhesion of monocytes to the endothelial surface via adhesion molecules expressed on the endothelium (Van Gils *et al*, 2009). Normal endothelium expresses these molecules in limited quantities. During inflammatory processes, the total expression and turnover of adhesion molecules are markedly increased (Golias *et al*, 2007). Activated/inflamed monocytes and endothelial cells also increase secretion of chemokines such as monocyte chemotactic protein-1 (MCP-1), which attracts monocytes to the site of inflammation (Libby, 2012). Injury and inflammation lead to increased expression of three key adhesion molecules: P-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Wateridge, 2005). P-selectin binds

monocytes weekly and transiently and allows them to slowly roll over the endothelial layer; ICAM-1 firmly binds retarded monocytes and also activates monocytes through this binding; VCAM-1 mediates the migration of monocytes into the subendothelial layer through the endothelial interstitial spaces generated by matrix metalloproteinase (MMP) activity (Libby *et al*, 2010). Activated monocytes produce superoxide radicals, which cause oxidative damage to low-density lipoprotein (LDL) particles (Khatana *et al*, 2020). Oxidized LDL is then internalized by the scavenging receptor, CD36, on the surface of monocytes (Di Pietro *et al*, 2016).

In order to ascertain the role of adipokines on diabetes and its complications, we incubated MIN6 cells in high and low glucose media concentrations. The media was treated with different concentrations of adipokine (WISP1). Another cell line called BRIN BD11 was also used to see the effect of eNAMPT/visfatin on insulin release at low and high glucose concentrations. Another adipokine called sFRP4 was also tested to ascertain their effects in insulin secretions or release. This work yielded a positive result as it was able to establish a relationship between adipokines and diabetes. All adipokines tested, showed in one way or the other their effects or role in insulin secretions or release. Abdul-Waheb *et al* (2014) in their research were able to secrete insulin from the skin of the frog Rana palustris were pseudin-2 stimulates insulin secretion from BRIN-BD11 cells through a mechanism involving Ca^{2+}-independent pathways. Pseudin-2 was identified as a peptide that may have potential for developing as a therapeutically beneficial insulinotropic agent for the treatment of type 2 diabetes (Abdul-Waheb *et al*, 2014). Ethanol extract of *A. squamosa* leaves (EEAS) possesses antidiabetic activity by enhancing insulin secretion both in vitro in clonal pancreatic BRIN BD11 β-cells and in isolated mouse islets, as well as in vivo in high-fat-fed (HFF) obese rats (Ansari *et al*, 2022). Scullion *et al.* (2012) in their research showed reason why BRIN-BD11 cells can become less responsive to stimulation of insulin release at physiological glucose concentrations. Increased hexokinase activity after exposure of the cells to homocysteine (HC) self-

sufficient from the presence of alloxan (Alx) can be taken as the de-differentiating effect of HC, describing why Higher hexokinase activity allows survival of the cells of this tissue culture line.

Using the adipokine eNAMPT/visfatin on a MIN6 cell line shows a U shape effect at certain concentrations of glucose indicating that eNAMPT is likely to be more monomer (ability to combine with other molecules to form a larger one) than dimer at high concentrations. Visfatin/NAMPT seems to be a unique molecule with clinical importance, with a potential promising of a diagnostic, prognostic, and therapeutic effects in many cardiovascular and metabolic disorders. The development of sensitive immunoassays with broader detection ranges to detect serum visfatin precisely may be essential to explain those contentious observations and unravel any possible correlation. The levels of plasma visfatin are not certainly accurate representatives of the tissue activity (Dakroub *et al,* 2020). eNAMPT/visfatin is capable of inhibiting IGF-1 function by activating the extracellular signal-regulated kinase (ERK)/mitogen activated protein kinase (MAPK) pathway without IGF-1 receptor activation, suggesting a novel mechanism for IGF-1 resistance in OA. eNAMPT mimics insulin function after binding to the insulin receptor (Yammani *et al*, 2012). Adipose tissue which is the natural source of eNampt/visfatin/PBEF could regulate β-cell function via secretion of eNampt and extracellular biosynthesis of nicotinamide mononucleotide (NMN) (Rabe *et al*, 2008). Nampt/visfatin which is an adipokine can be secreted due to cellular stress. In cellular inflammatory response Nampt might contribute positively but the loss of iNampt or the presence of eNampt/visfatin overwhelms the cell viability. (Lin *et al*, 2015).

WISP1 appears to have a significant effect on insulin release. WISP1 may play a role in linking obesity to inflammation and insulin resistance and could be a novel therapeutic target for obesity. The visceral fat from obese subjects overexpressed WISP1 a novel adipokine considerably and speculate insulin resistance and adipose tissue inflammation. Changes in weight regulate circulating WISP1 levels as well as WISP1

expression in adipose tissue (Murahovschi *et al,* 2015). WISP1 can be considered as one of the essential adipokines involved in glucose homeostasis. Increasing evidence supports the hypothesis that WISP1 plays a role in the complex domain, including excess body weight, impaired insulin sensitivity, and ultimately type 2 diabetes. Circulating levels of WISP1 are higher in obese than normal-weight subjects and are secreted by differentiated human adipocytes. The ability of WISP-1 to induce a pro-inflammatory state has been demonstrated, which may be one of the mechanisms that enhance endocrine dysfunction in adipose tissue. Furthermore, WISP-1 expression in adipocytes is negatively correlated with insulin sensitivity (Hörbelt *et al,* 2018).

WISP-1 mechanisms of action that impairs insulin sensitivity include inhibiting glycogen synthesis, interfering with insulin signaling, and promoting the inflammatory state. Here are two conflicting reports on the relationship between WISP-1 circulating levels and type 2 diabetes that need clarification. WISP-1 promotes human beta cell proliferation, making it a candidate for future therapeutic applications. However, the mitogenic, profibrotic and angiogenic potential of WISP-1 in various tissues carries the risk of adverse effects (Mirr *et al*, 2021).

sFRP4 appears to have a significant effect on insulin release, albeit in the opposite way compared to previous studies.

Adipose tissue is not the only source of systemic sFRP4, it is also expressed or secreted in other tissues such as liver, pancreas and skeletal muscle and selectively inhibits sFRP4 signaling insulin, which stimulates the Regulates glucose metabolism while enhancing DNL stimulation. The effect of insulin is further enhanced by sFRP4, and its levels increase even before the onset of type 2 diabetes. It may be interesting to target sFRP4 to mitigate pathophysiological changes that occur during diabetes mellitus development of type 2 diabetes and fatty liver disease (Hörbelt *et al*, 2019). According to Brix *et al.* (2016),

the type of diabetes does not matter because sFRP4 is significantly elevated in patients with different types of diabetes, i.e. this protein is generally involved in islet cell dysfunction and possibly in subclinical inflammation. Getting a standardised assays for the necessary studies to further evaluate the role of sFRP4 in diabetes will arrest these differences. In well-treated patients with coronary artery disease higher sFRP4 concentrations are associated with T2DM and the metabolic syndrome. sFRP4 concentrations though a novel marker of impaired glucose and triglyceride metabolism, it does not predict cardiovascular outcome in patients with steady coronary artery disease (Hoffmann *et al*, 2014). When expressed above average levels, secreted frizzled-related protein 4 (sFRP4) is known to cause five times more risk of diabetes. The levels of sFRP4 are seen to be high in diseases which results in less insulin production, thereby leading to reduced carbohydrate metabolism. The *in-silico* approach was used to discover some potential inhibitors against sFRP4. This could pave a way for diabetes mellitus cures in the future by reducing the expression of sFRP4 and increasing insulin production. Using the inhibitors cyclothiazide, clopamide and perindopril to inhibit sFRP4, it could lead to possible treatment of T2D (Bukhari *et al*, 2014). Anand *et al.* (2016) in their research found that increased sFRP4 levels were positively correlated with fasting glucose, 2hr postprandial glucose, glycated haemoglobin, and HOMA-IR and inversely correlated with ODI (oral disposition index). Raised sFRP4 could be a good marker of β-cell dysfunction and insulin resistance. sFRP4 in serum has been found to be associated with raised fasting glucose and reduced disposition index. Nevertheless, it was also associated with impaired insulin sensitivity, signifying that the protein could have excess of metabolic effects and could be released from numerous tissues involved in glucose homeostasis. These findings mark the protein as a possible biomarker for the low-grade islet inflammation that is commonly seen in T2D. This could be useful for early disease detection in combination with glucose and other biomarkers. sFRP4 could possibly be a therapeutic target for specific treatment of islet dysfunction (Mahdi *et al,*2012)

5.3 Contribution to knowledge

This research work has been able to show that FN3K is capable of inducing the secretion of insulin. It is very important in preventing glycation of proteins thereby preventing AGEs formation and the resulting diabetes and its complications. FN3K is associated with the GGap of diabetic patients and could be a therapeutic target. This work has also been able to show that adipokines have effects in insulin secretions or release (novel adipokine, WISP1 and others like, visfatin and sFRP4) and thereby plays a role in diabetes and its complications. And finally, GGap is significantly correlated with diabetes (positive GGap higher with T2DM) and GlycA a better biomarker of inflammatory response and its usefulness in linking GGap to diabetes and its complications. 1H-NMR was also used to show the relevant of GlycA and GlycB to GGap in diabetic patients. It also measures the lipoproteins and show the significant of measuring lipoprotein particles which could reveal patients who are at risk of CVD. Both positive and negative GGap are significantly correlated with lipoprotein particles size and diameter. Overall, this is the first research work that has looked into the use of glycoproteins analysis to establish a relationship between GGap and diabetes and its complications. This is also the first research work to show the role or effects of adipokines in diabetes and its complications. This work has exposed more areas of interest for further studies.

5.4 Recommendation

GlycA should be a conventional diagnostic requirement for diabetic patients. Lipoprotein analysis should be carried out for patients with diabetes and its complicated.

This may require carrying out in a larger population because the sample size for this research work was relatively small (54 samples). In that case the beauty and sensitivity of this work will be confirmed and much appreciated.

5.5 Conclusion

WISP1 appears to have a significant effect on insulin release. WISP1 may play a role in linking obesity to inflammation and insulin resistance and could be a novel therapeutic target for obesity. Knowing the importance of FN3K, it could be a useful tool to look out for in order to avoid AGEs and diabetes complications. GGap is significantly correlated with diabetes and GlycA is linked with it as well as diabetes. GlycA and GlycB are potent inflammatory markers, but GlycA is more potent. 1H-NMR is capable of analysing glycoproteins and lipoproteins and revealing important diagnostic information like measurement of lipid particles that could reveal CVD risk. This could help not only as target areas for treatments but for early and accurate diagnosis, disease treatment and proper management. It could also help to guide the clinicians in their judgment and prognosis. All these will lead to a reduce infection, diabetes and its complication, cost of treatment and management, and above all reduce morbidity and mortality rate.

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APPENDIX

LIPOPROTEIN STATISTICAL ANALYSIS

TABULAR REPRESENTATION OF LIPOPROTEINS RESULTS

G 0 = Neg gap

G 1 = Pos gap

Reference Range (mg/dL)

Cholesterol: VLDL (2-30), LDL (<130), HDL (35-65), IDL (<15)

Triglyceride: VLDL (<98), LDL (<19), HDL (<12), IDL (<12)

Group Statistics (Cholesterol)

The table below shows the main classes of cholesterol, their mean, standard deviation, and standard error mean. VLDL is higher in positive GGap while HDL is lower in positive GGap.

Table

	GGAP_C	N	Mean	Std. Deviation	Std. Error Mean
VLDL-C	0	34	14.98342	7.272021	1.247141
	1	20	20.31477	8.887832	1.987380
IDL-C	0	34	9.72908	3.275501	.561744
	1	20	11.42285	3.172612	.709418
LDL-C	0	34	102.57868	13.134488	2.252546
	1	20	94.21354	20.563644	4.598171
HDL-C	0	34	58.60958	8.427874	1.445368
	1	20	53.10303	6.464137	1.445425

Table shows the statistical representation of Cholester

The table below shows that VLDL-C is significant as well as HDL-C with GGap, but IDL-C and LDL-C were not significant.

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
Table		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VLDL-C	Equal variances assumed	2.126	.151	-2.395	52	.020	-5.331349	2.226458	-9.799064	-.863633
	Equal variances not assumed			-2.272	33.885	.030	-5.331349	2.346282	-10.100163	-.562534
IDL-C	Equal variances assumed	.169	.683	-1.856	52	.069	-1.693776	.912552	-3.524946	.137393
	Equal variances not assumed			-1.872	41.013	.068	-1.693776	.904892	-3.521226	.133673
LDL-C	Equal variances assumed	5.617	.022	1.827	52	.073	8.365135	4.578622	-.822542	17.552812
	Equal variances not assumed			1.634	28.276	.113	8.365135	5.120267	-2.118648	18.848918
HDL-C	Equal variances assumed	1.115	.296	2.515	52	.015	5.506543	2.189065	1.113864	9.899223
	Equal variances not assumed			2.694	48.230	.010	5.506543	2.044100	1.397111	9.615975

Table shows significant levels (2-tailed) of cholesterol p<0.05.

Group Statistics (Triglyceride)

The table below shows the sub classes of triglyceride, their mean, standard deviation, and standard error mean. VLDL-TG is higher in positive GGap while HDL-TG is higher in positive GGap.

Table

	GGAP_C	N	Mean	Std. Deviation	Std. Error Mean
VLDL-TG	0	34	58.76990	26.263444	4.504143
	1	20	81.89614	31.999824	7.155378
IDL-TG	0	34	10.50286	2.953224	.506474
	1	20	12.43095	2.946345	.658823
LDL-TG	0	34	12.84591	3.181709	.545659
	1	20	13.53462	3.465993	.775020
HDL-TG	0	34	16.36506	5.147549	.882797
	1	20	18.60968	6.014927	1.344979

Table shows the statistical representation of Triglyceride.

The table below shows that VLDL-TG is significant (p<0.006), and IDL-TG is significant (p<0.024), but LDL-TG and HDL were not significant.

Independent Samples Test

Table		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference Lower	Upper
VLDL-TG	Equal variances assumed	2.130	.150	-2.880	52	.006	-23.126241	8.029534	-39.238679	-7.013803
	Equal variances not assumed			-2.735	33.970	.010	-23.126241	8.454983	-40.309403	-5.943079
IDL-TG	Equal variances assumed	.145	.705	-2.319	52	.024	-1.928087	.831513	-3.596641	-.259534
	Equal variances not assumed			-2.320	40.041	.026	-1.928087	.831001	-3.607549	-.248626
LDL-TG	Equal variances assumed	.082	.775	-.743	52	.461	-.688710	.926683	-2.548237	1.170816
	Equal variances not assumed			-.727	37.237	.472	-.688710	.947839	-2.608802	1.231381
HDL-TG	Equal variances assumed	1.020	.317	-1.453	52	.152	-2.244620	1.544386	-5.343657	.854417
	Equal variances not assumed			-1.395	35.142	.172	-2.244620	1.608819	-5.510224	1.020984

Table shows significant levels (2-tailed) of Triglyceride $p<0.05$.

G 0 = neg gap

Cholesterol

VLDL higher HDL lower in +ve GGap

Group Statistics

	GGAP_C	N	Mean	Std. Deviation	Std. Error Mean
VLDL-C	0	34	14.98342	7.272021	1.247141
	1	20	20.31477	8.887832	1.987380
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	1	20	11.42285	3.172612	.709418
LDL-C	0	34	102.57868	13.134488	2.252546
	1	20	94.21354	20.563644	4.598171
HDL-C	0	34	58.60958	8.427874	1.445368
	1	20	53.10303	6.464137	1.445425

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
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	Equal variances not assumed			-2.272	33.885	.030	-5.331349	2.346282	-10.100163	-.562534
IDL-C	Equal variances assumed	.169	.683	-1.856	52	.069	-1.693776	.912552	-3.524946	.137393
	Equal variances not assumed			-1.872	41.013	.068	-1.693776	.904892	-3.521226	.133673
LDL-C	Equal variances assumed	5.617	.022	1.827	52	.073	8.365135	4.578622	-.822542	17.552812
	Equal variances not assumed			1.634	28.276	.113	8.365135	5.120267	-2.118648	18.848918
HDL-C	Equal variances assumed	1.115	.296	2.515	52	.015	5.506543	2.189065	1.113864	9.899223
	Equal variances not assumed			2.694	48.230	.010	5.506543	2.044100	1.397111	9.615975

Triglyceride

VLDL higher HDL lower in +ve GGap

Group Statistics

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	1	20	81.89614	31.999824	7.155378
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	1	20	12.43095	2.946345	.658823
LDL-TG	0	34	12.84591	3.181709	.545659
	1	20	13.53462	3.465993	.775020
HDL-TG	0	34	16.36506	5.147549	.882797
	1	20	18.60968	6.014927	1.344979

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
VLDL-TG	Equal variances assumed	2.130	.150	-2.880	52	.006	-23.126241	8.029534	-39.238679	-7.013803
	Equal variances not assumed			-2.735	33.970	.010	-23.126241	8.454983	-40.309403	-5.943079
IDL-TG	Equal variances assumed	.145	.705	-2.319	52	.024	-1.928087	.831513	-3.596641	-.259534
	Equal variances not assumed			-2.320	40.041	.026	-1.928087	.831001	-3.607549	-.248626
LDL-TG	Equal variances assumed	.082	.775	-.743	52	.461	-.688710	.926683	-2.548237	1.170816
	Equal variances not assumed			-.727	37.237	.472	-.688710	.947839	-2.608802	1.231381
HDL-TG	Equal variances assumed	1.020	.317	-1.453	52	.152	-2.244620	1.544386	-5.343657	.854417
	Equal variances not assumed			-1.395	35.142	.172	-2.244620	1.608819	-5.510224	1.020984

Particle size

A lot of diffs her as highlighted

Group Statistics

	GGAP_C	N	Mean	Std. Deviation	Std. Error Mean
VLDL-P (nmol/L)	0	34	43.68538	19.551020	3.352972
	1	20	59.33857	23.800470	5.321947
Large VLDL-P (nmol/L)	0	34	1.21573	.491066	.084217
	1	20	1.58218	.473328	.105839
Medium VLDL-P (nmol/L)	0	34	4.32247	2.091055	.358613
	1	20	6.54821	2.795620	.625120
Small VLDL-P (nmol/L)	0	34	38.14718	17.362162	2.977586
	1	20	51.20818	20.848956	4.661968
LDL-P (nmol/L)	0	34	1016.05115	114.551936	19.645495
	1	20	975.00688	182.635650	40.838573
Large LDL-P (nmol/L)	0	34	174.23917	20.206606	3.465404
	1	20	154.96839	25.957814	5.804344
Medium LDL-P (nmol/L)	0	34	285.86711	56.928558	9.763167
	1	20	254.07150	70.519626	15.768668
Small LDL-P (nmol/L)	0	34	555.94487	72.585298	12.448276
	1	20	565.96699	99.098399	22.159076
HDL-P (µmol/L)	0	34	29.54554	4.027080	.690638
	1	20	29.10964	4.436039	.991928
Large HDL-P (µmol/L)	0	34	.28205	.033029	.005664
	1	20	.28064	.021710	.004854
Medium HDL-P (µmol/L)	0	34	11.29356	1.887761	.323748
	1	20	10.09162	1.116979	.249764
Small HDL-P (µmol/L)	0	34	17.96993	3.085222	.529111
	1	20	18.73738	3.650294	.816231

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VLDL-P (nmol/L)	Equal variances assumed	2.198	.144	-2.620	52	.012	-15.653196	5.974934	-27.642779	-3.663613
	Equal variances not assumed			-2.489	33.993	.018	-15.653196	6.290115	-28.436338	-2.870053
Large VLDL-P (nmol/L)	Equal variances assumed	.143	.707	-2.683	52	.010	-.366457	.136578	-.640520	-.092394
	Equal variances not assumed			-2.709	41.173	.010	-.366457	.135257	-.639579	-.093335
Medium VLDL-P (nmol/L)	Equal variances assumed	5.296	.025	-3.329	52	.002	-2.225743	.668678	-3.567543	-.883943
	Equal variances not assumed			-3.088	31.593	.004	-2.225743	.720679	-3.694459	-.757028
Small VLDL-P (nmol/L)	Equal variances assumed	1.914	.172	-2.477	52	.017	-13.060996	5.272965	-23.641974	-2.480018
	Equal variances not assumed			-2.361	34.370	.024	-13.060996	5.531724	-24.298352	-1.823639
LDL-P (nmol/L)	Equal variances assumed	7.122	.010	1.017	52	.314	41.044270	40.362713	-39.949440	122.037979
	Equal variances not assumed			.906	27.949	.373	41.044270	45.318148	-51.793326	133.881865
Large LDL-P (nmol/L)	Equal variances assumed	.656	.422	3.042	52	.004	19.270780	6.334675	6.559323	31.982236
	Equal variances not assumed			2.851	32.576	.008	19.270780	6.760136	5.510380	33.031179
Medium LDL-P (nmol/L)	Equal variances assumed	.297	.588	1.813	52	.076	31.795607	17.539169	-3.399310	66.990525
	Equal variances not assumed			1.714	33.523	.096	31.795607	18.546437	-5.915054	69.506269
Small LDL-P (nmol/L)	Equal variances assumed	5.915	.018	-.427	52	.671	-10.022118	23.462035	-57.102135	37.057900
	Equal variances not assumed			-.394	31.101	.696	-10.022118	25.416219	-61.852011	41.807776
HDL-P (µmol/L)	Equal variances assumed	.272	.604	.370	52	.713	.435901	1.178251	-1.928433	2.800235
	Equal variances not assumed			.361	36.895	.720	.435901	1.208678	-2.013350	2.885152
Large HDL-P (µmol/L)	Equal variances assumed	2.530	.118	.170	52	.866	.001406	.008286	-.015221	.018032
	Equal variances not assumed			.188	51.255	.851	.001406	.007460	-.013569	.016380
Medium HDL-P (µmol/L)	Equal variances assumed	3.154	.082	2.587	52	.013	1.201941	.464537	.269780	2.134102
	Equal variances not assumed			2.939	51.987	.005	1.201941	.408895	.381429	2.022454
Small HDL-P (µmol/L)	Equal variances assumed	.930	.339	-.825	52	.413	-.767446	.930765	-2.635163	1.100272
	Equal variances not assumed			-.789	34.786	.435	-.767446	.972724	-2.742613	1.207721

Particle diameter and other measures

See tables

Group Statistics

	GGAP_C	N	Mean	Std. Deviation	Std. Error Mean
VLDL-Z (nm)	0	34	42.16672	.198520	.034046
	1	20	42.24413	.173511	.038798
LDL-Z (nm)	0	34	21.14279	.234049	.040139
	1	20	20.95391	.137742	.030800
HDL-Z (nm)	0	34	8.30122	.066868	.011468
	1	20	8.25814	.048254	.010790
Non-HDL-P (nmol/L)	0	34	1059.73653	112.004804	19.208665
	1	20	1034.34546	173.674598	38.834821
Total-P/HDL-P	0	34	1.03651	.006031	.001034
	1	20	1.03637	.007944	.001776
LDL-P/HDL-P	0	34	.03503	.006035	.001035
	1	20	.03436	.008142	.001821

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
VLDL-Z (nm)	Equal variances assumed	.210	.649	-1.448	52	.154	-.077416	.053476	-.184723	.029891
	Equal variances not assumed			-1.500	44.377	.141	-.077416	.051618	-.181421	.026588
LDL-Z (nm)	Equal variances assumed	4.152	.047	3.282	52	.002	.188881	.057543	.073413	.304348
	Equal variances not assumed			3.733	51.994	.000	.188881	.050594	.087355	.290406
HDL-Z (nm)	Equal variances assumed	.439	.511	2.517	52	.015	.043081	.017114	.008739	.077423
	Equal variances not assumed			2.736	49.674	.009	.043081	.015746	.011449	.074713
Non-HDL-P (nmol/L)	Equal variances assumed	5.638	.021	.654	52	.516	25.391074	38.825527	-52.518046	103.300194
	Equal variances not assumed			.586	28.454	.562	25.391074	43.325698	-63.293911	114.076059
Total-P/HDL-P	Equal variances assumed	.942	.336	.073	52	.942	.000139	.001914	-.003702	.003980
	Equal variances not assumed			.068	31.951	.946	.000139	.002055	-.004048	.004326
LDL-P/HDL-P	Equal variances assumed	1.345	.251	.348	52	.730	.000674	.001939	-.003217	.004565
	Equal variances not assumed			.322	31.380	.750	.000674	.002094	-.003595	.004943