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Introduction

Sweet taste is one of the five fundamental tastes and is associated with pleasurable sensation. Evolutionarily, animals used sweetness to evaluate the energy content of foods. Today our lifestyle has changed but our metabolism has remained the same. And satisfying sweet taste has led us into consuming large quantities of refined sugars.

Until the 18th century, sugar was a luxury and because of profits made from its sales, it was called “white gold”. The first record of sugar was made in England in 1100AD. The first sugar refinery was set up in Germany in 1573. In 1700 an average individual consumed about 2kgs of sugar per year. This increased to about 9kg/year in 1800s and 45kgs/year in 1900s. In 2009, the upper 50% of the people consumed 250gm/day. The worldwide consumption of sugar is about 167 million tons per year in 2010-11. India is the second largest production of sugar after Brazil.

Sugar is an essential metabolite for prokaryotes as well as eukaryotes. The word’ sugar’ referred to sucrose. But it also includes the milk sugars lactose, malt sugar maltose and other mono and disaccharides, of all these sugars, sucrose is the sweetest.

Sucrose is hydrolyzed to glucose and fructose by the enzyme sucrase (EC 3.2.1.10) which exits as a complex called sucrase-isomaltase complex on the tip of the microvilli. Glucose produced from the sucrose is absorbed in the intestine by GLUT 4 mediated transport whereas fructose is transported through GLUT5 (Uldry and Thoren 2004).

The monosaccharides that are absorbed are essentially three major monosaccharaides: namely glucose, Fructose and galactose. In a typical 24hrs diet, sugar accounts for 2-3% of total food consumed (Southgate 1995). All the monosaccharides are eventually oxidized to CO₂ and water via the TCA cycle. The absorbed monosaccharides pass into the portal circulation and into the liver. All of fructose and galactose and most of the glucose are removed. The remaining glucose enters the peripheral tissue under the influence of insulin.

Although glucose is the fuel for the metabolism of the whole body, it is not a major fuel for the beating heart or muscle doing mild exercise (Andres et al 1956:

Erythrocytes of all mammalian species are highly permeable to glucose. In adult humans the plasma glucose and erythrocytes glucose is almost the same. However when erythrocyte glucose transporters are saturated, the plasma glucose can be very high (McKay 1932). Erythrocyte serves as buffer of glucose concentration in blood. The blood glucose concentration is not always constant but can vary depending on the state of feeding, exercise and availability of free fatty acids and emotional state. The glucose concentrations become also low as 3mM (54mg/dl) to a high of 9mM (162mg/dl) (Somogyi 1948).

**Fig. 1.1:** Major metabolic pathways of glucose Fructose and Galactose in normal humans.
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The major metabolic pathways of three major sugars in animal cells is shown in Fig. 1.1. The fructose leads to formation of increased amount of pyruvate which in turn leads to decrease in FA oxidation and an increase in FA esterification leading to the synthesis of VLDL (Mayes 1993).

**Glucose toxicity**

Glucose comes from glycogen breakdown, gluconeogenesis from pyruvate and non-carbohydrate sources and from the diet. After a carbohydrate rich meal the blood glucose level will rise. This will lead to release of insulin. 70% of the glucose will then be taken up by insulin responsive tissues (Triplett et al. 2012). About 50% of the glucose in the post absorptive phase utilized by the brain. About 25% of the glucose is utilized by insulin dependent tissues like muscles and adipose tissues and the remaining 25% by the liver and gastrointestinal issue (DeFronzo 2004). While liver accounts for 85% of the total glucose production; the remaining 15% is produced by the kidneys (DeFronzo 2004). The blood glucose level is tightly controlled by at least three different types of hormones namely Insulin, Glucogon and the Incretins (Triplett et al. 2012). The reason for this tight control is obvious. Glucose though an essential nutrient can be toxic to multiple organs particularly the pancreas. The LD₅₀ of sucrose fed orally to rats was 38.4±7.0 g/Kg in males and 29.7±3.7 g/kg in females (Boyd et al. 1965).

The fructose in sucrose is metabolized differently than glucose. Most of the toxic effect of sugar can be attributed to those of fructose consumption. Fructose is 7 times more likely than glucose to cause AGE, it suppresses the hunger hormone Ghrelin. Fructose does not have receptor on β cells of the pancreas and hence does not induce insulin secretion. Fructose has been shown to be metabolized in the liver to glycerol which enters into VLDL synthesis.

In 1957, W.C. Martin classified refined sugar as a ‘Poison’ because of the toxic effects exhibited by consuming large quantities of refined sugar. The toxic effect of refined sugar was observed as early as the 18th century. A ship carrying a cargo of sugar was ship wrecked in 1793. Five surviving sailors were rescued after 9 days. They were in a wasted condition, since the only food they had was sugar. French
physiologist F. Magendie conducted experiments on dogs. He fed them a diet of sugar and water. All the dogs wasted and died.

Is sugar toxicity the cause of Diabetes?

The prevalence of diabetes is rapidly rising all over the globe at an alarming rate in the past two decades. The status of diabetes has changed from being considered as a mild disorder of the elderly to one of the major causes of morbidity and mortality affecting the youth and middle aged people. More common form of diabetes, namely type 2 diabetes, accounts for more than 90 per cent of all diabetes cases. Incidence of Diabetes has paralleled the increase in consumption of refined sugar all over the world. However, correlation is not causation. Hence health scientists have been searching a direct link between sugar consumption and induction of diabetes.

It is now well known that fructose is metabolized differently than glucose. In animals, or at least in laboratory rats and mice, it is clear that the liver will convert much it to fat. This is the reason for insulin resistance, which is now considered the fundamental problem in obesity, and the underlying defect in heart disease and in type 2 diabetes. It might also be the underlying defect in many cancers.

Diabetes and its complications

India leads the world with largest number of diabetic subjects which is being termed as the “diabetes capital of the world”. According to the Diabetes Atlas 2006 published by the International Diabetes Federation, the number of people with diabetes in India currently around 40.9 million is expected to rise to 69.9 million by 2025 unless urgent preventive steps are taken. The “Asian Indian Phenotype” refers to certain unique clinical and biochemical abnormalities which include increased insulin resistance, greater abdominal adiposity i.e., higher waist circumference despite lower body mass index, lower adiponectin and higher high sensitive C-reactive protein levels. This phenotype makes Asian Indians more prone to diabetes and premature coronary artery disease. At least a part of this is due to genetic factors. However, the primary driver of the epidemic of diabetes is the rapid epidemiological transition associated with changes in dietary patterns and decreased physical activity as evident from the higher prevalence of diabetes in the urban population.
Antecedent factors, such as toxins, abnormal energy storage, and hypertension, may also contribute to the development of both diabetes and cardiovascular disease. Cardiovascular disease is the major cause of morbidity and mortality associated with diabetes and diabetes is considered as a risk factor for cardiovascular disease. Diabetics have a 2 to 4 fold higher risk of developing atherosclerosis than non-diabetics (Kannel 1979). Atherosclerosis accounts for virtually 80% of all deaths among diabetic patients. Hyperglycemia induces a large number of alterations at the cellular level of vascular tissue that potentially accelerate the atherosclerotic process. Both type I and type II diabetes are powerful and independent risk factors for coronary artery disease (CAD), stroke, and peripheral arterial disease (Stamler et al 1993: Schwartz et al 1992]. Glucose and its metabolites in diabetics have direct toxic effects on vascular endothelium. Abnormal glucose is evidence of absolute or relative insulin deficiency, which can predispose patients to cardiovascular disease via endothelial dysfunction, lipid abnormalities, and inflammation.

Hyperglycemia in diabetics induces nonenzymatic glycation of proteins and lipids, leading to the accumulation of advanced glycation end-products (AGEs). Nonenzymatic glycosylation of proteins and lipids interfere with their normal function by disrupting molecular conformation, alter enzymatic activity, reduce degradative capacity, and interfere with receptor recognition. In addition, glycosylated proteins interact with a specific receptor present on all cells relevant to the atherosclerotic process, including monocyte-derived macrophages, endothelial cells, and smooth muscle cells. The interaction of glycosylated proteins with their receptor results in the induction of oxidative stress and pro-inflammatory responses. Oxidative stress also involved in the activation of protein kinase C (PKC) ( Takeshi Nishikawa 2000), with subsequent alteration in growth factor expression. Importantly, these mechanisms may be interrelated.

Diabetes is known to alter the phenotype of lipoproteins (Singh et al 1995). Lipoproteins are circulating molecules in plasma which work interlinked to each other ultimately to maintain cholesterol homeostasis. LDL is required to transport cholesterol from the liver to tissues, and HDL is involved in the reverse transport of
cholesterol from the tissues back to the liver. LDL is the harmful form of cholesterol, and its levels are directly related to the risk of developing atherosclerotic disease. Whereas the HDL is known as beneficial form of cholesterol, with levels inversely related to risk of atherosclerotic diseases. The cardio-protective role of HDL is performed by the enzymes and the proteins associated with HDL. HDL removes cholesterol from the peripheral cells with the aid of Apo A1, a major protein present on HDL followed by the action of LCAT enzyme associated with HDL. The primary protective effect of HDL is believed to be its pivotal role in reverse-cholesterol transport; however, HDL also has anti-oxidative, anti-inflammatory and anti-thrombotic properties (Mackness 2000).The anti-inflammatory effects are implicated in its anti-atherogenic properties. Apo lipoprotein (Apo) A-I, and paraoxonase enzyme present on HDL can remove oxidized lipids from LDL, rendering LDL highly resistant to oxidation. HDL associated Paraoxonase (PON1) is primarily responsible for the anti-oxidative properties of HDL in retarding the oxidation of LDL and cell membranes (Mackness 1991, 1993; Watson 1995), which retards the lipid oxidation by preventing the generation of lipid peroxides (Furlong et al 1998).

Human serum paraoxonase (PON) is a calcium-dependent HDL-associated ester hydrolase that catalyzes the hydrolysis of organophosphates, aromatic carboxylic acid esters, and carbamates (La Du 1992). PON is tightly associated with apolipoprotein A-I in HDL and has the highest activity in the liver and blood (La Du 1992). HDL associated PON has been reported to inhibit copper-induced lipid peroxide generation in LDL (Mackness 1991). PON activity in serum can be varied by 40 fold in a given population. The modulation of PON activity could be due to many factors which include environmental and genetic. PON1 activity can vary depending on different physiological conditions or pathological states. PON1 is low in subjects with Type 1 or Type 2 diabetes (Abbott 1995: Boemi et al 2001), leading to dysfunctional HDL with impaired antioxidant capacity (Boemi 2001). Serum PON1 activity decreased over time, by 36%, in streptozotocin-treated diabetic rats (Patel et al 1990 ), this low PON1 activity is independent of genotype, and may be due to altered glycation of HDL and/or PON1, lower rate of synthesis or higher rate of catabolism, or increased oxidative stress (Ferretti et al 2004).
Individuals with familial hypercholesterolemia and insulin-dependent diabetes mellitus have significantly lower serum levels of PON than do control individuals (Mackness et al 1991). Increased glycation of HDL leads to the impairment of its anti-atherosclerotic properties (Hedrick et al 2000). In vitro glycation of HDL appears to partially inhibit PON1 (Feretti et al 2001). HDL from people with Type 1 diabetes was significantly less efficient at metabolizing erythrocyte membrane hydroperoxides than HDL from normal control subjects (Ferretti et al 2004). It is therefore conceivable that HDL from Type 2 diabetic subjects will be defective in metabolizing cell membrane oxidized lipids and that this could contribute to increased atherosclerosis in Type 2 diabetes. The non-enzymatic glycation of proteins affects its structural and a functional property which is reflected in both type I and type II diabetes. Hyperglycemia in diabetic patients results in non-enzymatic glycation of plasma proteins including lipoprotein such as HDL. Glycation of HDL decrease several important functions of HDL particles, rendering the anti-atherogenic HDL to a more pro-atherogenic HDL.

Several studies demonstrate that both LDL and HDL undergo specific modifications. These modifications are biochemical in nature which may include oxidation of phospholipids, structural modulation of proteins or reduction of enzyme activity. Hence it is very important to understand the type and the site of modification of these lipoproteins and their enzymes. This could lead to the development of therapeutic approaches. Detection of the modifications of enzymes is important to know the functionality of lipoproteins. Hence the present study investigates the non-enzymatic glycation of HDL and its implication on the function of HDL associated Paraoxonase. The study also involves the development immunodetective method for the identification of glycated proteins in the serum.

**Literature survey**

**Diabetes mellitus**

Diabetes mellitus is a diverse metabolic disorder is characterized by elevated blood glucose level called hyperglycemia and distinctive complications which results from defects in insulin secretion, in insulin action, or both. Diabetes mellitus is not a
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pathogenic entity but a group of etiologically different metabolic defects. Common symptoms of diabetes are lethargy from marked hyperglycemia, polyuria, polydipsia, weight loss, blurred vision and susceptibility to certain infections. Severe hyperglycemia may lead to hyper-osmolar syndrome and insulin deficiency to life-threatening ketoacidosis. Chronic hyperglycemia causes long-term damage, dysfunction and failures of various cells, tissues and organs. Long-term complications of diabetes include premature atherosclerotic cardiovascular disease and small vessel disease manifested as retinopathy with potential loss of vision; nephropathy leading to renal failure; and peripheral neuropathy with a high risk of foot ulcer and amputation.

Classification of Diabetes

Type 1 Diabetes

Insulin dependent Juvenile Diabetes (IDDM-Type 1) is an autoimmune disease, and this form of diabetes accounts for 5-10% of all cases. It results from a progressive cellular mediated autoimmune destruction of β-cell, which causes the pancreas to produce little or no insulin. Patients with type 1 diabetes are severely insulin deficient and are dependent on insulin treatment for their survival. Type 1 diabetes develops mostly in children and young adults but can appear at any age. Clinical diagnosis of type 1 diabetes can be made by use of markers for immune β-cell destruction which include autoantibodies to islet cells, tyrosine phosphatases IA-2α and IA-2β.

Type 2 Diabetes

Non-Insulin Dependent Adult Onset Diabetes (NIDDM-Type 2) is the most common form of diabetes in which the pancreas fails to produce enough insulin, characterized by abnormal glucose homeostasis (Gupta et al 2003). Hence it is insufficient for reducing the blood glucose to normal levels. About 90 to 95 percent of people with diabetes have Type 2 Diabetes. It is often associated with central or visceral obesity as well as other cardiovascular risk factors such as hypertension and abnormalities of lipoprotein metabolism with characteristic of elevated level of triglyceride and LDL cholesterol. Its disorders are characterized by hyperglycemia
and associated with microvascular (i.e., retinal, renal, possibly neuropathic), macrovascular (i.e., coronary, peripheral vascular), and neuropathic (i.e., autonomic, peripheral) complications. Type 2 diabetes is the major risk factor for premature cardiovascular morbidity and mortality.

**Other specific types of diabetes**

There are many other specific types of diabetes accounts for only a minor portion of all cases of diabetes. Genetic defects of the β-cell function leads to hyperglycemia at an early age and are characterized by impaired insulin secretion with minimal or no defects in insulin. These forms of diabetes are frequently characterized by onset of hyperglycemia at an early age. They are referred to as maturity onset diabetes of the young (MODY). It is a monogenic disorder in which a mutation causes hyperglycemia by producing a defect in glucose sensing or insulin secretion.

Abnormalities in exocrine pancreas which include pancreatitis, trauma and cystic fibrosis is one of the cause for diabetes. Hyperglycemia can also be induced by drugs such as Dilantin, α-interferon, diazoxide etc. Viral infections such as adenovirus, cytomegalovirus and mumps are also associated with β-cell dysfunction resulting in hyperglycemia. Several hormones like growth hormone, cortisol, glucagon epinephrine antagonize insulin action. Excess of these hormones can cause diabetes. There are also uncommon forms of immunomediated diabetes and genetic syndromes that may lead to hyperglycemia are included in these groups.

**Gestational diabetes**

Gestational diabetes is similar to Type 2 diabetes and is developed by some women during late in pregnancy. If given proper medical attention during pregnancy period, this form of diabetes disappears after delivery. About 20-50 percent of women affected by gestational diabetes develop Type 2 diabetes within 5 to 10 years.
Pathophysiology of diabetes complications

The DCCT (Diabetes Control and Complications Trial) and the UKPDS (U.K. Prospective Diabetes Study) established that hyperglycemia, is the initiating cause of the diabetic tissue damage which may be influenced by either genetic susceptibility or by other factors like hypertension, hyperlipidemia, diet, life style etc. The reason may be many but all that leads to abnormal metabolism of glucose by different mechanism by which prolonged hyperglycemia ultimately results in chronic micro and macrovasular complications.

The osmotic glucose concentration in most of the cells in our body is constantly maintained. They reduce glucose transport inside the cell when they are exposed to hyperglycemia. Whereas a particular subset of cell types: capillary endothelial cells in the retina, mesangial cells in the renal glomerulus, and neurons and Schwann cells in peripheral nerves which makes them so vulnerable to hyperglycemia. In these cells glucose transport inside the cell is not maintained constant leading to high glucose concentration and gets damaged by hyperglycemia induced diabetes. The different pathways which lead to the cell damage and diabetic complications have been explained.

The sorbitol (polyol) pathway

One of the consequences of hyperglycemia in human diabetes mellitus is increased metabolism of glucose by the sorbitol pathway. Aldose reductase present in human brain, nerves, aorta, muscle, erythrocytes and ocular lens reduces glucose to polyalcohol sorbitol, followed by oxidation of sorbitol to fructose by sorbitol dehydrogenase. Sorbitol is not permeable to cell membranes and tends to accumulate in the cell resulting in osmotic damage to microvascular cells. Conversion of glucose to sorbitol by aldose reductase requires NADPH and forms NADP+ (Fig.1.2) and thereby competes with other NADPH-requiring reactions. NADPH is required for the conversion of oxidized to reduced glutathione, a powerful antioxidant which protects cellular components from oxidative damage, inducing intracellular oxidative stress. NADPH is also required for fatty acid and cholesterol biosynthesis. The pentose phosphate pathway is the major source of NADPH in most tissues and its flux is
generally determined by the NADP+/NADPH ratio. Conversion of sorbitol to fructose is coupled to reduction of NAD+ to NADH and this competes with glycolysis at the glyceraldehyde dehydrogenase step for NAD+. An increase in the NADH/NAD+ ratio favors increased conversion of dihydroxyacetone phosphate to glycerol 3-phosphate (GAPDH), and increasing concentrations of triose phosphate (Williamson et al 1993). Raised triose phosphate concentrations could increase formation of both methylglyoxal, a precursor of AGEs, and diacylglycerol (DAG) (through α-glycerol-3-phosphate), thus activating PKC.

![Fig 1.2: Hyperglycemia induced polyol pathway](image)

**Activation of protein kinase C**

Intracellular hyperglycemia increases the synthesis of molecule called diacyl glycerol a lipid second messenger which is a critical activating cofactor for the isoforms of protein kinase-C,-β - , δ and α (Koya et al 1997: Koya and King 1998). Increased de novo synthesis of DAG activates PKC both in cultured vascular cells (Xia et al 1994) and in retina and glomeruli of diabetic animals (Koya and King 1998). Hyperglycemia may also activate PKC isoforms indirectly through both ligations of AGE receptors (Portilla et al 2000) and increased activity of the polyol pathway (Keogh 1997), presumably by increasing reactive oxygen species. When
PKC is activated by intracellular hyperglycemia, it has a variety of effects on gene expression. Activation of PKC in hyperglycemia induces abnormalities of blood flow and permeability. The vasodilator producing endothelial nitric oxide (NO) Synthase (eNOS) is decreased, while the vasoconstrictor endothelin-1 is increased (Fig.1.3).

**Fig.1.3 Consequences of hyperglycemia-induced activation of protein kinase C (PKC).**

Activation of PKC by raised glucose also induces expression of the permeability enhancing factor VEGF in smooth muscle cells (Williams 1997). The increased permeability of endothelial cells induced by high glucose in cultured cells is mediated by activation of PKC-α, it also induces expression of the permeability enhancing factor VEGF in smooth muscle cells (Williams 1997). Activation of PKC contributes to increased microvascular matrix protein accumulation by inducing expression of TGF-β1, fibronectin and type IV collagen in cultured mesangial cells (Studer 1993). Hyperglycemia-induced activation of PKC has also been implicated in the overexpression of the fibrinolytic inhibitor PAI-1 (Feener. et al 1996), the activation of NF-κB a pro inflammatory factor in cultured endothelial cells and vascular smooth muscle cells (Pieper and Riaz-ul-Haq 1997;Yerneni. et al, 1999) and
in the regulation and activation of various membrane-associated NAD (P) H-dependent oxidases.

**Intracellular production of AGE precursors**

Hyperglycemia induces nonenzymatic reactions between extracellular proteins and glucose which gradually form advanced glycation end product (AGEs). AGEs can arise from intracellular auto-oxidation of glucose to glyoxal (Wells-Knecht et al 1995), decomposition of the Amadori product (glucose-derived 1-amino-1-deoxyfructose lysine adducts) to 3-deoxyglucosone, and fragmentation of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate to methylglyoxal (Thornalley 1990). These reactive intracellular dicarbonyls — glyoxal, methylglyoxal and 3-deoxyglucosone — react with amino groups of intracellular and extracellular proteins to form AGEs. Production of intracellular AGE precursors damages target cells by three general mechanisms. First, intracellular proteins modified by AGEs have altered function. Second, extracellular matrix components modified by AGE precursors interact abnormally with other matrix components and with the receptors for matrix proteins (integrins) on cells. Third, plasma proteins modified by AGE precursors bind to AGE receptors on endothelial cells, mesangial cells and macrophages, inducing receptor-mediated production of reactive oxygen species. This AGE receptor ligation activates the pleiotropic transcription factor NF-κB, causing pathological changes in gene expression (Thornalley 1990).

**Increased flux through the hexosamine pathway**

When glucose is high inside a cell, the glycolytic pathway is diverted into a signaling pathway by an enzyme called glutamine: fructose-6 phosphate aminotransferase (GFAT) where an intermediate fructose-6-phosphate gets converted into glucosamine-6-phosphate finally to UDP (uridine diphosphate) N-acetyl glucosamine. The N-acetyl glucosamine gets put onto serine and threonine residues of transcription factors, and often results in pathologic changes in gene expression (Kolm-Litty Et al 1998: Sayeski and Kudlow 1996 ). Increased modification of the transcription factor Sp1 results in increase in transcription of TGF-α, TGF-β1, and PAI-1 and has been implicated in insulin resistance (Brownlee et al 2001: Du et al
which are bad for diabetic blood vessels (Du et al 2000). It has been shown to play a role both in hyperglycemia-induced abnormalities of glomerular cell gene expression (Kolm-Litty 1998) and in hyperglycemia-induced cardiomyocyte dysfunction in cell culture (Clark 2003). In carotid artery plaques from type 2 diabetic subjects, modification of endothelial cell proteins by the hexosamine pathway is also significantly increased (Federici 2002). eNOS activity is also known to reduce by hyperglycemia-induced O-acetylglucosaminylatation at the AKT site of the eNOS protein (Brownlee et al 2001: Marshall et al 1991). Adenovirus mediated overexpression of GFAT was reported to impair glucose-stimulated insulin secretion and to reduce expression levels of the insulin, GLUT2, and glucokinase genes (Kaneto et al 2001). Thus, activation of the hexosamine pathway by hyperglycemia may result in many changes in both gene expression and protein function, which together contribute to the pathogenesis of diabetic complications (Fig.1.4).

Fig.1.4: Hyperglycemia induced hexosamine pathway
Glyceraldehyde Autoxidation

Under hyperglycemic condition the glycolytic pathway of glucose takes an alternative pathway. Where glyceraldehyde 3-phosphate gets autoxidized. Autoxidation of these α-hydroxyaldehydes generates from two potentially toxic substances hydrogen peroxide ($H_2O_2$) and α-ketoaldehydes. In the presence of redox active metals, $H_2O_2$ can form the highly toxic hydroxyl radical and the hydroxyl radical, a reactive oxygen species that can cause mutagenic alterations in DNA. α-ketoaldehydes contribute to glycosylation-related protein chromophore development. Glyceraldehyde when present in excess inhibits insulin secretion. Long term exposure to high glucose concentrations decreases GAPDH activity in islets (Sakai et al 2003), which favors excess glyceraldehyde accumulation.

Disorders in lipid metabolism

Due to hyperglycemia not only glucose but also lipids and protein metabolism gets adversely affected. Decreased glycolysis due to Insulin resistance or lack of insulin effects the ATP generation hence hampers the biosynthesis of lipids, fatty acids and proteins. Lipolysis occurs in the adipose tissues where triacyl glycerol gets converted into more of free fatty acids by elevation of cAMP. Fatty acid reaching the liver in high concentration blocks further fatty acid synthesis by a feedback mechanism. Increased acetyl Co.A by the fatty acid degradation activates the gluconeogenic enzymes pyruvate carboxylase. They finally retard tricarboxylic acid (TCA) cycle at the level of citrate synthetase. The Acetyl Co A thus formed instead of going in to the TCA cycle or biosynthesis of fatty acids in diabetics and forms keto acids like acetoacetic acids and beta hydroxyl butyric acid and acetone. These are known as ketone bodies which accumulate in blood and secreted in urine (Ketouria). In a severe keto acidosis with high blood sugar diabetic patients may go into coma.

The excess of fatty acid in the liver also gets converted into very Low Density Lipoproteins rich in tryacyl glycerol (TG) which leads to the increased TG in the blood. It also increases blood cholesterol level.
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Hyperglycemia-induced Oxidative Stress

Hyperglycemia induced pathological mechanisms are all linked to a common upstream event. This single unifying process is the overproduction of superoxide by the mitochondrial electron-transport chain (Nishikawa et al 2000) (Fig 1.5). Many studies have shown that diabetes and hyperglycemia increase oxidative stress (Giugliano et al 1996). Du et al. have found that hyperglycemia increases the proton gradient above threshold value as a result of overproduction of electron donors by the TCA cycle. This, in turn, causes a marked increase in the production of superoxide by endothelial cells. Overexpression of mitochondrial manganese superoxide dismutase (MnSOD), abolished the signal generated by reactive oxygen species, and overexpression of uncoupling protein-1 (UCP-1) collapsed the proton electrochemical gradient and prevented hyperglycemia-induced overproduction of reactive oxygen species.

Fig. 1.5: Biochemical pathways of glucose metabolism derived ROS
Inhibition by MnSOD or UCP-1 of hyperglycaemia-induced overproduction of mitochondrial superoxide completely prevented an increase in polyol pathway flux, increased intracellular AGE formation, increased PKC activation and an increase in hexosamine pathway activity in endothelial cells. These damaging pathways are activated by the inhibition of a key glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The GAPDH activity gets modified or decreased by the enzyme poly (ADP-ribose) polymerase (PARP). This enzyme is involved in ROS induced DNA damage. Once activated, PARP splits the NAD$^+$ molecule into its two component parts: nicotinic acid and ADP-ribose. PARP then proceeds to make polymers of ADP-ribose, which accumulate on nuclear proteins and GAPDH which shuttles in and out of the nucleus, where it plays a critical role in DNA repair (Brownlee 2001) (Fig.1.6).

![Fig.1.6: ROS-induced DNA damage activates PARP and modifies GAPDH](image)

**Consequences of Oxidative Stress on Beta Cell Function**

Continuous exposure of the beta cell to increased concentrations of glucose causes defective insulin gene expression accompanied by marked decreases in insulin content and abnormal insulin secretion (Robertson et al 1992). The defect in insulin gene expression is due to the loss of major two critical proteins that activate the insulin promoter. One is PDX-1 (Olson et al 1993, 1995), and the other is RIPE-3b1 activator (Sharma et al. 1995), recently identified as MafA (Poitout et al 1996: ...)
Matsuoka et al 2003). Prolonged exposure to high glucose concentrations also upregulates the levels of transcription factor C/EBPβ, a repressor for insulin promoter activity (Lu et al 1997). One potential central mechanism for glucose toxicity is the formation of excess reactive oxygen substances (ROS) levels, which takes place within multiple mitochondrial and non-mitochondrial pathways. The islet is especially vulnerable to ROS because of its low intrinsic level of antioxidant enzymes. Chronically excessive glucose and ROS levels can cause decreased insulin gene expression via loss of the transcription factors PDX-1 and MafA and can also accelerate rates of apoptosis.

Hyperglycemia induced unfavoured biochemical pathways in diabetic patients abolishes structure and functions of important proteins and enzymes in the physiological condition. And these pathways results in generation of free radicles which end up in oxidative stress. All these mechanisms ultimately results in microvascular and macrovascular complications. Hyperglycaemia and insulin resistance both seem to have important roles in the pathogenesis of macrovascular complications.

**Complications of Diabetes Mellitus**

Diabetes mellitus Complications develop as a result of uncontrolled blood glucose levels. Complications of diabetes mellitus can be classified into acute and chronic. Acute complications arise as an immediate response to increase in blood glucose levels. On the other hand chronic complications are a result of prolonged hyperglycemia for prolonged period of time.

**Acute complications**

Acute complications of diabetes mellitus develop either immediately or over a period of few days, and are treated as medical emergencies. The acute metabolic complications of diabetes consist of diabetic ketoacidosis (DKA), hyperosmolar non-kenotic coma (HNC), lactic acidosis (LA), and hypoglycemia (Howard Fishbein 1995). DKA is due to absolute insulin deficiency (IDDM), in non-insulin dependent diabetes mellitus diabetic ketoacidosis occurs under the stress of acute illness with increased lipolysis, increased ketone production, hyperketonemia. HNC is the
presence of relative insulin deficiency and hyperglycemia, usually >1,000 mg/dl with associated elevated serum osmolality (>300 mosm/kg), dehydration, and stupor, progressing to coma if uncorrected, without the presence of ketosis or acidosis. LA consists of elevated lactic acid and without ketoacidosis. There may be low levels of ketones present.

**Chronic complications**

Metabolic, genetic and other factors lead to major diabetic complications. Chronic complications may take months or years to manifest and progress. Chronic complications are vascular (involving blood vessels) and are classified as microvascular complications (involving small vessels) and macrovascular complications (involving large vessels).

Microvascular complications involves thickening of the walls of small arteries and capillaries, which initially renders them permeable (leaky) to fluids and subsequently renders them prone to obstruction (thrombosis). These changes occur primarily in the retina (diabetic retinopathy) and kidneys (diabetic nephropathy), also in nervous system (diabetic nephropathy) (Singh et al 1987; Rudderman et al 1992).

Diabetic retinopathy occurs when the microvasculature that nourishes the retina is damaged, leading to the leakage of blood components through the thin vessel walls. Retinopathy is extremely common among persons with diabetes, and is the leading cause of blindness in type 2 diabetes (David et al 2012). Diabetic nephropathy is a significant complication in individuals with diabetes, increasing the risk of hypertension which in turn exacerbates the nephropathy and frequently develops into clinically significant renal disease. Diabetic nephropathy is the most common cause of renal failure (also known as end-stage renal disease or ESRD). In diabetic nephropathy number of functional nephrons decrease and thickening of the basement membrane resulting in increased glomerular filtration rate (Avodee 2004). Profound changes occurs in extracellular matrix components due to non enzymatic glycation (Dyck et al 1982). Diabetic neuropathy is a descriptive term for a clinical or sub-clinical disorder (Said G 2007) that occurs in as many as 75% of all patients with diabetes. Diabetic neuropathy comprises a large group of sensory and autonomic
syndromes with a wide range of manifestation diffused microangiopathy of endoneural capillaries with ischemia and alteration of blood nerve barrier and also biochemical derangements, insulin deficiency or phospholipid deficiency may ultimately leads to diabetic neuropathy (Dyck et al 1982).

Macrovascular complications involve large vessels and generally presents as atherosclerotic vascular disease (Atherosclerosis). Adults with diabetes mellitus frequently develop macrovascular complications. Vascular disease is a frequent cause of morbidity and mortality among patients with diabetes. Atherosclerotic vascular disease of the coronary vessels develops at an earlier age in patients with diabetes, and involves coronary arteries, the cerebral arteries, and the large arteries (iliac and femoral arteries) that supply blood to the legs (Michael and Fowler 2008). Thus, nonfatal and fatal myocardial infraction (heart attack), stroke, and ulceration and gangrene of the feet, often necessitating amputation, are common in patients with diabetes.

**Non enzymatic glycation with glucose**

Non enzymatic glycation occurs through the covalent binding of aldehyde or ketone groups of reducing carbohydrates (e.g., glucose, fructose, and ribose) or derivatives (ascorbic acid etc) with either terminal or epsilon amino groups in lysine or guanidine groups in arginine residues of amino acids, peptides, and proteins, ultimately resulting in the formation of complex brown pigments and protein-protein crosslinks i.e. labile Schiff’s base.

The mechanism of the glycation reaction was firstly tested by Millard at the beginning of the 20th century by incubation of amino acids with different sugars. The non enzymatic glycation can be explained in a three different stages.

**Biochemical mechanism of Non enzymatic glycation**

- Reaction between reducing sugar and amino group- it is initiated with the reversible formation of an adduct known as Schiff base by conversion of the aldehydic carbon –oxygen double bond of the sugar to a carbon- nitrogen double bond with the amine.
Formation of the Schiff base from sugar and amine is relatively fast. Schiff’s base is a thermodynamically unstable form in relation to the equilibrium cycled pyranose or furanose forms. Therefore, the Schiff’s base give rise to an enaminol intermediate by rearrangement and subsequently to a relatively stable ketoamine compound called amadoric compound. Formation of Amadori product from the Schiff base is slower but much faster than the reverse reaction, so that the Amadori glycation product tends to accumulate on proteins. The Amadori rearrangement of a lysine-glucose Schiff base is thought to be facilitated if there is a histidine side-chain or another lysine amino group on which the Schiff base has formed, due to localized acid-base catalysis (Acosta et al 2000).

In the second stage the Amadori compound further undergoes a series of dehydration and fragmentation reactions generating a variety of carbonyl compounds which are generally more reactive than the original carbohydrates and act as propagators by reaction with free amino groups. Among the most active of the enhancers are α-dicarbonyls such as methyl glyoxal, glyoxal, (Takeuchi et al 2001) glucosones, deoxyglucosones and dehydroascorbates. Glyoxal and methylglyoxal can be also formed by glucose auto-oxidation and by products from glycolipids (Thornalley et al 1999: Miyata et al 2000).

In the final step these initial and intermediate glycation products slowly undergo a complex series of further chemical rearrangements, to yield irreversible advanced glycation end products (AGEs) a structure of yellow-brown color and fluorescence. AGEs are characterized by a wide structural and physiochemical diversity. An extensive variety of AGE has been discovered like pentosidine, pyrraline, argpyrimidine, tetrahydropyrimidine, carboxymethyllysine(CML), carboxyethyllysine, a number of imidazolones and lysine-lysine crosslinks such as glyoxal-lysine dimer (GOLD), methyl-glyoxal-lysine dimer,(MOLD) and imidazoysine (Vlassara et al 1996 Bucala et al 1994). Maillard reaction and AGE formation is shown in Fig.1.7.
AGEs are spontaneously generated and circulate as a part of normal metabolism. However, the biological, non-enzymatic reaction of the reducing sugars with amino groups of amino acids, peptides, and proteins takes place in excess during aging. In pathophysiological condition like hyperglycemia it happens at an accelerated rate. Non enzymatic reaction finally ends up in the formation of highly reactive structures called AGE . Additionally, when oxidative damage occurs reactive oxygen species produced within an organism can react with lipids to form AGEs in lipid peroxidation. Notably, a major portion of AGEs can be generated from exogenous sources. AGEs are fluorescent molecules with a propensity to generate reactive oxygen species (ROS) and interact with specific cell surface structures (Brownlee et al 1988).

AGEs can also be incorporated through diet. Studies show that diet in particular the modern Western diet supplies a vast portion of AGE-precursors and
preformed AGE. Chemical derivatives of protein and lipid glycoxidation, which are AGE derivatives can be found in most foods today. Food processing and cooking methods can affect the rate of AGE production. Tobacco smoke is another main exogenous source of AGEs.

**How AGES promote atherosclerosis: molecular mechanisms**

In type-2 diabetic patients with coronary heart disease, elevated levels of AGEs and CML have been reported (Kilhovd et al 1999). AGE deposition in macrophages and vascular smooth muscle cells have been observed in human atherosclerotic lesions using monoclonal anti-AGE antibodies (Nakamura et al 1993; Vlassara et al 1996). Tissue AGE concentration correlates with the severity of atherosclerotic lesions and with the accumulation of plasma proteins, lipoproteins and lipids in the vessel wall (Kilhovd et al 1999; Sims et al 1996).

AGES interferes in the integrity and function of blood vessel walls in several ways. AGEs forms cross bridges among vessel wall macromolecules (Sell and Monnier 1989). Accumulation of AGEs in blood cells causes them to adhere to the vessel wall. Receptors for AGEs have been identified on various cell types which include macrophages, endothelial cells, and smooth muscle cells, renal and neuronal cells which damage the cellular function (Hori et al 1995; Ritthaler et al 1995).

AGE formation may thus accelerate the atherosclerotic process through two general mechanisms which can be classified as non-receptor-dependent and receptor-mediated.

**Non receptor mediated mechanism**

Glycosylation of proteins and lipoproteins can interfere with their normal function by disrupting molecular conformation, alter enzymatic activity, reduce degradative capacity, and interfere with receptor recognition, thus changes in the normal physiology of proteins that are relevant to atherogenesis, may promote atherosclerosis in diabetic individuals.
Non enzymatic glycation interfere in the normal physiology of low-density lipoprotein (LDL) particle. The glycosylation process occurs both on the Apo protein B (Bucala et al 1995) and phospholipid (Bucala et al 1993) components of LDL, leading to both functional alternations in LDL clearance and increased susceptibility to oxidative modifications. Glycosylation of LDL Apo B occurs mainly on a positively charged lysine residues within the putative LDL receptor binding domain which are essential for the specific recognition of LDL by the LDL receptor (Bucala et al 1994) which results in a significant impairment of LDL-receptor-mediated uptake decreasing the in vivo clearance of glycated LDL compared to native LDL (Steinbrecher and Witztum 1984).

Human monocyte derived Macrophages recognize glycated LDL to a greater extent than native LDL (Klein et al 1995). The uptake of glycated LDL by these cells however, is not mediated by the LDL receptor pathway, but by a high-capacity, low-affinity receptor pathway (Klein et al 1995). Thus, glycated LDL are poorly recognized by the specific LDL receptor and are preferentially recognized by a nonspecific (scavenger) receptor present on human macrophages. Because LDL glycosylation enhances its uptake by human aortic intimal cells and monocyte-derived macrophages (Klein et al 1995) with stimulation of foam cells formation, the recognition of glycated LDL by these scavenger receptor pathways is thought to promote intracellular accumulation of cholesteryl esters and promote atherosclerosis.

Oxidation reactions occur normally during glycation can oxidize the amine-containing phospholipids component of LDL. Advanced glycosylation of an amine-containing phospholipids component of LDL is accompanied by progressive oxidative modification of unsaturated fatty acid residues (Steinbrecher and Witztum 1984). Thus, glycation confers increased susceptibility of LDL to oxidative modification (Bowie et al 1993) which is considered a critical step in its atherogenicity.

AGEs on matrix also alter the normal interactions of trans membrane integrin receptors with three specific matrix ligands. For example, modification of the cell binding domains of type IV collagen causes decreased endothelial cell adhesion (Brownlee et al 1988).
AGE formation alters the functional properties of several important matrix molecules. Collagen undergoes significant nonenzymatic glycation, which may have a considerable bearing on atherosclerosis (Brownlee et al 1986). Soluble plasma proteins, such as LDL and immunoglobulins (Ig)G, are also entrapped and covalently cross-linked by AGEs on collagen (Vlassara 1996; Meng et al 1998). AGE formation on type-IV collagen from the basement membrane inhibits lateral association of these molecules. These AGE-induced abnormalities in the function of extracellular matrix alter structure and function of intact vessels.

Glycation of Apo B and phospholipids components of LDL leads the functional alterations of LDL. This modified LDL is now more susceptible for oxidative modifications (Bucala et al 1994). Intermediates such as glyoxals, glycolaldehydes, hydroxyaldehydes or other carbonyl group-containing compounds can be formed in the oxidation of both carbohydrates and poly-unsaturated fatty acids (Glomb and Monnier 1995). Further, the uptake of glycated LDL by human monocyte derived macrophages occurs to a greater extent than for native LDL by a low-affinity nonspecific (‘‘scavenger’’) receptor with the resulting stimulation of “foam cell” formation, characteristic of the early atherosclerotic lesion (Klein et al 1995).

**Receptor-mediated mechanisms**

The cellular interactions of AGEs are mediated through a specific receptor for AGE determinants on cell surfaces (Schmidt et al 1994). The receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin superfamily of receptors (Neeper 1992). It has been demonstrated in all cells involved in atherosclerotic process including monocyte-derived macrophages, endothelial cells, and smooth muscle cells (Schmidt et al 1994; Brett et al 1993). AGE interaction with RAGE on endothelial cells results in the induction of oxidative stress and consequently of the transcription factor NF-κB (Yan et al 1994; Wautier et al 1994) and VCAM-1 (Schmidt et al 1995).

AGEs with their specific receptors increases permeability of endothelial cell monolayers (Esposito et al 1989). This in turn increases the lipid entry into the sub endothelium. Monocyte-macrophage interaction with AGEs also results in the
production of mediators such as interleukin-1, tumor necrosis factor-α, platelet-derived growth factor, and insulin growth factor (Kirstein et al 1990; Vlassara et al 1988; Kirstein et al 1992) which have a pivotal role in the pathogenesis of atherosclerosis (Ross 1999). Fig.1.8 shows the schematic representation of AGE action, and Fig.1.9 shows the extracellular and intracellular effects of AGEs.
In smooth muscle cells RAGE increases cellular proliferation (Vlassara et al 1994) which is mediated by the cytokine or growth-factor. Thus, under conditions of enhanced tissue AGE deposition, receptor-mediated interaction of AGE-proteins with vascular wall cells facilitates the migration of inflammatory cells into the lesion with the subsequent release of growth-promoting cytokines.

**Metabolism of glyoxal and methyl glyoxal**

Glyoxal (O=CH–CH=O) is a α-oxoaldehyde, and it is often grouped with two similar α-oxoaldehydes, methylglyoxal and 3-deoxyglucosone. All three compounds are products of various metabolic and oxidative reactions and are capable of causing cellular damage and apoptosis (Abordo et al 1999). Early stage reactions in glycation of protein by glucose lead to the formation of fructosyl-lysine (FL) and N-terminal amino acid residue-derived fructosamines. Later stage of glycation leads to advanced glycation end-products (AGEs) which have been linked to long-term complications of chronic diseases such as diabetic retinopathy, neuropathy, and nephropathy (Shangari and Brien 2004).

Glyoxal originates from both exogenous and endogenous pathways. Exogenous sources of glyoxal include environmental and dietary sources (Kielhorn et al 2004). Endogenously it is produced via autoxidation of carbohydrates and ascorbate, degradation of glycated proteins and lipid peroxidation (Brien et al 2005). Glyoxal can be formed through various pathways. It is created directly from glucose via retroaldol condensation, and it is formed indirectly from glucose via a glycoaldehyde intermediate that undergoes autoxidation. The autoxidation reaction is promoted by the presence of phosphate buffer and trace metal ions (Fe3+ and Cu2+) in solution. During the Non enzymatic glycation of glucose auto oxidation of AGE generates glyoxal (Thornalley et al 1999) (Fig.1.10).

Early stage reactions in glycation of protein by glucose lead to the formation of fructosyl-lysine (FL) and N-terminal amino acid residue-derived fructosamines. FL degrades slowly to form AGEs and also glyoxal and methylglyoxal (Thornalley et al 1999). Glyoxal and methylglyoxal further react with proteins to form AGE residues directly and relatively rapidly. There is concurrent formation of minor lysine derived
adducts – \( \text{N}^\epsilon - \text{carboxymethyl-lysine (CML)} \) and \( \text{N}^\epsilon - \text{carboxyethyl-lysine (CEL)} \) residues, and bis (lysyl) crosslinks – GOLD and MOLD (Ahmed et al 2002). Glyoxal, further form a minor arginine-derived adduct – \( \text{N}^\omega - \text{carboxymethylarginine (CMA)} \).

Fig.1.10: Formation of glyoxal from different sources

Glyoxal is primarily detoxified by the glyoxalase system present in the cells of bacteria, protozoa, fungi, plants, animals, and humans (Thornalley 1993). T Neuberg discovered the glyoxalase system and metabolism of methylglyoxal (Neuberg 1913). Glyoxalase I also exists in the endoplasmic reticulum of cells, while glyoxalase II can additionally be found in the mitochondria. The bulk of the glyoxal formed in tissues is converted to glycolate by the glyoxalase system which consists of glyoxalase I and glyoxalase II located in the cytosol of all cells (Abordo et al 1999). Glyoxal conversion to glycolate requires glutathione (GSH). In situations where GSH is depleted, as might occur with oxidative stress, other enzymes such as aldehyde reductase, aldose reductase, carbonyl reductase, aldehyde dehydrogenase, and 2-oxoaldehyde dehydrogenase may be involved in the metabolism of glyoxal (Shangari and Brien 2004: Kraemer and Deitrich 1968). Glyoxal is also known to involve in
endogenous oxalate synthesis. And is studied in diabetics where urinary oxalate excretion was found (Lapolla et al 2003). Incubation of liver homogenates with glyoxal further revealed that glyoxal can be converted to glyoxylate, the major precursor of oxalate, by an NAD+-dependent reaction catalyzed by an aldehyde dehydrogenase (Fig.1.11)

Elevated intracellular glyoxal levels may inhibit aldehyde reductase, glutathione reductase, and NADPH-producing enzymes. In addition, high glyoxal concentrations produce reactive oxygen species (ROS) and formaldehyde, they increase cell susceptibility to hydrogen peroxide, and they disrupt the mitochondrial membrane potential (Shangari et al 2003: Shangari and Brien 2004).

**Fig.1.11: Potential mechanisms for oxalate synthesis from glyoxal**

Methylglyoxal is formed spontaneously from triose phosphates, that is glyceraldehyde 3-phosphate and dihydroxyacetone phosphate in all organisms with anaerobic glycolysis (Phillips and Thornalley 1993) and from other non-enzymatic and enzymatic pathways of differing reactions. (Thornalley 1993). Other sources of methylglyoxal in mammalian metabolism are, from the oxidation of acetol in the metabolism of acetone (Casazza et al 1984; Reichard et al 1986) and from aminoacetone formed in threonine catabolism by amine oxidase (Ray and Ray 1983, 1987).
The glyoxalase system catalyses the conversion of methylglyoxal to D-lactic acid via the intermediate S-D-lactoylglutathione. It comprises two enzymes, glyoxalase I and glyoxalase II, and a catalytic amount of reduced glutathione (Thornalley and Atkins 1989). Glyoxalase I (EC 4.4.1.5, lactoylglutathione lyase) catalyses the formation of S-D-lactoylglutathione from the hemithioacetal formed nonenzymatically from methylglyoxal and reduced glutathione (Mannervik 1980: Sellin et al 1983). Glyoxalase-II (EC3.1.2.6, hydroxyl acylglutathione hydrolase) catalyses the hydrolysis of S-D-lactoylglutathione to D-lactic acid and regenerates the reduced glutathione consumed in the glyoxalase I-catalysed reaction (Fig.1.12).

**The Glyoxalase System**

![Diagram of the glyoxalase system]

**Fig.1.12: The glyoxalase system.**

During the early glycation processes of glucose, many parallel glycation pathways leads to the subsequent formation of AGEs. The α-Oxoaldehydes formed during glycation reacts with lysine and arginine residues in proteins to form AGEs. Where, glyoxal forms Ne-carboxymethyl-lysine, the glyoxal-derived bis(lysyl)imidazolium crosslink GOLD and hydro-imidazolone; methylglyoxal forms Ne-carboxy ethyl-lysine, methylglyoxal-derived bis(lysyl)- imidazolium crosslink MOLD, hydroimidazolone and argpyrimidine; and 3-DG forms pyrraline, the 3-DG-derived bis- (lysyl)imidazolium crosslink DOLD and hydroimidazolone (Thornalley 1999).
Various AGEs that are identified in the physiological system alter the structural and functional properties of proteins. They form cross links in basement membrane proteins and disrupts the membrane elasticity. DNA is susceptible to glycation by glyoxal and methylglyoxal. The major nucleotide AGEs imidazopurinone derivatives are formed from the glyoxal and methylglyoxal. DNA suffers continuous damage from glycation by methylglyoxal and glyoxal: The nucleotide derived AGEs are associated with increased mutation frequency, DNA strand breaks and cytotoxicity. The formation of crosslinks of protein to DNA by glycation with methylglyoxal has been known for many years (Brambilla et al 1985).

AGEs are risk markers and risk predictors of diabetic complications. A number of experimental evidence supports the hypothesis that AGEs formed from glyoxal, methylglyoxal and 3-DG have an aetiological role in the development of diabetic complications and other diseases (Thornalley et al 1999; Thornalley 1996)

**Modification of Proteins by glycation and its consequences**

**Glycated Hemoglobin**

Human hemoglobin is an oxygen-carrying pigment and the most extensively studied protein among all other protein for its structural and functional features. Hemoglobin (Hb) is composed of four globin chains. Adult hemoglobin (HbA0) is the most abundant form and consists of two α and two β chains. Fetal hemoglobin (HbF), which is the predominant species present at birth, consists of two α and two γ chains. HbF is a minor form in normal adults. HbA₂ is minor Hb after birth and consists of two α and two δ chains.

Adult hemoglobin (HbA₀) the most abundant form comprises over 90% of the total protein. Approximately 8% of hemoglobin A₀ is made up of minor components that are chemically slightly different. These minor components include hemoglobin A₁c, A₁b, A₁a₁, and A₁a₂. Hemoglobin A₁c (HbA₁c) is the most abundant minor component arising from post translational modification at the α-amino terminal of valine of each β chain in HbA₀. HbA₁c is referred to as glycosylated or glucosylated
hemoglobin. Rahbar (Rahbar 1968) first discovered the association of increased HbA1c levels with diabetes mellitus in 1968.

HbA1c constitutes the major portion of the glycated hemoglobins. HbA1c, is a biochemical marker that is used routinely in the management of individuals with diabetes mellitus to monitor long-term glycemic control and assess the risk of developing complications (DCCT 1993). HbA1c is measured as the ratio of glycosylated to non-glycosylated hemoglobin (Peterson et al 1998). Higher levels of glucose in the blood contribute to more binding and consequent higher levels of glycosylated hemoglobin. Glycation occurs over the entire 90-120 day life span of the red blood cell (Kilpatrick 2000). HbA1c can consequently be interpreted as an average of the blood glucose present over the past 3-4 months (Peterson et al. 1998). Measurement of HbA1c is accepted as a useful index of mean blood glucose in the treatment of patients with diabetes (Rohlfing et al 2000). The relation of glycated hemoglobin to glycemic control was first convincingly shown by Koenig et al, who examined the relationship between HbA1c and glycaemic control in five poorly controlled diabetic patients (Koenig et al 1976). Improvement in glycaemic control caused a reduction in the levels of HbA1c after approximately four weeks. Levels of HbA1c are not influenced by daily fluctuations in the blood glucose concentration but reflect the average glucose levels over the prior six to eight weeks. It can be used to monitor the effects of diet, exercise, and drug therapy on blood glucose in diabetic patients.

HbA1c concentration is associated with diabetic microvascular complications (Rohlfing et al 2000, 2002), macrovascular complications, risk of death and cardiovascular disease (Khaw et al. 2001). Positive correlations with metabolic syndrome are also suggested (Grant et al. 2004).

The Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS) demonstrated conclusively that risks for complications are related directly to glycemic control, as measured by HbA1c (DCCT 1993: UKPDS 1998). Many diabetes organizations worldwide recommend specific HbA1c targets in terms of DCCT/UKPDS HbA1c (ADA 2008). According to
the American Diabetes Association (ADA) Guidelines 2007, the value of HbA1c should be kept below 7% in all diabetics. Values greater than 7% indicate an increased chance of progression to diabetic complications, especially microvascular ones.

**Lipoproteins**

Human serum lipoproteins are soluble complexes of proteins (apolipoproteins) and lipids that represent the major cholesterol transport vehicles in both the intravascular and extravascular compartments. Lipoprotein particles are synthesized by the liver and intestine and mediate lipid transport from the intestine to the liver, and between the liver and cells in the peripheral tissue of the body. Mature lipoproteins containing a core of neutral lipids (triacylglycerol (TAG), cholesteryl ester (CE) and cholesterol) stabilized by a surface monomolecular film of phospholipids (PL), cholesterol and apolipoproteins (apo). Plasma lipoprotein concentration measured about 700mg/dl. Lipoprotein particles are fractionated on the basis of their densities weight and by electrophoretic mobility (Jonas and Phillips 2008) namely chylomicrons, very low density lipoprotein (VLDL), low density lipoproteins (LDL), intermediate density lipoproteins (IDL), high density lipoprotein (HDL) and lipoprotein LP(a).

**An Overview of HDL**

HDL is a class of heterogeneous lipoproteins containing approximately equal amounts of lipids and proteins. HDL is a small lipoprotein (Stoke’s diameter 5 to 17 nm) with high density (1.063 g/mL) and contains the least amount of lipids. HDL contains a lipid core of cholesteryl esters (CE) and tryglycerides (TG) surrounded by phospholipids and specialized proteins known as apolipoproteins. Apolipoproteins are a specialized group of proteins that associate with lipids and mediate several biochemical steps associated with plasma lipid metabolism. About 50% of HDL by weight is composed of several apoproteins (ApoHDL). The HDL consists of two major proteins ApoA₁ (R-Gln-I) and ApoA-II (R-Gln-II). HDL also contains another class of apolipoproteins, ApoC, which is comprised of three proteins ApoC-I (R-Ser), ApoC-II (R-Glu), and ApoC-III (R-Ala).
Among several apoproteins, the apolipoproteins ApoA₁ is present on the majority of HDL particles and constitutes 70% of the apolipoprotein content of HDL particles; as a result, plasma ApoA₁ concentrations correlate closely with plasma HDL-C. Hence this fraction of plasma Apoa₁ concentration is quantified in the clinical laboratory as HDL-C.

The HDL has various subclasses which vary in quantitative and qualitative content of lipids, apolipoproteins, enzymes, and lipid transfer proteins, resulting in differences in shape, density, size, charge, and antigenicity. HDL can be further fractionated by density into HDL₂ and HDL₃, by size, or by apolipoprotein composition.

**Cardio protective function of HDL**

In vitro and in vivo studies have suggested several potential protective actions of HDL. The cardiovascular-protective effects of HDL have mainly been attributed to its role in reverse cholesterol transport, i.e. the transport of excess cholesterol from peripheral tissue towards the liver for excretion into bile or to steroidogenic organs for steroid hormone synthesis. The participation of HDL in the reverse cholesterol transport (RCT) from peripheral cells to the liver is critical for the anti-atherogenic properties of this lipoprotein. However HDL perform many other protective functions. It perform anti-oxidative function by preventing the oxidation of LDL or neutralizing the atherogenic effects of oxidized LDL (oxLDL) in the artery wall (Mackness and Durrington 1995; Maier et al 1994) Paraoxonase and platelet activating factor-acetyl hydrolase (PAF-AH) – enzymes carried on the surface of HDL – can cleave the oxidized fatty acids off oxLDL in vitro and render oxLDL less atherogenic. ApoA₁ itself may also have antioxidant potential by reducing lipid hydroperoxides in LDL and HDL (Hayek et al 1995; Garner et al 1998). HDL also functions as anti-inflammatory molecule by inhibiting the formation of platelet aggregates at sites of endothelial injury (Yui et al 1998: Cockerill et al 2001) or by inhibiting the production of monocyte adhesion molecules by endothelial cells in the first stage of atherogenesis (Ashby et al 1998). HDL also has anti-apoptotic features (Sugano et al 2000).
**HDL and Reverse Cholesterol Transport**

Peripheral (nonhepatic) cells obtain their cholesterol from a combination of local synthesis and the uptake of preformed sterol from low and very low density lipoproteins (LDL and VLDL). Reverse cholesterol transport (Fielding and Fielding 1991) is a mechanism in which the cholesterol in these extra hepatic cells will be transported to the liver for excretion from the body in bile.

The reverse cholesterol transport pathway is initiated by the interaction of nascent or small HDL (HDL₃) particles with peripheral cells. HDL particles can penetrate from plasma into the interstitial fluid in sites such as the intima of the artery wall (Sloop et al 1987). At such sites, HDL or free apolipoproteins (e.g. ApoA₁) dissociated off from the surface of HDL and interact with cells such as cholesterol-loaded macrophages and smooth muscle cells, and promote the removal of cholesterol. HDL then return to the plasma via lymphatic channels (Fielding and Fielding 1995: Wong L et al 1992), will be acted upon by lecithin cholesterol acyltransferase (LCAT) to esterify cholesterol on the HDL surface, which is activated by ApoA₁ (Breslow 1995). The hydrophobic cholesteryl esters are sequestered in the particle core, depleting cholesterol on the HDL surface so that the HDL can accept more cellular cholesterol upon recirculation into the interstitium. Further esterification of this cholesterol results in larger HDL₂ particles, which, beyond a certain size are no longer efficient acceptor particles. A portion of the cholesteryl ester in HDL is transferred to ApoB100-containing lipoproteins very low density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), and (LDL) in exchange for triglycerides by the plasma enzyme cholesteryl ester transfer protein (CETP). Cholesterol originally removed from peripheral cells by HDL is thereby returned to the liver largely via uptake of VLDL, IDL and LDL. Removal of these particles by the LDL receptor and the LDL receptor-related protein (LRP) seems to be more efficient than the uptake of cholesterol from HDL itself (Francis et al 1991) (Fig.1.13).
Fig. 1. 13: Pathways involved in the generation and conversion of HDL

The selective uptake of cholesteryl esters from HDL through the independent and possibly coordinated actions of hepatic lipase and the human homologue of the recently discovered scavenger receptor B1 (SR-BI) (Acton et al 1996: Wang et al 1996), also returns some cholesterol to the liver. SR-BI is located on the surface of hepatocytes mediates the selective uptake of FC and CE from HDL. The lipolytic action of hepatic lipase on HDL also regenerates HDL and lipid-free or lipid-poor ApoA1, which can then recirculate in the reverse cholesterol transport pathway. A small fraction (15%) of HDL that contain ApoE (‘HDL’) may also be taken up as whole particles by the LDL receptor or LRP (Mahley 1998: Fagan et al 1996). This FC and CE is then released into bile as either FC or bile acid and then, in the last step of RCT, excreted from the body in feces.

**HDL and peripheral cell interaction during cholesterol efflux**

Cholesterol efflux from the cells involves both a nonspecific, passive and specific active mechanism. Cholesterol efflux from macrophages to HDL can occur by passive diffusion (Yancey et al 2003) by interaction with the SR-BI receptor (Williams et al 1999) or by binding to the ABCA1 transporter (Remaley et al 2001: Liu et al 2003).
Passive or ‘non-specific’ cholesterol efflux occurs when cholesterol moves by aqueous diffusion down a concentration gradient from the cell to the HDL surface (Costet et al 2000; Schwartz et al 2000). HDL particles that contain fully lipidated Apo A₁ molecules participate in the aqueous diffusion process; this involves the desorption of cholesterol molecules from the cell plasma membrane and their diffusion through the aqueous phase where they can become absorbed by the HDL acceptor particles. Lecithin:cholesterol acyl transferase (LCAT) activated by ApoA₁ protein esterify cholesterol on the HDL surface, creating concentration gradient in the plasma space, now the cholesterol rich HDL returns to the interstitial space and receive more cholesterol from the cell (Fielding and Fielding 1995).

The second mechanism of cholesterol efflux involves SR-BI pathway, where free cholesterol is transported to mature spherical α-HDL. A lipid-free ApoA₁or lipid-poor pre-β-HDL particle promotes cholesterol efflux by interaction with cell surface sites. ApoA₁solubilizes cholesterol and phospholipid directly through reversible interactions with the plasma membrane and stimulates mobilization of pools of cholesterol that are readily accessible to esterification by acyl CoA:cholesterol acyl-transferase, an enzyme localized to the rough endoplasmic reticulum. A member of the scavenger receptor family, the class B scavenger receptor SR-BI, bind to HDL with high affinity (Bryan Brewer 2004) and mediate selective cholesteryl ester uptake, leaving the HDL particles largely intact. HDL-PL can modulate the functional interaction of HDL to SR-BI (Christopher and Phoebe 1995).

The third pathway is an active process specifically stimulated by the amphipathic, alpha-helical apolipoprotein components of HDL (Hara and Yokoyama 1991; Oram et al 1991). The efflux of cellular cholesterol initiated by apoA₁ appears to require energy (Mendez 1997) involves protein kinase C (Mendez et al 1991) and a Golgi apparatus-dependent pathway for the delivery of stored cholesterol to the cell surface (Mendez and Uint 1996). This pathway involves the ABCA1 transporter. ApoA₁and ApoE possess detergent-like properties in that their lipid-binding capabilities permit them to solubilize vesicular phospholipid and form discoidal HDL particles. In the ABCA1 transporter pathway, the preferred acceptor of cellular cholesterol is poorly lipidated ApoA₁, which binds to the ABCA1 transporter and
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facilitates the efflux of cellular cholesterol from the late endocytic compartment (Neufeld et al 2001) thereby decreasing the cholesterol content of the cell. The lipid-binding and lipid-solubilizing properties of ApoA1 and ApoE underlie their abilities to interact with ABCA1, efflux cellular lipids and create nascent HDL particles. The efflux of cholesterol and phospholipids from macrophages and other peripheral tissues results in the formation of preβ-HDL, which is ultimately converted to mature spherical α-HDL after the esterification of FC to CE by LCAT (Fig.1.14).

![Fig.1.14: Three major pathways of cholesterol efflux](image)

Both the SR-BI and ABCA1 transporter pathways are regulated by the oxycholesterol content of the cell. Excess cellular cholesterol is converted at least in part to 27-hydroxycholesterol by 27-hydroxylase (Fu et al 2001). 27-hydroxycholesterol binds to the ligand-stimulated transcription factor LXR, which, after dimerization with RXR, binds to the LXRE promoter element and increases the expression of SR-BI and the ABCA1 transporter genes. Thus, both mature and preβ-HDL facilitates the efflux of cellular cholesterol and participates in reverse cholesterol transport to the liver.
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Non reverse cholesterol transport mechanism

In recent times HDL is recognized to have functions besides RCT which may be equally responsible for the anti atherogenic properties. The anti-inflammatory effects of HDL are well documented and include:

Inhibition of low-density lipoprotein (LDL) oxidation,

ApoA₁ is a major HDL protein with a binding domain for LDL deposition in the extracellular matrix of many tissues, especially arteries susceptible to atherosclerosis. LDL binding to the sub endothelial space causes cells to oxidize LDL lipids evoking the cells to secrete monocyte chemo attractant protein (MCP-1) and inducing an inflammatory response (Navab et al 1991). HDL in normal state abolishes the extracellular transport of lipids by preventing LDL oxidation, secretion of MCP-1, and the inflammatory response. Furthermore, HDL comprises a series of antioxidant enzymes which protect LDL from oxidation. Oxidized lipids are transferred to HDL from LDL and are hydrolyzed by HDL-associated PON1, LCAT, and PAF-AH enzymes (Navab et al J 2001: Marathe et al 2003).

HDL, ApoA₁ is capable of removing LDL lipid hydroperoxides in vitro, after injection into mice in vivo, and after infusion into humans in vivo (Navab et al 2000: Navab et al 2000). HDLs are also carriers of enzymes that destroy the lipid hydroperoxides that oxidize LDL phospholipids (Navab et al 2001). These enzymes include paraoxonase-1 (Mackness et al 2004) and paraoxoanse-3 (Reddy et al 2001) and possibly glutathione phospholipid peroxidase (Navab et al.2001). In addition, it has been shown that HDL phospholipid hydroperoxides are reduced to corresponding hydroxides with a concomitant oxidation of ApoA₁ methionine residues. HDL also transport enzymes such as platelet-activating factor acetyl hydrolase (Watson et al 1995) and lecithin cholesterol ester acyltransferase (Forte et al 2002 ) that are able to remove oxidized phospholipids.
Reduction of inflammatory cytokines and vascular leukocyte adhesion molecules

HDL functions as anti-inflammatory which selectively decrease endothelial cell adhesion molecules which facilitate the binding of monocytes to the vessel wall and promote lesion development, thereby protecting against CAD (Fogelman 2004). HDL limits expression of cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 that mediate up regulation of leukocyte-endothelial adhesion molecules. The ability of HDL to inhibit adhesion molecule expression could be mediated by apolipoproteins, and also by phospholipids, including sphingosine-1-phosphate and sphingosyl phosphorylcholine (Baker et al 1999; Recalde et al 2004). However, HDL has multiple additional endothelial and antithrombotic actions that may also afford cardiovascular protection which were discussed in detail in some recent reviews (Saemann et al in press).

HDL modulates endothelial function, probably by stimulating endothelial nitric oxide (NO) production which is an atheroprotective signaling molecule. HDL stimulates NO production by upregulating endothelial NO synthase (eNOS) expression by maintaining the lipid environment in caveolae, where eNOS is colocalized with partner signaling molecules and by stimulating eNOS as a result of kinase cascade activation by the high-affinity HDL receptor SR-BI. HDL also protects endothelial cells from apoptosis and promotes their growth and their migration via SR-BI initiated signaling.

Reversal of Endothelial Cell Apoptosis

Endothelial cell apoptosis is one of the major events which promote the pathogenesis of apoptosis. An intact endothelial cell monolayer plays a critical role in normal homeostasis in the vascular wall, and apoptosis of endothelial cells can occur on exposure to circulating factors and inflammatory cells, leading to disruption of endothelial monolayer integrity (Dimmeler et al 2002). Multiple pro-atherogenic factors promote apoptosis in endothelium, and these include OxLDL (Li et al 1998; Choy et al 2001) tumor necrosis factor-α (TNF-α) (Dimmeler et al 1999), homocysteine, (Welch and Loscalzo 1998) and angiotensin II(Strawn and Ferrario
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Introduction

2002). OxLDL causes a delayed but sustained increase in intracellular Ca\(^{2+}\) in endothelial cells, which results in cell death.

Endothelial cell apoptosis is reversed by the HDL by much mechanism where it prevents the sustained increase of Ca\(^{2+}\). TNF-\(\alpha\) induced endothelial cell apoptosis is also inhibited by HDL, and this is associated with attenuated induction of CPP32-like protease (caspase 3), which is a component of all primary apoptotic pathways (Sugano et al 2000). The HDL-associated lysophospholipids protect endothelial cells from growth factor deprivation–related apoptosis via parallel mechanisms (Nofer et al 2001), where lysophospholipids are involved in signaling. In Chinese hamster ovary cells SR-BI induced novel ligand-independent apoptotic pathway is demonstrated the pro-apoptotic effects of SR-BI were reversed by HDL and by eNOS.

Participation in innate immunity

HDL plays an important role in host defense as part of the innate immune system. It binds microorganisms or compounds derived from microorganisms. When either endotoxin (lipopolysaccharide, LPS), lipoteichoic acid (LTA), are incubated with whole blood from healthy humans, the majority of the LPS and LTA are bound to HDL. This binding to HDL inhibits the ability of LPS and LTA to interact with toll like receptors (TLR) and activate macrophages (Khovidhunkit et al 2004). TLR activation of macrophages stimulates the production and secretion of cytokines and other signaling molecules, which if produced in excess can lead to septic shock and death (Beutler et al 2003; Parrillo 1993). In addition to binding LPS, studies have shown that HDL also facilitates the release of LPS that is already bound to macrophages, reducing macrophage activation (Kitchens et al.1999) In addition to binding bacterial products, HDL also binds a wide variety of viruses and neutralizes their activity (Khovidhunkit et al 2004). Both the lipid and proteins that comprise HDL contribute to the neutralization of LPS. Apolipoprotein A-1 alone can neutralize LPS and this interaction can be altered by changing the structure of apolipoprotein A-1 (Wang et al. 2008). The phospholipid content of lipoproteins correlates with the ability of lipoproteins to neutralize LPS, whereas the content of cholesterol or triglycerides does not (Khovidhunkit et al.2004). Additionally, phospholipids alone have been shown to
protect animals from LPS induced toxicity. Thus both apolipoproteins and phospholipids can play important roles in the ability of HDL to neutralize LPS (Khovidhunkit et al. 2004).

**Inhibition of thrombosis**

Thrombus formation during atherosclerosis includes three major mechanisms. Namely, dysfunction of the cells within the vascular wall, particularly the endothelium, disturbed blood flow; and dysfunction of blood components. HDL exhibit antithrombotic effect by its multiple actions. HDL increases blood flow by increasing NO and prostacyclin production. HDL causes enhanced prostacyclin synthesis which acts synergistically with NO to induce VSM relaxation, inhibit platelet activation, and diminish the release of growth factors that stimulate the local proliferation of VSM cells (Vane and Botting 1995). Prostacyclin is synthesized from arachidonate derived from phospholipids of cellular membranes or from exogenous sources that include phospholipids and cholesteryl esters associated with circulating lipoproteins. Cyclooxygenase (Cox), promote prostacyclin synthesis in endothelial cells. (Caughey et al 2001) Recently, it has been shown that HDL3 induces Cox-2 expression and prostacyclin release via a p38 MAP kinase/CREB-dependent pathway in endothelium that mimics the mechanism that occurs in VSM (Escudero et al 2003: Martinez-Gonzalez et al 2004). Prostacyclin release increases when isolated rabbit and rat hearts are infused with HDL (Calabresi et al 2003).

The pro-thrombotic factors, tissue factor and the selectins, P-selectin and E-selectin present on the surfaces of platelets, endothelial cells and cell-derived, circulating microparticles contributes to the thrombus formation and progression. The adhesive reactions between microparticles and endothelium or blood cells in a growing thrombus are critical to the thrombotic process; down regulation of the expression of selectins or of tissue factor inhibit thrombus formation. The phospholipid components of HDL contribute to the down regulation of E-selectin expression on endothelial cell surfaces (Barter et al 2004: Wadham et al 2004). HDL may decrease tissue factor induction on endothelial cells indirectly by increasing the
synthesis of NO (Landmesser et al 2004). HDL inhibit thrombus formation by preventing the endothelial cell apoptosis

Thrombin generation via blood coagulation pathways induces platelet activation and platelet-derived growth factor release and directly causes fibrin clot formation by cleaving fibrinogen. The protein C pathway provides a major physiological anticoagulant mechanism to down regulate thrombin formation by inactivating factors Va and VIIIa in plasma. HDL modifies thrombin generation under normal conditions. HDL up regulates endothelial cell thrombomodulin, which is an anticoagulant factor that supports the generation of APC and the suppression of thrombin generation (Nicholls et al 2005).

HDL transports various sphingolipids that are present in plasma. These sphingolipids contribute antithrombotic activity in HDL (Norata et al 2004). HDL-bound glycosphingolipids have clinical significance as antithrombotic lipid cofactors for APC and protein S. Sphingosine inhibits pro-thrombin activation on platelet surfaces, also sphingosine appears to inhibit pro-coagulant interactions between factors Xa and Va (Deguchi et al 2004). Lysosphingolipids exert potent effects on cells via a family of G protein–coupled receptors. The anti-apoptotic activity of HDL mediated by both lysosphingolipids and NO (Gong et al 2003) may reduce the risk of thrombosis by reducing endothelial cell apoptosis. HDL also, down regulates endothelial cell adhesive reactions, through the HDL-associated lysosphingolipids showing antithrombotic function.

HDL may promote fibrinolysis by down regulating plasminogen activator inhibitor-I (PAI-I) and by up regulating tissue plasminogen activator (t-PA) (Connell and Genest 1983: Eren et al 2002). HDL indirectly regulate platelet function by down regulating the release of platelet activating factor or by up regulating NO synthesis and release from endothelial cells which can decrease platelet aggregation as well as blunt leukocyte-endothelial cell interactions and thereby prevent the initiation and progression of atherogenesis. (Connell and Genest 1983: Kobayashi et al 2004).
It was believed that HDL cannot undergo modifications and it was only the LDL that underwent oxidative modification that caused heart diseases. However this picture is rapidly changing and several mechanisms for the modification of HDL have been identified.

**Modifications of HDL**

**Oxidation**

High density lipoproteins play a key role in the protection against oxidative damage of membranes. They are susceptible to structural modifications mediated by various mechanisms including oxidation, glycation, homocysteinylation or enzymatic degradation. Modifications in lipid and apolipoprotein composition results in altered physicochemical properties and also significant decrease in enzyme activities especially paraoxonase. HDL undergoes oxidation by a variety of oxidants such as metal ions, peroxyl and hydroxyl radicles, aldehydes, peroxidase generated tyrosil radicle lipo-oxygenase, cigarette smokes and hypchlorous acid. The majority of oxidation of HDL takes place in the inflammatory microenvironments, like atherosclerotic lesions, interstitial fluid of arterial intima and other peripheral tissues (Francis 2000).

The potential candidates which are involved in the HDL-oxidations are myeloperoxidase, superoxide radicles (O$_2^-$) and H$_2$O$_2$ secreted by the activated phagocytes (Panzenboeck et al.1997). The polyunsaturated fatty acids of phospholipids in lipoproteins undergo lipid peroxidation by the transition metal ions producing oxidative HDL (Esterbauer et al.1992). Oxidation induces various structural and compositional modifications in lipids and apolipoproteins of HDL. ApoA$_1$ the major protein present on HDL forms dimer, trimer, or heterodimers with other lipoproteins like Apo-II to form higher molecular aggregates (Ferretti et al. 2006). A decreased ability of HDL to promote the reverse cholesterol transport has been shown in HDL-oxidized in vitro (Salmon et al.1992). Oxidation impairs HDL anti-inflammatory properties and exerts cytotoxic effect (Girona et al.1997). Lipid peroxidation of HDL alters the activity of enzymes associated with HDL, where a
significant reduction of paraoxonase enzyme has been observed (Jaouad et al 2003). Other enzymes which are involved in lipoprotein metabolism like LCAT and PLTP are also modified in oxidized HDL (Ferretti et al 2006). Amyloid-β an antioxidant can become a pro oxidant under increased oxidative stress (Kontus 2000) and contributes Alzheimer’s disease. The HDL present in cerebro-spinal fluid (CSF) contains Polyunsaturated fatty acids are susceptible for oxidative modification which could participate degeneration associated with oxidative stress in Alzheimer’s disease. Oxidation of HDL is followed by an increase in the levels of lipid peroxidation markers including conjugated dienes, lipid hydroperoxides, thiobarbuturic acids reactive substances and aldehydes. The compositional changes which occur due to oxidation are associated with alteration of physicochemical properties of HDL such as fluidity, mass and charge. And the functional changes makes HDL into a proatherogenic.

**Homocysteinylation**

Homocysteine (Hcy) is a non-protein sulfur-containing amino acid which is an intermediate product of methionine metabolism. It was noted in the 1960s that the risk of atherosclerosis is markedly increased in patients with homocystinuria. homocysteine thiolactone (HCTL), one of its metabolites, specifically damages proteins by reacting with free –NH2 groups of lysine residues; the process referred to as protein N-homocysteinylation. HCTL is formed in all cell types as a result of error-editing met-tRNA synthetase when there is excess homocysteine.

The interaction of HCTL with proteins leads to protein homocysteinylation and loss of function (Jakubowski et al 2000). Homocysteinylation of protein lysine residues results in the incorporation of additional thiole groups, which has several consequences for the physicochemical properties of the affected protein tertiary structure and protein aggregation, respectively. Additional –SH groups may also make the protein more susceptible to oxidative damage.

An elevated level of total homocysteine (tHcy) in blood, denoted hyper-homocysteinemia, is emerging as a prevalent and strong risk factor for atherosclerotic vascular disease. The interaction between Hcy-thiolactone and amino groups of ApoB
Lysyl residues of low density lipoproteins cause the formation of Homocysteimide-LDL (Hcy-LDL) (Ferguson E et al 1999). This reaction induces the LDL aggregation and a higher uptake of homocysteinylated LDL by cultured macrophages (Jakubowski 1997). Hcy-LDL induces atherogenic modification of LDL as bringing about functional alterations and oxidative damage in human endothelial cells (Ferretti et al 2004; Vigniniet al 2004). Incubation of Hcy-thiolactone with plasma HDL is shown to form Hcy-HDL adducts. Also homocysteinylation of HDL induces an increase of sulfhydryl group at lipoprotein surface and it is not accompanied by oxidative damage (Ferretti et al 2003).

Human serum paraoxonase is studied for its homocysteine thiolactose hydrolyzing activity (HCTL). Elevated levels of vitreous HCTL and PON-HCTLase activity in proliferative diabetic retinopathy (PDR) subjects were seen indicating the protective effect of PON to eliminate HCTL, which mediates endothelial cell dysfunction (Subramaniam et al 2010). However Serum PON-HCTLase and PON-AREase activity of PON were reported to be significantly lowered in diabetic patients (Sonoki et al 2009), this could be due to the compositional changes induced by homocysteintlation on HDL reflect in a significant decrease of paraoxonase activity.

Tyrosylation

Myeloperoxidase and the H₂O₂ generated during inflammation together induce the generation of diffusible cytotoxic radicles called tyrosyl radicals from L-tyrosine. In vitro studies have shown that a these tyrosyl radical induces the tyrosylation of HDL, with the formation of lipid peroxidation products, protein dityrosine as well as crosslinking of HDL proteins (Francis et al 1996; Banka 1996). The formation of theses tyrosylation induces the formation of dimers and trimmers as well as heterodimers of ApoA₁ (ApoA₁,ApoA_III) (Francis et al 1993). It has been shown that tyrosylation of HDL enhances cellular cholesterol mobilization by increasing translocation of cholesterol from an intracellular pool to the sites on the cell surface available for a similar action by acceptors including ApoA1, suggesting that a similar action in-vitro could enhances the lipidiation of ApA₁ and the maturation of pre-beta HDL increasing plasma HDL levels (Francis et al 1993).
Non-enzymatic Glycation

Hyperglycemia in diabetic patients results in non-enzymatic glycation of plasma proteins including lipoprotein such as HDL. Glycation/glyoxidations of HDL decrease several important functions of HDL particles, rendering the anti-atherogenic HDL to a more pro-atherogenic HDL. The protective role of HDL is performed by the proteins and the enzymes arranged on HDL particles. HDL protein modified by non-enzymatic glycation is shown to more susceptible for oxidation (Hedrick et al 2000). Ferritin and others have shown that HDL incubated for three days with varying concentrations of glucose had significance increase in TBARS and conjugated dienes, which was higher in subjects with low PON activity with respect to the subjects with higher PON activity suggesting the sensitivity of HDL to glucose induced lipid peroxidation.

NON enzymatic glycation generated oxygen free radicles such as superoxides (Wolff and Dean: 1997; Huntet al 1988) which auto oxidizes glucose and stimulates lipoprotein peroxidation. In support of this, experiments have also been done where lipid peroxidations of LDL is enhanced by high concentration of glucose. (Kawamura et al 1994). Peroxides and free radicles are known to cause protein conformational changes which can be observed by altered fluorescence (Dousset et al 1994: Shoukry et al 1994) HDL has also shown modification in apoprotein and conformational changes when subjected to glycation with glucose.

Incubation of HDL and purified PON with 25mM glucose has shown reduction in PON activity by 65% and 40% respectively (Hedrick et al 2000). Also the enzyme activity decreased in a greater extent in the presence of 50mM and 100mM glucose (Ferretti et al 2001). Type 2 diabetic pateints have shown 40% decreased PON activity with higher coronary artery (Hedrick et al 2000). The decrease in the PON activity could be explained by many reasons, like it could be due to free radical induced oxidative damage of proteins which might be generated by glyoxidation of proteins and many other which will be explained later.

HDL has diverse mechanism of action in relation to its cardio-protective functions. Plasma HDL is known to inhibit monocyte adhesion to the endothelial cells
a first step in atherosclerosis response to MM-LDL in-vitro (Navab et al 1991). In
against to this Hedric and others have shown that the glycated HDL did not inhibit
adhesion to human aortic endothelial cells in response to oxidized LDL in-vitro. Glycative modifications of HDL have been shown to increased hepatic lipase activity
due to enhanced inactivation of glycated HDL with the enzyme (Hedrick et al 2000).

Gly-ox-HDL shows several functional alterations with the decreased enzyme
activity associated to surface of HDL such as PON (Jaouan et al 2003: 48, Ferretti et
al 2001), CEPT and LCAT (Lemkadem et al 1999). In vitro glycation of HDL and its
LCAT activity was studied showing the decreased LCAT activity (Fournier et al
1995). The decrease in LCAT activity could be correlated with the diabetic patients.
(Fournier et al 1995). Several studies have certified that HDL also exerts potent anti-
inflammatory effects to reduce the endothelial inflammation by inhibiting the
expression of endothelial adhesion molecules induced by lipopolysaccharide (LPS) or
cytokines both in vitro and in vivo (Cockerill et al 1995: Barter et al 2004). Diabetic
HDL and HDL glycated in vitro both partly lose their protective effects to inhibit
cytokines release induced by LPS in macrophages (Donghui et al.2012).

**Dysfunctional and Proinflammatory HDL**

Even though HDL and cardiovascular disease show an inverse correlation, recent studies indicate that higher HDL levels may not always be protective and can become dysfunctional losing their cardio protective effects (Barter et al 2002). HDL is measured as HDL-C but not in terms of its functionality but HDL function is not always accurately predicted by HDL cholesterol levels. Functional HDL shows high levels of antioxidants, active antioxidant proteins, and antioxidant enzymes with anti-
inflammatory activity. In chronic illnesses such as diabetes that are characterized by
systemic oxidative stress and inflammation antioxidant and anti-inflammatory functions of HDL are converted into a dysfunctional, pro-inflammatory particle that cannot promote cholesterol efflux or prevent LDL oxidation (Navab et al 2009). This
dysfunctional HDL shows decreased levels and activities of anti-inflammatory and
antioxidant factors, such as Apo A1 and PON1. The acute-phase HDLs are depleted in
cholesterol esters but enriched in free cholesterol, triglycerides, and free fatty acids,
with subsequent decreased activity of lipoprotein lipase, hepatic lipase, LCAT, or a combination of these. All these metabolic alterations are frequently observed in the acute phase and during inflammation (Cabana et al. 1996) but none of them can participate in reverse cholesterol transport or antioxidation (Khovidhunkit et al. 2000; Navab et al. 2001).

Dysfunctional HDL contains oxidized phospholipids and pro-inflammatory proteins, such as serum amyloid A (SAA) and ceruloplasmin. SAA is a pro-oxidant acute-phase reactant associated disabling the anti-inflammatory role of HDL with creation of pro-inflammatory HDL (Khovidhunkit et al. 2004; Esteve et al. 2005). Replacement of Apo A1 by SAA during inflammation occurs, which will then, have a significant impact on RCT mechanism. Enrichment of HDL with SAA results in increased HDL binding to macrophages, decreased cholesterol efflux from macrophages, and increased selective uptake of CE by macrophages (Fig. 1.15a,b) (Banka et al. 1995; Artl et al. 2000).

Fig. 1.15a: Characteristics of normal, protective HDL (left panel) and non-protective, pro-inflammatory HDL (right panel).
Fig.1.15b: Model of bidirectional conversion of HDL from anti-inflammatory to proinflammatory

**HDL metabolism in Diabetes**

Several studies show that HDL-C is decreased in diabetes. However there is a debate whether it is due to decreased synthesis of HDL or its increased degradation or both. Measurement of ApoA\textsubscript{1} kinetics has shown a decreased synthesis of HDL (Golay et al 1985). A decrease in ApoA\textsubscript{1} content of HDL has also been reported (Briones et al 1984). In addition to its decrease in concentration alterations its phenotype has also been reported. Decrease in HDL\textsubscript{2} has been reported. (Howard et al 1986). Increases in HDL- associated triglycerides have also been reported. In summary in Diabetes HDL can be affected in a variety of ways leading ultimately to the increased risk of cardiovascular diseases.

**Aim and scope of the present investigation**

It is now becoming increasingly evident that pure sugar and not fat is the cause of major non communicable disease like diabetes, cardiovascular disease and cancer. Sugar when purified is devoid of all nutrients except the sugar consisting of one molecule of glucose linked to one molecule of fructose.

While glucose is an essential nutrient for all cells, it has tight metabolic control. Whereas fructose does not have the same control. Elevated fructose in blood
does not cause insulin release. Whether glucose or fructose, when elevated in blood, can bring about a series of toxic reactions.

Sugars can increase oxidative stress by a variety of pathways. They can increase the synthesis of diacyl glycerol which is an activator of protein Kinase C. Alternate metabolites of glucose namely glucosone, glyoxal and methyglyoxal can non-enzymatically glycate proteins. In fact a diet of pure sucrose can be lethal to humans. A major impact of sugar toxicity is on the pancreas and its response to elevated blood glucose level. Elevated sugars can reduce apoptosis of β cells. When about 30-50% of the β cells have been destroyed, the ability of pancreas to regulate glucose homeostasis is lost. This leads to insulin resistance, resulting in the establishment of diabetes mellitus.

Diabetes is the major epidemic of the world and a leading cause of cardiovascular disease. One common mechanism linking the various disorders including cardiovascular disorders in diabetes is the ability of sugars, whether fructose or glucose or some of their metabolites, to non-enzymatically glycate proteins.

This thesis investigates the consequences of non-enzymatic glycation to serum lipoproteins, particularly High Density Lipoprotein. The major focus of this study was to investigate the role of glucose and glyoxal in non-enzymatic glycation of HDL, since it is the only negative risk factor of atherosclerosis. We wanted to study on the loss of antioxidant function of HDL associated paraoxonase enzyme.

Since HbA1c is a well-recognized marker for non-enzymatic glycation of Hemoglobin one aim of this study was to see whether HbA1c can be used as a marker for non-enzymatic glycation of HDL associated paraoxonase. Since loss of PON activity would also imply that HDL functionality would be lost including its cardio-protective functions like anti-inflammatory properties also.