CHAPTER 2

LITERATURE REVIEW

2.1 Type 2 Diabetes Mellitus

2.1.1 Definition and Classification

The term diabetes mellitus describes a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion and/or insulin action. According to World Health Organization (WHO, 1985), diabetes can be classified into two major classes: type 1 diabetes (T1DM) and type 2 diabetes (T2DM). T1DM is the classical form of diabetes and these subjects cannot survive without insulin treatment. In most cases, this is a disease of autoimmune origin in which the insulin producing $\beta$-cells in the pancreas are destroyed leading to total exhaustion of insulin secretion. Genetic screening seems to be essential in the identification of individuals at increased risk for T1DM (Åkerblom, et al., 1997). T2DM is a group of genetically determined diseases which may be controlled by diet and/or hypoglycemic agents and/or exogenous insulin (Groop & Tuomi, 1997). T2DM is mainly characterized by insulin resistance, but impairment in insulin secretion also occurs in type 2 diabetes (Weyer, et al., 1999; Lebovitz, 2001).

Subjects with T2DM cannot compensate for insulin resistance at hyperglycemic levels by increasing insulin secretion (Groop & Tuomi, 1997). Several human monogenic forms of diabetes have been identified including: maturity-onset diabetes of the young (MODY) (Owen & Hattersley, 2001), which can be caused by mutations in the glucokinase gene. MODY is characterized by $\beta$-cells dysfunction and young age at
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diagnosis, usually less than 25 years, leading to early-onset T2DM. In addition, other minor classes include gestational diabetes, extreme insulin resistance caused by a defective insulin receptor gene, the diabetes-deafness and optic atrophy syndrome which is due to defects in mitochondrial genes (Kahn, 1996), and latent autoimmune diabetes in adults (LADA) (Tuomi, et al., 1999; Pozzilli & Di Mario, 2001) which was introduced to define adult diabetic patients who initially present as type 2 diabetics but with immune markers of type 1 diabetes which, in a number of cases, progress to insulin dependency. However, LADA more closely resembles and shares common characteristics of T1DM including genetics, metabolic dysfunction, and autoimmune features, but LADA does not affect children and is classified distinctly as being separate from juvenile diabetes. T1DM and the minor classes of diabetes will not be discussed any further in this thesis.

2.1.2 Diagnostic Criteria

Type 2 diabetes is diagnosed on the basis of an elevated blood glucose concentration. The diagnosis of diabetes is based on the criteria defined by the World Health Organization in 1985 (WHO, 1985). According to these criteria, a fasting venous plasma glucose concentration of less than 6.4 mmol/l and a 2-h value in 2-hours glucose load test of less than 7.8 mmol/l is considered normal. A fasting plasma glucose concentration of less than 7.8 mmol/l and a 2-hours glucose load test value of between 7.8 mmol/l and 11.0 mmol/l refers to impaired glucose tolerance. Diabetes is diagnosed when the fasting plasma glucose concentration is 7.8 mmol/l or more and the 2-hours glucose load test value is 11.1 mmol/l or more (WHO, 1985). To further simplify the diagnosing of diabetes, new criteria highlighting the use of fasting glucose concentrations, were proposed by the American Diabetes Association in 1997. The WHO adopted the fasting
criterion (fasting blood glucose above or equal to 6.1 mmol/l) from the ADA in 1999 and the criterion for fasting plasma glucose to indicate diabetes was decreased to 7.0 mmol/l (WHO, 1999).

2.1.3 Epidemiology

The most common form of diabetes is type 2 diabetes which constitutes about 85 to 95% of all diabetes in developed countries (WHO, 1994), and accounts for an even higher percentage in developing countries. The prevalence of diabetes is increasing worldwide. The increasing prevalence of T2DM is a global health problem and closely related to the increasing prevalence of obesity due to western lifestyles (Songer & Zimmet, 1995). Amos, et al. (1997) estimated that there were 124 million persons with diabetes in the world in 1997 and predicted this number would grow to 221 million in 2010. Another study group estimated that the number of persons with diabetes was 150 million in 2000 and this number is expected to double by 2025 (King, et al., 1998). In 2003, it was estimated that approximately 194 million people worldwide, or 5.1% in the age group 20-79, have diabetes.

The largest increase in the prevalence numbers is thought likely to appear in India, China and other developing countries. This estimate is expected to increase to 6.3% in the adult population, by 2025. In the United States, the National Health and Nutrition Examination Surveys (NHANES) I and II showed that the prevalence of DM between 1976 and 1994 among American adults increased from 6.6% to 7.8% (Harris, et al., 1987; Harris, et al., 1998). Although the absolute increase is relatively small, when the U.S. population growth during this period is considered, the number of patients with DM almost doubled from an estimated 8 million to 15.6 million people. Similar pictures have
been observed in Europe, in which DM affects about 8.5% of the adult population (DECODE study group, 1998). The European Region with 48 million and Western Pacific Region with 43 million currently have the highest number of people with diabetes. However, the prevalence rate of 3.1% for the Western Pacific Region is significantly lower than 7.9% in the North American Region and 7.8% in the European Region. By 2025, the region with the greatest number of persons with diabetes is expected to change to the South-East Asian Region with about 82 million. The region’s prevalence of 7.5% will however continue to be lower than that of North America, estimated at 9.7%, and Europe at 9.1%. (Sicree, et al., 2003).

Table 2.1: List of countries with the highest numbers of estimated cases of diabetes for 2000 and 2030

<table>
<thead>
<tr>
<th>Ranking</th>
<th>2000 Country</th>
<th>People with diabetes (millions)</th>
<th>2030 Country</th>
<th>People with diabetes (millions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>India</td>
<td>31.7</td>
<td>India</td>
<td>79.4</td>
</tr>
<tr>
<td>2</td>
<td>China</td>
<td>20.8</td>
<td>China</td>
<td>42.3</td>
</tr>
<tr>
<td>3</td>
<td>U.S.</td>
<td>17.7</td>
<td>U.S.</td>
<td>30.3</td>
</tr>
<tr>
<td>4</td>
<td>Indonesia</td>
<td>8.4</td>
<td>Indonesia</td>
<td>21.3</td>
</tr>
<tr>
<td>5</td>
<td>Japan</td>
<td>6.8</td>
<td>Pakistan</td>
<td>13.9</td>
</tr>
<tr>
<td>6</td>
<td>Pakistan</td>
<td>5.2</td>
<td>Brazil</td>
<td>11.3</td>
</tr>
<tr>
<td>7</td>
<td>Russia</td>
<td>4.6</td>
<td>Bangladesh</td>
<td>11.1</td>
</tr>
<tr>
<td>8</td>
<td>Brazil</td>
<td>4.6</td>
<td>Japan</td>
<td>8.9</td>
</tr>
<tr>
<td>9</td>
<td>Italy</td>
<td>4.3</td>
<td>Philippines</td>
<td>7.8</td>
</tr>
<tr>
<td>10</td>
<td>Bangladesh</td>
<td>3.2</td>
<td>Egypt</td>
<td>6.7</td>
</tr>
</tbody>
</table>

The 10 countries (Wild, et al., 2004) estimated to have the highest numbers of people with diabetes in 2000 and 2030 are listed in Table 2.1. The 40-59 age group currently has the greatest number of persons with diabetes. By 2025, because of the aging of the world’s population, there will be 146 million aged 40-59 and 147 million aged 60
or older with diabetes. As estimated for both 2003 and 2025, females showed predominance in the number of persons with diabetes. The numbers were about 10% higher for females than males. In developing countries, the majority of people with diabetes are in the 45- to 64-year age range. In contrast, the majority of people with diabetes in developed countries are older than 64 years of age (Sicree, et al., 2003).

2.1.4 Pathophysiology and Major Risk Factors

Impaired insulin action and impaired pancreatic insulin secretion represent the principal pathophysiological abnormalities leading to increase blood glucose levels (Taylor, et al., 1994; Kahn & Rossetti, 1998). These are present to varying degrees in almost all patients with the common form of T2DM. Insulin resistance is a common pathologic state in which target cells fail to respond to the physiological effects of insulin occurring in peripheral organs and leading to abnormalities in glucose, lipid and protein metabolism (Kahn, 1994). When the target tissue does not respond to even high levels of insulin, glucose builds up in the blood resulting in high blood glucose or type 2 diabetes. In fact, insulin resistance is present in the majority of patients with impaired glucose tolerance or T2DM, and it is also found in up to 25 % of the general, apparently healthy population (Reaven, 1988).

Insulin secretion from pancreatic β-cells in the islets of Langerhans is in two-phases and is mainly regulated by glucose entry via its transporters. In nondiabetic subjects after glucose administration, the insulin secretion is abundant and rapid, lasting approximately 10 minutes, to compensate for an acute postprandial glucose peak and this state is defined as the first-phase response. The second-phase insulin secretion is the subsequent sustained increase in insulin secretion which is slower and lasts longer. In
T2DM, the first-phase secretion of insulin as a response to glucose is missing or considerably weakened (Pratley & Weyer, 2001). A secretory defect in the second phase is also characteristic of T2DM, and the ability of glucose to potentiate the effects of other stimulants of insulin secretion is diminished (Ward, et al., 1984; Roder, et al., 1998). In response to elevated blood glucose concentration due to insulin resistance, β-cells need to increase the insulin secretion to maintain homeostasis in glucose levels. Finally, β-cells become unresponsive to glucose due to pancreatic β-cells dysfunction and eventually type 2 diabetes develops. Although the etiology of the β-cell dysfunction of diabetes is incompletely understood, it is thought to result from both genetic and environmental factors (Kahn & Porte, 2001; Pratley & Weyer, 2001). Eriksson and co-workers (2001) have postulated that, among individuals with type 2 diabetes, roughly half of their disease risk can be attributed to environmental exposure and half to genetics.

Family history, diet, and lack of physical activity are all major risk factors for developing T2DM. Dyslipidemia and high blood pressure are other risk factors that often appear before the clinical disease is evident (Groop, et al., 1997). Elevated levels of free fatty acids are also a strong predictor of diabetes and correlate with hepatic glucose output, a major cause of diabetic hyperglycemia (Bergman Ader, 2000; Saltiel & Olefsky, 1996), high glucose levels, and obesity. Virtanen and Aro (1994) demonstrated in a case-control study that 60–80% of persons with T2DM are obese.

2.1.5 Type 2 Diabetes and Metabolic Syndrome

T2DM is responsible for the explosive increase in the prevalence of diabetes in many parts of the world (King, et al., 1993). A majority of patients with T2DM have features of the so-called metabolic syndrome, which has also been called “Syndrome X”
The syndrome has since gained a number of different names including Reaven’s syndrome, insulin resistance syndrome, metabolic syndrome, and cardiovascular risk syndrome. The name insulin resistance syndrome has been widely used and refers to insulin resistance as a common denominator of the syndrome (DeFronzo, et al., 1991; Haffner, et al., 1992). The major components of the metabolic syndrome include abdominal obesity, glucose intolerance/T2DM, dyslipidemia and hypertension (Hauner, 2002). Gerald Reaven suggested that insulin resistance and compensatory hyperinsulinemia underlie the clustering of cardiovascular risk factors like glucose intolerance, hypertension and dyslipidemia (Reaven, 1988). The prevalence of the metabolic syndrome has varied markedly between different studies, most likely because of the lack of accepted criteria for the definition of the syndrome (Bonora, et al., 1998; Rantala, et al., 1999). WHO proposed a unifying definition for the syndrome and chose to call it the metabolic syndrome, rather than the insulin resistance syndrome (Alberti & Zimmet, 1998). The main reason was that it was not established that insulin resistance is the cause of all the components of the syndrome.

### 2.2 Cardiovascular Disease

Cardiovascular disease (CVD) has for a long time been among the worldwide public health problem. Cardiovascular diseases are important causes of illness, disability, and death (Wenger, 1988; Murray & Lopez, 1996; Sans, et al., 1997). The incidence and prevalence of CVD increase markedly with age, and so does the prevalence of disability (Guralnik & Simonsick, 1993; Andersen-Ranberg, et al., 1999). It may therefore be assumed that the burden on the health-care system resulting from CVD and related disability will increase as the population ages. Thus, the burden of CVD on society
depends on the incidence and prevalence of CVD in each age group, the degree of
disability caused by CVD, and the age structure of the population. Although the problems
associated with CVD are severe in all parts of the world, the manifestations vary between
different countries. In China, Japan and many Africans countries for example, stroke is
more common than coronary heart disease whereas, among Caucasian populations,
coronary heart disease is more common. In some developed nations, such as the USA,
Australia and Europe, where coronary heart disease rates were previously very high,
mortality has fallen in recent decades (International Diabetes Federation, 2001).
However, in other areas such as Eastern Europe and the Middle East, the opposite is true.
The “top ten” countries for both coronary and cerebrovascular disease mortality rates are
now mainly from Eastern Europe and the former Soviet Union.

The clinical manifestations of CVD include coronary artery disease (CAD),
cerebrovascular disease, and peripheral vascular disease. The underlying disease
mechanism is accelerated atherosclerosis. The atherosclerotic process starts from fatty
streaks, consisting of intimal deposits of lipids and macrophages with lipid droplets (foam
cells), gradually developing into more advanced plaques. The process ends up in
complicated atherosclerotic lesions, which through a plaque rupture and thrombosis can
cause an acute myocardial infarction (Stary, et al., 1994; Stary, et al., 1995).
2.3 Type 2 Diabetic Complications

2.3.1 Pathogenesis and Major Risk Factors of Diabetic Complications

2.3.1.1 Hyperglycemia

A strong consistent relationship has been postulated between hyperglycemia and the incidence and progression of micro- and macrovascular complications in people with diabetes (Klein, 1995; Hanssen, 1997). Studies on nondiabetic subjects have observed that even slightly elevated serum glucose concentrations increase risk for cardiovascular disease (Laakso, 2000). Epidemiological data have revealed hyperglycemia to be a major player in the development of the macrovascular complications such as CAD and stroke (Laakso, 1999). Prospective clinical studies in T2DM patients have shown an association between level of hyperglycemia and increased risk for mortality due to macrovascular disease (Uusitupa, *et al*., 1993; Standl, *et al*., 1996; Lehto, *et al*., 1997). The San Antonio Heart Study demonstrated that hyperglycemia is a risk factor not only in Caucasians, but also in other ethnic groups (Wei, *et al*., 1998). The data of the UK prospective diabetes study (UKPDS) suggest that any improvement in glycemic control among patients with T2DM is likely to reduce the risk of diabetic complications (Stratton, *et al*., 2000).

2.3.1.2 Protein Kinase C

Protein kinase C (PKC) is a family of serine-threonine kinases that plays an important role in signal transduction mechanisms (Lee, *et al*., 1989; Ron & Kazanietz, 1999). The PKC pathway is activated in diabetes as a result of hyperglycemia (*Figure 2.1A*). In this pathway, PKC is activated by the increased amounts of diacylglycerol (DAG), which are synthesized directly from glycolytic intermediates such as dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (King, *et al*., 1997). It is
possible that also advanced glycation end products and oxidants increase formation of DAG and activate PKC (Koya & King, 1998). PKC appears to be activated in a range of diabetic tissues including the retina, kidney, heart, and aorta (Inoguchi, et al., 1992). An activation of PKC has been implicated in many processes relevant to diabetic complications, including regulation of vascular permeability and flow, increased production of cytokines, and increased synthesis of basement membranes (Giardino, et al., 1997). In diabetes microvascular complications, for example, PKC affects the activation of a number of growth factors and changes the function of vasoactive factors. These vasoactive factors include vasodilators such as nitric oxide (NO) as well as vasoconstrictors such as angiotensin II and endothelin-1 (Takagi, et al., 1996; Candido, et al., 2002).

**Figure 2.1**: Scheme represents protein kinase C and polyol pathways in the pathogenesis of diabetic complications. GAP, glyceraldehyde-3-phosphate; DAG, diacylglycerol; PKC, protein kinase C; AR, aldose reductase; SDH, sorbitol dehydrogenase.
2.3.1.3 Polyol Pathway

Only a small proportion of glucose is metabolized to sorbitol during normoglycemia, while in hyperglycemia the enzyme aldose reductase is activated, leading to an accumulation of intracellular sorbitol and fructose that increases the flux through the polyol pathway (Hawthorne, et al., 1989). Sorbitols and other polyols accumulate intracellularly, leading to osmotic damage and swelling. Aldose reductase (AR) is the first and rate-limiting enzyme of the polyol pathway, which converts monosaccharides (e.g. glucose) to their polyols or sugar alcohols (e.g. sorbitol) as seen in Figure 2.1B. This enzyme is widely distributed throughout the body, including those tissues that are susceptible to chronic diabetic complications (e.g. retina, lens, cornea, glomerulus, nervous system and the blood vessels) (Greene, et al., 1987; Harrison, et al., 1989). In fact, alterations in sorbitol and fructose metabolism are implicated as factors contributing to vascular complications in diabetes mellitus (Greene, et al., 1987).

2.3.1.4 Advanced Glycation End Products

Non-enzymatic glycation has recently attracted increasing interest as a crucial pathophysiologic event behind hyperglycemia-related alterations and in the pathophysiology of the development of diabetic complications. Proteins and lipids exposed to aldose sugars go through reactions, which are not enzyme-dependent, and generation of reversible Schiff bases or Amadori products take place. These early glycation products can serve as a starting point for further rearrangements, which ultimately lead to the formation of advanced glycation end products (AGEs), a heterogeneous mixture of complex structures (Harding, 1985).
The series of reactions producing AGEs occurs at a very slow rate under normal circumstances. This process also takes place during normal ageing, but in diabetes their formation is accelerated to an extent related to the level and duration of hyperglycemia. (Reiser, 1991; Vlassara, et al., 1994; Vlassara, 1997). The potential pathophysiological significance of AGEs is associated with their accumulation in plasma, cells and tissues and their contribution to the formation of cross-links, generation of reactive oxygen intermediates, and interactions with particular receptors on cellular surfaces (Schmidt, et al., 1996; Vlassara & Bucala, 1996). More details about AGEs will be discussed elsewhere in this chapter.

2.3.1.5 Genetic Factors

Despite the crucial role of hyperglycemia on diabetic complications, other factors such as hypertension, obesity, and hyperlipidemia undoubtedly contribute. There are patients with longstanding hyperglycemia without complications and patients with short duration of disease who seem to be very prone to complications. Genetic factors are obviously important as the prevalence is affected by the background population (WHO Multinational Study of Vascular Disease in Diabetics, 1985). An important variability in the incidence of diabetes-derived chronic complications exists, which may indicate the existence of a predetermined genetic susceptibility in its onset.

The majority of studies have focused their attention on the search for useful genetic markers for the detection of subjects with a greater risk of developing diabetic retinopathy or nephropathy. Diabetic nephropathy has been found to aggregate in families (Borch-Johnsen, et al., 1992; Fioretto, et al., 1999) and ethnic differences seem to exist in

2.3.2 How Big is the Problem?

Normally properly treated diabetes is symptomless, but continuing hyperglycemia seen in type 2 diabetes can give rise to chronic complications (United Kingdom Prospective Diabetes Study Group 33, 1998) including retinopathy, neuropathy and nephropathy (Vinik, et al., 2003), and macrovascular complications (Pyorala, et al., 1987), which have been related to premature mortality and morbidity (Uusitupa, et al., 1993). A common denominator for all microvascular and macrovascular complications is extensive vascular damage. Both of these conditions are life threatening and may result in an altered mental state, loss of consciousness, and possibly death; therefore prompt medical attention is necessary to avoid adverse outcomes. Microvascular complications comprise changes in the small blood vessels of the eye that result in diabetic retinopathy, in the peripheral nerves, causing neuropathy, and finally in the kidney, causing diabetic glomerulosclerosis or diabetic nephropathy. As a consequence, diabetes is the most common cause of blindness, end-stage renal disease (Creager, et al., 2003), and limb amputation (Beckman, et al., 2002).

In macrovascular complications, accelerated atherosclerosis results in cardiovascular disease (CVD) such as coronary heart disease (CHD) and acute myocardial infarction (AMI). Through its effects on cardiovascular disease (70-80% of people with diabetes die of cardiovascular disease), diabetes is also now one of the leading causes of death. While the pathogenesis of these complications has been extensively studied for the past 50 years, no single etiology exists to explain all types of
complications. Instead, multiple etiologies exist that are specific to each. The cost to care for patients with DM in the U.S. was approximately $132 billion. Of those costs, $40 billion was indirect medical expenses (disability, work loss, and premature deaths), and $92 billion dollars was direct medical expenses (those attributable to the disease itself, i.e. microvascular and macrovascular complications) (Centers for Disease Control and Prevention, 2003). These are the chronic complications that significantly impact the cost of health care. In fact, approximately 25% of the total Medicare budget is used for the treatment of DM and its complications (Finkelstein, et al., 2003; Finkelstein, et al., 2004).

2.3.3 Type 2 Diabetes and Microvascular Complications

2.3.3.1 Diabetic Retinopathy

Diabetes results in characteristic lesions in the retinal blood vessels. Diabetic retinopathy is still the most common cause of acquired blindness in the Western world (Klein, et al., 1992a). Its prevalence increases most steeply between 5 to 15 years of diabetes duration, being about 60% after 20 years in the European population. This can result in formation of microaneurysms (minimal retinopathy), haemorrhages and increased leakage, causing retinal edema and lipid exudates (background retinopathy). These changes do not threaten visual acuity unless they are located in the macular region, where they can cause macular edema. When pathological development of new vessels in the retina or abnormal blood vessels and fibrous tissue (i.e. neovascularisation), occurs, the retinopathy is classified as proliferative retinopathy (Aiello, et al., 1998). Microaneurysm may be reversible (Feman, 1994) even if a higher count is predictive of higher rates of proliferative retinopathy and macular oedema in the coming years (Klein, et al., 1995a). The formation of fibrous tissue may eventually cause retinal detachment.
and severe visual impairment (Forrester, et al., 1997). Also, an excess of glucose activates the polyol pathway, which causes accumulation of sorbitol in the lens and is accompanied by cataracts. (Kinoshita, et al., 1990; American Diabetes Association, 1998a). The etiology of retinopathy includes hyperglycemia-associated biochemical, anatomical and functional changes.

2.3.3.2 Diabetic Nephropathy

Diabetic nephropathy is estimated to develop in one third of both main types of diabetes (O'Bryan & Hostetter, 1997). Nephropathy is characterised by glomerular basement membrane thickening and arteriosclerosis of small arterioles. The mechanisms proposed to induce glomerulosclerosis include hyperglycemia, a hyperfiltration-related increase of glomerular pressure, and increased blood viscosity. The hallmark of renal damage in diabetes is increased excretion of albumin in the urine. The natural history of diabetic nephropathy has been viewed as a descending path from normoalbuminuria to microalbuminuria, clinically overt diabetic nephropathy; i.e macroalbuminuria, and eventually to end-stage renal disease. The term microalbuminuria; i.e. incipient diabetic nephropathy, has been defined as urine albumin excretion rate 20-200 µg/min in a timed overnight or 30-300mg/24h urine collection (Mogensen, et al.,1986) as determined by sensitive laboratory measurements. Urine albumin excretion rate exceeding these values is called macroalbuminuria and considered a sign of manifest diabetic nephropathy. It has been estimated that approximately half the patients with microalbuminuria will progress to overt nephropathy (Krolewski, et al., 1996). The rate of the development of diabetic nephropathy varies in individual patients (Viberti, et al., 1982) and improvement in glycemic control may result in the disappearance of microalbuminuria (Bojestig, et al.,
Along with macroalbuminuria the glomerular filtration rate falls consistently. Nephropathy may culminate in uremia and, in fact, most of the hemodialysis patients and the patients receiving renal transplants have diabetes. (Friedman, 1990; Nelson, et al., 1995; American Diabetes Association, 1998b).

### 2.3.3.3 Diabetic Neuropathy

The term diabetic neuropathy includes either a clinical or subclinical disorder without any additional causes of peripheral neuropathy other than diabetes (Report and recommendations of the San Antonio conference on diabetic neuropathy, 1988). In fact, damage to the microvasculature in peripheral nerves is now becoming recognized as a major pathogenic factor in diabetic neuropathy (Dyck, et al., 1985; Vinik, et al., 1992). It may affect both sensory and autonomic nerves, but distal symmetric polyneuropathy is probably the most common consequence which, together with peripheral vascular disease, is an important etiologic factor for foot ulcerations and lower limb amputations. Autonomic dysfunction is common in people with diabetes, but is only clinically apparent in a small percentage. Diabetic neuropathy is encountered in about half of all people with diabetes either as a polyneuropathy or mononeuropathy (Sheetz & King, 2002; Dyck, et al., 1993) especially in patients over 60 years age with T2DM (King’s Fund, 1996). Although exact prevalence depends on the diagnostic criteria used to identify neuropathy, most studies suggest that 50% of patients with a 20-years history of either type 1 or type 2 diabetes have neuropathy (Feldman, et al., 2001; Apfel, 1999). Around 10% of these cases of neuropathy are associated with abnormal sensations and pain (Calcutt, 2002). The incidence of neuropathy increases with duration of diabetes and is accelerated by
poor control (Feldman, 2002). Additionally, the death rate is as high as 50% at three years after diagnosis of overt autonomic neuropathy (King’s Fund, 1996).

2.3.4 Type 2 Diabetes and Macrovascular Complications

Macrovascular complications of DM are due to accelerated atherosclerosis and have an important role in the increased morbidity and mortality suffered by these individuals (Wingard & Barrett-Connor, 1995). People with diabetes are at a high risk of suffering from cerebrovascular disease, peripheral vascular disease and cardiovascular complications such as myocardial infarction, coronary heart failure, and stroke (Cooper, et al., 1997; McMillan, 1997; Stehouwer, et al., 1997) and these chronic illnesses take 10–20 years to manifest. Observations from the United Kingdom Prospective Diabetes Study (UKPDS, 1998a) demonstrated that the 10-year risk for macrovascular complications is more than four-fold higher than the risk for microvascular complications. However, patients with T2DM are more at risk to develop macrovascular disease, both at an earlier age and in a higher frequency as compared to the general non-diabetic population.

The presence of diabetes, in addition to any or all the other risk factors (such as smoking, hypertension, dyslipidemia, and genetic factors), approximately doubles the probability of developing macrovascular diseases and the available evidence suggests that strict diabetic control does not prevent or delay these complications (Davidson, 1998). Coronary heart disease, cerebrovascular accidents and peripheral vascular disease are the most important complications in T2DM. They are responsible for at least 50-60% of all deaths in subjects with type 2 diabetes (Panzram, 1987; Pyorala, et al., 1987). Cardiovascular disease accounts for about 70% of all deaths in patients with diabetes.
Epidemiological studies show that the risk of cardiovascular mortality is two to three times higher in men and three to five times higher in women with diabetes than in non-diabetic subjects (Kannel, 1979; Barrett-Connor, et al., 1991; Stamler, et al., 1993). Cardiovascular complications are often present already at the time of diagnosis of T2DM (Pyorala, et al., 1987). Subjects with impaired glucose tolerance (IGT) have an approximately two-fold increase in the risk of macrovascular diseases (Pyorala, et al., 1987).

2.4 Type 2 diabetes and Coronary Artery Disease

2.4.1 Coronary Artery Disease: General Overview

The term coronary artery disease (CAD) refers to the consequences of oxygen deficiency in the myocardium caused by the decrease or complete interruption of the blood supply, generally originating from reduced blood flow from coronary arteries and usually caused by atherosclerotic changes (Miles, et al., 1990). CAD is the major cause of mortality and morbidity in the industrialized world (Braunwald, 1997). Atherosclerosis is a process starting early in life, slowly and silently progressing for decades (Ross, 1999).

Vascular injury and thrombus formation are key events in the origin and progression of atherosclerosis. The process of athereogenesis was previously considered by many to consist mainly of lipid accumulation within the artery wall. Other processes, as inflammation, are also involved (Ross, 1999). CAD, the most important manifestation of CVD, represents a wide spectrum from angina pectoris, myocardial infarction and sudden death to silent myocardial ischemia (Naka, et al., 1992). The risk for developing CAD depends on multiple factors and each factor influences the risk over a wide range (Fraser, 1986; Kannel & McGee, 1987). The multivariate synergistic nature of risk factors
implies that minor elevations of several risk factors will cause important elevations in the risk of CAD (Fraser, 1986; Kannel & McGee, 1987). On the other hand, inappropriate emphasis on a single elevated risk factor may lead to undue identification of people at low risk (Hulley, et al., 1992).

### 2.4.2 Type 2 Diabetes and the Risk of Coronary Artery Disease

It has been suggested that type 2 diabetes be considered as “a state of premature cardiovascular death which is associated with chronic hyperglycemia and may also be associated with blindness and renal failure” (Fisher, 1998). Diabetes predisposes to CVD in a number of ways. People with diabetes are at increased risk of atherosclerosis, and, to make matters worse, atherosclerosis in people with diabetes is accelerated in development, more widespread and more severe. The same traditional risk factors for CVD are operative in type 2 diabetic as in non-diabetic individuals. However, the effect of any given risk factor on the incidence of CVD is greater in diabetic than non-diabetic populations (Stamler, et al., 1993). One of the major vascular beds where atherosclerosis clinically manifests is the coronary arteries (Beckman, et al., 2002) (Figure 2.2). The risk for subsequent coronary events in type 2 diabetic patients is equally high as in non-diabetic subjects with previous myocardial infarction (Haffner, et al., 1998). Diabetic patients with myocardial infarction also have a worse prognosis than non-diabetic patients with myocardial infarction (Abbott, et al., 1988; Miettinen, et al., 1998).

T2DM and CAD share several important characteristics (Pyorala, et al., 1987). Both conditions become more prevalent with age, both are associated with obesity, an abnormal serum lipid profile and a sedentary lifestyle. Both conditions are insulin resistant states associated with atherosclerosis (Howard, et al., 1996). However, insulin
resistance is the common denominator in the cluster of abnormalities, which together comprise the insulin resistance syndrome or metabolic syndrome X (Defronzo & Ferrannini 1991; Reaven & Laws, 1994). Having the insulin resistance syndrome implies a higher risk for CAD (Kuusisto, et al., 2001). Therefore, T2DM has been defined as a coronary artery disease risk equivalent by the Adult Treatment Panel III of the National Cholesterol Education Program (NCEP) (Adult Treatment Panel III, 2001).

\[\text{Figure 2.2: A Schematic model of an atherosclerotic lesion in the coronary artery.}\]

2.4.3 **Epidemiology in Type 2 Diabetes**

Approximately 17 million people in the United States or 6.2% of the population are diagnosed as having diabetes mellitus (Cowie, et al., 2003). The risks of developing coronary artery disease (Fox, et al., 2004) as well as long-term mortality as a result of CAD are higher in individuals with diabetes than in those without diabetes (Chyun, et al.,
For example, a recent meta-analysis showed that the rate of fatal CAD is higher in diabetic patients than in non-diabetic individuals (5.4 vs. 1.6%) (Huxley, et al., 2006). In fact, coronary artery disease is the major cause of mortality in type 2 diabetes. The overall prevalence of CAD, detected by a variety of diagnostic methods, is reported to be as high as 55% in individuals with diabetes compared with 2% to 4% in the general population (Hammoud, et al., 2000). CAD is not only more prevalent in diabetic patients compared with the rest of the population but tends to be more extensive, involving multiple vessels and is rapidly progressive (Cariou, et al., 2000). About 70% of deaths among type 2 diabetics result from CAD (Gu, et al., 1998). Compared with CAD in nondiabetic persons, CAD in patients with diabetes is more advanced at diagnosis and is generally characterized by more extensive atherosclerosis and with higher rates of left ventricular dysfunction and cardiac events (Hammoud, et al., 2000; Giri, et al., 2002).

Mortality from cardiovascular disease and the incidence of non-fatal coronary artery disease is 2 to 4 times higher in patients with T2DM than in non-diabetic subjects even after adjusting for the classic risk factors (Haffner, et al., 1998; Kannel & McGee, 1979; Pyorala, et al., 1987). Diabetic women have even a higher risk for CAD than diabetic men when compared to non-diabetic counterparts (Haffner, et al., 1997; Laakso, et al., 1995). Since we are facing a dramatic, worldwide increase in the incidence of type 2 diabetes (Zimmet, et al., 2001), the cost for healthcare is high and increasing (Massi-Benedetti, 2002). The complications associated with T2DM account for the majority of these expenditures and the cardiovascular complications make significant contribution to the cost of diabetes care (American Diabetes Association, 1998c).
2.4.4 Pathophysiology of CAD in Type 2 Diabetes

2.4.4.1 Vascular Endothelium Disturbances

Links between endothelial dysfunction, atherosclerosis and diabetes have been increasingly recognized. Atherosclerosis in the coronary arteries of diabetic patients is a complex process that includes a series of changes affecting the endothelium, smooth muscle cells and platelets (Beckman, et al., 2002). One of the earliest discernible atherogenic changes in diabetes is endothelial dysfunction, which is characterized by inhibited vasodilation, vascular smooth-muscle proliferation, increased thrombogenesis and proatherogenic cellular processes (Glasser, et al., 1996). One of the functions of the endothelium is the synthesis of nitric oxide (NO) which is responsible for the endothelial vascular relaxation, vasodilatation, and inhibition of platelet adhesion. Production of reactive oxygen species is increased due to inhibition of the production of nitric oxide by insulin resistance, hyperglycemia and elevated free fatty acid (FFA) levels in T2DM (De Vriese, et al., 2000) leading to oxidative stress (Inoguchi, et al., 2000) and a hypercoagulable state.

Accelerated atherosclerosis, thrombosis, hypertension and hyperlipidemia all participate in the pathogenesis of vascular disease in patients with diabetes, and endothelial dysfunction is probably involved in each of these vascular abnormalities (Cohen, 1993). It is well known that vascular endothelium plays a key role in the balance between the coagulation and fibrinolytic systems. Type 2 diabetic patients have increased platelet surface expression of glycoprotein Ib, mediating binding to von Willebrand factor, and glycoprotein IIb/IIIa, mediating platelet-fibrin interaction (Vinik, et al., 2001). Moreover, the levels of regulators of fibrinolysis and fibrinolytic activity, tissue plasminogen activator and plasminogen activator inhibitor type 1, are increased in
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patients with type 2 diabetes (Vague & Juhan-Vague, 1997). Hyperglycemia causes glycosylation of virtually all proteins, inducing collagen cross-linking with other extracellular matrix proteins in the arterial wall (Haffner, 1998). Long-term exposure to elevated glucose levels alone can cause the endothelial cell dysfunction observed in diabetes.

2.4.4.2 Atherogenic Dyslipidemia

Dyslipidemias are disorders of lipid metabolism that results in lipoprotein imbalances that are characterized by elevations in serum total cholesterol, low density lipoprotein (LDL), and triacylglycerol, as well as a decrease in high density lipoprotein (HDL) concentrations. Researchers have demonstrated that overweight men and women have a common pattern of dyslipidemia characterized by elevated triglycerides, elevated LDL cholesterol and low HDL levels (Denke, et al., 1994; Sundquis, et al., 2001; Denke, 2001). Dyslipidemia has predicted the incidence of T2DM in several studies (Haffner, et al., 1990; McPhillips, et al., 1990; Perry, et al., 1995). A number of recent clinical trials have also documented the benefits of lipid-lowering therapies in diabetic patients. Insulin resistance and compensatory hyperinsulinemia contribute to hyperglycemia in T2DM and may play a pathophysiologic role in a variety of other metabolic abnormalities including dyslipidemia, hypertension, abnormal fibrinolysis, and coronary heart disease (Adult Treatment Panel III, 2001; Steinberg, et al., 2000). This diabetic dyslipidemia independently correlates with increased CVD risk (Garg, 1997).

Dyslipidemia is generally a clinically symptomless condition, which over time contributes to the development of atherosclerosis or hardening of the walls and narrowing of the lumen of the blood vessels. Atherosclerosis is a major cause of cardiovascular
disease that begins early in life and progresses silently for decades. Although the classical diabetic dyslipidemia is characterized by high serum triglyceride levels, low levels of HDL-C, and an increased number of small, dense LDL particles, there are additional lipid abnormalities as well (Taskinen & Smith, 1998; Garg, 1998). LDL cholesterol is the major cholesterol-rich lipoprotein that mediates the link between serum cholesterol and atherosclerosis. This interrelated group of lipoprotein abnormalities, collectively known as “atherogenic dyslipidemia”, predisposes patients to develop CAD. The risk of heart disease associated with these lipoprotein abnormalities equals or exceeds the risk from an LDL cholesterol concentration of 150 to 220 mg/dL (3.90 to 5.70 mmol/L) (Grundy, 1997). Type 2 diabetic patients often have key elements of this condition.

Atherosclerosis starts with extracellular lipid accumulation, where lipoprotein particles accumulate in the intima of the coronary arteries because of the atherogenic-rich diet (Kruth, 1997a). Monocytes then enter the area, moving towards the artery endothelium and intima and begin to transform into foam cells by absorbing lipids (Gimbrone, et al., 1995). Later, smooth muscle cells migrate into intimal lesions and strengthen the atheroma (Stary, et al., 1995). Diabetic patients have abnormal vascular smooth muscle cell function due to impaired nitric oxide-mediated vasodilatation and increased endothelin-1. Smooth muscle cells are stimulated by growth factors and this increases the production of collagen, which is an important substance in the plaque formation (Amento, et al., 1991). The lipid core forms a cellular mass within the collagen matrix of the plaque. After foam cell death, the lipid core of plaques prone to rupture has a high concentration of cholesteryl esters (CE) with a high proportion of polyunsaturated fatty acids leading to rapid changes in severity of stenosis and resulting in subtotal or total vessel occlusion.
2.4.4.3 Oxidative Stress

Oxidative stress has been defined as an imbalance between pro-oxidants (free radicals and other reactive species) and antioxidants in favor of the former leading to potential damage (Steinberg & Baron, 2002; Halliwell, 1997; Sies, 1997; Betteridge, 2000). This imbalance can be due to depletion of endogenous antioxidants, low dietary intake of antioxidants and/or increased formation of free radicals and other reactive species. Diabetes causes increased oxidative stress which can lead to increased production of reactive oxygen species (ROS) as well as an impaired endogenous capacity to scavenge free radicals (van Dam, et al., 1995; Baynes, 1991; Rosen, et al., 2001; Sundaram, et al., 1996). A higher production of ROS leads to increased peroxidation of lipid membranes, proteins and DNA with important consequences for cell structure and function. A notable target for increased pro-oxidant activity in diabetes is the vascular system which is in part explaining the increased propensity for atherogenesis and cardiovascular disease (Cameron, 1999).

In addition to its critical role in the modulation of vascular tone, oxidative stress is also related to cellular growth, hypertrophy, remodeling, inflammation, and lipid oxidation, (Alexander, 1995; Kunsch & Medford, 1999; Griendling, et al., 2000). ROS can act as intracellular signaling molecules in vascular cells controlling growth, survival, and apoptosis (Mervaala, et al., 2000). The specific response is dictated by the intracellular targets involved. Hyperglycemia causes oxidative stress not only due to increased production of mitochondrial ROS (Brownlee, 2001), but also due to glucose autooxidation (Wolff, et al., 1991), and non-enzymatic glycation of proteins which alters their structure and function. (Brownlee, 2000). These altered proteins, advanced glycation
end products, accumulate in patients with chronic glucose levels elevations (Brownlee, 1995). AGEs contribute to diabetic vasculopathy (Schmidt, et al., 1994) and initiate transcriptional activation of vascular cell adhesion molecule-1 (VCAM-1) (Marui, et al., 1993) and monocyte chemotactic protein-1 (MCP-1), which promotes monocyte entry into the vessel wall (Gimbrone, 1995). The pathogenesis of atherosclerosis also involves oxidation of low density lipoprotein cholesterol (Witztum, 1994). Exposure to glycation end products can prolong the half-life of LDL cholesterol, increasing the likelihood that it will be trapped in the vascular wall where it is more susceptible to oxidation (Haffner, 1998).

### 2.4.5 Major Risk Factors

#### 2.4.5.1 Hypertension

The standard definition of hypertension is a blood pressure ≥140/90 mmHg (Joint National Committee VI, 1997). Hypertension is a major conventional risk factor for CHD (Stamler, et al., 1993) and successful treatment with antihypertensive drugs reduces the incidence of CHD (Insua, et al., 1994). Up to 70% of adults with type 2 diabetes have raised blood pressure (Cowie & Harris, 1995). Together, hypertension and overt diabetes double the risk of cardiovascular disease (Grundy, et al., 1999; Joint National Committee VI, 1997). Raised blood pressure is more common in people with T2DM than in the general population (Manson, et al., 1991; Barrett-Connor, et al., 1991; Hypertension in Diabetes Study Group, 1993). For example, an epidemiological analysis of the UKPDS Group data demonstrated that, with each 10 mm Hg increase in systolic blood pressure, the risk of a person with type 2 diabetes sustaining a myocardial infarction was increased by 11% (Adler, et al., 2000). In addition to assessing the effect of improving blood
glucose control in people with T2DM, the UKPDS also compared the effects of tight blood pressure control to poor blood pressure control (UKPDS, 1998b; UKPDS, 1998c).

2.4.5.2 Smoking

Smoking is a major conventional risk factor for CHD (Stamler, et al., 1993). Smoking increases the risk of coronary heart disease and stroke in people with diabetes and also increases their overall mortality (UKPDS, 1991; Yudkin, 1993). People with diabetes who stop smoking can substantially reduce their risk of developing complications (Chaturvedi, et al., 1997; Haire-Joshu, 1991). Indeed, stopping smoking is one of the most effective ways of reducing the risk of developing the long-term complications of diabetes, particularly cardiovascular disease.

2.4.5.3 Low Density Lipoprotein: the Major Player

In type 2 diabetes lipid abnormalities are almost the rule. LDL cholesterol is a well-known risk factor for coronary heart disease and is now recognized as the primary target of lipid lowering therapy (Adult Treatment Panel III, 2001). Elevated LDL cholesterol has been associated with CHD in follow-up studies (Turner, et al., 1998; Forsblom, et al., 1998). Increased LDL cholesterol predicts CHD in patients without macroangiopathy at baseline indicating that elevated LDL cholesterol becomes important after exclusion of high-risk patients with CHD at baseline. More than 70% of people with type 2 diabetes have raised LDL cholesterol levels (Mykkänen, et al., 1993). The risk of a cardiovascular event increases as the level LDL cholesterol level increases. In the UKPDS, the risk of either angina or a myocardial infarction in people with type 2 diabetes increased 1.57 fold for every 1 mmol/l increase in LDL cholesterol. Those with
an LDL cholesterol > 3.89 mmol/l were 2.3 times more likely to develop angina or a myocardial infarction than people with an LDL cholesterol < 3 mmol/l (Turner, *et al*., 1998). People with diabetes also tend to have a combination of raised triglyceride levels and decreased high density lipoprotein (HDL) cholesterol levels, both of which are also risk factors for cardiovascular disease (Dean & Durrington, 1996).

2.5  **LDL Metabolism, Glycoxidation and Diabetic Complications**

2.5.1  **Overview of LDL and Cholesterol Metabolism**

2.5.1.1  **Low Density Lipoprotein Particles Synthesis**

Fat absorbed from the diet and lipids synthesized by the liver and adipose tissue will be transported to be utilized and stored in various tissues. As lipids are insoluble in water, they are transported in plasma as lipoproteins. Lipoprotein particles contain a central core of non-polar lipids, mainly triglycerides and cholesteryl esters, and a surface monolayer of polar lipids, mainly phospholipids, apolipoproteins and free cholesterol. LDL is mainly formed as an end product of very low density lipoprotein (VLDL) metabolism (Sigurdsson, *et al*., 1975; Thompson, *et al*., 1987; Demant, *et al*., 1996) and it contains predominantly cholesterol esters added to small amounts of triglycerides, phospholipids and free cholesterol. When VLDL is hydrolyzed by lipoprotein lipase, smaller VLDL remnants, intermediate density lipoprotein (IDL) particles are generated and surface lipids and apolipoproteins are transferred to HDL as in hydrolysis of chylomicrons. Remnant particles are partially removed by the liver via receptor-mediated pathway, or IDL is further converted to LDL by hepatic lipase.

Triglyceride-rich VLDL particles are synthesized in the liver and they transport triglycerides to adipose tissue and muscles. LDL production rate depends both on the rate
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at which VLDL is produced and the rate at which VLDL remnants and IDL are removed from the circulation via LDL receptors, and also on the lipolytic activity in the transformation of VLDL and IDL into LDL. In addition, some LDL can be directly secreted by hepatocytes, especially in hyperlipidemic states (Janus, et al., 1980; Kissebah, et al., 1984; Fisher, et al., 1994; Gaw, et al., 1995), but also in normolipidemia (Cohn, et al., 1990). Apolipoprotein B-100 (apoB-100), the exclusive apolipoprotein in the LDL particle, is produced by the liver and secreted in VLDL and availability of lipids regulates whether apoB is secreted or degraded. Moreover, production of apoB is correlated with LDL cholesterol level. In the percent mass composition, each LDL particle consists of 20-25% protein, 7-10% free cholesterol, 35-45% cholesterol ester, 7-10% triglycerides, and 15-20% phospholipids (Deckelbaum, et al., 1987; Schultz & Liebman, 1997). ApoB-100 is a large (513 kDa), single chain glycoprotein composed of 4536 amino acid residues with a coding gene residing on the short arm of chromosome 2 (Knott, et al., 1986; Yang, et al., 1986). There is only one apoB-100 molecule in each LDL particle (Tikkanen & Schonfeld, 1985; Cladaras, et al., 1986).

2.5.1.2 Receptor Mediated Uptake of LDL

Low density lipoprotein, normally accounting for about two-thirds of plasma total cholesterol, is the most abundant cholesterol-carrying lipoprotein in the circulation and plays the key roles in the cholesterol transfer and metabolism. LDL is cleared from the circulation mainly by the specific LDL receptor (Goldstein, et al., 1995; Kesaniemi, et al., 1983), which is strictly regulated by the in-flowing cholesterol and therefore does not generally lead to intracellular accumulation of cholesterol. The LDL receptor, a single-chain transmembrane glycoprotein highly expressed in the liver, is located on the surface
of hepatocytes and nearly all normal cells where the uptake of plasma LDL provides cholesterol for membrane synthesis and other requirements of these cells.

LDL can pass through the junctions between capillary endothelial cells and bind to LDL receptor on cell membranes that recognize apoB-100 (Marshall, 1995). The strong binding interaction between LDL apoB and the LDL receptor is responsible for the receptor-mediated uptake and clearance of LDL from the circulation. The liver plays a crucial role in this process: about 75% of the LDL particles removed from the circulation are mediated by the liver (Brown & Goldstein, 1986). Of these, 75% of the clearance is LDL receptor-mediated and the remainder is by a nonspecific, receptor-independent low affinity process (Pittman, et al., 1982; Billheimer, et al., 1984).

Following the binding of LDL to its receptors, the receptor-lipoprotein complex is internalized by endocytosis and LDL dissociates from the receptor, which returns to the cell surface and is again able to bind lipoproteins or alternatively, degraded to prevent further influx. After internalization, LDL is transported to the lysosome where its cholesterol esters are converted to free cholesterol by acid lipase and apolipoproteins are degraded to amino acids. The liberated cholesterol is then used by the cell for the synthesis of plasma membranes, bile acids, and steroid hormones, or stored in the ester form. The synthesis of LDL receptors in the cell is suppressed by the increased intracellular cholesterol levels derived from LDL, and this phenomenon regulates the amount of cholesterol entering the cell (Brown, et al., 1981; Goldstein, et al., 1995). Subsequently, the number of synthesized LDL receptors decreases when the cellular cholesterol content increases and vice versa. The defects of LDL receptor and its function cause familial hypercholesterolemia, a genetic disorder in which the LDL receptor
activity is reduced either because of a reduced number of LDL receptors or formation of structurally altered LDL receptors (Brown & Goldstein, 1986).

2.5.1.3 Intracellular Cholesterol Metabolism

Cholesterol is essential for homeostasis and requires lipoproteins for *in vivo* transportation. Cells require cholesterol in their membranes for fluidity, and cholesterol is also used for steroid hormone and bile production. The properties of cholesterol that make it localize in cell membranes, which includes its insolubility, makes it problematic to transport about the body and when it accumulates in the body in places such as in the artery wall it cannot be removed easily (Brown & Goldstein, 1986). Cells can obtain cholesterol either by de novo synthesis in the endoplasmic reticulum or from exogenous sources, usually in the form of plasma-derived lipoproteins depending on the availability of exogenous cholesterol and the cell type. Most cell types are able to synthesize cholesterol themselves. Some cell types (for example hepatic cells and macrophages) have specialized in the uptake of lipoproteins, (Simons & Ikonen, 2000). On the other hand, central nervous system relies practically solely on de novo synthesis for cholesterol supply (Dietschy & Turley, 2001).

Since too much free cholesterol is toxic to the cells (Tabas, 2002), the cellular levels of both free and esterified cholesterol are under tight regulation. The primary mode of regulation is the control of expression of LDL receptors and the rate limiting enzyme in endogenous cholesterol biosynthesis; 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (Brown & Goldstein, 1986). Therefore, the cell can control cholesterol content by lowering the LDL uptake by its receptor and consequently results in elevated plasma LDL cholesterol levels and accumulation of cholesterol in peripheral cells. Free
cholesterol can also activate the cholesterol esterifying enzyme acyl-Coenzyme A: cholesterol acyltransferase (ACAT).

Excess of cellular cholesterol, for example after high intake of cholesterol and dietary saturated fat, is stored in the cytoplasmic lipid droplets as cholesteryl ester (Goldstein, *et al.*, 1974) or effluxed out of the cell to extracellular acceptors such as LDL receptors. Cholesterol excess also induces suppression of HMG-CoA reductase and cellular cholesterol biosynthesis is decreased. Conversely, cholesterol-lowering treatments with bile acid-binding resins or statins, inhibitors of HMG-CoA reductase, and other causes of low cellular cholesterol stimulate the expression of hepatic LDL receptors and decrease plasma LDL cholesterol levels. As cells are not able to break down cholesterol, only the liver can remove cholesterol from the circulation, by converting it to bile acids. Thus, continuous overloading of cells with cholesterol may result in exceeding their capacity to handle it. One very common and very dangerous example of such failure is atherosclerosis (Tabas, 2002).

### 2.5.2 Advanced Glycation End Products

#### 2.5.2.1 Synthesis and Function

The glycation process occurs when glucose and other circulating reducing sugars react non-enzymatically with the amino groups of macromolecules including proteins, lipids and nucleic acids (Bucala, *et al.*, 1994). The extent of this spontaneous reaction is dependent on the duration of exposure to the modifying species (Zeng & Davies, 2005). Glycation is a natural biochemical event occurring as a consequence of the glucose-rich milieu that constantly permeates living tissues. As shown in *Figure 2.3*, reducing sugars such as glucose and fructose form a Schiff base linkage by covalent binding to free amino
groups of the proteins (Watanabe, 1992). These aldehyde groups of free sugars react with free amino groups of proteins. This reaction further undergoes various rearrangements and free radical mediated oxidation to generate a group of adducts collectively known as advanced glycation end products (Avigad, et al., 1996; Lal, et al., 1996). The rearrangement of the Schiff bases gives rise to Amadori products, such as fructose-lysine. The Amadori product subsequently degrades into α-keto aldehyde compounds. Early glycated products are still in equilibrium with plasma glucose, but when glucose levels fall, they can dissociate to the native proteins. Alternatively, if glycation continues, further molecular rearrangements (glycation, glycoxidation, and autooxidative glycosylation) occur to generate the irreversible, heterogeneous crosslinks as well as chromo/fluorophoric adducts called Maillard products or AGEs (Friedman, 1999). Glycation is a common post-translational modification of proteins.

![Figure 2.3](image_url)

**Figure 2.3:** Scheme of advanced glycation end products formation. Equilibrium levels of the reversible Schiff base and Amadori products are reached within hours and days, respectively. AGEs form over a longer period of time but remain irreversibly bound to amino groups. R, amino acid or lipid backbone; AFGP, antifreeze glycoprotein; CML, Nε-(carboxymethyl)lysine. (Adopted from Bierhaus, et al., 1998)
2.5.2.2 AGEs and Diabetic Macroangiopathy

There is considerable evidence linking hyperglycemia, the most obvious metabolic abnormality in diabetes, with enhanced (non-enzymatic) formation of irreversible AGEs (van Boekel, 1991; Brownlee, 1995; Lalla, et al., 1998; Nawroth, et al., 1999). AGEs have been implicated in most complications of diabetes (Vlassara & Palace, 2002). In diabetic patients increased AGEs levels have been found in many tissues including dermal connective tissue, small blood capillary walls and vessel walls of arterioles and arteries (Schleicher, et al., 1997; Makita, et al., 1991). AGEs were identified in mesenteric vessels of streptozotocin-treated diabetic rats within 3 weeks (Rumble, et al., 1997) and in their skeletal muscle arteries within 4-6 weeks of diabetes induction (Hill & Ege, 1994).

It was postulated that AGEs contribute to the development of vascular diseases associated with diabetes (Chibber, et al., 1997; Makita, et al., 1995; Wautier & Guillausseau, 1998). Vlassara, et al. (1992) administered AGE-modified albumin to healthy nondiabetic rats and rabbits. After 2-4 weeks of AGE-administration, animals displayed diabetes-like vascular complications: a significant increase in vascular permeability, significant mononuclear cell migration in subendothelial and periarteriolar spaces and a defective endothelium-dependent and -independent vasodilatation. Moreover, it has been shown in diabetic rats that some AGEs were found in the aortic collagen after 4 and 12 weeks but this was significantly increased by 20 weeks (Turk, et al., 1999).

Advanced glycation end products formation is a slow process under normal ambient sugar concentrations, but is enhanced in the presence of hyperglycemia in diabetes. The formation, deposition and accumulation of AGEs are irreversible processes
In addition to hyperglycemia, AGE formation is enhanced by hyperlipidemia in atherosclerosis, and by oxidative stress in chronic diseases, inflammation, neurodegenerative disorders such as Alzheimer’s disease, and renal failure and under conditions where the turnover of lipids and proteins is prolonged. AGE products accumulate in plasma (Ahmed & Thornalley, 2003), body fluids, cells, and tissues. In the cardiovascular system, AGEs accumulation contributes to arterial stiffening, myocardial relaxation abnormalities, atherosclerotic plaque formation, and endothelial dysfunction. Physiological AGEs in blood plasma had high renal clearances in normal healthy subjects (Ahmed & Thornalley, 2003).

Immunohistochemical studies (Horiuchi, et al., 1991) of human atherosclerotic lesions have demonstrated intracellular AGEs deposition in smooth muscle cells-derived foam cells in fatty streak and atherosclerotic plaques in human aorta. Significant extracellular AGEs accumulation was also observed in advanced lesions (Kume, et al., 1995). AGEs formation on the extracellular matrix component of the vessel wall can cause structural damage by decreasing elasticity, increasing thickness, rigidity, and narrowing of the vessel lumen (Bierhaus, et al., 1998; Turk, et al., 1999). Table 2.2 summarizes some of the pathogenic effects of AGEs relevant in diabetic vascular dysfunction. Airaksinen and co-workers have showed that the diminished arterial elasticity in humans with diabetes was related to the enhanced AGEs formation (Airaksinen, et al., 1993). AGEs increase collagen cross-linking leading to the arterial stiffness that is commonly observed in normal ageing but at an accelerated rate in diabetes (Odetti, et al., 2000; Wolffenbuttel, et al., 1998).

As reported by Bucala, et al. (1991), AGEs formed on vascular matrix proteins mediate defective endothelium-dependent vasodilatation by quenching nitric oxide. The
above mentioned alterations, seen specifically in diabetes where prolonged hyperglycemia is the major trigger for the accelerated formation of AGEs (Brownlee, 1995), have been observed to cause changes in the function and/or structure of almost all cells and tissues explored. The extent of these alterations was found to be dependent on the level and duration of the abnormal metabolic state. Some of the changes are reversible and return to normal when the glucose balance is corrected. Some, however, turn out to be irreversible and seem to accumulate, especially in tissues with a long half-life.

Commonly found epitopes in AGEs include $N^ε$-(carboxymethyl)lysine (CML), pentosidine and pyralline (Singh, et al., 2001; Bucala & Vlassara 1995). It seems that CML is the major epitope of AGEs which is recognized by experimentally induced polyclonal CML-antibodies (Ikeda, et al., 1996). Recently, the concept of AGEs has widened considerably.

**Table 2.2: Pathogenic effects of AGEs in the Macrovasculature**

<table>
<thead>
<tr>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Increased vascular matrix formation and narrowing of the vessel lumen</td>
<td>Rumble, <em>et al.</em>, 1997</td>
</tr>
<tr>
<td>- Increased basement membrane thickening</td>
<td>Brownlee, <em>et al.</em>, 1988</td>
</tr>
<tr>
<td>- Increased endothelial permeability</td>
<td>Esposito, <em>et al.</em>, 1989</td>
</tr>
<tr>
<td>- Induction of smooth muscle cell proliferation</td>
<td>Hogan, <em>et al.</em>, 1992</td>
</tr>
<tr>
<td>- Induction of fibroblast proliferation</td>
<td>Vlassara, <em>et al.</em>, 1994</td>
</tr>
<tr>
<td>- Increased oxidative stress</td>
<td>Dobrian, <em>et al.</em>, 1996</td>
</tr>
</tbody>
</table>
2.5.2.3 Role of AGEs Receptors

In view of the close association of AGEs with cells in the body and their known adverse effects on cells, many studies have focused on searching for AGE binding proteins. Binding and internalization of AGE-modified proteins is facilitated through several AGE-specific cell surface receptors (Li, et al., 1998; Schmidt & Stern, 2000). This interaction results in oxidant stress of the target cells, inducing production of different patterns of cytokines and growth factors, depending on the type of cells involved. Excess production of growth factors and cytokines plays an essential role in both micro- and macrovascular alterations (Witztum, 1997).

Normally, AGE-modified proteins are either repaired or replaced and degraded in vivo. The recognition and degradation of such proteins is mediated by cellular receptors for AGEs (RAGEs), which are present on certain critical target cells such as monocytes, macrophages, endothelial cells, mesangial cells and fibroblasts (Vlassara, et al., 1994; Lamster & Lalla, 2001). Interaction of AGE with RAGE on macrophages stimulates these cells to produce and release cytokines, growth factors, proteolytic enzymes, and increase expression of extracellular matrix proteins and vascular adhesion molecules, which are all required for normal tissue remodeling (Nawroth, et al., 1999). Functional alterations or saturation of the macrophage system would allow anomalous tissue accumulation of AGEs leading to reduced structural protein turnover, increased collagen cross-links, and excessive degeneration and/or proliferation of tissue components as observed in ageing and diabetes (Bierhaus, et al., 1998; Grossi & Genco, 1998; Sensi, et al., 1991).

In addition to RAGEs, several other cell surface receptors for AGEs have been identified. These include macrophage scavenger receptor types I and II, oligosaccharyl transferase-48 (AGE-R1), 80K-H phosphoprotein (AGE-R2) and galectin-3 (AGE-R3)
(Singh, *et al*., 2001). From these, RAGE has attracted most attention, as it is to date the only AGE receptor reported to have signal-transducing properties. An abundant CML epitope in structurally heterogenous AGEs is specifically recognized by the RAGE V-domain (Kislinger, *et al*., 1999). In addition, as RAGE is expressed in endothelial cells, vascular smooth muscle cells and monocytes, it is centrally positioned to contribute to the pathogenesis of diabetic vascular complications.

Binding of AGEs to endothelial cells increases expression of adhesion molecules (VCAM-1 and ICAM-1) and tissue factor in a RAGE-dependent manner resulting in vascular hyperpermeability (Schmidt, *et al*., 1995; Bierhaus, *et al*., 1997). RAGE is a member of the immunoglobulin superfamily and exists in two forms, 35kDa and 45kDa (Thornalley, 1998). The 45kDa form of RAGE is known as soluble RAGE (sRAGE) with proteolytic property. It is used as a pharmacological agent to prevent the vascular effects of AGEs (acts as an antagonist) in experimental diabetes (Renard, *et al*., 1997). For example, vascular hyperpermeability in diabetic rats was inhibited in the presence of sRAGE in a dose-dependent manner (Wautier, *et al*., 1996). In a murine model of accelerated diabetic atherosclerosis, a six-week treatment with sRAGE suppressed development of atherosclerosis in a glycemia- and lipid-independent manner (Park, *et al*., 1998). Furthermore, vascular lesions that formed in animals receiving sRAGE were arrested at the fatty streak stage and the number of complex atherosclerotic lesions was strikingly reduced. These findings implicate that RAGE plays a central role in the development of diabetic vascular complications.

A two-hit model for RAGE-mediated perturbation of vascular function has been suggested (Schmidt & Stern, 2000; Schmidt, *et al*., 2001). In the first hit, prolonged presence of RAGE ligands changes vascular properties priming the vasculature for a basal
level of activation. In the second hit, additional perturbation, such as oxidized lipoproteins, ischemia, physical stress or inflammatory stimuli, results in exaggerated cellular response promoting formation of vascular lesions rather than restitution of vascular homeostasis. Furthermore, Anti-RAGE IgG can also inhibit AGE-RAGE interaction and subsequently the rise of vascular complications seen in diabetes (Schmidt, et al., 1995; Wautier, et al., 1994; Yan, et al., 1994).

2.5.3 LDL Glycoxidation and Diabetes-Induced Atherosclerosis

2.5.3.1 Oxidative Modification of LDL and Atherosclerosis

High serum total cholesterol concentration has been strongly connected with atherosclerosis in numerous studies (e.g. Keys, 1970; Martin, et al., 1986). Being the main carrier of cholesterol in blood, LDL is also the principal lipoprotein causing atherosclerosis (Grundy, 1995). Oxidized LDL in particular is thought to be a key molecule in atherogenesis (Ross, 1993; Witztum, 1994). Several lines of evidence separately indicate that oxidized LDL (ox-LDL) is present in atherosclerotic lesions in vivo. Firstly, LDL isolated from atherosclerotic lesions is in part oxidatively modified (Ylä-Herttuala, et al., 1989; Ylä-Herttuala, et al., 1990). Secondly, immunological techniques have demonstrated that atherosclerotic lesions contain materials reactive with antibodies generated against ox-LDL (Haberland, et al., 1988; Palinski, et al., 1989; Rosenfeld, et al., 1990). Thirdly, autoantibodies reactive with ox-LDL are present in plasma and lesions of humans and animals (Palinski, et al., 1994; Ylä-Herttuala, et al., 1994; Salonen, et al., 1992). Fourthly, administration of antioxidants prevents oxidative modification of LDL and slows the progression of atherosclerosis in several animal models (Carew, et al., 1987; Kita, et al., 1987; Steinberg, 1997a).
More recently, Nishi, et al. (2002) have documented that vulnerable carotid plaques from humans are greatly enriched in ox-LDL and that plaque content of ox-LDL was 70 times the plasma concentration. Increased levels of ox-LDL are also associated with increased carotid intima-media thickness (Hulthe & Fagerberg, 2002). In addition, there have been numerous studies that have shown that oxidized LDL is not recognized by its normal receptors. Therefore, it is preferably taken up via scavenger receptors on macrophages of extrahepatic tissues, including artery walls, resulting in cholesteryl esters deposition and foam cells formation.

Oxidation of LDL enhances atherogenesis by a number of different mechanisms, in particular by attracting the monocytes into the vascular intima and transforming them into foam cells (Steinberg, et al., 1989) and subsequent endothelial dysfunction as illustrated in Figure 2.4. Oxidative modification of LDL has also been shown to increase the ability of LDL to bind to the extracellular matrix (Wang, et al., 2001). Therefore, oxidation of LDL plays an important role not only in allowing LDL to be taken up by macrophages leading to the formation of foam cells, but also in promoting entry of monocytes/macrophages to the sub-intimal space where the process of LDL uptake occurs. However, oxidation of LDL apparently leads to possibly a very large array of consequences other than the generation of foam cells thought to be important in atherogenesis. For example, the oxidative modification of LDL has also been shown to be a chemoattractant for monocytes and to be cytotoxic to endothelial cells and mitogenic for macrophages and smooth muscle cells (SMC), as well as to inhibit nitric oxide-induced vasodilation (Steinberg, 1997b). Protection of LDL from oxidation could increase nitric oxide bioactivity and bioavailability and improve endothelium-dependent vasomotor, anti-inflammatory, and anticoagulant properties of the endothelium (Guetta &
Cannon, 1996). Additional pro-atherogenic effects of oxidized LDL are summarized in

*Table 2.3* (Stocker & Keaney, 2004).

*Figure 2.4*: A schematic illustration of foam cell formation in atherosclerosis. Circulating LDL cholesterol moves into the subendothelial space. Trapped LDL is subjected to oxidative modification by vascular cells such as smooth muscle cells, endothelial cells, and macrophages. Macrophages uptake modified LDL (ox-LDL) by scavenger receptors. In LDL overload, macrophages transform themselves into foam cells which become necrotic due to the accumulation of oxidized LDL. Oxidized LDL also results in endothelial dysfunction and injury. Local inflammation increases the entry of LDL and monocytes. (Adapted from Diaz, *et al.*, 1997).
Table 2.3: Potential proatherogenic activities of oxidized LDL

- Cytotoxic to endothelial cells and can induce apoptosis
- Alters inflammatory gene expression in vascular cells
- Increases expression of macrophage scavenger receptors
- Is immunogenic and elicits autoantibody formation and activated T cells
- Undergoes aggregation, which independently leads to enhanced uptake
- A substrate for sphingomyelinase
- Induces tissue factor expression and platelet aggregation
- Binds C-reactive protein leading to activation of the complement pathway.

A prototypical protein target for oxidants in cardiovascular disease is the LDL and oxidation of LDL has been considered as an important mechanism for the development of atherosclerosis (Heinecke, 2002; Brennan & Hazen, 2003; Heinecke, 2003; Shishehbor & Hazen, 2004). The major target in oxidation of lipoproteins is the lipid moiety due to the abundance of polyunsaturated fatty acids. It is noteworthy that oxidized lipoproteins have been implicated in the development of diseases ranging from diabetes to arthritis. Consequently, there has been a vast amount of interest in evaluating factors that influence the LDL oxidation, as well as development of pharmacological agents and antioxidants that could reduce the oxidative modification of LDL.

2.5.3.2 LDL Peroxidation

Lipid peroxidation is probably the most extensively investigated free radical-induced chain reaction (Gutteridge, 1995; Moore & Roberts, 1998; De Zwart, et al., 1999). The earliest step in the generation of oxidative modified LDL is peroxidation of polyunsaturated fatty acids (PUFAs) in the LDL phospholipids. Thus, PUFAs are particularly susceptible to peroxidation and once the process is initiated, it proceeds as a free radical-mediated chain reaction involving initiation, propagation and termination.
An early event in the peroxidation of PUFAs with at least two methylene-interrupted double bonds is the formation of a conjugated diene system (Porter, 1984). The PUFAs undergo extensive breakdown yielding an array of reactive aldehydes, some of which can become covalently attached to apolipoprotein B (apoB) moiety of the LDL (Steinbrecher, 1987; Ylä-Herttuala, *et al.*, 1989; Palinski, *et al.*, 1989).

Each single substrate radical may result in conversion of multiple fatty acid side chains into lipid hydroperoxides. The length of the propagation chain before termination depends on several factors such as the oxygen concentration and the amount of chain-breaking antioxidants present. Hydroperoxides are fairly stable molecules, but their decomposition can be stimulated by high temperatures or by exposure to transition metal ions (iron and copper ions). Decomposition of hydroperoxides generates a complex mixture of secondary lipid peroxidation products such as malondialdehyde (MDA) and 4-hydroxynonenal.

There is a variety of methods may be used for measuring the rate and levels of oxidation *in vitro* and *in vivo*. One of the methods used for *in vivo* LDL oxidation determination is by measuring the autoantibodies to ox-LDL immunologically (Jialal & Devaraj, 1996). For *in vitro* estimation of LDL oxidation, spectrophotometric method to evaluate the increase of thiobarbituric acid-reactive substances (TBARS) by measuring malondialdehyde is widely used (Puhl, *et al.*, 1994; Valkonen & Kuusi, 1997), in which thiobarbituric acid (TBA) reacts with MDA at low pH to form a chromophore that absorbs UV light at 532 nm. However, a convenient and very frequently used method for continuously monitoring the process of copper-induced LDL oxidation is to measure the conjugated diene formation at 234 nm as a time course in a UV spectrophotometer (Esterbauer, *et al.*, 1989).
2.5.3.3 LDL Glycation and Glycoxidation

The oxidative modifications of the LDL play an important role in the pathogenesis of atherosclerosis in the general population and its study has received considerable attention (Witztum, 1994; Bucala, 1995; Palinski & Witztum, 1995). A multitude of secondary complications of T2DM have been attributed to nonenzymatic glycation. LDL has a special place in studies of glycation and oxidation in diabetes because it is a strong risk factor for atherosclerotic vascular diseases. Increased levels of glycated LDL have been detected in diabetic patients (Lyons, et al., 1986). LDL of diabetic patients with poor glycemic control is more susceptible to oxidation than is LDL of normal subjects (Tsai, et al., 1994).

Non-enzymatic glycation of LDL particles can alter their structure, function, and their susceptibility to oxidation and hence affect their atherogenic potential. One of the most compelling lines of evidence, which allows us to consider glycated LDL atherogenic, is the in vitro and in vivo studies which suggested the altered biological activity of glycated lipoproteins. Incubation of LDL with glucose leads to AGE formation on both the lipid and apoprotein components. It has been shown that glycated LDL uptake by normal human fibroblasts, which only have classical LDL receptors, was defective (Witztum, et al., 1982). Later, Klein, et al. (1992b) showed that recognition of LDL from diabetic patients in poor glycemic control by human fibroblasts was also impaired, supporting the role of glycation in altering recognition of LDL by classical LDL receptor. Subsequent results showed that incubation of macrophages with glycated LDL resulted in transformation of these macrophages into cholesterol loaded foam cells (Lopes-Virella, et al., 1988). Glycated LDL uptake resulted in increased cholestryl ester (CE) synthesis in macrophages leading to increased intracellular CE accumulation. Human macrophages in
culture also showed increased in CE accumulation and synthesis when exposed to LDL from diabetic subjects (Lopes-Virella, et al., 1988). Thus, the increase in LDL glycation causes a reduction in the uptake and degradation of LDL by tissue fibroblasts and macrophages, contributing to premature atherosclerosis in diabetics (Vlassara, 1996). Lipid peroxidation is known to induce cross-linking of collagen with a high rate in the presence of high glucose (Fu, et al., 1994). Additionally, advanced glycated collagen was shown to be capable of covalently trapping LDL (e.g. in the arterial wall) causing it to be oxidatively modified by free radicals (Mullarkey, et al., 1990). Table 2.4 summarizes the mechanisms by which lipids are accumulated in the atherosclerotic lesions (Kruth, 1997b).

Table 2.4: Mechanisms of lipid accumulation in atherosclerosis

- Increased entry of lipid into the artery wall due to:
  - increased passive diffusion from elevated levels of LDL in plasma
  - uptake and transport of lipid into the subendothelial space by endothelial cells
  - uptake of lipid through intercellular spaces as a result of dysfunctional endothelium
- Altered metabolism/flux of lipid in the artery wall
- Decreased removal of lipids from the intima because of the following:
  - the lipid is trapped in the intima (i.e. cannot pass through the internal lamina)
  - dysfunctional endothelium fails to transport intimal lipid back to the plasma
  - lipid becomes sequestered in foam cells
  - lipid becomes bound to connective tissues components
  - lipid becomes aggregated and physically trapped by lipoprotein components

The increase of the plasma levels of LDL and/or changes in their subfractions are associated with an increase of atherogenic risk (Austin, et al., 1990). In the diabetic patients, although this level often stays within the normal range, alterations take place in the lipoproteins components such as diameter, density, and lipid composition that make it
more atherogenic (Haffner, 1995). To them the nonenzymatic glycation that modifies the LDL and affects its metabolism resulting in increase in its potential atherogenicity must be added. In this sense, the glycation is directly linked to the oxidation of the LDL in the arterial wall (Lyons & Jenkins, 1997; Palinski & Witztum, 1995). Modifications to LDL such as oxidation and glycation are strongly implicated in the pathogenesis and progression of atherosclerosis (Tabas, 1999; Stocker & Keaney, 2004). Accumulated evidences in the last years suggest the combination of processes of nonenzymatic glycation and oxidation by free radicals acting on the lipoproteins, would contribute to generate a greater atherogenic risk in the diabetic patients, as proposed by Bayne (Baynes, 1991). The combinations of these two reactions, collectively known as glycoxidation, generate products that can be especially atherogenic (Lyons & Jenkins, 1997; Baynes & Thorpe, 1999). The interaction between glycation and oxidation provides a probable explanation for the increase of atherosclerosis frequently associated with diabetes.

The development of atherosclerosis is accelerated in patients with diabetes mellitus, and LDL in a diabetic state is susceptible not only to oxidation (Kobayashi, et al., 1995; Bowie, et al., 1993) but also to glycation (Cohen, et al., 1993; Klein, et al., 1995b). In the diabetic patients several types of modified LDL molecules have been detected, including glycated LDL, oxidized LDL, and glycoxidized LDL. Immunohistochemical studies on human atherosclerotic lesions have shown that AGEs and oxidized LDL are colocalized in the cytoplasm of macrophage-derived foam cells (Imanaga, et al., 2000). Therefore, it is possible that glycoxidized LDL (GO-LDL) may contribute to the development and progression of atherosclerotic lesions in diabetes mellitus.
The contribution of advanced glycation to the oxidative modification of LDL was first observed in an *in vitro* study (Bucala, *et al*., 1993). Isolated human LDL was incubated with glucose (in presence of metal ions) and analyzed for both advanced glycation and oxidative modification. Incubation of LDL with 200 mM glucose for 3 days resulted in the formation of readily measurable levels of AGEs on both apoprotein and lipid. These studies indicated that lipid-linked AGEs formed more rapidly than ApoB-AGEs and measurements of oxidative modification further showed that LDL was oxidized concomitantly with the formation of AGEs (Bucala, *et al*., 1993). Moreover, increased levels of peroxidation products are detected in glycated or cell-modified LDL, which have been known as minimally modified LDL (Berliner, *et al*., 1990; Lyons, *et al*., 2000). For better explanation the relationship between advanced glycation and LDL oxidation *in vivo*, Bucala, *et al.* (1993) have analyzed LDL from both diabetic and nondiabetic individuals by an AGE-specific ELISA. Their investigations revealed increased levels of both phospholipids-AGEs and apoB-AGEs of the LDL molecules from diabetic patients compared to healthy controls.

### 2.5.3.4 Role of Scavenger Receptors

Atherosclerosis is an inflammatory disease (Li & Glass, 2002) and macrophages are present at all stages of the disease (Takahashi, *et al*., 2002). Animal studies, using several different models of atherosclerosis, support a key role for macrophages in the development and progression of atherosclerosis. In addition to their contribution in the modification of LDL (Stocker & Keaney, 2004; Li & Glass, 2002), macrophages, in their role of scavenging tissue and cellular debris, can take up the modified LDL via scavenger receptors (Trigatti, *et al*., 2000). Scavenger receptors recognize chemically and
biologically modified lipoproteins, typically acetylated LDL (Brown & Goldstein, 1983; Goldstein, et al., 1979) and oxidized LDL (Steinberg, et al., 1989). Unlike LDL receptor, the expression of scavenger receptor is not down regulated by cellular cholesterol content (Goldstein, et al., 1979) and, therefore, results in the intracellular lipid accumulation and consequently induce foam cell formation. Recent studies showed that, at least in macrophages, macropinocytosis has been shown to operate in the uptake of native LDL too, in the process leading to foam cell formation (Kruth, et al., 2005). However, important in this respect is that modified LDL is taken up faster by macrophages than native LDL.

Monocyte-derived macrophages endocytose modified LDL via multiple receptors, including both class A (SR-A) and class B (SR-BI and CD36) scavenger receptors (Gough, et al., 1999; Kapinsky, et al., 2001), class E receptor (lectin-like oxidized LDL receptor-1), and lipoprotein lipase (Zimmermann, et al., 2001; Kataoka, et al., 1999). Other scavenger receptors include class D receptor (CD68) and scavenger receptors for phosphatidylserine and oxidized lipoprotein (SR-PSOX) that mediate binding and uptake of ox-LDL (Boullier, et al., 2000; Febbraio, et al., 2001; Linton & Fazio, 2001). Uptake of lipoproteins in excess of its metabolism results in the formation of cholesterol esters in macrophage foam cells. In addition, foam cells can develop similarly from vascular smooth muscle cells by the uptake of oxidized LDL via class A and class B scavenger receptors (Ohgami, et al., 2001; Mietus-Snyder, et al., 2000). Lipid-laden macrophages have been shown to exhibit strong immunoreactivity to CD36, but only low or moderate levels of immunoreactivity to SR-A (Nakata, et al., 1999), suggesting that CD36 could be the predominant macrophage receptor for ox-LDL in human atherosclerotic lesions.
Diabetes exacerbates the uptake of modified LDL by foam cells via several mechanisms. Firstly, diabetes causes a proatherogenic dyslipidemia resulting in reduced HDL levels and increased triglycerides, which can reduce reverse cholesterol transport and contribute to increased levels of small dense LDL particles (Sibley, et al., 1999). Secondly, diabetes increases the expression of macrophage class B scavenger receptor CD36 (Griffin, et al., 2001), which enhances oxidized LDL endocytosis. Thirdly, hyperglycemia increases glycoxidation, resulting in increased levels of oxidized and glycated LDL (Lyons, et al., 1986; Bucala, et al., 1993), which increases ligand availability for scavenger receptors and lipoprotein lipase. Modification of proteins by oxidation, glycation, or glycoxidation has been proposed to impair binding of LDL to the LDL receptor, and increase binding and endocytosis by unregulated scavenger receptor pathways.

### 2.6 Pathophysiologic Role of CML Accumulations in Diabetes and Chronic Diseases

#### 2.6.1 Nε-(Carboxymethyl)lysine: Formation and Chemical Properties

AGEs form a large group of heterogeneous compounds of which only a few have been identified. CML seems to be the major epitope of AGEs which is recognized by experimentally induced polyclonal AGE-antibodies. CML, a product of glycation and oxidation in vivo, is generated by oxidative cleavage of the Amadori product threulosyl-lysine and is also a product of metal-catalysed oxidation of LDL or peroxidation of polyunsaturated fatty acids in the presence of fructose-lysine (Figure 2.5). Thus, CML is known to be a glycoxidation product which means that it requires oxidation reactions for their formation from glucose (Baynes, 1991). Although CML is the smallest one of the
AGE modifications, it might be functionally relevant. It leads to a change in charge, since, after modification, the former positively charged lysine residues carry a negatively charged carboxylic group.

![Diagram of multiple pathways of Nε-(carboxymethyl)lysine formation.](image)

**Figure 2.5:** Multiple pathways of Nε-(carboxymethyl)lysine formation.

CML is an irreversible protein modification which is stable to acid hydrolysis and can be quantified from protein hydrolysates. It has been reported that CML binds to RAGE and activates cell-signaling pathways (Kislinger, *et al.*, 1999). Moreover, co-localization of CML with adducts derived from products of lipid peroxidation, such as 4-hydroxy-2-nonenal and malondialdehyde, supports the concept that lipid peroxidation itself, in addition to and apart from advanced glycation, triggers the formation of CML (Fu, *et al.*, 1996). CML formation is a slow process and the accumulation of CML
epitopes in the extracellular matrix (ECM) is probably exacerbated by the slow turnover of these proteins. CML contents of proteins exist in several tissues such as lung, skin, bone, and blood vessels. However, CML epitopes are not uniformly distributed in these tissues, with the elastic fibres of blood vessels and skin staining more intensely than surrounding tissues (Schleicher, et al., 1997).

2.6.2 $N^\epsilon$-(Carboxymethyl)lysine and Diabetic Complications

Inefficient clearance of degraded low molecular weight AGE-rich peptides and recirculation of these 'toxic' molecules might be responsible for vascular damage in diabetic patients. $N^\epsilon$-(carboxymethyl)lysine (CML) is the major AGE that accumulates in vivo as suggested by immunohistochemical and biochemical studies (Dunn, et al., 1991; Reddy, et al., 1995; Schleicher, et al., 1997) and can be recognized by experimentally induced polyclonal anti-AGE antibodies (Ikeda, et al., 1996).

Since specific CML antibodies have become available, several studies have demonstrated the accumulation and distribution of CML in atheromatous lesions (Nerlich & Schleicher, 1999; Horiuchi, et al., 1996; Takayama, et al., 1998; Sakata, et al., 1998; Kume, et al., 1995; Meng, et al., 1998; Horie, et al., 1997) and observed within atherosclerotic plaques, in foam cells (Nerlich & Schleicher, 1999; Schleicher, et al., 1997), in a variety of chronic degenerative, chronic inflammatory diseases (Schleicher, et al., 2005), and recently in human heart valves (Baidoshvili, et al., 2004) as well as in the heart tissue of diabetic patients (Schalkwijk, et al., 2004). CML has become a key marker of protein modification in response to glyoxidative, lipoxidative and carbonyl stress in vitro. This has led to suggestion that it could also represent a biomarker for systemic or local oxidative stress in tissue lesions in vivo (Nerlich & Schleicher, 1999; Shaw, et al.,...
CML content increases with the chronological age of proteins (Dunn, et al., 1989; Dunn, et al., 1991; Dyer, et al., 1993) and detected in patients with diabetes mellitus (Schleicher, et al., 1997). CML can be formed on low density lipoproteins in the blood circulation and trapped CML-LDL as well as CML-LDL formed in extracellular matrix will be taken up by macrophages through scavenger receptors. Macrophages will be transformed into foam cells leading to organ damage seen in diabetic complications as illustrated in Figure 2.6.

![Figure 2.6: Proposed model of CML formation on LDL in the blood circulation and in the subendothelial space. LDL is subjected to glycoxidation and CML formation. CML-LDL is recognized by endothelial cell through AGE receptor (RAGE) leading to reactive oxygen species (ROS) formation. Monocytes and CML-LDL crosses the endothelium and can become trapped in the extracellular matrix followed by binding to scavenger receptors on the macrophages, which undergo foam cell formation and, thus, atherosclerosis lesions.](image-url)
In diabetes, the rate of CML formation is accelerated as compared to that seen in aging and in some studies, CML seems to correlate with the severity of diabetic complications independent of age (Wells-Knecht, *et al*., 1996; Hammes, *et al*., 1999). Areas of intimal thickening and atherosclerotic plaques express more CML epitopes than areas with less severe morphologic alterations (Schleicher, *et al*., 1997). CML has been reported to stimulate superoxide radical and $\text{H}_2\text{O}_2$ generation via activation of NADPH oxidase in human endothelial cells (Wautier, *et al*., 2001). The cytoplasmic domains of foam cells also contain the CML epitopes, which may be due to receptor-mediated uptake and degradation of AGE-modified proteins by macrophages within plaques (Imanaga, *et al*., 2000).

Since CML can be formed from glycation and lipid peroxidation reactions, the observed increase in CML in diabetes may be accounted for either an increase glycation or increased oxidative stress. However, it has been reported that CML can be formed by a nonoxidative mechanism and its elevations in diabetic serum supporting the notion that the nonoxidative glycation of proteins may contribute to AGE accumulation in diabetes (Dunn, *et al*., 1991; Portero-Otin, *et al*., 1997). Longstanding diabetes mellitus is associated with micro- and macroangiopathic changes. The observation of CML accumulation in diabetic muscle is in good accordance with this general notion. The vasculature is the preferred environment for localisation of CML accumulation in diabetic macro- and microangiopathy. Although several studies showed CML elevations in diabetic microvascular complications, there is ongoing debate about the effect of CML on macrovascular diseases resulting from T2DM.
2.7 **Rationale of the Thesis**

In comparison to the general population, individuals with diabetes mellitus suffer a 3- to 4-fold increased risk for developing the complications of atherosclerosis and vascular insufficiency. Since diabetes has been estimated to affect at least 10 million people in the United States alone, the contribution of diabetes to the overall mortality of heart disease and stroke is significant. This fact should be taken into account to develop a suitable determinant for the early detection of these complications and subsequently reduce the adverse effect of T2DM. *In vitro* experiments have shown that the products of glucose auto-oxidation and Amadori-adducts are both potential sources of CML and their contributions to CML formation depend on their relative concentrations and on the local oxidative environment. Low density lipoprotein modification leads to intracellular accumulation of lipoprotein-derived cholesterol and this may be due to resistance of the AGE-LDL or CML-LDL modifications to lysosomal degradation. Thus, excessive formation of CML on LDL has been proposed to be an important mechanism for the dyslipidemia and accelerated atherogenesis that is observed in patients with type 2 diabetes. The precise molecular basis for this uptake abnormality is still elusive. We hypothesized that glycoxidation of LDL leads to CML production and that CML may be suitable for use as an endogenous biomarker for the impaired LDL uptake and altered endothelial function in the atherosclerotic arteries resulting from type 2 diabetes mellitus.
2.8 **Objectives of the Study**

The aims of this thesis are:

1. To estimate the serum levels of \( \text{N}^e \text{-(carboxymethyl)lysine (CML)} \) in the study populations; CAD patients without type 2 diabetes, type 2 diabetes patients with and without CAD, and nondiabetic healthy subjects, and to establish whether circulating CML levels are significantly different in patients with type 2 diabetes with or without CAD.

2. To examine the correlation between CML with other clinical parameters and/or risk factors of CAD in diabetic patients.

3. To explore the predictive power of CML in patients with increased risk for CAD in type 2 diabetes and to allow for detection of the disease in its early stages.

4. To evaluate CML as a major component of glycoxidated LDL which might be used as a biomarker of oxidative stress in diabetic patients with CAD and to explore potential synergistic interactions among CML and lipid peroxidation?

5. To test the hypothesis that altered LDL metabolism is enhanced by CML-LDL rather than glycated- or oxidized-LDL alone in diabetes-induced atherosclerosis.

6. To establish a pathway and mechanism by which CML could lead to coronary artery complications result from type 2 diabetes by studying the impact of CML-LDL in altering the metabolism and recognition of LDL by the classical LDL receptor.