CHAPTER 1

INTRODUCTION

1.1. Diabetes

Diabetes mellitus (DM) or simply diabetes is a metabolic disorder clinically characterized by hyperglycemia due to defective insulin secretion, defective insulin action or both. The chronic hyperglycaemia of diabetes is associated with long-term damage, dysfunction and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels (ADA, 2010). It is the most common severe metabolic disorder and is considered to be one of the five top causes of death in the world (Chandramohan *et al.,* 2008).

1.2. History of diabetes: discovery, description and treatment

History of diabetes, starting from its earliest known records in 3500 BCE to current stem cell research is filled with near achievements, agregious errors, serendipities, unsuccessful labors, victories and defeats.

1.2.1. Ancient records of clinical features of diabetes

The history of diabetes unfolds during the age of antiquity and it comprised a remarkable chronicle of discovery, description, management and treatment covering more than 3500 years of medical history (Sanders, 2002). Ancient Egypt physicians, in an effort to understand the ailment of diabetes and its discussion, recorded their observations. The earliest known record of clinical features similar to diabetes was found written on Egyptian Ebers Papyrus (1552 BCE), where physician Hesy-Ra mentioned the passing of too much urine (polyuria) as a symptom (Diana and Richard, 2009).

Two Indian physicians Sushruta and Charuka, sometime around 1000 to 600 BCE, first reported the sweet taste in the urine of patients characterized by excessive passage of urine that attracts ants and are sticky. Sushruta described this ailment as madhumeha (literally means excessive urine with sweet taste like honey) in his Sushruta Samhita (text on medicine) (Singh and Bhavna, 2013). Indeed, the diagnosis was made by testing the urine or noting that ants congregated round it. Charaka and Sushruta noted that the disease was most prevalent in those who have sedentary life style, indolent, overweight and who indulged in sweet and fatty foods (Girish and Shridhar, 2007). Sushruta was the first to differentiate between the two types of diabetes mellitus. Sahaja which could be interpreted as type 1 developed mostly in

thin people at a younger age and apatarpana which could be interpreted as type 2 developed in obese persons (Richard, 2013).

In 250 BCE Apollonius of Memphis probably coined the term "diabetes", meaning to go through or siphon which explained the liquefaction of flesh and bones into urine. Hippocrates didn't specifically mention diabetes in his writings, but there are certain accounts similar to signs and symptoms of diabetes (Lasker *et al.,* 2010). Aretaeus of Cappadocia, a disciple of Hippocrates, first used the term "diabetes" in 2nd century AD in his work "Acute and Chronic Diseases". He gave a recognizably clearer description of what would now be called diabetes. "Diabetes is a dreadful affliction, not very frequent among men, being a melting down of the flesh and limbs into urine....." (Tattersall, 2010).

In 164 AD Galen another disciple of Hippocrates attributed the excessive loss of urine to disease of kidney and this view remained in vogue for centuries to come and name the ailment as diarrhoea of the urine (Zajac *et al.,* 2010).

1.2.2. Diagnostic period

In the long history of understanding diabetes, there was no progress until Thomas Willis in 1674 reiterated the sweet taste of the patient's urine and added mellitus (sweet honey in Latin) to the word diabetes (Lasker *et al.,* 2010).

It was William Cullen, who recognizably distinguished between diabetes mellitus and diabetes insipidus. In 1769 in his classification of human disease, diabetes was first classified as diabetes with the urine "the smell, colour and flavour of honey," and diabetes with limpid but not sweet urine (Sanders, 2002).

Mathew Dobson in 1776 found out that serum also tasted sweet apart from the urine of his patient Peter Dickinson. Dobson concluded that the kidneys excreted sugar and that it was not formed in the organ but previously existed in the serum of the blood. Provided experimental evidence of sweet urine, Mathew Dobson heated two quarts of urine to dryness. The remaining was a whitish cake like residue which smelled sweet like brown sugar; neither could it be distinguished from sugar, except that the sweetness left a slight sense of coolness on the palate. Mathew Dobson conclusively established the diagnosis of diabetes as the presence of sugar in the urine and blood in a paper presented to medical society of London in 1776 (Ali *et al.*, 2006).

John Rollo in 1797 first applied the discovery of Dobson in metabolic study of diabetes. He recorded the amount and kinds of food (animal and vegetable matters) consumed by a patient and weighed the sugar cake obtained by boiling the urine. Weight of sugar cake varied depending on the kind of food consumed. Animal matters resulted in comparatively lower excretion of sugar than vegetable matters. Based on his findings he came up with the first approach to the dietary treatment of diabetes (Kahn *et al.,* 2005).

Thamos Cawley, in 1788 observed shrunken pancreas riddled with stones in a diabetic patient at an autopsy. This might have been the first evidence of pancreas in connection to diabetes (Tripathy *et al,* 2012).

Glucose metabolism was clarified by the work of Claude Bernard. In his work in 1843, he was surprised to find glucose in blood samples of animals in spite of several days fasting. He questioned if the glucose was being synthesized in the body itself. But it was in contrary to that time's prevailing theory that only plants can synthesize nutrients. He further discovered high concentration of glucose in the hepatic vein leaving the liver which gave him a clue that liver probably had to be the source of glucose. He logically started to analyze liver tissue samples and found enormous quantities of a starch - like substance in the liver which could be readily converted into sugar. He called this "glycogen" in his publication in 1849. Another impressive discovery of Bernard was that a lesion in the floor of the fourth ventricle produced temporary hyperglycaemia (Holt *et al.,* 2010).

1.2.3. Experimental road to discovery of insulin.

Till the first part of the 19th century, the cause of diabetes remained as a mystery. A major breakthrough came in 1889 when Oskar Minskowski and Josef Von Mering while investigating fat metabolism serendipitously found out that pancreatectomy in the dog caused severe diabetes. The possible hypothetical explanations were either it removed a diabetogenic toxin, or produced an internal secretion that controlled carbohydrate metabolism (Luft, 1989).

In 1893, Gustave Laguesse suggested that the putative internal secretion of the pancreas was probably produced by the "islands" of cells, which had been discovered in 1869 by Paul Langerhans and name it as Islets of Langerhans (Holt *et al.,* 2010).

Minskowski's experiment failed to immediately establish the key role of pancreas. Researchers believed that the damage to the nerve upon removal of the pancreas was the reason for developing diabetes. It was Jean De Meyer's who strongly believed that diabetes was a direct outcome of loss of the internal secretion of the pancreas. He wrote in 1909 that "the internal secretion of the pancreas (not as yet named) and which, if derived, as we believe, from the islets of Langerhans, could be called insulin (Latin: insula, island)". De Meyer, making his hypothetical suggestion more specific, showed that addition of pancreatic extract to inactive serum gave it glucose lowering properties. He even suggested that hepatic glycogen formation was directly promoted by the infusion of pancreatic extract (Meyts, 2014).

Isolating the elusive internal secretion became the target for the treatment of diabetes and was a challenging task. Repetitive failures in isolating, over the next three decades make the researchers regrettably to retreat from the likelihood of discovering antidiabetic internal secretion. Researchers started giving more attention to diet and lifestyle as the only option to control diabetes. The best known was the Frederick Madison Allen's starvation diet regulation "Total Dietary Regulation in the Treatment of Diabetes" published in 1919. He described the importance of Low carbohydrate diet and fat diet in controlling diabetes (Mazur, 2011).

Among those who attempted to isolate internal secretion of pancreas, few of them were closest – Georg Zuelzer, a Berlin physician in 1907, Ernest Scott in Chicago in 1911, and Nicolas Paulesco in Romania in 1920 – 1921 (Lasker, 2010).

The discovery of insulin at the University of Toronto in 1921 was one of the greatest achievements in the history of diabetes. Destructive effect of the pancreatic juices was the major problems in isolating the internal secretion of the pancreas. Frederick Grant Banting got an influencing idea from Moses Barron's article where he reported a case of stone blocking the main pancreatic duct leading to degeneration of acinar cells but not the islet cells. Banting's idea was – if he ligated the pancreatic ducts (basically to stop the flow of nutrients to the pancreas) and make the acinar cells to degenerate, then pancreas would no longer able to secrete digestive juices. With this idea Banting consulted John James Rickard Macleod, professor at the University of Toronto. Macleod provided a small Laboratory, research animals and a medical student Charles Best to assist his research work. Banting and Best surgically ligated the pancreas of a dog, remove the pancreas after a while, sliced, frozen it, grounded up and filtered. The

name “isletin” was given to the extracted substance. The extracted isletin was injected to a diabetic dog. The dog was recovered from diabetic symptoms. The word isletin was replaced by insulin as suggested by Macleod. The first clinical trial of insulin took place on fourteen year old Leonard Thompson. His blood glucose level fell slightly, but he developed a sterile abscess. Later Macleod approached James Bertram Collip for the purification of the extract. Thompson was injected another extract purified by Collip and his blood glucose level was normalized with marked clinical improvement. In 1923, Eli Lilly started the mass production of insulin. In 1923, Banting and Macleod were awarded Nobel Prize in Physiology or Medicine. Best and Collip were also acknowledged as all four of them contributed equally to discovery of insulin (Banting and Macleod, 1925).

In 1939, Harold Himsworth, using insulin-glucose tolerance test distinguish insulin-sensitive and insulin-insensitive types of diabetes. His observation was that insulin-sensitive patients did not differ from healthy controls in their sensitivity to insulin. His work formed the scientific basis for the distinction of type 1 and 2 diabetes (Krentz and Hitman, 2011).

1.2.4. Chemical and structural identification of insulin

Few years after discovery of insulin, researchers became curious about chemical structure of insulin. John Jacob Abel was the first to contribute to chemical identification of insulin. Abel, while working on the sulphur content of the insulin was luckily rewarded with insulin crystals forming on the sides of a test tube and reported in 1926 that it was a protein. Abel’s idea was opposed by some researchers claiming that the protein might be a carrier of the active part of insulin. Abel in countering his opponents unfortunately failed to crystallize insulin again. The whole confusion was apparently solved in 1934 by David Scott in Toronto when he reported that the crystalline insulin contains zinc and when zinc is removed, insulin cannot be crystallized again. Vincent du Vigneaud of Chicago was inspired by Abel’s work and in collaboration with Hans Jensen and Oskar Wintersteiner isolated different amino acids from insulin and showed that insulin is a protein or large peptide composed of amino acids (Wieland and Bodanszky, 1991).

Scientists believed that amino acids in insulin were randomly arranged and patternless. The idea of exact chemical structure of insulin was brought in forth by Frederick Sanger. All proteins contained roughly the same amino acids but differed in

both physical and biological properties. Sanger pointed out that the difference must be due to different patterns of arrangement of amino acid residues. Sanger determined that insulin molecule was composed of two polypeptide chains held together by disulphide bridges in 1945 and discovered the exact amino acid sequence of insulin in 1955. For his work Sanger was awarded Nobel Prize in1958 in chemistry (Sanger, 1958).

The radioimmunoassay technique developed by Rosalyn Yalow and Solomon Berson in1960 revolutionized the field of biology and medicine. Arthur Mirsky hypothesized that maturity-onset diabetes might be due to abnormally rapid degradation of insulin by hepatic insulinase. In an attempt to prove this hypothesis they studied the I-labelled insulin metabolism following intravenous administration to non-diabetic and diabetic subjects. On contrary to Mirsky's hypothesis they found out that insulin degraded rapidly in normal patients and suspected that the reason was due to antigenic property of the insulin. They developed radio-immunoassay technique by exploiting the idea that insulin binds to anti-insulin antibodies to form an antigen antibody complex. Yalow was honored with a Novel Prize in 1977 (Kahn and Roth, 2004). Dorothy Hodgkin in 1969 determined the three dimensional structure of insulin by the X-ray crystallographic method (Dodson, 2014).

1.2.5. The birth of Human Insulin

The synthesis of biosynthetic human insulin (BHI) was the first health-care product derived from rDNA technology. The history of production of biosynthetic human insulin can be traced back to 1950s, with the classic structural studies on DNA and on insulin. Many research ideas and rDNA technology were discussed at 16th Lilly Insulin Symposium in 1976. The insulin biosynthesis was the central theme of discussion. Scientists were interested in rDNA technology as it might provide a non pancreatic source of insulin that could eliminate the insulin shortage (Chance and Frank, 1993). Axel Ullrich described construction of plasmids containing the coding sequences of rat insulin genes in 1977 (Ullrich *et al.*, 1977). Pierre Freychet described the existence of insulin receptors based on the specific binding of I-labelled insulin to liver plasma membrane (Freychet *et al.*, 1971). The sequence of the human insulin gene was deduced by Graeme I. Bell in 1980 (Bell *et al.*, 1980). The first successful laboratory production of human insulin was announced in 1978 by Genentech and City of Hope National Medical Center, California (Genentech, 1978). The human

insulin was synthesized by joining the purified A and B chains expressed in *E. Coli* with cloned synthetic genes (Goeddel *et al.,* 1979). Axel Ullrich deduced the entire 1,370 amino acid sequence of the human insulin receptor precursor in 1985 (Ullrich *et al.,* 1985). Mueckler M in 1985 deduced the amino acid sequence of glucose transport protein from human HepG2 hepatoma cells (Mueckler *et al.,* 1985).

1.2.6. Stem cell research: future insulin production

Researchers have tried to generate human pancreatic β-cells in vitro that can produce insulin since the year 2000. Human pluripotent stem cells can generate cells and tissues that could be effectively used for the treatment of diseases. Researchers like Nadya Lumelsky, Takahisa Fujikawa and Suheir Assady are worth mentioning who have worked in the area of generation of human pancreatic beta cells from embryonic stem cells (Lumelsky *et al.,* 2001; Fujikawa *et al.,* 2005; Assady *et al.,* 2001). Harverd stem cell researchers led by Doug Melton announced the successful large-scale generation of differentiated human β-cells that mimic their normal in vivo counterpart by using pluripotent stem cell lines as a starting point.

1.3. Glucose homeostasis: Glucose metabolism and regulation

Glucose metabolism is critical to normal physiological functioning. The elevation of glucose in the circulation depends on the rate of gastric emptying during fed state and hepatic processes like glycogenolysis and gluconeogenesis during fasting state (Shrayyef and Gerich, 2010).

Insulin and glucagon are glucoregulatory hormones of the body that maintain circulating glucose concentrations in a narrow range. The post meal blood glucose level gradually increases and reaches a peak. Insulin is the key anabolic hormone that is secreted in response to increased blood glucose and control postprandial glucose in three ways. Initially, insulin signals the cell of insulin sensitive peripheral tissues and skeletal muscle to increase their uptake of glucose. Secondly insulin acts on the liver to promote glucogenesis. Finally, insulin simultaneously inhibits glucagon secretion thus signaling the liver to stop producing glucose. Insulin secretion stops when the plasma glucose level returned back to fasting level and eventually glucagon secretion starts. Glucagon is a catabolic hormone secreted by the pancreatic α-cells that helps in sustaining plasma glucose during fasting conditions by stimulating hepatic glucose production (Aronoff *et al.,* 2004).

1.4. Classification of diabetes

DM is a heterogeneous group of clinical conditions that share certain common features. Assigning a type of diabetes to a patient depends on the diagnosis and the current knowledge about the disease. An appropriate uniform terminology and a functional, working classification of diabetes is required as to understand the pathogenesis of hyperglycemia and to treat it effectively (ADA, 2010).

The first classification of diabetes was published in 1979 by the National Diabetes Data Group (NDDG) based on the pharmacologic therapy applied into two major groups: Insulin Dependent Diabetes Mellitus (IDDM) and Non Insulin Dependent Diabetes Mellitus (NIDDM). The classification by NDDG became popular during 1980s and 1090s, but with time, the misclassification of patients became evident. Several patients classified as with NIDDM needed insulin to control diabetes making it unfit either in IDDM or NIDDM (Maraschin, 2013). More meaningful classification of DM favoring more adequate treatment was proposed by American Diabetes Association in 1997 based on the pathogenesis of the disease. The classification was approved by WHO in 2006 and it comprises of four categories: Type 1 DM, Type 2 DM, other types and gestational diabetes (Alberti and Zimmet, 1998).

1.4.1. Type 1 Diabetes Mellitus (β-cell destruction, usually leading to absolute insulin deficiency)

Type 1 diabetes mellitus previously recognized by the terms insulin dependent diabetes, juvenile-onset diabetes, is a chronic illness characterized by the body's inability to produce insulin due to autoimmune destruction of the β-cells of the pancreas. Type 1 Diabetes mellitus accounts for 5-10% of those with diabetes (Cnop *et al.,* 2005).

1.4.2. Type 2 Diabetes Mellitus (ranging from predominantly insulin resistance with relative insulin deficiency to predominantly an insulin secretory defect with insulin resistance).

Type 2 diabetes mellitus (T2DM) is a heterogeneous disorder, characterized by defects in insulin sensitivity and usually have relative (rather than absolute) insulin deficiency. Type 2 diabetes is the predominant form of diabetes and accounts for at least 90% of all cases of diabetes mellitus (Nyenwea *et al.*, 2011).

1.4.3. Other specific types of diabetes mellitus

Other specific types of diabetes mellitus include-

a) Genetic defects of the β-cell: Associated with monogenetic defects in β-cell function. Also known as maturity-onset diabetes of the young (MODY) characterized by impaired insulin secretion with minimal or no defects in insulin action.
b) Genetic defects in insulin action: That results from genetically determined abnormalities of insulin action due to mutations of the insulin receptor.
c) Disease of the exocrine pancreas: That results from any process that injures the pancreas like pancreatitis, trauma, infection, pancreatectomy and pancreatic carcinoma.
d) Endocrinopathies: Diabetes due to excess amount of some hormones that antagonize insulin action.
e) Drug- or chemical-induce diabetes: Diabetes due to drugs/chemicals that impair insulin secretion or action.
f) Infections: Due to certain viruses that have been associated with β-cell destructions.
g) Uncommon forms of immune mediated diabetes: Example anti-insulin receptor antibodies.
h) Other genetic syndromes sometimes associated with diabetes: Down syndrome (ADA, 2010).

1.4.4. Gestational diabetes mellitus

Gestational diabetes mellitus (GDM) represents a heterogeneous group of metabolic disorders, which result in varying degrees of maternal hyperglycemia and pregnancy-associated risk (Landon and Gabbe, 2011).

1.5. Etiology and pathogenesis of Diabetes Mellitus

Type 1 and type 2 diabetes are characterized by progressive β-cell failure. Apoptosis is probably the main form of β-cell death in both the types of the disease and the mechanisms leading to β-cell loss may be quite diverse in the various subtypes of the disease (Cnop *et al.,* 2005). The pathogenetic pathways for these two major types of diabetes appear to be distinct and separate. Both forms of diabetes have a genetic as well as environmental component in their pathogenesis (Tan and Cheah, 1990).

1.5.1. Etiology and pathogenesis of Type 1 Diabetes Mellitus

The etiology or cause of type 1 DM is the autoimmune destruction of the β-cells leading to progressive β-cell death. It is also thought that T1D is caused by one or more environmental factors interacting with a relatively common genetic background (ADA, 2010).

Type 1 diabetes is immune-mediated diabetes with pancreatic β-cells being the target of autoimmune assault. Infiltration of islets by CD4+ and CD8+ T cells (CD4+ T cells are sufficient to induce insulitis while CD8+ T cells contribute to the severity of the damage) and macrophages leads to an inflammation reaction termed "insulitis", leading to loss of most β-cells after prolonged periods of disease. The β-cell death in the course of insulitis is caused by either direct contact with activated T-cells or macrophages or by exposure to mediators (cytokines, nitric oxide, oxygen free radicals, etc) secreted by these cells. Apoptosis, the main cause of β-cell death at the onset of type 1 diabetes, is a highly regulated process. Cytokines like IL-1β and/or TNF-α plus IFN-γ activate β-cell gene networks which are under the control of transcription factors NF-κB and STAT-1 and induce β-cell apoptosis (Cnop *et al.,* 2005). Markers of the immune destruction of the β-cell include islet cell autoantibodies, autoantibodies to insulin, autoantibodies to GAD (GAD65), and autoantibodies to the tyrosine phosphatases IA-2 and IA-2β. The detection of these markers in serum is currently the most reliable diagnostic test for type 1 diabetes (ADA, 2010). Islet autoantibodies are not thought to cause direct damage to the beta cells however, autoantibodies have been shown to enhance accumulation of islet-reactive CD4+ T cells and promote diabetes **(**Nokoff and Rewers, 2013).

Environmental factors that may influence Type 1 diabetes include drugs/chemical agents, food/diet and viral/bacterial infections. The mechanisms by which infectious environmental factors that cause Type 1 diabetes are probably by - molecular mimicry: some pathogen proteins share sequence or structural homology with self-proteins, for example, the rubella virus capsid protein which shares homology with a 52-kD pancreatic islet antigen can induce autoimmune reaction; bystander activation: The body's natural response to pathogenic agents could indirectly provide an environment conducive to the activation of autoreactive T lymphocytes and development of autoimmune disease; direct cellular injury; superantigens; immunoregulations **(**Kraine and Tisch, 1999).

1.5.2. Etiology and pathogenesis of Type 2 Diabetes Mellitus

The etiology of type 2 diabetes mellitus (T2DM) involves the induction of insulin resistance along with the disruption of pancreatic β-cell function and the loss of β-cell mass. The cause of type 2 diabetes is multifactorial. In addition to a genetic predisposition, lifestyle factors and environmental elements seem to have an important role (Alonso-Magdalena *et al.*, 2011).

The pathogenesis of type 2 diabetes is more variable, comprising different degrees of β-cell failure relative to varying degrees of insulin resistance (Cnop *et al.*, 2005). β-cell dysfunction and reduced insulin sensitivity play important roles to the pathogenesis of Type 2 diabetes. However, the mechanisms controlling the interplay of these two impairments are unclear. A majority of individuals suffering from type 2 diabetes are obese, with central visceral adiposity. Therefore, the adipose tissue should play a crucial role in the pathogenesis of type 2 diabetes (Scheen, 2003).

The impaired glucose transport into skeletal muscle and adipose tissues can result from a variety of mechanisms involving insulin receptor defects. Over expression of tumor necrosis factor α (TNF α) in muscle cells of obese individuals has been implicated as an inducer of insulin resistance. TNF α have direct effects on insulin signaling cascade. TNF α induce serine phosphorylation of insulin receptor substrate (IRS-1) and (IRS-2), resulting in the reduction in the ability of the IRS molecules to dock with receptor and interact with downstream pathway (Roith and Zick, 2001). Interleukin-6 (IL-6) also played an important role in the induction of insulin resistance in adipocytes by cross reacting its signal transduction with the insulin signaling network (Lagathu *et al.*, 2003). Chronic treatment with IL-6 to adipocyte can diminish expression of β subunit receptor, IRS-1 and GLUT4, resulting in reduced glucose transport. IL-6 also found to inhibit the insulin induced activation of β-subunit of insulin receptor, extracellular signal regulated kinases (ERK-1) and (ERK-2) (Jager *et al.*, 2007). Another mechanism leading to hyperglycemia in patients with Type-2 diabetes involves the inability to produce hepatic glucose (Jensen *et al.*, 2011).

1.6. Molecular genetics of Diabetes Mellitus

Diabetes mellitus both type 1 and type 2 are, in part, genetically determined. The degree of the genetic contribution to type 1 and type 2 diabetes differs considerably and genes that may contribute to risk for type 1 diabetes may differ from those that contribute to risk for type 2 diabetes (Rich, 2006). A large body of evidence suggests

that inherited genetic factors influence both susceptibility and resistance to the disease. Genetic susceptibility is clearly dependent on the degree of genetic identity with the proband. The highest risk of T1D in families is observed in monozygotic twins (100% sharing) followed by first and second degree relatives (50% and 25% sharing respectively) (Pociot and McDermott, 2002).

About 18 regions of the genome have been linked with influencing type 1 diabetes risk, labeled as IDDM1 to IDDM18. The best studied is IDDM1 next is IDDM2 and then other Type 1 Diabetes Susceptibility Loci: IDDM3–IDDM18. IDDM1 contains a cluster of HLA genes on chromosome 6 that encode immune response proteins (MHC). There are three classes of MHC molecules- MHC class I, class II and class III. The genes encoding class II MHC proteins are strongly linked with diabetes, and these genes are called HLA-DR (DR2, DR3 and DR4), HLA-DQ, and HLA-DP. Type 1 diabetes is unique among the autoimmune diseases in that HLA alleles may increase the risk of diabetes, have no effect, or even be protective. Individuals with both DR3 and DR4 are particularly susceptible to type 1 diabetes. On the contrary, the DR2 allele is protective. Similar to the DR gene, certain alleles of the DQ gene are risk factors for developing the disease, whereas other alleles of DQ are protective. There is also a tendency for people who inherit DR3 or DR4 to inherit DQ, which adds to their genetic risk of developing diabetes. Conversely, the protective alleles of DR and DQ tend to be inherited together. These tendencies have complicated the study of the effects of individual HLA-DR or HLA-DQ genes (Dean and McEntyre, 2004).

IDDM2 locus contains the insulin gene (INS). IDDM2 has been mapped to chromosome 11p15.5 close to CTLA4, which has a regulatory role in the immune response. Mutations of INS cause a rare form of diabetes that is similar to MODY (Maturity Onset Diabetes in the Young). Other variations of the insulin gene (variable number tandem repeats and SNPs) may play a role in susceptibility to type 1 and type 2 diabetes. The shortest (class I) variable number of tandem repeat (VNTR) alleles were found to increase, whereas the longest (class III) alleles were observed to decrease in the patients in comparison to the controls (Awata *et al.*, 2013).

Ample evidence suggested that T2DM has a complex genetic etiology. The concordance of type 2 diabetes in monozygotic twins is ~70% compared with 20–30% in dizygotic twins. The lifetime risk of developing the disease is ~40% in offspring of one parent with type 2 diabetes, greater if the mother is affected and

approaching 70% if both parents have diabetes (Lyssenko and Laakso, 2009). Genome wide scans have identified several potential chromosomal T2DM susceptibility regions. The best studied is a novel T2DM susceptibility gene calpain-10 (CAPN10) located at chromosome 2q37.3, identified through positional cloning and partitioning linkage. Three single-nucleotide polymorphisms (SNPs) (UCSNP43, UCSNP19, and UCSNP63), all located in intronic sequences, were found to be involved in increased risk of the disease (Tsai *et al.*, 2001). Variation in the non-coding region of the CAPN10 gene is associated with a threefold increased risk of type 2 diabetes in Mexican Americans. A genetic variant of CAPN10 may alter insulin secretion, insulin action, and the production of glucose by the liver. Other identified T2DM susceptibility genes are GCGR gene that encodes glucagon receptor, GLUT2 gene that encodes glucose transporter, INSR (the insulin receptor gene), the potassium channel gene (KCNJ11), PIK3RI gene that encodes lipid kinase that has a key role in insulin signaling, etc. (Dean and McEntyre, 2004).

1.7. Complications of diabetes

Hyperglycemia, in fact, leads to some metabolic abnormalities generating complications of the body system. Although long term complications of diabetes develop gradually, they can eventually be disabling or even life-threatening. The risk of complications of diabetes depends on both the duration and the severity of the diabetes (ADA, 2009).

The cells damaged by hyperglycemia are those that cannot reduce the transport of glucose inside the cell when exposed to high glucose level. The exact mechanisms that cause complications of diabetes are still unclear, but many theories have been postulated to explain the typical cause. Some well studied theories involved in cellular destruction are- a). The increased flux through the polyol pathway: Under high glucose concentration, aldose reductase reduces glucose to sorbitol consuming the cofactor NADPH which is required for generating intracellular antioxidant reduced glutathione. The polyol pathway thus makes the cells vulnerable to intracellular oxidative stress by reducing the amount of reduced glutathione. b). The intracellular production of advanced glycosylated end products (AGE) precursors: AGE precursors generated inside the cell under high glucose concentration can diffuse out of the cell and modify extracellular matrix molecules nearby and cause cellular dysfunction by changing signaling between the matrix and the cell. Also it can modify the circulating

proteins that bind to AGE receptors of a cell activating them, thereby producing inflammatory cytokines which in turn cause vascular pathology. c). The PKC (Protein Kinase-C) activation: Hyperglycemia inside the cell increases the synthesis of diacylglycerol molecule which is an activator of PKC that affects the normal gene expression. The consistent differentiating feature common to all cell types that are damaged by hyperglycemia is an increased production of reactive oxygen species (ROS) (Brownlee, 2005).

Generally the complications of diabetes can be separated into microvascular complications (involving small vessels such as capillaries) and macrovascular complications (involving large vessels such as arteries and veins). Both microvascular and macrovascular complications have similar etiologic characteristics (Cade, 2008).

1.7.1. The microvascular complications: It includes long-term complications of diabetes affecting small blood vessels. These classically have included retinopathy, nephropathy, and neuropathy (Zimmerman, 2010).

A). Diabetic Retinopathy (DR): Diabetic Retinopathy (DR) is a leading cause of visual disability and blindness in people with diabetes. The severity ranges from nonproliferative or priproliferative to proliferative DR. Nonproliferative DR is indicated by the presence of hemorrhages, microaneurysms and hard exudates. Proliferative DR is characterized by preretinal hemorrhages that appeared between the retina and the posterior hyaloids face, hemorrhages into the vitreous as clumps of blood dots that can cause total or partial vision loss, fibrovascular tissue proliferation, retinal detachment, impairment of retinal blood flow, increased inflammatory cell adhesion to retinal blood vessels and capillary blockage (Cade, 2008).

B). Diabetic Neuropathy: Diabetic Neuropathy is defined as signs and symptoms of peripheral nerve dysfunction in a patient with diabetes (Bansal *et al.*, 2006). Several types of nerves can be affected, like large-fiber sensory, small-fiber sensory, autonomic and motor, distal nerves, nerve roots, cranial nerves, etc. Some common syndromes of Diabetic Neuropathy are Diabetic Sensorimotor Polyneuropathy (DSP): mild distal sensory abnormalities and distal weakness due to nerve and blood vessel changes. In severe case, footdrop as well as other distal lower extremity weakness can occur; Diabetic Autonomic Neuropathy (DAN): results in many troublesome symptoms, including orthostatic hypotension, incontinence and erectile dysfunction; Acute Painful Diabetic Neuropathy with Weight Loss: also known as diabetic

cachexia. The illness begins with sudden, profound weight loss followed by severe pain, often burning, and excessive sensitivity to touch (allodynia) of the lower legs and feet (Tracy and Dyck, 2008).

C). Diabetic Nephropathy (DN): The first manifestation of DN is typically microalbuminuria, which progresses to macroalbuminuria and eventually to renal failure and is the cause of end-stage renal disease (Gross *et al.,* 2005). Diabetic nephropathy is characterized by excessive growth of extracellular matrix (ECM) with thickening of glomerular and tubular basement membranes and increased amount of mesangial matrix, which ultimately progress to glomerulosclerosis and tubulo-interstitial fibrosis (Kanwar *et al.,* 2008).

1.7.2. Macrovascular complications: Atherosclerosis (narrowing of the arterial walls reducing the space for blood to flow) is the central mechanism in macrovascular disease. Atherosclerosis results from chronic inflammation and injury to the arterial wall in the peripheral or coronary vascular system. Cardiovascular Disease (CVD) is collectively the disease of the heart and the blood vessels and it is the primary cause of death in people with diabetes. CVD is the cause of heart attack and stroke. Stroke is due to damage to blood vessels in the brain that may lead to disability, brain death, or death (Fowler, 2008).

1.8. Prevalence of diabetes

According to Diabetes Atlas published by International Diabetes Federation (IDF), the global prevalence of diabetes in 2014 is 8.3% (387 million people) out of which 46.3% is undiagnosed. The number of diabetic patient is estimated to reach 592 million by the year 2035. The highest prevalence of 11.4% is found in North America and Caribbean followed by Middle East and North Africa (9.7%), Western Pacific (8.5%), South East Asia (8.3%) and the lowest prevalence of 5.1% is found in Africa. Plate 1 presents the details of prevalence of diabetes. **77%** of people with diabetes live in low and middle income countries (IDF, 2014). The prevalence of diabetes is predicted to double globally in 2030 with a maximum increase in India. It is predicted that by 2030 diabetes mellitus may afflict up to 79.4 million individuals in India (Kaveeshwar and Cornwall, 2014).

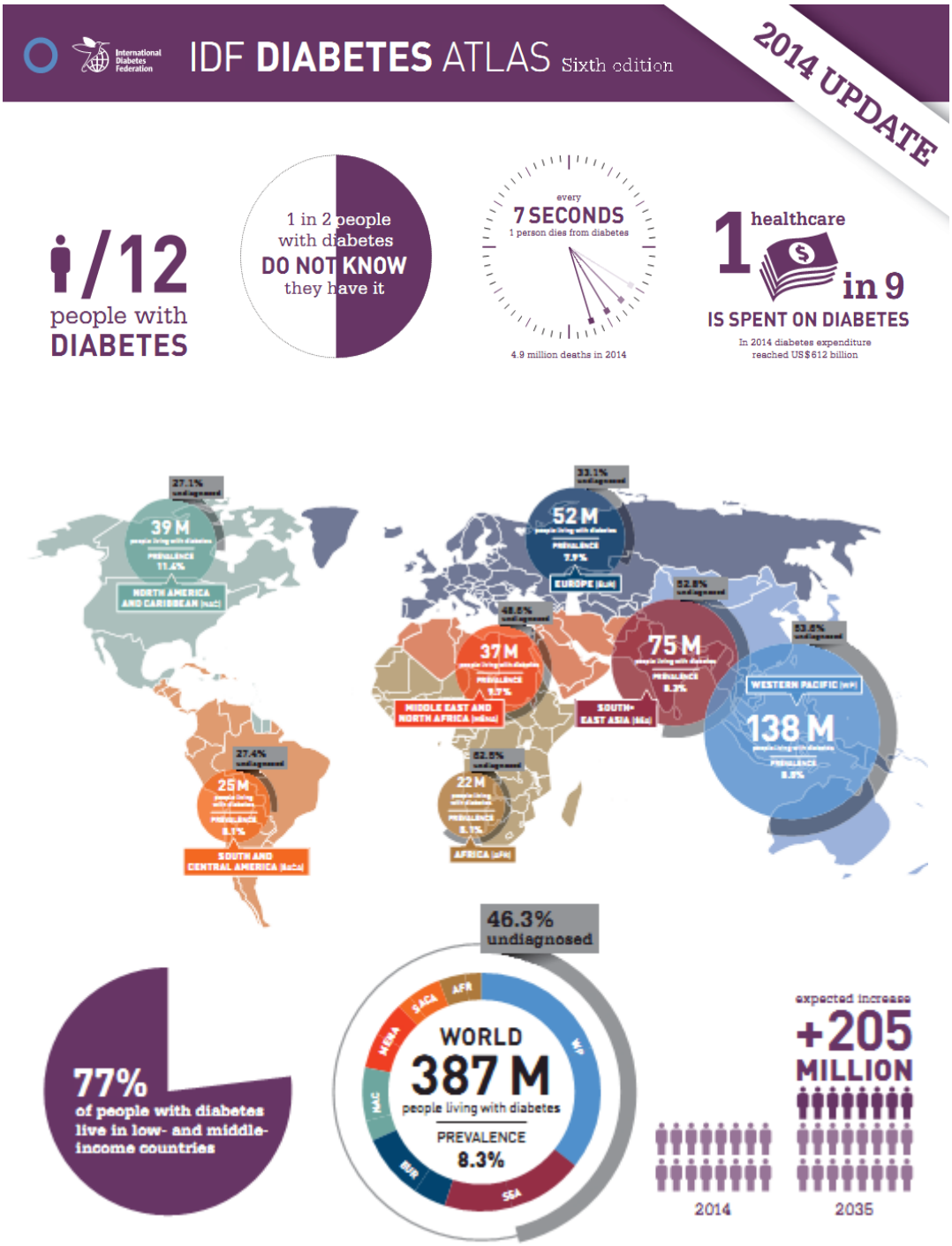

Plate 1: World prevalence of diabetes. Source: IDF Diabetes Atlas Sixth Edition Update, International Diabetes Federation 2014

1.9. Current treatment approaches to diabetes mellitus: success and shortcomings

The recommended first line therapeutic options for treating hyperglycemia include oral hypoglycemic agents and insulin sensitizers like metformin a biguanide that

reduces insulin resistance. Other effective medications include non-sulfonylurea secretagogues, thiazolidinediones, alpha glucosidase inhibitors, and insulin. Recent research into the pathophysiology of type 2 DM has led to the introduction of new medications like glucagon-like peptide 1 analogoues: dipeptidyl peptidase-IV inhibitors, inhibitors of the sodium-glucose cotransporter 2 and 11ß-hydroxysteroid dehydrogenase 1, insulin-releasing glucokinase activators and pancreatic-G-protein-coupled fatty-acid-receptor agonists, glucagon-receptor antagonists, metabolic inhibitors of hepatic glucose output and quick-release bromocriptine (Olokoba *et al.,* 2012). Antidiabetic drugs operate through diverse physiological pathways, for instance by stimulating insulin secretion through AMP-dependent kinase (AMPK) signalling pathway, activation of the peroxisome proliferator-activated receptor γ(PPARγ), a nuclear transcription protein that belongs to the family of PPARs, which regulate genes involved in lipid and glucose metabolism. Some drugs directly increase insulin secretion in the pancreas by inhibiting the ATP-sensitive potassium channel in β-cells (Boyda *et al.,* 2012). Although the Oral antidiabetic drugs are somehow successful in managing the diabetes, they are regrettably associated with unavoidable side effects, sometimes even life threatening. Sulfonylureas increase cardiovascular risk presumably by preventing protective ischemic cardiac preconditioning. Rosiglitazone increases risk of myocardial infarction and death possibly by increasing serum triglycerides and LDL-cholesterol levels. Muraglitazar increased risk of cardiovascular death, myocardial infarction, or stroke due to as yet unidentified reasons (Panicker *et al.,* 2012). Insulin sensitizing drugs like metformin however, reduce cardiovascular risk to some extent but may trigger microvascular complications (Panicker *et al.,* 2012, Kooy *et al.,* 2009). There is a high possibility of hypoglycemic risks among T2DM patients on OAD therapy with an impact on the long-term prognosis (Chen *et al.,* 2011). Certain drawbacks of current therapies like deleterious effects and high cost and poor availability of the current treatment for DM necessitates the identification of novel drugs which might act in mechanistically distinct way compared to existing drug targets (Palsamy and Subramanian, 2008). Scientific investigation on traditional herbal remedies for diabetes may provide valuable leads for the development of alternative drugs and therapeutic approaches. Hence the ethnomedicinal hypoglycaemic plants are being hunted vigorously by the scientific community (Marles and Farnsworth, 1995).

1.10. Antihyperglycemic plants: Source for better antidiabetic drugs of tomorrow

Herbs and plants have been used as a traditional form of medicine since time immemorial. They have been proven to be a good source of biological active compounds which are the foundations for the development of new chemicals for pharmaceuticals (Hotwani *et al.,* 2014). The use of medicinal plants to treat diabetes can be traced back to the era of discovery of diabetes. The oldest known records of treatment of diabetes using plants were found in Ebers Papyrus (3400 BCE) where elderberry, fibres of the asit plant, flower of the cucumber and green dates were described as part of the mixture for the treatment of diabetes (Sanders, 2002). In India antidiabetic plants are known to mankind since ancient times. Earliest historic records of antidiabetic plants in India are found in ancient medicinal book Samhita written as early as 4^{th} to 5^{th} century BCE. Sushruta Samhita, describes 760 species of antidiabetic plants, while Charaka Samhita describes 500 species (Saravanamuttu and Sudarsanam, 2012).

Ethnobotanical studies of traditional herbal remedies used for diabetes around the world have identified more than 1200 species of plants with hypoglycemic activity. These plants are broadly distributed throughout 725 different genera (Shekelle *et al.,* 2005). One third of the total plants identified i.e. around 410 plants, are experimentally proven to have antidiabetic properties but the complete mechanism of actions available only for about 113 plants (Prabhakar and Doble, 2008). Antidiabetic plants and compounds exert their antihyperglycemic effect through targeting one single mechanism or multiple mechanisms, some by improving insulin sensitivity, inhibiting carbohydrate digesting enzymes (Gray and Flatt, 1999), augmenting insulin secretion, β-cell rejuvenation, insulin-secretagogue activity (Patel *et al.,* 2012).

In developing countries like India, almost 90% of the people in rural areas still rely on traditional medicines for the primary health care (Marles and Farnsworth, 1995). Consequently, scientific investigation of traditionally used antihyperglycemic plant is needed for the better antidiabetic drugs and to ensure safety use of the plants.

1.11. Cassia alata Linn.: *An ethnomedicinal antihyperglycemic plant*

Cassia alata Linn. is commonly known as candle bush and is native of tropical America. The plant is a well recognised ethnomedicinal plant for antidiabetic activity and is practiced by different ethnic communities of North East India (Devi *et al.,* 2011, Prodyut *et al.,* 2013, Mohd and Yadava, 2010). Traditionally aqueous extract of

leaves and flowers are used to treat diabetes. Methanolic extract (Palanichamy *et al.*, 1988) and ethyl acetate extract (Villasenor *et al.*, 2002) of the plant have been revalidated to have antidiabetic activity. Very few literature of antidiabetic activity of *Cassia alata* is available worldwide. So far the scientific investigation of the plant is concerned; there is no report from North East India. Given this as background, the present study investigates the antihyperglycemic activity of the plant to generate a stronger biochemical rationale for confirming *Cassia alata* Linn. as a good source of antidiabetic drugs.

1.11.1. Taxonomic hierarchy of Cassia alata Linn.

Kingdom	Plantae
Division	Tracheophyta
Class	Magnoliopsida
Order	Fabales
Family	Caesalpiniaceae
Genus	*Cassia*
Species	*alata* (L.)

Plate 2: *Cassia alata* Linn.

1.12. Objectives of the work

The present study consist of the following objectives

a) Phytochemical screening of the plant's bioactive compounds.
b) Study of antihyperglycemic activity of the plant extracts –
    In vitro methods
    In vivo method
c) Molecular characterization of the plant.