Universidade de Lisboa
Faculdade de Ciências
Departamento de Biologia Animal

Family History of Diabetes: A risk analysis

Pedro André Mendes Mataloto

Dissertação

Mestrado em Biologia Humana e Ambiente

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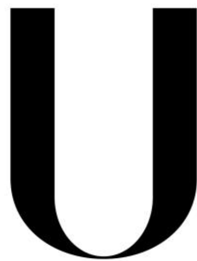

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2014

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List of abbreviations

ADA – American Diabetes Association

APDP – *Associação Protectora dos Diabéticos Portugueses* / Portuguese Diabetes Association

AUC – Area under the curve

BMI – Body mass index

CKD - chronic kidney disease

CVD - cardiovascular disease

DM – Diabetes mellitus

DPP-IV – Inhibitors of dipeptidyl peptidase 4

EGP - endogenous glucose production

ER - endoplasmic reticulum

FADH - Flavin adenine dinucleotide

FDR - first degree relatives

FFA - Free fatty acids

FHD – Family History of diabetes

FLI – Fatty liver index

FPG – Fasting Plasma Glucose

GADAs - autoantibodies to glutamic acid decarboxylase

GIP - glucose-dependent insulinotropic peptide

GLP-1 - Glucagon-like peptide-1

HDL - high-density lipoprotein

HIC – Hepatic insulin clearance

HIEC - Hyperinsulinemic Euglycemic Clamp

HOMA-IR - homeostatic model assessment homeostasis model assessment-estimated insulin resistance index

IA2As - autoantibodies to transmembrane tyrosine phosphatase

IAAs - insulin autoantibodies

ICAs - islet cell autoantibodies

IDE - insulin-degrading enzyme

IFG - Impaired fasting glucose

IFG+IGT – Impaired fasting glucose plus impaired glucose tolerance

IGT – Impaired glucose tolerance

IR - Insulin resistance

IVGTT - intravenous glucose tolerance test

LADA - Latent autoimmune diabetes of the adult

LDL - Low-density lipoprotein

NADH - Nicotinamide adenine dinucleotide

NAFLD - nonalcoholic fatty liver disease

NAFLD – Nonalcoholic fatty liver disease

NASH - nonalcoholic steatohepatitis

NFS – NAFLD fibrosis score

NGT – Normal glucose tolerance

OGIS – Oral Glucose Insulin Sensitivity

OGTT – Oral glucose tolerance test

OR - odds ratio

PDI - protein disulfide isomerase

T1D – Type 1 diabetes

T2D – Type 2 diabetes

T2D-siblings – Siblings of type 2 diabetics

TAG - triacylglycerol

TCA - tricarboxylic acid

WHO – World Health Organization:

ZnT8As - antibodies against the ZnT8 molecule

Abstract

Family history of diabetes (FHD) has been recognized over the years as an important risk factor of the disease. Thus herein we evaluate the importance of FHD in the development of the three more prevalent types of diabetes, namely type 1 diabetes (T1D), Latent autoimmune diabetes of adults (LADA) and type 2 diabetes.

Our research study can be divided in two different parts: study 1, a clinico-epidemiological study with a sample of 16.874 clinic files of people with diabetes (T1D, LADA and T2D); study 2, a preliminary clinic study with 23 siblings of type 2 diabetic patients. The metabolic profile of siblings was assessed with the performance of a modified OGTT with blood collections (0, 30, 90 and 120 mins).

The results of study 1 indicated that the presence of a sibling with T1D or a sibling with T2D is the high risk factor among FHD to develop T1D (14-fold) or T2D (6-fold), respectively. The high risk factor to develop LADA is the presence of different types of diabetes in parents (11-fold). The study 1 also indicated that type 2 diabetes was the prevalent type of diabetes among the siblings of each type of people with diabetes evaluated. Thus, in study 2 we addressed the hypothesis that siblings of type 2 diabetics have a risk to develop diabetes and for that we evaluated their metabolic profile. The results of this study indicated a high prevalence of obesity among the siblings-T2D, mainly visceral, high levels of LDL-cholesterol, low levels of HDL-cholesterol as well as high prevalence of fatty liver disease. These features can be related with a lower decrease of hepatic insulin clearance in the first 30 minutes of the OGTT.

It was concluding that presence of diabetes in siblings is the high risk factor to develop T1D and T2D, and this fact can be related with impairment in hepatic insulin clearance process.

Keywords

Family History of Diabetes; First-degree relatives ; Siblings; Diabetes Mellitus; Insulin Clearance; Modified OGTT.

Sumário

A presença de história familiar de diabetes (HFD) está descrita como um factor de risco importante para o desenvolvimento da doença. A HFD representa uma informação genómica valiosa porque caracteriza as interacções combinadas entre os factores ambientais, comportamentais e genéticos. Este é também um factor que tem apresentado elevada variabilidade entre populações, sendo de esta forma fundamental caracterizar a HFD de uma amostra da população portuguesa - pacientes da maior clínica de diabetes em Portugal, a Associação Protectora dos Diabéticos de Portugal (APDP).

O presente trabalho de investigação cientifica pode ser dividido em duas partes: estudo 1, foi um estudo clínico/epidemiológico, que consistiu na analise da HFD dos pacientes acompanhados na APDP num período de 5 anos (2009-2013). A amostra foi composta por um total de 16.874 fichas clínicas, englobando pessoas com diabetes tipo 1 (n=3013), pessoas com diabetes Latente Autoimune do Adulto (n=373) e pessoas com diabetes tipo 2 (n = 13488); e estudo 2, - um estudo clinico preliminar, com uma amostra de 23 irmãos de pessoas com diabetes tipo 2. O perfil metabólico de resposta a uma prova modificada de tolerância oral à glicose foi avalaliado ao longo do tempo em com 4 pontos escolhidos previamente (0, 30, 90, 120 minutos).

No estudo 1, foi observado que o maior risco em termos de HFD de primeiro grau para o desenvolvimento de diabetes tipo 1 é a presença de um irmão com diabetes tipo 1 (14 vezes), enquanto o factor familiar de maior risco para o desenvolvimento de diabetes tipo 2 é a presença de um irmão com diabetes tipo 2 (6 vezes). Nos indivíduos com LADA foi detectado que o maior risco de desenvolver este tipo de diabetes, ocorre na presença de pais com diabetes de diferentes tipos. Foi também interessante observar as respetivas médias de idade do aparecimento dos três tipos de diabetes que se ajustam ao já observado na literatura. Tendo em conta estes resultados, nomeadamente a importância do parentesco entre irmãos constituir um fator de risco para a ocorrência de diabetes, e ainda pelo facto de a prevalência de irmãos com diabetes tipo 2 ser mais elevada em todos os tipos de pacientes analisados, foi decidido proceder-se ao estudo 2. Ou seja, com o estudo 1 foi possível perceber quais os grupos de maior risco para desenvolver a doença, enquanto que com o estudo 2 foram analisadas possíveis alterações metabólicas associadas a um elevado risco de desenvolver diabetes que pode ser detectado nos irmãos de pessoas com diabetes tipo 2. Os resultados dos estudos

revelaram uma elevada prevalência de obesidade, principalmente de tipo visceral, níveis elevados de colesterol LDL e níveis baixos de colesterol HDL, bem como uma prevalência de perto de 100 % de esteatose hepática. Estas alterações metabólicas que estão intrinsecamente ligadas à diabetes tipo 2 parecem estar relacionadas com uma menor diminuição da *clearance* nos primeiros 30 minutos da prova de tolerância à glicose oral.

Este estudo permitiu concluir que os irmãos das pessoas com diabetes tipo 2 são de facto um grupo de risco para o desenvolvimento da doença, e que as diversas alterações metabólicas parecem estar relacionadas com um funcionamento inadequado do processo de *clearance* da insulina.

Palavras-chave

Historia Familiar de Diabetes; Parentes de primeiro grau; Irmãos; Diabetes Mellitus; *Clearance* da insulina; PTGO

1. State of the art

1.1 Glucose Homeostasis

Glucose is an essential metabolic substrate of all mammalian cells. Glucose is the major carbohydrate presented to the cell for energy production and many other metabolic requirements. Glucose and other monosaccharides are transported across the intestinal wall to the hepatic portal vein and then to liver cells and other tissues. Then, they are converted to fatty acids, amino acids, and glycogen, or are oxidized by the various catabolic pathways of cells to generate energy necessary to supply all the metabolic process. The low blood concentrations of glucose can causes seizures, loss of consciousness, and death. However, long lasting elevation of blood glucose concentrations, can result in, renal failure, blindness, neuropathy and vascular disease. Therefore, blood glucose concentrations need to be maintained within narrow limits. The process of maintaining blood glucose at a steady-state level is called glucose homeostasis (Wood et al., 2003).

Although several factors are involved in regulation of carbohydrate metabolism (Fig 1), this physiological process is mainly maintained through the antagonistic action of two polypeptide hormones: glucagon and insulin. The liver is the major metabolic regulatory organ. About 80% of all circulating glucose that not derived directly from the diet comes from the liver. Glycogenolysis, the breakdown of glycogen (the polymerized storage form of glucose) and gluconeogenesis (the formation of glucose primarily from lactate and amino acids during the fasting state) are hepatic processes under the control of glucagon resulting in elevated plasma glucose levels. Insulin has the opposite effect by promoting the uptake of glucose by cells, mainly in liver, muscle and adipose tissue, with consequently decrease of glucose levels. There are a number of external signals that can contribute to insulin release, particularly increase in plasma glucose levels. There is considerable clinical interest in the mechanism and control of insulin secretion because this process normally declines during the onset of insulin resistance that leads to develop of diabetes (Huang et al., 2007).

Plasma glucose concentration is a function that depends on the glucose entering in the circulation balanced by the rate of glucose that is remove from the circulation. Circulating glucose is derived essentially from three sources: intestinal absorption during the fed state, glycogenolysis, and gluconeogenesis. The factor determinant of how quickly glucose appears in the circulation during the fed state is the rate of gastric

emptying. During the fasting state glycogenolysis and gluconeogenesis are partly under the control of glucagon. After a meal, the glucose levels depend especially of insulin action in the several tissues. Thus, all these processes in order to maintain glucose within a narrow range, are regulated by the effect of different glucoregulatory hormones that include insulin, glucagon, amylin, glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic peptide (GIP), epinephrine, cortisol, and growth hormone. Of these, insulin and amylin are derived from the β-cells, glucagon from the α-cells of the pancreas, and GLP-1 and GIP from the L-cells of the intestine (Shrayyef, 2010).

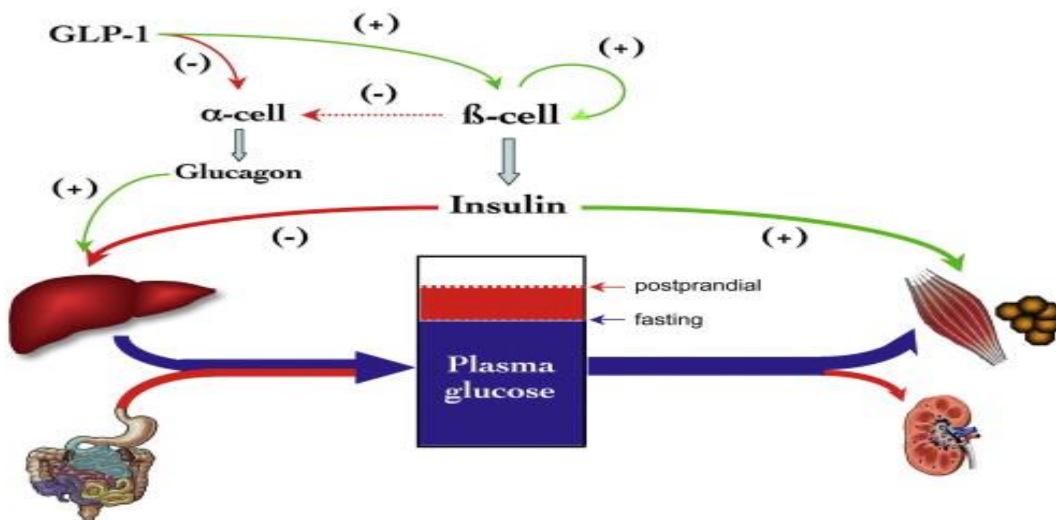

Figure 1– Plasma glucose: primary hormonal control. GLP-1 inhibits the production of glucagon in α-cell and stimulates the production of insulin by β-cell. Insulin inhibits the production of glucose by liver and promotes the uptake of glucose in insulin-dependent tissues like adipose tissue and skeletal muscle. Glucagon promotes the production of glucose in liver by glycogenolysis and gluconeogenesis (Adapted from Drucker, 2006).

In the fasting state, glucose leaves the circulation at a constant rate. With the aim to compensate the decrease of glucose levels, endogenous glucose need to be produce. The liver is responsible for approximately 80% of glucose release into the circulation in the post-absorptive state, via glycogenolysis and gluconeogenesis. Under these conditions, and in an initial phase ~50% of the glucose entering the circulation is due to glycogenolysis and the reminder (~5.0 µmol/kg/min) to gluconeogenesis, whose the major precursors are lactate, glycerol, glutamine, and alanine. The majority amino acids released from skeletal muscle protein are converted to alanine and glutamine for

transport through plasma to liver and kidney: alanine being selectively used by liver, glutamine being preferentially used in the kidney, while lactate and glycerol used to roughly comparable extent by both organs. In the resting post-absorptive state, lactate is the major gluconeogenic precursor, accounting for about half of all gluconeogenesis (Gerich J, 1993). The proportion owing to gluconeogenesis rapidly increases with the duration of fasting, as glycogen stores become depleted; 24 h after the last meal, gluconeogenesis accounts for about 70% of all glucose released into the circulation, whereas by 48 hours it accounts for over 90%. Although the kidney be able to store glycogen, renal cells lack glucose-6-phosphatase to produce glucose. Thus, all the glucose released by the kidney is the result of gluconeogenesis. Under normal conditions gluconeogenesis in the kidney provides only a small contribution to the total circulating glucose; however, during prolonged starvation, the kidney contribution may approach that of the liver. Kidney have yet another function in the maintenance of glucose homeostasis once that some of the glucose that passes by this organ need to be efficiently reabsorbed to prevent losses (Landau et al., 1996; Shrayyef & Landau , 2010).

The muscle is not able to release glucose into circulation; however, its ability to rapidly increase its glucose uptake is critical decrease plasma glucose after a meal. Skeletal muscle has an additional role in maintaining plasma glucose levels: it releases free amino acids into circulation to serve as substrates for liver gluconeogenesis. The muscle can use glucose, fatty acids, and ketone bodies for energy. The muscle normally maintains significant amounts of stored glycogen, small amounts of fatty acids, and contains a large pool of protein that can be broken down in emergencies. The resting muscle uses fatty acids as its primary energy source; however, glucose (from its own glycogen stores and from circulation), is preferred for rapid energy generation (e.g. in sudden exercise). Adipose tissue can give too an important contribution in fed state, supplying free fatty acids and glycerol to the circulation to be taken up by the liver as substrate to gluconeogenesis. As was mention glucagon is the main hormone involve in this process, however epinephrine has too an important role in this process, for instance Glucagon, which increases both glycogenolysis and gluconeogenesis in the liver, however, has no effect on the kidney. Epinephrine, which can directly activate hepatic glycogenolysis, appears to increase glucose release, predominantly by directly stimulating renal gluconeogenesis and, to a lesser extent, by increasing availability of gluconeogenic precursors/activators (e.g., glycerol and free fatty acids) (Brant, 1999). .

In the immediate post-feeding state, the glucose is remove manily into skeletal muscle and adipose tissue, being this process regulated by insulin. At the same time, endogenous glucose production need to be suppressed, what occur by 1) the direct action of insulin, delivered via the portal vein, on the liver, and 2) the paracrine effect or direct communication within the pancreas between the β- and α-cells, which results in glucagon suppression. There are various factors that can affect glucose levels after a meal ingestion. These include the time and the degree of physical activity since the last meal; the composition and form (liquid vs. solid); rate of gastric emptying; digestion within the lumen of the small intestine; absorption into the portal vein; extraction by the liver; suppression of endogenous glucose release; and finally the uptake, storage, oxidation, and glycolysis of glucose in post-hepatic tissues (Aronoff, 2004). Glucose taken up by tissues postprandially can be driven by two ways: immediately stored or undergoes glycolysis. Of the glucose undergoing glycolysis, some will be oxidized; the remainder will undergo non-oxidative glycolysis leading to the formation of pyruvate, lactate, and alanine. These 3-carbon compounds will then be available to undergo gluconeogenesis and either be stored in glycogen via the indirect pathway or be released into plasma as glucose. In figure 2 is possible analyze the pathways for disposal of a mixed meal containing 78 g of glucose. During the 6-h postprandial period, a total of ~98 g is available, that is more than the glucose contained in the meal. This occur due to persistent endogenous glucose release (~21 g): Splanchnic tissues initially took up ~23 g, and an additional ~75 g were removed from the systemic circulation. Direct glucose storage accounted for ~32 g and glycolysis ~66 g (oxidative ~43 g and non-oxidative ~23 g). About 11 g of glucose appeared in plasma as a result of gluconeogenesis. This indicates that glycolysis is the main initial postprandial source of glucose, accounting for ~66% of overall disposal. Oxidation and storage each account for about 45% (Shrayyef, & Gerich , 2010).

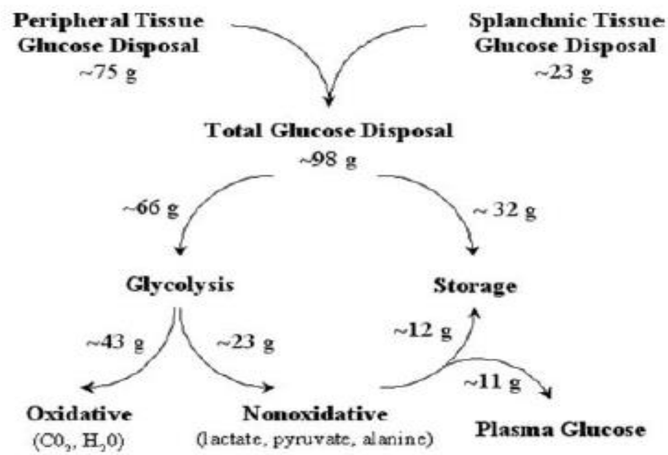

Figure 2 - Summary of sites and routes of postprandial glucose disposal (adapted from Woerle et al. 2003)

1.1.1 Glucose Utilization

The majority of glucose is metabolized mainly in six tissues: the brain (45–60%), skeletal muscle (15–20%), kidney (10–15%), blood cells (5–10%), splanchnic organs (3–6%), and adipose tissue (2–4%). Glucose taken up by the brain is completely oxidized whereas that taken up by the kidney, blood cells, splanchnic tissues, and muscle mainly undergoes glycolysis (Boden, 1997). A summarize of glucose utilization in the postabsortive state can be viewed in Figure 3 and Table 1.

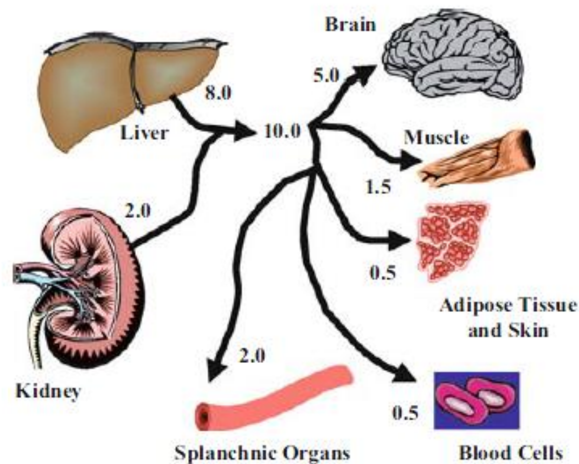

Figure 3 - Glucose utilization and production in the postabsorptive state. The liver and kidney contribute approximately 8.0 and2.0 µmol/kg/min, respectively; top, the total release of glucose into the circulation (10 µmol/kg/min); the brain, splanchnic tissue, muscle, adipose tissue, and blood cells account for approximately 5.0, 2.0, 1.5, 0.5, and 0.5 µmol/kg/min, respectively. (From Gerich, 2010)

Table 1- Glucose disposal in the postabsorptive state (Shrayyef, & Gerich , 2010).

	Rate (µmol/kg/min)	% of total
Overall	10	100
Oxidation	~7	~70
Glycolysis	~3	~30
Tissues		
Brain	5	~50
Skeletal muscle	2	~20
Splanchnic organs	1	~10
Kidney	1	~10
Adipose Tissue	0.5	~5
Blood cells	0.5	~5

Not all glucose uptake is mediated by the action of insulin - brain, blood cells, renal medulla, and splanchnic tissue occurs largely independent of insulin, and plasma insulin concentrations are low in the post-absorptive state (<10 µU/ml). Under these conditions, amount of glucose removed from the circulation is determined almost exclusively by different factors like tissue demands, the mass action effect of the plasma glucose concentration per se, and the number and characteristics of the glucose transporters in specific tissue rather than by insulin. Insulin may be viewed as playing a permissive role, while counter-regulatory hormones that antagonize the action of insulin (e.g., cortisol, growth hormone, epinephrine, and thyroid hormones) can be viewed as modulating the sensitivity of tissue to the effect of insulin on tissue glucose uptake and utilization (Berridje, 2014).

1.1.2 Glucose Uptake

The uptake of glucose by the cells is a fundamental process mediated by two classes of transporter: (1) GLUT family: These transporters facilitated the diffusion of glucose, a process that is not energy dependent and that follows Michaelis–Menton kinetics. The high-affinity transporters (GLUT 1, 3, 4) have a Michaelis–Menton constant (Km) below the normal range of blood glucose concentrations and are capable of providing glucose transport under basal conditions for many cells. GLUT3 is localized mainly in neurons (lowest Km) whereas GLUT4 mediates insulin stimulated glucose uptake by skeletal muscle, heart, and adipose tissues. Insulin and exercise promote GLUT3 expression on cell surface. The low-affinity transporters (GLUT2) are present on ß-cells and in tissues exposed to large glucose fluxes, such as intestine, liver, and kidney. (Bouche et al., 2004) (2) SGLT family: These transporters utilize he

electrochemical sodium gradient to transport glucose against concentration gradients and are prominent in intestine and kidney. SGLT1 is responsible for the dietary uptake of glucose from the small intestine lumen whereas SGLT2 plays a major role in glucose reabsorption from proximal renal tubule (Moran et al., 2010).

In the last years the number of these glucose, and other sugar, transporters identified has increased considerably, mainly the GLUT, with major implications for the control of the delivery of glucose to mammalian cells. Among these facilitative transporters, GLUT 4 can be highlighted especially because is the major mediator of glucose removal from the circulation and a key regulator of whole-body glucose homeostasis, being its action stimulated by insulin. The way how insulin stimulates the translocation of GLUT 4 for plasma membrane in order to allow the glucose transport to inside of cells have been further studied: Insulin binding to its receptor leading to receptor autophosphorylation on tyrosine residues and the tyrosine phosphorylation of insulin receptor substrates (IRS-1, IRS-2 and IRS-3) by the insulin receptor tyrosine kinase. This allows association of IRSs with the regulatory subunit of phosphoinositide 3-kinase (PI3K) through its SRC homology 2 (SH2) domains. Once activated, the catalytic subunit phosphorylates phosphoinositides at the 3¢ position of the inositol ring or proteins at serine residues. PI3K activates PtdIns(3,4)P2 / PtdIns(3,4,5)P3-dependent kinase 1 (PDK1), which activates PKB/Akt, a serine kinase. PKB in turn deactivates glycogen synthase kinase 3 (GSK-3), leading to activation of glycogen synthase and thus glycogen synthesis. Activation of PKB also results in the translocation of GLUT-4 vesicles from their intracellular pool to the plasma membrane, where they allow uptake of glucose into the cell. PKB also leads to mTOR-mediated activation of protein synthesis by PHAS/elf4 and p70s6k (Fröjdö et al., 2009).

1.1.3 Insulin

Insulin, is a small protein composed of two polypeptide chains containing 51 amino acids. The monomeric structure of insulin consists of the 21 amino acid residue “A” chain and 30 amino acid residue “B” chain bound by disulfide linkages. The monomer presents three disulfide linkages, including two between the A and B chains (A7-B7, A20-B19) and one within the A chain (A7-A11). Insulin regulates glucose metabolism by direct and indirect actions. Through binding to its receptors in the liver, kidney, muscle, and adipose tissue, insulin activates its signaling pathway which involves a complex cascade of protein kinases and regulatory proteins of which IRS-1

and IRS-2 are the most important. The primary action of insulin is to stimulate glucose clearance. Insulin helps control post-prandial glucose in multiple ways. Initially, insulin signals the cells of insulin-sensitive peripheral tissues, promoting mainly the translocation of glucose transporters in muscle and adipose tissue; Also, insulin acts on the liver to promote glycogenesis; Insulin simultaneously inhibits glucagon secretion from pancreatic α-cells, thus signaling the liver to stop producing glucose via glycogenolysis and gluconeogenesis (Alarcón et al., 2002). Insulin acts too, promoting the inhibition of release of FFA into the circulation due to suppression of the activity of hormone-sensitive lipase and a simultaneous increase in their clearance from the circulation. Although insulin does not increase glucose transport into liver, it promotes glycogen accumulation by inhibiting glucose-6-phosphatase and phosphorylase (glycogenolysis enzymes) while stimulating glycogen synthase. All of these actions reduce blood glucose. Other actions of insulin include the stimulation of fat synthesis, promotion of triglyceride storage in fat cells, promotion of protein synthesis in the liver and muscle, and cell growth. Insulin action is carefully regulated in response to circulating glucose levels. Insulin is not secreted if the blood glucose concentration is lower than 3.3 mmol/l, but is secreted in increasing amounts as glucose concentrations increase beyond this threshold. The process of secretion of insulin occurs in two phases: an initial rapid release of preformed insulin, followed by a second longer phase that imply new insulin synthesis and its release. Long-term release of insulin occurs if glucose concentrations remain high. Beside glucose is the most potent stimulus of insulin, there are other factors that stimulate insulin secretion. These additional stimuli include increased plasma concentrations of some amino acids, especially arginine, leucine, and lysine; incretines; and parasympathetic stimulation via the vagus nerve (Fu, 2013).

1.1.3.1 Insulin Biosynthesis

The secreted insulin consists of 51 amino acids with a molecular weight of 5.8 kDa. However, the insulin gene encodes a 110-amino acid precursor known as preproinsulin. As with other secreted proteins, preproinsulin contains a hydrophobic N-terminal signal peptide, which interacts with cytosolic ribonucleoprotein signal recognition particles (SRP). SRP facilitates preproinsulin translocation across the rough endoplasmic reticulum (rER) membrane into the lumen. This process occurs via the peptide-conducting channel, where the signal peptide from preproinsulin is cleaved by a

signal peptidase leading to a production of proinsulin. Proinsulin then undergoes folding and formation of three disulfide bonds, a process requiring a diverse range of endoplasmic reticulum (ER) chaperone proteins. Subsequent to maturation of the three dimensional conformation, the folded proinsulin is transported from the ER to the Golgi apparatus where proinsulin enters immature secretary vesicles and is cleaved to yield insulin and C-peptide. Insulin and C-peptide are then stored in these secretory granules together with islet amyloid polypeptide (IAPP or amylin) and other less abundant β-cell secretary products. Although insulin biosynthesis is controlled by multiple factors, glucose metabolism is the most important physiological event that stimulates insulin gene transcription and mRNA translation. Insulin biosynthesis is regulated both at transcriptional and translational levels. Insulin content in β-cells is highly dynamic. Insulin accumulates in the presence of nutrients and decreases in response to nutrient deprivation. The ability of β-cells to quickly respond to cellular signals is generally due to transcriptional regulation (Suckale et al., 2008; Huang &Arvan, 1995).

1.1.3.2 Regulation of insulin secretion

The signal that leads to insulin secretion is absolutely depend on glucose levels. Glucose is internalized by the pancreatic β-cell through the plasma membrane transporter GLUT2. Once in the β-cell cytosol, glucose promotes glycolysis to generate ATP, NADH and pyruvate. NADH can be shuttled into the mitochondria to produce ATP at the electron transport chain. Pyruvate is directly transported into the mitochondria, where it is metabolize by the tricarboxylic acid (TCA) cycle to generate NADH and FADH equivalents that produce additional ATP. The increase in cytosolic ATP levels, leads to a increase in ATP/ADP ratio, a trigger essential to insulin exocytosis. The increase in the ATP/ADP ratio causes the closure of ATP-sensitive K+-channel, which depolarizes the β-cell plasma membrane, with a subsequent opening of voltage sensitive L-type Ca2+-channels, and the influx of extracellular Ca2+. The rise in intracellular cytosolic Ca2+ concentration acts as a major signal to trigger insulin exocytosis (Alarcón et al., 2002).

1.1.3.3 Insulin secretion

Hyperglycemic clamps and experiments in isolated pancreatic islets have demonstrated that glucose induces insulin secretion in a biphasic pattern: an initial

component (first phase), which occurring within the first 10 minutes, followed by a sustained component (second phase) with a progressively slow increase in insulin secretion reaching a plateau in 2-3 hours as seen in rats and humans (Gerich, 2002). Loss of first-phase secretion and reduced second-phase secretion are characteristic features of type 2 diabetes mellitus (T2D); it is well known that a decrease in the first phase of insulin secretion is found in the early stage of T2D and also in impaired glucose tolerance (IGT) (Davis et al. 1993). Thus, in order to understand the pathogenesis and pathophysiology of these diseases, it is important to clarify the cellular and molecular mechanisms responsible for the alterations in the dynamics of insulin secretion. Biochemical experiments and capacitance measurement have suggested that secretory vesicles generally exist in functionally distinct pools and that sequential release of these pools underlies the dynamically separable components of exocytosis (Rorsman & Renström, 2003). Pancreatic β cells contain at least two pools of insulin secretory granules that differ in release competence: a reserve pool (RP), which accounts for the vast majority of granules, and a readily releasable pool (RRP), which accounts for the remaining granules (less than 5%). The prevailing hypothesis is that the release of RRP granules accounts for the first phase of insulin secretion and that mobilization of a subsequent supply of new granules for release by mobilization accounts for the second phase. Thus, the initial amount of insulin released upon glucose absorption is dependent on the amounts available in storage. Once depleted, a second phase of insulin release is initiated. This latter release is prolonged since insulin has to be synthesized, processed, and secreted for the duration of the increase of blood glucose. Furthermore, beta cells also have to regenerate the stores of insulin initially depleted in the fast response phase (Seino et al., 2011).

1.1.4 Glucagon

Glucagon is a key catabolic hormone constitute by 29 amino acids. It is secreted from pancreatic α-cells and is particularly important in stimulating glycogenolysis, gluconeogenesis, and ketogenesis. Described by Roger Unger in the 1950s, glucagon was characterized as opposing the effects of insulin. Glucagon plays a major role in sustaining plasma glucose during fasting conditions by stimulating hepatic glucose production (Berridge, 2014).

Like insulin, glucagon is secreted first into the portal blood and is therefore anatomically in a favorable position to regulate hepatic metabolism. Glucagon act mainly in the liver, however it appear that have some glycogenolytic action on cardiac and skeletal muscle and lipolytic action on adipose tissue, and it promotes the breakdown of protein by several tissues. However, these effects on protein tissue breakdown appear to be more prominent when tissues are exposed to pharmacological concentrations of glucagon. At more physiological concentration, the liver appears to be the major target tissue. In many circumstances, the actions of glucagon antagonize those of insulin. However unlike the cellular mechanism of action of insulin, the mechanism of glucagon action is not so completely understood.

Hepatic glucose production, which is primarily regulated by glucagon, is fundamental to maintain the basal blood glucose levels within a normal range during the fasting state. When plasma glucose decrease below the normal range, glucagon secretion increases, resulting in hepatic glucose production and return of plasma glucose to the normal range. This endogenous source of glucose is not necessary during and immediately following a meal, and glucagon secretion is suppressed. When coupled with insulin's direct effect on the liver, glucagon suppression results in a near-total suppression of hepatic glucose output. In the diabetic state, there is inadequate suppression of postprandial glucagon secretion (hyperglucagonemia) which results in elevated hepatic glucose production, contributing determinately to hyperglycemia (Fu, 2013).

1.1.5 Incretin hormones: GLP-1 AND GIP

By the late 1960s, Perley and Kipnis and others demonstrated that ingested food caused a more potent release of insulin than when glucose was infused intravenously. This effect, named "incretin effect," suggested that exist a signal from the gut that are important in the hormonal regulation of glucose clearance. Further, these hormonal signals from the proximal gut seemed to help regulate gastric emptying and gut motility. Several incretin hormones have been characterized, however the most well studied and characterized are GIP and GLP-1. GIP stimulates insulin secretion and regulates fat metabolism, but does not inhibit glucagon secretion or gastric emptying. GIP levels are normal or slightly elevated in people with type 2 diabetes.

GLP-1 is secreted in greater concentrations and is more physiologically relevant in humans. GLP-1 also stimulates glucose dependent insulin secretion but is significantly reduced post-prandially in people with type 2 diabetes or impaired glucose tolerance. In contrast to GIP, GLP-1 inhibits glucagon secretion and slows gastric emptying. GLP-1 has many glucoregulatory effects. In the pancreas, GLP-1 stimulates insulin secretion in a glucose-dependent manner while inhibiting glucagon secretion (become this away a target to therapies). Both GIP and GLP-1 are rapidly inactivated by inhibitors of dipeptidyl peptidase 4 (DPP-IV) in vivo (Campbell & Drucker, 2013).

1.2 Diabetes mellitus

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in the main processes involved in regulation of insulin levels: insulin secretion, insulin action, and insulin clearance (Al Ali et al., 2013; Duckworth, 1998). The prevalence of diabetes is increasing rapidly worldwide and the International Diabetes Federation has predicted that by 2030 the number of adults with diabetes would have almost doubled worldwide, from 387 million in 2013 to 592 million (IDF, 2013). Diabetes is also costly to health care systems. People with diabetes have more outpatient visits, use more medications, have a higher probability of being hospitalized, and are more likely to require emergency and long-term care than people without the disease. The global health expenditure on diabetes was 612 billion dollars in 2014, 11% of total spending on adults (IDF, 2013). Diabetes is now one of the most common non-communicable diseases globally. It is the fourth or fifth leading cause of death in most high-income countries and there is substantial evidence that it is epidemic in many low and middle income countries. Complications from diabetes are resulting in increasing disability, reduced life expectancy and enormous health costs for virtually every society. Diabetes is certain to be one of the most challenging health problems in the 21st century. Thus, it is important channeling health resources, emphasize the importance of lifestyle changes, and encourage other measures to counteract trends for increasing prevalence of diabetes (Dieren et al.2010).

The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels. Several pathogenic processes are involved in the development of diabetes. These range from autoimmune destruction of the β-cells of the pancreas with

consequent insulin deficiency to abnormalities that result in resistance to insulin action. In this disease, both carbohydrate, lipid and protein metabolism are impaired, which leads to development of specific microvascular complications and of non-specific macrovascular disease. The basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes is the result deficient action of insulin on target tissues. Deficient insulin action results from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action. Impairment of insulin secretion and defects in insulin action frequently coexist in the same patient, and it is often nuclear which abnormality, if either alone, is the primary cause of the hyperglycemia. Symptoms of marked hyperglycemia include polyuria, polydipsia, weight loss, sometimes with polyphagia, and blurred vision. Impairment of growth and susceptibility to certain infections may also accompany chronic hyperglycemia. Acute, life-threatening consequences of uncontrolled diabetes are hyperglycemia with ketoacidosis or the nonketotic hyperosmolar syndrome. Long-term complications of diabetes include retinopathy with potential loss of vision; nephropathy leading to renal failure; peripheral neuropathy with risk of foot ulcers, amputations, and Charcot joints; and autonomic neuropathy causing gastrointestinal, genitourinary, and cardiovascular symptoms and sexual dysfunction (Ozougwu, 2013 & Valdez et al., 2007). Patients with diabetes have an increased incidence of atherosclerotic cardiovascular, peripheral arterial, and cerebrovascular disease. Hypertension and abnormalities of lipoprotein metabolism are often found in people with diabetes (Lüscher et al, 2003). Insulin clearance is an integral component of insulin metabolism, as it regulates the cellular response to the hormone by decreasing insulin availability and mediates certain aspects of insulin action. The liver is the primary site of insulin clearance. Approximately 80% of endogenous insulin is removed by the liver, and the remainder is cleared by the kidneys and muscles. Clearance rates for insulin decrease in glucose intolerance, obesity, in particular abdominal obesity, hypertension, hepatic cirrhosis, and nonalcoholic fatty liver disease (Lee et al, 2013). Despite its potential role in the etiology of diabetes (especially in type 2 diabetes), little is known about the factors that are independently associated with decreased of insulin clearance in T2D and clearly need further investigation.

There are a number of different types of diabetes, some of which are more prevalent than others. The most common forms of diabetes in the general population are type 1 diabetes (T1D) and type 2 diabetes (Ozougwu, 2013). Nevertheless, exists a type

of diabetes that is often misdiagnosed as T2D, mainly because share features with T2D and with type 1, titled as Latent autoimmune diabetes in adults (LADA) that presents a high prevalence (Naik et al., 2009). The common characteristic among these types of diabetes are the hyperglycemia, however the causes/pathophysiology are different: i) Type 1 diabetes, is caused by lack of insulin secretion by beta cells due to an autoimmune reaction to proteins of the islets cells of the pancreas. The pathogenesis of selective β-cell destruction within the islet in type 1 diabetes mellitus is difficult to follow due to marked heterogeneity of the pancreatic lesions. The autoimmune destruction of pancreatic β cells leads to a deficiency of insulin secretion that leads to the metabolic derangements associated with type 1 diabetes (Vlad &Timar, 2011); ii) Type 2 diabetes, is the more prevalent and is caused by a combination of genetic factors related to impaired insulin secretion, insulin resistance, likely insulin clearance, and environmental factors such as obesity, overeating, lack of exercise and stress, as well as aging. The impairment of pancreatic β cell function notably shows progression overtime in type 2 diabetes although aging, obesity, insufficient energy consumption, alcohol drinking, smoking, etc are independent risk factors of pathogenesis of type 2 diabetes mellitus (Fonseca, 2009); iii) The pathophysiology of LADA is not yet clearly understood, but these patients are typically diagnosed after 35 years of age and are often misdiagnosed as type 2 Diabetes. Glycemic control is initially achieved with sulfonylureas but patients eventually become insulin dependent more rapidly than with T2D patients. Although they have a type 2 DM phenotype, patients have circulating beta (β) cell autoantibodies, a hallmark of T1D. With regards to its autoimmune basis and rapid requirement for insulin, it has been suggested that LADA is a slowly progressive form of T1D (Naik et al., 2009).

The actually numbers of people with diabetes reveals that is necessary implement measures in order to decrease this epidemic. Among these possible actions the prevention is yet the principal method that can reduce this disease. Thus, it is clear that developing strategies to screen and identify high-risk individuals should be an important public health goal. For prevention efforts to be most effective, public health programs must recognize the factors involved in diabetes susceptibility.

Family history of diabetes (FHD) has been recognized as an important risk factor of the disease. Family medical history represents valuable genomic information because it characterizes the combined interactions between environmental, behavioral, and genetic factors. Evidence for strong genetic element diabetes susceptibility is

suggested by the high incidence in certain racial/ethnic populations, high concordance in monozygotic twins compared with dizygotic twins, and high incidence among first-degree relatives of persons with diabetes. The complex pathophysiologic nature of diabetes supports the idea that multiple biologic and/or chemical pathways are implicated in the development and progression of the disease, and numerous genetic loci have been investigated in the search for genetic determinants of the disease. The use of family history as part of a comprehensive risk assessment for an individual can be crucial in the prevention, early detection, and treatment of diabetes. On a population level, family history may help tailor health promotion messages for specific population groups (Valdez et al., 2007; Annis et al., 2005).

1.2.1 Types of diabetes

1.2.1.1 Type 1 Diabetes

Type 1 diabetes is a chronic inflammatory disease caused by a selective destruction of the insulin producing β-cells in the islets of Langerhans, characterized by an impaired (or total loss) insulin production. Type 1 diabetes is associated with the appearance of humoral and cellular islet autoimmunity, and a defective immunoregulation appears to be involved. The exact etiology and pathogenesis of type 1 diabetes, however, is still unknown. The model of the natural history of type 1 diabetes suggests stages that commence with a genetic susceptibility, autoimmunity without clinical disease, and finally clinical diabetes. Type 1 diabetes is responsible by 5% to 10% of all cases of diabetes, affecting approximately 20 million people worldwide (American Diabetes Association, 2008). Associated risk factors include autoimmune, genetic, and environmental factors. Until the present time, known solutions to prevent diabetes have not been discovered (Deshpande 2008, 1255).

Type 1 diabetes affects all age groups, but presents two peaks for onset, the first occur in childhood between 5 and 7 yr of age, and the other occur at or near puberty (Harjutsalo et al. 2008). Worldwide, the incidence of T1D is increasing, particularly in the under-5-years age group (Narendran et al., 2005). However, have the first signs of islet autoimmunity very early in life, with the majority by 2 years of age. Children who develop autoantibodies within the first 2 years of life are those who most often develop multiple islet autoantibodies and progress to type 1 diabetes in childhood. Autoantibodies do not exclusively develop before age 2 years, but children who develop

autoantibodies later have a slower progression to multiple antibodies and type 1 diabetes (Kimpimaki et al, 2002). Type 1 diabetes is possible the result of an interplay between genetic susceptibility (polygenic) and a triggering environmental agent that was thought to provide the fundamental elements for disease formation and, additionally, form potential targets for both improved disease prediction and prevention autoimmune reaction to proteins of the islets cells of the pancreas (Vlad & Timar, 2012). Genes clearly determine the likelihood of developing islet autoantibodies and progression to multiple islet autoantibodies. It remains controversial, however, if progression from multiple islet autoantibodies to type 1 diabetes is influenced by genetic factors. It is clear at this point that multiple genes can have predisposing, as well as protective effects, resulting in a complex interaction. Overall contribution of genetic factors will not explain the etiology of disease alone, since there is a significant discordance in monozygotic twins . Other environmental factors are therefore important, and many potential candidates were under evaluation; among them dietary factors, cow's milk in young infants, viral infections and psychological stress (Steck, & Rewers,,2011).

Genes for type 1 diabetes provide both susceptibility towards, and protection from, the disease. Although many chromosomal loci associated with such activities have been located, few true genes have been identified. The genetics of type 1 diabetes cannot be classified according to a specific model of dominant, recessive, or intermediate inheritance of a specific set of genes. The most important genes are located within the major histocompatibility complex (MHC) HLA class II region on chromosome 6p21, accounting for about 45% of genetic susceptibility for the disease. The function of these genes in terms of an immune response is well known (ie, presentation of antigenic peptides to T lymphocytes), yet their specific contribution to the pathogenesis of type 1 diabetes remains unclear (Steck, & Rewers,,2011).

1.2.1.2 Pathophysiology of T1D

T1D as autoimmune disease, the end stage culminating in destruction of the pancreatic b cells, characterized histologically by insulitis (i.e., islet cell inflammation) and associated β-cell damage. It remains unclear why the autoimmunity in T1D is

specific to the insulin-producing β cells. Beyond this, the specific mechanisms responsible for inducing the autoimmunity in T1D also have yet to be elucidated. The inflammatory lesion within islets of those with T1D is typically characterized by a decrease (or absence) of insulin-producing β-cells along with a pancreatic islet cell infiltrate composed of T-lymphocytes, B-lymphocytes, macrophages, and lesser numbers of other cells representing the immune response. It is import refer that the study of this process is not easy mainly because pancreatic biopsies in humans is not considered, in most of the cases an ethical procedure. Therefore, the majority of analysis of this process is realized in animal models or in autopsies. Autoimmunity in T1D has typically been identified by the presence of autoantibodies to islet and/or b-cell antigens, which in addition to their presence at the time of diagnosis, can often be detected long before the disease becomes clinically evident (Atkinson, 2012). Among a list of T1D-associated autoantibodies that actually has more than two dozen members are islet cell autoantibodies (ICAs), autoantibodies to glutamic acid decarboxylase (GADAs), insulin autoantibodie (IAAs), and autoantibodies to transmembrane tyrosine phosphatase (IA2As), as well as those against the ZnT8 molecule (ZnT8As). Although these are the five most prevalent and best characterized, the potential for other autoantibody/ autoantigen combinations remains. Other point that yet still controversial is whether such antibodies play a pathogenic role. For decades, the predominant dogma was that autoantibodies possessed no known etiological role in the disease (Zhang et al. 2008).

Over the past three decades, the ability to understand the natural history of T1D has improved dramatically through the combined use of genetic, autoantibody, and metabolic markers of the disease. In the mid-1980s, a model was develop integrating these three features. This model for the natural history of T1D suggests that genetically susceptible individuals are exposed to an environmental trigger, which induces b-cell autoimmunity. This process, initiate by the development of islet reactive autoantibodies, portends the development of activated autoreactive T cells capable of destroying b cells, resulting in a progressive and predicable loss in insulin secretory function. According to this model, the symptoms of T1D do not present until 80%–90% of the β cells have been destroyed, and there is a marked gap between the onset of autoimmunity and the onset of diabetes (Atkinson, 2005). This model has been a guide line for several years, but some aspects of the classical model have been modified to update knowledge gains (Fig. 1.3) (Atkinson, 2012). Among these updates, can be highlighted for instance that

are data to suggest that pancreatic β-cells may persist in some individuals with T1D for an extended period of time (i.e., never reaching zero in many T1D patients) (Meier et al. 2005). In addition, the degree of b-cell destruction required for symptomatic onset has been also debated, with recent studies suggesting that 40%–50% b-cell viability may be present at the onset of hyperglycemia. This may explain why, despite persistent autoimmunity, insulin secretory function can remain stable for long periods of time in persons with T1D. The inflammation and a decrease of β-cell mass leads then to a loss of first-phase insulin response, followed by a period of glucose intolerance and a period of clinically "silent" diabetes (Sosenko et al. 2010). The "slope" reflective of b-cell loss in the pre-diabetic that leads to diabetes period has also recently been subject to considerable debate, with some proposing that the disorder may see its symptomatic onset only following a period of relapsing/remitting like autoimmunity (Fig 4). Thus, this means that the onset of clinical disease represents the end stage of β-cell destruction (Atkinson, 2012).

In addition to the loss of insulin secretion, the function of pancreatic α-cells is also abnormal and there is excessive secretion of glucagons in T1D patients. The resultant inappropriately elevated glucagons levels exacerbate the metabolic defects due to insulin deficiency. The most pronounced example of this metabolic disruption is that patients with T1D rapidly develop diabetic ketoacidosis in the absence of insulin administration. Deficiency in insulin leads to uncontrolled lipolysis and elevated levels of free fatty acids in the plasma, which suppresses glucose metabolism in peripheral tissues such as skeletal muscle. This impairs glucose utilization and insulin deficiency also decreases the expression of a number of genes necessary for target tissues to respond normally to insulin such as glucokinase in liver and the GLUT 4 class of glucose transporters in adipose tissue (Ozougwu et al., 2013).

Thus, the combination of increased hepatic glucose production and reduced peripheral tissues metabolism leads to elevated plasma glucose levels. When the capacity of the kidneys to absorb glucose is suppressed, glucosuria occurs. Glucose is an osmotic diuretic and an increase in renal loss of glucose is accompanied by loss of water and electrolyte. The result of the loss of water (and overall volume) leads to polydipsia. The negative caloric balance, which results from the glucosuria and tissue catabolism leads to an increase in appetite and food intake that is polyphagia (Ozougwu et al., 2013).

Other effect that is noted in T1D is the loss of weight. In uncontrolled T1D there is a rapid mobilization of triglycerides leading to increased levels of plasma free fatty acids. The free fatty acids are taken up by numerous tissues (except the brain) and metabolized to provide energy. In the absence of insulin, malonyl COA levels fall, and transport of fatty acyl-COA into the mitochondria increases. Mitochondrial oxidation of fatty acids generates acetyl COA that can be further oxidized in the TCA cycle. However, in hepatocytes the majority of the acetyl COA is not oxidized by the TCA cycle but is metabolized into the ketone bodies (acetoacetate and b-hydroxybutyrate). These ketone bodies are used for energy production by the brain, heart and skeletal muscle. In T1D, the increased availability of free fatty acids and ketone bodies exacerbates the reduced utilization of glucose, furthering the ensuing hyperglycemia. Production of ketone bodies in excess of the body's ability to utilize them leads to ketoacidosis (Achenbach et al., 2005).

Beyond all this information that is already known about the disease, there are features that need to be clarify: identification of genes controlling disease susceptibility, improved understanding of autoimmunity/mechanisms underlying loss of immune regulation, and further identification of environmental agents influencing the disease are all examples of information needed to impact efforts toward the goal of disease prevention; each is discussed below. Likewise, understanding events (e.g., rate of C-peptide loss, the presence of residual b cells, etc.) following symptomatic onset are also of importance because many ongoing efforts are actively seeking to reverse the disorder in those previously diagnosed with the disease. Other important factor that clearly needs to be clarified is the importance of family history of diabetes as risk factor for T1D.

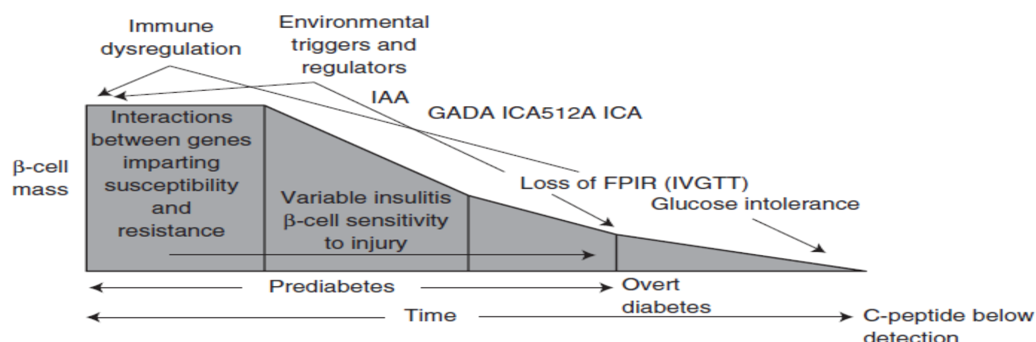

Figure 4 - Model of the pathogenesis and natural history of type 1 diabetes. The modern model expands and updates the traditional model by inclusion of information gained through an improved understanding of the roles for genetics, immunology, and environment in the natural history of T1D. FPIR – First phase of Insulin release; IVGTT - intravenous glucose tolerance test. The time it takes to the onset of T1D is

variable, being shorter when T1D appear in child and longer when T1D only is diagnosed on adult phase. (From Atkinson, 2012).

1.2.2.1 LADA

Latent autoimmune diabetes of the adult (LADA) is a common form of diabetes that presents several features of both type 1 and type 2 diabetes. Lada can be categorized as an autoimmune diabetes form, being the pathology characterized by the presence of diabetes-associated autoantibodies against β-cell. Unlike type 1 diabetes, these patients are normally detected in adulthood and are non-insulin requiring at time of diagnosis. Regarding to metabolic dysregulations involved, LADA presents similarity with type 2 diabetes (insulin resistance), demonstrating the high heterogeneity of this pathology (Carlsson et al., 2007).

The term latent autoimmune diabetes in adults (LADA) was introduced by Zimmet (Zimmet, 1995). to define adult diabetic patients initially non–insulin-requiring but with immune markers of type 1 diabetes that, in a number of cases, progress to insulin dependency. First, this type of diabetes was called slow-progressing type 1 diabetes, but a slow progression of b-cell destruction can be considered as only one of the possible explanations. Several terms like 1.5 diabetes, latent type 1 diabetes, or youth onset diabetes of maturity have been suggested to classify the pathology instead LADA. However, the term autoimmune diabetes not requiring insulin at diagnosis (or LADA) seems to be the more appropriate because the concept of latency indicates patients of adult age who do not require insulin at least for 6 months after diagnosis and who possess immunological and genetic features typical of type 1 diabetes (Nambam et al., 2010).

As expected all these characteristics leads to an often misdiagnosed of type diabetes presented, it is not easily discernible signs and symptoms. Lada patients typically have some features that distinguishes it from T1D and T2D (Table 2), but a diagnosis of LADA is still a challenge. This can have significantly influence in the epidemiologic numbers but mainly in the type of the therapy to apply. Due to the latent nature of the disease, LADA patients are often diagnosed as T2D and started on oral hypoglycemic. However, glycemic control deteriorates after a few months/years of therapy and by the time insulin therapy is started, the disease often progresses to the morbid stage which could have been delayed with timely initiation of insulin therapy dependency. The progression to insulin dependence in LADA patients is believed to be

more rapid than in type 2 diabetes subjects. The usual clinical features of these patients include weight loss, ketosis proneness, unstable blood glucose levels, and an extremely diminished C-peptide reserve (Naik et al., 2009).

Table 2- Diagnostic features of type 2, LADA and type 1 diabetes mellitus (From Ola et al., 2006).

Features	Type 1 Diabetes	LADA	Type 2 Diabetes
Metabolic syndrome	Present	Reduced frequency	Absent
Ketoacidosis	Absent	Usually absent	Present
Cardiovascular Complications	Present	Present	Present
Microvascular Complications	Present	Present	Present
Islet cell autoantibodies	Negative	Positive	Positive
Treatment with insulin	Required late in course of disease	Required at least 6 months after diagnosis	Required at diagnosis

As T1D, the epidemiology of LADA is influenced by geography, genetic susceptibility, environmental factors, gender and age at diagnosis, but a fact already related that can lead to significant bias of results is the misdiagnosed. The onset of diabetes in adulthood result in a high percentage of patients that are misdiagnosed with type 2 patients and are not submitted to an autoantibody test – essentially to a diagnosis of LADA. Thus, it´s absolutely important determine who should be subjected to this type of test. Many clinicians only asks for the antibody assay if exists a suspicion of LADA based on BMI ($< 25kg/m^2$). Obese, adult onset diabetics are often categorized as type 2 DM and not tested for LADA while adults with normal BMI are potentially suspected for LADA and hence tested. Nevertheless, the prevalence of LADA has been estimated in a number of studies worldwide. A wide variation has been described, partly depending on the markers chosen to define the condition but also on the characteristics of the patients (e.g., newly diagnosed or previously diagnosed), but these studies have identified a prevalence of 10–20% of non–insulin requiring diabetic patients with ICA and GAD antibodies, therefore with LADA (Nambam et al., 2010; Pozzilli & Di Mario, 2001).

1.2.2.2 Pathophysiology of LADA

As described below, LADA is defined as a latent form of T1D. There are several evidences that can explain the age of onset of this autoimmune diabetes in adults. Some studies suggests that in LADA, the typical HLA genetic predisposition to type 1 diabetes is less marked than in patients diagnosed in younger age. Others explanation is

that the interaction between environmental and genetic features is less acute in LADA patients. T-cell “insulitis” is also observed in LADA patients like in T1D, however the immune tolerance to b-cell antigens is probably higher in LADA, which can contribute to protect these patients from extensive T-cell destruction of b-cells (Poudel, 2012).

The process that leads to b-cell destruction varies according to the age when hyperglycemia is diagnosed, as indicated by the residual β-cell function found at age of diagnosis. It is possible that in the childhood, a linear and rapid progression toward exhaustion of the b-cell function occur, while in the adolescent, a longer followed by an acute precipitating factor (maybe viral) may be more common, based on available data. In LADA, it is believed that numerous events can hit the b-cells in genetically susceptible subjects, promoting the decline of b-cell function. This could be one of the possible explanations of pathogenic mechanisms. Therefore, the age at diagnosis is influenced by the amount of b-cell remainder, which is clearly more elevated in patients with LADA unlike the adolescent or the very young with TID.

Although are both autoimmune diseases there are differences in antibodies between LADA and type 1 diabetes. Many researchers have demonstrated that anti-GAD and ICA are much more common than IAA, IA-2A, and ZnT8 antibodies in LADA patients vs. type 1 patients (Hosszúfalusi et al., 2003; Cervin et al., 2008). These autoantibodies can distinguish between acute-onset type 1 diabetes and LADA because GAD antibodies and ICA indicate slow disease progression, whereas the presence of IA-2 antibodies is associated with an acute-onset clinical phenotype. The presence and quantity of single or a multiple antibodies in patients are also factors that clearly influence the progression of this disease. Presence of multiple autoantibodies and/or a high titer of anti-GAD autoantibodies, compared with single and low-titer autoantibody, was associated with an earlier age at onset, lower fasting C-peptide values, and a higher likelihood for future insulin requirement (Van Deutekom et al., 2008). It also has been observed that first degree relatives of patients that present multiple autoantibodies have a higher risk of developing T1D. The presence of circulating autoantibodies as well as the early requirement of insulin LADA patients has led to workers suggesting that LADA is a spectrum of type I DM with a much slower progression. The slower progression has been attributed to a more restricted antigen spreading in LADA than in type I DM leading to a more aggressive disease in the latter (Nambam et al., 2012).

Cell dysfunction in LADA has been reported to be intermediate between type 1 and type 2 diabetes. LADA subjects appear to have a faster decline in C-peptide levels

compared with people with type 2 diabetes. In comparison, a greater rate of decline in C-peptide has been reported in adult type 1 diabetes compared with LADA. Other investigators have also observed differences in insulin secretion between type 1 diabetes, LADA, and type 2 diabetes (Naik et al., 2009). A study revealed that the level of insulin secretion in LADA was intermediate between type 1 and type 2 diabetes and that fasting and stimulated C-peptide were reduced in LADA compared with type 2 diabetes (Gottsater et al. 1993).

Other feature that has been studied over the years is the role of insulin resistance and obesity; Because LADA subjects span the spectrum from lean to obese, differences in insulin sensitivity could be an important variable in their physiology. According to several studies the degree of insulin resistance in LADA has been reported to be less than in type 2 diabetes and comparable to type 1 diabetes. However how insulin resistance can contribute to the pathophysiology of LADA is a question that is yet not solved. A recent study in adult European diabetes patients has shown that the prevalence of metabolic syndrome is significantly higher in type 2 diabetic patients than in patients with LADA or adults with type 1 diabetes; it was further shown that metabolic syndrome is not more prevalent in patients with autoimmune diabetes than in control subjects, and metabolic syndrome is not a characteristic of autoimmune diabetes (Hawa et al., 2009).

1.2.3.1 Type 2 Diabetes

Type 2 diabetes is the most common form of diabetes. The World Health Organization (WHO) estimates that 90 percent of people with diabetes suffer from type 2 diabetes (~ 348 million). T2D is non-autoimmune, unlike LADA and T1D, and is characterized by hyperglycemia, insulin resistance, relative insulin deficiency and impaired insulin clearance. T2D results from an interaction between genetic, environmental and behavioral risk factors, like obesity, over eating, lack of exercise, and stress and aging. It is typically a multifactorial disease involving multiple genes and environmental factors to varying extents (Al li, et al., 2013).

T2D is now an epidemic disease and the number of people with this disease is increasing in every country, with 80% of people with DM living in low and middle income countries. It is estimated that 439 million people would have T2D by the year 2030. The incidence of T2D varies substantially from one geographical region to the

other as a result of environmental and lifestyle risk factors (International Diabetes Federation, 2013). The population of Portugal has not been spared from the global surge in T2D as vividly described by the first study of diabetes prevalence in Portugal conducted by the Portuguese Diabetes Association (APDP). The overall diabetes incidence is 11.7%, of which 5.1% were newly diagnosed during the study. The prevalence of impaired fasting glucose and/or impaired glucose tolerance (pre-diabetes) was 23.3% (Gardete-Correia et al., 2010).

Although all the great progressions that have been done in the last years, the current knowledge about the etiology of diabetes and pre-diabetes is insufficient to decrease the alarming numbers observed all over the world. The increase in type 2 diabetes is related to lifestyle changes that have resulted in overweight, obesity, and decreased physical activity levels. A westernised lifestyle, which involves a high-energy diet and reduced physical activity, is certainly linked to the pandemics of obesity and type 2 diabetes. Rates of overweight, obesity, and diabetes rise suddenly in populations that move from traditional rural to urban environments. Dietary changes are typically from unprocessed, low-energy, high-fiber foods to processed, energy-dense foods characterized by high sugar and fat contents. Micronutrient imbalances, including deficiency in concentrations of vitamin D, vitamin B12 in individuals replete with folic acid, and increased body iron stores have been implicated in the pathogenesis of type 2 diabetes (Nolan et al.,2011). Evidence also suggests that exposure to some synthetic organic pollutants (eg, pesticides) affects endocrine cells and increases the risk of developing type 2 diabetes (Casals-Casas et al., 2011). Thus, all these environmental and behavioral features, along with genetic predisposition, increase insulin resistance, which, in concert with progressive β-cell failure, results in rising glycemia in the nondiabetic range. In addition to the risk for diabetes, insulin resistance and impaired insulin secretion and clearance are accompanied by a host of major cardiovascular disease (CVD) risk factors including hypertension and dyslipidemia. Further reduction in insulin secretion over time results in increasing glycemia and the development of diabetes. The transition from the early metabolic abnormalities that precede diabetes, impaired fasting glucose (IFG) and impaired glucose tolerance (IGT), to diabetes may take many years; however, current estimates indicate that most individuals (perhaps up to 70%) with these pre-diabetic states eventually develop diabetes (Tabák et al., 2014).

Although the main metabolic defects of type 2 diabetes are present to some degree in most patients, this disorder is highly heterogeneous. Many different

susceptibility genes have been identified that interact with environmental factors, during gestation, early childhood, and later in life. Nevertheless the exact significance of individual genes for disease onset is still only partly resolved although previous twin studies applying quantitative genetic models suggested a substantial genetic component behind this disease. A hereditary component is suggested from a number of studies that assessed the influence of a family history of diabetes on risk of type 2 diabetes; most studies report a two- to six fold increased relative risk of type 2 diabetes and the associations appear to be independent of lifestyle factors, highlighting the importance of FHD (Wikner et al., 2013).

It is believed that the decline of β-cell function is the main factor that contributes to disease progression, being therefore determinant the acute insulin response to define the glucose tolerance status over time. Among Pima Indians, over a mean of 5.1 years, progressors (from normal glucose tolerance to IGT and then diabetes) differed significantly from non-progressors in their acute insulin response. Acute insulin response decreased by 27% during the transition from normal to impaired glucose tolerance and by 51% during the transition from impaired glucose tolerance to diabetes, and in nonprogressors, it actually increased by 30% (Weyer et al., 2001). Longitudinal changes in β -cell function were assessed over 5 years. Again, the main determinant of glucose tolerance status during follow-up was the change in acute insulin response. Normal glucose tolerance was maintained by a compensatory increase in insulin secretion, whereas failure to increase insulin secretion led to impaired glucose tolerance, and a decrease in insulin secretion led to overt diabetes. Thus, the progressive decrease in β-cell insulin secretion, particularly the first phase insulin secretion that occurs acutely after an increase in glycaemia, is likely the most critical functional β -cell defect in the development of type 2 diabetes (Festa et al.,2006).

1.2.3.2 Prediabetes

Prediabetes is an intermediate stage in between normal glucose regulation and diabetes and can be characterized by either impaired fasting glucose and/or impaired glucose tolerance. IFG and IGT subjects should not be viewed as clinical entities in their own right but rather risk factors for diabetes as well as cardiovascular disease. The term prediabetes itself has been further discuss on the basis that many people with

prediabetes do not progress to diabetes, the term may imply that no intervention is needed as no disease is present, and diabetes risk does not necessarily differ between people with prediabetes and those with a combination of other diabetes risk factors. Indeed, the WHO used the term 'Intermediate Hyperglycemia' and an International Expert Committee convened by the ADA the 'High Risk State of Developing Diabetes' rather than 'prediabetes" (Tabák et al., 2014). However the most conventional way to refer this intermediate hyperglycemic state is prediabetes, and this way will be used in the present work. The transition from the early metabolic anomalies that precede prediabetes to diabetes may take many years; however, recent estimates indicate that most individuals (~ 70%) with these prediabetic states eventually develop diabetes. During the prediabetic state, the risk of develop CVD event is modestly increased. However with the progression to diabetes, there is a large increase in risk for CVD, as well as for long-term complications affecting the eyes, kidneys, and nervous system (Nathan et al., 2007; Levitan et al., 2004). The complications associated with diabetes, which are the cause of major morbidity and mortality, are related to its duration, chronic level of glycaemia, and other risk factors. As expected, several evidences show that the risk for DM in prediabetic subjects is much higher compared with those with normal glucose tolerance (NGT). When several prospective epidemiological studies were analyzed, the incidence of T2D in isolated IFG and IGT subjects was estimated at 4 to 6% per year, and this value was significantly higher than NGT subjects (<0.5% per year). In the subjects who were diagnosed with IFG and IGT in combination, the annual percentage for the risk of developing T2D increased by 10% (Rhee & Woo, 2011). However several studies have demonstrated too that after lifestyle and drug-based interventions, the cases of prediabetes can be reverted to normal glucose tolerance. In a population-based observational study of the natural history of diabetes in England, 55%–80% of the participants with IFG at the initial phase, had achieve normal fasting glucose after a 10-year follow-up (Forouhi et. Al, 2012).

IFG is currently defined by an elevated fasting plasma glucose (FPG) concentration (≥ 100 and < 126 mg/dl) (29). IGT is defined by an elevated 2-h plasma glucose concentration (≥140 and < 200 mg/dl) after a 75-g glucose load on the oral glucose tolerance test (OGTT) in the presence of an FPG concentration <126 mg/dl. With the definitions above, is detected an overlap between these two groups. To study the separate characteristics of prediabetic states, classifications of isolated IFG and isolated IGT that are mutually exclusive have been created (isolated IFG = FPG of 100–

125 mg/dl with the 2-h value <140 mg/dl; isolated IGT = 2-h value of 140– 199 mg/dl with the fasting level < 100 mg/dl). The combined characteristics of IFG and IGT have been studied by identifying populations that accomplish both criteria (FPG = 100 –125 mg/dl and 2-h value = 140–199 mg/dl). Thus, normal glucose tolerance (NGT) is defined as FPG < 100 mg/dl and 2-h plasma glucose < 140 mg/dl (Table 3) (American Diabetes Association, 2010).

Table 3 - Classification of glucose tolerance states (from American Diabetes Association, 2010)

State	FPG	2-H plasma glucose in OGTT (mg/dl)*
IFG	100 - 125	< 200
Isolated IFG	100 - 125	< 140
IGT	< 126	140 – 199
Isolated IGT	< 100	140 – 199
Combined IFG/IGT	100 – 125	140 – 199
NGT	< 100	< 140
T2D	≥ 126	≥ 200

*Standard 75 – g OGTT

According to the IDF Diabetes Atlas, the number of cases of IGT in 2010, worldwide is estimated to be approximately 340 million. By 2030, the global prevalence of IGT is estimated to reach 8.4%, which will be approximately 462 million people (IDF, 2013). The highest prevalence of IGT in the world is in North America, with 10.4%. For Europe and the Middle East, the values are 8.9% and 8.2%, respectively, which is also relatively high versus other parts of the world. In Southeast Asia the prevalence is 6.2% and in the Western Pacific Region is 7.7%. Generally, the prevalence of IGT is known to be higher than that of IFG; however, these data were mostly based on the previous ADA/WHO criteria. According to the new ADA criteria, when the IFG cut-off value is adjusted from 110mg/dL to 100 mg/dL, IFG prevalence increases dramatically. In this case, the increase in IFG prevalence is greater than that of IGT (Rhee & Woo, 2011). For example, by changing the IFG diagnostic criteria, the Danish IFG prevalence increased from 11.8% to 37.6% (Glumer et al., 2003). Further, by changing the diagnostic criteria in DETECT-2 study subjects, IFG prevalence increased from 12.7% to 28.7% in Chinese subjects, from 11.0% to 38.6% in Asian Indians, from 16.3% to 45.7% in French subjects, and from 12.1% to 32.0% in the Unites States (Borch-Johnsen et al., 2003).

Both IFG and IGT have a heterogeneous pathogenesis, and this reason may contribute to different rates of progression to diabetes. Several studies tried to quantify

the risk of prediabetics to develop diabetes and others diseases associated in comparison with normal glucose tolerance subjects. Individuals with both IFG and IGT have approximately double the rate of developing diabetes compared with individuals with just one of them. Numerous longitudinal studies indicate that both IFG and IGT are associated with a modest increase in the hazard ratio (~1.1–1.4) for CVD, with IGT being a slightly stronger risk predictor. Many cardiovascular risk factors (e.g., low HDL cholesterol, hypertension, and elevated triglycerides) are prevalent in IFG and IGT, but it is unclear whether they occur more frequently in one state than the other. However, after adjustment for known cardiovascular risk factors, both IFG and IGT remain as independent, albeit weak, risk factors for CVD in some studies but not in others. Even so, it is unclear whether the CVD risk associated with IFG or IGT can be attributed to the development of diabetes during follow-up or whether these states per se convey such risk (Nathan et al., 2007).

1.2.3.3 The pathophysiology of prediabetes

Fasting plasma glucose levels are determined by endogenous glucose production (EGP) that is mostly dependent on the liver. The product of EGP and fasting insulin is used as a marker of hepatic insulin resistance and it shows a relatively strong relationship with fasting glycaemia.

During absorption of a glucose-containing meal, changes in glucose levels are determined by intestinal absorption, suppression of EGP and by total body glucose clearance. After glucose ingestion, EGP is markedly suppressed in people with NGT, while this suppression is less pronounced in prediabetes and even less in diabetes. In type 2 diabetes, total body glucose clearance is also decreased and 85–90% of this impairment is related to muscle insulin resistance. If the secretion of insulin by β-cells were able to compensate for insulin resistance perfectly, no observable changes in glucose levels would occur. Therefore, this apparently suggests that some β-cell dysfunction is already present in the prediabetic phase (Tabák et al, 2009). In several studies that used different measures of β-cell function, was reported severely abnormal (up to 80% decreased) insulin secretion in prediabetic people (Abdul-Ghani et al., 2006; Ferrannini et al., 2007). This observation is supported by autopsies reporting a 50% decrease in β-cell volume among those with glucose values within the IFG range (Butler et al., 2003). The epidemiologic differences between IFG and IGT suggest that different pathophysiologic mechanisms are involved, contributing to these disturbances in

maintenance of glucose homeostasis. These differences can be observed, for instance, during an OGTT with glucose dose of 75-g, where the subjects with different states of glucose tolerance present different response curves. People with isolated IGT have, by definition, FPG levels that are similar to those with NGT. However, following glucose ingestion the plasma glucose concentration rises excessively at all-time points and remains elevated (by definition 140–199 mg/dl) after 120 min (Fig. 5). On the other hand, in isolated IFG, the FPG is higher (by definition 100 –125 mg/dl) than in NGT and isolated IGT, and the plasma glucose concentrations at 30–60 min in the OGTT are greater than in both NGT and isolated IGT. Thereafter, the plasma glucose concentration in IFG declines to near-baseline values at 120 min. These two very distinct oral glucose tolerance curves reflect different pathophysiologic disturbances in glucose homeostasis in isolated IFG and isolated IGT. The plasma glucose curves in people with both IFG and IGT reflect the characteristics of both (Nathan et al., 2007).

Although both isolated IFG and isolated IGT are considered insulin-resistant states, are detected differences in their site of insulin resistance. People with isolated IFG predominantly have hepatic insulin resistance and normal muscle insulin sensitivity, whereas individuals with isolated IGT have normal to slightly reduced hepatic insulin sensitivity and moderate to severe muscle insulin resistance. As expected, subjects with both IFG and IGT have both muscle and hepatic insulin resistance. The pattern of insulin secretion also is different between IFG and IGT. People with isolated IFG have a decrease in first-phase (0–10 min) of insulin secretion in response to an intravenous glucose and a reduced early phase (first 30 min) insulin response to oral glucose. However, the late-phase (60–120min) plasma insulin response during the OGTT is normal in isolated IFG. Isolated IGT also has a defect in early-phase insulin secretion in response to an oral glucose load and in addition has a severe deficit in late phase insulin secretion. The combination of hepatic insulin resistance and defective insulin secretion in isolated IFG results in excessive fasting hepatic glucose production accounting for fasting hyperglycemia. The impairment in early insulin response in combination with hepatic insulin resistance results in the excessive early rise of plasma glucose in the 1st hour of the OGTT. However, the preservation of late insulin secretion combined with normal muscle insulin sensitivity allows glucose levels to return to the preload value in isolated IFG. In contrast, in isolated IGT the defective late insulin secretion, combined with muscle and hepatic insulin resistance, results in

prolonged hyperglycemia after a glucose load (Nathan et al., 2007; Tabák et al., 2012; Rhee & Woo, 2011) .

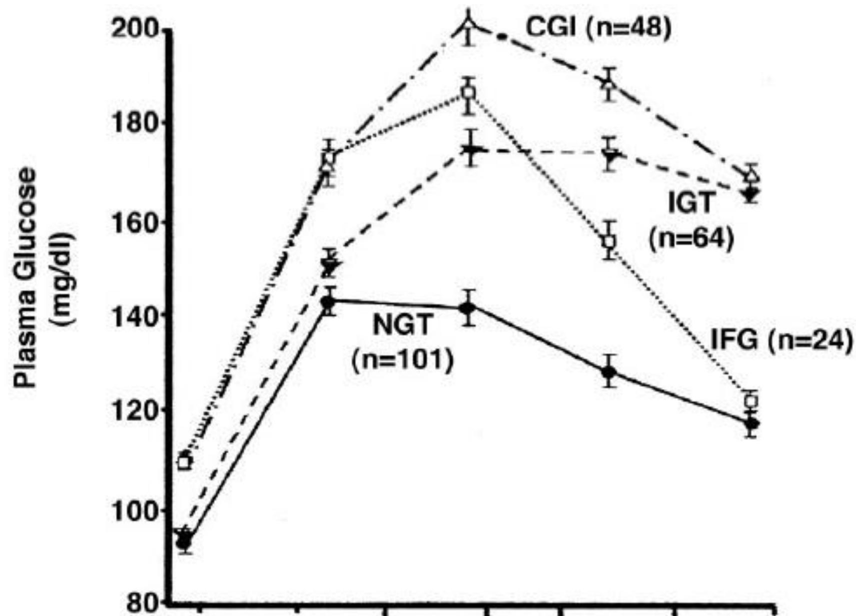

Figure 5 - Plasma glucose concentration during an OGTT performed in subjects with IFG, IGT, NGT, or combined IFG/IGT (CGI) (From Abdul-Ghani et al., 2006)

1.2.3.4 Pathophysiology of T2D

T2D is characterized by insulin insensitivity as a result of insulin resistance, declining insulin production, impaired insulin clearance pancreatic beta-cell failure. This leads to a decrease in glucose transport into the liver, muscle cells, and fat cells. Regarding to insulin clearance, this mechanism over the years has been viewed as a compensatory mechanism, however recently this concept was changed being this process linked to T2D pathology - some studies suggesting that increased insulin resistance is associated with reduced insulin clearance. The involvement of impaired alpha-cell function has recently been too recognized in the pathophysiology of T2D. As a result of this dysfunction, glucagon and hepatic glucose levels that rise during fasting are not suppressed with a meal. Given inadequate levels of insulin and increased insulin resistance, hyperglycemia results (Wikner et al, 2013; Lee et al., 2013).

The role of incretins in T2D must be considered once these represent a possible target to therapies. In healthy people, up to 70% of post‑glucose insulin secretion is mediated by incretins. However, in T2D patients, the insulin response to oral glucose is

blunted in comparison to non‑diabetic control subjects, suggesting impairment of the incretin effect. In T2D patients during hyperglycemic clamp studies, infusion of GLP‑1, but not GIP, stimulates insulin secretion, establishing that the insulinotropic effect of GLP‑1 is relatively well‑preserved in T2D, despite possibly lower levels, when compared to non‑diabetic subjects. On the other hand, GIP levels are essentially normal in T2D but GIP‑stimulated second‑phase insulin secretion is markedly diminished (although it has recently been reported that reversal of poor glycemic control in T2D improves the insulin response to both GIP and GLP‑1). However, like GIP, GLP-1 is rapidly inactivated by DPP-IV in vivo. Thus, the therapies involving incretines has been mainly focus on inhibitors of DPP-IV, with special attention to GLP-1 due to its effect on glucagon (Nolan et al., 2011; Campbell & Drucker, 2013).

Pancreatic β-cells normally respond to insulin resistance by increasing their output of insulin to meet the needs of tissues. Development of type 2 diabetes essentially results from a failure of the β-cell to adequately compensate for insulin resistance. The β-cell dysfunction progresses over time and is well advanced by the time that glucose level is in the diabetic range and continues to worsen after diabetes develops. Many obese individuals, who tend to have insulin resistance, progress to diabetes. However this doesn´t occur in all, with β-cells remaining to function adequately and this away being able to maintain glucose homeostasis and compensate for increasing insulin resistance with increasing insulin secretion. This mechanism can occur through increased insulin secretion from each β-cell and/or an increase in the β-cell mass. Some individuals have a reduced insulin secretion or reduced β -cell mass but normal glucose levels; they have sufficient insulin sensitivity to ensure adequate insulin secretion. In insulin-resistant subjects or subjects with type 2 diabetes, there is inadequate insulin secretion from each β-cell or an inadequate β-cell mass for the levels of prevailing insulin sensitivity (Fonseca, 2009).

One of the mains questions that remains it is the physiological process that leads to decrease of β-cell function in T2D, being at this point the glucotoxicity, whereby β-cells become sensitized to the presence of glucose, and lipotoxicity, whereby accumulated fatty acids and their metabolic products deleteriously affect β-cells, the mains hypothesis. In glucotoxicity, chronic hyperglycemia depletes insulin secretory granules from β-cells, lessening the amount of insulin available to be released in response to new glucose stimuli. Lowering glucose levels permits regranulation of β-

cells and a better acute insulin response follows. In lipotoxicity, prolonged increases in free fatty acid levels adversely affect the conversion of pro-insulin to insulin and eventually affect insulin secretion. Fatty infiltration of pancreatic islets may also contribute to β-cell dysfunction, and pancreatic fat correlates negatively with β-cell function. The concepts of gluco- and lipotoxicity remain hypotheses; the exact mechanisms responsible for impaired β-cell function have yet to be conclusively proved. In addition to glucose and lipid deposition in the pancreas, pancreatic islets from type 2 diabetic patients are known to have amyloid deposits, fibrosis, and increased cell death, associated with an inflammatory response (exacerbates insulin resistance), which has long been associated with the development and progression of type 2 diabetes (Fonseca, 2009; Ozougwu, 2013).

Thus, in type 2 diabetes, excessive carbohydrate and fat intake causes hyperinsulinemia in association with increased hepatic lipoprotein secretion, adipose tissue growth, and increased free fatty acid levels in genetically susceptible individuals. Together with postprandial hyperglycemia, elevated free fatty acid levels cause muscle and liver insulin resistance and increase hepatic glucose production. The same stimuli also facilitate β-cell compensation by promoting insulin secretion and biosynthesis as well as β-cell growth. In late stages, however, the progressive rise in insulin resistance, combined with alterations in β-cell gene expression and signaling induced by rising levels of free fatty acids, cause β-cell failure. Overt diabetes occurs as a result of this β-cell decompensation, with altered insulin secretion and apoptosis as possible contributing factors (Shan et al., 2014).

1.2.3.5 Main features of Type 2 diabetes

The role of impaired insulin secretion and insulin resistance has been discussed all over the years, being pointed as the main causes of T2D. However in the last years has emerged the contribution of impaired insulin clearance to development of T2D.

Insulin resistance (IR) is defined clinically as the inability of exogenous or endogenous insulin to increase glucose uptake and utilization in an individual as much as it does in a normal population. IR is linked to several diseases that represent tremendous burdens to health system resources, like obesity, metabolic syndrome, nonalcoholic fatty liver disease (NAFLD), T2D, and atherosclerotic heart disease. Insulin resistance may be either peripheral (which refers to diminished insulin-mediated

uptake of glucose by skeletal muscle and depends primarily on the failure of glucose transporter type 4 (GLUT4) expression and translocation to the plasma membrane) or hepatic insulin resistance (which describes impaired suppression of hepatic glucose production, and largely accounts for hyperglycemia and glucose intolerance) (El-Zayadi, 2010; Sansbury & Hill, 2014). Insulin action is the consequence of insulin binding to its plasma membrane receptor and is transmitted through the cell by a series of protein-protein interactions. Two major cascades of protein-protein interactions mediate intracellular insulin action: one pathway is involved in regulating intermediary metabolism and the other plays a role in controlling growth processes and mitoses. The regulation of these two distinct pathways can be dissociated. Indeed, some data suggest that the pathway regulating intermediary metabolism is diminished in type 2 diabetes while that regulating growth processes and mitoses is normal. Several mechanisms have been proposed as possible causes underlying the development of insulin resistance and the insulin resistance syndrome. These include: (1) genetic abnormalities of one or more proteins of the insulin action cascade (2) fetal malnutrition (3) increases in visceral adiposity (Samuel & Shulman, 2012).

The earliest detectable defect in β-cell function is frequently a reduction in first-phase of insulin secretion. First-phase insulin release is normally reduced in individuals with plasma glucose in upper ranges of normal and is essentially absent in people with fasting hyperglycemia (Gerich, 2002). The concept received further support from studies of subjects with impaired glucose tolerance (IGT), showing that these generally had reduced plasma insulin levels at 30 min after glucose ingestion and "normal or increased" plasma insulin levels at 120 min (Gerich, 1997). The assumption has been made assuming that the 30-min response reflected first phase insulin release, whereas the 120-min response reflected second-phase insulin release. Thus, it is accepted that reduced first-phase insulin release is responsible for the development of IGT (Natham et al., 2007).

A problematic that have receive further discussion during the years related to the pathogenesis of type 2 diabetes is the debate whether is insulin resistance or impaired β-cell function the primary defect in this pathology. By primary defect, the underlying genetic defect is meant. It seems well established that T2D is a polygenic disorder in which both hereditary and environmental or acquired factors are involved, and both of these factors can affect β-cell function and insulin sensitivity (Shah & Vella, 2014). It is know that the normal islet adjusts its function in order to compensate for insulin

resistance. However in interpreting the appropriateness of insulin secretion, it should take into consideration not only the stimulus (i.e., plasma glucose level), but also the prevailing insulin sensitivity. For instance, during a hyperglycemic clamp experiment in which plasma glucose was increased to comparable levels in a lean and obese subjects, plasma insulin responses in the lean individual compared with those of the obese individual would be considered inappropriate and would suggest an impaired β-cell function. In the past, these variables were generally not taken into consideration and because of this, the concept that insulin resistance precedes β-cell failure in the progression to type 2 diabetes became widely accepted and consequently so did the concept that insulin resistance was the primary genetic component of type 2 diabetes (Gerich, 2002). This concept does not explain why the majority of obese individuals, who obviously are insulin resistant, do not develop diabetes. If one accepts that the normal β-cell adjusts its function to compensate for insulin resistance, then one could explain the development of IGT and type 2 diabetes as a failure of β-cell compensation and that this may be the genetic basis for type 2 diabetes. Acceptance of this proposition does not exclude the effect of environmental/acquired factors (e.g., glucose toxicity, lipotoxicity, and amyloid accumulation in islets) might also be involved.

1.3.2.5.1 Insulin clearance

Insulin clearance is a feature of all insulin sensitive tissues and an integral component of insulin metabolism. This process regulates the cellular response to the hormone by decreasing insulin availability and mediating certain aspects of insulin action. At physiological concentrations, uptake is mediated primarily by the insulin receptor with a smaller contribution from nonspecific processes. At higher concentrations, nonreceptor processes start to assume greater importance. Insulin has a short plasma half-life (4–6 min), as would be expected from the necessity to respond rapidly to changes in blood glucose. The modeling of insulin kinetics is a technically difficult process, and the mathematical ramifications of whole-body systems are extremely complex and are been development. The liver is the primary site of insulin clearance. Although C-peptide passes through the liver without significant extraction, ~ 70% of the secreted insulin is cleared by the hepatocytes before entering the systemic circulation being the remainder cleared by the kidneys and muscles. Therefore, the rate of hepatic insulin clearance is an important regulator of peripheral glucose metabolism

(Bojsen-Møller et al., 2013). A reduction in hepatic insulin clearance is typically found after oral glucose or meal ingestion, thereby increasing the systemic availability of insulin to respond an increase in of glucose levels. The mechanisms underlying this phenomenon are largely unknown (Meier et al., 2007). Changes in insulin clearance can occur under different physiological conditions and can be related with pathological states. Clearance rates for insulin decrease in glucose intolerance, obesity, in particular abdominal obesity, hypertension, hepatic cirrhosis, and nonalcoholic fatty liver disease. Although the plasma concentration of insulin is largely determined by its rate of secretion and clearance, some evidences suggest that increased insulin resistance is associated with reduced insulin clearance (Lee et al., 2013). Reduced insulin clearance has important physiological functions; for example, animal models have shown that decreased insulin clearance serves as a compensatory mechanism to preserve b-cell function and to maintain peripheral insulin levels in the states of insulin resistance (Kim et al., 2007). In addition, insulin clearance has been found to be a highly heritable trait in Mexican Americans, and specific haplotypes in the AMPD1 gene were associated with variation in insulin clearance (Goodarzi et al., 2005).

Despite the potential role of insulin clearance in the etiology of diabetes, little is known about the factors that are independently associated with decreased insulin clearance and need to be further investigated. As mentioned above the liver is the primary site of insulin clearance, when this is release in the portal vein. A higher percentage of portal insulin is removed during first pass transit, but this percentage varies widely under different conditions. Hepatic uptake is not a static process, but rather is influenced by both physiological and pathophysiological factors. Prolonged increases in portal insulin levels also result in reduced clearance due to receptor down-regulation. Removal of insulin from the circulation does not imply immediate destruction of the hormone. A significant amount of receptor-bound insulin is released from the cell and reenters the circulation with possible biological functions. Nutrient intake alters insulin clearance. The glucose-induced increase in insulin secretion may decrease hepatic fractional extraction (Duckworth, 1998). Given the importance of the liver in insulin clearance, it is expected that liver disease may result in a decrease or at least an impairment in insulin clearance process, although not all studies agree (Antoniello et al., 1989). The decreased clearance is probably due to reduced hepatic function. The reduced hepatic clearance is also associated with reduced insulin sensitivity, again supporting the relationship between insulin degradation and insulin

resistance. The primary cellular mechanism for hepatic uptake and degradation of insulin is a receptor-mediated process. Most hepatic uptake is due to hepatocytes, with Kupfer cells contributing about 15% to the total (Duckworth, 1998).

The kidney is the major site of insulin clearance from the systemic circulation, removing around 50% of peripheral insulin. In addition, the kidney removes 50% of circulating proinsulin and 70% of c-peptide by glomerular filtration. Insulin analogs are also cleared by kidney. Insulin not cleared by liver and kidney is ultimately removed by other tissues. All insulin-sensitive cells remove and degrade the hormone. After liver and kidney, muscle plays the major role in insulin removal (Duckworth, 1998). The main enzymes involve in this process are the insulin-degrading enzyme (IDE), an insulinase, which degrades insulin with a high degree of specificity and protein disulfide isomerase (PDI) whose the main role is to catalyze protein folding in the endoplasmic reticulum (Hwang et al., 2007; Xu et al., 2014; Duckworth, 1998). It is believed that the participation of these two enzymes occur in a in a sequential manner with disulfide cleavage and subsequent proteolysis. The mechanism proposed is that the initial degradative step occurs in endosomes with two or more cleavages in the B chain of insulin by IDE. This is followed by reduction of the disulfide bonds by PDI, or a related enzyme, yielding an intact A chain and several B chain fragments. The insulin fragments are then further cleaved, probably by multiple proteolytic systems, including lysosomes and produce intracellular fragments of insulin with potential biological activity (Duckworth, 1998).

1.3.2.5.2 Nonalcoholic Fatty Liver Disease and Type 2 Diabetes

Nonalcoholic fatty liver disease (NAFLD) involves a spectrum of pathological conditions, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis, in the absence of excessive alcohol consumption (typically a threshold of<20 g/d for women and < 30 g/d for men is adopted). NAFLD has been becoming a growing public health problem worldwide reaching epidemic proportions, and is the most common cause of chronic liver disease in many developed countries. NAFLD increases the risk of end-stage liver disease, and NAFLD-induced liver failure is one of the most important reasons for liver transplantation (Targher& Byrne, 2013). Because NAFLD and T2D share pathogenic abnormalities of excess adipose tissue and insulin resistance,

both frequently co-occur, and the several outcomes of each disease can overlap in affected individuals. T2D is a risk factor for progressive liver disease and liver-related death in patients with NAFLD, and NAFLD may be a marker of increased cardiovascular risk and mortality in subjects with T2D, whereas patients with both NAFLD and T2D have poorer prognoses in terms of increased cardiovascular and liver-related mortality. Insulin-resistant patients with liver steatosis, compared with insulin-sensitive individuals, have higher insulin responses and lower hepatic insulin clearance, leading to hyperinsulinemia (Chai et al., 2014).

NAFLD is a manifestation of pathological ectopic fat accumulation coupled with a low-grade chronic inflammatory state in liver, which is an organ not able to accumulate fat. This condition is still poorly recognized by endocrinologists and general physicians, and recent work is now suggesting putative mechanisms by which NAFLD increases risk of developing T2D and worsens glycemic control, contributing to the pathogenesis of major chronic complications of T2D, such as cardiovascular disease and chronic kidney disease (CKD) (Targher & Byrne, 2013). The amount of intrahepatic fat closely correlates with serum liver enzyme activity and the number of metabolic syndrome features (Kotronen et al., 2007). Patients with T2D have approximately 80% more intrahepatic fat content than age-, sex-, and body weight-matched nondiabetic controls, and their serum liver enzymes are less representative of the severity of intrahepatic fat accumulation. The prevalence of NAFLD in people with T2D ranges from approximately 50 to 75%. A recent study with ultrasound of near of 3000 unselected patients with T2D reported a prevalence of NAFLD of 69.5% (Targher et al., 2007). Notably, patients with NAFLD and T2D are also more likely to develop the more advanced forms of NAFLD, such as NASH, advanced fibrosis, cirrhosis, and in some cases hepatocellular carcinoma. It is known that T2D along with obesity and older age are among the strongest risk factors for advanced fibrosis and cirrhosis. The presence of NAFLD among patients with T2D appears to be an important risk factor for all-cause mortality. In a study with T2D patients was reported that those with NAFLD had a 2.2-fold increased risk of all-cause mortality compared with those without NAFLD (Adams et al., 2010); the most common causes of death were malignancy, CVD, and liver-related complications. However the possible underlying mechanisms leading to CVD and CKD in patients with T2D and NAFLD are complex and not yet fully understood (Obika & Noguchi, 2012).

However, the most accepted underlying mechanisms linking NAFLD with T2D and poor glycemic control may originate from the development of an expanded and inflamed visceral adipose tissue mass. In this situation, the liver may function as both the target organ and the source of the resulting systemic abnormalities that promote not only increased risk of T2D but also increased risk of chronic vascular complications. Fat accumulation in the liver is influenced (in order of importance) by the delivery of extrahepatic free fatty acids (FFA) (principally from adipose tissue), hepatic de novo lipogenesis, and the supply of dietary fat to the liver. Importantly, the main processes regulating hepatic fat metabolism also have the potential to affect hepatic glucose production. For example, with respect to the link between hepatic steatosis, IR, and dysglycemia, dietary carbohydrate intake increases glucose and insulin levels, which in turn activates 2 key hepatic transcription factors that promote hepatic de novo lipogenesis and hepatic glucose production (carbohydrate response element binding protein and sterol response element binding protein-1c). Whether IR causes intrahepatic triglyceride accumulation or vice versa is the subject of much research in nowadays (Obika & Noguchi, 2012; Targher & Byrne, 2013).

Liver biopsy is considered the gold standard for the diagnosis and assessment of both NAFLD and NASH but has several limitations, such as sampling variability, invasiveness and expense. Thus the diagnosis is mainly realized based on equation that has in count several parameters that can indicate liver disease.

1.3 Family History of Diabetes

Family history of diabetes has been recognized over the years as an important risk factor of the disease. Family medical history represents valuable genomic information because it characterizes the combined interactions between environmental, behavioral, and genetic factors. However the results regarding to this risk factor have presented highly heterogeneity, being inconsistent among the different population analyzed. Thus it is fundamental keep studying the importance of FHD in the development of the three more prevalent types of diabetes, namely T1D, LADA and T2D.

Although more than 85% of patients with T1D do not present a positive family history for the disease, one of the major risk factor is a presence of first-degree relative type 1 diabetic in the family. Familial aggregation of type 1 diabetes has been

recognized for many years, and ~ 10–15 % of newly diagnosed children have a first-degree relative affected with type 1 diabetes. The risk of developing islet autoimmunity varies depending on which relative(s) have type 1 diabetes. The risk also depends on the number of relatives with type 1 diabetes (Redondo & Eisenbarth, 2002). Analysis of the BABYDIAB cohort found that children's risk for islet autoantibodies was markedly increased if both parents or a parent and a sibling had type 1 diabetes compared with a single affected family member. Among first-degree relatives, siblings are at a higher risk (5%–10% risk by age 20) than offspring (the risk for T1D in siblings of patients is 15-fold higher than the risk for T1D in the general population); offspring of diabetic fathers are at a higher risk (approximately 12%) than offspring of diabetic mothers (approximately 6%) (Ziegler et al.,1999).

Regarding to importance of family history of diabetes to development of LADA, this has been a subject understudied. The majority of the studies have been focus on type 1 and type 2. Interesting recent reports have shown familial clustering of type 1 and type 2 diabetes genes and have suggested that selected susceptibility gene variants may be involved in the pathogenesis of type 1 and type 2 diabetes. The results of the Nord-Trøndelag Health Study showed that family history of diabetes, although the type of diabetes in the relatives was unknown, was also a strong risk factor for the development of LADA, highlighting the importance of use FHD as a tool to screen the presence of diabetes (Carlsson et al., 2007)

With respect to importance of family history of diabetes for development of T2D, evidence for a strong genetic element of type 2 diabetes susceptibility is suggested by the high incidence in certain racial/ethnic populations, high concordance in monozygotic twins compared with dizygotic twins, and high incidence among first-degree relatives of persons with type 2 diabetes. The complex pathophysiologic nature of diabetes supports the idea that multiple biologic and/or chemical pathways are implicated in the development and progression of the disease, and numerous genetic loci have been investigated in the search for genetic determinants of the disease (Olokoba et al., 2012). Identifying susceptibility loci for diabetes, however, has been difficult because of the multiple genes involved and strong environmental contributing factors. Individuals who have a family history of diabetes presents a risk two to six times higher of develop type 2 diabetes compared with individuals with no family history of the disease. In this field is detected too, not in all, but in some studies an excess of maternal transmission of type 2 diabetes (i.e., greater likelihood of diabetes in

offspring of affected mothers than affected fathers, unlike what occurred in type 1 diabetes (Valdez et al., 2007; Kaeter et al.,1999) .

Therefore, the use of family history as part of a comprehensive risk assessment for an individual can be crucial in the prevention, early detection, and treatment of diabetes. On a population level, family history may help tailor health promotion messages for specific population groups who are included in this high risk group. Beside many studies have been made analyzing the influence of FHD to risk of diabetes, according to our knowledge no one have been focus in analyzing the type of diabetes that people are in risk to develop according to type of diabetes presented in first relatives. Thus, this work elaborated with a large population, is expected to bring important new evidences especially for clinics, in order to become FHD a higher robust tool to analyze the risk of develop diabetes mellitus.

2. Objectives

This study will be focused on the risk of developing diabetes in the presence of first-degree relatives with diabetes.

Objective 1 – characterize the Family History of people with Diabetes according to the type of diabetes

Objective 2 – Assess the risk of developing diabetes in different first degree relatives of people with diabetes

Objective 3 – Differentiate dysglycemic states in the high risk population of relatives of people with diabetes.

Our hypotheses are:

Hypothesis 1 - First-degree relatives of people with diabetes represent a high risk factor group to develop diabetes.

Hypothesis 2 – First degree relatives of people with diabetes are a heterogeneous group for the risk of developing diabetes

Hypothesis 3 – Insulin clearance during an OGTT differentiates metabolic states between dysglycemic categories

3. Subjects and Methods

The present work was performed in Portuguese Diabetes Association (APDP - *Associação Protectora dos Diabéticos de Portugal*), Lisbon. APDP is the largest clinical of diabetes in Portugal, with over 125,000 registered patients, thus it is a privileged institution to realize investigations studies having access to a higher number of population. The study realized in this master thesis can be divided into two different parts: the first one, a clinico-epidemiological study, that aimed to analyze the relationship between the family history of diabetes and the prevalence of diabetes among the patients of APDP in the last five years (2009-2013); the second one, a clinic study that aimed to analyze the possible metabolic alterations in a high risk group among first degree relatives of type 2 diabetic patients. Both parts of this project were realized based on evidences that a family history of diabetes is indeed a major risk factor to develop the disease. Thus, this work pretends to highlight the importance of FHD as a preventive tool for diabetes, expecting to provide new important evidences that allow an earlier intervention in this risk group in order to decrease the epidemic numbers of diabetes observed all over the word. All the procedures here realized were previously reported and approved by the ethics committee of APDP.

3.1 Study Group

3.1.1 Study 1

The group study chosen was comprised by the patients of APDP with T1D, LADA and T2D within a period of five years (2007-2013).These three types of diabetes are the most prevalent among the patients of APDP. The patients of APDP are citizens from Portugal, natives and/or immigrants, thus it is possible that the present group has some heterogeneity. We make an analysis of the FHD available in the clinic file of diabetics, which was elaborated by the doctor in the first appointment in APDP based on information provided by the patients. In an initial phase 24.788 clinic files were analyzed, however 7.914 were incomplete, thus these were removed, being the viable population for analyzes comprised by 16.874 clinic files of people with diabetes. The main reason that explains the incomplete files is the fact that some patients demonstrated lack of knowledge about their family history of diabetes. The analyzed

population for the present study included 3013 patients (17.86%) with T1D, 373 with LADA (2.21%) , and 13488 (79.93%) with T2D.

3.1.2 Study 2

The chosen study group to this work was the non-diabetic siblings of T2D subjects, because they are a low hanging fruit since they are at an inherently higher risk of progressing to T2D and they are also likely more motivated to undergo testing and lifestyle changes to prevent T2D. The recruitment of T2D-siblings was made in two different parts: 1) in waiting rooms we inquired the T2D patients about the existence of non-diabetic siblings and if they would be available to come to APDP in order to realize the present study. In case of positive answer, we asked the name and the telephone number of T2D-sibling; 2) then, we contacted the T2D-siblings by telephone, explaining all the details involved in the study, and in the case of those who matched all the conditions, we scheduled the analysis. The analyzed population in the present study was composed by 23 siblings of type 2 diabetics, 8 were males and 15 were females.

3.2 Study design

3.2.1 Study 1

It was selected the data from 2009 to 2013, mainly because within this range of time it was possible to achieve a significant amount of clinic files in order to aim our goal. The first step in data proceeding (after exclude all the incomplete files) was to evaluate the prevalence of each type of diabetes, calculating the number of men and women with T1D, LADA and T2D, as well as the average age observed in each group. After this, the patients' files among each group of diabetic were separated in individuals with FHD and those without FHD. The people with FHD were categorized in two different groups according to the type of relatives that had diabetes: 1) at least first degree relatives 2) only second degree relatives. It is important to bear in mind that the same process of all analysis realized in each type of diabetic group without distinguishing between gender and age was applied.

Next, we analyzed the prevalence of diabetes in first-degree relatives, therefore parents, siblings and offspring. We started to analyze the presence of diabetes in parents, separating the patients in three groups: 1) only mothers with diabetes, 2) only

fathers with diabetes 3) both parents with diabetes. Within each group, it was distinguished the type of diabetes presented by parents. After, we analyzed the presence and the type of diabetes present in offspring and siblings, completing the process of analysis of first degree relatives. We just had access to complete information about FHD of patients with first degree relatives with diabetes, therefore it was only possible for us to realize a further exploration in this group. We categorized the clinic process of these subjects according to different combinations of FHD, achieving the following categories: 1) only parents; 2) only siblings; 3) only siblings with T1D; 4) only siblings with T2D; 5) only siblings with T1D and T2D; 6) parents and siblings.

In these entire categories that were here described, it was calculated the prevalence. The odds ratio was calculated too, enabling to realize directly comparisons among the different types of diabetes analyzed in this research project.

3.2.2 Study 2

The analyses were carried out in the fasting state. Thus, it was asked to siblings-T2D not to eat or drink anything except water for 8 to 10 hours before the OGTT modified. In the day of analysis, after the subjects had been conducted to the blood collecting room, the procedures were explained and the informed consent was signed. Then, the clinic information (e.g. previous diseases, medication), weight, height were obtained and the waist circumference fasting state was measured before the beginning of OGTT modified. The first blood sample was collected in the fasting state, and then the subject ingested the glucose solution (75g of glucose /200mL of H_2O). In this first collection 1 serum tube (9ml) and two tubes (3mL per tube) of whole blood (1 for plasma, and 1 for whole blood) were obtained. The next blood collections were realized at 30, 90 and 120 minutes after the ingestion of glucose solution. At this three last points, only was necessary collected 2 different tubes, 1 tube for serum (9ml) and 1 tube for plasma (3 ml). After all the process conclude, the serum samples were analyzed by Olympus AU640 and Liaison auto-analyzers and the whole blood samples were assessed in Automated Hematology Analyzer Sysmex XT-1800i and Variant II Turbo. This process was conduct by the specialized laboratory technicians of APDP. After all the parameters analyzed, the blood samples were stored in a biobank at – 80ºc, in order to be used in further investigations that are not determined yet.

This work was an observational study involved in a large project already approved by FCT, namely “Anticipating diabetes and diabetic wounding for siblings of

Type 2 diabetics”. However it allows us an effective detection of people with unknown type 2 diabetes and people in a state of prediabetes. Thus, to these people with deregulation at glycaemia metabolisms we applied some interventional measures: for prediabetic and unknown type 2 diabetic subjects, we scheduled an appointment with the project coordinator, Prof. Doctor João Filipe Raposo; for subjects with normal glucose tolerance we sent the results to their homes giving information that all the parameters were within a normal range.

3.3 Analysed parameters

3.3.1 Study 1

In order to analyze the relationship between FHD and the prevalence of diabetes, was solicited to informatics of APDP a file with appropriate information. The file contained the gender, age, number of process, and information about FHD. The information about FHD was obtained in the first appointment at APDP, in which each patient was inquired about the presence of diabetes in first degree relatives. Thus, it was possible to analyze all the information about the presence of diabetes in parents, siblings and offspring, including the type of diabetes present on them. Regarding to the presence of diabetes in second degree relatives, the computer system that will allow the security of these data is in development, thus we only had access if the patients had or not knowledge about the presence of second degree relatives with diabetes (indiscriminate).

3.3.2 Study 2

The parameters that were analyzed in blood serum and in whole blood are described in table 4:

Table 4 - Parameters analyzed and methods used on serum and whole blood samples at different times.

Serum 0’
Insulin (Chemiluminescence, according to Liaison Insulin Kit, DiaSorin)
C-Peptide (Chemiluminescence, according to Liaison C-peptide Kit, DiaSorin)
Glycose (Hexokinase method UV, according to Olympus AU640 Glucose Kit, Beckman Coulter)
Free Fatty Acids (Enzymatic endpoint method, according to NEFA FS Kit, DiaSys)
Triglycerides (Colorimetric enzymatic method, according to Olympus AU640 triglyceride kit, Beckman Coulter)
Alanine transaminase (ALT) (UV cinetic assay, according to Olympus AU640 ALT Kit, Beckman Coulter)
Aspartate aminotransferase (AST)

(UV cinetic assay, according to Olympus AU640 AST Kit, Beckman Coulter) **Gamma-glutamyl transpeptidase (γ-GT)** (colorimetric cinetic assay, according to Olympus AU640 γ-GT Kit, Beckman Coulter) **Albumin** (colorimetric method, according to Olympus AU640 Albumin Kit, Beckman Coulter) **HDL cholesterol** colorimetric method, according to Olympus AU640 **HDL-Cholesterol** Kit, Beckman Coulter **LDL cholesterol** colorimetric method, according to Olympus AU640 **HDL-Cholesterol** Kit, Beckman Coulter **Total cholesterol** colorimetric method, according to Olympus AU640 **t**otal cholesterol Kit, Beckman Coulter
Whole blood 0'
Platelets (Hydrodynamic Focusing Direct Corrent Detection Method, in Automated Hematology Analyzer Sysmex XT-1800i) **Glycated hemoglobin (HbA1c)** (HPLC, according to Variant II Turbo Hba_{1c} Kit – 2.0, Bio-Rad Laboratories)

In the 30, 90 and 120 minutes the following parameters were analyzed in serum:

Serum (30',90',120')
Glycose Free Fatty Acids Insulin C-Peptide

Based on the OGTT the T2D-siblings were categorized according to glucose tolerance status in impaired glucose tolerance (IGT), impaired fasting glucose (IFG), impaired fasting glucose plus impaired glucose tolerance (IFG+IGT), normal glucose tolerance (NGT), and T2D. After this, the three main physiological processes involved in the regulation of insulin levels were evaluated: insulin secretion, insulin action and insulin clearance. The measure of insulin and the c-peptide levels allow us the evaluation of insulin secretion. The action of insulin was assessed by the application of HOMA-IR and OGIS, whereas insulin clearance was estimated from the molar C-peptide-to-insulin ratio and from the respective areas under the curve. Hepatic steatosis and development of fibrosis are features that are often found in patients with type 2 diabetes, thus we decided to evaluate the fatty liver index (FLI) and the NAFLD fibrosis score (NFS).

3.4 Techniques and methods

3.4.1 Study 1

3.4.1.1 Classification of the types of diabetes in APDP patients

This process was exclusively realized by clinics of APDP. The first step was evaluated if the disease was present in the subject. The criteria used in this process are presented in the table 5:

Table 5- Criteria for the diagnosis of diabetes (From American Diabetes Association, 2010).

Criteria
A1C≥ 6.5%. The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay*   Or
FPG ≥ 126 mg/dl (7.0mmol/l). Fasting is defined as no caloric intake for at least 8h.*   Or
2-h plasma glucose ≥ 200mg/dl (11.1mmol/l) during an OGTT. The test should be performed as described by the world Health Organization, using a glucose load containing the equivalent of 75g anhydrous glucose dissolved in water*   Or
In a patient with classic symptoms of hyperglycemia or hyperglycemia crisis, a random plasma glucose ≥ 200 mg/dl (11.1 mmol/l)
In the absence of equivocal hyperglycemia, criteria 1 – 3 should be confirmed by repeat testing

After confirmation of diabetes diagnosis, the individuals were categorized according to the type of diabetes. The main criteria for classification of T1D, LADA and T2D are the following (table 6):

Table 6 - Criteria followed to distinguish among the different types of diabetes.

Type of Diabetes	Some criteria
Type 1 Diabetes	Decreased C peptide levels and/or presence of GADA and ICA and/or treatment with insulin required at diagnosis and/or loss of weight and/or polyuria, polyphagia

LADA	Adult age of onset (> 30 years of age) and/or presence of at least one circulating autoantibodies (GADA/ICA/ IAA/IA-2) and/or initial insulin independence (for the first six months)
Type 2 Diabetes	Insulin resistance and/or no islet cell autoantibodies and/or obesity and/or sedentary lifestyle

Note: The majority of the cases were diagnosed only according to the characteristic clinic symptoms of each type of diabetes. In the cases that presented some uncertainty laboratorial exams were realized to confirm the diagnosis.

3.4.1.2 Prevalence calculation

In order to calculate the prevalence in each category, the number of individuals was divided by the total number of the individuals with that type of diabetes. For instance, all the categories created in analysis of type 1 diabetic were divided by 3006 and obviously multiplied by 100% to obtain a percentage.

3.4.1.3 Calculation of the Odds Ratio

The odds ratio (OR) is a measure of association between an exposure and an outcome. The OR represents the odds that an outcome will occur given a particular exposure, compared to the odds of the outcome occurring in the absence of that exposure. Odds ratios are most commonly used in case-control studies, however they can also be used in cross-sectional and cohort study designs as well (with some modifications and/or assumptions).

Odds ratios are used to compare the relative probability of the occurrence of the outcome of interest (e.g. disease or disorder), giving exposure to the variable of interest (e.g. health characteristic, aspect of medical history). The odds ratio can also be used to determine whether a particular exposure is a risk factor for a particular outcome, and to compare the magnitude of various risk factors for that outcome. According to the results obtained when the OR is used, the following conclusions can be assumed:

OR=1 Exposure does not affect odds of outcome

OR>1 Exposure associated with higher odds of outcome

OR<1 Exposure associated with lower odds of outcome

The 95% confidence interval (CI) is used to estimate the precision of the OR. A large CI indicates a low level of precision of the OR, whereas a small CI indicates a higher precision of the OR. It is important to note however, that unlike the p value, the 95% CI does not report a measure's statistical significance. In practice, the 95% CI is often used as a proxy for the presence of statistical significance if it does not overlap the null value (e.g. OR=1) - evidence for lack of association between the exposure and outcome (Szumilas, 2010).

The necessary formula to calculate OR is the following:

		Outcome Status	
		+	–
Exposure status	+	a	b
	–	c	d

Where

a = Number of exposed cases
b = Number of exposed non-cases
c = Number of unexposed cases
d = Number of unexposed non-cases

$$OR = \frac{a/c}{b/d} = \frac{ad}{bc}$$

Confidence intervals are calculated using the formula that is shown below:

$$\text{Upper } 95\% \text{ CI} = e \wedge [\ln(OR) + 1.96 \sqrt{(1/a + 1/b + 1/c + 1/d)}]$$

$$\text{Lower } 95\% \text{ CI} = e \wedge [\ln(OR) - 1.96 \sqrt{(1/a + 1/b + 1/c + 1/d)}]$$

Normally, the OR is calculated with the resource of a control group, however in our case we just had access to diabetic individuals, thus we did a direct comparison among the different types of diabetes, something that as far as we know hadn't been done before. This away, the estimation based on the concept of Odds Ratio that we obtained shows the risk of developing one type of diabetes instead the other analyzed types of

diabetes. The following example will explain the process that we use to calculate odds ratio in this work:

1) What type of diabetes is more likely to develop, having a mother with Diabetes (not discriminate):

	T1D	LADA	T2D
Only mother with diabetes	N = 264/3013 8.76%	N = 61/373 16.35%	N = 2765/13488 20.50%

Let´s begin to calculate the risk of develop T2D instead T1D or LADA, having a mother with Diabetes:

a = the exposed cases are the type 2 diabetic with mother with diabetes (a=2765)

b = the exposed non-cases are the 2 diabetic that do not have a diabetic mother (b= 13488 – 2765 ⇔ b = 10723)

c = exposed non-cases are the people with a diabetic mother that do not have type 2 diabetes (c = 264 + 61 ⇔ c = 325)

d = unexposed non-cases are the people that do not have a diabetic mother among type 1 and LADA and do not have type 2 diabetes.

(d = (3013+373) – (264+61) ⇔ d = 3061

$$OR(T2D) = \frac{2765 \ x \ 3061}{10723 \ x \ 325} \Leftrightarrow OR = 2.43 \ (95\% \ C.I = 2.15 - 2.74)$$

We can repeat the same process for T1D and LADA, in order to compare the OR and in this away achieve which type is more probably to develop, having a mother with diabetes:

$$OR \ (LADA) = \frac{61 \ x \ 13472}{3029 \ x \ 312} \Leftrightarrow OR \ (LADA) = 0.87 \ (95\% \ C.I=0.66-1.15)$$

Note: Non significant because it spans the null value.

$$\text{OR (T1D)} = \frac{264 \; X \; 11035}{2826 \; X \; 2749} \Leftrightarrow \text{OR (T1D)} = 0.375 \; (95\% \; \text{C.I} = 0.33 - 0.43)$$

Comparing the three OR obtained we can thus conclude that the risk of developing T2D instead T1D or LADA is higher, having a mother with diabetes mellitus.

3.4.2 Study 2

3.4.2.1 Oral Glucose Tolerance Test (OGTT)

The glucose tolerance test was first described in 1923 by Jerome W. Conn. This test was based on the fact that after carbohydrate ingestion, normal patient will rapidly return to normal levels of blood glucose after an initial spike. Thus, OGTT has the capacity to identify defects in the regulation of glucose metabolism after a meal, in this case after the ingestion of a higher concentrate glucose solution, allowing the identification of IGT subjects. The OGTT is currently the gold standard for the diagnosis of diabetes. The interpretation is based on venous plasma glucose results before and 2 hours after a 75 g oral glucose load (Harris et al., 2013). However the number of blood collections during the test is variable, depending on the purpose of the study. In our case, we decided four times because previous studies show us that the 30 and 90 minutes are essential points to evaluate the three processes in regulation of insulin levels, something that is essential in order to maintain glucose homeostasis. The Royal Australian College of General Practitioners (RACGP) recommends an OGTT when the results of fasting or random blood glucose are equivocal (Figure 2.1): fasting 100 mg/dL–125 mg/dL; random 100 mg/dL–199 mg /dL. An OGTT is unnecessary if fasting or random blood glucose values are clearly in the nondiabetic or diabetic range: fasting or random <100 mg/dL; fasting ≥ 125 or random ≥ 200mg/dL respectively. However, diagnosis should only be based on laboratory results, not results from a glucose meter. The test is also recommended to pregnant woman in order to detect gestational diabetes (Phillips, 2012).

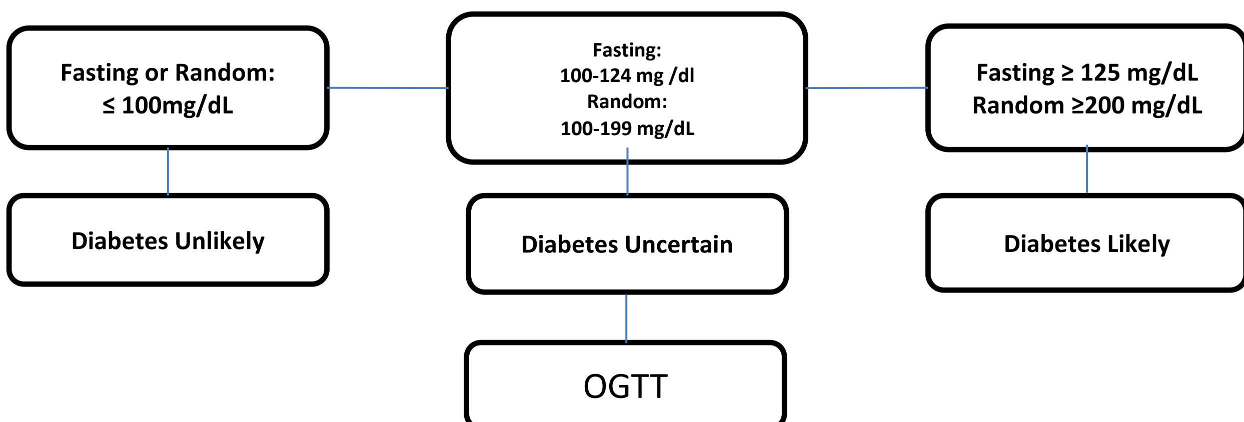

Figure 6 - The RACGP recommendations for when to perform an OGTT (from Harris et al., 2013)

3.4.2.2 Serum preparation

The whole blood was collected to a vaccuette tube (9 ml) containing no anticoagulant and then centrifuged at 3500 rpm during 10 minutes at room temperature. The resulting supernatant is designated serum and it was used in automatic auto analyzers to assess several parameters.

3.4.2.3 Whole blood preparation

The whole blood was collected to a vaccuette tube (3 ml) containing anticoagulant (EDTA) and it was used to assess platelets and HbA1c.

3.4.2.4 Categorizing glucose tolerance in T2D siblings

The criteria adopted to categorize T2D siblings were based on American Diabetes Association (American Diabetes association, 2010). Regarding to fasting plasma glucose, the subject was categorized according to the following parameters in the table 7:

Table 7- Parameters used to classify glucose tolerance according to fasting plasma glucose levels.

Result	Fasting Plasma Glucose (FPG)
Normal Glucose Tolerance (NGT)	less than 100 mg/dl
Impaired Fasting Glucose (IFG)	100 mg/dl to 125 mg/dl
Type 2 Diabetes (T2D)	126 mg/dl or higher

After the OGTT, the subjects were categorized according to the following parameters (Table 8):

Table 8 - Parameters used to classify glucose tolerance according to glucose levels after an OGTT (75g glycose).

Result	Oral Glucose Tolerance Test (OGTT)
Normal Glucose Tolerance (NGT)	less than 140 mg/dl
Impaired glucose tolerance (IGT)	140 mg/dl to 199 mg/dl
IFG + IGT	100 mg/dl to 125 mg/dl in fasting and 140 mg/dl to 199 mg/dl after the OGTT
Type 2 Diabetes (T2D)	200 mg/dl or higher

3.4.2.5 Homa-IR

Homeostasis model assessment as an index of insulin resistance (HOMA-IR), developed in 1985 by Matthews and coworkers, was used in this study as it is a simple and appropriate method in epidemiological studies where dynamic studies like the euglycaemic glucose clamp technique, though the gold standard, may not be feasible due to the degree of sophistication and cost of necessary apparatus. The HOMA-IR method requires measuring a single fasting plasma glucose and the corresponding fasting plasma insulin level and it is the most commonly used surrogate measure of insulin resistance in vivo, being the ability of identifying a proportion of subjects with insulin resistance without directly measuring insulin action. In terms of precision (reproducibility of measure), HOMA-IR is comparable to the glucose clamp technique. Although the HOMA-IR has been widely used, its cut-off for insulin resistance has not been conclusive. In addition, the HOMA-IR cut-off points to diagnose insulin resistance cannot be readily applied to all populations and may vary from race to race. In this work we used HOMA-IR to do a comparison among the different types groups of glucose tolerance. The formula was used to estimate the HOMA – IR is the following: $\frac{insulin\left(\frac{mU}{L}\right) X\ glucose\left(\frac{mg}{dL}\right)}{405}$(Esteghamati et al., 2012; Matthews et al.,1985).

3.4.2.6 OGIS

OGIS (an acronym for Oral Glucose Insulin Sensitivity) is a method for the assessment of insulin sensitivity from the oral glucose tolerance test. OGIS provides an index which is analogous to the index of insulin sensitivity obtained from the glucose clamp. This complex mathematic model was developed in 2001 by Andrea Mari and has the advantage of considering the effect of insulin clearance on that. Unlike HOMA-IR allows us to evaluate the insulin sensitivity during the OGTT, therefore during the fed state and not only in the fasting state. The necessary equations to determine OGIS Insulin sensitivity index are described in (Mari et al., 2001) or can be downloaded on http://webmet.pd.cnr.it/ogis/. This method calculates insulin sensitivity with a model-derived equation of the form: OGIS = f (G_0, G_{90}, G_{120}, I_0, I_{90}, I_{120}, D_O), where G and I are glucose and insulin concentrations (subscripts represent time instant) and D_O is the oral glucose dose (g/m2 body surface area). The expression of f contains some parameters, chosen to maximize the agreement with the Hyperinsulinemic Euglycemic Clamp (HIEC). OGIS is a predictor of the HIEC insulin sensitivity, expressed as glucose clearance M/G, normalized to body surface area. The units of OGIS are thus

ml/min/m2 of body surface area. OGIS has been validated against a 120 mU/min/m2 insulin infusion HIEC (by direct comparison of the glucose clearance values), instead of the most standard 40mU/min/m2 used in the previous methods. Formulas for a 3 h and 2 h OGTT are also available (Patarrão et al., 2014).

3.4.2.7 Fatty Liver Index (FLI)

Diagnosis of NAFLD is regarded as clinically problematic due to the invasive character of the gold-standard method of liver biopsy. Bedogni and co-workers introduced the FattyLiver Index (FLI), a multivariate model including biomarkers to accurately estimate presence of fatty liver. This estimation has already been applied by scientific investigations including large populations to determine the prevalence of fatty liver. Presence of fatty liver was evaluated using the recently validated FLI. The index uses an algorithm based on body-mass-index (BMI), waist circumference (WCF), triglycerides (TG), gamma-glutamyl transferase (GGT), and natural logarithm (ln) as it follows:

$$FLI = (e^{0.953*loge\ (triglycerides) + 0.139*BMI + 0.718*loge\ (ggt) + 0.053*waist\ circumference - 15.745}) / (1 + e^{0.953*loge\ (triglycerides) + 0.139*BMI + 0.718*loge\ (ggt) + 0.053*waist\ circumference - 15.745}) * 100$$

The subjects were classified into three groups: with FLI $\leq$ 20, who have a very low risk for fatty liver; with 20<FLI<60, an intermediate group; and the high risk group with FLI $\geq$ 60(Bedogni et al., 2006).

3.4.2.8 NAFLD fibrosis score (NFS)

Patients with NASH can have a significant progression of fibrosis within a few years. Recently, a simple, noninvasive tool used for liver fibrosis assessment has been developed. This new scoring system, the NAFLD fibrosis score (NFS), is a composite score of age, hyperglycemia, body mass index, platelet count, albumin, and aspartate aminotransferase and alanine aminotransferase (AST/ALT) ratio and was found to independently identify NAFLD patients with and without advanced fibrosis at initial NAFLD diagnosis. Thus, the NFS is composed of 6 variables, including age, hyperglycemia, BMI, platelet count, albumin, and AST/ALT ratio as independent indicators of advanced liver fibrosis. NAFLD fibrosis score = -1.675 + 0.037 $\times$ age (year) + 0.094 $\times$ BMI (kg/m2) + 1.13 $\times$ IFG/diabetes (yes = 1, no = 0) + 0.99 $\times$ AST/ALT ratio - 0.013 $\times$ platelet count ($\times 10^9$/L) - 0.66 $\times$ albumin(g/dL). According to the results obtained, subjects were differentiated according the following parameters:

NFS <-1.5 for low, NFS ≥ -1.5 to NFS < 0.67 for intermediate and NFS ≥ 0.67 for high probability of fibrosis (Angulo et al., 2007)

3.4.2.9 Hepatic Insulin Clearance

Hepatic insulin clearance can be estimated using simple measurements of the ratio of C-peptide to insulin, based on the assumption that C-peptide is secreted by pancreatic β-cells in equal amounts with insulin; however, unlike insulin, C-peptide is not extracted by the liver and has a constant peripheral clearance. Due to the different elimination kinetics of insulin and C-peptide, the C-peptide/insulin ratio can be used to estimate the amount of insulin that was clear in the liver. However this estimation is more feasible during steady-state conditions, eg, in the fasting state, whereas the estimation of insulin clearance during non-steady-state conditions requires modeling of C-peptide kinetics to calculate pre-hepatic insulin secretion rates to which peripheral insulin can be compared. However the models that will allow us to do that evaluation have been being developed. For estimating insulin clearance, the molar ratio of C-peptide over insulin was calculated from the plasma levels at each time and from the total areas under the respective concentration curves (Meier et al., 2007).

3.4.2.10 BioBank

In order to realize future investigations, all the blood samples analyzed were distributed by different Eppendorfs according to the amount of serum or whole blood remained after the analysis, and stored at – 80ºc.

3.5 Statistical analysis

In study 1 the calculation of odds ratio was performed to access to the risk of developing diabetes according to type of F.H.D. In order to evaluate if the analyzed populations displayed a gaussian distribution a Shapiro–Wilknormality test was performed ($p<0.05$). The average age between the different groups of diabetics as well as between the genders within the LADA, T1D and T2D subjects was analyzed by Mann Whitney test ($p<0.05$). In study 2 due to the small size of population was not possible to perform statistical analysis.

4. Results

4.1 Study 1

Analyzing the age distribution in the three different populations it was possible to observe that in T1D (Fig. 7) the higher density of population was located below the 50 years (72.52%), while in T2D (Fig 8) the majority of individuals were located after 60 years (76.45%) . The LADA population (Fig 9) has shown an intermediate behavior with the higher group above 70 years and (76.41%).

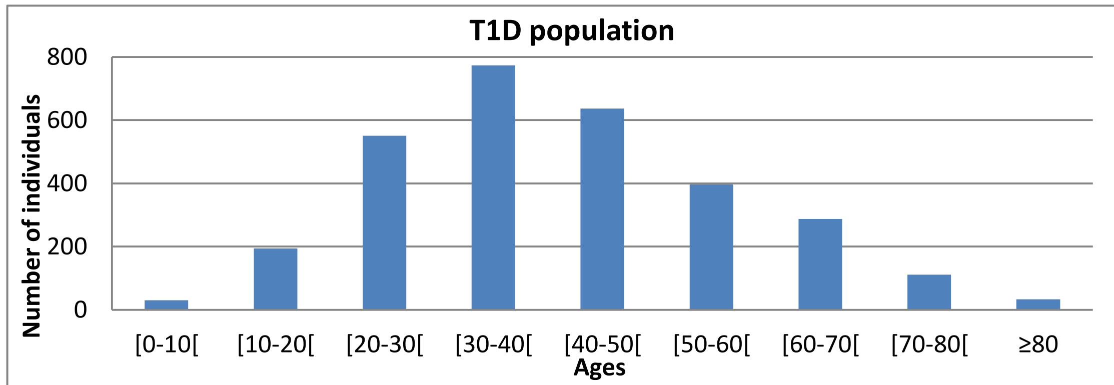

Figure 7 - Age distribution of T1D subjects. Data are presented in number of individuals distributed in 10 years categories.

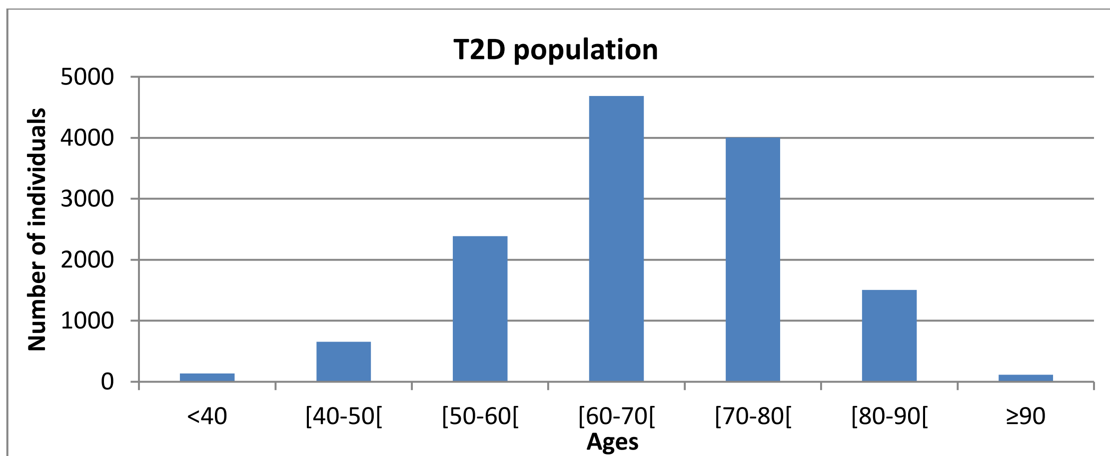

Figure 8 - Age distribution of T2D subjects. Data are presented in number of individuals distributed in 10 years categories.

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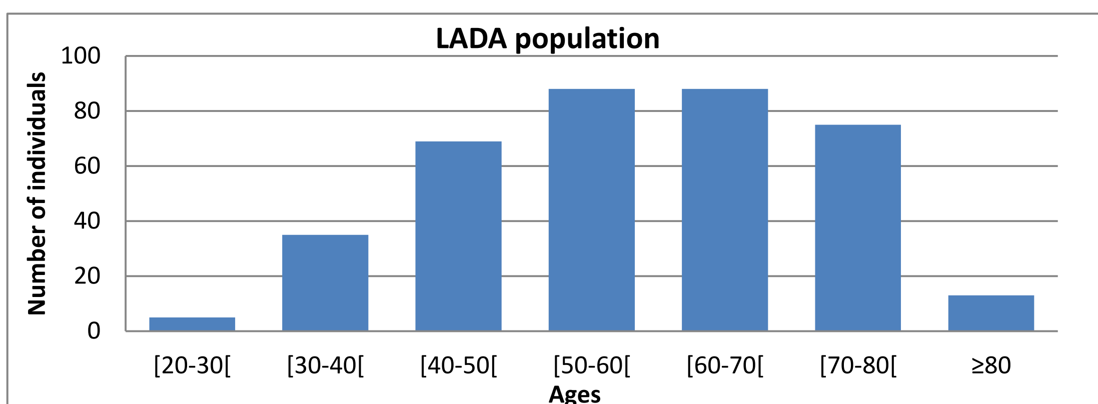


Figure 9 - Age distribution of LADA subjects. Data are presented in number of individuals distributed in 10 years categories.

As the three populations analyzed do not follow Gaussian or Normal distribution in the Mann-Whitney test (T1D: $p < 0.001$; LADA $p<0.001$; T2D $p<0.001$), we had to use non-parametric tests for the analysis process.

The type 1 diabetes group was constituted by 1649 males and 1364 females with an average age of 40.73 ± 15.53 (3 – 90 yr. old) and 40.66 ± 16.32 (2 – 98 yr. old), respectively. No statistical differences were found between the average ages of the genders.

The LADA population was the smallest analyzed, with 180 males and 193 females with an average age of 55.40 ± 13.68 (28 – 85 yr. old) and 60.51± 13.15 (22 – 100 yr. old), respectively. It was found a statistical difference between these average ages of the genders ($p < 0.01$).

The type 2 diabetes population was comprised by 7047 males and 6441 females, with an average age of 65.94 ± 10.56 (18 – 97 yr. old) and 67.88 ± 10.81 (19 – 102 yr. old), respectively – with a significant difference between these averages. The three populations analyzed were statistical different regarding the average age (T1D: 40.70 ± 15.89 vs LADA: 58.04 ± 13.63 vs T2D: 66.86 ± 10.73; $p < 0.001$).

The T1D population had the lowest prevalence of FHD among populations analyzed (~50%), with LADA and T2D subjects presenting a prevalence of FHD ~60 %.

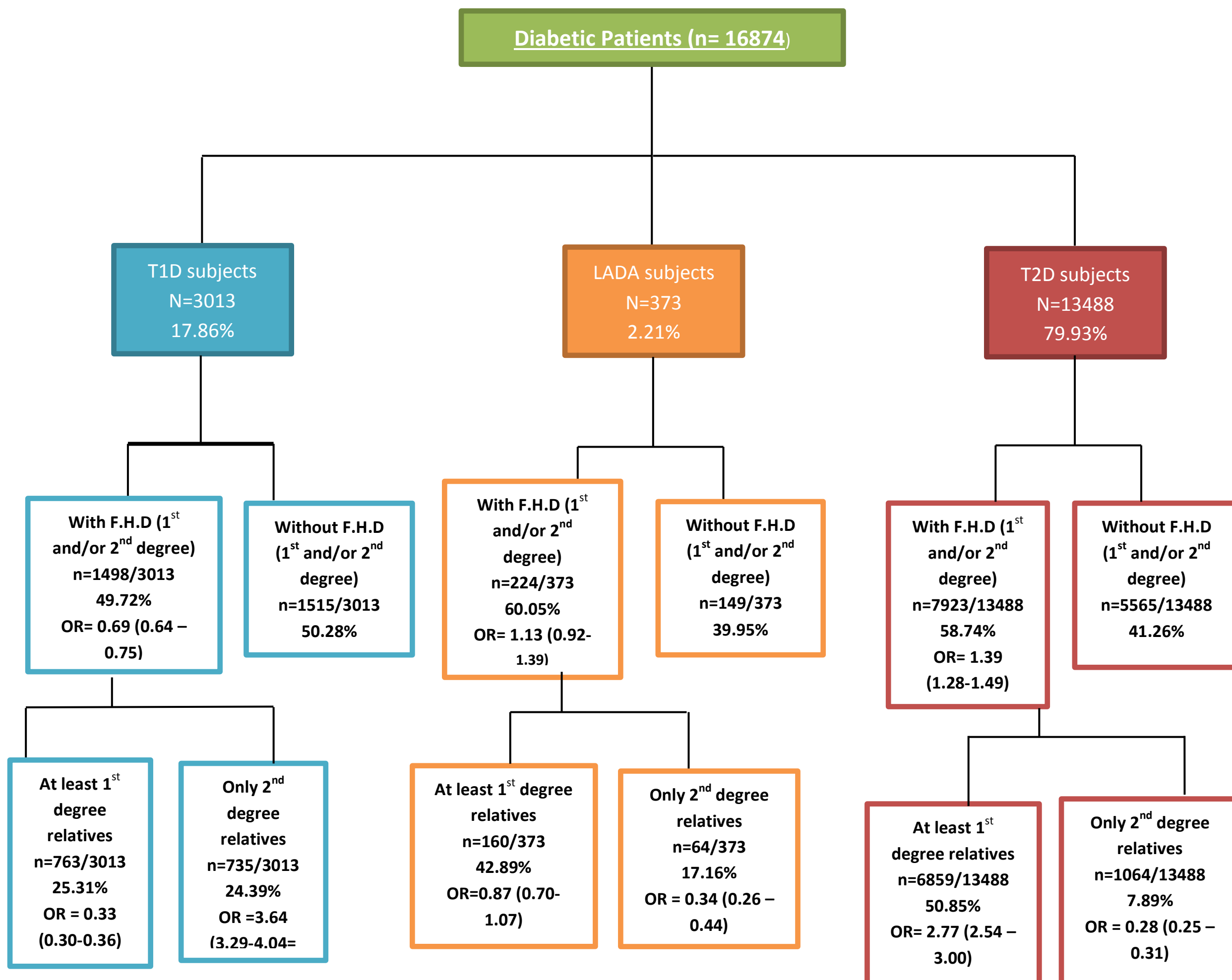


Figure 10 - Prevalence of FHD and odds ratio in the three populations analyzed. The prevalence is presented in %, while the odds ratio is presented with a 95% confidence interval.

In TID subjects with F.H.D, second degree relatives were the more prevalent group and those who increased most the risk to develop T1D, while for T2D group the first-degree relatives showed the same pattern. LADA population displayed a high prevalence of first-degree and second degree relatives with diabetes in the family (Fig 10). T2D was the most prevalent type of diabetes in the relatives of the three types of diabetics, however the OR indicated that the type of FHD clearly influence the type of diabetes that the subjects are in risk to develop in the future. Presence of T1D in relatives increased the risk for T1D, and presence of type 2 relatives increased the risk for T2D. It was possible observed too that a FHD that includes relatives with type 1 and type 2, therefore a combination of this two types of diabetes in the family, increased the risk of develop LADA instead type 1 or type 2 diabetes (Table 9).

Table 9- Type of F.H.D present on T1D, LADA, and T2D subjects. The prevalence is presented in %, while the odds ratio is presented with a 95% confidence interval. Only T1D relatives – patient of APDP with a FHD only composed by type 1 diabetic. Only T1D relatives – patient of APDP with a FHD composed only by type 1 diabetics. T1D and T2D relatives - patient of APDP with FHD composed by

relatives with T1D and T2D. Only T2D relatives - patient of APDP with a FHD composed only by type 2 diabetics.

Family history of diabetes	Only T1D Relatives			T1D and T2D relatives			T2D Relatives		
	n	**%**	**OR (95% CI)**	**n**	**%**	**OR (95% CI)**	**n**	**%**	**OR (CI 95%)**
T1D patients (n=763)	177	5.87	7.66 (6.03 – 9.74)	69	2.29	2.03 (1.53-2.70)	517	17.16	0.22 (0.20- 0.24)
LADA patients (n=160)	13	3.49	2.12 (1.21 – 3.74)	12	3.22	2.52 (1.39-4.55)	135	36.19	0.74 (0.59-0.91)
T2D patients (n=6859)	99	0.73	0.12 (0.10 – 0.16)	146	1.08	0.44 (0.34-0.59)	6614	49.04	4.04 (3.69-4.43)

The prevalence and type of diabetes in the parents was a parameter evaluated (table 10). The higher prevalence of diabetic parents was found in type 2 patients; followed by LADA and type 1 patients. LADA and T2D subjects displayed similar results regarding to prevalence, with higher percentage of diabetes in only mothers than only fathers (twice times higher) and both parents. In type 1 diabetics, the prevalence was nearly equal for only mothers and only fathers with diabetes, and lowest for cases with both parents with the disease. The OR indicated for T1D and T2D that just the fact that having only one parent with the respective type of diabetes increased the risk of development the disease. However for type 1 the risk was higher having only father with T1D, while in the case of T2D the risk was higher having only mother with type T2D. Having both parents with the same type of diabetes is also a risk factor to develop T1D or T2D, depending on the type of diabetes present in these parents. Regarding to LADA, it was only found significant OR in the case of having parents with different types of diabetes, which was in concordance with results obtained in table 9.

Table 10 - Prevalence of diabetes in parents with respective odds ratio. The prevalence is presented in %, while the odds ratio is presented with a 95% confidence interval. The data represent the number of patients of APDP with different parental history.

Type of diabetics	T1D patients			LADA patients			T2D patients		
Parental history	N	%	OR	N	%	OR	n	%	OR
Without parental diabetics	2428	80.58	-	257	68.90	-	8301	61.54	-
With Parental Diabetics	585	19.42	0.39(0.35-0.43)	116	31.10	0.84 (0.67 – 1.05)	5187	38.46	2.39 (2.19 – 2.62)
Only father with DM	252	8.36	0.14 (0.12-0.16)	31	8.31	0.73 (0.50- 1.05)	1580	11.71	1.46(1.28-1.66)
T1D	42	1.39	8.89 (5.30-14.91)	2	0.54	1.43 (0.35 – 5.87)	20	0.15	0.11 (0.07-0.19)
T2D	210	6.97	0.58 (0.50 - 0.67)	29	7.7	0.70 (0.48 -1.03)	1560	11.57	1.72 (1.49-1.98)
Only mother with DM	264	8.76	0.38 (0.33 - 0.43)	61	16.35	0.87 (0.66-1.15)	2765	20.50	2.43 (2.15-2.74)
T1D	29	0.96	3.63 (2.23 – 5.91)	2	0.54	1.38 (0.34 – 5.68)	35	0.26	0.28 (0.17 – 0.48)
T2D	235	7.80	0.34 (0.29 – 0.39)	59	15.82	0.86 (0.64 – 1.14)	2730	20.24	2.67 (2.35 – 3.03)
Both parents with diabetes	69	2.29	0.35 (0.27 – 0.45)	24	6.43	1.18 (0.77 – 1.79)	842	6.24	2.36 (1.89 – 2.93)
T1D	4	0.13	3.68(0.99-13.72)	-	-	-	5	0.04	0.31 (0.08 – 1.17)
T2D	59	1.96	0.30 (0.23 – 0.40)	22	5.90	1.09 (0.71 – 1.79)	836	6.20	2.69 (2.14 – 3.40)
With different types of DM	6	0.20	3.46 (1.20-9.97)	2	0.54	11.11(2.35-52.52)	6	0.04	0.19 (0.07 – 0.54)

It was detected a higher prevalence of diabetes in offspring of people with LADA than in the offspring of the other patients with T1D and T2D. Regarding to diabetic siblings, the prevalence of those was higher in type 2 diabetic population. It was noticed too that the number of siblings affected with diabetes was always larger than the offspring in all diabetic groups analyzed, demonstrating a higher prevalence of diabetes in siblings than in offspring. Analyzing the offspring diabetic of people with type 1 diabetes, it was possible to observe that type 1 diabetes was more prevalent, while in siblings it was found an equal incidence of type 1 or type 2 diabetes. In LADA group, the majority of offspring with diabetes were detected with T1D whereas the majority of siblings presented type 2 diabetes. Regarding to offspring and siblings diabetics of T2D population, it was clearly observed a higher prevalence of T2D in both relatives (Fig 11).

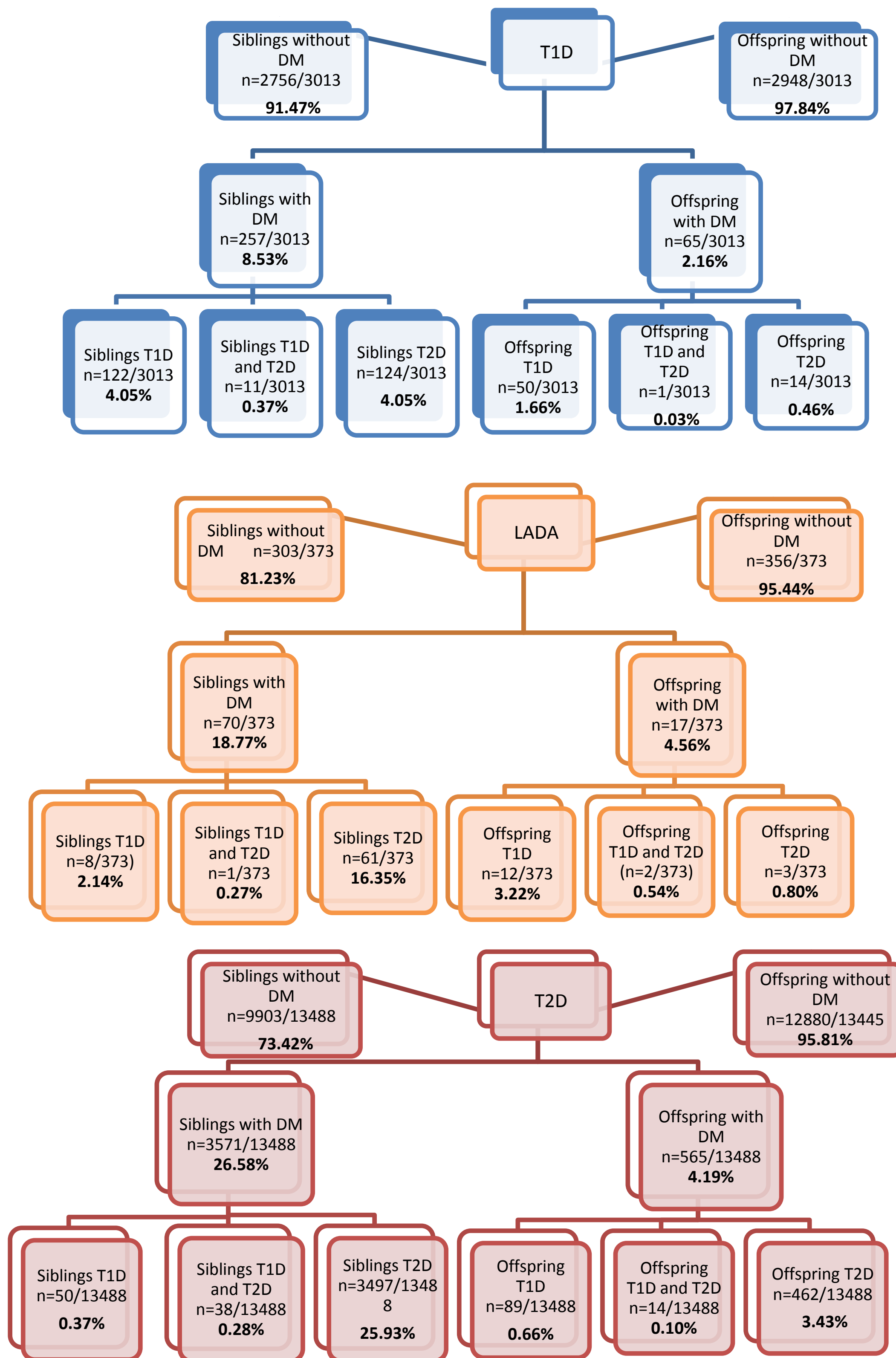


Figure 11- Prevalence of diabetes in siblings and offspring of people with T1D, LADA or T2D. The prevalence is presented in %. Siblings T1D – patient of APDP with type 1 diabetic siblings; Siblings T1D and T2D – patient of APDP with at least two siblings, one with T1D and other with T2D; Siblings T2D – patient of APDP with type 2 diabetic siblings.

Table 11 – Prevalence of diabetes and OR in relatives of diabetics with FHD composed by only first-degree relatives. The prevalence is presented in %, while the odds ratio is presented with a 95% confidence interval.

Relatives with Diabetes	**T1D**			**LADA**			**T2D**		
	(n)	**%**	**OR (95% IC)**	**(n)**	**%**	**OR (95% IC)**	**(n)**	**%**	**OR (95% IC)**
Only parents	214	7,10	0.47 (0.41 – 0.54)	51	13,67	1.09 (0.81 – 1.46)	1887	13,99	1.92 (1.67 – 2.19)
Only Siblings	82	2,72	0.36 (0.29 -0.45)	17	4,56	0.70 (0.43 – 1.14)	971	7,20	2.58 (2.09 – 3.18)
Only siblings with T1D	48	1,59	14.01 (7.94 – 24.70)	3	0.80	2.19 (0.54 – 6.23)	13	0.10	0.06 (0.03-0.12)
Only Siblings with T2D	31	1,03	0.14 (0.10 – 0.20)	14	3.75	0.62 (0.36 – 1.06)	950	7.04	5.63 (4.16 – 7.60)
Only Siblings with T1D and T2D	2	0,07	1.15 (0.24 – 5.42)	0	0	-	8	0.06	1.01 (0.21 – 4.75)
Parents and Siblings	59	1,96	0.20 (0.16 – 0.26)	22	1,07	0.74 (0.48 – 1.15)	1222	9,06	4.06 (3.24 – 5.11)

In order to further evaluate the impact of first-degree relatives with diabetes as risk factor to develop T1D, LADA or T2D, we analyzed specifically only siblings and parents. As was explained below, the subjects that we had access to all information regarding to F.H.D were the people only with first-degree relatives with diabetes. Thus, we decided access this variable only in this people, removing possible alterations caused by the presence of second-degree relatives with diabetes. The results indicated that the simply presence of first-degree relative increased the risk of develop diabetes. However, once again, the type of diabetes present in relatives clearly influences the disease that people are in risk to develop: first-degree relatives with T1D increase the risk to develop T1D while the presences of T2D in first-degree relatives increase the risk to develop T2D. No significant OR was found in LADA population, probably due the small size of the sample. The prevalence was higher in only parents group in both types of diabetes, but regarding OR, was noticed that among the first-degree relatives of people with diabetes, the presence of diabetes in siblings are indeed the higher risk factor to develop the disease (Table 3.3). This effect was specially observed in T1D population with an OR of 14.01 (95 %IC: 7.94 – 24.70).

4.2 Study 2

After the first part of this study, it was concluded that in fact the siblings of type 2 diabetics are a high risk group to develop the disease, not only due the OR obtained

but mainly due the higher prevalence of the disease. However, the previous results were totally achieved with basis on self-report of patients. Thus, the next step was naturally evaluated possible metabolic alterations that could be associated to this higher risk of develop diabetes by siblings of type 2 diabetics. In order to achieve this purpose we decided to perform an OGTT modified on healthy siblings of T2D patients. This procedure allowed in first, categorizes the siblings according to dysglycemic state. It was obtained 12 siblings with NGT, 4 with IFG, 1 with IGT, 3 with IFG+IGT and 3 with T2D. Therefore, among the 23 subjects analyzed, around 50% displayed some degree of dysregulation of glucose metabolism. The others parameters assessed in this population are described in table 12.

Table 12 – Characteristics of the Study Population (23 adults aged >18 categorized according to glucose tolerance in normal glucose tolerance (NGT), impaired fasting glucose(IFG), impaired glucose tolerance (IGT), impaired fasting glucose plus impaired glucose tolerance (IFG+IGT), Type 2 Diabetes (T2D). Abbreviations: BMI - Body mass index; HbA1c - Glycated hemoglobin; LDL - Low-density lipoprotein; HDL - high-density lipoprotein; TAG - Triacylglycerols.

Variable	All subjects	NGT	IFG	IGT	IFG+IGT	T2D
Number of subjects (%)	23 (100)	12 (52.17)	4 (17.39)	1 (4.35)	3 (13.04)	3 (13.04)
Age (yrs)*	56.48 ± 11.13	55.67 ± 10.01	56.5 ± 15.61	69	63.67 ± 6.81	48.33 ± 11.84
Gender (M/F)	8/15	3/9	1/3	1/0	1/2	2/1
Waist Circumference (cm) (M/F)*	107.38 ± 11.02 / 88.87 ± 12.36	101 ± 15.13 / 83.39 ± 6.76	110 / 94 ± 14.73	105/0	111/92.3±20.79	115 ± 11.31/116
BMI(Kg/m^2)*	29.70 ± 5.32	29.92 ± 3.66	30.11 ± 6.16	30.78	32.27 ± 4.31	37.38 ± 4.06
HbA1c %*	5.56 ± 0.89	5.19 ± 0.49	5.48 ± 0.53	5	5.73 ± 0.06	7.2 ± 1.4
[glucose]$_{0\ minutes}$ (mg/dL)*	108.63 ± 31.40	92.83 ± 3.79	107.28 ± 6.90	93.5	115.73 ± 4.47	175.8 ± 51.36
[glucose]$_{120\ minutes}$ (mg/dL)*	138.2 ± 73.27	106.23 ± 16.55	103.05 ± 13.91	152.60	174.27 ± 13.23	298.4 ± 86.53
LDL (mg/dL)	138.74± 34.35	125.25 ± 30.15	150 ± 33.80	171	151.33 ± 25.32	154.3 ± 56.16
Total cholesterol (mg/dL)*	194.29± 39.21	180.07 ± 34.28	207.8 ± 39.73	225.2	212.63 ± 35.02	204.5 ± 64.29
HDL (mg/dL)*	59.38 ± 33.96	68.86 ± 45.05	51.23 ± 5.91	43	55.33 ± 9.45	41.73 ± 9.21
TAG (mg/dL)*	125.58 ± 54.12	119.59 ± 55.26	124.45 ± 69.64	200.9	129.97 ± 70.01	121.57 ± 12

* The values are expressed in mean ± standard error of mean (SEM)

The number of individuals with obesity, central obesity, decreased HDL cholesterol level, increased LDL cholesterol levels, higher levels of total cholesterol and hypertriglyceridemia in the different groups are represented in table 13.

Table 13 - Prevalence of obesity and lipid profile in the overall population and in each dysglycemic categories.

	Overall (n=23)	NGT (n=12)	IFG (n=4)	IGT (n=1)	IFG+IGT (n=3)	T2D (n= 3)
Overweight	6	4	1	0	1	0
Obesity	11	3	2	1	2	3
Central obesity (n)	21	10	4	1	3	3
Decreased HDL cholesterol (n)	19	9	4	1	2	3
Increased LDL cholesterol (n)	22	11	4	1	3	3
Higher levels of total cholesterol (n)	9	2	2	1	2	2
Hypertriglyceridemia (n)	8	4	2	1	1	0

In this study, we examined siblings of diabetic patients aged between 41 and 68 years old and the average age of the siblings was 56 years old. Analyzing the values of waist circumference, and taking in count the cutoffs of IDF (Central obesity for Male: ≥ 94 cm and for Female ≥ 80 cm), it was possible observe that all the subjects with dysregulation of glucose metabolism (prediabetes or diabetes) present values of waist-circumference above the cutoffs, being this away considered as having central obesity. In NGT group, 2 males and 7 females presented central obesity while 1 men and 2 women presented a waist circumference normal. The BMI average in all groups were above the normal values (healthy weight - from 18.5 to 25 Kg/m^2), with NGT group being the only sub-group with values within the excess of weight category and the remainders within the obesity category. These results indicated a tendency in siblings to have dysregulation of weight control with high levels of obesity, mainly visceral (table 3.4 and 3.5).

Although HbA1c testing is mainly used for monitoring blood sugar control in patients with diabetes, the World Health Organization (WHO) now recommends that HbA1c can be used as a diagnostic test for diabetes, provided that stringent quality assurance tests are in place and assays are standardized to criteria aligned to the

international reference values. An HbA1c of 6.5% is recommended as the cut-off point for diagnosing diabetes. A value less than 6.5% does not exclude diabetes diagnosed using glucose tests. In our study the values of HbA1c, as expected, were above the cut-off in diabetic patients. Among the individuals in prediabetes state the values were higher for IFG+IGT sub-group.

Other interesting point that can be highlighting on these results was the fact that glucose levels on fasting state for overall population are within the range of prediabetes (IFG), while the values at 120 minutes are within the normal range. This is probably due the fact that exist a higher prevalence of IFG than IGT among the T2D-siblings.

Dyslipidemia is defined as an abnormal amount of lipids (e.g. cholesterol and/or fat) in the blood. Dyslipidemias may be manifested by deregulation of several parameters as elevation of total cholesterol, low-density lipoprotein (LDL) cholesterol and the triglyceride (TAG) concentrations, and a decrease in high-density lipoprotein (HDL) cholesterol concentration in the blood. This metabolic disorder contributes directly to a high risk of cardiovascular diseases (CVD). The National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) stablished the follow criteria to dyslipidemia: LDL$\geq$ 100mg/dL; total cholesterol $\geq$ 200 mg/dL; HDL $\leq$ 60 mg/dL; TAG $\geq$ 150 mg/dL. Taking in consideration these criteria, the overall population presented values above the range for LDL and slightly below the recommended for HDL. It was in NGT subjects that less deregulation were detected, with only the levels of LDL below the cutoff points. In prediabetic and diabetic state, it was observed a tendency to increase the levels of TAG, LDL and total cholesterol, and a decrease in HDL levels (table 3.5).

In the figure 12, 13, 14 it is showed the variation of glucose, insulin and c-peptide, respectively, during the OGTT. Through the analyses of insulin and c-peptide plasma levels it was possible understand the different profiles of insulin secretion in different dysglycemic states.

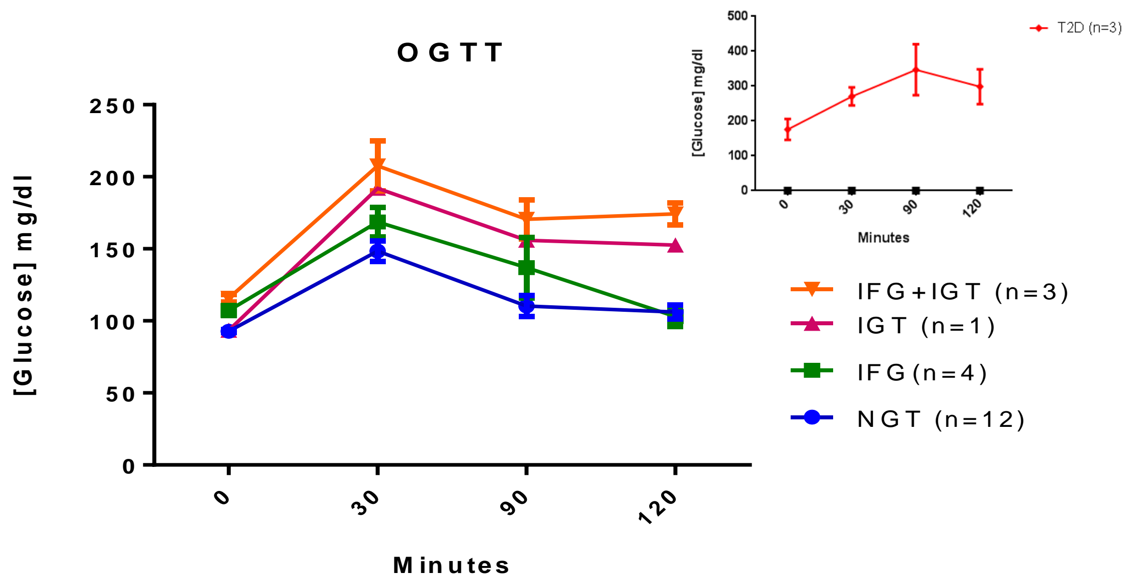


Figure 12 - Plasma glucose concentration during the OGTT in different categories. Data are presented as mean ± SEM.

The fasting plasma glucose levels of IFG were higher than the glucose levels observed in both NGT and IGT sub-groups (IFG: 107.26 vs IGT: 93.5 vs NGT: 92.83 mg/dl). The variation of glucose during the OGTT in the IFG group was intermediate between that of the normal and impaired glucose-tolerant groups. However, the 2-h plasma glucose concentration in the IFG group had returned to values observed in NGT and was markedly lower than in the impaired glucose tolerant group. Subjects with IFG+IGT had elevated plasma glucose and insulin concentrations, similar to those with IFG, and elevated glucose and insulin levels, similar to IGT. Thus, individuals with IFG+IGT shared the characteristics of both IFG and IGT.

In NGT sub- group, the regular secretion of insulin is showed, with a first phase of insulin release occurring in the initial minutes and the correspondent second phase occurring in the last minutes of OGTT (Fig 13 and 14).

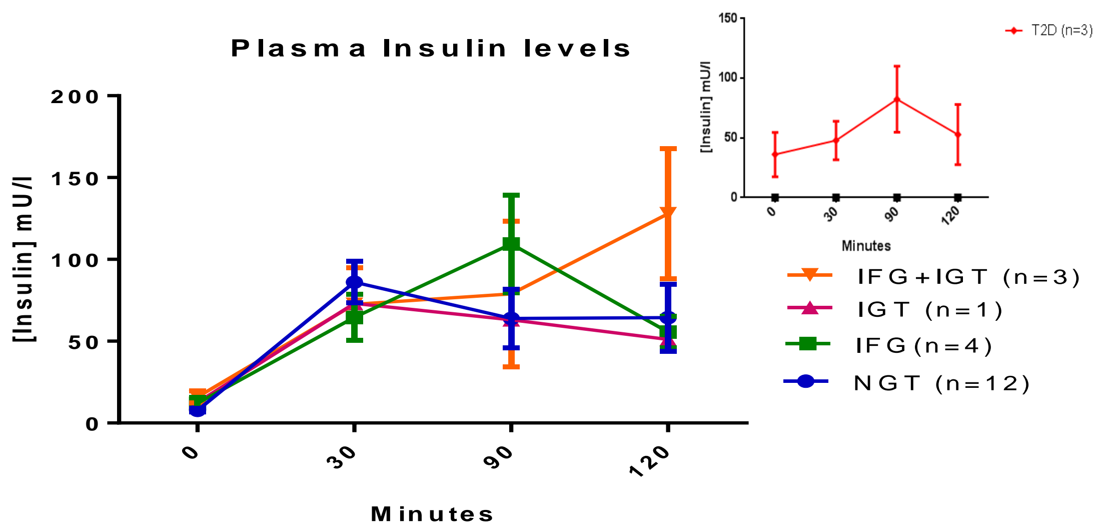


Figure 13 - Plasma insulin concentration during the OGTT in different categories. Data are presented as means ± SEM.

In IFG subjects it was possible observe a slightly increase of insulin and c-peptide levels in the first 30 minutes, however the higher increase occur in the minute 90 (probably last phase of insulin release or a delay in first-phase) allowing compensate the impaired first-phase and thus decrease glucose levels to normal ranges. In IGT group did not occurred such a higher increase as in IFG group at last minutes (Fig 13 and 14), which could have, possibly, as consequence higher glucose levels at minute 120 (Fig 12).

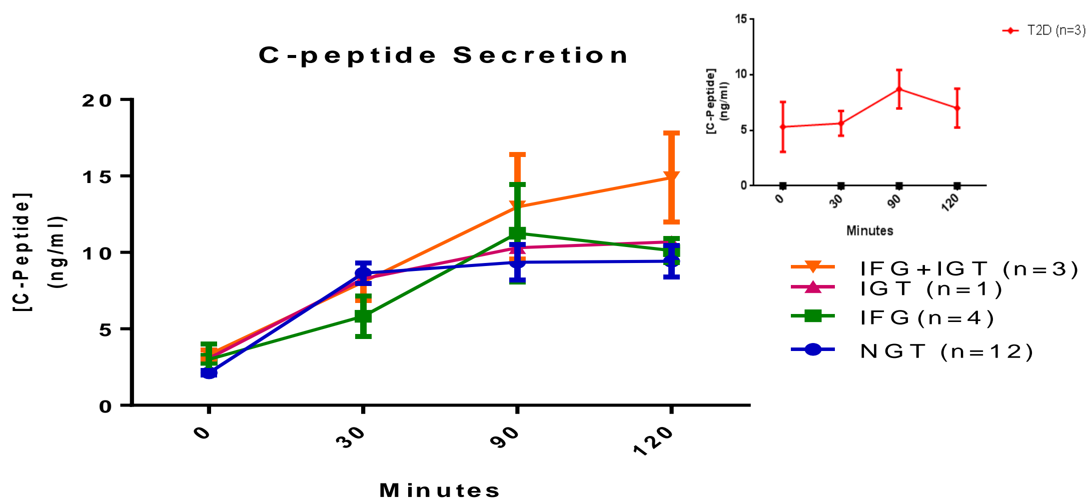


Figure 14 - C-peptide secretion during the OGTT in different categories. Data are presented as means ± SEM.

It was on IFG+IGT group that was achieved the higher levels of insulin and c-peptide secretion, what indicated that being the subjects with the worst glucose regulation among the prediabetic individuals, β-cell was probably trying to compensate the higher

levels of insulin resistance in order to maintain glucose levels below the diabetic range. In T2D group it was observed an expected lack of insulin and c-peptide secretion which it is a factor responsible for the occurrence of diabetes.

The other important process in regulation of insulin levels assessed in this project was insulin clearance. Due the fact that c-peptide (kidney) and insulin (liver) are cleared in different organs, and due to fact that both are secreted at same time and rate, it was possible have an estimation of insulin clearance through the calculation of c-peptide/insulin ratio plus estimation of area under the curve (AUC).

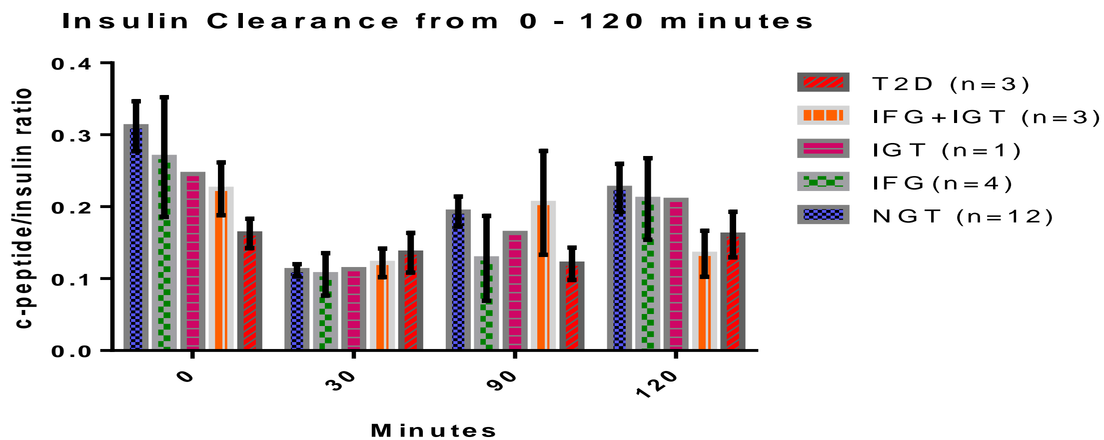


Figure 15 - Plasma C-peptide/insulin ratios during the OGTT in different categories. All results are presented as means ± SEM.

It was aimed that differences in insulin clearance would be related with different dysglycemic categories. Analyzing the values of c-peptide/insulin ration during the OGTT, it was observed that in feeding state the values of insulin clearance was characteristic of each dysglicemic category. It was also detected that the main alteration of this process occurred at minute 30 with a higher decrease of HIC, mainly in NGT and IFG group (Fig 15). This slope was not so noted in IGT and IGT+IFG and practically did not occur on type 2 diabetic (Fig 16). Nevertheless, all the groups diminished the hepatic insulin clearance to a value similar.

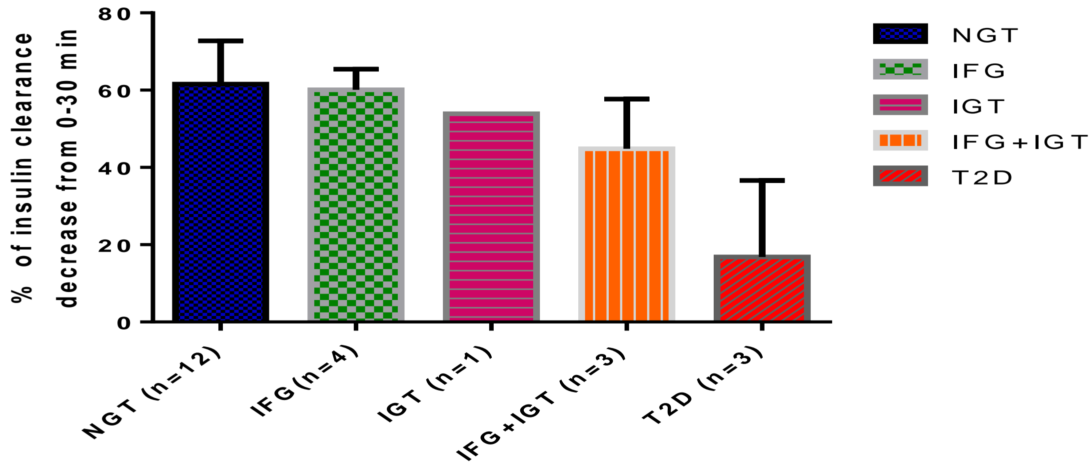


Figure 16 - % of insulin clearance decreased during the first 30 minutes of OGTT in different categories. All results are presented as means ± SEM.

Beside the decrease on insulin clearance had be larger on NGT and IFG group, it is important understand that a higher ratio of insulin clearance is associated with less insulin resistance. The rational of this is just the simple fact that on a NGT subject, higher levels of IC means less requirement of insulin to achieve normal glucose levels. Thus, our results are in accordance with this theory, with higher levels of insulin clearance in NGT group per minute at 30 and 120 minutes and concomitant lower rates of this process in prediabetics and mainly in diabetics (Fig 17).

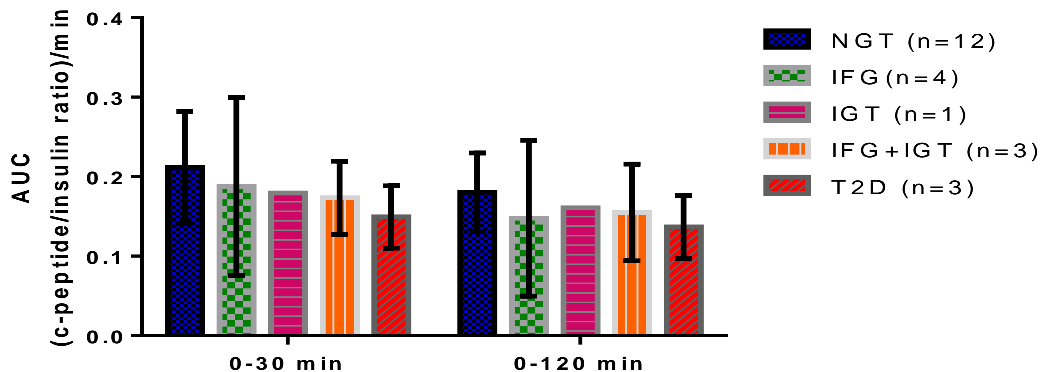


Figure 17- Insulin clearance per minute during the first 30 minutes and the overall OGTT for different categories. All results are presented as means ± SEM.

During the remainder minutes of OGTT, it was observed an increased on insulin clearance in all groups excepted in IFG+IGT group. However the tendency obtained in the first 30 minutes was sustained in the course of OGTT, with NGT group having the higher values of IC while the ratios of IC were smaller in subjects with abnormal states of glucose levels (Fig 15 and Fig 17). Among prediabetics, the AUC_{120min}/min of IFG

unlike was observed on AUC_{30min}/min, become lower than the values of clearance of IGT and IFG+IGT. Comparing the values of AUC_{30min}/min and the values of AUC_{120min}/min it was reported that the percentage of hepatic insulin clearance per minute was higher during the first 30 min than in the 120 minutes of OGTT, highlighting the importance of the decrease of this process in the first 30 minutes of OGTT (Fig 3.11).

The action of insulin was evaluated by the utilization of OGIS and HOMA-IR. According to HOMA-IR (Fig 18), IFG+IGT and IFG group exhibited the highest levels of insulin resistance among the prediabetics groups.

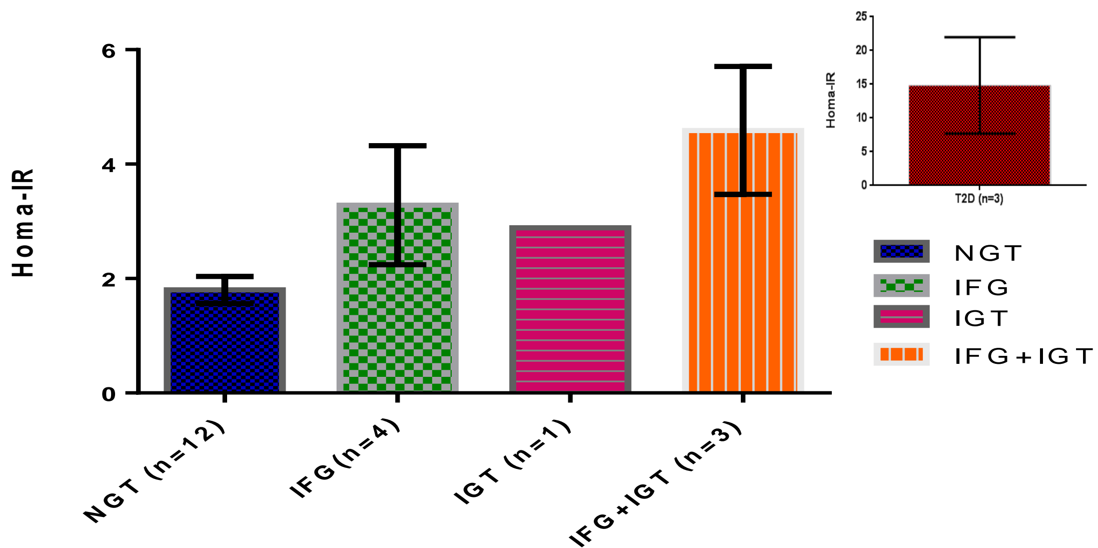


Figure 18 - Homeostasis model assessment as an index of insulin resistance (HOMA-IR) for different categories. Data are presented as means ± SEM.

The results obtained in OGIS (Fig 19) were in concordance with HOMA-IR, with the IFG and IFG+IGT group presenting the lower values of insulin sensibility of prediabetics. The diabetic subjects displayed the higher values of insulin resistance and the lower values of insulin sensitivity, while the NGT were the less resistant and those with more insulin sensitivity.

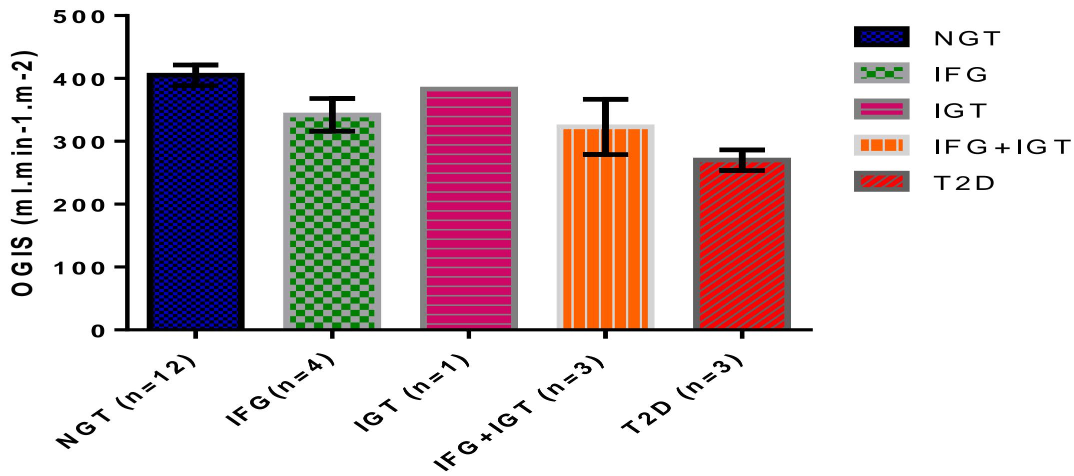


Figure 19 - Oral Glucose Insulin Sensitivity (OGIS) for different categories. Data are presented as means ± SEM.

Elevated plasma FFA is a common dysregulation in type 2 diabetes and early changes may be predictive for the transition of patients from prediabetes states to type 2 diabetes. These increase on FFA levels on circulation leads to an ectopic accumulation on several organs, like liver. Ectopic fat accumulation in the liver is responsible for abnormalities like nonalcoholic fatty liver disease (NAFLD), which is the most common cause of chronic liver disease in Western countries. Ectopic liver lipid, exacerbates hepatic insulin resistance, promotes systemic inflammation, and increases risk of developing both type 2 diabetes mellitus and cardiovascular disease. In our results, the levels of FFA in the fasting state were high mainly in IFG+IGT group.

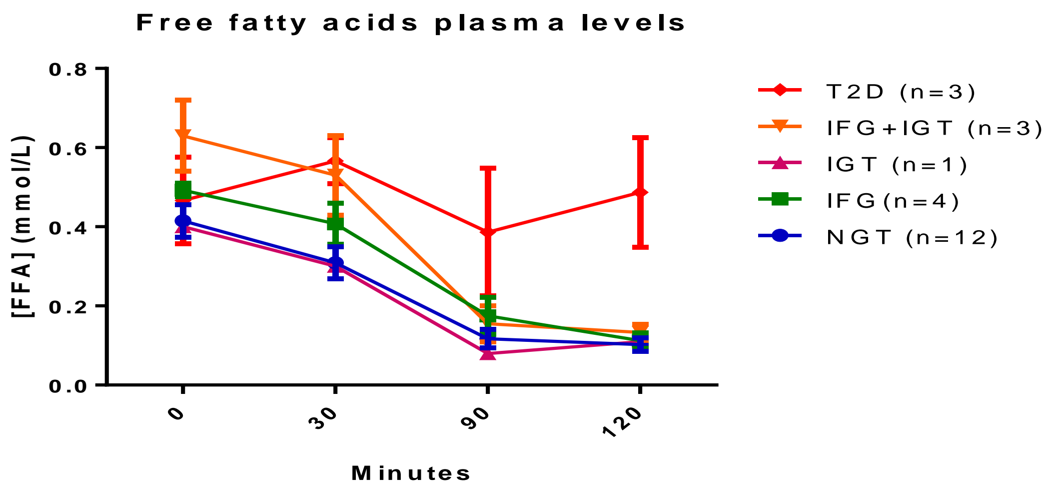


Figure 20 - Free Fatty Acid plasma levels during the OGTT in different categories. Data are presented as means ± SEM.

After the first 30 minutes, it was reported a higher increase of FFA levels on type 2 diabetes, while the levels of FFA on the other subjects decreased. Nevertheless at this point the FFA levels of IFG+IGT and IFG group were yet higher than IGT and NGT. With the continuation of OGTT, the levels of FFA decreased in all groups to similar levels while the levels of T2D remained high (Fig 20).

In order to assess the risk of fatty liver and liver fibrosis it was used the fatty liver index (FLI) and NAFLD fibrosis score (NFS), respectively. The results of FLI indicated that only 4 out of 23 siblings analyzed had low risk of fatty liver. The group with the lower FLI average was NGT subjects, followed by IFG, IFG+IGT, IGT subjects and finally with the higher FLI values the type 2 diabetics. The tendency here observed with these preliminary results was that with deterioration of glucose levels the number of subjects with high risk of fatty liver disease increases (Fig 21).

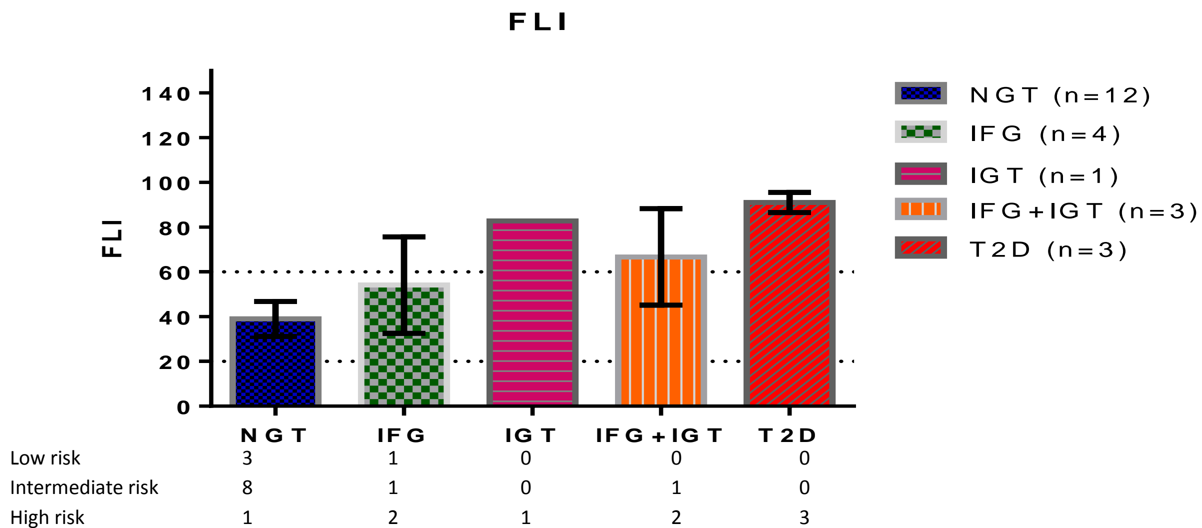
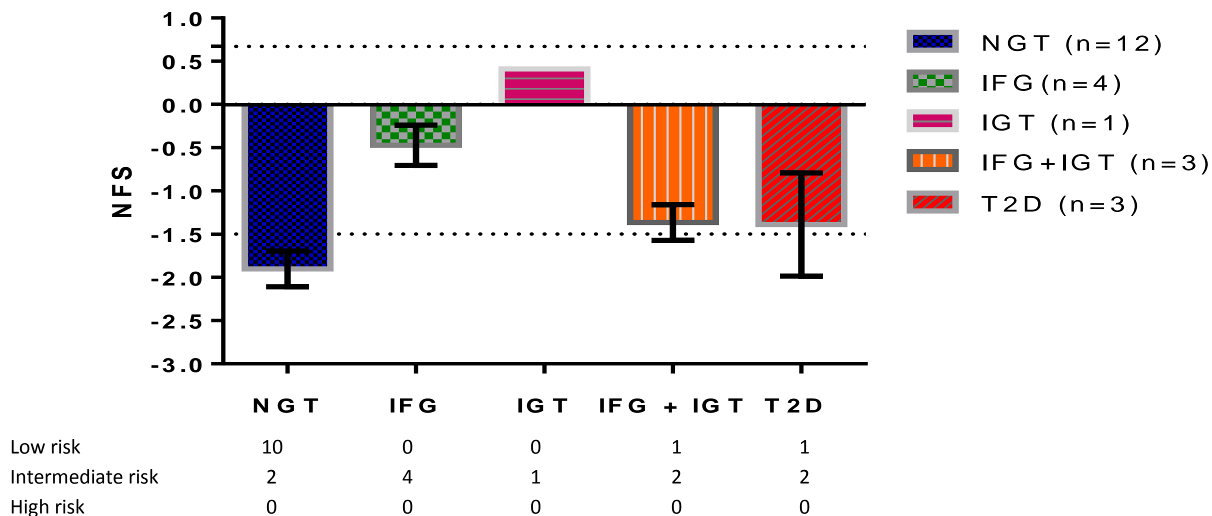


Figure 21 - Fatty liver index (FLI) for different categories . This data are presented as means ± SEM. The number of individuals within each category of FLI is presented in the figure. $FLI \leq 20$, with a very low risk for fatty liver; $20 < FLI < 60$, with an intermediate risk for fatty liver; and $FLI \geq 60$ with the high risk for fatty liver.

Regarding to NFS, it was observed a high number of NGT individuals (10 out of 12) within a range of low probability of liver fibrosis. In the remains groups the majority of individuals were located in the middle probability of liver fibrosis range (Fig 22).



	NGT	IFG	IGT	IFG + IGT	T2D
Low risk	10	0	0	1	1
Intermediate risk	2	4	1	2	2
High risk	0	0	0	0	0

Figure 22 - NAFLD fibrosis score (NFS) for different categories. This data are presented as means ± SEM. The number of individuals within each category of NFS is presented in the figure. NFS ≤ -1.5 for low probability of fibrosis, - 1.5 < NFS < 0.67 for Intermediate probability of fibrosis, and NFS > 0.67 for high probability of fibrosis.

5. Discussion

Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, and insulin clearance. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels. There are several risk factors that could lead to dysregulations of glucose metabolism with the worst scenario being the development of diabetes mellitus. Among these, family history of diabetes can be highlighted. A hereditary component of diabetes is suggested from a number of studies that assessed the influence of family history of diabetes on risk of diabetes (Joseph et al., 2010; Annis et al., 2005). However it has been hypothesized that the development of diabetes results from interactions between genetic and environmental factors. Family history of diabetes may reflect both genetic susceptibility and exposures to possible hazard environmental factors that are shared within the family. In our study we did two different approaches to analyze the importance of relatives with diabetes as a risk factor to develop the disease. Firstly, we used the clinic files of APDP patients to study the prevalence of FHD on the three types of diabetes more prevalent in our clinic, type 1 diabetes, LADA and type 2 diabetes. Several studies had assess the risk of FHD on these three types of diabetes, however to the best of our knowledge no studies had compared directly the risk between them

according to type of FHD present in relatives. Therefore the OR calculated represented the probability of determinate subjects develop one type of diabetes instead the others types of diabetes evaluated. The study 2 was performed with basis on the results obtained in study 1. It was concluded that in fact the siblings of T1D and T2D are a high risk group to develop the disease, however it was reported a higher prevalence of siblings with T2D in all patients analyzed. Furthermore, all the results obtained in the first part were self-reported, which naturally were associated with some percentage of bias. Thus, we further evaluated possible metabolic alterations on siblings of type 2 diabetics that could be associated to a higher risk of these group develop diabetes.

5.1 Study 1

It was detected significant differences regarding to average age of three types of diabetics analyzed. This difference can be explained by the different age of onset characteristic of each disease, with type 1 diabetes occurring mainly during the childhood (Lebenthal et al., 2010), LADA occurring after the 35 years (Naik et al., 2009), and T2D developing mainly in advanced age (Wikner et al., 2013). Therefore the results are in accordance with the expected, being the T2D population the older, follow by LADA and T1D population. Among the genders, it was detected significant differences regarding to average age in LADA and T2D group, however our goal was evaluate FHD in overall population and not separately, in spite of these differences have been found. Nevertheless, these possible differences among genders will be a subject explored in a next future study.

As expected the majority of people with diabetes were type 2 diabetics (79.93 %), with type 1 diabetics (17.86%) being the second more prevalent and LADA (2.21%) the lesser. The prevalence of T2D and LADA was lower while the prevalence of T1D was higher compared with other studies (Carlsson et al., 2007; Dabelea et al., 2014). This lower prevalence of LADA was probably due the difficulty of the diagnosis of this disease. The T1D and T2D results can be explained by the fact that our population was a convenience sample; the subjects attended at APDP are all advanced cases of diabetes. Thus, it is normal that the prevalence had some variability when comparing with prevalence existence in general populations that includes no distinguish cases of diabetes. In our findings it was observed a high prevalence of FHD for all diabetics populations which is in accordance with what is found in literature (Carlson et al., 2007) (T1D:49.72% vs LADA: 60.05 % vs T2D: 58.84). Regarding FHD in T1D

population, was observed a higher percentage of individuals with second-degree relatives, whereas in T2D a higher prevalence of subjects with first-degree relatives was found. Concerning to diabetic relatives of LADA individuals, it was observed a high prevalence of patients with first-degree relatives although the second-degree relatives had high prevalence too when compared with prevalence of second-degree diabetic subjects in T2D. The odds ratio indicated a high risk to develop T1D for people with second degree relatives affected by diabetes while the risk to develop T2D was elevated in the presence of first degree relatives with diabetes. No significant OR was found on LADA population likely due the small size of this population. In type 1 diabetic population, the larger prevalence of subjects with second-degree relatives was a result that is not in accordance with is described in literature (Parkkola et al., 2013). The same fact was reported in OR, with the literature pointing the presence of diabetes in first relatives as the high risk factor to development of T1D (Weires et al., 2007). However we did not had access to information related with the type of second-degree relatives involved in FHD, thus in order to better understand this results we will access the presence of diabetes in second degree relatives of APDP patients with higher levels of specificity. For T2D the prevalence and OR obtained is in accordance with the previous publications (Weires et al., 2007).

When analyzed the type of diabetes in relatives of each population, it was clear that type 2 diabetes was the more prevalent in all the groups analyzed. These results are in concordance with other already published, which may indicate a genetic background between type 1, type 2 diabetes and LADA (Li et al., 2000). The higher prevalence of type 2 diabetics in relatives of LADA patients is also a feature already described (Castleden et al., 2006). Nonetheless the important find that can be highlighted in these results is the fact that presence of relatives with type 1 and type 2 increases the risk of develop LADA. This result enforces the idea already stablished that LADA share common features with T1D and T2D.

After this general analysis to the population, we analyzed specifically the prevalence of diabetes in first-degree relatives, namely parents, siblings and offspring. The results regarding to parents confirmed some of information already published. The higher prevalence of diabetic parents was found in T2D subjects (38.46%), followed by LADA (31.10%) and T1D subjects (19.42%) (Carlson et al., 2007). In T1D population it was reported that the higher risk factor to develop T1D was the presence of a type 1 diabetic father, which was also observed in other studies (Gale & Gillespie, 2010). For

type 2 diabetes, on the other hand, it was found that a presence of a mother with T2D increase the risk of develop the disease. This maternal effect, thus the higher risk of diabetes associated with having a diabetic mother compared to when only the father was affected, already was found in several populations (Meiloud et al., 2013; Benrahma et al., 2011). For LADA population the main important achievement was again the fact that the risk increase in the presence of relatives, in this particular case parents, with different types of diabetes. No additional effect were observed in when both parents are diabetes in, this is in contrast with previous studies (Wikner et al., 2013). Considering the assessment of other first degrees relatives, it was observed a high prevalence of diabetes in siblings and offspring of T2D and LADA patients, being the percentage of offspring with diabetes even higher in LADA than in type 2 population. These results indicated that although LADA is an autoimmune disease like T1D, the inheritability of this type of diabetes is more similar with T2D.

The evaluation of parents and siblings of subjects with only first-degree relatives with diabetes, brought important information. The prevalence was higher in parents for both groups of diabetics. However it was noticed that the presence of diabetic siblings was the greater risk factor to develop the disease: type 1 diabetic sibling causes approximately a 14-fold increased risk of T1D (Redondo et al.,2008), whereas type 2 diabetic siblings causes approximately a 6-fold increased risk to develop T2D (Hemminki et al., 2010). In LADA results due the small size no OR with significant were found, but once again regarding to prevalence it was observed similar results with T2D subjects.

The main limitation in this study was the lack of a control group in order to evaluate the risk of diabetes in comparison with healthy subjects. The fact that all the subjects had diabetes did not allow us realize statistical processing in prevalence results, except the calculation of odds ratio. However the fact that we compared directly the three types of diabetes makes this research unique.

5.2 Study 2

Our results are clearly preliminary and the sample needs to be increased in order to perform statistical analyses that allow us to achieve significant comparisons among the different groups. Nevertheless, it was obtained several important evidences that may be used to understand possible metabolic alterations that are associated with high risk of diabetes in T2D-siblings.

It is important to understand that the results of family history of diabetes in study 1 was obtained basis on an interview realized during their first appointment at APDP. Thus, it is likely that these can contain some recall bias, once no one of relatives were in fact evaluated and the presence of diabetes was only assumed based on a questionnaire. In study 2 we performed a metabolic characterization of a high risk group to develop diabetes among the first relatives, namely the siblings. First degree relatives are a suitable group to perform metabolic studies since they are at inherently higher risk of progressing to T2D and are also likely to be more motivated to undergo testing and lifestyle changes to prevent T2D. There is evidence that the glucose intolerance of first degree relatives is more highly associated with decreased insulin secretion compared to the glucose intolerance of subjects without a T2D relative (Janghorbani & Amini, 2010) suggesting that glucose dysmetabolism in first degree relatives are more susceptible to develop T2D via b-cell failure. However, less is known about the other factors involved in glucose dysmetabolism, notably hepatic and peripheral insulin resistance and how they interact with defective insulin secretion to generate the T2D phenotype in these relatives. Here, hepatic insulin clearance may play an important but hitherto uncharacterized role in the coupling of insulin secretion to its hepatic and peripheral actions. In first degree relatives there is a fractional decrease in HIC (Emerson et al., 2009) possibly in response to a reduced b-cell secretion capacity but no clear mechanism is unveil. This is expected to potentiate any underlying hepatic insulin resistance and further erode the suppression of hepatic glucose production during feeding. The additional input of hepatic glucose into the bloodstream places a further burden on peripheral insulin actions above and beyond compensating for peripheral insulin resistance. In short, the secretion and clearance of insulin and the metabolic responses of liver and peripheral tissues are all interconnected components of glycemic control and therefore they all contribute directly or indirectly to a given T2D phenotype.

The prevalence of NGT in our study was 52.17%, while prediabetes was 34.78% (IFG:18.18%; IGT:4.55%; IFG+IGT:13.64%) and diabetes was 13.04%. Comparing our results with the results obtained in PREVADIAB study (Gardete-Correia et al., 2010), was detected a higher prevalence of prediabetes (34.78 vs 23.20%); and among the prediabetics it was observed a higher prevalence of IFG (18.18 vs 8.2%) and IFT+IGT (13.64 vs 2.64%), while the percentage of IGT was lower in our findings than in Portuguese population (4.55 vs 12.6%); the prevalence of diabetes in our subjects was slightly higher (13.04 vs 11.70%). Regarding to HbA1c results obtained, there are some

considerations that need to be explored. A consensus statement from an International Expert Committee recommended the use of HbA1C levels 6.5% for the diagnosis of diabetes. HbA1C levels between 6.0 and 6.5% are proposed to identify individuals at high risk of developing diabetes. It may, however, be questioned whether HbA1C is a good indicator of glucose in individuals with normal or moderately elevated glucose levels and whether it can therefore be used to identify those with intermediate hyperglycemia or undiagnosed diabetes (International Expert Committee,2009) . The values of HbA1c for the 23 siblings analyzed are not in accordance with the proposed values by the International Expert Committee. Among the diabetics diagnosed in our study, two had present an HbA1c result of 8.2% and 7.6%, while one of diabetics presented a value of 5.8%. This means that in this specific case by the simple analyzes of HbA1c would be difficult achieve the diagnosis of diabetes. Nevertheless, in these cases is recommended the repetition of HbA1c measure, procedure that was not realized. In the other hand, among the 8 prediabetics siblings in this study, only one case presented a HbA1c value above 6.0 cutoff, the remainders were all below this value. These results apparently indicated that the utilization of HbA1c to access individuals with prediabetes need to be better evaluated.

In our findings it was detected a higher BMI and waist circumference average than in other preliminary study realized with siblings of T2D. The lipid profile in the siblings analyzed in this study was also worse than in that previous study (Purnamasari, et al., 2010). Our results showed a high prevalence of overweight (26.09%) and obesity (47.82%). As expected the higher prevalence of obesity were observed in prediabetics and diabetics, strengthening the already know connection between obesity and T2D (Tharkar & Viswanathan, 2010). The values of obesity obtained in our study are clearly higher than the prevalence of obesity in Portuguese population (47.82 vs 24.00%) (WHO, 2013). Thus, along with the reported that the majority of the individuals analyzed had central obesity (91.30%), may be concluded that the siblings of type 2 diabetics have a susceptibility to develop obesity, mainly visceral (related with insulin resistance and fatty liver). Regarding to lipid profile, the major deregulations detected in overall population were the high levels of LDL cholesterol (95.65%) and the low levels of HDL cholesterol (82.61%). When the average of each lipid parameter was evaluated, it was noticed in prediabetics and in diabetics, a tendency to increase the levels LDL and total cholesterol, as well as a decrease in HDL levels when compared with NGT

group. These results demonstrated the risk of CVD in prediabetic and diabetic states (Pfister et al., 2011).

An interesting fact reported was the average glucose level at 0 and 120 minutes for overall population, with these values being compatible with the IFG known profile. As the IFG are more related with a hepatic insulin resistance, this result seems to suggest that the insulin resistance in siblings of type 2 diabetics is mostly hepatic.

The glucose levels obtained for the different degrees of glucose tolerance in OGTT are the mirror of different defects involve in each glucose dysregulation. It is known that people with isolated IFG predominantly have hepatic insulin resistance and normal muscle insulin sensitivity, whereas individuals with isolated IGT have normal to slightly reduced hepatic insulin sensitivity and moderate to severe muscle insulin resistance. Not surprisingly, individuals with both IFG and IGT manifest both muscle and hepatic insulin resistance. The pattern of insulin levels also differs between IFG and IGT. IFG subjects have a decrease in insulin bioavailability in the first-phase (0-30 min) throughout the oral glucose tolerance test. However, the later phase of plasma insulin secretion (60-120) during the OGTT remains preserved, allowing the decrease of insulin levels to normal range. Isolated IGT also has a defect in early- insulin levels in response to an oral glucose load and in addition has a severe deficit in insulin bioavailable in the last phase. The high levels of hepatic insulin resistance in isolated IFG results in excessive fasting hepatic glucose production accounting for fasting hyperglycemia. However, in the OGTT preservation of late insulin response combined with normal muscle insulin sensitivity allows glucose levels to return to the preload value in isolated IFG. In contrast, in isolated IGT the defective late insulin secretion, combined with muscle and hepatic insulin resistance, results in prolonged hyperglycemia after a glucose load. In order to evaluate the bioavailability of insulin in our patients we had access the levels of insulin and c-peptide in the blood. In spite of insulin and c-peptide are equimolar releases, the longer half-life (5 min vs 30 min) and renal clearance of c-peptide, make it an attractive and more reliable parameter to estimate insulin secretion and ß-cell function(Nathan, et al.,2007). In IFG and IGT group the increase in insulin levels in the first 30 minutes was lower compared with NGT group (IFG: ratio $[\text{Insulin}]_{30min}/[\text{Insulin}]_{0\ min}$ = 4.49 vs IGT= 5.84 vs NGT = 11-fold; IFG: $[\text{c-peptide}]_{30min}/[\text{c-peptide}]_{0min}$=1.93 vs IGT=2.69 vs NGT = 4.06-fold). By definition, subjects with IFG had significantly elevated fasting plasma glucose compared with normal and impaired glucose-tolerant subjects, and their fasting plasma insulin

concentration was higher than the value in NGT and IGT(Abdul-Ghani et al., 2006; Rhee & Woo, 2011). In our results the insulin and c-peptide levels were similar with IGT, however we are not able to make any conclusion in relation to this group as there is the need to evaluate an higher number of subjects. After the minute 30, a high released of insulin in IFG group along with normal muscle insulin sensitivity, allowed compensate the lowers initial levels of insulin release and thus decrease glucose levels. This increase was not so marked in IGT and NGT (IFG: ratio $[Insulin]_{90min}/[Insulin]_{30\ min}$ = 1.70 vs IGT = 0.86 vs NGT = 0.74; IFG: ratio $[c\text{-}peptide]_{90min}/[c\text{-}peptide]_{30\ min}$=1.93 vs IGT=1.24 vs NGT=1.08). In IGT subject due to a not enough level of insulin bioavailable and/or high levels of muscle insulin resistance, the plasma glucose concentration continued to increase after 30 min and remained elevated at 120 min. In IFG+IGT group it was noted an initial increased of insulin levels similar with IGT group (IFG+IGT: ratio $[Insulin]_{30min}/[Insulin]_{0\ min}$=4.55; $[c\text{-}peptide]_{30min}/[c\text{-}peptide]_{0min}$= 2.43), and a later insulin release higher than in the others groups evaluated. However, even with this larger secretion of insulin, it was observed that the glucose levels remain high. This likely had happen due the higher levels of insulin resistance (as demonstrated by HOMA-IR and OGIS) in comparison with IFG and IGT group. Individuals with IFG+IGT, as can be viewed in our results, start with a high fasting plasma glucose concentration because of insulin resistance (see HOMA-IR). It was also noted the greatest rise (among prediabetics) in plasma glucose concentration during the OGTT likely due a muscle (and hepatic) insulin resistance (Abdul-Ghani et al., 2006). Thus, in order to maintain glucoses levels below the diabetic range, the organism possible tried to over the resistance by increasing the insulin bioavailability. In type 2 diabetics as expected it was observed lowers levels of insulin secretion, as well as lowers levels of c-peptide, demonstrating the loss of capacity of β-cell to maintain glucose homeostasis (Kanat et al., 2012).

The insulin action was also a parameter evaluated in our study, with the utilization of HOMA-IR and OGIS. The HOMA-IR index is derived from the product of the fasting plasma glucose and insulin concentrations. Since hepatic glucose production is the primary determinant of the fasting plasma glucose concentration, and the fasting plasma insulin concentration is a regulator of hepatic glucose production, the product of fasting plasma glucose and FPI primarily reflects hepatic insulin resistance (Esteghamati et al., 2011). The OGIS is a method for the assessment of insulin sensitivity from the OGTT. OGIS provides an index that correlates to the index of

insulin sensitivity obtained from the the Hyperinsulinemic Euglycemic Clamp. OGIS exploits the known quantitative relationships between the observed data and the HIEC insulin sensitivity to attempt a genuine insulin sensitivity prediction. Thus, this method allow evaluated the sensitivity peripheral to insulin action not only in the fasting state but also during the OGGT (Patarrão et al., 2014). When HOMA-IR was used to access the insulin resistance, IFG and IFG+IGT subjects were found to be markedly insulin resistant compared with subjects with IGT and NGT. This result is in accordance with other studies (Kanat et al., 2012; Abdul-Ghani et al., 2006), with IFG and IFG+IGT having high levels of hepatic insulin resistance, contributing this way to a higher insulin and glucose fasting levels, increasing the HOMA-IR value. As the resistance in IGT is essentially in skeletal muscle, the levels of glucose in fasting are lower as well as insulin, leading to a smaller value of HOMA-IR. The NGT as expected presented the lower value of resistance whereas the type 2 diabetics presented the higher values. Regarding to OGIS, the same pattern were observed, agreeing with the values obtained in HOMA-IR. The IFG+IGT and IFG presented lowest sensibility when compared with IGT. The NGT was the group with higher sensibility and the type 2 diabetics the lowest sensibility to insulin action.

Regarding to insulin clearance, this is a theme that have been underexplored by scientific community, but we believe that its evaluation is essential to further understand the metabolic alterations involved in type 2 diabetes. Insulin clearance is a highly heritable trait (Goodarzi et al., 2005), raising the possibility that genetic determinants of insulin clearance may affect risk for hyperinsulinemic disorders such as diabetes mellitus or polycystic ovary syndrome. We aimed that alterations on insulin clearance during an OGTT would allow differentiates metabolic states between dysglycemic categories. The importance of this process is fundamentally supported by the fact that hepatic insulin clearance regulates the insulin bioavailable. A higher rate of HIC is responsible for lower insulin levels, while a decrease on clearance leads to higher levels of insulin in circulation. These alterations in different physiological or physiopatological states can be absolutely fundamental to maintain glucose homeostasis and possible are characteristic of each dysglycemic categories. At fasting state the values of HIC was different in all groups, with higher levels of clearance in NGT group and lower levels in people with type 2 diabetes (suppressed 47.76%). In prediabetic states the suppression comparing with NGT group was 13.78, 21.15 and 27.88% for IFG, IGT and IFG+IGT group. These results it is in accordance with other studies that

related the reduction of HIC with an impaired glycemic control and type 2 diabetes (Kotronen et al., 2008). However the reasons that are involve in the reduction of this process need yet to be further investigated.

In our results, the main alteration in hepatic insulin clearance was observed at minute 30, with a decrease in all groups. This reduction is explained by regulation of insulin levels according to requirements of organism: in a fasting state there is no such necessity of insulin, and thus the hepatic insulin clearance is higher; however after a load of 75g glucose, the requirement of insulin naturally increases which is achieve by a reduction of insulin clearance, allowing a higher bioavailability of insulin levels (Lee et al., 2013). This reduction was higher in NGT and IFG group, while the percentage of HIC decreased was lower in IGT and IFG+IGT and was minimal in type 2 diabetics. Therefore the groups with higher reductions at 30 min were those that presented lower glucose levels in the end of OGTT. These results suggest that a lower decrease of IC in the first 30 min is related with abnormal glucose tolerance states. In T2D the reduction was minimal; fact that again reinforces that lowers reductions of IC in the first 30 min of OGTT are associated with impaired glucose regulation. These results are in concordance with our unpublished results. The reason why lower reductions of IC at minute 30 are apparently related with dysregulations in glucose homeostasis is not yet understood and need further investigation. Nevertheless, this fact can be related with lost of the first phase of insulin release, that was already be reported has an important defect first defect in T2D (Seino et al, 2011). The importance of HIC in the first 30 minutes was further reinforced when compared the values of $AUC_{0\text{-}30min}$/min and $AUC_{0\text{-}120min}$/min, with higher percentage of hepatic insulin clearance per minute during the first 30 min than in the overall OGTT. The main difference that is noted between the values of $AUC_{0\text{-}30\ minutes/min}$ and $AUC_{0\text{-}120\ minutes/min}$ is that IFG in this analyzes is the prediabetic group with the lower rate of HIC, unlike in $AUC_{0\text{-}30\ minutes/min}$. This reduction was due the fact that HIC almost did not increased between 30 and 90 min, process that contributed to an insulin spike at 90 min, thus allowing decrease the glucose levels to a normal range. In IGT this reduction was not so markedly. However in this subject was observed a reduction in comparison with NGT group, which may be related with attempt of increase insulin levels in order to compensate resistance state.

Our results are in accordance with several publications that relate lower rates of HIC to insulin resistance (Kotronen et al., 2008). Among the prediabetics groups it was noted a higher $AUC_{0\text{-}30\ minutes}$/min value on IFG group; this fact explains the lowers

levels of insulin bioavailable in IFG subjects at minute 30 comparing with IFG and IFG+IGT group. The type 2 diabetic displayed the lower rate of insulin clearance, which indicates a possible mechanism compensatory to maintain insulin levels as higher as possible, taking in count that secretion of β-cell at this point is impaired. In the rest of OGTT it was noticed in the majority of the groups an increase in hepatic insulin clearance, due the fact that insulin requirement decreased (similar to our unpublished results).

Insulin is a critical regulator of virtually all aspects of adipocyte biology, and adipocytes are one of the most highly insulin-responsive cell types. Insulin promotes adipocyte triglyceride stores by a number of mechanisms, including fostering the differentiation of preadipocytes to adipocytes and, in mature adipocytes, stimulating glucose transport and triglyceride synthesis (lipogenesis), as well as inhibiting lipolysis. Insulin also increases the uptake of fatty acids derived from circulating lipoproteins by stimulating lipoprotein lipase activity in adipose tissue. Thus, after a meal, insulin suppresses lipolysis through the activation of its downstream kinase. During insulin resistance, this process is ineffective, and levels of FFA remains higher. This is a process that has been related with deteriorating of insulin resistance. The increase in FFA in the blood stream could promote ectopic fat accumulation in several organs (Choi et al., 2010). The fasting FFA levels in our data were higher in IGT+IFG group, followed by IFG. Normally, the IGT subjects have higher values of fasting FFA than the IFG subjects due the higher levels of fasting insulinemia usually found on IFG group allowing a decrease FFA levels (Abdul-Ghani et al., 2008). However in our findings, IFG did not present high levels of fasting insulinemia. Thus, this factor along with lower values of insulin sensitivity on IFG group may be the reason why fasting FFA levels were higher in IFG than in IGT group. Other result are not in agreement with the literature regarding the lower FFA levels of the type 2 diabetics (Mai et al.,2013). During the rest of OGTT it was possible to report that FFA in prediabetics with more insulin resistance (IFG, IFG+IGT) were hardly suppressed. However at 120 minutes all the prediabetics and the NGT group presented similar values. In type 2 diabetics due the high insulin resistance the suppression of FFA was not achieve, with levels remaining high at the end of OGTT.

Nonalcoholic fatty liver disease (NAFLD) is a condition which is characterized by abnormal lipid infiltration in liver (steatosis) in the absence of excess alcohol intake; it encompasses a spectrum of conditions associated with lipid deposition in hepatocytes.

It ranges from steatosis (simple fatty liver), to nonalcoholic steatohepatitis (NASH—fatty changes with inflammation and hepatocellular injury or fibrosis), to advanced fibrosis and cirrhosis. In order to assess the risk of fatty liver and liver fibrosis it was used the fatty liver index (FLI) and NAFLD fibrosis score (NFS), respectively. About 66% of type 2 diabetic patients are reported to have fatty liver (Mai et al., 2013). All our patients with type 2 had high probability of fatty liver. In prediabetics, the higher FLI value was detected in IGT subject, due the high levels of TAG and BMI compared with the others groups. In literature normally is reported a higher FLI in IFG+IGT among the prediabetics due the worst lipid profile that these present. In our findings that did not was observed. Nevertheless the average FLI of IGT, IFG+IGT and T2D group were situated within the range of high risk of fatty liver, fact that agreed with other studies that report the risk of fatty liver on these groups (Faghihimani et al., 2013; Rückert et al., 2011). Regarding to NFS, no subjects with higher risk of fibrosis were reported in our study, which suggest that although exist a higher prevalence of fatty liver, the siblings of type 2 diabetics does not seem to progress to fibrosis.

Limitations in this study were (1) this study is a preliminary study with a sample of only 23 people, which do not allowed perform statistical relationships (2) the results displayed higher variability, which created higher difficult in the date analysis (3) the results obtained in IGT, with only one subject in this group, were obviously the less reliable.

Conclusion/Future work

Several studies have evaluated the risk of having a positive family history of diabetes (FHD) to develop the disease. The Portuguese Diabetes Association (APDP), as the main outpatient clinic of diabetes in Portugal, has a large database originated from a large number of clinic files, including the FHD of its patients.

Due to the existence of this database, it was possible to have a high volume sample available. In order to achieve a further knowledge about F.H.D of APDP patients we decided to evaluate this variable in a 5 year period (2009 – 2013). Furthermore, we directly compared the risk between three different types of diabetes, something that to the best of our knowledge is a completely new approach in the evaluation of the effect of first-degree diabetic relatives.

With this work it was possible to reinforce the idea that clearly there is a background shared between the three types of diabetes, with FHD in T1D, LADA and T2D. Type 1 and type 2 diabetes in FHD contribute with different weights to the risk of developing diabetes.

Type 2 diabetes was the more prevalent either in T1D, LADA and T2D relatives, highlighting both the fact that T2D has a much higher prevalence in the general population but also is the more inherited type.

It was reported too in our study that the type of diabetes present in relatives clearly influences the type of diabetes that people are in risk to develop. Regarding to the presence of diabetes in parents, it was observed that having a father with T1D significantly increased the risk of develop T1D, while having a mother with T2D increased the risk of develop T2D. We have found also significant associations for other first degree relatives. Thus, it was clearly demonstrated that first degree relatives of people with diabetes represent a high risk factor to develop diabetes. However, among first degree relatives of this large group of patients it was observed that in T1D and T2D the factor that provide the higher risk to develop diabetes is the presence of a diabetic sibling, with a type 1 siblings increasing the risk 11 fold of develop T1D and type 2 siblings increasing the risk 6 fold of develop T2D. Regarding the LADA group results, it was reported a high heterogeneity with the odds ratio being similar to T1D and prevalence being similar to T2D. This confirms that LADA shares in fact features with T1D and T2D. The higher risk factor to develop this type of diabetes is the presence of parents with different types of diabetes (T1D and T2D).

Our second hypothesis was confirmed with first degree relatives of people with diabetes providing different weight representation of risk for developing diabetes. All this information will help the clinicians of APDP to evaluate future cases of diabetes according to type of F.H.D observed.

In the future the same analysis could be further exploited using a different methodological approach - specifically the utilization of a control group without diabetes and analyze possible differences between genders.

The second study was performed based on the results reported in study 1. It was *a priori* established that the study 2 would be the analysis of a higher risk group for develop diabetes among the first-degree relatives. After the results obtained in study 1, we decided to evaluate possible metabolic alterations in siblings of type 2 diabetics. The results presented are just a preliminary analysis of a first group of people of what is

expected to be a larger sample. So we have considered this as a preliminary study, and all the results should be interpreted on this basis. It is necessary to increase the number of siblings analyzed, mainly in prediabetics and diabetics group in order to clarify all the results here reported. Nevertheless, we believe that our findings provided some important indicators in the study of this high risk population. It was observed that the T2D-siblings have a higher tendency to have obesity, mainly visceral, than the population in general. As it is well-known, visceral obesity is the type of obesity more related with insulin resistance and type 2 diabetes.

Our population displayed too, a high percentage of IFG as well as high glucose levels at 0 min. This fact may indicate a prevalence of hepatic insulin resistance among the siblings of type 2 diabetics.

Regarding to lipid profile, it was observed a high prevalence of decreased HDL cholesterol and increased LDL cholesterol in siblings of T2D. The levels of FFA detected were also higher in siblings, mainly in prediabetics with IFG and IFG+IGT. This occurrence may be related with the visceral obesity – associated with high levels of lipolysis probably due insulin resistance. All this circumstances may have contributed to the high prevalence of fatty liver reporting in our population. Nevertheless, the siblings do not seem to progress to liver fibrosis.

The analysis of hepatic insulin clearance, indicated that this process is related with an impaired glucose regulation; mainly the reduction reported at minute 30, being detected a higher decrease in HIC in NGT and IFG - only those who have normal glucose tolerance; and a lesser decrease in IGT, IFG+IGT and mainly in type 2 diabetics. The reason why this decrease is so determinant needs yet to be further investigated.

Due the small population and high variability of our results, the hypothesis 3 was not confirmed. However, insulin clearance, which is the regulatory process of insulin concentration less explored by scientific community, apparently has a promisor role in the etiology of different dysglicemic states, and thus may be related with several issues found in our results like insulin resistance, visceral obesity, higher percentage of free fatty acids and T2D.

The next step in this work could be to define glucometabolic and insulin secretion and clearance kinetics in more detail for the T2D sibling populations that has been categorized as normal, glucose intolerant or T2D by the OGTT to see if it could be possible to identify the phenotype that is highly predictive for T2D progression.

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Appendices

Appendix A – Parameters obtained in the study 2.

Patient	Gender	Age (years)	LDL (mg/dL)	Total cholesterol (mg/dL)	HDL (mg/dL)	TAG (mg/dL)	AST (U/L)	ALT (U/L)	Gama-GT (U/L)	[Glucose] 0 mim (mg/dL)	[Glucose] 30 min (mg/dL)	[Glucose] 90min (mg/dL)	[Glucose] 120min (mg/dL)	[FFA] 0min (mmol/L)	[FFA] 30 min (mmol/L)	[FFA] 90 min (mmol/L)	[FFA] 120 min (mmol/L)	[Insuline] 0 min (µU/L)	[Insuline] 30 min (µU/L)	[Insuline] 90 min (µU/L)	[Insuline] 120min (µU/L)	[C-peptide] 0 min (ng/ml)	[C-peptide] 30 min (ng/ml)	[C-peptide] 90 min (ng/ml)	[C-peptide] 120 min (ng/ml)	HbA1c (%)	Height (m)	Weight (Kg)	Waist circumference (cm)
1	M	68	101	164	56,3	161,9	25	19	20	98,5	199,7	137,7	110,4	0,55	0,55	0,25	0,22	4,2	62,5	36,9	20,9	1,97	7,27	8,39	7,27	5,5	1,65	80	106
2	F	60	101	178,2	75	94,7	19	16	11	90,3	150,5	109,5	84,3	0,42	0,27	0,1	0,09	3,8	71,7	74,9	23,5	2,08	9,41	13,8	8,75	5,5	1,62	55	80
3	M	49	167	232	54,9	247,9	32	34	73	96,5	135,7	154,6	85,7	0,73	0,58	0,28	0,22	4,2	19,9	72,7	11,6	1,97	3,71	10,5	5,21	5,1	1,69	68	84
4	M	48	129	182,2	49,9	115,2	26	28	30	94,9	157	106,1	120,1	0,47	0,37	0,14	0,11	11,3	77,2	27,1	28,7	2,29	8,46	6,95	7,18	5,1	1,82	110	113
5	M	58	183	236,7	44,2	159,3	33	47	32	116,7	170,4	155,7	102,8	0,46	0,51	0,27	0,16	22	72,3	183,1	66,9	5,65	8,83	17,7	12,2	5,2	1,78	103	110
6	F	55	110	160,7	44,1	152,7	22	26	41	88,7	136,1		95,6	0,39	0,33		0,1	9,8	107,3		57,4	2,77	10,2		10,8	5,5	1,57	61,2	85
7	M	56	180	250,5	59,6	210,8	34	44	64	117,8	191		186,6	0,78	0,72		0,17	22,4	55,8		144	3,84	6,58		13,9	5,7	1,72	100,2	111
8	F	57	113	191,1	76	91,5	27	28	20	96	160,2	102,8	105	0,5	0,36	0,06	0,06	10,8	83,5	55,6	66,1	1,97	8,71	8,54	9,65	5,1	1,51	61	85
9	F	41	87	137,9	51,5	53,3	12	11	10	86,4	109,8	70,5	96,6	0,3	0,2	0,08	0,07	3,8	77,7	20,2	62,6	1,14	7,62	4,15	7,42	3,9	1,6	63	74
10	F	43	104	159,2	55,9	50,1	20	13	8	88,8	136,6	87,4	88,8	0,33	0,3	0,05	0,06	3,6	57,8	17,1	22,2	1,22	5,93	5,37	5,55	5,2	1,61	60,5	76
11	F	58	175	246,9	56	203,2	39	49	29	106,1	141,5	176,9	119,5	0,48	0,48	0,24	0,13	9,6	43,1	129,8	62,6	2,49	4,4	9,84	8,37	5,7	1,53	88,3	110
12	F	64	135	135	208,2	157,6	18	17	11	94,2	116,3	88,1	139	0,38	0,19	0,07	0,09	8,9	86,3	24,7	49	2,3	10,2	6,4	10	5,3	1,65	71,8	81,5
13	M	42	201	253	47	108,5	18	36	34	233	321,9	473,3	383,5	0,56	0,58	0,69	0,55	15,5	18,2	32,7	29,4	3,13	3,4	5,32	5,05	8,6	1,73	100	107
14	F	69	132	4	44,5	90,1	22	21	21	118,8	242,7	156,9	160,3	0,64	0,49	0,2	0,13	15,7	116,7	123,3	187,3	3,27	10,5	16,4	20,4	5,8	1,58	68,4	81,6
15	F	36	125	179,3	56,3	49,2	19	11	12	100,1	170,7	136,7	104,4	0,49	0,34	0,09	0,07	10,5	101,1	76,9	66,1	0,81	2,91	2,96	9,84	4,9	1,62	65,4	81
16	M	62	92	131,6	31,1	124,1	20	30	53	160,6	251,8	347,3	301,2	0,25	0,66	0,33	0,69	73,2	52,1	86,6	26	9,8	6,74	9,84	5,46	7,2	1,59	105	123
17	F	47	174	222,6	43,4	86	48	63	53	94,9	162,9	114,5	118,6	0,41	0,25	0,1	0,08	10,7	115,1	86,4	96,4	2,48	10,2	10,5	10,9	4,9	1,54	66,1	83
18	F	66	142	206	61,9	89	29	27	33	110,6	189,2	184,1	175,9	0,47	0,38	0,11	0,1	9,7	45,3	34,4	52,5	2,86	7,16	9,55	10,4	5,7	1,5	80	103
19	F	67	171	235,7	59,1	90,9	25	33	22	94,7	173,8	112,7	115,4	0,15	0,13	0,07	0,07	11,4	202,8	226,5	276,9	2,39	12,9	17,3	18,3	5,3	1,5	68,6	90
20	M	69	171	225,2	43	200,9	19	23	29	93,5	192	156	152,6	0,4	0,3	0,08	0,11	12,5	73	63	51,1	3,07	8,27	10,3	10,7	5	1,71	90	105
21	F	69	111	162,2	52	133,3	19	17	17	90,1	142,1	129,7	115,2	0,35	0,18	0,09	0,06	11,4	71,1	60,4	57,5	2,85	9,02	11,1	12	5,9	1,49	69,8	96
22	F	74	117	168,3	48,6	86,1	25	23	21	106,2	191,7	78,5	85,5	0,54	0,3	0,1	0,09	6,4	41,7	48,1	26,7	3,09	7,14	14,5	10,1	6,1	1,7	73,3	91
23	F	41	170	229	47,1	132,1	22	37	23	133,7	237,4	220,3	210,5	0,59	0,46	0,14	0,22	19,8	73,5	128,1	103,2	3,02	6,77	11	10,5	5,8	1,56	90,5	116

Appendix B - Parameters calculated in the study 2.

Patient	Glucose Tolerance	BMI (Kg/m^2)	c-peptide/insulin ratio (ng/ **µU**) **0 min**	c-peptide/insulin ratio (ng/ **µU**) **30 min**	c-peptide/insulin ratio (ng/ **µU**) **90 min**	c-peptide/insulin ratio (ng/ **µU**) **120 min**	AUC (ng c-peptide/mU Insulin)$_{30\ min}$	AUC (ng c-peptide/mU Insulin)$_{120\ min}$	%of insulin decrease from 0-30 min	OGIS (ml.min^{-1}.m^{-2})	HOMA-IR	FLI	NFS
1	NGT	29,38	0,47	0,12	0,23	0,35	8,78	27,72	75,20	414	1,02	72,24	-0,33
2	NGT	20,96	0,55	0,13	0,18	0,37	10,18	27,99	76,02	427	0,85	7,35	-2,01
3	NGT	23,81	0,47	0,19	0,14	0,45	9,83	28,66	60,25	415	1,00	58,68	-1,77
4	NGT	33,21	0,20	0,11	0,26	0,25	4,68	23,26	45,93	396	2,65	86,12	-1,99
5	IFG	32,51	0,26	0,12	0,10	0,18	5,68	16,43	52,44	275	6,34	87,26	-0,43
6	NGT	24,83	0,28	0,10		0,19	5,67	18,41	66,37		2,15	41,82	-3,01
7	IFG+IGT	33,87	0,17	0,12		0,10	4,34	13,99	31,21		6,52	94,94	-1,47
8	NGT	26,75	0,18	0,10	0,15	0,15	4,30	16,53	42,81	409	2,56	25,61	-2,33
9	NGT	24,61	0,30	0,10	0,21	0,12	5,97	19,94	67,31	466	0,81	4,93	-1,78
10	NGT	23,34	0,34	0,10	0,31	0,25	6,62	27,58	69,73	485	0,79	3,73	-2,57
11	IFG	37,72	0,26	0,10	0,08	0,13	5,42	13,90	60,64	327	2,51	94,32	0,17
12	NGT	26,37	0,26	0,12	0,26	0,20	5,65	23,92	54,26	376	2,07	22,87	-2,02
13	T2D	33,41	0,20	0,197	0,16	0,17	5,83	21,33	7,49	252	8,92	82,76	-2,42
14	IFG+IGT	27,40	0,21	0,09	0,13	0,11	4,47	14,79	56,80	279	4,61	24,30	-1,66
15	IFG	24,92	0,08	0,03	0,04	0,15	1,59	6,42	62,69	386	2,60	7,65	-0,82
16	T2D	41,53	0,13	0,13	0,11	0,21	3,95	16,09	3,37	303	29,03	98,19	-1,39
17	NGT	27,87	0,23	0,09	0,12	0,11	4,81	14,63	61,77	369	2,51	40,70	-2,30
18	IFG+IGT	35,56	0,29	0,16	0,28	0,20	6,79	27,00	46,39	367	2,65	80,91	-0,97
19	NGT	30,49	0,21	0,06	0,08	0,07	4,10	10,44	69,66	276	2,67	44,53	-1,81
20	IGT	30,78	0,25	0,11	0,16	0,21	5,38	19,28	53,87	383	2,89	82,77	0,41
21	NGT	31,40	0,25	0,13	0,18	0,21	5,65	20,86	49,25	423	2,54	59,98	-0,91
22	IFG	25,30	0,48	0,17	0,30	0,38	9,81	34,19	64,54	380	1,68	27,42	-0,80
23	T2D	37,19	0,15	0,09	0,09	0,10	3,67	11,82	39,61	255	6,54	92,25	-0,35