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CICLO XXII

TITOLO DELLA TESI DI DOTTORATO

Influence of genetic factors in newly diagnosed type 2 diabetic patients: the TCF7L2 and GENETIC LOAD studies.

S.S.D. MED/13

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ABSTRACT

Background. Genome wide association studies (GWAS) have played a primary role in demonstrating that genetic variation in a number of loci, as assessed by single nucleotide polymorphisms (SNPs), affects the risk of type 2 diabetes mellitus (T2DM). Among these, rs7903146, an intronic variant of the *TCF7L2* (Transcription Factor 7 Like 2) gene, is possibly the strongest known genetic risk factor for T2DM (O.R.=1.37). Each risk variant, however, per se contributes quantitatively little to the overall risk and is often of questionable biological significance in affecting the determinants of glucose regulation.

Aim(s). To elucidate the effects of several T2DM risk genetic loci on clinical and pathophysiological (beta-cell function and insulin sensitivity) phenotypes of patients with newly diagnosed T2DM.

Methods. We studied 464 patients of Italian ancestry with newly diagnosed, GAD-antibody negative, type 2 diabetes mellitus. Standard clinical phenotyping was carried out by classical methods. Beta-cell function and insulin sensitivity were assessed by mathematical modeling of glucose and C-peptide curves during a 240' frequently sampled OGTT and by euglycemic insulin clamp, respectively. Beta-cell function is described as the sum of two components: i. first phase of insulin secretion or derivative control (DC), presented as the pulse secretory response to a unit rate of change in glucose concentration; ii. second phase of insulin secretion or proportional control (PC), presented as the insulin secretion rate at glucose concentrations of 5.5, 8.0, 11.0, 15.0 and 20.0 mM, respectively. Insulin sensitivity is presented as the amount of glucose infused which is metabolized in the last 60' of the euglycemic clamp (M value). The following SNPs (related gene in brackets), already known to be risk loci of T2DM, were genotyped: rs7901695 (*TCF7L2*), rs7903146 (*TCF7L2*), rs11196205 (*TCF7L2*), rs12255372 (*TCF7L2*), rs679931 (*CACNA1E*), rs1801282 (*PPARG*), rs1044498 (*ENPP1*), rs10946398 (*CDKAL1*), rs1111875 (*HHEX/IDE*) rs10010131 (*WFS1*), rs4430796 (*TCF2*), rs4402960 (*IGF2BP2*).

Results.

     TCF7L2. The risk alleles of 3 (rs7901695, rs7903146, rs11196205) out of 4 *TCF7L2* SNPs were associated with higher fasting plasma glucose (p<0.01, p<0.03 and p<0.01 respectively). The risk alleles of the first two SNPs

(rs7901695, rs7903146) were associated to a decrease in the proportional control of beta-cell function (p<0.02 and p<0.03 respectively). Four TCF7L2 haplotypes were detected, two of which (haplo4, frequency: 0.038; and haplo9, frequency: 0.086) had a strong impact on beta-cell function. Haplo4 was associated with the lowest proportional control of beta-cell function while haplo9 showed the highest.

Other genetic variants. None of the 8 remaining SNPs (rs679931, rs1801282, rs1044498, rs10946398, rs1111875, rs10010131, rs4430796, rs4402960) showed any significant independent association with insulin sensitivity or beta-cell function. We computed a genetic risk score of this variants, by summing the number of the T2DM risk alleles present in each patient (excluding *TCF7L2*). The patients were divided into three groups: 6 or less risk alleles (group A, n=76), 7-9 risk alleles (group B, n=226), 10 or more risk alleles (group C, n=69). The porportional control of beta-cell function was significantly impaired (P=0.05) in group C than in the other two groups, i.e. the higher the number of T2DM risk variants the lower beta cell function.

Conclusions. These data show that several genetic variants play a significant role in determining the pathophysiological phenotype of patients with newly diagnosed type 2 diabetes, with most of the influence exerted on beta-cell function. Thus, assessment of T2DM risk genotype may turn to be useful for diagnostic, prognostic and therapeutic purposes in patients with newly diagnosed T2DM.

ABSTRACT

Introduzione. Negli ultimi anni sono stati individuati molti nuovi polimorfismi (SNP, Single Nucleotide Polymorphisms) associati allo sviluppo di diabete di tipo 2 (T2DM), soprattutto grazie all'avvento di nuove tecniche di genotipizzazione su larga scala, GWAS (Genome Wide Association Scan).

Ogni SNP però contribuisce da solo in modo marginale al rischio di sviluppare la malattia e spesso è poco chiara la funzione biologica di queste varianti geniche nella regolazione dell'omeostasi del glucosio. Tra questi polimorfismi, la variante intronica rs7903146 del gene *TCF7L2* (Transcription Factor 7 Like 2) è al momento il più forte fattore di rischio per il diabete di tipo 2 (O.R.= 1.37).

Scopo dello studio. Chiarire l'effetto di diversi loci associati allo sviluppo di T2DM su fenotipi clinici e patofisiologici (funzione beta-cellulare e sensibilità insulinica) in pazienti con T2DM neo-diagnosticato.

Metodi. Abbiamo analizzato 464 pazienti di discendenza italiana con T2DM neo-diagnosticato, anti-GAD negativi. La caratterizzazione clinica standard è stata effettuata mediante metodi classici.

La funzione beta-cellulare è stata valutata applicando un modello matematico alle curve di glucosio e di C-peptide derivanti da prelievi eseguiti durante OGTT, ed è differenziabile in due componenti: prima fase di secrezione insulinica o controllo derivativo, rappresentato dalla quantità di insulina secreta in risposta all'incremento unitario della concentrazione di glucosio, e la seconda fase di secrezione o controllo proporzionale, rappresentato come la velocità di secrezione insulinica a concentrazioni crescenti di glucosio (5.5, 8.0, 11.0, 15.0 and 20.0 mM). La misura della sensibilità insulinica deriva dalla quantità di glucosio infuso che viene metabolizzato negli ultimi 60 minuti del clamp euglicemica, tale valore è detto M.

Sono stati genotipizzati i seguenti SNP, già visti essere associati allo sviluppo di T2DM: rs7901695 (*TCF7L2)*, rs7903146 (*TCF7L2)*, rs11196205 (*TCF7L2)*, rs12255372 (*TCF7L2),* rs679931 (*CACNA1E*), rs1801282 (*PPARG*), rs1044498 (*ENPP1*), rs10946398 (*CDKAL1*), rs1111875 (*HHEX/IDE*) rs10010131 (*WFS1*), rs4430796 (*TCF2*), rs4402960 (*IGF2BP2*).

Abstract

Risultati.

  TCF7L2. Gli alleli di rischio di 3 su 4 polimorfismi di *TCF7L2* (rs7901695, rs7903146, rs11196205) sono associati a più alti valori plasmatici di glucosio a digiuno ($p<0.001$, $p<0.03$ e $p<0.01$ rispettivamente). Gli allele di rischio dei primi due SNP (rs7901695 e rs7903146) sono associati ad un aumento del controllo proporzionale della funzione beta cellulare ($p<0.02$ e $p<0.03$ rispettivamente). Tramite l'analisi degli aplotipi sono stati individuati 4 aplotipi rappresentati nella popolazione in esame e due di questi (haplo4, frequenza: 0.038 e haplo9, frequenza: 0.086) hanno un forte impatto sulla funzione beta-cellulare.

  Altre varianti geniche. Nessuno dei rimanenti 8 polimorfismi (rs679931, rs1801282, rs1044498, rs10946398, rs1111875, rs10010131, rs4430796, rs4402960) ha mostrato associazione indipendente con alterazioni della funzione beta-cellulare o della sensibilità insulinica. Abbiamo calcolato uno score genetico di queste varianti sommando il numero di alleli di rischio presenti in ogni paziente (escludendo *TCF7L2*). I pazienti sono stati divisi in tre gruppi: portatori di un numero di alleli di rischio ≤ 6 (gruppo A, n=76), pazienti con un numero di alleli di rischio compresa tra 7 e 9 (gruppo B, n=226) e soggetti con un numero di alleli di rischio ≥ 10 (gruppo C, n=69). Il controllo proporzionale della funzione beta-cellulare risultava alterato in modo statisticamente significativo ($p=0.05$) nel gruppo C rispetto agli altri due gruppi. Maggiore è il numero di alleli di rischio associato a T2DM, peggiore è la funzione beta-cellulare.

Conclusioni. Questi dati mostrano come diverse varianti geniche giocano un ruolo significativo nel determinare il fenotipo patofisiologico dei pazienti con T2DM neo-diagnosticato, influenzando soprattutto la funzione beta-cellulare. Perciò, la valutazione dei genotipi di rischio per il T2DM potrebbe tornare utile per scopi diagnostici, prognostici e terapeutici in pazienti con T2DM neo-diagnosticato.

INTRODUCTION

INTRODUCTION

Diabetes mellitus affects approximately 200 million people around the world and it is forecasted that by 2025 this number would be doubled. Because of its widespread distribution and increasing prevalence in modern society, great efforts have been made to understand the underlying causes of the disease.

Approximately 95-98% of patients with diabetes mellitus are affected by either type 1 (3-7%) or type 2 diabetes (T1DM and T2DM respectively). By definition, they are both characterised by raised plasma glucose levels.

Normal glucose homeostasis depends on the balance between glucose production by the liver and glucose absorption by the gut, on one side, and glucose uptake by the brain, kidney, liver itself, muscle, and adipose tissue, on the other side. Insulin, the predominant anabolic hormone involved, increases the uptake of glucose from the blood, enhances its conversion to glycogen and triglycerides, and also increases glucose oxidation. Plasma glucose levels are normally kept within a small range (4 to 6 mmol/l) by multiple mechanisms. After a meal, a small increase in plasma glucose will lead to an increased insulin secretion by the pancreatic β-cells. This is associated with a decrease in glucose production by the liver and enhanced glucose uptake in muscle and adipose tissue. These actions result from a combination of short term rapid effects and longer term slow effects, which involve changes in gene transcription and in the rate of translation of enzymes involved in glycogen synthesis, the glycolytic pathway, and lipid metabolism. There are a number of glucose counterregulatory hormones, the main one is glucagon, and the balance between the insulin action and the effects of the counterregulatory hormones ensures normal glucose homeostasis.

Both type 1 and type 2 diabetes mellitus show a familial predisposition, which is a strong indication for the involvement of genes in people's susceptibility to the disease. However, the aetiology underlying types 1 and 2 is different and different genes are likely to be involved in each type of diabetes mellitus.

As the major cause of Type 1 diabetes mellitus is the autoimmune destruction of the pancreatic β cells, this form of diabetes usually appears or is first diagnosed in young people and was known as "juvenile diabetes". Type 2 diabetes, which accounts for around 90% of all cases of diabetes mellitus, usually develops after

the age of 40, and it was referred to as "adult onset diabetes mellitus". Unlike Type 1, Type 2 diabetes is not caused by autoimmune destruction of the pancreatic β-cells, but appears to be caused by a number of different defects in both insulin action and insulin secretion.

There are many long term complications associated to diabetes mellitus, generally divided into micro- and macrovascular complications. These long term chronic complications have a severe impact on health and quality of life of patients and represent a quite important problem for the entire society. Microvascular complications include retinopathy, neuropathy, and nephropathy, with type 2 diabetes mellitus being one of the main causes of blindness, lower limb amputations, and renal failure in adults. As to macrovascular complications, suffice it to say that type 2 diabetes mellitus is a major risk factor for cardiovascular morbidity and mortality. These chronic complications have a high socioeconomic cost and put a heavy burden on public health services [1].

Since type 2 diabetes mellitus (T2DM) is a complex disease, both environmental and genetic factors are involved in the development of the disease. Environmental factors of particular importance include overweight/obesity, increased amount of body fat, hypertension, dyslipidemia, lack of physical exercise. Regarding the genetic factors, there is ample evidence that T2DM has a strong genetic component, as long appreciated by physicians because of the role as risk indicators of family history of diabetes and of ethnicity.

Despite strenuous efforts over the past two decades to identify the genetic variants that contribute to individual differences in predisposition to T2DM, slow progress and limited success have been obtained until recently. Two different methods have been used to discover genes that are related to a disease: analysis of genomic regions shared by relatives more often than expected (so called linkage analysis using polymorphic markers such as microsatellites or tandem repeats) and candidate gene studies, particularly by attempts to correlate biological variation (phenotype) with variation in DNA sequences (genotype) in the form of a single nucleotide polymorphism (SNP) [2]. The advent of genome-wide association (GWA) analysis has allowed researchers to uncover variants influencing complex, common phenotypes – including T2DM – and has resulted in the identification of a growing number of trait susceptibility loci [3].

PATHOPHYSIOLOGY OF TYPE 2 DIABETES MELLITUS.

The main pathophysiologic factors in type 2 diabetes are defects in both β cell function and insulin sensitivity.

β-cell function and insulin action.

Insulin is produced by β-cells located in the pancreatic islets. A human pancreas contains ~1 million pancreatic islets that are distributed throughout the exocrine parenchyma of the gland. Every pancreatic islet contains ~1,000 endocrine cells of which 75% are insulin-producing β-cells. Insulin is synthesised as pro-insulin in the endoplasmic reticulum and is processed to the biologically active form inside the secretory granules. The granules are small membrane-bound secretory granules with a diameter of ~0.35 μm. Insulin can be stored within the granules for several days prior to its release. Within the granules, insulin is present as a Zn^{2+}-insulin crystal, the stability of which depends on an acidic intragranular pH. Upon fusion of the secretory granule with the plasma membrane, the pH within the granule equilibrates with that in the exterior resulting in the dissolution of the crystal and the liberation of insulin. The β-cells receive a rich vascular supply which empties into the portal vein and the liver is the first organ to be exposed to the newly-released insulin.

The β-cell is electrically excitable and uses changes in membrane potential to couple variations in blood glucose to changes in insulin secretion. Stimulation-secretion coupling in β-cells is different from other cell types because, instead of being mediated by receptor binding, glucose needs to be transported (GLUT2-glucose transporter 2) into the cytoplasm and metabolized to stimulate exocytosis. The β-cell contains many different ion channel proteins (each present in 100–5,000 copies per β-cell) but two types of ion channels are particularly important for the initiation of insulin secretion, K_{ATP}-channels and Ca^{2+}-channels.

The Kir6.2-SUR1 complex, now known as the K_{ATP} channel, acts as a regulated K^+channel, binding ATP. K_{ATP}-channels are active at low glucose concentrations, presumably because of the high intracellular ADP levels, which stimulate channel activity. During fasting, basal glucose levels are ~4-5 mM, and the β-cell membrane is polarized at resting potential. The resting potential in β-cells is mainly due to the activity of ATP-sensitive potassium (K_{ATP}) channels and an

electrogenic Na^+-K^+-ATPase pump that preserves the cationic gradient. When glucose is low or absent in the extracellular medium, the resting potential of isolated β-cells is around -70 to -80 mV.

It is well accepted that when glucose concentration rises a first phase of coupling is observed, where glucose enters the cell and is metabolized, leading to a rise in the ratio of ATP to ADP and closure of K_{ATP} channels. These channels act as metabolic sensors, because they close in response to changes in the nucleotide concentration (ATP/ADP) that result from glucose metabolism. K_{ATP} closure initiates a slow depolarization of the membrane potential that, when it reaches around -40 mV, increases the open probability of Na^+ and $Ca2^+$ voltage-dependent channels, increasing Na^+ and $Ca2^+$ entry into the cells and causing further depolarization. Around -20 mV, high-voltage $Ca2^+$-dependent channels open, and this leads to a larger increase in $[Ca2^+]i$ and subsequent insulin exocytosis (Fig. 1).
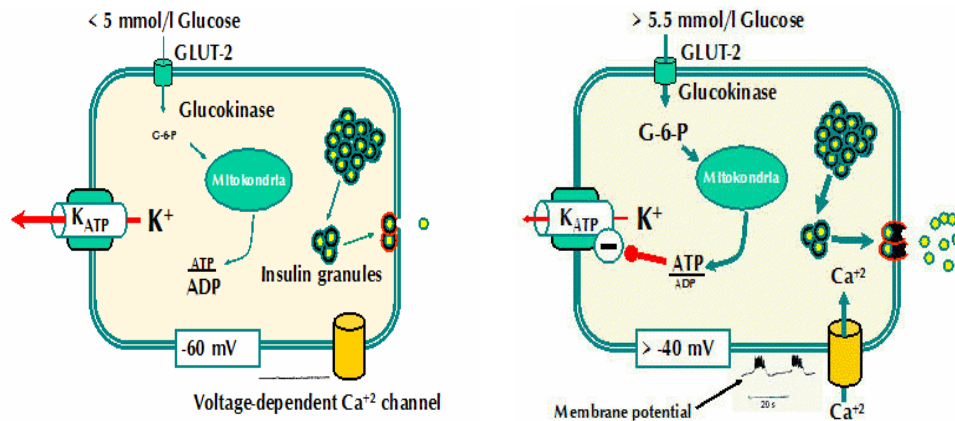

Fig. 1 At low glucose concentrations, ATP-sensitive potassium (K_{ATP}) channels are open and are responsible for the membrane potential. At high glucose concentration K_{ATP} channels are close and calcium arises inside the cell, causing membrane depolarization and consequently insulin release from the granules.

Insulin secretion in response to changes in extracellular glucose concentrations is biphasic both in vitro (Fig.2) and in vivo.

The first phase results from exocytose of granules from the "Readily Released Pool" (RRP). This glucose-induced first-phase insulin release is considered to be due to the fact that the K_{ATP} channel–dependent elevation of $[Ca^{2+}]ic$ (intracellular) promotes fusion of the β-granules already in the RRP. This phase of insulin secretion accounts for only about 5-10% of the total released after a meal.

The second phase, characterized by a gradual increase of insulin release following the initial peak, requires ATP and leads to the movement of the granules from the Reserve Pool (RP) to the RRP. β-granules in the cell do not constitute a uniform population. A majority of them remain inside the cell and cannot immediately be releasable, thus forming a reserve pool, whereas a small portion of them constitute the RRP, which is available for immediate secretion. This slow phase is the major contributor to insulin release. Maximal quantities of insulin are released after about 60 minutes.

Upon elevation of extracellular glucose, Ca^{2+}-induced fusion of granules already in the RRP occurs within minutes, and expansion and/or replenishment of the RRP from the RP is in progress several minutes later [4].
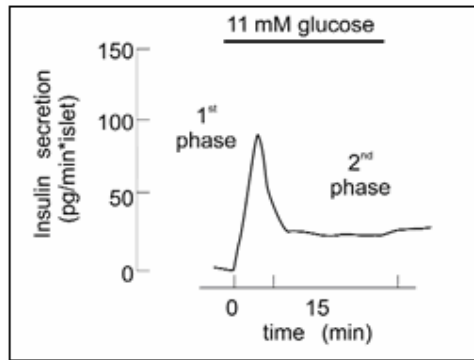

Fig. 2 Profile of glucose-induced insulin secretion. Glucose was elevated to 11 mM as indicated by horizontal bar. There is a rapid first phase (lasting ~10 min) and a slower second phase [5].

Insulin action.

After being secreted by the pancreas, insulin is released in to the bloodstream, it is delivered to its target cells throughout the body, and it binds to specific receptors to elicit insulin-dependent biological responses.

The insulin receptor is a tyrosine kinase. It functions as an enzyme that transfers phosphate groups from ATP to tyrosine residues on intracellular target proteins. Binding of insulin to the alpha subunits of the receptor causes the beta subunits to phosphorylate themselves (autophosphorylation), thus activating the catalytic activity of the receptor. The activated receptor then phosphorylates a number of intracellular proteins, which in turn alter their activity, thereby generating a biological response.

- Insulin and carbohydrate metabolism

Insulin facilitates entry of glucose into muscle, adipose and several other tissues. When insulin concentrations are low, the glucose transporters GLUT4 are located in cytoplasmic vesicles, where they are useless for transporting glucose. Binding of insulin to receptors on such cells leads rapidly to fusion of those vesicles with the plasma membrane and insertion of the glucose transporters, thereby giving the cell an ability to efficiently take up glucose, which can be used as a source of energy for the cells. When blood levels of insulin decrease and insulin receptors are no longer occupied, the glucose transporters are recycled back into the cytoplasm.

There are some tissues that do not require insulin for efficient uptake of glucose: important examples are brain, liver, red blood cells ad kidney. This is because these cells do not use GLUT4 for importing glucose, but rather, other transporters which are not insulin-dependent.

Insulin also stimulates liver and muscle to store glucose in the form of glycogen. So when the supply of glucose is abundant, insulin "tells" the liver and the muscle to bank as much of it as possible in order to use it later.

- Insulin and lipid metabolism.

Insulin promotes synthesis of fatty acids in the liver. Indeed when the liver is saturated with glycogen, any additional glucose taken up by hepatocytes is

shunted into pathways leading to synthesis of fatty acids, which are exported from the liver as lipoproteins. These lipoproteins, after being released in the circulation, provide free fatty acids for use in other tissues, including adipocytes, which use them to synthesize triglyceride.

Insulin also inhibits breakdown of fat in adipose tissue by inhibiting the intracellular hormone sensitive lipase which hydrolyzes triglycerides to release fatty acids and glycerol. It also facilitates entry of glucose into adipocytes, and within those cells, glucose can be used to synthesize glycerol. Glycerol, along with the fatty acids delivered from the liver, is used to synthesize triglycerides within the adipocyte. By these mechanisms, insulin is involved in further accumulation of triglycerides in fat cells.

Beta-cell function in T2DM.

Type 2 diabetes involves defects of insulin secretion, manifested as a loss of first phase and a reduction of second phase. The insulin secretory defect could result from either defects of β-cell function or a reduction in β-cell mass or both.

It is well known that patients with type 2 diabetes and those with impaired glucose tolerance exhibit a delayed response to a glucose load. While these patients produce apparently sufficient quantities of insulin in response to a challenge, secretion is not properly timed. The initial rapid rise in blood glucose does not elicit the usual "insulin spike"; the rapid first phase is reduced or lacking. Because of the lack of correlation between carbohydrate uptake from meals and timing of insulin secretion, these patients experience hyperglycemic periods after meals.

Type 2 diabetes has been proposed to result from a combination of a genetic predisposition, aging, lifestyle and obesity. The gene variants predisposing to type 2 diabetes may be expected to have mild effects on β-cell function and even in the case several negative polymorphisms are present simultaneously, they may not influence β-cell function sufficiently to produce type 2 diabetes unless other factors come into play.

Elevated plasma levels of both FFAs (Free Fatty Acid) and glucose represent hallmarks of type 2 diabetes. Both have been postulated to contribute to the insulin secretion defect. Chronic exposure of islets to an excess of either glucose

or lipids impairs insulin secretion. There is a strong negative correlation between plasma glucose levels and insulin secretion.

This complex relationship between glucose and beta-cell function may be underlied also by a dedifferentiation of the β-cell so that key regulatory genes become either down- or up-regulated. Once insulin secretion show initial impairments, a vicious cycle comprising a slight increase in plasma glucose levels with subsequent progressive impairment of glucose-induced insulin secretion results.

The molecular mechanisms by which high FFAs interfere with glucose-induced insulin secretion remain obscure. It is important to emphasise that obesity and elevated FFA levels do not result in type 2 diabetes by default. It is therefore evident that other factors must be important, including a strong genetic component (see the section *Genetics of type 2 diabetes*). A very significant observation in this context is that a sustained increase in plasma FFAs (by lipid infusion) resulted in marked suppression (1st phase insulin secretion reduced by 60%) of insulin secretion only in individuals with a family history of type 2 diabetes [6].

Insulin sensitivity.

Insulin sensitivity could be defined as the systemic response of the body to the glucose lowering action of insulin. It represents the ability of endogenous insulin to reduce glucose in extracellular fluids by inhibiting glucose release from the liver and stimulating the uptake of glucose from peripheral tissues.

As I mentioned above, insulin acts at the surface of insulin-dependent cells to cause the insertion in the plasma membrane, from within the cell, of special protein molecules – the so-called GLUT-4 transporters. The GLUT-4 transporters rise to the cells' outer membranes where they grab hold of the glucose molecule and transport it into the interior of the cell. Here the glucose is used to produce energy and any excess is converted into fat.

This process functions perfectly in non-diabetic persons resulting in a steady glucose level in the blood stream usually between 60 and 115 mg/dL with an average of 85 mg/dL. Patients with type 2 diabetes usually produce an adequate amount of insulin, but for some reason the mechanism whereby the insulin

summons the GLUT-4 transporters does not function. This state is called insulin resistance and is characterized by an inability of insulin to lower plasma glucose levels through suppression of hepatic glucose production and stimulation of glucose utilization in skeletal muscle and adipose tissue. The result is that both glucose and insulin levels in the blood remain high (Fig. 3).

In diabetics glucose levels are not under control and can reach levels of 250 mg/dL or higher. Up to 75% of insulin-dependent glucose disposal occurs in skeletal muscle, whereas adipose tissue accounts for only a small fraction.
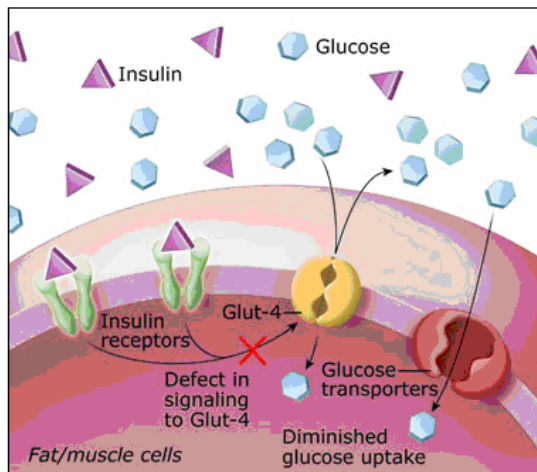
Fig. 3 Defects in insulin signaling to GLUT4 could cause insulin resistance.

Both obesity and lipoatrophy also cause insulin resistance and predisposition to type 2 diabetes, demonstrating that adipose tissue is crucial in regulating metabolism beyond its ability to take up glucose. In the liver, insulin blocks glycogenolysis and gluconeogenesis, and stimulates glycogen synthesis, thereby regulating fasting glucose levels.

Obesity is clearly a major risk factor, but in some cases of extreme obesity in humans and animals, insulin sensitivity is normal.

Adipose tissue has a special role in insulin resistance. Circulating FFAs derived from adipocytes are elevated in many insulin-resistant states and have been suggested to contribute to the insulin resistance of diabetes and obesity by inhibiting glucose uptake, glycogen synthesis and glucose oxidation, and by increasing hepatic glucose output. Elevated FFAs are also associated with a

reduction in insulin-stimulated IRS-1 phosphorylation and IRS-1-associated PI(3)K (phosphatidylinositol-3-OH kinase) activity [7].

Not long ago, it seemed plausible that insulin resistance resulted from a defect inherent in the insulin signaling pathway, indeed there is only a single insulin receptor, and individuals who lack it or have antibodies against it develop severe insulin resistance. However intrinsic receptor defect does not explain the link between obesity and insulin resistance; indeed, as insulin enhances fat storage, insulin resistance in adipose tissue might be expected to mitigate obesity.

An alternative theory postulates that insulin resistance arises when pathological levels of humoral factors disrupt insulin signaling in responsive tissues. This humoral theory emerged from the recently recognized role of adipose tissue as a secretory organ, and provided an obvious link between obesity and insulin resistance. It now seems that the adipose tissue secretes many factors— collectively termed 'adipokines'—that include adiponectin, resistin, retinol binding protein 4 and numerous classical cytokines including interleukin IL-6. In obesity, secretion of these factors is dysregulated in a way that is generally detrimental to insulin action on peripheral tissues. Some adipokines are involved in innate immunity and are also produced by macrophages. Remarkably, bone marrow–derived macrophages home to adipose tissue in obesity. Increased secretion of resistin and cytokines by adipocytes and macrophages in adipose tissue, together with decreased adipocyte secretion of the insulin-sensitizing hormone adiponectin, all contribute to the development of insulin resistance in muscle and liver.

Another hypothesis is that inflammation can cause insulin resistance. In fact it has been reported that increased NF-κB (a transcription factor involved in inflammatory events) activity is associated with increased expression of the genes that encode several cytokines and causes insulin resistance. These observations strongly support the idea that the inflamed liver can be the primary source of humoral factors that cause systemic insulin resistance. So the liver, in addition to adipose tissue, can be the primary source of factors that cause insulin resistance [8].

In figure 4 it is shown how β-cell dysfunction and insulin resistance could lead to hyperglycaemia and increased circulating fatty acid in type 2 diabetes.

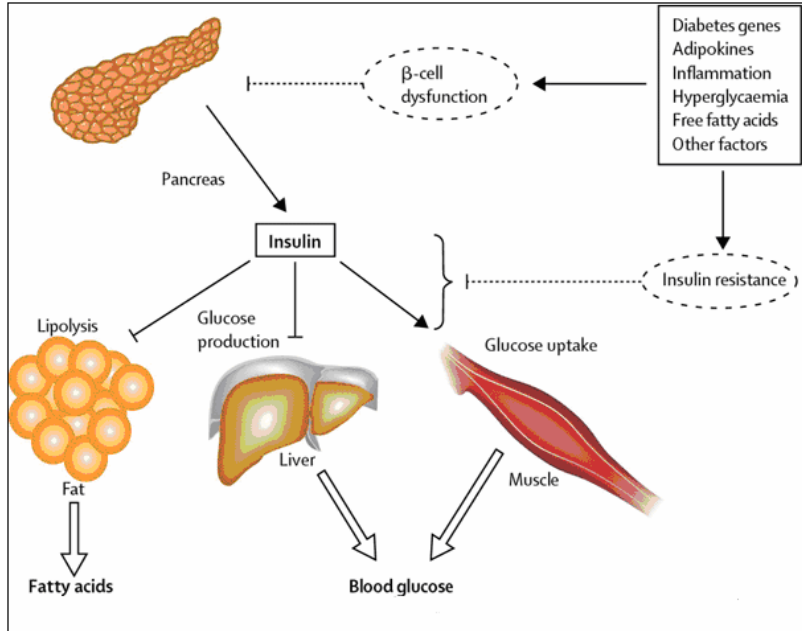**Fig. 4** Decreased insulin secretion will reduce insulin signalling in its target tissues. Insulin resistance pathways affect the action of insulin in each of the major target tissues, leading to increased circulating fatty acids and hyperglycaemia of diabetes. In turn, the raised concentrations of glucose and fatty acids in the bloodstream will feed back to worsen both insulin secretion and insulin resistance

GENETIC FACTORS INVOLVED IN TYPE 2 DIABETES.

The field of type 2 diabetes genetics had until recently succeeded in identifying few genuine disease susceptibility variants. A susceptibility locus is a genomic fragment of DNA that could be considered a genetic factor involved in the development of a complex and multigenic disease like T2DM. The presence of a susceptibility variant in this locus, together with environmental factors, increases the risk of T2DM.

There is ample evidence that T2DM has a strong genetic component. The concordance of T2DM in monozygotic twins is approximately 70% compared with 20–30% in dizygotic twins. The life-time risk of developing the disease is about 40% in offspring of one parent with T2DM the risk approaching 70% if both parents have diabetes. Large ethnic differences in the prevalence of T2DM have also been ascribed to a genetic component [2].

Traditionally, two different methods have been used to discover genes that are related to a disease: analysis of genomic regions shared by relatives more often than expected, the so called linkage analysis, and the candidate gene studies, but, as to T2DM, little results have been obtained from the use of both of them. The advent of genome-wide association (GWA) scans has transformed the situation, leading to an expansion in the number of established, robustly replicated T2DM loci to almost 30 [9, 10].

Linkage analysis.

Genetic linkage occurs when particular genetic loci or alleles for genes are inherited jointly. Genetic loci on the same chromosome which are physically close to one another tend to stay together during meiosis, and are thus genetically linked. Because there is some crossing over of DNA when the chromosomes segregate, alleles on the same chromosome can be separated and go to different daughter cells. There is a greater probability of this happening if the alleles are far apart on the chromosome, as it is more likely that a cross-over will occur between them. By working out the number of recombinants it is possible to obtain a measure for the distance between the genes. This distance is called a genetic map unit (m.u.), or a centimorgan, and is defined as the distance between genes for

which one product of meiosis in 100 is recombinant. A recombinant frequency (RF) of 1 % is equivalent to 1 m.u.

A linkage map is created by finding the map distances between a number of traits that are present on the same chromosome. Linkage mapping is critical for identifying the location of genes that cause genetic diseases. The success of identifying T2DM susceptibility genes by linkage has been restricted to the story of one single gene: *CAPN10*, which was found to be associated with type 2 diabetes mellitus in Mexican Americans, in the *NIDDM1* region. The mechanisms by which calpain10 is involved in the development of T2DM is not yet clear, but it could be possible that polymorphisms located in this gene alter the calpain-regulated gene expression [11, 12]

Second to *CAPN10, TCF7L2* (Trascription Factor 7 Like 2) is one of the earliest examples of a gene discovered to be associated with T2DM without prior knowledge of its function as a basis for its candidacy. *TCF7L2* was identified in a follow-up analysis of a region on chromosome 10q identified through linkage analysis. *TCF7L2* is involved in Wnt signaling and in the transcription of a number of genes. [2, 13]

TCF7L2 variants and their association with T2DM will be discuss later in this section.

Candidate gene approach.

The starting point for the candidate gene approach is the potential implications that either altered expression and/or function of a particular gene product (conferred by intronic or exonic genetic variants) may have on a biological function or disease. Defects in genes encoding proteins that play a role in pathways involved in insulin control and glucose homeostasis are excellent candidates for type 2 diabetes mellitus. To date, many candidate genes have been studied for their role in type 2 diabetes. The majority of these studies have failed to uncover any association [1-3, 9]. The first two genes identified through this approach that have been confirmed to be associated with T2DM are *PPARG* (Peroxisome Proliferator-Activated Receptor Gamma) [14] and *KCNJ11* (Potassium Channel, Inwardly Rectifying, Subfamily J, Member 11) [15].

More recently other few genes have been demonstrated to be associated with T2DM through the gene-candidate approach: *WFS1* (Wolfram Syndrome 1, involved in the Wolfram Syndrome) [16], *TCF2* (Transcription Factor 2, involved in the Maturity-Onset Diabetes of the Young (MODY) 5) [17] and *ENPP1* (Ectonucleotide Pyrophosphatase/Phosphodiesterase1, the product of which is known to modulate insulin-receptor function) [18].

GWAS (Genome Wide Association Scan)

The last few years have seen substantial changes in the way genetic association studies are conducted. The advent of the GWA approach was the result of at least three components. The first was delivery of a catalogue of patterns of human genome-sequence variation through the efforts of the International HapMap Consortium (http://www.hapmap.org) [19].

In the Hapmap website all the SNPs identified until now are collected and also information is provided about them, such as allele frequency in 4 different population (Yoruba, Chinese, Japanese and individuals with ancestry from Northern and western Europe) and Linkage Disequilibrium (LD) values between different SNPs. The presence of LD among SNPs allows to reduce the number of independent genetic tests required to survey the genome, such that about 80% of all common variation could be sampled with as few as 500,000 carefully chosen SNPs.

The second important factor is represented by the new genotyping methods that allow to perform massively parallel SNP-typing at high accuracy and low cost.

Finally the availability of large sample collections has led to increased power in both discovery and replication studies.

In addition to *TCF7L2*, *PPARG*, *KCJN11* and *WSF1* the first GWAS have identified at least six novel genes/loci for T2DM: *CDKN2A* and *CDKN2B* encoding the tumor suppressors cyclin-dependent kinase inhibitor-2A and -2B which may play a role in pancreatic islet regenerative capacity through inhibition of CDK4 and CDK6 (cyclindependent kinase 4 and 6), respectively; *IGF2BP2* which encodes insulin-like growth factor 2 binding protein 2, an mRNA binding protein implicated in transport of RNA targets to enable protein synthesis

important for pancreas development as judged by studies in *Xenopus* and transgenic mice; *CDKAL1* which is homologous to *CDK5RAP1*, an inhibitor of cyclin-dependent kinase CDK5 which transduces glucotoxicity signals in pancreatic beta- cells; *SLC30A8* (solute carrier family 30,member 8) encoding a pancreatic beta-cell specific zinc transporter which may affect insulin stability, storage, or secretion; and the *HHEX* (hematopoietically expressed homeobox) region which also harbors *IDE*, encoding insulin-degrading enzyme which has been implicated in both insulin signal and islet function. Both *HHEX*, critical for ventral pancreas development, and *IDE* are powerful biological candidates for T2DM. Moreover *FTO* (Fat Mass- and Obesity-Associated Gene), already seen to be associated with obesity, has been confirmed to be also a T2D-associated locus [20-25].

GWAS	First scan				Replication			Confirmed T2DM associated loci
	Cases	Controls	Population		Cases	Controls	Population	
Sladek et al [20]	661	614	French		2617	1894	French	**TCF7L2, SLC30A8, HHEX/IDE**
WTCCC [21]	1924	2938	UK	**Zeggini et al [22]**	3757	5346	UK	**TCF7L2, CDKAL1, CDKN2A/B,HHEX/IDE, SLC30A8, FTO, PPARG, KCNJ11**
Scott et al [23]	1161	1174	Finnish		1215	1258	Finnish	**TCF7L2, CDKAL1, CDKN2A/B,HHEX/IDE, SLC30A8, FTO, PPARG, KCNJ11**
DGI [24]	1467	1464	Finnish Swedish		5065	5758	European	**TCF7L2, CDKAL1, CDKN2A/B,HHEX/IDE, SLC30A8, FTO, PPARG, KCNJ11**
Steinthorsdottir et al [25]	1399	5275	Icelandic		3826	12562	European	**TCF7L2, CDKAL1, CDKN2A/B, HHEX/IDE, SLC30A8**
					1457	986	Hong Kong	
					865	1106	West Africa	

Table 1 T2DM associated loci identified in 2007 by GWAS.

Recent meta-analysis [26] efforts such as DIAGRAM have identified 6 further candidate SNPs: *JAZF1* (juxtaposed with another zinc finger gene 1) encodes a transcriptional repressor of *NR2C2* (nuclear receptor subfamily 2, group C, member 2); *CDC123* (cell division cycle 123 homolog) and *CAMK1D* (calcium/calmodulin-dependent protein kinase 1D), both involved in the cell cycle; *TSPAN8* (tetraspanin 8) which encodes a cell-surface glycoprotein

expressed in carcinomas of the colon, liver and pancreas; *THADA* (thyroid adenoma associated) gene; *ADAMTS9* (ADAM metallopeptidase with thrombospondin type 1 motif, 9), encoding a widely expressed and secreted metalloprotease that cleaves the proteoglycans versican and aggrecan; *NOTCH2* (Notch homolog 2 Drosophila) encoding a type 1 transmembrane receptor expressed in embryonic ductal cells of branching pancreatic buds during pancreatic organogenesis in mice and *ADAM30* (ADAM metallopeptidase domain 30) that represents the same signal [2].

Between the end of 2008 and the beginning of 2009 other two loci have been identified to be associated with T2DM: *KCNQ1* (Potassium channel, voltage-gated, KQT-like, member 1) identified in the Japanese population and confirmed in European, encoding the pore-forming subunit of a voltage-gated K^+ channel (KvLQT1) that is essential for the repolarization phase of the action potential in cardiac muscle and involved in the onset of T2DM, but through yet unknown mechanism [27, 28]; *MTNR1B* (Melatonin Receptor 1 B), involved in the regulation of the circadian rhythms, it has also a role in the regulation of glucose homeostasis [29-32].

A recent meta-analysis [10], focused on loci implicated in fasting glucose homeostasis, identified other 5 new loci associated with higher levels of fasting glucose and also with increased risk of T2DM: *ADCY5* (adenylate cyclase 5) catalyzes the generation of cAMP, important for the activation of protein kinase A (PKA) and for the insulin secretion process; *PROX1* (prospero homeobox protein 1) encodes a novel co-repressor of hepatocyte nuclear factor 4 (*HNF4A*) that plays a crucial role in beta-cell development; The *DGKB-TMEM195* locus, *DGKB* (diacylglycerol kinase catalytic domain β) regulates the intracellular concentration of the second messenger diacylglycerol and TMEM195 (transmembrane protein 195) encodes an integral membrane phosphoprotein highly expressed in liver; *GCK* (glukokinase 4) catalyzes the initial step in utilization of glucose by the beta-cell and liver at physiological glucose concentration; *GCKR* (glucokinase 4 regulator) is a regulatory protein that inhibits glucokinase in liver and pancreatic islet cells by binding non-covalently to form an inactive complex with the enzyme.

Until now about 30 genes have been identified, but this is an in-progress field and other new T2DM associated loci will be found out in the future.

TCF7L2

TCF7L2 has been an important topic of my doctorate studies, so in the following chapter I will stop a little bit on it.

TCF7L2 (Transcription Factor 7 Like 2), also known as TCF-4, is a transcription factor involved in the Wnt pathway (Fig. 5) and regulates the transcription of a large number of genes.

There are three known pathways of Wnt signalling; the Wnt/β-catenin (canonical), the Wnt/Ca^{2+} and the Wnt/polarity pathway, which activate distinct intracellular signaling pathways, but I will focus just on the first one. In the absence of Wnt signaling, glycogen synthase kinase 3β (GSK3b), adenomatous polyposis coli protein (APC), axin and β-catenin form components of the 'destruction complex', enabling GSK3β to phosphorylate β-catenin at serine and threonine residues, targeting β-catenin for ubiquitination and degradation by the proteosome. When Wnt ligands bind to their receptors (the Frizzled receptors and the LDL receptor-related protein 5/6 (LRP5/6) co-receptors) Wnt signals are transmitted by the association of Wnt receptors with Dishevelled protein (Dvl). This event triggers the disruption of the destructive complex, preventing the phosphorylation-dependent degradation of β-cat, which then enters the nucleus and interacts with a member of the TCF family, the protein product deriving from *TCF7L2*, to form a complex that stimulates the β-cat/TCF (or WNT) downstream target genes [33, 34].
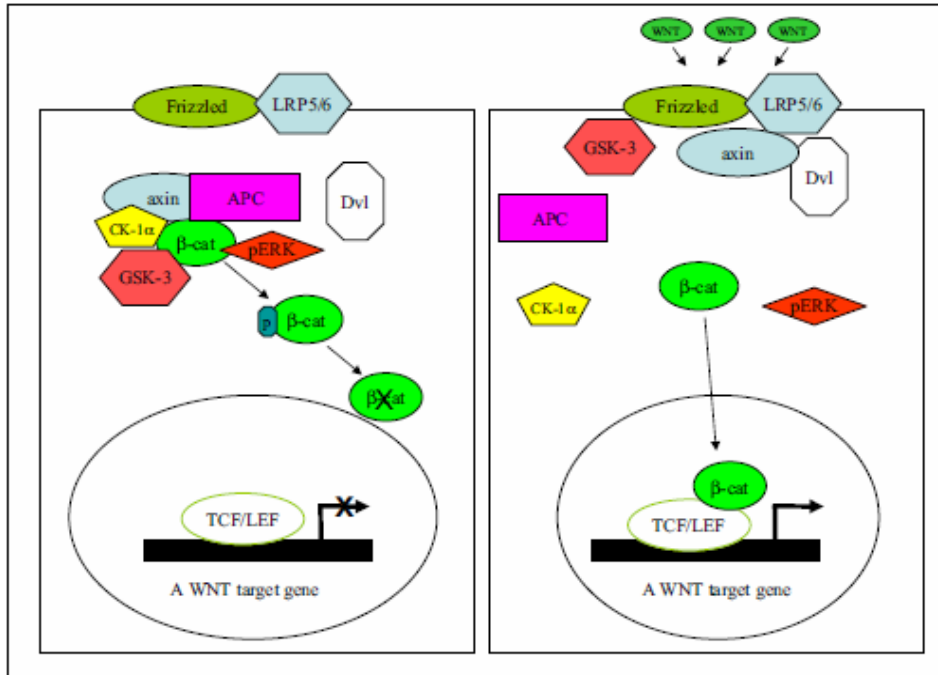
Fig. 5 Wnt/β-catenin signalling pathway [34].

Wnt signaling is a key pathway in cell proliferation and embryogenesis, and it also regulates myogenesis and adipogenesis. Defects in this pathway are involved also in tumorigenesis.

Wnt signaling has also been reported to be critical for development of pancreas and islets during the embryonic growth, but these findings are quite controversial. Indeed, while it is well-known now that this pathway is very important in exocrine pancreas development, it is still uncertain its role in the islets development, and different studies have been performed in order to point it out. At first it seems that Wnt signaling would not be involved in the endocrine pancreas development, while other studies showed that the absence of beta-catenin causes defects in endocrine pancreas development [34]. Finally a more recent study [35] suggested that the conflicting results could be resolved if we assumed that β-cat and β-cat/TCF exert different functions in a very precise dose-dependent manner at different developmental stages. So regulation of Wnt signaling's timing and stimulus is crucial for effects on pancreas development.

On the basis of these findings it may be less surprising than it was at the time that in 2006 a linkage analysis study by Grant and colleagues [13], who were

searching for microsatellite associations across a large region of chromosome 10 previously implicated in T2DM susceptibility, led to the identification of 5 variants associated with T2DM. This result has been then replicated in many different populations [36-41] and also the GWAS published in 2007 [22-24] confirmed *TCF7L2* association with T2DM.

Among all the T2D-associated loci discovered until now *TCF7L2* is still the one with the highest OR (~1,4) and its intronic variant rs7903146, located in exon 3, is still the most informative SNP and the one with the strongest association signal. Since this variant is intronic it seems that there is no obvious mechanism by which it affects the activity of *TCF7L2* and there is the possibility that the causal variant acts by affecting expression of *TCF7L2* rather than altering the structure of the expressed protein [42]

Several potential mechanisms have been proposed to underlie the association between *TCF7L2* variants and T2DM, including decreased β-cell mass, impaired insulin processing or release, impaired GLP1 (Glucagon Like Peptide 1, involved in the glucose-stimulated insulin secretion) signalling in β-cells, liver insulin resistance and altered chromatin state in T carriers [43-45].

Notably, most of the evidence linking *TCF7L2* variation to diabetes-related intermediate phenotypes in humans has been obtained in nondiabetic individuals. Furthermore, with few exceptions [46-49], surrogate markers of beta-cell function and/or insulin sensitivity have been used in these human studies. Since both insulin sensitivity and β-cell function traits display a significant spread in patients with T2DM, the question arises whether *TCF7L2* variants may affect beta-cell function and/or insulin sensitivity also in diabetic patients, thereby potentially being a determinant of T2DM heterogeneity, and/or a marker of the metabolic phenotype and/or a tool to select optimal therapy and to better define prognosis.

Part of my thesis will focus on the studies performed in order to test whether risk polymorphisms and/or haplotypes of *TCF7L2* are associated with the clinical and metabolic (beta-cell function and/or insulin sensitivity) phenotypes of patients with newly diagnosed T2DM.

To achieve this goal we used the database of the Verona Newly Diagnosed Type 2 Diabetes Study (Verona NDDS), an ongoing investigation which aims at building a biobank of patients with newly diagnosed T2DM. This cohort was deemed useful for our purposes because of the absence of the potentially confounding effects of long-lasting antidiabetic treatments and of the limited impact of duration and severity of hyperglycemia *per se* on the metabolic phenotypes.

"GENETIC LOAD"

The second part of my thesis will focus on the combined analysis of several T2DM-associated loci and follows the footprints of previous studies dealing with the additive role played by gene risk variants in shaping T2DM risk.

As I said above until now *TCF7L2* is the locus with the highest OR and its variant rs7903146 has the strongest association signal, but by itself it can explain only a small part of the phenotypic and metabolic impairments which characterize T2DM. And this observation is even more true for all the other identified T2D-associated polymorphisms with ORs lower than the *TCF7L2*'s one. However, it has been demonstrated that these variants are at least additive in determining T2DM susceptibility. These studies, indeed, have been conducted in order to test whether considering several T2D-associated variants together could explain the role of the genetic variants in determining the risk of T2DM [50-54]. It seems that genetic information improve very little the knowledge coming from common phenotypic and clinical risk factors and they provide only a slightly better prediction of risk. However, they could be more helpful if we consider the T2DM pathophysiological traits, such as impaired β-cell function and insulin sensitivity, instead of the risk of disease. A study by Pascoe and colleagues [47] has showed that in nondiabetic subjects, while individual susceptibility alleles only moderately alter pancreatic β-cell function, their effect is additively increased when they are combined together.

From these considerations comes the idea to assess the joint gene influence of several T2D-associated SNPs on pathophysiological T2DM traits, like β-cell function and insulin sensitivity. In agreement with previous studies of T2DM genetic epidemiology, we named this putative joint gene influence "genetic load".

In the second part of my thesis, therefore, I will describe the results that we obtained considering the combined effect of 8 T2D-associated variants (table 2), each of which per se devoid of any detectable effect, in determining impairments in β-cell function and/or insulin sensitivity in patients with newly diagnosed T2DM.

SNP	NEAREST GENE (chr)	ACRONYM	FUNCTION
rs679931	CACNA1E (1)	Calcium channel, voltage-dependent, alpha 1E subunit	Voltage-dependent Ca^{2+} channel
rs1801282	PPARG (3)	Peroxisome proliferator-activated receptor-gamma	The heterodimer PPARG/RXRA is a nuclear receptor that regulates glucose and lipid homeostasis
rs1044498	ENPP1 (6)	Ectonucleotide pyrophosphatase/ phosphodiesterase 1	It modulates insulin-receptor function
rs10946398	CDKAL1 (6)	CDK5 Regulatory subunit associated protein 1-like 1	Unknown
rs1111875	HHEX (10)	Haematopoietically expressed homeobox	Involved in Wnt/β-catenin pathway, regulation of pancreas organogenesis
rs10010131	WFS1 (4)	Wolfram syndrome 1	Putative transmembrane protein highly expressed in pancreatic islets,with loss of function by mutations in Wolfram syndrome
rs4430796	TCF2 (17)	Transcription factor 2	Nuclear transcription factor
rs4402960	IGF2BP2 (3)	Insulin-like growth factor 2 mRNA binding protein 2	It binds to the 5'-UTR of the insulin-like growth factor 2 (IGF2) mRNAs

Table 2 List of the considered SNPs, with their location and function.

PATIENTS AND METHODS

PATIENTS AND METHODS

Patients.

Verona Newly Diagnosed Type 2 Diabetes Study (Verona NDDS)

The Verona NDDS is an ongoing study aiming at building a biobank of patients with newly diagnosed T2DM. As of Jan 1 2002, all patients referred to the Division of Endocrinology and Metabolic Diseases of University of Verona School of Medicine, whose diabetes has been diagnosed in the last six months, are asked to participate in this research. The clinical evidence on which the diagnosis of type 2 diabetes has been made is reviewed and the diagnosis confirmed [55]. Patients already treated with antidiabetic drugs are put on therapy washout for at least one week before metabolic tests are performed. Among the exclusion criteria are age>75 years, non-Italian ancestry, insulin treatment, presence of GAD antibodies, malignancies, and any condition severely impairing liver and/or kidney function. The clinical features of the population considered in the two studies (*TCF7L2* and GENETIC LOAD studies) are reported in table 3 and 9 in the *RESULTS* section.

Verona Newly Diagnosed Type 2 Diabetes Study

All subjects consumed a weight-maintaining diet containing 200-250 g of carbohydrate/day for at least three days before studies. Body weight was stable in all subjects for at least 1 month before studies. No subject participated in any heavy exercise. Each subject gave informed written consent before participating in the research, which was approved by the Human Investigation Committee of the Verona City Hospital. Measurements of standard clinical phenotypes were collected in all patients. Metabolic tests were carried out on two separate days in random order. On both days, patients were admitted to the Metabolic Clinic Research Center at 07:30 after an overnight fast. All studies were carried out in a quiet, temperature controlled (22° C) room.

On one day an OGTT (75 g) was performed to assess beta-cell function. During the entire test patients were sitting in a comfortable cardiac chair. One teflon (21 g) venous catheter was inserted into an antecubital vein for blood sampling and kept patent with heparinized normal saline solution. After a 30' rest to establish

baseline and after collecting a 20 cc blood sample for leukocyte DNA extraction, at time = 0' subjects ingested 75 g of glucose in 300 ml of water over 5 min. Blood samples to measure glucose, C-peptide and insulin concentrations were collected at times -10', 0', +15', +30', +45', +60', +90', +120', +150', +180', +210' and +240'. In some patients further blood samples were collected at +270' and +300'. Urines were collected to measure glycosuria.

On a separate day, an euglycemic insulin clamp was performed to assess insulin sensitivity [56].During the entire test patients were lying in bed. One teflon catheter was introduced into an antecubital vein for the infusion of test substances. Another teflon catheter was placed retrogradely into a wrist vein for sampling arterialized venous blood, according to the "hot box" technique. After a 30' rest in bed to establish baseline, indirect calorimetry (at least 40') was performed as previously described, for a companion study [57].At the end of calorimetric measures, baseline blood samples were collected and a standard euglycemic insulin (intravenous prime: 4.8 $nmol^.min^{-1.}m^{-2}$ BSA; continuous infusion: 240 $pmol^.min^{-1.}m^{-2}$ BSA) clamp was performed [56]. Plasma glucose was allowed to decline until it reached 5.5 mmol/l, after which glucose clamping started with a glucose concentration goal of 5 mmol/l. The duration of the glucose clamp was at least of 120', but it was prolonged, if and as needed, to ensure at least 60' of insulin clamp at euglycemia in each patient. Timed blood samples were collected to measure hormone and substrate levels. In the last 45' of the clamp indirect calorimetry was repeated to assess substrate oxidation and energy production rates for a companion study. Urines were collected to measure urea excretion rate.

In both metabolic tests, all blood samples were collected in pre-chilled tubes and readily spun at 1,500 g. Plasma and serum specimens were stored at –80° C.

Analytical procedures

Plasma glucose concentration was measured in duplicate on a Beckman Glucose Analyzer II (Beckman Instruments, Fullerton, CA, USA) at bedside. Serum C-peptide and insulin concentrations were measured by chemiluminescence as previously described [58]. Glycated hemoglobin and serum lipids were measured by standard in-house methods.

Calculations

The amount of glucose metabolized during the last 60 min of the clamp (M value, reference insulin sensitivity) was computed with standard formulae [56].

The following classical indexes of beta-cell function were computed [59]:

- Insulinogenic Index: $(\text{Insulin}_{30'} - \text{Insulin}_{0'})/(\text{Glucose}_{30'} - \text{Glucose}_{0'})$, units: mU/mmol;

- $CIR_{120'}$: $\text{Insulin}_{120'}/[\text{Glucose}_{120'}*(\text{Glucose}_{120'} - 3.89)]$; units: $mU*L/mmol^2$.

Further insights in beta-cell function were sought by mathematical modeling (see below).

Substrate oxidation rates and energy production rate were computed by standard formulae as previously described [57].

Modeling of Beta-Cell Function

The analysis of the glucose and C-peptide curves during the OGTTs of the Verona NDDS follows the general strategy described in previous publications [60, 61] with some modifications and builds upon previous works from other laboratories [62, 63]. The kinetics of C-peptide is described with a two-compartment model, in which the two pools (1 and 2) exchange with each other and the irreversible loss of the hormone is from pool 1, the same where C-peptide concentration is measured. C-peptide kinetic parameters are computed according to the equations by Van Cauter et al.[64].

Herein are the equations describing the model of glucose induced insulin secretion during an OGTT:

$d\text{cp}_1(t)/dt = \text{ISR}(t) + \text{cp}_2 \cdot k_{12} - (k_{01} + k_{21}) \cdot \text{cp}_1$ (*Eq.1*)

where ISR = insulin secretion rate, cp_1 = C-peptide mass in the sampling (accessible) compartment, cp_2 = C-peptide mass in the remote compartment, k_{12} and k_{21} = rate constants of the exchange between the two C-peptide compartments, and k_{01} = rate constant of the irreversible loss of C-peptide from the accessible compartment. Note that the values of the volume of distribution of C-peptide pool 1 (accessible compartment), k_{12}, k_{21}, and k_{01} are computed according to the equations by Van Cauter et al.[64]

$\text{ISR}(t) = \text{BSR} + \text{DSR}(t) + \text{PSR}(t)$ (*Eq.2*)

where BSR = basal insulin secretion rate, DSR = insulin secretion rate due to the derivative (or dynamic) component, and PSR = insulin secretion rate due the proportional (or static) component.

$BSR = CP_{ss} \cdot V_1 \cdot k_{01}$ (*Eq. 3*)

where CP_{ss} is basal C-peptide concentration and V_1 is the volume of the accessible compartment of C-peptide.

From the modeling viewpoint, DSR(t) and PSR(t) are the components which in intravenous glucose tolerance tests or hyperglycemic clamps describe classical first phase insulin secretion and second phase insulin secretion, respectively. Furthermore, from a physiological viewpoint, the sum of BSR and PSR(t) describes the relationship linking glucose concentration and insulin secretion rate, in the absence of the derivative component (DSR).

DSR(t) and PSR(t) are mathematically defined as follows:

$DSR(t) = X1(t) \cdot \tau^{-1}$ (*Eq. 4*)

$dX1(t)/dt = \sigma1 \cdot [dG(t)/dt]/[\log(1.1+t)] - X1(t) \cdot \tau^{-1}$ if $dG(t)/dt > 0$ (*Eq. 5*)

$dX1(t)/dt = - X1(t) \cdot \tau^{-1}$ if $dG(t)/dt \leq 0$ (*Eq. 6*)

where $\sigma1$ = glucose sensitivity of derivative control of insulin secretion, G = plasma glucose concentration, X1 = C-peptide (insulin) mass made available for the derivative component of insulin secretion, τ = time constant of the derivative component of insulin secretion, and the term $\log(1.1 + t)$ accomodates the time-associated decline of $\sigma1$ documented in humans during a hyperglycemic stimulus [65].

$PSR(t) = X2(t) \cdot \delta^{-1}$ (*Eq. 7*)

$dX2(t)/dt = \sigma2 \cdot [G(t) - \theta] - X2(t) \cdot \delta^{-1}$ (*Eq. 8*)

where $\sigma2$ = glucose sensitivity of the proportional component of insulin secretion, X2 = C-peptide (insulin) mass made available for the proportional component of insulin secretion, δ = time constant of the proportional component of insulin secretion, θ = glucose threshold above which the beta-cell responds with the proportional component of insulin secretion to plasma glucose concentration.

This model was implemented in the SAAM 1.2 software (SAAM Institute, Seattle, WA) [66] to estimate its unknown parameters. Numerical values of the unknown parameters were estimated by using nonlinear least squares. Weights were chosen optimally, i.e., equal to the inverse of the variance of the

measurement errors, which were assumed to be additive, uncorrelated, with zero mean, and a coefficient of variation (CV) of 6-8%. The unknown parameters of the model are: CP_{ss}, $\sigma1$, τ, $\sigma2$, θ, and δ.

There are two main physiological outputs of the model:

1. derivative control [pmol·m^{-2} BSA] · [mmol·l^{-1}·min^{-1}] $^{-1}$: it is presented as the amount of insulin secreted in response to a rate of glucose increase of 1 mmol/l per min which lasts for 1 minute;

2. stimulus-response curve linking glucose concentration (x axis) to insulin secretion rate (ISR; y axis), when the derivative control is absent: as explained above, ISR is the sum of BSR and PSR, and it depends on the following three parameters estimated by the model: CP_{ss}, $\sigma2$, and θ. With the purpose of avoiding artifactual increases in the power of statistical analyses, we used the stimulus-response curve at the pre-selected glucose concentrations of 5.5, 8.0, 11.0, 15.0 and 20.0 mM.

Genotyping.

A leukocyte DNA sample was collected for each patient and the DNA extraction was made through standard salting out method. Different genotyping methods have been used. All the DNA extraction and the genotyping have been performed at the Department of Mother and Child, Biology and Genetics – Section of Biology and Genetics – University of Verona, directed by Prof. Pinatti.

RFLP-PCR (Restrinction Fragment Length Polymorphism – Polymerase Chain Reaction)

The genomic DNA fragment containing the SNP of interest is amplified through PCR. The PCR product is then processed with a properly chosen restriction enzyme recognizing a specific binding site, including only one of the two allelic variants of the SNP. In this way it is possible to discriminate the genotype (Fig 6).
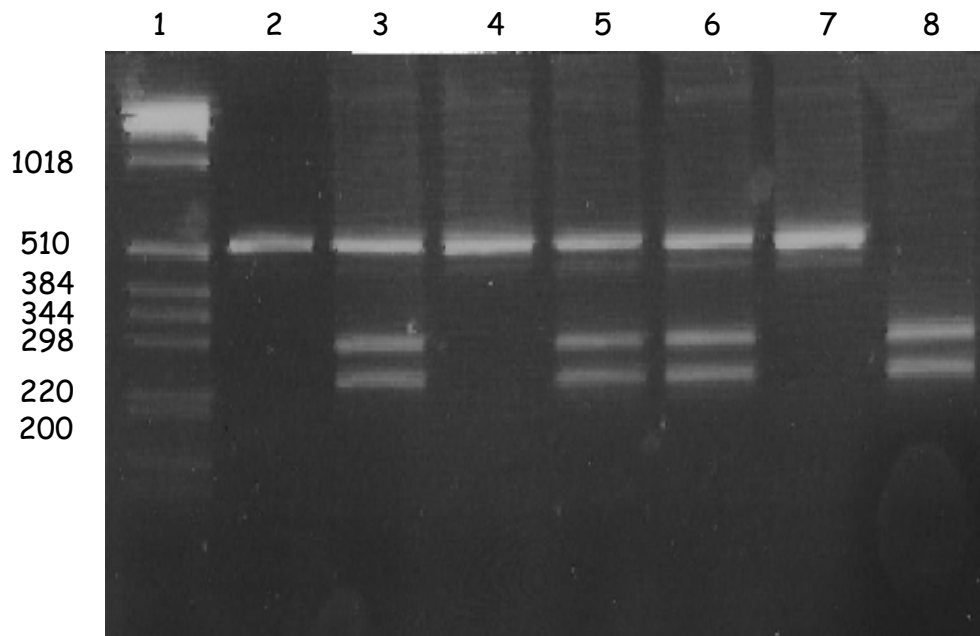
Fig. 6. RFLP. The restriction enzyme identified only one of the two allelelic variants of the SNP. In lane 3, 5 and 6 both the variants are present (heterozygous NM); in lane 8 only the allele not recognized by the enzyme is present and the PCR product is completely cut (homozygous NN); in lane 2 and 7 the enzyme did not identify its specific binding site and it did not cut (homozygous MM). On the Y axis the molecular weights references are reported.

SNAPSHOT METHOD (ABI Prism Genetic Analyzer 310)

This method is based on the single nucleotide primer extension technique and it allows to genotype up to 10 SNPs together.

The genomic region bearing the SNP of interest is amplified through PCR followed by purification of the PCR product (see below steps 1-2). After that a mini-sequencing reaction is performed using fluorescent ddNTPs and a special primer, complementary to the region of the amplified DNA located just before the polymorphism (step 3). In this way only one single nucleotide, the one in correspondence to the SNP of interest, can be add to the primers. After another purification step, the reaction is loaded on a ABI Prism Genetic Analyzer 310 and a capillary electrophoresis is performed (Fig.7). The different alleles of the SNPs are characterized by a slightly different weight, which can be detected by the instrument. Moreover the probes have been designed of different length for the different contemporary analyzed polymorphisms in order to easily recognize them on the basis of the size of the fragment.

Herein is the protocol of the entire procedure:

1. Amplify genomic DNA (tot 15 μl)

2. Remove dNTPs and primers through SAP (5U) and ExoI (2U), 37°C 1h and 72°C 15min

3. Obtain purified template and quantify it

4. Prepare sample reaction: template (0,01-0,04 pmol)

     Primers (0,5-1 pmol)

     SNaPshot Multiplex (2X)

5. Perform thermal cycling

96°C
10''
50°C
5''
60°C
30''
4°C
∞

25 cycles
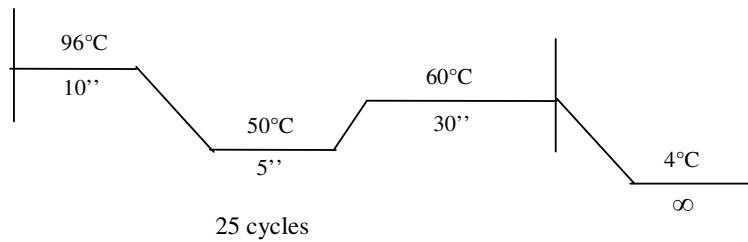
6. Remove unincorporated ddNTPs (SAP 1U)

7. Samples electrophoresis on 310 ABI Prism SNaPshot Multiplex Kit

8. Analyze data with GeneScan software
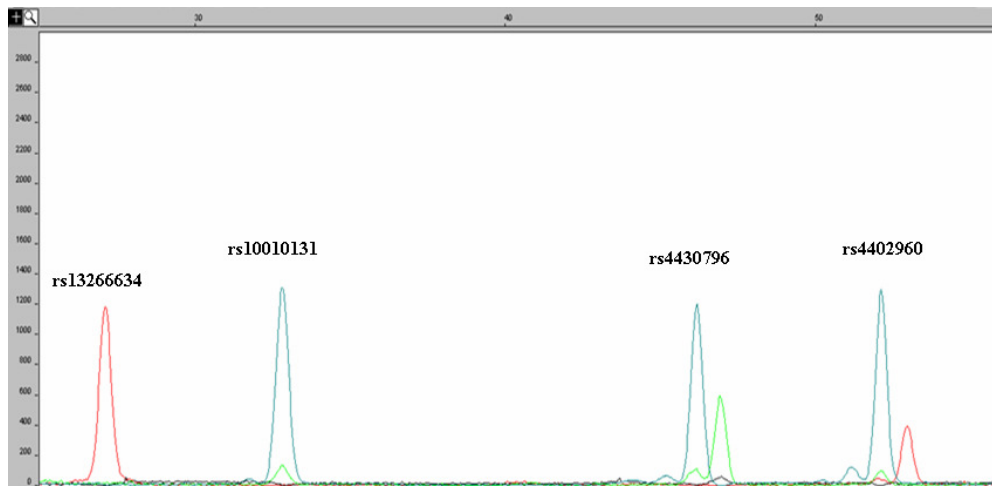
Fig. 7. Electropherogram. Each polymorphism is represented by a single (homozygous) or a doubled peak (heterozygous) of different colours according to the nucleotide.

This method is the most commonly used for genotyping because it is reliable, reproducible and quick.

A commercial assay is used for TaqMan genotyping and it contains both primers and fluorescent probes. There are two probes with two different fluorescent molecule, one for each alleles, at one side and a quencher at the other side for every SNP and they are complementary to the region surrounding the SNP, the polymorphism lays in the middle of the probe. According to the genotype of the SNP the probes could perfectly match or not. During the elongation phase the polymerase will find the probes on its way. If the probe perfectly matches, the enzyme will disrupt it, the fluorophore will be moved away from the quencher and it will emit fluorescence. However, if the matching is not perfect the whole probe will be moved away from the DNA strand but no fluorescence will be released. (fig.8).
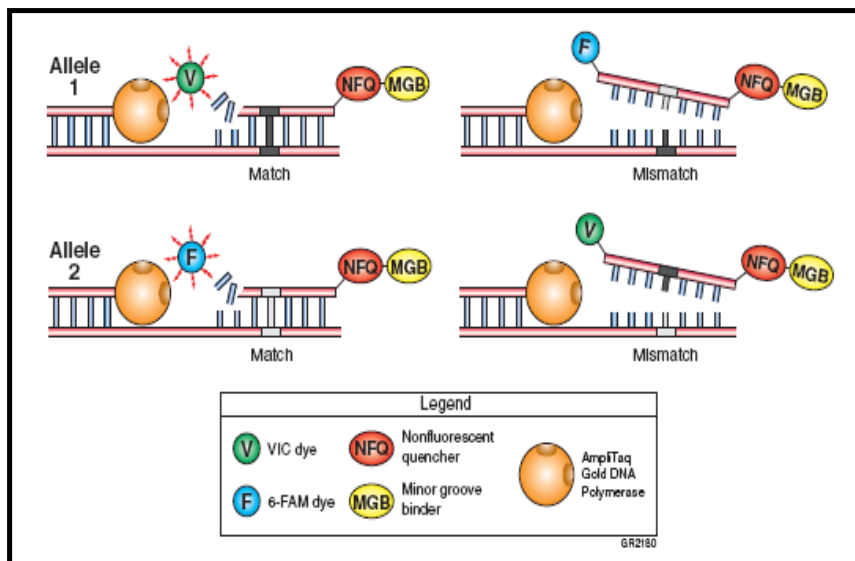
Fig. 8 TaqMan genotyping principles.

Herein is the protocol of the procedure and the PCR programme.

-       6-30 ng of genomic DNA

-       Master mix (Applied Biosystem) 2X

-       TaqMan Assay (Applied Biosystem) 40X

-       Water

-       Perform the following thermal cycling
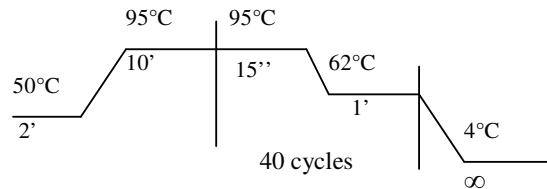
Statistical analysis.

Data are presented as medians and interquartile range, unless otherwise indicated. Before performing statistical contrasts, skewed variables were log-transformed to improve the approximation to the Gaussian distribution and reported p-values are two-sided.

The associations of *TCF7L2* single variants with phenotypic traits have been explored by ANOVA generalized linear models (GLM) as implemented in SPSS. Since the logarithmic transformation was insufficient to improve the distribution of derivative control of beta-cell function, the latter was analyzed by Kruskal-Wallis test. These statistical analyses were carried out with the software SPSS 12.0.

Haplotype association analysis was performed by generalized linear models (GLM) as implemented in the library haplo.stats [67] of the R package [68]. Associations between traits and haplotypes, allowing for ambiguous haplotypes, were investigated also by including relevant covariates such as age, gender and BMI. The statistical significance of global test and of single haplotype effects was ascertained by a Monte Carlo procedure in which the value of the trait was permuted among individuals and studied for association with the genetic factor in 1000 replicates.11.0.

The associations between the genetic load and phenotypic traits were sought by generalized linear models (GLM) as implemented in SPSS. In each patient we calculated a genetic load score by summing up all the risk alleles of which he/she is a carrier. Patients were divided in 3 groups on the basis of this score: low (LGL: ≤6, n=76), intermediate (IGL: 7-9, n=226) and high (HGL: ≥10, n=69) "genetic load" score. Parametric and nonparametric statistical methods were used as appropriate. Data were adjusted for age, gender and BMI if appropriate. Statistical significance was declared at $p<0.05$.

TCF7L2 STUDY

TCF7L2: RESULTS

Clinical Features of the Study Population

The clinical and metabolic features of the study sample are shown in table 3. They are referred to the population considered in the *TCF7L2* study. Most patients were overweight or obese, but less than 50% were obese. However, abdominal obesity was present in 50% of males and in >75% of female patients. Glucose control, as measured by HbA1c, was fairly good with only 25% patients with HbA1c>7.4%. High triglycerides and low HDL-cholesterol were common, especially the latter one in the female patients. Systolic and diastolic hypertensive values were detected in 50% and 25% of the patients, respectively.

Variable	Males	Females	All
Number	315	149	464
Age (yrs)	59 [51-65]	61 [53.5-66.5]	59 [52-65]
BMI (kg/m^2)	28.8 [26.2-32.3]	30 [27.1-34.3]	29.3 [26.5-32.9]
Waist (cm)	102 [95-11.7]	97 [90-104]	100 [94-109]
Fasting P-glucose (mmol/l)	7 [6.1-8]	7.2 [6.1-8]	7 [6.1-8]
2-h P-glucose (mmol/l)	13.3 [10.6-16.2]	12.8 [10.2-16.1]	13.3 [10.5-16.2]
HbA1c (%)	6.7 [6.1-7.5]	6.7 [6.2-7.4]	6.7 [6.1-7.4]
Triglycerides (mmol/l)	1.4 [1-2]	1.3 [1-2]	1.4 [1-2]
HDL-cholesterol (mmol/l)	1.1 [0.9-1.3]	1.2 [1-1.4]	1.1 [1-1.3]
Total Cholesterol (mmol/l)	4.9 [4.3-5.6]	5.2 [4.5-5.8]	5 [4.3-5.7]
SBP (mmHg)	134 [124-150]	140 [130-150]	139 [125-150]
DBP (mmHg)	85 [80-90]	85 [80-90]	85 [80-90]
Insulin Sensitivity (µmol/min/m^2 BSA)	572 [317-819]	536 [396-754.5]	562 [356-790]
Insulinogenic Index (mU/mmol)	3.4 [2.0-5.9]	4.3 [2.3-7.5]	3.5 [2.0-6.1]
CIR$_{120'}$ (mUxL/mmol^2)	0.4 [0.2-1.1]	0.7 [0.2 – 1.4]	0.5 [0.2-1.2]

Table 3. Clinical characterization of *TCF7L2* study population. The values are reported as median [interquartile range].

Effects of the risk alleles of TCF7L2

We analyzed 4 SNPs in *TCF7L2*: rs7901965T/C and rs7903146C/T (intron 3), rs11196205G/C and rs12255372G/T (intron 4) (the second allele is the risk allele). The genotype frequencies for all 4 SNPs were in Hardy-Weinberg equilibrium and their frequencies are reported in table 4. The location of the 4 SNPs and the linkage disequilibrium block they form are shown in fig. 9. The 4 adjacent SNPs are in strong LD (D'=0.91;0.88;0.87).
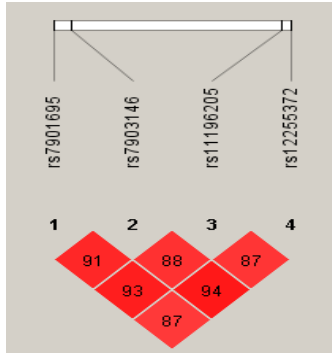
Fig. 9 SNPs location and LD plot elaborated with Haploview. LD values are reported as D'.

	TCF7L2										
SNP	**rs7901695**			**rs7903146**			**rs11196205**			**rs12255372**		
GENOTYPE	TT	TC	CC	CC	CT	TT	GG	GC	CC	GG	GT	TT
NUMBER	144	234	86	146	233	85	108	226	129	167	225	72
RISK ALLELE FREQUENCY	0,44			0,43			0,52			0,39		

Table 4 Risk allele frequencies are reported for each SNP, the orange allele is the risk one.

The clinical characteristics of the study population stratified according to rs7901695, rs7903146, rs1196205 and rs1225372 are shown in tables A to D in the *Appendix*.

The diabetes risk alleles of rs7901695, rs7903146 and rs12255372 were associated to lower BMI, but only when analyzed under the hypothesis of a dominant model (*Appendix*, tables A, B and D). The risk alleles of rs7901695, rs7903146 and rs11196205 were associated to higher fasting plasma glucose, but not to 2-hour plasma glucose during the OGTT or to HbA1c (*Appendix*, tables A, B and C). The risk allele of rs7903146 was associated with lower triglycerides (*Appendix*, table B). This association was still significant after adjusting for age, gender and BMI, but not after adjusting for fasting insulin.

None of the four *TCF7L2* variants influenced insulin sensitivity, as measured by the euglycemic insulin clamp (*Appendix*, tables A, B, C and D).

Neither the insulinogenic index nor CIR_{120} were significantly changed in the carriers of any risk allele (*Appendix*, tables A, B, C and D), although trends for reduction were observed for all 4 SNPs.

State-of-art analysis of beta-cell function showed a different pattern. The derivative control of beta-cell function showed no significant changes in the presence of the risk alleles of *TCF7L2* (Table 5). However, the stimulus-response

curve relating plasma glucose concentration to insulin secretion rate, after correcting for age, gender and BMI, showed a significant, graded downward shift in the carriers of the risk alleles (fig. 10) of both rs7901695 and rs7903146, but not of rs1196205 or rs1225372.

	n	Risk allele frequency	Not risk allele	1 risk allele	2 risk alleles	P Value
rs7901695	464	0.44	525 [2-921]	376 [0.6-954]	530 [219-888]	0.38
rs7903146	464	0.43	525 [0.5-939]	382 [0.2-957]	528 [227-873]	0.46
rs11196205	464	0.52	423 [0-1031]	388 [0.5-893]	523 [166-913]	0.36
rs12255372	464	0.39	529 [56-921]	376 [0-935]	572 [257-906]	0.15

Table 5 Derivative control of beta-cell function in patients of the Verona NDDS according to genotype of TCF7L2 variants. Data are presented as median [I.Q. Range]; units are $pmol^. m^{-2.}BSA$ $per^. min^{-1}$
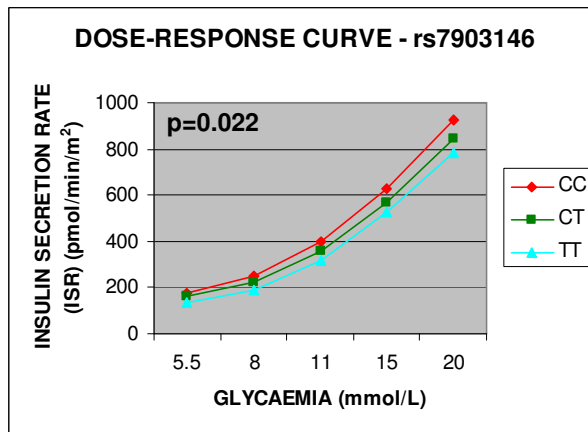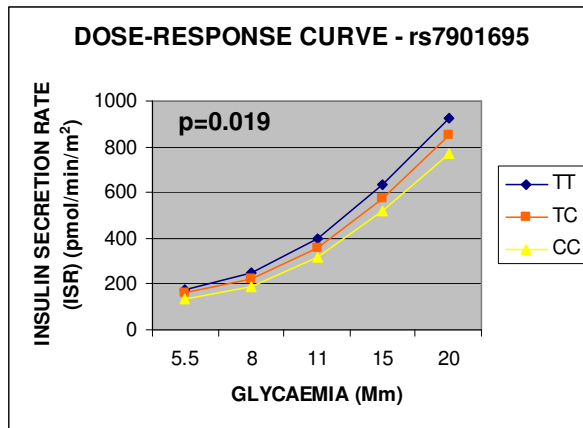

Fig. 10 The proportional control of β-cell function decreases at the increasing number of rs7901695 risk alleles. The same result is obtained for rs7903146.

Effects of TCF7L2 Haplotypes

Haplotype analysis showed that 4 main haplotypes could be detected in our study population. They were denominated arbitrarily after their approximate percent frequency. Additional rare (frequency<3%) haplotypes were grouped together and accounted for ~7% of the total number of haplotypes (table 6).

Two haplotypes, haplo.46 and haplo.35, accounted for 80% of all haplotypes. Haplo.46 was formed by all nonrisk alleles, whereas haplo.35 was composed by all risk alleles. Haplo.35 had higher fasting plasma glucose (table 11). Furthermore, haplo.35 was associated to a lower curve relating plasma glucose concentration to insulin secretion rate at all glucose levels (p<0.01 or less) (fig. 3). Also haplo.9 and haplo.4 showed an interesting pattern. Haplo.9 was associated with the best beta-cell function compared to the other haplotypes, as manifested by an upward shift in the stimulus-response curve relating plasma glucose to insulin secretion rate (fig. 11). At the highest glucose concentration (20 mM) haplotype 9 was associated with ~25% higher insulin secretion rate than the two most prevalent haplotypes. Haplo.4 was associated with higher fasting plasma glucose (table 7) and with the worst beta-cell function compared to the other haplotypes, as manifested by a downward shift in the stimulus-response curve relating plasma glucose to insulin secretion rate (fig. 11). At the highest glucose concentration (20 mM) Haplo.4 was associated with ~30% lower insulin secretion rate than the two most prevalent haplotypes. The effect of Haplo.4 on beta cell function was no longer significant after correcting for fasting plasma glucose. The p-values related to the stimulus-response curves (Fig.11) are reported in table 8.

Of the insulin-derived indexes of beta-cell function, CIR120', but not the insulinogenic index, was significantly related to *TCF7L2* haplotypes (table 7), with Haplo.9 showing the best response and Haplo.4 the worst response. No other haplotype associated effects were detected in any other tested phenotype.

Haplotypes:					
	rs7901695	rs7903146	rs11196205	rs12255372	hap.freq
haplo.4	C	T	C	G	0,0383
haplo.36	C	T	C	T	0,3479
haplo.9	T	C	C	G	0,0864
haplo.rare	*	*	*	*	0,0715
haplo.45	T	C	G	G	0,4559

Table 6 Haplotype composition and distribution in the studied population.

Variable	N	Mean value ± SEM	Coefficients ± SEM			P-Value
		Haplo.45 (reference)	**Haplo.36**	**Haplo.9**	**Haplo.4**	
Frequency (%)		44.8	36.1	8.6	3.7	
Age (yrs)	460	56.9 ± 0.89	+1.31 ± 0.76	+0.69 ± 1.3	+0.67 ± 1.9	0.5
BMI (kg/m^2)	460	30.26 ± 0.44	-0.39 ± 0.38	+0.32 ± 0.65	-0.89 ± 0.95	0.43
Waist (cm)	451	101.99 ± 1.07	-1.2 ± 0.92	+0.32 ± 1.57	-0.62 ± 2.27	0.33
Fasting P-glucose (mmol/l)	459	6.98 ± 0.15	+0.33 ± 0.13	+0.05 ± 0.22	+0.57 ± 0.32	**0.03**
2-h P-glucose (mmol/l)	459	13.45 ± 0.35	+0.3 ± 0.3	-0.13 ± 0.5	+1.3 ± 0.75	0.12
HbA1c (%)	453	6.97 ± 0.11	+0.05 ± 0.09	+0.04 ± 0.16	-0.02 ± 0.24	0.68
Triglycerides (mmol/l)	457	1.64 ± 0.09	-0.05 ± 0.082	+0.13 ± 0.14	+0.1 ± 0.23	0.3
HDL-cholesterol (mmol/l)	454	1.19 ± 0.03	+0.02 ± 0.027	-0.08 ± 0.046	+0.05 ± 0.068	0.15
Total Cholesterol (mmol/l)	457	5.04 ± 0.08	-0.05 ± 0.07	-0.02 ± 0.12	-0.23 ± 0.18	0.38
SBP (mmHg)	452	139.03 ± 1.6	-2.01 ± 1.43	+0.84 ± 2.4	-1.44 ± 3.47	0.6
DBP (mmHg)	452	85.08 ± 0.85	-0.4 ± 9.73	+0.03 ± 1.23	-0.6 ± 1.79	0.96
Insulin Sensitivity (µmol/min/m^2 BSA)	452	609 ± 26.9	+19.8 ± 24.9	-53.3 ± 44.2	-13.5 ± 13.2	0.7
Insulinogenic Index (mU/mmol)	411	4.84 ± 0.79	+0.15 ± 0.68	+1.1 ± 1.1	-0.13 ± 1.5	0.23
CIR$_{120'}$ (mUxL/mmol^2)	427	0.89 ± 0.097	-0.05 ± 0.082	+0.24 ± 0.14	-0.30 ± 0.19	**0.045**
Derivative Control [(pmol/m^{2}BSA)/(mM/min)]	460	585 ± 17.53	+9.5 ± 18.18	+103 ± 2.88	-84.1 ± 1.11	0.16

Table 7. Effects of *TCF7L2* haplotypes on clinical and metabolic features of the Verona NDDS population. Data are presented as mean±SEM for the reference haplotype, while for the other haplotype coefficients ± SEM are reported.
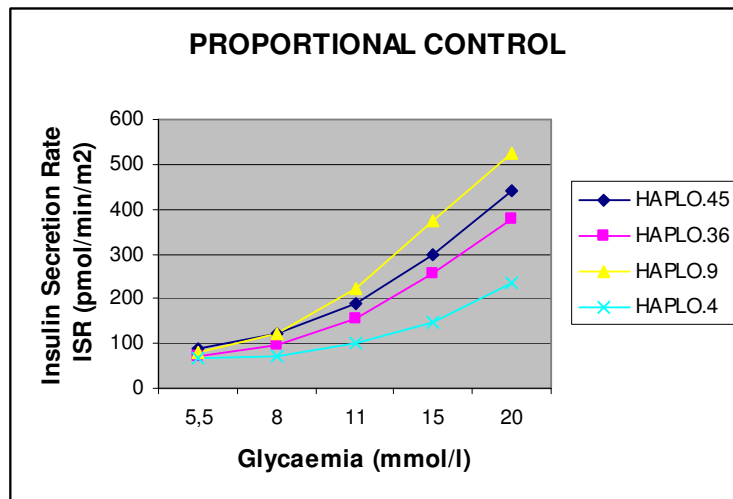
Fig. 11 Proportional control of the β-cell function is represented as the stimulus-response curve, with insulin secretion rate (ISR) on the Y axis and glucose concentration on the X axis. Haplo.4 is associated with a decreased ISR, while Haplo.9 shows increased ISR, both in comparison with the reference haplotype, Haplo.45.

Glycaemia (mmol/l)	HAPLOTYPES											Overall p-vaue
	HAPLO.45		HAPLO.36			HAPLO.9			HAPLO.4			
	mean	sem	Coef	sem	p-value	coef	sem	p-value	Coef	sem	p-value	
5,5	86,83	5,76	-15,85	4,91	**0,001**	-5,25	8,5	0,5	-21,4	12,4	0,08	**0,009**
8	120,76	9,6	-25,36	8,17	**0,001**	-0,3	14,2	0,9	-49,9	20,4	**0,015**	**0,003**
11	190,75	13,8	-36	15,2	**0,009**	32	23,8	0,17	-90,7	37.9	**0,02**	**0,005**
15	299,65	26,2	-45,6	26,4	**0,003**	57,5	46,5	**0,03**	-152,1	67,2	**$5*10^{-4}$**	**0,021**
20	439,8	41,8	-60,8	43,3	**$<10^{-4}$**	83,9	74,4	**0,0014**	-203,7	106	**$<10^{-4}$**	0,064

Table 8 The overall p-value refers to any haplotype influence on the different levels of the stimulus-response curve. The other p-values refer to individual haplotype related differences in the stimulus-response curve when compared to Haplo.45 (reference haplotype).

TCF7L2: DISCUSSION

In this study we assessed the associations of 4 variants in *TCF7L2*, investigated as single SNPs as well as specific haplotypes, with main clinical and pathophysiological features in patients with newly diagnosed T2DM. *TCF7L2* variants still represent the strongest genetic signal associated to an increased risk of T2DM and they may play a causal role through several mechanisms, including decreased beta-cell mass, impaired insulin processing or release, impaired GLP-1 signalling in beta cells, abnormal glucagon secretion, liver insulin resistance and altered chromatin state in T carriers.

Novelties of the present study are: 1. it was carried out in patients with newly diagnosed T2DM; 2. it applied state-of-the art methods to assess the two main physiological traits responsible for glucose homeostasis, i.e. beta-cell function and insulin sensitivity; 3. it explored the association of *TCF7L2* haplotypes with main clinical and pathophysiological phenotypes.

We observed that risk alleles of 3 out of 4 *TCF7L2* SNPs were associated with significantly lower BMI and significantly higher fasting plasma glucose, although it should be noted that the SNP triplet was not the same in the two cases. The first finding is somewhat reminiscent of an earlier study [69] in which a risk haplotype of *TCF7L2* was associated with lower BMI in patients with T2DM, and it may imply that the genetic risk conferred by *TCF7L2* leads to overt diabetes for lower BMI thresholds, all other clinical features being equal. Higher fasting plasma glucose in our study was not accompanied by higher 2-hour plasma glucose or HbA1c. Thus, it does not reflect poorer glucose control, but, perhaps, it may be related to some *TCF7L2* related pathogenetic mechanisms with a particularly relevant impact on fasting glucose (see below).

Only the risk allele of rs7903146 showed a significant association with lower triglyceride levels (*Appendix*, table B). Beyond the possibility of being only a finding due to the chance, it should be noted that the association between rs7903146 and triglyceride levels was lost after adjusting for fasting insulin levels, thus suggesting that it is probably mediated by the latter. Indeed, it is well known that hyperinsulinemia is an important co-determinant of hypertriglyceridemia. The most likely interpretation, therefore, is that rs7903146 risk allele exerts an effect on triglyceride levels through its effect on beta-cell function (see below). Further

support to this interpretation comes form recent data obtained in individuals belonging to families with familial combined hyperlipidemia [70] and in European Americans of the Genetics of Lipid Lowering Drugs and Diet Network Study [71] In both cases, the risk allele of rs7903146 was found related to high triglycerides and VLDL levels.

In this study, the risk alleles of rs7901695 and rs7903146 were associated with reduced beta-cell function, as assessed by state-of-the art techniques (Fig. 10). Interestingly, these risk alleles are associated with reduced beta-cell function not only in nondiabetic individuals, as previously reported, but also in patients with newly diagnosed T2DM. In nondiabetic individuals the association between *TCF7L2* variants and beta-cell function is used to explain the higher risk of T2DM in subjects with these variants. Our findings that the same allelic variants are associated with a poorer beta-cell function also in newly diagnosed T2DM subjects strongly support the conclusion that *TCF7L2* plays a major role in the development of diabetes and in contributing to the leading physiologic abnormality of the disease, i.e. beta-cell dysfunction.

Recent evidence indicates that in nondiabetic individuals the risk allele of rs7903146 marks insulin resistance in the liver, as manifested by an increased endogenous glucose output both in the postabsorptive state and during a euglycemic insulinemic clamp (performed at the same insulin dose employed in our study). We herein report no effect of rs7903146, or other SNPs, on insulin sensitivity as assessed by the euglycemic insulinemic clamp. However, the risk alleles of 3 *TCF7L2* SNPs, including rs7903146, were associated with higher fasting plasma glucose, a major determinant of which is endogenous glucose output and its sensitivity to insulin. Furthermore, in our study we did not measure liver but rather whole body insulin sensitivity, i.e. the algebraic sum of liver and peripheral insulin sensitivities. In the very same study which reported liver insulin resistance in rs7903146T carriers, whole body insulin sensitivity was slightly, albeit non significantly, increased, not decreased, in rs7903146T carriers. Thus, our data are not inconsistent with Pilgaard's findings [37, 40, 46, 49]

Previous studies have endeavoured to investigate the role played by *TCF7L2* haplotypes in determining clinical and metabolic phenotypes. Helgason et al reported that the haplotype $HapB_{T2D}$, which includes rs7903146T, was associated

with lower BMI in subjects with T2DM [69]. In another study, 11 tag SNPs in *TCF7L2* were genotyped [72] and 15 haplotypes (frequency from 0.12 to 40%) were identified by using four diabetes risk SNPs. Haplotype hap2 (frequency 26%), which lacks the risk alleles for all four SNPs, was associated with a strong protection against T2DM, higher age of onset of the disease and lower 2-h glucose values.

To the best of our knowledge, our study is the first in which *TCF7L2* haplotypes are assessed together with state-of-the-art assessment of beta-cell function and insulin sensitivity. In our study, several *TCF7L2* haplotypes had low frequency (i.e. <3%), accounted altogether for 7% of all haplotypes, and will not be discussed further. Two haplotypes together reached a frequency of ~80%. One of them (frequency ~35%) was formed only by the diabetes risk alleles of the 4 SNPs. The other (frequency ~46%) was formed only by non-risk alleles of the 4 SNPs. Accordingly, the latter was associated to a statistically significant 14-21% reduction in the stimulus-response curve relating glucose concentration to insulin secretion (Fig. 11). These findings seem to support the view that, as to *TCF7L2*, the pathophysiologic counterpart of at-risk variants is beta-cell function.

The other two haplotypes display a frequency of about 9% and 4%. The former is associated with better, the latter with poorer beta-cell function than the two most prevalent haplotypes. Again, the effect of *TCF7L2* genetic variants was selective for the stimulus-response curve linking glucose concentration to insulin secretion rate. The derivative control of beta-cell function was unaffected (table 5).

The size of the haplotype effect on beta-cell function was relevant: Haplo.9, the "good haplotype", had almost twice higher beta-cell response to glucose than Haplo.4, the "bad haplotype". This difference may be of clinical relevance from the diagnostic, prognostic and therapeutic viewpoints. Furthermore, both haplotypes might deserve to be considered when assessing beta-cell function and T2DM risk in nondiabetic individuals. To the extent that beta-cell function is a phenotypic marker of genetically-determined diabetes risk, the "bad haplotype" might be one of those low-frequency genetic risk regions with large size impact on diabetes risk which are currently searched in order to expand our knowledge on the genetic basis of T2DM. Conversely, the "good haplotype" might be used to identify those who are protected against T2DM.

It is noteworthy that the roles exerted by *TCF7L2* variants and haplotypes on beta-cell function may hint that through the genotype one might capture a sizeable fraction of the variability in beta-cell function of the patient with newly diagnosed T2DM. This possibility deserves to be explored thoroughly in the future.

GENETIC LOAD STUDY

GENETIC LOAD: RESULTS

The clinical features of subjects included in the GENETIC LOAD study are very similar to the *TCF7L2* study. They are reported in table 9.

Variable	Males	Females	All
Number	248	123	371
Age (yrs)	58 [51-64]	61 [54-67]	59 [52-65]
BMI (kg/m^2)	28.9 [26.1-32]	30 [27.2-34]	29.2 [26.4-32.6]
Waist (cm)	102 [95-110]	97 [90-104]	100 [93-108]
Fasting P-glucose (mmol/l)	7 [6.1-7.9]	7.1 [6.1-8.3]	7.1 [6.1-8]
2-h P-glucose (mmol/l)	13.3 [10.9-16.2]	13.1 [10.3-16.4]	13.3 [10.7-16.3]
HbA1c (%)	6.7 [6.2-7.5]	6.8 [6.1-7.4]	6.7 [6.1-7.5]
Triglycerides (mmol/l)	1.4 [1-2]	1.4 [1-2]	1.4 [1-2]
HDL-cholesterol (mmol/l)	1.1 [1-1.3]	1.2 [1-1.4]	1.1 [1-1.4]
Total Cholesterol (mmol/l)	4.9 [4.3-5.5]	5.3 [4.4-5.8]	5 [4.3-5.6]
SBP (mmHg)	140 [128-150]	140 [130-150]	140 [128-150]
DBP (mmHg)	85 [80-90]	86 [80-90]	85 [80-90]
Insulin Sensitivity (µmol/min/m^2 BSA)	578 [318-821]	535 [387-768]	565 [352-796]
Insulinogenic Index (mU/mmol)	3.3 [1.9-5.9]	4.3 [2.1-6.9]	3.7 [2-6.2]
$CIR_{120'}$ (mUxL/mmol^2)	0.4 [0.2-1]	0.6 [0.2-1.3]	0.5 [0.2-1.1]

Table 9 Clinical features of the genetic load study population. The values are reported as median [interquartile range].

In this study we aimed to assess the joint effect of 8 T2DM associated polymorphisms in determining clinical and pathophysiological traits of interest. The 8 analyzed SNPs are listed in table 2 and 10.

Preliminary to this analysis was the assessment of the effects exerted by each single variant, but none of them reached statistical significance individually, either for clinical and pathophysiological parameters (data not shown).

We then carried on assessing the joint effect of these SNPs ("genetic load") on key metabolic phenotypes, i.e. beta-cell function and insulin sensitivity, and also on clinical features. We gave different values to the alleles for each SNP: **0** to the not-risk allele and **1** to the risk allele. In this way we were able to calculate a sort of "genetic score", summing up all the risk alleles values for each patients. Since we considered 8 SNPs, the genetic load score potentially ranges between 0 (patients with no risk alleles) and 16 (patients homozygous for risk alleles in all SNPs). We then divided the studied population into three groups on the basis of the number of risk alleles: Low (LGL: ≤6, n=76), Intermediate (IGL: 7-9, n=226) and High (HGL: ≥10, n=69) "Genetic Load" score. Table 10 lists the SNPs under study, related genes, risk alleles and frequency.

SNP	Nearest gene	Not-risk/risk alleles	MAF
rs679931	CACNA1E	A̲/T	0.37
rs1801282	PPARG	T̲/C	0.07
rs1044498	ENPP1	A/C̲	0.16
rs10946398	CDKAKL1	A/C̲	0.30
rs1111875	HHEX	G/A̲	0.38
rs10010131	WFS1	A̲/G	0.35
rs4430796	TCF2	A̲/G	0.43
rs4402960	IGF2BP2	G/T̲	0.35

Table 10. Genotyped SNPs and alleles frequencies are reported. The minor allele is underlined, in orange the risk one. MAF: Minor Allele Frequency. CACNA1E: Calcium channel, voltage-dependent, R type, alpha 1E subunit; PPARG: Peroxisome proliferator-activated receptor-gamma; ENPP1: Ectonucleotide pyrophosphatase/phosphodiesterase 1; CDKAL1: CDK5 regulatory subunit-associated protein1-like1; HHEX: Haematopoietically expressed homeobox; WFS1: Wolfram syndrome 1; TCF2: Transcription factor 2; IGF2BP2: Insulin-like growth factor 2 mRNA binding protein 2

Effects of genetic load on clinical and phatophysiological T2DM phenotypes.

In table 11 the clinical features of the three groups (low, intermediate and high genetic load score) are reported. No significant differences were detected among the three groups, although a trend of the insulinogenic index to decrease with increasing genetic load scores was noticed. Similarly, no differences were detected in the derivative control of beta-cell function (table 12). However, the stimulus-response curve relating glucose to insulin secretion rate (proportional control) showed a statistically significant graded reduction with higher genetic load scores (fig. 12).

Variable	Genetic Load Score			p-value
	LGL	IGL	HGL	
Number	76	226	69	
Age (yrs)	57,8±10,8	58.2±9.6	57.2±10.7	0.8
BMI (kg/m^2)	30.1±5.5	30.3±5.1	28.4±3.8	0.07
Waist (cm)	101.2±12.6	102.2±12.3	98.9±11.1	0.1
Fasting P-glucose (mmol/l)	7.2±1,4	7.2±1.9	7.4±1.7	0.6
2-h P-glucose (mmol/l)	12. 8±4	13.6±4	14.3±4.1	0.09
HbA1c (%)	6,8±1,1	7±1.2	7±1.1	0.4
Triglycerides (mmol/l)	1.6±0.7	1.6±1.1	1.8±1.4	0.4
HDL-cholesterol (mmol/l)	1.1±0.3	1.2±0.3	1.2±0.5	0.8
Total Cholesterol (mmol/l)	5±0.9	5±1	5.2±0.9	0.3
SBP (mmHg)	138.7±17.9	139±18.6	138.3±17.6	0.9
DBP (mmHg)	83.6±9	85.9±9.1	84.5±10.1	0.2
Insulin Sensitivity (μmol/min/m^2 BSA)	634±373	597±318	634±383	0.6
Insulinogenic Index (mU/mmol)	5.6±5	5±4.6	4.2±4.1	0.2
CIR$_{120'}$ (mUxL/mmol^2)	1±1.2	0.8±1	0.7±0.7	0.2

Table 11 Clinical and phatophysiological features stratified by the genetic load score are reported in this table as mean±DS. There are no statistically significant differences among the three groups.

	N	Median [range IQ]	P Value
LGL	76	432 [0-911]	
IGL	226	420 [71-888]	0.8
HGL	69	330 [0-780]	

Table 12. Derivative control of beta-cell function in patients of the Verona NDDS according to the genetic load score groups. Data are presented as median [I.Q. Range]; units are pmol$\cdot$m$^{-2}\cdot$BSA per mM$\cdot$min^{-1}
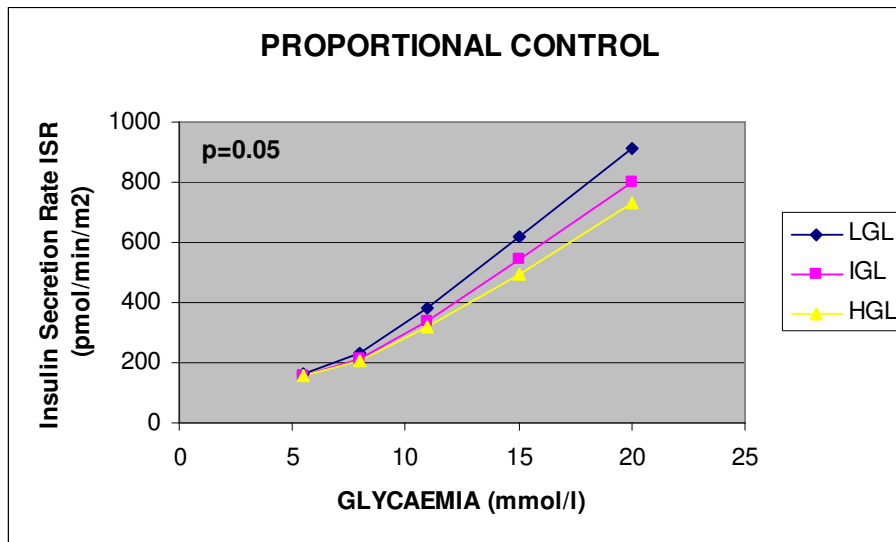

Fig. 12. The proportional control of beta-cell function is plotted as the stimulus-response curve, with insulin secretion rate (ISR) on the Y axis and glucose concentration on the X axis. In this analysis the higher is the genetic load score the lower the response of beta-cell to plasma glucose.

GENETIC LOAD: DISCUSSION

In type 2 diabetes. each of the recently discovered risk loci per se contributes quantitatively little to the overall risk. Not surprisingly, they are often of questionable biological significance in affecting beta-cell function and insulin sensitivity. To the present day the highest odd ratio is attributable to the *TCF7L2* variant rs7903146 (OR~1.4).

Since T2DM is a complex disease, multiple genetic factors are involved in its development. For this reason we tried to assess the joint effect of different risk alleles in our patients with newly-diagnosed T2DM.

We chose 8 loci already seen to be associated with increasing risk of T2DM. We deliberately did not include *TCF7L2* variants, to be sure that the effect, if any, is not depending on this strong diabetogene at all.

We found no differences among the three genetic load score groups for any of the standard clinical features, including indexes of obesity and of glucose control.

A trend for a worse insulinogenic index with higher genetic load score was not statistically significant. However, the proportional control of beta-cell function showed a graded worsening moving from the low to the intermediate to the high genetic load score group.

Many other research groups have previously investigated the combined effect of different risk alleles, but they all mainly analyzed the genetic load in relation to the risk of T2DM.

The first two studies that used this kind of combined analysis were carried out before the identification of the new T2DM associated polymorphisms, so they considered just 2 or 3 SNPs (PPARG, KCNJ11 and *TCF7L2*) [51, 73]. They both found that each additional risk allele increases the risk of T2DM in an additive manner. They tested also the interaction among the different variants but they found no evidence of it.

Even though these two studies were conducted in a small number of gene variants, the overall risk could be considered as the sum of the effects of each SNP.

After the development of the GWAS, a number of studies considering the combined effect of several T2DM associated polymorphisms have been published [50, 52-54, 74]. They all mainly analyzed the risk of type 2 diabetes and concluded that the higher is the number of risk alleles the higher is the risk of

developing the disease, but they came to different conclusions regarding the usefulness of genetic factors in improving the ROC (Receiver Operating Characteristic) curve of disease prediction. This curve indicates how useful adding further risk factors could be in predicting risk of future diabetes. Adding the genetic data to clinical risk factors, Cauchi et al. [52] found an improvement in the prediction of the disease, while other groups [53, 54] found that genetic factors improved very little the capacity of clinical parameters to predict T2DM.

These different results could reflect different strategies in selecting the study samples: a case control study in the French paper [52] and population-based studies in the ones by Meigs and Lyssenko [53, 54].

In our study we did not consider the ROC curve of disease prediction, because our study sample was formed by patients with diabetes. We instead focused on the influence of the genetic load score on the pathophysiological parameters. In the Swedish group paper [53] also this aspect was considered and they found that in nondiabetic subjects a higher number of risk alleles is associated with worse beta-cell function, measured as Disposition Index and insulin secretion. This result is comparable with the decreased proportional control that we found in our population, even though the methods of assessment of the beta-cell function are different.

Two recently published papers focused on the cumulative effect of several T2DM associated polymorphisms on beta-cell function [47, 75] and both of them concluded that the higher is the number of risk alleles, the worse is the beta-cell function.

These two studies are similar to ours for several reasons: - pathophysiological traits of T2DM and not the risk for the disease have been studied; - they are neither a case control nor a population based study; - they analyzed only non-diabetic subjects, whereas we studied newly-diagnosed diabetic patients; - all three studies found an association between the genetic load score and beta-cell function.

CONCLUSIONS

CONCLUSIONS

The novelty of this study is rooted in the accurate characterization, with state-of-art methods, of typically the two main pathophysiological traits characterizing type 2 diabetes: beta-cell function and insulin sensitivity.

Commonly used indexes of insulin secretion were not significantly associated to *TCF7L2* variants (*Appendix* tables from A to D). This points out that these surrogate indexes are less sensitive than parameters generated by state-of-the art techniques. Indeed, both insulinogenic index and CIR120' encompass several physiological facets of the insulin system, some of which, e.g. insulin clearance, are not related to beta-cell secretory function. Furthermore, only the stimulus-response curve between glucose concentration and insulin secretion rate was found to be selectively impaired by *TCF7L2* genetic variants (fig. 10), whereas no changes in the derivative control were observed (table 5).

Similar conclusions can be drawn for the GENETIC LOAD study. Also in this case the surrogate indexes disclosed no associations with genetic factors, but the analysis of proportional control of beta-cell function highlighted a reduction in beta-cell function in subjects with a greater number of risk alleles.

Our study has a number of limitations. First, the Verona NDDS is not a population based study, although subjects in the cohort are fairly representative of Italian patients with T2DM. Nonetheless, the Verona NDDS cohort has a somewhat higher than expected prevalence of males, possibly reflecting a gender related referral bias. Second, although we used state-of-the art methods to assess beta-cell function and insulin sensitivity, many relevant pathophysiological facets were unexplored, e.g. incretin axis and liver vs. peripheral insulin resistance. Third, our study was performed in Italian patients and transferability of our results to other ethnic groups should be made with caution. Fourth, subjects with severe glycemic decompensation were excluded for ethical reasons and this might have biased our findings. Finally, as in all association studies and particularly for the *TCF7L2* study, our findings need to be replicated.

In summary, we have reported evidence that genetic variants of *TCF7L2*, both at the single SNP and at the haplotype level, may play a significant role in determining beta-cell function in patients with newly diagnosed T2DM. Two low

frequency *TCF7L2* haplotypes that we have identified may deserve to be further investigated, also in nondiabetic individuals.

Regarding the GENETIC LOAD study, its results emphasize the value of investigating the combined effect of several T2DM associated polymorphisms. Indeed, even though many studies have shown that the genetic load has a measurable impact on T2DM risk, our findings suggest that it could have an important role in determining clinical and pathophysiological phenotypes in diabetic patients.

Genetics of type 2 diabetes is a continuously evolving field. Most recent papers have identified novel additional loci associated to T2DM, only some of which with known function, such as KCNQ1 (Potassium Channel, Voltage-Gated, KQT-like subfamily, member 1) [27, 28] and MNTR1B (Melaton Receptor 1B) [29-31]. For most of the T2DM risk loci, biological function and role in the disease are still unknown. Many further studies will be necessary to elucidate the role of genetics in this disease.

We propose that our findings support the conclusion that, in order to define metabolic phenotypes and, perhaps, personalized treatment and prognosis of T2DM, genotyping might be a simpler and cheaper approach than using the gold-standard techniques of clinical physiology.

APPENDIX

		rs7901695			Additive mode (Dominant model)		
Phenotype	Number	TT	TC	CC	BETA	SE	*P* Value
Risk (A) allele frequency, %		0.44					
Number (M/F)	464 (315/149)	144	234	86			
Age (yrs)	464	59 [52.2-65]	59 [51-65]	61 [51.7-67]	0.76	0.67	0.258
BMI* §(kg/m^2)	464	30.4 [27.3-33.5]	28.7 [26.2-32.5]	28.4 [26.2-32.2]	-0.03	0.01	**(0.047)**
Fasting P-glucose § (mmol/l)	463	6.8 [6.1-7.7]	7.2 [6.1-7.9]	7.4 [6.1-8.6]	0.3	0.11	**0.009**
2hr P-glucose (mmol/l)	463	13.3 [10.7-16.1]	12.7 [10.1-16.2]	14.3 [10.8-16.9]	0.33	0.28	0.22
Fasting insulin* (mU/l)	459	12.1 [7.5-17.2]	11.5 [7.7-16.9]	9.8 [6.9-13.7]	-0.06	0.04	0.11
2hr insulin* (mU/l)	426	74.3 [43-105.9]	61 [40.4-95.1]	58.7 [30.7-104.7]	-0.09	0.05	0.07
Insulinogenic Index* (mU/mmol)	411	4.2[2.1-6.3]	3.7 [2-6.8]	3.5 [1.8-5.7]	-0.09	0.07	0.19
CIR$_{120}$*(mUxL/mmol^2)	425	0.5 [0.2 –1.2]	0.5 [0.2-1.3]	0.4 [0.2-0.9]	-0.11	0.08	0.18
HbA1c	453	6.7 [6.1-7.5]	6.7 [6.1-7.4]	6.7 [6.1-7.6]	0.06	0.09	0.47
Triglycerides * (mmol/l)	456	1.4 [1-2]	1.4 [1.1-2.1]	1.4 [0.9-1.8]	-0.05	0.03	0.16
HDL-cholesterol (mmol/l)	452	1.1 [1-1.4]	1.1 [1-1.3]	1.2 [1-1.4]	0.015	0.02	0.5
Cholesterol (mmol/l)	455	5.1 [4.2-5.7]	5 [4.4-5.7]	4.8 [4.4-5.5]	-0.05	0.07	0.5
SBP (mmHg)	448	140 [130-150]	138 [124-150]	134 [120-150]	-1.7	1.2	0.17
DBP (mmHg)	448	85 [80-90]	85 [80-90]	84 [80-90]	-0.2	0.65	0.7
Insulin Sensitivity* (µmol/min/m^2 BSA)	449	577 [317-753]]	553 [366-840]	585.5 [387-819]	0.06	0.04	0.17

Table A. Influence of Rs7901695 genotype on clinical and metabolic variables of patients with newly diagnosed type 2 diabetes in the Verona NDDS. *Variables were log-transformed before statistical analysis to approximate a gaussian distribution; the coefficient beta and its S.E. refer to log-transformed values. § p-value still significant after adjustment for age, sex and BMI.

Phenotype	Number	rs7903146			Additive model (Dominant model)		
		CC	CT	TT	BETA	SE	*P* Value
Risk (T) allele frequency, %		**0.43**					
Number (M/F)	464 (315/149)	146	233	85			
Age (yrs)	464	59 [52-65]	59[52-65]	60[50-67]	0.5	0.7	0.44
BMI* § (kg/m^2)	464	30.5 [27.4-33.5]	28.7 [26.3-32.5]	28.3 [26.0-32.9]	-0.01	0.01	**(0.036)**
Fasting P-glucose § (mmol/l)	463	6.8 [5.9-7.7]	7.2[6.1-8]	7.3 [6.2-8.6]	0.25	0.11	**0.027**
2hr P-glucose (mmol/l)	463	13.3 [10.6-16.1]	12.8 [10.2-16.3]	14.0 [10.6-16.6]	0.22	0.28	0.41
Fasting insulin* (mU/l)	459	12.2 [7.5-17.4]	11.5 [7.8-16.8]	9.6 [6.9-13.8]	-0.07	0.04	0.085
2hr insulin* (mU/l)	426	74.1 [43.8-106.3]	60.0 [40.3-94.7]	61.7 [31.2-107.9]	-0.09	0.05	0.09
Insulinogenic Index* (mU/mmol)	411	4.0 [2.0-6.2]	3.5 [2.0-6.3]	3.3 [1.6-5.2]	-0.097	0.07	0.15
$CIR_{120'}$ * (mUxL/mmol^2)	425	0.5 [0.2-1.3]	0.5 [0.2-1.3]	0.4 [0.2-1.0]	-0.1	0.083	0.21
HbA1c	453	6.6 [6.1-7.5]	6.7 [6.1-7.5]	6.6 [6.1-7.4]	0.047	0.89	0.59
Triglycerides* § (mmol/l)	456	1.5 [1-2.1]	1.4 [1.1-2.1]	1.2 [0.8-1.7]	-0.08	0.03	**0.015**
HDL-cholesterol* (mmol/l)	452	1.1 [0.9-1.4]	1.1 [0.9-1.3]	1.2 [1-1.4]	0.02	0.02	0.23
Cholesterol (mmol/l)	455	5.1 [4.2-5.7]	4.9 [4.4-5.7]	4.8 [4.3-5.5]	-0.06	0.06	0.35
SBP (mmHg)	448	140 [127.5-150]	138 [125-150]	134.5 [120-150]	-1.3	1.2	0.30
DBP (mmHg)	448	81 [80-90]	86 [80-90]	84 [80-90]	0.25	0.65	0.69
Insulin Sensitivity* (µmol/min/m^2 BSA)	449	562.5[315.2-753.2]	545.5[369.2-810.5]	623[382-857]	0.05	0.04	0.24

Table B. Influence of rs7903146 genotype on clinical and metabolic variables of patients with newly diagnosed type 2 diabetes in the Verona NDDS. *Variables were log-transformed before statistical analysis to approximate a gaussian distribution; the coefficient beta and its S.E. refer to log-transformed values. § p-value still significant after adjustment for age, sex and BMI.

		rs11196205			Additive model		
Phenotype	Number	GG	GT	TT	BETA	SE	*P* Value
Risk (A) allele frequency, %		0.52					
Number (M/F)	464 (315/149)	108	226	129			
Age (yrs)	464	58 [51.2-64]	60 [52-65]	60 [52-67]	1.2	0.65	0.06
BMI* (kg/m^2)	464	30.3 [26.8-33.5]	29.4 [26.4-32.9]	28.5 [26.4-32.3]	-0.02	0.02	0.21
Fasting P-glucose [§] (mmol/l)	463	6.7 [5.9-7.6]	7.2 [6-7.9]	7.3 [6.3-8.5]	0.3	0.11	**0.009**
2hr P-glucose (mmol/l)	463	13.1 [10.7-15.9]	13.1 [10.1-16.4]	14.3 [11.1-16.5]	0.41	0.27	0.12
Fasting insulin* (mU/l)	459	11.7 [6.9-16.8]	11.6 [7.9-17.3]	11 [7.7-15.]	-0.01	0.04	0.85
2hr insulin* (mU/l)	426	61.4 [35-99.7]	66.1 [44.1-100]	61.7 [35.2-93.2]	-0.02	0.05	0.7
Insulinogenic Index (mU/mmol)*	411	4.3[2.1-6.4]	3.7 [2-6.7]	3.5 [2-6.8]	-0.04	0.07	0.51
CIR$_{120'}$ *(mUxL/mmol^2)	425	0.5 [0.24 – 1.1]	0.6 [0.3-1.4]	0.4 [0.2-1.1]	-0.06	0.08	0.5
HbA1c	453	6.6 [6-7.4]	6.7 [6.1-7.5]	6.6 [6.2-7.6]	0.04	0.09	0.66
Triglycerides* (mmol/l)	456	1.4 [1-2]	1.4 [1-2.1]	1.4 [0.9-1.8]	-0.02	0.03	0.58
HDL-cholesterol* (mmol/l)	452	1.1 [1-1.4]	1.1 [0.9-1.3]	1.1 [1-1.4]	-0.002	0.02	0.92
Cholesterol* (mmol/l)	455	5.1 [4.2-5.9]	5 [4.3-5.6]	4.9 [4.4-5.4]	-0.09	0.06	0.15
SBP (mmHg)	448	140 [126-150]	138 [128-150]	140 [120-150]	-1.3	1.2	0.29
DBP (mmHg)	448	85 [80-90]	85 [80-90]	84 [80-90]	-0.17	0.62	0.77
Insulin Sensitivity* (µmol/min/m^2 BSA)	449	610 [351-781]	532 [328-788]	598 [383-830]	0.02	0.04	0.52

Table C. Influence of rs11196205 genotype on clinical and metabolic variables of patients with newly diagnosed type 2 diabetes in the Verona NDDS. *Variables were log-transformed before statistical analysis to approximate a gaussian distribution; the coefficient beta and its S.E. refer to log-transformed values. [§] p-value still significant after adjustment for age, sex and BMI.

Phenotype	Number	rs12255372			Additive model (dominant model)		
		GG	GT	TT	BETA	SE	*P* Value
Risk (A) allele frequency, %		**0.39**					
Number (M/F)	464 (315/149)	167	225	72			
Age (yrs)	464	59 [51-65]	59 [52-65]	60 [52-67]	0.85	0.67	0.20
BMI* § (kg/m^2)	464	30.1 [26.9-33.5]	28.7 [26.1-32.4]	28.7 [26.1-32.9]	-0.03	0.01	**(0.023)**
Fasting P-glucose (mmol/l)	463	6.8 [5.9-7.8]	7.2 [6.3-8.0]	7.3 [6-8.7]	0.21	0.11	0.073
2hr P-glucose (mmol/l)	463	13.1 [10.7-16.1]	13.4 [10.2-16.2]	13.2 [10.5-16.7]	0.14	0.28	0.6
Fasting insulin* (mU/l)	459	11.8 [7.0-17.1]	11.6 [8.0-16.8]	10 [6.8-13.8]	-0.03	0.04	0.44
2hr insulin* (mU/l)	426	69.8 [38.2-103.5]	61.4 [41.8-97.3]	64 [32.6-92.6]	-0.06	0.05	0.24
Insulinogenic Index* (mU/mmol)	411	4.1[2.1-6.4]	3.6 [1.9-6.9]	3.7 [2-6.1]	-0.07	0.07	0.33
CIR$_{120'}$ (mUxL/mmol^2)	425	0.5 [0.24 – 1.2]	0.5 [0.2-1.3]	0.4 [0.2-1]	-0.04	0.08	0.64
HbA1c	453	6.7 [6.1-7.5]	6.7 [6.1-7.4]	6.6 [6.1-7.5]	0.05	0.9	0.53
Triglycerides* (mmol/l)	456	1.4 [1-2]	1.4 [1-2.1]	1.4 [0.9-1.8]	-0.04	0.03	0.24
HDL-cholesterol* (mmol/l)	452	1.1 [0.9-1.4]	1.1 [1-1.3]	1.1 [1-1.4]	-0.003	0.02	0.87
Cholesterol* (mmol/l)	455	5.1 [4.2-5.7]	5 [4.4-5.7]	4.8 [4.2-5.5]	-0.06	0.07	0.34
SBP (mmHg)	448	140 [130-150]	138 [124-150]	130 [120-150]	-2	1.2	0.1
DBP (mmHg)	448	85 [80-90]	85 [80-90]	84 [80-90]	-0.4	0.65	0.5
Insulin Sensitivity* (μmol/min/m^2 BSA)	449	547 [327-768]]	544 [355-797]	648 [409-875]	0.07	0.044	0.12

Table D. Influence of rs12255372 genotype on clinical and metabolic variables of patients with newly diagnosed type 2 diabetes in the Verona NDDS. *Variables were log-transformed before statistical analysis to approximate a gaussian distribution; the coefficient beta and its S.E. refer to log-transformed values. § p-value still significant after adjustment for age, sex and BMI.

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