The association of DNA methylation patterns in TCF7L2 and GIPR genes with Type 2 Diabetes

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To My Parents

To My Family

To My Friends

To My Director

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The present thesis is presented as a compendium of articles. No article presented in this thesis will be used for other thesis.

Article 1.

Silvia Canivell, Elena G.Ruano, Antoni Sisó-Almirall, Belchin Kostov, Luis González-de Paz, Eduardo Fernandez-Rebollo, Felicia A. Hanzu, Marcelina Párrizas, Anna Novials, Ramon Gomis.

Differential methylation of TCF7L2 promoter in peripheral blood DNA in newly diagnosed, drug-naïve patients with Type 2 diabetes.

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Article 2.

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Gastric Inhibitory Polypeptide Receptor Methylation in Newly Diagnosed, Drug-Naïve Patients with Type 2 Diabetes: A Case-Control Study.

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INDEX

ABBREVIATIONS

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ADA: American Diabetes Association,

BMI: Body mass index,

Bp: Base pair(s),

CpG: Cytosine and guanine separated by only one phosphate,

CRP: C-reactive protein,

CV: Cardiovascular,

CGI: CpG islands (genomic regions that contain a high frequency of CG dinucleotides),

DGF: Digital genomic footprints,

DHS: DNase hypersensitive sites,

DNA: Deoxyribonucleic acid,

DNMTs: DNA methyltransferase family of enzymes,

FFA: Free fatty acids,

GIP: Glucose-dependent insulinotropic polypeptide,

GIPR: GIP receptor,

GLP-1: Glucagon-like peptide,

GWAS: Genome-wide association study,

HbA1c: Glycated hemoglobin,

HDL-Cholesterol: High density lipoprotein cholesterol,

HOMA: Homeostatic model assessment,

HOMA-IR: HOMA insulin resistance score,

HOMA-B: HOMA β-cell function score,

IL: Interleukin,

Kb: Kilo-base pair, a unit of measurement of DNA or RNA length, equal to 1,000 nucleotides,

LDL-Cholesterol: Low density lipoprotein cholesterol,

lncRNA: Long non-coding RNAs,

LRES: Long-range epigenetic silencing,

miRNA: MicroRNA,

ncRNA: Non-coding RNAs,

OGTT: Oral glucose tolerance test,

piRNA: Piwi-interacting RNA,

siRNA: Short interfering RNA,

SNPs: Single-nucleotide polymorphisms,

T2D: Type 2 Diabetes,

TCF7L2: Transcription factor 7-like 2.

1 INTRODUCTION

1.1 Type 2 Diabetes, the disorder

1.1.1 Definition and diagnosis of Type 2 Diabetes

Diabetes mellitus is defined as the dysregulation of glucose metabolism resulting from defects in insulin secretion, decreased insulin sensitivity or a combination of both, which leads to chronic hyperglycemia and subsequent acute and chronic complications. The clinical symptoms are polydipsia, polyuria, unexplained loss of body weight, weakness and susceptibility to certain infections. Following the American Diabetes Association (ADA), the criteria for diabetes diagnosis are: HbA1c ≥6.5% , or, a fasting plasma glucose (FPG) ≥126 mgr/dL (7.0 mmol/L) (confirmed twice), or, a 2-h plasma glucose ≥200 mgr/dL (11.1mmol/L) during an oral glucose tolerance test (OGTT), or, the coexistence of clinical symptoms of hyperglycemia and a random plasma glucose ≥200 mgr/dL (11.1mmol/L)(1). Type 2 Diabetes (T2D) includes the major subgroup of diabetes mellitus, comprising the 90% of subjects affected by diabetes around the world(2). T2D, also previously named *"non-insulin-dependent diabetes"*, results from a progressive insulin secretory defect on the background of insulin resistance(1).

1.1.2 Epidemiology

The prevalence of T2D has been increasing over the past three decades around the world. In 2010, an estimated 285 million people worldwide had diabetes mellitus, 90% of whom had T2D(3). The number of people globally with diabetes mellitus is projected to rise to 439 million by 2030, which represents 7.7% of the total adult population of the world aged 20–79 years(4). Diabetes mellitus is actually categorized as a global pandemic, which affects both developed and developing countries(5). Data from the International Diabetes Federation show that Asia (China, India, Indonesia, and Japan) is the centre of the global epidemic of diabetes, mainly due as a result of rapid economic

development, urbanization, and nutritional transition(6). The major burden of diabetes is now in developing countries rather than developed countries. Indeed, about 80% of people with diabetes live in low-income and middle-income countries(5). This distribution presents an additional challenge for the correct clinical management of the subjects affected, since the lack of information and inadequate resources impede the correct prevention, diagnosis and treatment strategies. Globally, the main culprits of the increasing prevalence and already T2D-pandemic-like levels are the indirect consequences of urbanization and technology, particularly, the increasing of the sedentary behavior, and, in turn, obesity rates.

With reference to the situation in Spain, a recent population-based cross sectional study has been done to estimate the prevalence of T2D and glucose intolerance in the country(7). Results showed that almost 30% of the participants presented a carbohydrate disorder, the overall prevalence of diabetes mellitus was almost 14%, from which half of them were not aware of their condition, and the prevalence of "prediabetes" (including isolated impaired fasting glucose, isolated impaired glucose tolerance and both of them) ranged between 2% and 9%(7). Moreover, indices of the quality of life related to health outcomes have also been studied in relation to the degree of glucose tolerance in Spain. Results showed that, as expected, diabetes conferred a higher decrease in indices of quality of life as compared to a normal glucose tolerance state(8).

In addition, there is another novel characteristic in the current epidemiology of T2D. T2D was traditionally a disorder of middle-aged and elderly people, and almost exclusively an adult disorder. However, in the last few years, T2D has become more common in young adults, adolescents and children(3). T2D represents an increasing percentage of all incident cases of pediatric diabetes mellitus, with less than 4% of new-

onset cases among adolescents reported two decades ago and up to more than 80% today in some ethnic groups, such as American Indian, Asian and Pacific Islander populations(3). The increasing prevalence of T2D in young adults and children has important implications in terms of diabetes classification and diagnosis in childhood and adolescence. Children and adolescents, who are frequently overweight and obese, present some features of auto-immunity with accompanying insulin resistance, which has led to a new "subtype" of diabetes, named double diabetes, or, 1.5 diabetes(9). Moreover, the management of T2D in children and adolescents is different from that in adults(5). Lifestyle change and adherence to medication are difficult to achieve in this age group. In addition, the early onset of diabetes usually means a long period of disease which confers a higher risk of developing both micro- and macrovascular complications(5). Referring to data from Spain, there is no available data on the prevalence of T2D in children. The most recent population-based cross sectional study, the Di@betes study, excluded subjects younger than 18 years old(7). However, the incidence of diabetes adjusted by age and sex in a cohort from *Castilla y León* has recently been published(10). Authors concluded that the estimation of the incidence of diabetes in the age-group lower that 15 years old represents a good approximation of the incidence of Type 1 Diabetes(10). Thus, we can deduce that, in Spain, the incidence of T2D in children is not as high as in other countries. Reasons for this particularity are unknown. One reason might be that, in Spain, obesity rates in children are not as high as in other countries. In addition, the Mediterranean diet could confer a protective effect in this age-group.

The increase in the prevalence and incidence of T2D is tightly linked to increasing rates of obesity worldwide(3). The prevalence of obesity (i.e., Body Mass Index (BMI)$\geq$30kg/m^2) is expected to rise from 33% in 2005 to 58% in 2030, if secular trends

are maintained(11). Overweight and obesity are the principal predictors of T2D(3). Furthermore, the effect of obesity on lifetime risk of T2D is stronger in younger adults(12). However, since not all obese individuals present the disease, the concept of "metabolically obese" phenotype has emerged to explain the cases of T2D in normal-weight individuals(13). Indeed, a normal-weight subject who is also insulin resistant or presents the metabolic syndrome has a higher risk of T2D as compared to a metabolically "healthy" obese individual(14).

Apart from overweight and obesity, there are other risk factors for T2D, some of which are modifiable, while others are nonmodifiable (such as age, sex, ethnicity, family history of T2D, history of gestational diabetes and polycystic ovary syndrome)(3). Amongst the modifiable risk factors, the standard risk factors are physical inactivity, sedentary behavior, smoking, dietary factors, previously identified glucose intolerance (impaired fasting glucose or impaired glucose tolerance), abnormal lipid profile (high triglyceride levels and/or low HDL-cholesterol levels), hypertension and inflammation(3). Novel factors have been described such as sleeping disorders, depression, antidepressant medication use and environmental toxins (such as endocrine disruptors, as bisphenol A, and air pollution)(3). In addition, the role of the intrauterine environment in the future development of T2D is becoming increasingly important(15). Fetal under-nutrition and/or rapid postnatal growth, as well as fetal over-nutrition are associated with an increased risk for T2D(15).

1.1.3 Ethiopathogenesis

T2D is a complex metabolic disorder that results from multiple pathophysiologic abnormalities. Insulin resistance in muscle and liver, and β-cell failure represent the core effects(16), also named as the "triumvirate". β-cell failure occurs early in the natural history of diabetes, and is already present in the prediabetes state. Indeed,

subjects in the upper tertile of impaired glucose tolerance (IGT) are near-maximally insulin resistant and have lost >80% of their β -cell function(17). Insulin resistance in liver is manifested by glucose overproduction during the basal state despite fasting hyperinsulinaemia and impaired suppression of hepatic glucose production (HGP) by insulin, as occurs following a meal(17, 18). In muscle, insulin resistance is manifested by impaired glucose uptake after carbohydrate ingestion, resulting in postprandial hyperglycaemia(18). Although, insulin-resistant subjects can have an inherited genetic risk to become insulin-resistant(19, 20), the current epidemics of T2D results mainly from the increasing rates of obesity and physical inactivity, which are insulin-resistant states per se(21, 22). Within an insulin-resistant state, β-cells are stressed to produce more insulin to offset the insulin resistance(16). As long as β-cells are able to compensate the excess need for insulin due to the insulin resistance, glucose tolerance remains normal(16, 23-27). However, with time, β-cells start to fail resulting in a rise in postprandial plasma glucose levels and a rise in fasting plasma glucose, leading to overt T2D(16). In this sense, studies of insulin pulsatility and glucose oscillations during meals have shown that diabetic patients present: 1) decreased relative amplitudes of insulin pulses, 2) reduced frequency of glucose oscillations, 3) increased absolute amplitudes of glucose oscillations, 4) decreased temporal concomitance between peaks of insulin pulses and glucose oscillations, 5) reduced correlation between the relative amplitudes of glucose oscillations concomitant with insulin pulses, and 6) temporal disorganization of the insulin pulse profiles as compared to obese non diabetic subjects(28). These results reflect that β-cell responsiveness is reduced, and the regulation of insulin secretion is abnormal under physiological conditions in patients with T2D. Moreover, these abnormalities are not completely normalized with weight loss, even in patients who achieve metabolic control comparable to obese controls(28).

These results manifest the presence of an inherent β-cell defect that contributes to impaired insulin secretion in T2D patients. This abnormality is also present in subjects with glucose intolerance ("prediabetes") and may ultimately contribute to the development of overt T2D(28). Thus, the progressive β-cell failure might determine the rate of disease progression(17) (see Figure 1).

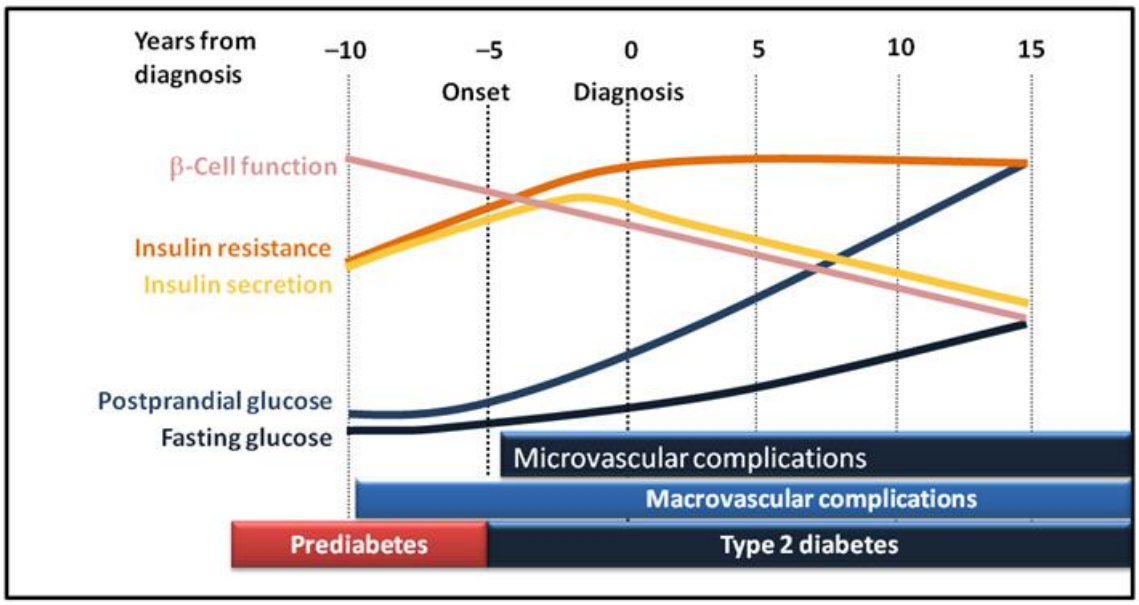

Figure 1: Scheme indicating the natural history of T2D over time from prediabetes to overt T2D and the development of vascular complications. Adapted from *Holman RR. Diabetes Res Clin Pract. 1998;40(suppl):S21-S25; Ramlo-Halsted BA, Edelman SV. Prim Care. 1999;26:771-789; Nathan DM. N Engl J Med. 2002;347:1342-1349; UKPDS Group. Diabetes. 1995;44:1249-1258.*

In addition to β-cell failure and insulin resistance in muscle and liver (what it is called as the "triumvirate"), other pathophysiologic factors contribute to the disease, globally known as the "ominous octet"(16). There is adipocyte resistance to insulin's antilipolytic effect, which leads to increased plasma free fatty acids (FFA) concentration

and elevated intracellular levels of toxic lipid metabolites in liver, muscle and β-cells that cause insulin resistance and β-cell failure(29). Incretin (glucagon-like peptide [GLP]-1/ glucose-dependent insulinotropic polypeptide [GIP]) effect is decreased due to impaired GLP-1 secretion(30). Moreover, there is severe β-cell resistance to the stimulatory effect of GLP-1 and GIP(31, 32). On another hand, glucagon secretion is increased in α cells and hepatic sensitivity to glucagon is enhanced leading to increased basal hepatic glucose production (HGP) and impaired HGP suppression by insulin(33, 34). In addition, renal glucose reabsorption is improved which contributes to the maintenance of elevated plasma glucose levels(35, 36). Lastly, the central nervous system becomes resistant to the anorectic effect of insulin and there is an alteration in the secretion of neurosynaptic hormones, which plays a role in appetite dysregulation, weight gain and insulin resistance in muscle and liver(37-39) (see Figure 2).

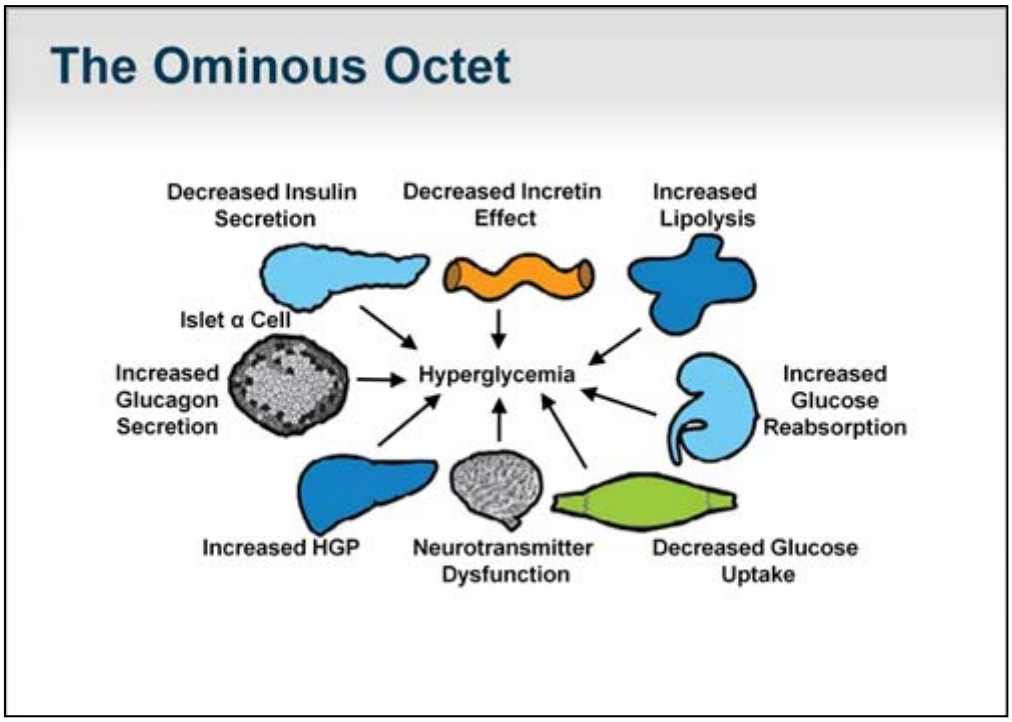

Figure 2: Image indicating the eight characteristics comprising the ominous octet in T2D. Adapted from *Defronzo RA. Diabetes. 2009 Apr;58(4):773-95.*

Progressive β-cell failure determines the progression to overt T2D, which is influenced by different physiopathologic factors, as discussed above. There is ongoing debate whether β-cell failure depends more on a reduction of β-cell mass or, instead, a reduction in β-cell function(40, 41). In fact, studies have shown that β-cell mass is decreased in T2D and that the mechanism underlying this is by an increase in β-cell apoptosis(42). One of the factors associated with these facts are the deposits of islet amyloid(43, 44) that are present in patients with T2D(44). Studies from human pancreas autopsies have confirmed that both β-cell mass and the number of β cells are increased in obese subjects(45). The causes and mechanisms underlying these facts remain unknown. In contrast, aging does not seem to have an effect on the number and mass of β-cells(45). Furthermore, there was no evidence of an increase in β-cell apoptosis neither in obesity, nor in advanced age in pancreas human samples(45). In contrast, as

said above, it has been described a 63% loss of β-cell mass in obese patients with T2D, and a 41% loss in lean patients with T2D, as compared with weight-matched non diabetic subjects(42). It has been proposed that the extent of the β-cell mass that develops during childhood may underlie subsequent successful or failed adaptation to insulin resistance in later life(46).

To summarize, both reduction of β-cell mass and reduction in β-cell function have been proved by distinct studies and what seems more likely is that a combination of the two processes determines β-cell failure(40). Recently, it has been postulated the following theory. β-cell dysfunction is sufficient to cause hyperglycemia; whereas reduced β-cell mass is not necessary but, if severe, can be sufficient. With time (several months or years), insulin resistance modulates β-cell capacity, at least in part, by driving β-cell mass expansion; the impact of obesity is partially mediated by insulin resistance. As a result, in obese subjects with normal glucose tolerance, β-cell mass is increased, β-cell capacity is upregulated, but glucose sensitivity is normal. Weight loss restores capacity, possibly with some effect on β-cell mass. In glucose-intolerant and diabetic subjects, mass may be reduced and β- cells are markedly dysfunctional in capacity as well as glucose sensitivity. Euglycemia and weight loss improves β- cell function, presumably without major changes in β-cell mass(47) (see Figure 3).

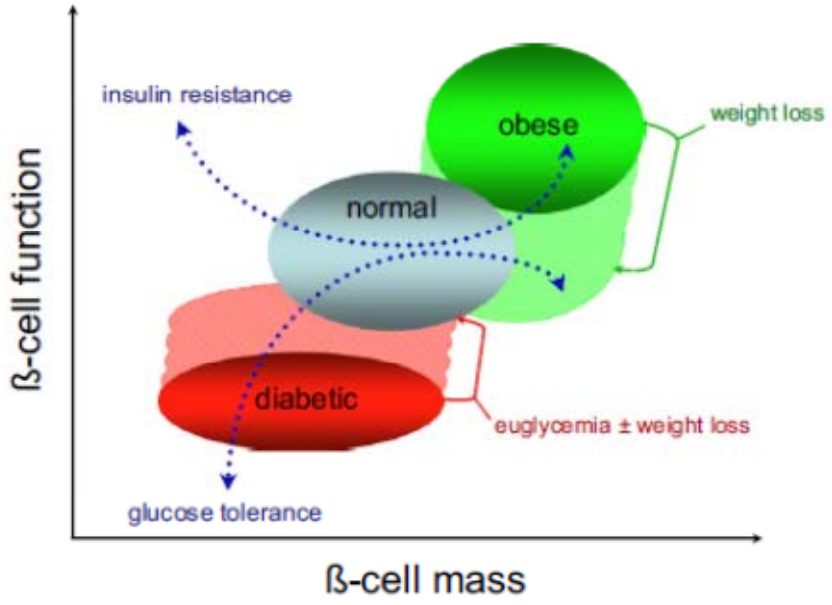

Figure 3: Schematic plot of β-cell function versus β-cell mass in normal, obese non-diabetic subjects and diabetic subjects.

- The obese non-diabetic subjects present a reversible degree of insulin resistance, a marginally impaired glucose tolerance, and a normal glucose sensitivity in expense to a higher β-cell capacity and a higher β-cell mass. Weight loss reduces β-cell capacity and does not change glucose sensitivity.
- The diabetic patients present a reduced β-cell mass and β-cell function, as well as glucose intolerance and insulin resistance (both reversible). Euglycemia and weight loss increases β-cell capacity and glucose sensitivity.

Adapted from *Ferrannini E. Cell Metab. 2010 May 5;11(5):349-52.*

Finally, a term that encloses the common pathophysiological elements present in T2D is what it is called the "stunned β-cell", i.e., a cell that is temporarily unable to appropriately sense its primary stimulus but may recover competence, at least in part(47). This topic has important implications for T2D therapeutics where the main objective would be to "rescue " the stunned β-cells.

1.1.4 Genetic risk associated with Type 2 Diabetes

Epidemiologic studies have demonstrated the hereditary load present in T2D. First, it was shown that if a child had a parent with T2D, he will have an approximately threefold increase in disease risk later in life(48). Then, family-based linkage analyses identified alleles or mutations responsible for rare monogenic forms of diabetes, including maturity onset diabetes of the young (MODY)(49). MODY encloses a heterogeneous group of autosomal dominantly inherited, young-onset β-cell disorders. In these disorders, diabetes is caused either by mutations in the glucokinase gene or by mutations in transcription factors. This led to molecular diagnoses of diabetes with demonstrable prognostic and therapeutic relevance. However, to identify common forms associated with T2D, the multifactorial nature of T2D made the task more complex. First, in order to ensure enough power, it was proposed to conduct association studies in large unrelated samples focusing on particular candidate genes(50). Subsequently, in 2007, the first wave of genome-wide association studies (GWAS) facilitated greatly the task by allowing the search for susceptibility variants across the entire genome in an unbiased, hypothesis-free manner(51).

Overall, linkage studies and GWAS have identified more than 40 genes associated with increased risk of T2D over the past decade(41, 52, 53). As briefly commented above, three main waves drove the discovery of susceptibility genes for T2D (53). The first wave consisted of family-based linkage analyses and focused on candidate-gene studies. These proved effective in identifying genes responsible for extreme forms of early-onset disease segregating as single-gene (Mendelian) disorders(54). These discoveries provided knowledge about processes critical for the maintenance of normal glucose homeostasis and energy balance and clues to the inner workings of the pancreatic β-cell and hypothalamus. The second wave of discovery involved a switch to tests of

association. Although intrinsically more powerful than linkage analysis, association analysis suffers from the disadvantage that the signal can be detected only if one examines the causal variant itself or a nearby marker with which it is tightly correlated(53). Researchers were therefore obliged to direct their attention to specific candidate variants or genes of interest(55). Common coding variants in *PPARG* and *KCNJ11* (each of which encodes a protein that acts as a target for classes of therapeutic agents widely used in diabetes management) were shown to have modest effects on the risk of T2D(56, 57). The third, and most successful, wave of discovery has been driven by systematic, large-scale surveys of association between common DNA sequence variants and disease. The first demonstration that unbiased discovery efforts could reveal new insights into the pathogenesis of T2D resulted from identification of the association between T2D and variants within *TCF7L2* (encoding transcription factor 7–like 2, a protein not previously identified as a biologic candidate)(58). *TCF7L2* has now been shown to modulate pancreatic islet function(59). The number of loci for which there is convincing evidence that they confer susceptibility to T2D started to grow in early 2007 with the publication of the first GWAS(60, 61). Since then, the dominant approach to discovery has involved larger aggregations of GWAS from multiple samples so as to improve the power to identify variants of modest effect(62). Though early studies were restricted to samples obtained from persons of European descent, GWAS conducted in other ethnic groups have emerged(63-65). The current total of approximately 60 loci have been confirmed for T2D(53, 65, 66) (see Table 1).

Table 1: Overview of gene loci that are associated with type 2 diabetes or related traits

Gene locus	Associated phenotype	Putative function(s)	References
Candidate gene studies			
PPARG	T2D	IR	(67)
KCNJ11	T2D	B	(67)
Large-scale association studies			
TCF7L2	T2D, glucose, HbA1c	B	(58, 61, 67, 68)
WFS1	T2D	B	(69)
HNF1B (TCF2)	T2D	B	(70)
GWAS for type 2 diabetes			
FTO	T2D, BMI	IR	(67)
SLC30A8	T2D, HbA1c	B	(61, 67, 71, 72)
HHEX/IDE/KIF11	T2D	B?	(61, 67, 71, 72)
CDKAL1	T2D	B	(67, 71, 72)
IGF2BP2	T2D	B	(67, 71, 72)
CDKN2A/CDKN2B	T2D	B	(67, 71, 72)
TSPAN8	T2D	?	(73)
ADAMTS9	T2D	B? IR?	(73)
NOTCH2	T2D	B	(73)
CDC123-CAMK1D	T2D	B	(73)
THADA	T2D	B?	(73)
JAZF1	T2D	B?	(73)
KCNQ1	T2D	B	(63, 74)
IRS1	T2D	IR	(75)
DUSP9	T2D	IR?	(74)
ZFAND6	T2D	?	(74)
PRC1	T2D	?	(74)
CENTD2	T2D	B?	(74)
TP53INP1	T2D	?	(74)
KLF14	T2D	IR?	(74)
ZBED3	T2D	?	(74)
BCL11A	T2D	?	(74)
HNF1A	T2D	?	(74)
CHCHD9	T2D	?	(74)
HMGA2	T2D	B?	(74)
GWAS for type 2 diabetes-related traits			
MTNR1B	T2D, glucose, HOMA-B, HbA1c	CR, B	(62, 76-78)
GCKR	T2D, glucose, insulin, HOMA-IR	GS	(62, 68)
DGKB-TMEM195	T2D, glucose, HOMA-B	B?	(62)
GCK	T2D, glucose, HOMA-B, HbA1c	GS	(62, 78)
PROX1	T2D, glucose	B	(62)

Table 1 *Continued*

Gene locus	Associated phenotype	Putative function(s)	References
ADCY5	T2D, glucose, HOMA-B, HbA1c	B?	(62, 68)
SLC2A2	Glucose	B	(62)
G6PC2	Glucose, HOMA-B, HbA1c	B	(62, 78)
GLIS3	Glucose, HOMA-B, HbA1c	B	(62)
ADRA2A	Glucose	B	(62)
CRY2	Glucose	CR	(62)
MADD	Glucose	B?	(62)
FADS1	Glucose, HOMA-B, HbA1c	?	(62)
IGF1	Insulin, HOMA-IR	IR	(62)
GIPR	Glucose	B	(68)
VPS13C	Glucose	B?	(68)
C2CD4B	Glucose	?	(62)
HK1	HbA1c	E?	(78)
FN3K	HbA1c	?	(78)
HFE	HbA1c	E	(78)
TMPRSS6	HbA1c	E	(78)
ANK1	HbA1c	E?	(78)
SPTA1	HbA1c	E?	(78)
ATP11A/TUBGCP3	HbA1c	?	(78)

Legend Table 1: Putative functions:

B, role in β-cell development, β-cell function, insulin secretion

CR, role in the regulation of circadian rhythm

E, erythrocyte physiology

GS, role in glucose sensing

GWAS, genome-wide association study

IR, role in insulin resistance

?, unknown

Adapted from *Herder C, Roden M., Eur J Clin Invest. 2011 Jun;41(6):679-92.*

Overall, most gene associations were inferred from single-nucleotide polymorphisms (SNPs) within noncoding regions of the gene, and in many cases the annotation is not yet conclusive. Thus, it remains uncertain whether diabetes is linked to the gene within whose intron the SNP resides or to genes that lie close by or are coregulated. With few exceptions, the genes identified were unexpected based on existing knowledge, and precisely how they predispose to T2D is not yet understood. However, many are believed to be important for β-cell function, β-cell development, or the regulation of β-cell mass(41). Others, such as *FTO*, predispose towards obesity and thus indirectly to T2D; when corrected for body mass index (BMI), the association with T2D disappears(41). Indeed, it is not easy to determine how the various SNPs predispose to T2D. Given that genes found in GWAS only cause a small increase in disease risk and that their effects manifest only later in life or in the face of obesity, disease-associated SNPs may only have small effects on β-cell function that will be hard to measure in human studies or even in vitro. As might be expected, disease risk is enhanced for individuals who carry multiple risk-associated SNPs(53). In fact, GWAS genes collectively appear to explain only 5%–10% of T2D. It has been suggested that there may be a large number of far less common mutations that carry a greatly enhanced disease risk(52). There is ongoing effort devoted to identifying these rare variants(41).

Attempts have been made to calculate a person risk for suffering from T2D taking into account the loci identified or confirmed by GWAS(79). However, a recent meta-analysis concluded that, although the identification of GWAS markers could help improve our understanding of the pathophysiology of T2D, their clinical utility in improving the prediction of T2D beyond that of conventional risk factors remains limited(79). In effect, there is actually no proof that genetic testing for the prediction of T2D in high risk individuals is of any value in clinical practice(80). This is mainly due

to: the small effect size of genetic loci, the low discriminative ability of the genetic test, the small added value of genetic information compared with the clinical risk factors, the questionable clinical relevance of some genetic variants in disease prediction, and the lack of appropriate models for studies of gene-gene and gene-environment interactions in the risk prediction(80).

1.2 *TCF7L2* gene and Type 2 Diabetes

1.2.1 *TCF7L2* gene and function

TCF7L2 gene, officially called "transcription factor 7-like 2 (T-cell specific, HMG-box)" is located in the long arm of chromosome 10 at position 25.3 (10q25.3). TCF proteins belong to a family of transcription factors that contain high mobility group box DNA-binding domains. TCF4, encoded by *TCF7L2*, plays a key role in the Wnt signalling pathway: its binding by β catenin after Wnt activation of its receptor (or in the absence of the inactivating molecule adenomatous polyposis coli (APC)) leads to the formation of a TCF4/β catenin transcriptional complex which induces the expression of TCF4 target genes. The controlled regulation of this signalling mechanism is thought to direct normal cellular proliferation and differentiation(81). Several transcript variants encoding multiple different isoforms have been found for this gene. This gene is highly expressed in pancreas, followed by colon, brain, small intestine, monocytes, and lung(82). There is lower expression in other tissues, and no expression in activated or resting T and B cells(82). The human *TCF7L2* gene consists of 14 exons and 13 introns (NCBI build 36.2). However, a previous study has shown that *TCF7L2* has 17 exons, of which five are alternative(81). There is tissue-specific expression of several splice variants(82). Prokunina-Olsson et(82) concluded that alternative splicing results in *TCF7L2* proteins that either repress or activate the WNT signaling pathway.

The Wnt-signalling pathway consists of extracellular ligands (Wnts), secreted antagonists, seven transmembrane cell surface receptors (Frizzled) and coreceptors (e.g., LRP 5/6), and intracellular signalling molecules, among which β-catenin is pivotal. Currently, 19 different Wnts and 9 different Frizzled receptors are characterised. Intracellular Wnt-signalling branches into the canonical (β-catenin) and the noncanonical pathways. Wnts are secreted signalling molecules which bind on cell surface receptors called Frizzled and LRP5/6. Upon ligand binding, intracellular signalling cascades are activated resulting in the induction of either noncanonical or canonical Wnt-signalling, the latter involving the transcriptional coactivator β-catenin. Activated β-catenin translocates to the nucleus where it is able to coactivate various transcription factors, including TCF / LEF(83) (see Figure 4).

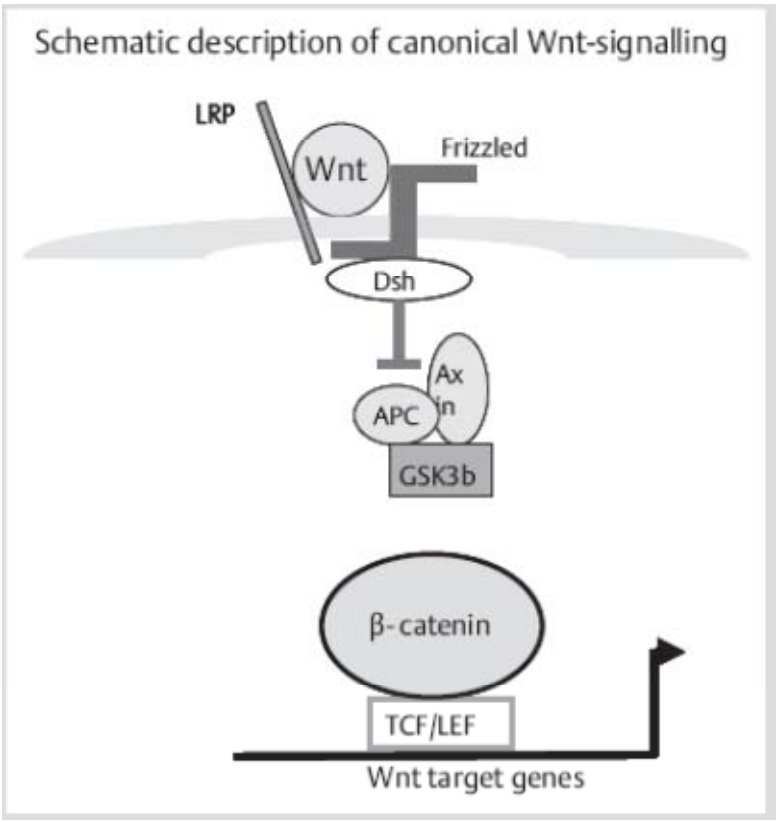

Figure 4: Schematic description of canonical Wnt-signalling.

- Binding of Wnt to the Frizzled and LRP coreceptors induces canonical Wnt-signalling by inhibiting GSK3b which allows β-catenin to translocate to the nucleus and coactivate transcription factors like TCF/LEF on target genes.

Adapted from *Schinner S. Horm Metab Res. 2009 Feb;41(2):159-63.*

The Wnt-signalling pathway plays a well-established role in embryogenesis and tumorigenesis. In addition, Wnt-signalling is a key factor in the context of metabolic disease. In vitro and in vivo data characterised the role of Wnt-signalling molecules in the regulation of adipocyte differentiation (adipogenesis)(83). In human and murine preadipocytes, a downregulation of canonical Wnt-signalling is a prerequisite in order to initiate adipogenesis. Furthermore, a pivotal role in regulating pancreatic β-cell function and mass has been attributed to the Wnt-signalling pathway.

Wnts induce glucose-stimulated insulin secretion and β-cell proliferation. Interestingly,

there is another indirect link between Wnt-signalling and β-cell function: canonical Wnt-signalling regulates the transcription of the proglucagon gene, eventually leading to GLP-1 expression. In addition, the intracellular effects of GLP-1 on pancreatic β-cells seem to be (at least partly) mediated by canonical Wnt signalling(83).

1.2.2 *TCF7L2* and Type 2 Diabetes

In 2006, two reports demonstrated an association of polymorphisms in the gene encoding *TCF7L2* with an increased risk to develop T2D. Grant et al. showed a 2.4-fold increase of T2D in homozygous carriers of the mutated *TCF7L2* variant(58). A similar study by Florez and colleagues confirmed the association of *TCF7L2* polymorphisms with T2D(84). Furthermore, they showed that progression from impaired glucose tolerance to diabetes mellitus is positively associated with polymorphisms in *TCF7L2*(84). These initial studies have been confirmed in large whole-genome scans and in numerous ethnic groups(85-90). From these studies *TCF7L2* is known to be the most powerful genetic contributor to T2D at present. However, from the data available at this stage we do not fully understand the mechanisms through which *TCF7L2* variants affect glucose metabolism. Heterodimerisation of *TCF7L2* with β-catenin induces transcription of a number of genes, including those coding for intestinal proglucagon (the prohormone of glucagon) and glucagon-like peptide one (GLP-1) and -2(91). In T2D, secretion of GLP-1 is impaired, whereas the effect of GLP-1 on insulin secretion is maintained (92). Also, suppression of glucagon by glucose is impaired in T2D(93). The pathophysiological background gathered from data in vitro and from animal studies suggest that *TCF7L2* may have effects on a) GLP-1 secretion, b) GLP-1 action within pancreatic β-cells, and c) GLP-1-independent β-cell function, namely proliferation and glucose-stimulated insulin secretion. There have also been a few studies in humans trying to unravel the role of *TCF7L2*. Subjects carrying *TCF7L2*

polymorphisms showed a blunted insulin response to both oral and intravenous glucose load(59). Another study investigated the effect of *TCF7L2* polymorphisms on GLP-1 levels in patients and found no difference in GLP-1 levels, but in glucose-stimulated insulin secretion between carriers and noncarriers(94). These data suggest defects within the β-cell in subjects carrying *TCF7L2* polymorphisms rather than defects in GLP-1 secretion.

Studies in human islets have shown that there is a 5-fold increase in *TCF7L2* expression in islets from T2D patients as compared to islets from non-diabetic donors(59). In addition, nondiabetic carriers of the risk genotype had the highest expression of *TCF7L2* in islets. These data are consistent with findings in rodent models for T2D(59). In contrast, a decreased *TCF7L2* expression was found in adipose tissue of obese T2D patients, suggesting a tissue especificity of *TCF7L2* expression(59, 85).

In conclusion, genetic variants in *TCF7L2* confer the stronger risk of future T2D known to date, possibly by influencing the expression of *TCF7L2* in pancreatic islets. Enhanced expression of *TCF7L2* reduces insulin but not glucagon secretion(59). The precise mechanisms by which alterations in *TCF7L2* expression relate to impaired insulin secretion as well as the potential involvement of impaired incretin effects are still not completely understood.

1.3 *GIPR* gene and Type 2 Diabetes

1.3.1 *GIPR* gene and function

GIPR gene, officially called “gastric inhibitory polypeptide receptor” is located in the long arm of the chromosome 19 at position 13.3 (19q13.3). *GIPR* gene encodes the gastric inhibitory polypeptide receptor (GIP-R) also known as the glucose-dependent insulinotropic polypeptide receptor. Gastric inhibitory polypeptide (GIP) is an incretin

hormone released from intestinal cells in response to feeding(95). GIP is synthesised in intestinal K-cells and its secretion is regulated largely by the ingestion of carbohydrate and fat(95). GIP exerts effects through GIP-R, which is expressed in various tissues including pancreatic islets, adipose tissue, and the brain(95, 96). The physiologic role of GIP is to stimulate insulin secretion after a glucose load(97). In addition, GIP has been shown to exert other effects on the pancreatic β cell, including stimulation of proinsulin gene transcription and translation plus enhancement of β cell growth, differentiation, proliferation and survival(98). Moreover, the presence of GIP receptors on adipocytes has prompted renewed awareness of GIP-mediated effects on lipid metabolism and fat deposition. Effects mediated by GIP receptor have been suggested as a key link between consumption of energy-rich high-fat diets and the development of obesity, insulin resistance and T2D(99). It has been suggested that inhibition of GIP signalling could be a potential avenue for the treatment of obesity and associated complications. In this sense, transgenic mice with knockout of the GIP receptor have been particularly useful in elucidating mechanisms that underlie beneficial effects of compromised GIP action on diet-induced obesity. Studies have shown that GIP receptor knockout in mice fed normal diet had little effect, other than small impairment of glucose tolerance and insulin secretion(95). However, inhibition of GIP receptor function in GIP receptor knockout mice fed a high-fat diet resulted in suppression of body weight gain, reduced adiposity, decreased tissue triacylglycerol stores, reduced insulin resistance and marked improvement of glucose tolerance(95). These observations were similar to those encountered with chemical GIP receptor blockade(95). GIP appears to directly link overnutrition to obesity and is considered as a potential novel target for anti-obesity-related diabetes drugs. The similar beneficial effect induced by a wide range of genetic and chemical approaches to impairment of GIP receptor signalling supports this belief.

In addition, interesting parallels also exist in clinically obese patients undergoing bypass surgery, with diversion of nutrient passage away from the gut section containing GIP-secreting cells. These patients display an unprecedented correction of hyperglycaemia and insulin sensitivity with and possibly even greater than that observed in animal models with disruption of GIP receptor signalling(95).

1.3.2 *GIPR* and Type 2 Diabetes

Variants at the *GIPR* locus (rs10423928 per A allele) were identified as being associated with 2-hour glucose levels(68). The *GIPR* A-allele carriers also showed decreased insulin secretion and diminished incretin effect(68). It has been shown that the incretin effect is impaired in individuals with T2D. Specifically, in individuals with T2D, stimulated GIP secretion appears normal and their insulinotropic response to GIP is reduced(100). *GIPR* is therefore a biologically plausible candidate for mediating insulin secretion after oral glucose challenge. *GIPR* variants were tested with indices of oral glucose–stimulated insulin secretion in up to 13 studies with samples measured at multiple times during the OGTT(68). The rs10423928 A allele associated with increased 2-h glucose was also associated with lower insulinogenic index, which represents a reduction in the early phase of insulin secretion(68). The rs10423928 A allele was also associated with a lower ratio of insulin to glucose area under the curve, which is an integrated measure of insulin response over the 2-h OGTT. Furthermore, the rs10423928 A allele was associated with lower 2-h insulin level(68). mRNA expression patterns of *GIPR* gene were assessed in a human tissue panel. *GIPR* gene was expressed in the pancreas, with strong specific mRNA expression in the sorted pancreatic β-cells, supporting the implication of *GIPR* in insulin secretion. No significant difference in *GIPR* mRNA expression in pancreatic islets was seen based on the rs10423928 genotype(68).

To sum up, it has been suggested a role for *GIPR* in the incretin effect and in early pathophysiologic pathways that could lead to impaired glucose tolerance and T2D in humans. In addition, it was hypothesized that patients with T2D might express a smaller amount of *GIPR* or defective *GIPR*(101). Meier *et al.* observed that individuals with T2D and a subgroup of the first-degree relatives of these individuals had a blunted insulin response to GIP, supporting the hypothesis that a defect of the *GIPR* could be part of the T2D pathophysiology(102).

1.4 Epigenetic mechanisms in diseases

1.4.1 Epigenetics, the concept and subtypes

All information required for the development and function of cells and complex organisms is encoded in the DNA molecule. However, access to the completed primary sequence of the human genome(103, 104) has highlighted the fact that genetic sequence alone cannot explain how the genome regulates the development and function of a wide assortment of cell types with highly specialized functions and different phenotypes, all of them arising from the same precursor cell and containing the same basic DNA sequence. In eukaryotic cells, both DNA itself and its associated proteins are targeted by an array of molecular modifications that influence gene expression without altering the primary sequence of DNA by either favoring or denying access of regulatory proteins to DNA(105). These additional layers of information piled over that of DNA are what constitute the field of study of epigenetics. In eukaryotes, gene expression and transcription are regulated at 3 levels. The first one is determined by the DNA nucleotide sequence in the promoters and other regulatory regions such as enhancers, silencers, and *locus* control regions, as well as by the proteins that bind to them, RNA polymerases, transcription factors, and coactivators/repressors of transcription. The

second one is mediated by the epigenetic mechanisms, which control chromatin condensation(106). The third and final level of organization is determined by the spatial organization of the genome in the interior of the cell nucleus.

The term “epigenetics” literally means: “beyond genetics”. It was initially crafted by Conrad Hal Waddington in 1941 to describe “the interaction of genes with the environment that brings the phenotype into being”. However, today this term describes the study of somatically, and sometimes transgenerationally, inherited changes in gene expression that take place without changes in the DNA sequence. Epigenetic inheritance participates in determining the gene expression pattern, and therefore, the fate of different cell types during embrionary development(107). The state of activation or inactivation of a gene in a four dimensional space explains how all the genes of the organism can be present in all cells, while only a selected set are expressed in a particular cell at a particular moment(108). Epigenetic mechanisms are also responsible for the inactivation of one of the two X chromosomes in all somatic cells of human females(109), as well as for genetic imprinting, which consists in the repression of particular alleles in function of their parent of origin(110).

Recent findings point out that epigenetic mechanisms are important not only during embrionary development, but also after birth including the adult life. Increasing evidence indicates that part of the gene–environment interactions relevant for complex diseases are mediated by epigenetic mechanisms. By regulating the accessibility to chromatin, epigenetic factors relay the effects of the environment to the transcriptional machinery, leading to changes in gene activity. Moreover, epigenetic mechanisms may permit the heredity of the environmental effects on transcription, even after the triggering signal has been eliminated. This so-called ‘epigenetic inheritance’ explains,

therefore, the relationship between the genetic background of an individual, environment and disease.

Currently, the term epigenetics encompasses a myriad of chemical changes to DNA or histone proteins, chromatin accessibility, small and long non-coding RNAs, and higher-order DNA organization (including nucleosome occupancy and positioning, and 3D chromatin interactions) (see Figure 5)(111). The epigenome (the total of epigenetic modifications present at a given time in a cell) determines the transcriptome (the set of all transcripts) of a cell. Epigenomic features influence the regulatory program of each gene's expression in several ways: they define the local environment of specific processes by regulating the chromatin architecture, determine access of transcription factors to DNA, as well as serve to keep a "memory" of specific features facilitating heritability of epigenetic characteristics(112).

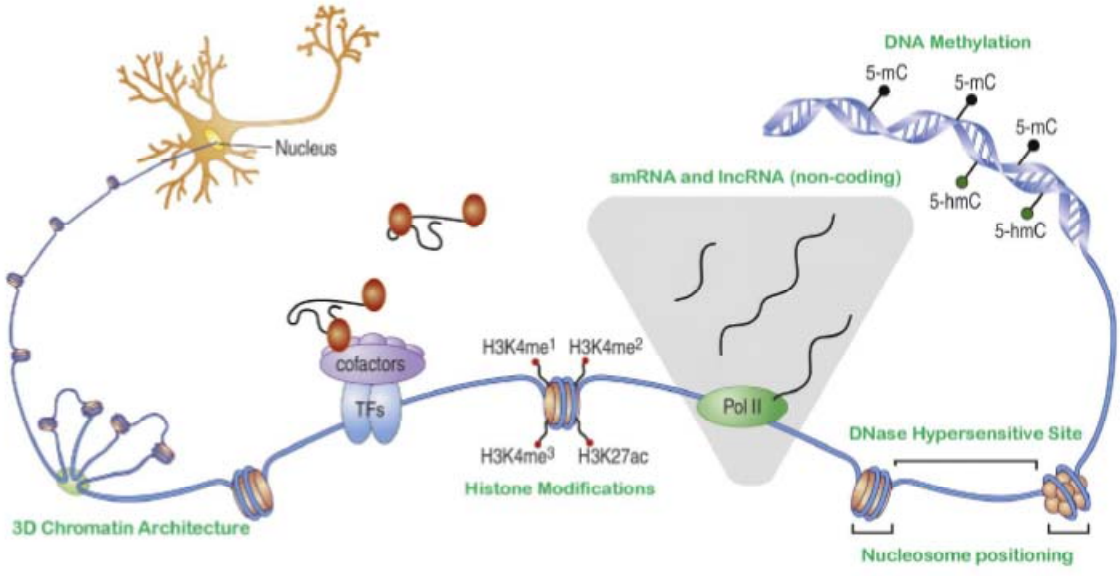

Figure 5: Schema showing the different subtypes of epigenetic marks on chromatin, ranging from DNA methylation, nucleosome positioning, small and long RNA, histone modifications and chromatin folding. Adapted from Figure 1, in

Sarda S, Hannenhalli S. Next-Generation Sequencing and Epigenomics Research: A Hammer in Search of Nails. Genomics Inform. 2014 Mar;12(1):2-11.

Two of the most important epigenetic mechanisms are DNA methylation (see next section) and the posttranslational modifications of histones. These mechanisms reorganize (remodel) the chromatin. The chromatin is the nucleoprotein complex structure where DNA is packaged and is highly dynamic, in that its "states" vary from one cell type to another. Chromatin remodeling is an active process in which inaccessible, compact and repressed chromatin is converted into an open, accessible form able of sustaining active gene transcription or vice versa(105, 107, 108).

Posttranslational modifications of histones

A nucleosome is the basic unit of DNA packaging in eukaryotes, consisting of a segment of DNA wound around the histone protein cores. The four core histones H2A, H2B, H3 and H4 are small, basic proteins (11-16 kDa) with an unstructured tail in the N-terminal region that protrudes out of the nucleosome. This tail is the main target of a number of transcriptional coregulators that have the capacity to enzymatically modify histones by acetylation, methylation, phosphorylation, sumoylation, deimination or ubiquitylation(113). These posttranslational covalent modifications affect the electrostatic charge of the proteins, and therefore their structure and association with DNA. On the other hand, these modifications create new binding sites for regulatory proteins, thus resulting in recruitment of specific activator or repressor complexes. Numerous reports have shown a clear link between the pattern of histone modifications in the chromatin of a given gene and its transcriptional status. Thus, histone lysine acetylation is usually related to gene activation(113), whereas arginine and lysine methylation result in different outcomes, depending on the modified residue. These

covalent modifications of histones can generate synergistic or antagonistic interactions with proteins associated to chromatin, and changes in nucleosome positioning, leading to dynamic changes between active and inactive transcriptional processes. The observed correlation between specific histone modifications and particular DNA-dependent processes, including gene expression, and the fact that the presence or absence of particular modifications often affect the presence or absence of other modifications led to the statement of the ***histone code hypothesis(114)***, which postulates that the pattern of histone postranslational modifications in a *locus* adds new layers of information to that conveyed by the genomic sequence alone, either by modulating access to the DNA or by actively recruiting transcriptional regulators. This notion has been further extended in more recent works into an ***'epigenomic code'*** to include epigenomic marks other than histone modifications(115).

Other types of epigenetic mechanisms are described below.

Nucleosome positioning and occupancy

The occupancy and periodic positioning of nucleosomes can control the accessibility of DNA to transcription factors(116) and DNases, as well as the transcription rate of active gene bodies(117), and are thus considered an epigenetic mark. Active regulatory regions are generally depleted of nucleosomes(118), whereas inactive repeat regions (heterochromatin) have higher affinity to form nucleosome structures(119).

Chromatin accessibility

Chromatin accessibility impacts transcription factor binding to DNA, and hence, transcriptional regulation. Open and easily accessible regions of DNA within the chromatin are indicative of local territories of transcriptional activity. Measuring "openness" of DNA at different regions genomewide has helped discover several

classes of functional elements, like promoters and enhancers. It has also aided in identifying cell-type specific behaviours by comparison of accessibility profiles(120).

The enzyme DNase I is capable of digesting DNA in nucleosome-depleted regions (i.e., free unwound DNA). Sequencing done post-digestion has identified large blocks of DNase hypersensitive sites (DHS) in chromatin (DNaseseq)(121, 122), which, upon further deep sequencing, can reveal up to 40-bp footprints of protected regions (potentially bound by transcription factors). These smaller regions are called ***digital genomic footprints*** (DGF)(123).

3D chromatin architecture

The 3D chromatin structure determines the range of DNA interactions. Indeed, chromatin conformation mediates a promoter's access to its enhancers, thereby determining the transcriptional fate of a gene(124). The tertiary structure of chromosomes has profound implications for cellular function and fate(124).

Non-coding RNA

Non-coding RNAs (ncRNA) are functional RNA molecules that are not translated into a protein. Recently, it has been shown that specific classes of non-coding RNA—short RNAs (including micro RNA [miRNA], short interfering RNA [siRNA], and piwi-interacting RNA [piRNA]) and long non-coding RNAs (lncRNA)—regulate gene expression through epigenetic mechanisms, influencing several cellular processes, like X chromosome inactivation(125), genomic imprinting(126), and cancer(127). For example, it has been recently shown that traumatic stress in early life altered mouse miRNA expression, and caused behavioural and metabolic responses in the progeny(128). Moreover, injection of sperm RNAs from traumatized males into fertilized wild-type oocytes reproduced the behavioral and metabolic alterations in the resulting offspring(128).

1.4.2 DNA methylation in physiology and pathology

DNA is constituted by combinations of four nucleotides, namely cytosine, guanine, thymine and adenine. DNA methylation in mammals is a post-replication modification that is predominantly found in cytosines of the dinucleotide sequence CpG(112).

DNA methylation is vital to healthy growth and development and is linked to various processes such as genomic imprinting, carcinogenesis and the suppression of repetitive elements. DNA methylation also enables the expression of retroviral genes to be suppressed, along with other potentially dangerous sequences of DNA that have entered and may damage the host. During embryogenesis, DNA methylation is highly regulated and establishes the pattern of gene expression of the cells as they differentiate. The change in gene expression is stable and the cell does not revert to a stem cell or another type of cell. The process of DNA methylation is stopped when a zygote is being formed but is restored as cell division occurs during development. Additionally, DNA methylation participates in the formation of the chromatin structure, which enables a single cell to grow into a complex multicellular organism made up of different tissues and organs.

Studies have shown that genes with a promoter region that contains a high concentration of 5-methylcytosine are transcriptionally silent. Aberrant methylation of DNA has been associated with an increased rate of malignancy. Methylation in particular gene regions, for example in promoters, can inhibit gene expression. This is done in part by the interaction of methylcytosine binding proteins with other structural components of chromatin, which , in turn, makes the DNA inaccessible to transcription factors through histone deacetylation and chromatin structure changes(129). In cancer research, it has been shown that there is a correlation between hypomethylation and increased

oncogenes expression. Case-control studies done in breast and ovary cancer demonstrated that an imprint can be found in the DNA of cells which indicates the predisposition to develop a cancer or to predict a progression of the disease(130, 131) .

In addition, DNA methylation also serves to integrate environmental signals for the cells to modulate the functional output of their genome. Complex human diseases such as cancer and T2D are believed to have a strong environmental component in addition to genetic causes. Thus, the study of changes in DNA methylation patterns may be useful in order to study the interactions between environment and genome in those diseases.

Gene-environment interactions are thought to be mediated by epigenetic modifications of the genome, and epigenetic changes of the genome often arise in response to changes in the environment(112). Unlike genetic changes, epigenetic changes are more dynamic and are often reversible, depending on the existence or removal of the inducing factors(132). Indeed, DNA methylation patterns fluctuate in response to changes in diet, inherited genetic polymorphisms and exposures to environmental chemicals. Methyl groups are acquired through the diet and are donated to DNA through the folate and methionine pathways. Changes in DNA methylation may occur as a result of low dietary levels of folate, methionine, or selenium, which can have profound clinical consequences (neural tube defects, cancer, atherosclerosis)(133). For example, hyperhomocysteinemia and global hypomethylation have been observed in vitro in atherosclerosis models as well as in vivo in humans(134).

As mentioned, DNA methylation involves the addition of a methyl group to cytosines within CpG (cytosine/guanine) pairs. Typically, unmethylated clusters of CpG pairs are located in tissue specific genes and in essential “housekeeping” genes, which are involved in routine maintenance roles and are expressed in most tissues(133). Clusters,

or CpG “islands”, are targets for proteins that bind to unmethylated CpGs and initiate gene transcription. In contrast, methylated CpGs are generally associated with silent DNA, can block methylation sensitive proteins and can be easily mutated.

DNA methylation is catalysed by a family of DNA methyltransferases (DNMTs) in eukaryotes. The cytosines in CpG dinucleotides appear to be the favorite substrate for these DNMTs (DNMT1, DNMT3A and DNMT3B, DNMT3L). DNMT1 is responsible for maintaining DNA methylation patterns during replication. DNMT3A and DNMT3B invoke de-novo methylation, particularly during embryogenesis. DNMTs are overexpressed in many tumor types and may be at least partly responsible for hypermethylation observed in tumour suppressor genes. However, it is becoming increasingly recognized that upregulation of DNMTs is only observed in subsets of patients. For example, in a study of 765 colorectal carcinomas, DNMT3B protein was increased in only 15% of cases(135) and, therefore, other mechanisms modulating DNMT activity must exist, such as by splice variants, factors that target DNMT mRNA or miRNAs(104). Although a major portion of the genome is unmethylated, CpG islands associated with gene promoters are subject to dynamic methylation modifications during development(136).

A previous study has suggested that there might be an epigenetic signature specific for each person and defined a striking strategy for identifying patients at risk of common disease(137). Using DNA extracted from nonimmortalized lymphocyte samples, 227 regions across the genome were identified as presenting extreme interindividual variability. These regions were enriched for development genes. Moreover, half of these variably methylated regions were stable within individuals over an average of 11 years. Four of these regions showed covariation with body mass index and were located near genes previously implicated in body weight regulation or diabetes. This study was done

in 74 individuals by performing a genome-scale analysis of near 4 million CpG sites using an array-based methylation analysis(137). Consequently, identifying variably methylated DNA regions from an accessible tissue as whole blood within individuals may give important information about the epigenetic mechanisms involved in the presence and progression of disease.

Moreover, another recent and promising use of DNA methylation profiles from accessible tissues is as an estimate of age(138). Indeed, a researcher developed an algorithm based on the methylation status of a set of diverse genomic positions that provides a remarkably accurate age estimate of the person(139). For example, white blood cells, which may be just a few days or weeks old, will carry the signature of the 50-year-old donor they came from, plus or minus a few years(138). As the investigator proposes, DNA methylation age measures the cumulative effect of an epigenetic maintenance system. This novel epigenetic clock has many potential diverse applications, from criminal investigation to developmental biology, cancer and aging research(139).

DNA methylation and disease

Disruption of DNA methylation patterns has been observed in a growing number of disease processes, cancer being the most rigorously investigated. The dogma was that gene-specific hypermethylation leads to transcriptional repression, which is generally the case for hypermethylation occurring in promoters. Recently, however, it is recognized that hypermethylation occurring in the body of genes can lead to transcriptional activation(104).

Cancer cells are characterized by global hypomethylation accompanied by de-novo hypermethylation in CpG islands (CGI) associated with genes, which can increase during progression from preneoplastic lesions to metastatic tumours, often leading to

silencing of tumour suppressor genes or miRNA genes. The list of tumour suppressor genes silenced by DNA methylation in neoplasia is ever-expanding, but an important unanswered question is why particular subsets of CGI become hypermethylated in cancer. One simple explanation could be that there are particular sequences in the genome that are more 'susceptible' to becoming methylated. A second explanation could be through ***Long-range epigenetic silencing*** (LRES) mechanisms. It is becoming increasingly recognized that epigenetic mechanisms can act over large megabase regions containing multiple genes that are coordinately suppressed(104). It is speculated to be a common phenomenon in malignancies. LRES is much more abundant in cancer than in normal cells and leads to a major reduction in the accessible genome potentially available for normal transcriptional regulation. For example, a recent report identified an 800 kb region spanning more than 50 transcripts, encompassing three clusters of protocadherin genes, on chromosome 5q31.3 that is hypermethylated in Wilms' tumours and was associated with transcriptional silencing(140).

DNA methylation plays a role in many other diseases such as autoimmunity, developmental and neurological disorders, and diseases related to imprinting or X-chromosome inactivation. DNA methylation also controls gene dosage reductions during X-chromosome inactivation in women and when disrupted can lead to developmental disorders such as fragile X syndrome(141).

DNA methylation and environment effects

Almost complete CpG demethylation occurs during embryogenesis, and must then be re-established during early development, which necessitates the availability of nutritionally derived methyl donors like methionine and co-factors like folic acid. Diseases such as coronary artery disease, schizophrenia, and other congenital abnormalities have been associated with inadequate establishment of DNA methylation

due to nutritional deficiency prenatally. In this sense, a recent work showed for the first time how the diet in pregnant women can lead to differences in methylation patterns in the offspring(142). In this study, they show that seasonal variations in methyl-donor nutrient intake of mothers around the time of conception influence 13 relevant plasma biomarkers. The level of several of these maternal biomarkers predicts increased/decreased methylation at metastable epialleles in DNA extracted from lymphocytes and hair follicles in infants postnatally(142). Metastable epialleles are genomic regions at which DNA methylation is established randomly in the early embryo and then stably maintained in differentiated tissues, which contributes to interindividual epigenetic variation in multiple cell types(143). Numerous other environmental factors, including stress or exposure to chemicals such as fungicides and pesticides can alter epigenetic components of the genome.

One of the largest groups of environmental factors that humans are exposed to daily is endocrine disrupters that alter hormone production and/or signalling, promoting conditions such as reproductive failure, infertility, or cancer. The distribution of DNA methylation in the developing embryo is tightly controlled, and disruption of normal methylation patterns by exposure to environmental factors such as endocrine disruptors during that time can result in developmental or transgenerational abnormalities, or adult-onset diseases.

Finally, it has been proposed that epigenetic mechanisms may play a critical role in the pathophysiology of T2D, as we are going to comment in the next section(144, 145) (see Figure 6).

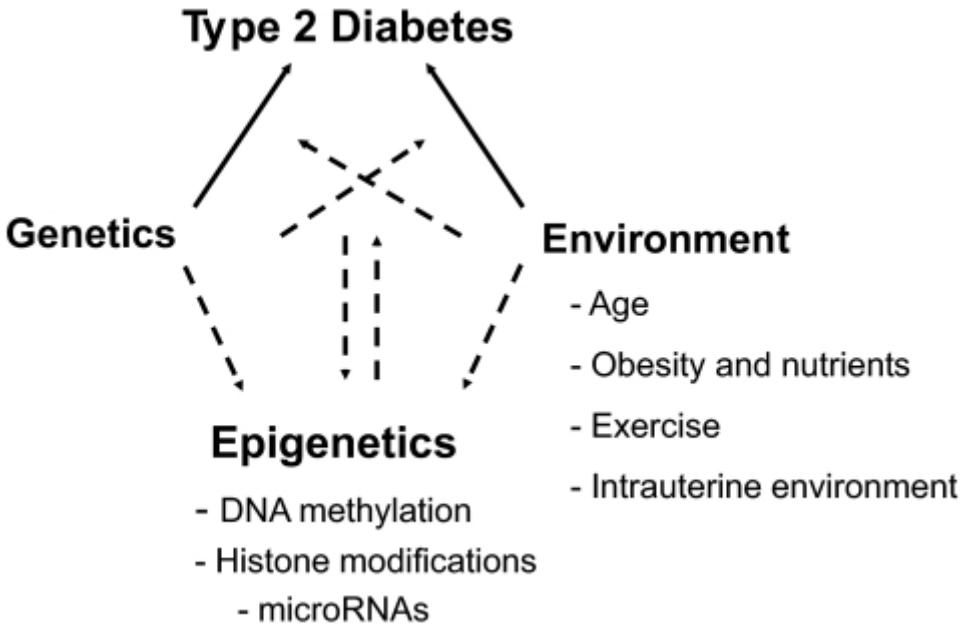

Figure 6: Schema proposing a role for epigenetic mechanisms in the pathophysiology of Type 2 Diabetes. Adapted from Figure 1 in *Ling C, Groop L, Diabetes. 2009 Dec;58(12):2718-25.*

1.4.3 DNA methylation in Type 2 Diabetes

T2D results from a complex interplay between environmental factors and genetics. The current approach is to study the interaction between the environment and the genetic susceptibility to improve the understanding of T2D physiopathology. Epigenetics mechanisms play a crucial role in relaying the effects of the environment to the genome, and thus heavily influence the risk of suffering a complex disease, such as T2D(146, 147). In the last few years, different studies have been done both in animal models and in humans showing the role and association of epigenetic alterations (such as DNA methylation patterns) and features of T2D(148-153). Human studies have been done studying the methylation profile of different genes in skeletal muscle (PGC-1α promoter)(150), pancreatic islets (CpG loci affiliated to promoters of 254 genes)(154) and peripheral blood (genome-wide survey)(155). These studies show the existence of differentially methylated regions in the genome that correlate with T2D in different

tissues. In terms of diabetes pathophysiology, alterations in DNA methylation patterns may also have important metabolic effects. A DNA methylation profile of human islets revealed significant changes in methylation patterns in islets from T2D patients as compared with islets from non diabetic donors(154). Specifically, this analysis revealed 276 CpG sites corresponding to 254 unique genes with altered CpG island methylation in diabetic islets compared with non-diabetic islets. Interestingly, only 10 sites were hypermethylated in T2D samples, compared with 266 sites that showed decreased methylation. Groups of proteins displaying altered methylation included genes related to three broad categories: signaling pathways essential for β-cell adaptation, pathways relating to survival or apoptosis of the β-cell, and pathways of unknown significance in the β-cell(154). Furthermore, these same patterns were not inducible in non-diabetic islets treated with high glucose, suggesting they may cause β-cell dysfunction as opposed to being a response to hyperglycemia. However, the diabetic milieu is also characterized by high levels of proinflammatory cytokines and free fatty acids(156). Whether changes in methylation patterns might result from these varied factors remains unclear.

There is also evidence that aberrant methylation can affect genes involved in β-cell survival and apoptosis in T2D islets, such as *CDK5R1*, *CASP10*, *BCL2*, *PP2R4*, and *GSTP1*(154, 157). Similarly, preclinical rodent models suggest that β-cell dedifferentiation plays a role in the pathophysiology of T2D(158). One possibility is that alterations in epigenetic pathways might lead to a loss of expression of key genes required for β-cell identity. In agreement with this hypothesis, it has been found that β-cell-specific transcription factor paired box 4 (Pax4) was hypermethylated and silenced in T2D human islets(154). Taken together, these data suggest that aberrant methylation

plays a role in the pathophysiology of T2D by altering key genes involved in β-cell survival, apoptosis and differentiation.

Also, other studies support the emerging idea that DNA methylation could be involved in the metabolic memory inherent to diabetes(152). ***"Hyperglycemic memory"*** , also named ***"metabolic memory"*** is a concept that refers to the progression of detrimental effects in different organs of diabetic patients after a finite period of hyperglycemia in spite of later presence of glycemic control (159-161). In diabetic patients, metabolic memory is responsible for the development of cardiovascular outcomes in spite of a good metabolic control. The mechanisms involved are not completely understood. However, there is emerging evidence that epigenetic processes play a role(162). Indeed, persisting epigenetic marks keep memory of previous transient hyperglycemic events. Recent studies are focusing in deciphering the mechanisms by which aberrant epigenetic pathways contribute to cardiovascular complications of diabetes, as nephropathy and retinopathy (163, 164).

Other studies have shown that epigenetic marks may serve to identify individuals at risk of suffering from T2D(153). In addition, recent data in rodent models indicate that in utero undernutrition results in altered promoter methylation patterns in the offspring influencing the later development of glucose intolerance(165).

Finally, since the possibility to work on target-tissues from human subjects is difficult and tedious for ethics reasons, several works have been done in order to identify specific methylation marks in patients as compared to controls taking DNA from an easily accessible tissue such as peripheral blood (148, 166-168).

In conclusion, there is evidence to suggest that DNA methylation patterns might have an effect in the development and future complications of T2D.

2 BACKGROUND

The incidence of Type 2 Diabetes Mellitus (T2D) is increasing worldwide. There are several explanations to this fact, such as the increased prevalence of obesity, population's ageing, and the lack of physical activity that is practised. However, not all obese and sedentary individuals become type 2 diabetic. Beyond a certain genetic susceptibility and a determined environment, some people will become diabetic, whereas others will not.

On the other hand, recent discoveries in the field of epigenetics have brought an insight in the molecular mechanisms underlying the interaction between environment and genome. Indeed, it is believed that specific changes in the epigenome are associated with the onset and/ or the progression of disease processes such as cancer or diabetes. DNA methylation is a reversible process that has important functions in cellular development and can be influenced by environmental factors. In view of the potential functional outcomes of methylation changes and the interaction between genetics and epigenetics, it becomes primordial to study whether known genomic regions that have a role increasing diabetes risk and /or in the pathophysiology of T2D present specific methylation patterns in T2D patients.

3 HYPOTHESIS

On the basis of the knowledge summarized above, we hypothesized that type 2 diabetic patients differ from age and BMI-matched non diabetic subjects in specific methylation patterns that might involve changes in gene expression in target tissues relevant to the physiopathology of Type 2 Diabetes.

In view of the interaction between environment and genetics, and, that methylation patterns can change in response to environmental factors, we hypothesized that the diabetic-related methylation patterns would be present in key genes implicated in the physiopathology of Type 2 Diabetes.

Amongst the genes potentially affected by methylation changes, we hypothesized that both *TCF7L2* and *GIPR* genes may present specific methylation patterns in type 2 diabetic patients as compared to age and BMI-matched non-diabetic subjects.

As methylation pattern might influence gene expression, we pinpoint the promoter regions of the selected genes.

Finally, as epigenetic marks can be detected from easily accessible tissues, we studied the methylation marks in DNA from whole blood in the two groups of subjects.

To complete, in order to avoid any confounding effect of the antidiabetic drugs on the methylation data, we selected a group of recently diagnosed, drug-naïve type 2 diabetic patients.

4 AIMS

The particular aims of this thesis were the following:

1st Aim. Design of the population at study (cases and controls).

A. Selection and inclusion of recently diagnosed type 2 diabetic patients treated only with diet (cases) from a primary health center.

B. Selection and inclusion of age- and BMI-matched non diabetic subjects (controls) from a primary health center.

C. Comparative study of the metabolic and cytokine profile of the two groups at study.

2nd Aim. Study of the DNA methylation pattern in *TCF7L2* promoter gene in type 2 diabetic patients (cases) and age and BMI-matched non-diabetic subjects (controls), using as source of DNA an easily accessible tissue (peripheral blood).

A. Comparative study of the methylation values in *TCF7L2* promoter gene between type 2 diabetic patients (cases) and age- and BMI-matched non-diabetic subjects (controls) in whole blood DNA.

B. Correlational study between the methylation values of *TCF7L2* promoter gene and clinical and biochemical parameters in type 2 diabetic patients (cases) and age- and BMI-matched non-diabetic subjects (controls).

3rd Aim. Study of the DNA methylation pattern in *GIPR* promoter gene in type 2 diabetic patients (cases) and age and BMI-matched non-diabetic subjects (controls), using as source of DNA an easily accessible tissue (peripheral blood).

A. Comparative study of the methylation values in *GIPR* promoter gene between type 2 diabetic patients (cases) and age- and BMI-matched non-diabetic subjects (controls) in whole blood DNA.

B. Correlational study between the methylation values of *GIPR* promoter gene and clinical and biochemical parameters in type 2 diabetic patients (cases) and age- and BMI-matched non-diabetic subjects (controls).

1ST AIM

1st Aim. Design of the population at study.

A. Selection and inclusion of recently diagnosed type 2 diabetic patients treated only with diet (cases) from a primary health care center.

B. Selection and inclusion of age- and BMI-matched non diabetic subjects (controls) from a primary health care center.

C. Comparative study of the metabolic and cytokine profile of the two groups at study.

A. Selection and inclusion of recently diagnosed type 2 diabetic patients treated only with diet (cases) from a primary health care center.

Initially, we presented the project to the Ethics Committee of the Hospital Clinic, Barcelona in order to have the ethics permission to proceed with the project.

We aimed to have a large enough group of T2D patients that were recently diagnosed and treated only with diet in order to study the methylation pattern of the selected genes in DNA from peripheral blood.

First, to ensure enough power, we made a power calculation of the sample size needed.

Sample size calculation

Since no previous studies were done on the subject, we assumed a difference of ten percent in the prevalence of DNA methylation differences in T2D patients compared to controls. Aiming a power of 80% and a significance level of 95%, 95 subjects were required in each group (EpiInfo software).

Thus, we first had to select 95 patients with T2D, that were recently diagnosed and only treated with diet. We reached the primary health centers that are assigned with the Hospital Clinic , Barcelona, in particular, Les Corts Primary Health care center. After discussing with the responsible of the center and presenting the research project to all the staff of the center, Les Corts Primary Health care center staff accepted to participate and we proceed to search for the patients for inclusion.

Recruitment and inclusion of the type 2 diabetic patients (cases)

A list enclosing the names and telephone numbers of the patients recently diagnosed with T2D in the previous year in Les Corts Primary Health care center was given to the researchers. After carefully examining the medical records of each patient from the list, potentially eligible patients were reached through telephone calls.

The inclusion and exclusion criteria of the T2D patients were the following:

Inclusion criteria:

1. Clinical diagnosis of T2D* between December 2010 until December 2011.
2. Adequate glycemic control after a period of minimum six months of low-carbohydrate diet and lifestyle interventions.
3. No pharmacological therapy for T2D needed to achieve the glycemic control.

* Diagnosis of T2D was done following ADA recommendations(1).

Exclusion criteria:

1. If antidiabetic medication was needed for optimal glycemic control.

Once the patients had accepted to participate in the study, a visit was scheduled with the doctor and research nurse for the following proceedings:

1. Signature of the written informed consent (attached below).
2. Blood extraction for posterior analysis (in fasting state).
3. Medical history and physical examination.

HOJA INFORMACIÓN PARA LOS PACIENTES

Proyecto de investigación Análisis epigenético de las diferencias en la metilación del DNA entre diabéticos tipo 2, obesos y controles.

Investigador principal Dr. Ramon Gomis
Servicio Endocrinología, Hospital Clínic Barcelona
Promotor Ayuda pública Ministerio de Ciencia e Innovación.

Objetivos:
Le solicitamos su participación en este proyecto de investigación cuyo objetivo principal es profundizar en el conocimiento de factores epigenéticos que puedan predisponer en el desarrollo de la diabetes tipo 2 (la diabetes de las personas mayores) que repercute en una disminución de la calidad de vida de las personas afectadas.

Beneficios:
Es posible que de su participación en este estudio no obtenga un beneficio directo. Sin embargo, la identificación de posibles factores relacionados con la diabetes tipo 2 podría beneficiar en un futuro a otros pacientes que la sufren y contribuir a un mejor conocimiento y tratamiento de esta enfermedad.

Procedimientos del estudio:
Si decide participar, se le realizará una historia clínica detallada y una exploración física, y se le extraerán cinco tubos de sangre (10mL en total) para poder realizar los análisis requeridos para éste estudio (análisis de la metilación en el ADN y determinación de parámetros inflamatorios en serum y plasma).

Molestias y posibles riesgos:
La toma de muestras de sangre se realizará por un/a enfermero/a y el procedimiento es idéntico a la extracción realizada durante su atención médica habitual (para saber el control de la diabetes, por ejemplo).
La toma de muestras de sangre le puede provocar una sensación de ardor en el punto en el que se introduce la aguja en la piel para hacer la extracción y ocasionar un pequeño hematoma o una leve infección que desaparece en pocos días. En muy raras ocasiones, podría marearse en el momento de la extracción.

Lugar de realización del análisis:
Su muestra de sangre se analizará en el laboratorio de Diabetes y Obesidad, IDIBAPS, Hospital Clínic, Barcelona.

Derecho de revocación del consentimiento:
Su participación en el estudio es totalmente voluntaria, y si decide no participar recibirá todos los cuidados médicos que necesite y la relación con el equipo médico que le atiende no se verá afectada.

Si cambia de opinión después de dar sangre para el estudio, puede pedir que se destruya su muestra.

Implicaciones de la información obtenida en el estudio:
Si decide participar en el estudio, es posible que en el análisis de su sangre se obtenga información relevante para su salud o la de su familia. De acuerdo con la legislación vigente, tiene derecho a ser informado de los datos que se obtengan en el curso del estudio.
Si quiere conocer los datos relevantes para su salud que se obtengan, infórmese a través de su médico sobre las implicaciones que esta información puede tener para su persona y su familia. Esta información se le comunicará si lo desea; en el caso de que prefiera no ser informado, su decisión se respetará.

De acuerdo con la Ley 15/1999 de Protección de Datos de Carácter Personal los datos personales que se obtengan serán los necesarios para cubrir los fines del estudio. En ninguno de los informes del estudio aparecerá su nombre, y su identidad no será revelada a persona alguna salvo para cumplir con los fines del estudio, y en el caso de urgencia médica o requerimiento legal.

Cualquier información de carácter personal que pueda ser identificable será conservada por métodos informáticos en condiciones de seguridad por el Biobanco, IDIBAPS. El acceso a dicha información quedará restringido al personal del IDIBAPS, Barcelona, designado al efecto o a otro personal autorizado que estará obligado a mantener la confidencialidad de la información.

De acuerdo con la ley vigente, tiene usted derecho al acceso de sus datos personales; asimismo, y si está justificado tiene derecho a su rectificación y cancelación. Si así lo desea, deberá solicitarlo al médico que le atiende en este estudio.

Además de los análisis realizados en contexto del presente estudio, se almacenará su muestra sanguínea en el Biobanco del Hospital Clínic de Barcelona-IDIBAPS (**www.clinicbiobanc.org**), la cual podría ser utilizada para futuros estudios de investigación biomédica (ver consentimiento informado específico del Biobanco).

Preguntas
Si tiene alguna duda o pregunta en relación al estudio, no dude comunicar lo a su médico o equipo. Si tiene dudas respecto a sus derechos como participante en el estudio, pregunte sus dudas a los principales investigadores responsables del estudio: Dr. Ramon Gomis y Dra. Silvia Canivell (números de teléfono 932279884, 932275400 ext. 4386).

CONSENTIMIENTO INFORMADO

Análisis epigenético de las diferencias en la metilación del DNA entre diabéticos tipo 2, obesos y controles.

Yo,……..(nombre y apellidos)

- He leído la información en el documento adjunto que se me ha entregado
- Acepto participar en el estudio
- He sido informado de todos los detalles y se me ha respondido a todas mis dudas al respecto.
- He sido informado por el Dr/Dra .....
- Entiendo que mi participación es voluntaria
- Entiendo que puedo abandonar el estudio:
  - Cuando quiera
  - Sin tener que dar explicaciones
  - Sin que esto afecte mi atención médica como paciente
- Entiendo que mis muestras personales y todos mis datos serán tratados anónimamente
- Entiendo que cualquier material residual del estudio será destruido

Doy libremente mi consentimiento de participar en éste estudio

_____ _____
Firma del participante Fecha

_____ _____
Firma del médico Fecha

B. Selection and inclusion of age- and BMI-matched non diabetic subjects (controls) from a primary health care center.

Once we had the 95 T2D patients selected and recruited, mean age and mean BMI of the patients was calculated. Then, we selected a group of 95 non diabetic subjects with similar mean and similar age ("*frequency matched*") than the T2D patients. The *frequency matching* (which is different from individual matching) refers to a population of controls such that the overall characteristics of the group match the overall characteristics of the cases. e.g. if 15% of cases are under age 20, 15% of the controls are also. It does not require using a matched analysis, because a random sample of controls in that cell (aged under 20 years) is taken. However, before selecting the controls, you have to wait until cases accumulate (unless the distribution of matching factors is known in advance). On the contrary, individual matching refers to the search for one (or more) controls who have the required matching criteria for each case(169). In our study, we performed a frequency matching on age and BMI to the cases.

The inclusion and exclusion criteria of the controls were the following:

Inclusion criteria:

1. A negative oral glucose tolerance test (OGTT) at recruitment.
2. No previous diagnosis of T2D or prediabetes.
3. No chronic treatment with oral steroids.

Exclusion criteria:

1. If any of the inclusion criteria is not present.

Same procedures done for the inclusion of the cases was performed in order to select and recruit the controls. At the end of the inclusion, we verified that mean age and mean BMI of both groups were not statistically different ($P>0.05$) by applying a t-Student test.

Data management, samples storage and DNA extraction from whole blood.

Same procedures were made for the two groups of subjects. Once the blood samples were extracted from the patients, they were brought to the Biobank-IDIBAPS, Hospital Clinic of Barcelona (http://www.clinicbiobanc.org/es_index.html) where serum and whole blood DNA were extracted from each sample using standards procedures of the Biobank for later analyses. Samples were stored in the Biobank as well.

All data collected from each subject was transferred into an excel file and then into a datasheet compatible for further statistical analyses using STATA software.

C. Comparative study of the metabolic and cytokine profile of the two groups at study.

We first compared and analyzed the characteristics of the patients included in terms of metabolic, hormonal and cytokine profiles in order to see what differed between them. Table 1 shows the results.

Table 1: Characteristics of the population at study.

Variable*	Type 2 diabetic patients (n=93)	Controls (n=93)	P Value†
Demographic characteristics			
Age, yr	69.1±9.2	66.6±11.7	0.099
BMI, kg/m^2	29.2±3.7	28.8±2.5	0.454
Waist circumference, cm	102.7±9.5	97.9±8.0	**0.002**
Male sex, (%)	66.7	53.8	0.072
Duration of diabetes, yr	5.4±4.1		
Physical inactivity, %	28.0%	53.8%	**<0.001**
Never smoked, %	50.5%	61.3%	0.261
Laboratory values			
Fasting glucose, (mmol/L)	6.4±1.2	4.6±0.3	**<0.001**
Glycated hemoglobin, (%)	5.8±0.6		
Fasting insulin , (pmol/L)	55.6±28.6	52.4±21.0	0.750
HOMA-IR §	2.6±1.5	1.8±0.7	**<0.001**
HOMA-B §§	75.7±51.1	113.6±510.6	**<0.001**
Alanine aminotransferase (ALT), (IU/liter)	13.5±7.9	14.6±7.3	0.486
Aspartate aminotransferase (AST), (IU/liter)	16.6±8.2	19.0±6.0	0.135
Total cholesterol (mmol/L)	4.8±1.0	5.2±1.1	**0.002**
LDL cholesterol (mmol/L)	2.8±0.8	2.9±0.8	0.782
HDL cholesterol (mmol/L)	1.3±0.3	1.4±0.3	0.262
Triglycerides (mmol/L)	1.4±0.9	1.3±0.8	0.338
Fasting leptin, (ng/mL)	18.0±16.7	25.4±26.8	0.066
Fasting adiponectin, (µg/mL)	7.0±3.8	10.0±4.2	**<0.001**

* Values shown are means ±SD, unless otherwise indicated.

† P values were calculated with the t test for quantitative variables or Chi-square test for categorical ones, except for HOMA-IR, HOMA-B, fasting insulin, fasting leptin and fasting adiponectin where non-parametric Mann-Whitney U test was applied.
§ HOMA-IR was calculated as [Insulin mlU/l x FSG: (mmol/l)/22.5].
§§ HOMA-B was calculated as (20 × FSI)/(FSG − 3.5), where FSI is the fasting serum insulin concentration (mU/l) and FSG is fasting serum glucose (mmol/l).

Overall, all patients were overweight and mean age of the whole group was 68 years. There were no significant differences in gender, with a majority of men. T2D patients had a higher waist circumference as compared to controls. Total cholesterol was lower in T2D patients as compared to controls. HOMA-IR was higher in T2D patients than in controls. HOMA-B was lower in T2D patients as compared to controls. T2D patients were less physically inactive as compared to controls.

With reference to hormones, we found that fasting adiponectin was lower in T2D patients as compared to controls. From the cytokines analyzed, significant differences were found for IL 10 (lower in T2D patients as compared to controls) and IL 12 (higher in T2D patients than in controls).

2nd AIM

2nd Aim. Study of the DNA methylation pattern in *TCF7L2* promoter gene in type 2 diabetic patients and non-diabetic subjects, using as source of DNA an easily accessible tissue (peripheral blood).

A. Comparative study of the methylation values in *TCF7L2* promoter gene between type 2 diabetic patients and age- and BMI-matched non-diabetic subjects in whole blood DNA.

B. Correlational study between the methylation values of *TCF7L2* promoter gene and clinical and biochemical parameters in type 2 diabetic patients and age- and BMI-matched non-diabetic subjects.

Article. **Silvia Canivell**, Elena G.Ruano, Antoni Sisó-Almirall, Belchin Kostov, Luis González-de Paz, Eduardo Fernandez-Rebollo, Felicia A. Hanzu, Marcelina Párrizas, Anna Novials, Ramon Gomis.

Differential methylation of TCF7L2 promoter in peripheral blood DNA in newly diagnosed, drug-naïve patients with Type 2 diabetes. *PLOS ONE*. Accepted: 13th May 2014. Impact factor 4.092. Q1.

2nd Aim. Study of the DNA methylation pattern in *TCF7L2* promoter gene in type 2 diabetic patients and non-diabetic subjects, using as source of DNA an easily accessible tissue (peripheral blood).

TCF7L2 is the susceptibility gene for Type 2 diabetes (T2D) with the largest effect on disease risk that has been discovered to date. However, the mechanisms by which *TCF7L2* contributes to the disease remain largely elusive. In addition, epigenetic mechanisms, such as changes in DNA methylation patterns, might have a role in the pathophysiology of T2D. This study aimed to investigate the differences in terms of DNA methylation profile of *TCF7L2* promoter gene between type 2 diabetic patients and age- and Body Mass Index (BMI)- matched controls. We included 93 type 2 diabetic patients that were recently diagnosed for T2D and exclusively on diet (without any pharmacological treatment). DNA was extracted from whole blood and DNA methylation was assessed using the Sequenom EpiTYPER system. Type 2 diabetic patients were more insulin resistant than their matched controls (mean HOMA IR 2.6 vs 1.8 in controls, $P<0.001$) and had a poorer beta-cell function (mean HOMA B 75.7 vs. 113.6 in controls , $P<0.001$). Results showed that 59% of the CpGs analyzed in *TCF7L2* promoter had significant differences between type 2 diabetic patients and matched controls. In addition, fasting glucose, HOMA-B, HOMA-IR, total cholesterol and LDL cholesterol correlated with methylation in specific CpG sites of *TCF7L2* promoter. After adjustment by age, BMI, gender, physical inactivity, waist circumference, smoking status and diabetes status uniquely fasting glucose, total cholesterol and LDL cholesterol remained significant. Taken together, newly diagnosed, drug-naïve type 2 diabetic patients display specific epigenetic changes at the *TCF7L2* promoter as compared to age- and BMI-matched controls. Methylation in *TCF7L2* promoter is further correlated with fasting glucose in peripheral blood DNA, which sheds new light on the role of epigenetic regulation of *TCF7L2* in T2D.

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PLOS ONE

Differential Methylation of *TCF7L2* Promoter in Peripheral Blood DNA in Newly Diagnosed, Drug-Naïve Patients with Type 2 Diabetes

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Abstract

TCF7L2 is the susceptibility gene for Type 2 diabetes (T2D) with the largest effect on disease risk that has been discovered to date. However, the mechanisms by which *TCF7L2* contributes to the disease remain largely elusive. In addition, epigenetic mechanisms, such as changes in DNA methylation patterns, might have a role in the pathophysiology of T2D. This study aimed to investigate the differences in terms of DNA methylation profile of *TCF7L2* promoter gene between type 2 diabetic patients and age- and Body Mass Index (BMI)- matched controls. We included 93 type 2 diabetic patients that were recently diagnosed for T2D and exclusively on diet (without any pharmacological treatment). DNA was extracted from whole blood and DNA methylation was assessed using the Sequenom EpiTYPER system. Type 2 diabetic patients were more insulin resistant than their matched controls (mean HOMA IR 2.6 vs 1.8 in controls, $P<0.001$) and had a poorer beta-cell function (mean HOMA B 75.7 vs. 113.6 in controls, $P<0.001$). Results showed that 59% of the CpGs analyzed in *TCF7L2* promoter had significant differences between type 2 diabetic patients and matched controls. In addition, fasting glucose, HOMA-B, HOMA-IR, total cholesterol and LDL-cholesterol correlated with methylation in specific CpG sites of *TCF7L2* promoter. After adjustment by age, BMI, gender, physical inactivity, waist circumference, smoking status and diabetes status uniquely fasting glucose, total cholesterol and LDL-cholesterol remained significant. Taken together, newly diagnosed, drug-naïve type 2 diabetic patients display specific epigenetic changes at the *TCF7L2* promoter as compared to age- and BMI-matched controls. Methylation in *TCF7L2* promoter is further correlated with fasting glucose in peripheral blood DNA, which sheds new light on the role of epigenetic regulation of *TCF7L2* in T2D.

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Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Type 2 diabetes (T2D) results from an interaction of genetic risk and environmental factors[1]. The heritability estimates for T2D range from 20% to 80%. The evidence for heritability has been proven with different studies, such as population, family and twin-based studies[2,3]. Through genome-wide association studies, over 60 loci have been associated with T2D risk[1]. However, the genetic loci discovered to date explain only a small part of the T2D heritability[1]. Reasons for the observed "missing heritability" in T2D include gene-environment interactions, the role of gene variants and epigenetics[1]. Epigenetics refer to heritable changes in gene function that occur without a change in nucleotide sequence. Epigenetic mechanisms could provide a molecular explanation for some unresolved issues in T2D[4], such as discordance within monozygotic twins[5], interindividual variation in age of onset, disease severity and effect of lifestyle factors on T2D risk. Indeed, recent studies propose that specific changes in the epigenome are associated with the onset and progression of diabetes[6,7,8,9]. DNA methylation is the best studied epigenetic modification and influences transcriptional regulation[10]. DNA methylation is a reversible process that can be modulated by both stochastic and environmental stimuli[11]. On the other hand, *TCF7L2* remains the most significant and consistently replicated gene linked to T2D[1,12]. *TCF7L2* has the strongest effect for T2D (average OR 1.37)[13]) and encodes a transcription factor implicated in wnt signaling and proglucacon transcription [14]. It has been shown that *TCF7L2* expression in human islets was increased 5-fold in T2D and overexpression of *TCF7L2* in human islets reduced glucose-stimulated insulin secretion[15]. However, the precise role of *TCF7L2* with regard to T2D risk is still under investigation. As DNA methylation influences gene expression, we speculated that *TCF7L2* gene could be affected by alterations in DNA methylation in type 2 diabetic patients. Considering that

DNA methylation occurs principally in the upstream regulatory regions of the genes[16], we concentrated on the promoter of *TCF7L2* gene. Previous studies have shown that disease-related methylation may be reflected in accessible tissues such as peripheral blood[17].

The aim of this study was to compare the epigenetic profile (defined here as the pattern of DNA methylation on *TCF7L2* promoter in DNA from peripheral blood) between type 2 diabetic patients and age- and BMI-matched controls.

Materials and Methods

Ethics Statement

This study was approved by the Clinical research ethical committee of the Hospital Clínic, Barcelona, Spain (25^{th} November 2010, register number 2010/6162) and complies with all laws and international ethics guidelines outlined in the Declaration of Helsinki. All human subjects provided written, informed consent. All samples and clinical data collected were anonymised at source.

Study design and subjects included

We conducted a case-control study where cases were defined as patients suffering from T2D that were treated only by diet. Cases and controls were recruited from the same primary health center. Eligibility criteria for inclusion of cases and controls were applied as previously cited[18]. Briefly, eligibility criteria for cases were the following: clinical diagnosis of T2D, adequate glycemic control after a period of minimum six months of low-carbohydrate diet and lifestyle interventions, no pharmacological therapy for T2D needed to achieve the glycemic control. In case oral medication was needed for optimal glycemic control, those patients were excluded from the study. Diagnosis of T2D was done following ADA recommendations[19]. Eligibility criteria for controls were as follows: a negative oral glucose tolerance test (OGTT) at recruitment, no previous diagnosis of T2D or prediabetes, no chronic treatment with oral steroids. All controls had an OGTT conducted to confirm they did not have any glucose intolerance. Controls were frequency matched (i.e. match on cell instead of individual[20]) on age and BMI to cases. Physical inactivity was assessed by asking the subjects if they practised at least 30 min of exercise by day. The subjects who answered "no" were classified as "physically inactive". Subjects addicted to alcoholism or with a history of alcoholism were excluded from the study. Metabolic profile and DNA methylation of *TCF7L2* promoter in peripheral blood DNA profile was studied for all subjects (93 cases and 93 controls).

Metabolic assessments

All subjects were examined by anthropometric measurements and had fasting metabolic assessments at recruitment. These assessments included fasting serum glucose, fasting serum insulin, glycohemoglobin A1 (HbA1), total cholesterol, triglycerides, high density level (HDL) cholesterol, low density level (LDL) cholesterol, hepatic profile, homeostatic model assessment to quantifiy insulin resistance (HOMA-IR) and homeostatic model assessment to quantifiy beta-cell function (HOMA-B). HOMA-IR was calculated as follows: HOMA-IR = (FSI × FSG)/22.5 [21]; HOMA-B $=(20\times FSI)/(FSG-3.5)$, where FSI is the fasting serum insulin concentration (mU/l) and FSG is fasting serum glucose (mmol/l)[22]. All laboratory analyses were performed at the central biochemical laboratory of the Hospital Clinic, Barcelona, Spain.

DNA methylation analysis

Whole blood samples were stored in the Biobank Hospital Clínic-IDIBAPS; Barcelona, Spain[23]. Genomic DNA was extracted from whole blood for all the subjects studied using standards procedures from the Biobank[23]. Sequenom's MassARRAY platform was used to perform quantitative methylation analysis[24]. This system utilizes MALDI-TOF mass spectrometry in combination with RNA base-specific cleavage (MassCLEAVE). A detectable pattern is then analyzed for methylation status. PCR primers for the amplification of the promoter of *TCF7L2* gene were designed using *Epidesigner* (See Appendix S1). Sequenom's EpiTYPER procedure and protocols include an intern quality control of the methylation data[25]. Bisulfite conversion was done for all samples (all cases and controls) together, with the same reactive preparation, and the same operator. The methylation analysis was done during the same day for all the samples (cases and controls). Methylation data was generated in duplicate for each CpG. There was one run for all cases and another one for all controls, and all were done by the same operator during the same day in the same machine. A fully methylated positive control was included for each run.

Statistical analysis

Methylation data are generated as β values between 0 and 1, indicating percentage methylation of the original template[26]. Due to the high variability of methylation data over the genomic region analyzed, we decided to do the analysis using each CpG site individually. Descriptive data are presented as the mean and standard deviation (SD) for continuous outcomes, or number and percentage (%) for categorical outcomes. HOMA-IR, HOMA-B, and insulin were compared using non-parametric Mann-Whitney U test because normality and equality of variance could not be assumed. Student's *t* test was used for the comparison of the rest of continuous outcomes and Chi-square test for categorical outcomes. Methylation differences between cases and controls were studied by comparing the methylation means in each CpG site using a non-parametric test (Mann-Whitney U test). Logistic regression models adjusting for age, BMI, gender, waist circumference, smoking status and physical inactivity were built to confirm the unadjusted results. Finally, to study the potential association of methylation data with clinical and biochemical parameters, we did a correlational analysis (calculating Spearman's rank correlation coefficients) and we performed multivariate lineal regression models adjusting for age, BMI, gender, waist circumference, smoking status, physical inactivity and diabetes status for each CpG site. Overall R^2 values for the models including CpG methylation values, sex, age, BMI, waist circumference, physical inactivity, smoking status and diabetes status are given as percentages. This was done to give an estimate of the association between outcome and methylation. False discovery rate (FDR) correction was used for multiple comparisons[27]. All significance tests were 2-tailed and values of $P<0.05$ were considered significant. All analyses were conducted using the statistical software package Stata version 11 and R Bioconductor.

Results

Metabolic profile of the type 2 diabetic patients and controls

Baseline characteristics of the patients included in the study are summarized in Table 1. All patients were overweight (mean BMI of 29.2 ± 3.7 in type 2 diabetic patients vs. mean BMI of 28.8 ± 2.5 in controls, $P=0.454$). Mean age of all patients was 68 years and there were no significant differences in gender (66.7% were men in

the group of cases vs 53.8% in the group of controls, $P=0.072$). Type 2 diabetic patients had a higher waist circumference as compared to controls (mean waist values of 102.7±9.5 cm vs. 97.9±8.0 cm, $P=0.002$). Total cholesterol was lower in cases as compared to controls (total cholesterol mean values of 4.8±1.0 mmol/L vs. 5.2±1.1 mmol/L $P=0.002$). HOMA-IR was higher in cases than in controls (2.6±1.5 vs. 1.8±0.7 in controls, $P<0.001$). HOMA-B was lower in type 2 diabetic patients as compared to controls (75.7±51.1 in type 2 diabetic patients vs 113.6±510.6 in controls, $P<0.001$). Type 2 diabetic patients were less physically inactive as compared to controls (28% vs. 53.8%, respectively $P<0.001$).

Quantitative DNA Methylation analysis in peripheral blood of *TCF7L2* promoter in type 2 diabetic patients and controls

Methylation levels in DNA from whole blood of 186 subjects were obtained for 22 sites covering the region between -497 bp and +186 bp according to the ATG position for the *TCF7L2* gene (ENSG00000148737). The heat map showing the methylation values (%) for each CpG site analyzed did not reveal a clearly distinct pattern of methylation between type 2 diabetic patients and controls in the region analyzed (Figure not shown), however some significant differences were actually found. Indeed, multivariate logistic regression models confirmed that 14 out of the 22 CpGs analyzed (64%) showed significant differences in DNA methylation values between type 2 diabetic patients and controls (see adjusted *P*-values in Table 2). When accounting for multiple testing in the multivariate logistic regression models, only 13 out of 22 (59%) remained significant (see adjusted *Q*-values in Table 2). The unadjusted correlational analysis showed that the methylation levels of 16 out of 22 CpG sites (73%) were associated with fasting glucose, 5 out of 22 CpG sites (23%) were associated with HOMA-IR, 9 out of 22 CpG sites (41%) were associated with HOMA-B, 6 out of 22 CpG sites (27%) with total-cholesterol and 2 out of 22 CpG sites (9%) with LDL-cholesterol (see Table 3). After further adjustment, only 4 CpG sites remained significantly correlated with fasting glucose and 1 CpG site with total-cholesterol and LDL-cholesterol (see Table 3). Explained variance of fasting glucose was 62% in CpG 9, CpG 17, CpG 25 and CpG 30, including only adjustment factors. These variances increased to 63%, 66%, 66% and 63%, respectively, after including *TCF7L2* methylation in the model, corresponding to an additional explained variance of 1%, 4%, 4% and 1%, respectively. The variance explained by CpG 27 methylation alone on total cholesterol was up to 5% and up to 4% on LDL-cholesterol.

Discussion

In this study, we report the methylation pattern of *TCF7L2* promoter from peripheral blood DNA in drug-naïve type 2 diabetic patients and age- and BMI-matched controls. We found that several CpGs had significant differences between type 2 diabetic patients and controls, although overall the methylation pattern did not show a clear differential pattern related to T2D. These results are consistent with previous data of promoter methylation patterns from peripheral blood DNA where a global

Table 1. Demographic and clinical characteristics of type 2 diabetic patients and age- and BMI-matched controls.

Variable*	Type 2 diabetic patients (n = 93)	Controls (n = 93)	P Value†
Demographic characteristics			
Age, yr	69.1±9.2	66.6±11.7	0.099
BMI, kg/m^2	29.2±3.7	28.8±2.5	0.454
Waist circumference, cm	102.7±9.5	97.9±8.0	**0.002**
Male sex, (%)	66.7	53.8	0.072
Duration of diabetes, yr	5.4±4.1		
Physical inactivity, %	28.0%	53.8%	**<0.001**
Never smoked, %	50.5%	61.3%	0.261
Laboratory values			
Fasting glucose, (mmol/L)	6.4±1.2	4.6±0.3	**<0.001**
Glycated hemoglobin, (%)	5.8±0.6		
Fasting insulin, (pmol/L)	55.6±28.6	52.4±21.0	0.750
HOMA-IR §	2.6±1.5	1.8±0.7	**<0.001**
HOMA-B §§	75.7±51.1	113.6±510.6	**<0.001**
Alanine aminotransferase (ALT), (IU/liter)	13.5±7.9	14.6±7.3	0.486
Aspartate aminotransferase (AST), (IU/liter)	16.6±8.2	19.0±6.0	0.135
Total cholesterol (mmol/L)	4.8±1.0	5.2±1.1	0.002
LDL cholesterol (mmol/L)	2.8±0.8	2.9±0.8	0.782
HDL cholesterol (mmol/L)	1.3±0.3	1.4±0.3	0.262
Triglycerides (mmol/L)	1.4±0.9	1.3±0.8	0.338

* Values shown are means ±SD, unless otherwise indicated.
†P values were calculated with the t test for quantitative variables or Chi-square test for categorical ones, except for HOMA-IR, HOMA-B, fasting insulin, where non-parametric Mann-Whitney U test was applied.
§HOMA-IR was calculated as [Insulin mIU/l x FSG: (mmol/l)/22.5].
§§HOMA-B was calculated as (20× FSI)/(FSG −3.5), where FSI is the fasting serum insulin concentration (mU/l) and FSG is fasting serum glucose (mmol/l).
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Table 2. Peripheral blood DNA methylation values (in %) for each CpG site analyzed in the *TCF7L2* promoter in type 2 diabetic patients and age- and BMI-matched controls*.

CpG site†	Position (bp) ‡	Type 2 diabetic patients (n = 93)	Controls (n = 93)	P-value	Q-value	Adjusted P-value	Adjusted Q-value
CpG 2	−497	27.8±5.3	31.4±5.6	<0.001	**<0.001**	**0.005**	**0.011**
CpG 3	−481	1.6±2.5	1.0±2.3	<0.001	**<0.001**	0.313	0.383
CpG 4	−473	1.6±2.5	1.0±2.3	<0.001	**<0.001**	0.313	0.383
CpG 5	−466	3.0±1.3	3.1±1.6	0.523	0.524	0.483	0.531
CpG 6	−437	92.8±5.8	90.5±4.8	**0.005**	**0.006**	**0.003**	**0.007**
CpG 7	−434	92.8±5.8	90.5±4.8	**0.005**	**0.006**	**0.003**	**0.007**
CpG 8	−386	89.1±4.3	89.8±5.0	0.345	0.389	0.451	0.522
CpG 9	−382	96.3±2.4	95.2±2.5	**0.002**	**0.003**	**0.035**	0.055
CpG 12	−254	3.0±1.6	7.6±3.3	<0.001	**<0.001**	**<0.001**	**0.004**
CpG 14	−214	1.1±1.8	0.7±1.0	0.494	0.524	0.800	0.800
CpG 15	−212	1.1±1.8	0.7±1.0	0.494	0.524	0.800	0.800
CpG 16	−114	36.2±11.2	29.2±16.2	**0.004**	0.006	**0.018**	**0.033**
CpG 17	+5	44.0±7.9	37.3±3.4	<0.001	**<0.001**	**<0.001**	**0.004**
CpG 18	+15	97.7±3.6	96.4±2.6	<0.001	**<0.001**	0.076	0.105
CpG 19	+18	97.7±3.6	96.4±2.6	<0.001	**<0.001**	0.076	0.105
CpG 20	+39	4.4±2.0	5.5±2.7	0.002	**0.003**	**0.002**	**0.006**
CpG 24	+75	52.6±5.6	47.7±6.1	<0.001	**<0.001**	**<0.001**	**0.004**
CpG 25	+96	44.0±7.9	37.3±3.4	<0.001	**<0.001**	**<0.001**	**0.004**
CpG 26	+107	90.0±4.4	96.4±2.3	<0.001	**<0.001**	**<0.001**	**0.004**
CpG 27	+137	14.8±4.3	13.3±4.1	0.065	0.078	**0.020**	**0.034**
CpG 29	+180	90.0±4.4	96.4±2.3	<0.001	**<0.001**	**<0.001**	**0.004**
CpG 30	+186	20.9±5.0	17.9±6.0	<0.001	**<0.001**	**0.006**	**0.012**

* Values shown are means ±SD.

P values were calculated using the Mann-Whitney U test.

Q values were calculated as estimates of the multiple-testing-corrected false discovery rate (FDR).

Adjusted P-values were calculated by performing a logistic regression analysis adjusting by age, gender, BMI, physical inactivity, smoking status and waist circumference.

Adjusted Q-values were calculated as estimates of the multiple-testing-corrected false discovery rate (FDR) on the adjusted P-values.

†CpG dinucleotides have been numbered relative to ATG.

‡CpG dinucleotide position has been determined according to the ATG position for the *TCF7L2* gene (ENSG00000148737).

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Table 3. Results of methylation correlation analysis between each CpG site analyzed in the *TCF7L2* promoter and the listed dependent variables*.

CpG site†	Fasting glucose	Fasting insulin	HOMA-IR	HOMA-B	GOT	GPT	Total cholesterol	HDL-cholesterol	LDL-cholesterol	Triglycerides
CpG 2	−0.231 **(0.002)**	−0.112 (0.132)	−0.163 **(0.028**)	0.089 (0.234)	0.003 (0.976)	−0.051 (0.581)	0.101 (0.174)	0.009 (0.902)	0.083 (0.267)	−0.012 (0.871)
CpG 3.4	0.19 **(0.011)**	−0.032 (0.665)	0.046 (0.539)	−0.187 **(0.012)**	−0.041 (0.661)	−0.015 (0.869)	0.017 (0.818)	0.021 (0.783)	0.051 (0.5)	−0.055 (0.461)
CpG 5	−0.021 (0.778)	−0.09 (0.227)	−0.095 (0.203)	−0.015 (0.844)	−0.032 (0.726)	0.003 (0.978)	0.109 (0.141)	0.079 (0.285)	0.064 (0.386)	0.089 (0.232)
CpG 6.7	0.161 **(0.03)**	−0.059 (0.431)	0.022 (0.764)	−0.203 **(0.006)**	−0.055 (0.548)	0.022 (0.808)	−0.07 (0.342)	−0.086 (0.244)	0.017 (0.824)	0.116 (0.116)
CpG 8	−0.116 (0.117)	−0.023 (0.76)	−0.073 (0.328)	0.065 (0.387)	−0.121 (0.183)	0.083 (0.369)	0.019 (0.798)	−0.01 (0.894)	0.001 (0.988)	−0.022 (0.771)
CpG 9	0.218 **(0.003)‡**	0.099 (0.183)	0.174 **(0.019)**	−0.096 (0.195)	−0.059 (0.52)	−0.028 (0.764)	−0.047 (0.528)	−0.046 (0.531)	0.032 (0.663)	0.088 (0.232)
CpG 12	−0.546 **(<0.001)**	−0.063 (0.398)	−0.249 **(0.001)**	0.367 **(<0.001)**	0.176 (0.053)	0.034 (0.716)	0.135 (0.067)	0.105 (0.157)	0.022 (0.763)	−0.129 (0.081)
CpG 14.15	0.044 (0.555)	0.008 (0.913)	0.035 (0.637)	−0.023 (0.759)	0.02 (0.826)	0.006 (0.946)	0.038 (0.613)	0.141 (0.058)	0.002 (0.981)	−0.13 (0.081)
CpG 16	0.108 (0.145)	−0.074 (0.318)	−0.01 (0.889)	−0.107 (0.149)	−0.036 (0.691)	0.014 (0.88)	−0.157 **(0.032)**	0.039 (0.6)	−0.153 **(0.038)**	−0.042 (0.571)
CpG 17	0.283 **(<0.001)‡**	−0.025 (0.733)	0.107 (0.15)	−0.219 **(0.003)**	−0.047 (0.601)	0.032 (0.725)	−0.163 **(0.026)**	−0.024 (0.743)	−0.094 (0.203)	0.037 (0.613)
CpG 18.19	0.213 **(0.004)**	0.013 (0.859)	0.089 (0.229)	−0.137 (0.064)	−0.131 (0.147)	−0.054 (0.558)	−0.117 (0.113)	−0.124 (0.093)	−0.04 (0.59)	0.058 (0.429)
CpG 20	−0.212 **(0.004)**	−0.036 (0.626)	−0.077 (0.298)	0.141 (0.056)	0 (0.997)	−0.063 (0.492)	0.039 (0.599)	0.003 (0.964)	−0.006 (0.933)	−0.008 (0.909)
CpG 24	0.286 **(<0.001)**	−0.074 (0.32)	0.054 (0.465)	−0.275 **(<0.001)**	−0.182 **(0.044)**	−0.092 (0.315)	−0.102 (0.166)	−0.054 (0.463)	−0.044 (0.557)	−0.014 (0.849)
CpG 25	0.283 **(<0.001)‡**	−0.025 (0.733)	0.107 (0.15)	−0.219 (0.003)	−0.047 (0.601)	0.032 (0.725)	−0.163 **(0.026)**	−0.024 (0.743)	−0.094 (0.203)	0.037 (0.613)
CpG 26	−0.504 **(<0.001)**	−0.056 (0.445)	−0.215 **(0.003)**	0.349 **(<0.001)**	0.124 (0.17)	0.076 (0.404)	0.18 **(0.014)**	0.033 (0.651)	0.102 (0.167)	−0.091 (0.215)
CpG 27	0.007 (0.922)	−0.015 (0.837)	0.003 (0.967)	−0.023 (0.759)	0.026 (0.776)	0.13 (0.153)	−0.234 **(0.001)‡**	−0.123 (0.093)	−0.231 **(0.002)‡**	−0.044 (0.555)
CpG 29	−0.504 **(<0.001)**	−0.056 (0.445)	−0.215 **(0.003)**	0.349 **(<0.001)**	0.124 (0.17)	0.076 (0.404)	0.18 **(0.014)**	0.033 (0.651)	0.102 (0.167)	−0.091 (0.215)
CpG 30	0.326 **(<0.001)‡**	−0.03 (0.684)	0.074 (0.316)	−0.24 **(0.001)**	−0.036 (0.687)	−0.001 (0.988)	−0.144 (0.05)	−0.025 (0.734)	−0.066 (0.37)	−0.045 (0.54)

*Values shown are Spearman correlation coefficients and p-values in brackets between each CpG site and the dependent variables. In bold are marked the variables that were significant in the unadjusted analysis.
‡Variables that remained significant after full adjustment by age, gender, BMI, physical inactivity, smoking status, waist circumference and diabetes status in linear regression analyses.
†CpG dinucleotides have been numbered relative to ATG.
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directional change in methylation levels that would affect all neighboring CpGs systematically and that would be characteristic of the disease has not been identified[18,28]. On the other hand, a recent study found some T2D-related methylation patterns in peripheral blood DNA[17] but their analysis did not cover the genomic region we studied. There is great interest to perform methylation profiling in peripheral blood to find methylation disease-related associations since specific methylated regions could be used as potent biomarkers[29]. However, to study how these differentially methylated regions may play a mechanistic role in the development of the disease of interest, the methylation analysis should focus in the tissues relevant for the genes studied. *TCF7L2* is highly expressed in beta-cells, followed by colon, brain, small intestine, monocytes, and lung[30], whereas no expression was detected in lymphocytes T or B. It has been shown that depletion of *TCF7L2* results in reduced GIP-Receptor levels in pancreatic islets and in impaired beta-cell function[31]. In our study, we found that methylation of specific CpG sites on *TCF7L2* promoter in blood was correlated with fasting glucose, total cholesterol and LDL-cholesterol. In line with our results, it has been recently shown that beta-cells cultured with high-glucose-lipid medium presented aberrant DNA methylation in different loci, among which was *TCF7L2* gene promoter[32]. Moreover, Hu et al showed that, while *TCF7L2* promoter was hypermethylated, *TCF7L2* mRNA expression increased, and, unexpectedly, the protein expression of *TCF7L2* was decreased in beta- cells[32]. The mechanisms of this opposite regulation remain unkown, although it could be speculated that DNA methylation may affect the *TCF7L2* splice variants[33], i.e., the increase in mRNA levels could represent transcripts of *TCF7L2* which would encode less active isoforms[32]. Methylation patterns are thought to be tissue-specific[10,34,35], thus we might not extrapolate the methylation patterns found in blood to those present in beta-cells. As *TCF7L2* gene is not expressed in blood lymphocytes, we did not perform mRNA expression analyses in peripheral blood. Nevertheless, the first methylome reference in human pancreatic islets has been just published[36]. Dayeh et al performed a genome-wide DNA methylation analysis of human pancreatic islets from type 2 diabetic and non-diabetic donors[36]. In this study, *TCF7L2* gene presented differential methylation values in diabetic pancreatic islets as compared to non-diabetic pancreatic islets. It should be noted though that the region they studied in *TCF7L2* gene is further downstream (3′) than the region we studied.

Type 2 diabetic patients and controls were similar in age and BMI to control for any confounder effect of age and obesity on the results. Moreover, none of the type 2 diabetic patients were on any pharmacological therapy for diabetes. Thus, no confounding effect of antidiabetic drugs or insulin therapy was possible, either. Type 2 diabetic patients received counselling about exercise and healthy diet in order to control their diabetes. This could explain why the % of physically inactive subjects was higher in the control group as compared to the type 2 diabetic patients. The majority of type 2 diabetic patients (67%) were on statins as compared to controls. This could explain the differences in mean total cholesterol between the two groups. Type 2 diabetic patients were in optimal glycemic control (mean glycated hemoglobin 5.8%) and had their clinical diagnosis of T2D recently (mean duration of diabetes was 5 years). Results showed that type 2 diabetic patients were more insulin-resistant than controls, since they presented higher values of HOMA-IR. In concordance to this, type 2 diabetic patients had a higher waist circumference as compared to controls. Higher waist circumference is one component used for the diagnosis of the metabolic syndrome and previous research showed that it correlates with poorer glucose control in type 2 diabetic patients[37]. In contrast, and as expected, beta-cell function was already impaired in type 2 diabetic patients as compared to controls (HOMA-B was significantly lower in type 2 diabetic patients as compared to controls). These data illustrates the fact that impairment of beta-cell function is worse in type 2 diabetic patients as compared to age- and BMI- matched controls. These results are in concordance with the existing literature[38,39,40].

The strength of our research is that we have demonstrated that type 2 diabetic patients have differences in concrete CpGs sites of *TCF7L2* promoter as compared to age- and BMI-matched controls. We also found new correlations between fasting glucose, total cholesterol and LDL-cholesterol with DNA methylation in specific CpG sites of *TCF7L2* promoter in DNA from peripheral blood. However, despite accounting for the major confounding factors (age, BMI, diabetes pharmacologic therapy), residual confounding and reverse causation remain possible[41]. As proposed by Relton et al[41], by applying a *"genetical epigenomics"* approach, we could overcome this issue. In our case, the approach would be to study the genetic variants related to the methylation patterns and then to verify whether the correlation with methylation values and fasting glucose and cholesterol remains. However, this was not the goal of the present study.

In conclusion, the targeted epigenetic analysis in DNA from peripheral blood identified differences in specific sites of the *TCF7L2* promoter between type 2 diabetic patients and matched controls. Lipid and glucose blood-parameters were correlated with methylation in specific CpG sites of the *TCF7L2* promoter. Further research should unveil the potential role of these data in the physiopathology of T2D. Our findings add to the growing understanding of the interplay between epigenetics and T2D susceptibility gene *TCF7L2* in the development of the disease.

Supporting Information

Appendix S1 Primers used for quantitative DNA methylation analysis.
(DOCX)

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Author Contributions

Conceived and designed the experiments: SC RG. Performed the experiments: ER EF MP. Analyzed the data: SC BK. Contributed reagents/materials/analysis tools: FH MP. Wrote the paper: SC. Recruited patients: SC AS LG. Critical review of the results: FH AN.

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Appendix S1. Primers used for quantitative DNA methylation analysis*

***TCF7L2* CpGs 1-15 LEFT**	aggaagagagGGTATTTTATTAAGGTAGTGTGTTTTTTT
***TCF7L2* CpGs 1-15 RIGHT**	cagtaatacgactcactatagggagaaggctTTTTTCTACTTAAAAATCTTTTTCTCC
***TCF7L2* CpGs 16-31 LEFT**	aggaagagagTTTTTAGGAGAAAAAGATTTTTAAGTAGA
***TCF7L2* CpGs 16-31 RIGHT**	cagtaatacgactcactatagggagaaggctCAAACCCAAAAAACAAATAAAAAAC

* LEFT: 10-mer tag sequence. RIGHT: T7 promoter tag with an 8 base pair insert (for prevention of abortive cycling and constant 5' fragment for RNaseA reaction).

Bold case indicate the actual sequence for the primers.

Methylation analysis.

Sequenom's MassARRAY platform was used to perform quantitative methylation analysis. This system utilizes MALDI-TOF mass spectrometry in combination with RNA base specific cleavage (MassCLEAVE). A detectable pattern is then analyzed for methylation status. PCR primers for amplification of the promoter of the gene *TCF7L2* have been designed by using *Epidesigner* (Sequenom). When it was feasible, amplicons were designed to cover CpG islands in the same region as the 5′ UTR. For each reverse primer, an additional T7 promoter tag for *in vivo* transcription has been added, as well as a 10-mer tag on the forward primer to adjust for melting-temperature differences. The primers used appear on the above table.

The PCRs have been carried out in a 5 μl format with10 ng/ml bisulfite-treated DNA, 0.2 units of *Taq*DNA polymerase (Sequenom), 1x supplied *Taq* buffer, and 200 mM PCR primers. Amplification for the PCR was done as follows: preactivation of 95°C for 15 min, 45 cycles of 95°C denaturation for 30 s, 56°C annealing for 30 s, and 72°C extension for 30 s, finishing with a 72°C incubation for 4 min. Dephosphorylation of

unincorporated dNTPs has been performed by adding 1.7 ml of H_2O and 0.3 units of shrimp alkaline phosphatase (Sequenom), incubating at 37°C for 40 min, and then for 10 min at 85°C to deactivate the enzyme. The MassCLEAVE biochemistry has been performed as follows: Next, *in vivo* transcription and RNA cleavage was achieved by adding 2 µl of PCR product to 5 µl of transcription/cleavage reaction and incubating at 37°C for 3 h. The transcription/cleavage reaction contains 27 units of T7 RNA&DNA polymerase (Sequenom), 0.64x of T7 R&DNA polymerase buffer, 0.22 µl T Cleavage Mix (Sequenom), 3.14 mM DTT, 3.21 µl H_2O, and 0.09 mg/ml RNaseA (Sequenom). The reactions have been additionally diluted with 20 ml of H_2O and conditioned with 6 mg of CLEAN Resin (Sequenom) for optimal mass-spectra analysis.

3rd AIM

3rd Aim. Study of the DNA methylation pattern in *GIPR* promoter gene in type 2 diabetic patients and non-diabetic subjects, using as source of DNA an easily accessible tissue (peripheral blood).

A. Comparative study of the methylation values in *GIPR* promoter gene between type 2 diabetic patients and age- and BMI-matched non-diabetic subjects in whole blood DNA.

B. Correlational study between the methylation values of *GIPR* promoter gene and clinical and biochemical parameters in type 2 diabetic patients and age- and BMI-matched non-diabetic subjects.

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GIP action in type 2 diabetic (T2D) patients is altered. We hypothesized that methylation changes could be present in GIP receptor of T2D patients. This study aimed to assess the differences in DNA methylation profile of *GIPR* promoter between T2D patients and age- and Body Mass Index (BMI)-matched controls. We included 93 T2D patients (cases) that were uniquely on diet (without any anti-diabetic pharmacological treatment). We matched one control (with oral glucose tolerance test negative, non diabetic), by age and BMI, for every case. Cytokines and hormones were determined by ELISA. DNA was extracted from whole blood and DNA methylation was assessed using the Sequenom EpiTYPER system. Our results showed that T2D patients were more insulin resistant and had a poorer β cell function than their controls. Fasting adiponectin was lower in T2D patients as compared to controls (7.0±3.8 μgr/mL vs. 10.0±4.2 μgr/mL). Levels of IL 12 in serum were almost double in T2D patients (52.8±58.3 pg/mL vs. 29.7±37.4 pg/mL). We found that *GIPR* promoter was hypomethylated in T2D patients as compared to controls. In addition, HOMA-IR and fasting glucose correlated negatively with mean methylation of *GIPR* promoter, especially in T2D patients. This case-control study confirms that newly diagnosed, drug-naïve T2D patients are more insulin resistant and have worse β cell function than age- and BMI-matched controls, which is partly related to changes in the insulin-sensitizing metabolites (adiponectin), in the proinflammatory profile (IL12) and we suggest in the methylation pattern of *GIPR*. Our study provides novel findings on *GIPR* promoter methylation profile which may improve our ability to understand type 2 diabetes pathogenesis.

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Gastric Inhibitory Polypeptide Receptor Methylation in Newly Diagnosed, Drug-Naïve Patients with Type 2 Diabetes: A Case-Control Study

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Abstract

GIP action in type 2 diabetic (T2D) patients is altered. We hypothesized that methylation changes could be present in GIP receptor of T2D patients. This study aimed to assess the differences in DNA methylation profile of *GIPR* promoter between T2D patients and age- and Body Mass Index (BMI)-matched controls. We included 93 T2D patients (cases) that were uniquely on diet (without any anti-diabetic pharmacological treatment). We matched one control (with oral glucose tolerance test negative, non diabetic), by age and BMI, for every case. Cytokines and hormones were determined by ELISA. DNA was extracted from whole blood and DNA methylation was assessed using the Sequenom EpiTYPER system. Our results showed that T2D patients were more insulin resistant and had a poorer β cell function than their controls. Fasting adiponectin was lower in T2D patients as compared to controls (7.0±3.8 μgr/mL vs. 10.0±4.2 μgr/mL). Levels of IL 12 in serum were almost double in T2D patients (52.8±58.3 pg/mL vs. 29.7±37.4 pg/mL). We found that *GIPR* promoter was hypomethylated in T2D patients as compared to controls. In addition, HOMA-IR and fasting glucose correlated negatively with mean methylation of *GIPR* promoter, especially in T2D patients. This case-control study confirms that newly diagnosed, drug-naïve T2D patients are more insulin resistant and have worse β cell function than age- and BMI-matched controls, which is partly related to changes in the insulin-sensitizing metabolites (adiponectin), in the proinflammatory profile (IL12) and we suggest in the methylation pattern of *GIPR*. Our study provides novel findings on *GIPR* promoter methylation profile which may improve our ability to understand type 2 diabetes pathogenesis.

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Introduction

Twin cohort studies have shown that shared genetic factors can only explain a fraction of the differences in incident type 2 diabetes (T2D) [1]. Behavioral (sedentary lifestyle, westernized food patterns) and environmental factors (organic pesticides, chemical exposures, and air pollutants) contribute to the development of T2D [2]. Moreover, inflammation induces inhibition of the insulin signalling pathway which can lead to insulin resistance and T2D. Recently, it has been proposed that epigenetic mechanisms could be involved in the complex interplay between genes and the environment [3]. Indeed, a recent study showed the presence of an epigenetic dysregulation in pancreatic islets from T2D patients [4]. Briefly, they found differences in DNA methylation profiles in several promoter regions in islets from T2D patients [4]. DNA methylation is the best studied epigenetic modification and influences transcripcional regulation [5]. DNA methylation is a reversible process that can be modulated by both stochastic and environmental stimuli [6]. On the other hand, *GIPR* gene codifies for the receptor of the incretin GIP, a gastrointestinal hormone that stimulates insulin response after an oral glucose challenge. In T2D patients, GIP action is reduced, whereas its secretion does not seem to be altered. There is increasing evidence supporting an important role for *GIPR* as a candidate for mediating insulin secretion after oral glucose challenge [7]. We speculated that *GIPR* gene could be affected by alterations in DNA methylation in T2D patients, which could explain the dysregulation of GIP action in T2D patients [8,9]. As DNA methylation occurs principally in the upstream regulatory regions of the genes [10], we concentrated on the promoter of *GIPR*. A previous study has shown that T2D-

related methylation may be reflected in accessible tissues such as peripheral blood [11].

The principal aim of this study was to compare the pattern of DNA methylation on *GIPR* promoter between T2D patients and age- and Body Mass Index (BMI)-matched controls. The secondary aims were to compare the metabolic and cytokine profiles between T2D patients and matched controls.

Materials and Methods

Ethics Statement

This study was approved by the ethics committees of the Hospital Clínic and complies with all laws and international ethics guidelines outlined in the Declaration of Helsinki. All human subjects provided written, informed consent.

Study Design and Subjects Included

We conducted a case-control study where cases were defined as patients suffering from T2D that were treated only by diet. Eligibility criteria for cases were the following: clinical diagnosis of T2D between December 2010 until December 2011, adequate glycemic control after a period of minimum six months of low-carbohydrate diet and lifestyle interventions, no pharmacological therapy for T2D needed to achieve the glycemic control. Diagnosis of T2D was done following ADA recommendations [12], by either a random elevated fasting glucose value (confirmed twice) and/or by performing an oral glucose tolerance test (OGTT). In case oral medication was needed for optimal glycemic control, those patients were excluded from the study. Cases and controls were recruited from the same primary health center. Eligibility criteria for controls were as follows: a negative OGTT at recruitment, no previous diagnosis of T2D or prediabetes, no chronic treatment with oral steroids. Controls were frequency matched on age and BMI to cases. Metabolic profile, cytokine profile and DNA methylation of *GIPR* promoter profile in peripheral blood DNA were studied for all subjects (93 cases and 93 controls).

Metabolic Assessments

All subjects were examined by anthropometric measurements and had fasting metabolic assessments at recruitment. These assessments included fasting glucose, fasting insulin, fasting leptin, fasting adiponectin, cytokines, glycohemoglobin A1 (HbA1) (only for the type 2 diabetic patients), HOMA-IR and HOMA-B. HOMA-IR was calculated as follows: HOMA-IR = (FPI × FPG)/22.5 [13]; homeostasis β-cell function (HOMA-B) = (20 × FPI)/(FPG − 3.5), where FPI is the fasting plasma insulin concentration (mU/l) and FPG is fasting plasma glucose (mmol/l) [14].

Hormone and Cytokine Measurements

Adiponectin, leptin and insulin were quantified from serum samples by ELISA (Mercodia), according to the manufacturer's instructions. Cytokines were measured from serum samples using CBA Human Inflammatory Cytokines kit (BD Bioscience), following the manufacturers instructions. Two-color flow cytometric analysis was performed using LSRFortessa (BD bioscience). Data were acquired and analyzed using FACS Diva and FCAP Array 1.01 Software. Hormone and cytokine measurements were performed at the Diabetes and Obesity Laboratory-IDIBAPS; Barcelona, Spain.

DNA Methylation Analysis

Whole blood samples were stored in the Biobank Hospital Clínic-IDIBAPS; Barcelona, Spain. Genomic DNA was extracted from whole blood for all the subjects studied using standards procedures from the Biobank. Sequenom's MassARRAY platform was used to perform quantitative methylation analysis [15]. This system utilizes MALDI-TOF mass spectrometry in combination with RNA base-specific cleavage (MassCLEAVE). A detectable pattern is then analyzed for methylation status. PCR primers for the amplification of the *GIPR* promoter gene were designed using *Epidesigner* (See Appendix S1).

Statistical Analysis

Descriptive data are presented as the mean and standard deviation (SD) for continuous outcomes, or number and percentage (%) for categorical outcomes. The methylation values (in %), cytokines, HOMA-IR, HOMA-B, insulin, leptin and adiponectin were compared using non-parametric Mann-Whitney U test, because normality and equality of variance could not be assumed. Student's *t* test was used for the comparison of the rest of continuous outcomes and Chi-square test for categorical outcomes. Correlation between methylation at all thirteen CpG sites was high ($P = 0.002$), therefore a mean of *GIPR* promoter methylation was generated. Spearman's rank correlation coefficient was used to assess correlation between mean *GIPR* promoter methylation and the different covariates (waist circumference, fasting glucose, fasting insulin, fasting adiponectin, fasting leptin, HOMA-IR, HOMA-B, cytokines). Linear regression was used to study the association between the mean *GIPR* promoter methylation (independent variable) and the covariates (dependent variables) that presented a significant correlation in the Spearman analysis, after adjustment for diabetis status (i.e, being case or control), sex, age and BMI. Mean *GIPR* promoter methylation was log-transformed for the regression analysis. Subgroup analyses (i.e, by disease status) were done for the variables that remained significant after the adjustment. Overall R^2 values for the models give the combined contribution of log-transformed mean *GIPR* promoter methylation, sex, age, BMI and diabetes status to the variability in dependent variables. Bonferroni correction was used for multiple comparisons. All significance tests were 2-tailed and values of $p<0.05$ were considered significant. All analyses were conducted using the statistical software package Stata version 11.

Results

Metabolic and Cytokine Profile of Type 2 Diabetic Patients and Controls

Baseline characteristics of the patients included in the study are summarized in Table 1. T2D patients had a higher waist circumference as compared to controls (mean waist values of 102.7±9.5 cm vs. 97.9±8.0 cm, $P<0.01$). Fasting adiponectin was lower in cases as compared to controls (mean values of 7.0±3.8 μgr/mL vs. 10.0±4.2 μgr/mL, $P<0.0001$). HOMA-IR was higher in cases (2.6±1.5 vs. 1.8±0.7 in controls, $P<0.0001$). HOMA-B was higher in controls as compared to T2D patients (113.6±510.6 vs. 75.7±51.1 in type 2 diabetic patients, $P<0.0001$). From the cytokines analyzed, significant differences were found for IL 10 (4.1±3.0 pg/mL in cases vs. 5.2±3.7 pg/mL in controls, $P<0.05$) and IL 12 (52.8±58.3 pg/mL in cases vs. 29.7±37.4 pg/mL in controls, $P<0.0001$). No differences were found between cases and controls in the routine laboratory measures (blood cell count, hepatic profile, lipid profile, renal function, data not shown).

Table 1. Demographic and metabolic characteristics of type 2 diabetic patients and age- and BMI-matched controls.

Variable*	Type 2 diabetic patients (n = 93)	Controls (n = 93)	P Value†
Demographic characteristics			
Age, yr	69.1±9.2	66.6±11.7	Matching variable
BMI, kg/m^2	29.2±3.7	28.8±2.5	Matching variable
Waist circumference, cm	102.7±9.5	97.9±8.0	**<0.01**
Male sex, (%)	66.7	53.8	0.07
Laboratory values			
Fasting glucose, (mmol/L)	6.4±1.2	4.6±0.4	**<0.0001**
Glycated hemoglobin, (%)	5·8±0.6	–	
Fasting insulin, (pmol/L)	55.6±28.6	52.4±21.1	0.39
HOMA-IR ‡	2.6±1.5	1.8±0.7	**<0.0001**
HOMA-B §	75.7±51.1	113.6±510.6	**<0.0001**
Fasting leptin, (ng/mL)	18.0±16.7	25.4±26.8	0.07
Fasting adiponectin, (μg/mL)	7.0±3.8	10.0±4.2	**<0.0001**

*Values shown are means ±SD, unless otherwise indicated.
†P values were calculated with the t test for quantitative variables or Chi-square test for categorical ones, except for HOMA-IR, HOMA-B, fasting insulin, fasting leptin and fasting adiponectin, where non-parametric Mann-Whitney U test was applied.
‡HOMA-IR was calculated as [Insulin mUI/l x Glycemia: (mmol/l)/22.5].
§HOMA-B was calculated as (20 × FPI)/(FPG − 3.5), where FPI is the fasting plasma insulin concentration (mU/l) and FPG is fasting plasma glucose (mmol/l).
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Quantitative DNA Methylation Analysis in Peripheral Blood of *GIPR* Promoter in Type 2 Diabetic Patients and Controls

Methylation levels in DNA from whole blood of 186 subjects were obtained for 13 CpG sites covering 1,000 bp upstream of the first exon of the human *GIPR* gene. The heatmap showing the values of methylation (%) for each CpG site analyzed did not reveal a clearly distinct pattern of methylation between T2D patients and controls (figure not shown); however some significant differences were found. Indeed, 9 out of 13 CpG sites studied (69%) showed significant differences between T2D patients and controls. There was a trend towards an hypomethylation in T2D patients as compared to controls (See Table S1). In fact, mean *GIPR* promoter methylation was lower in T2D patients as compared to controls (24.3±1.6 in cases vs. 26.2±1.5 in controls, $P<0.0001$). Mean methylation of *GIPR* promoter was correlated with waist circumference ($r=-0.26$, $P<0.01$), fasting glucose ($r=-0.50$, $P<0.0001$), HOMA-IR ($r=-0.29$, $P<0.001$), HOMA-B ($r=0.28$, $P<0.001$), fasting adiponectin ($r=0.23$, $P<0.01$) and IL-12 ($r=-0.22$, $P<0.01$) (see Table 2). After adjustment, increased *GIPR* promoter methylation was associated with decreasing fasting glucose [−2.4 (−4.5 to −0.2), $P<0.05$] and decreasing HOMA-IR [−4.6 (−7.5 to −1.8) $P<0.01$] (see Figure 1). Hence, following a 10% increase in log-transformed *GIPR* promoter methylation, fasting glucose and HOMA-IR decrease by 0.24 mmol/L and 0.46 units, respectively (see Table 2). The combined contribution of *GIPR* promoter methylation and diabetes status, age, sex and BMI to the variability in HOMA-IR was up to 23% and up to 53% regarding fasting glucose (see Table 2). Separate analyses of T2D patients and controls showed that the significant inverse correlation between mean *GIPR* methylation and HOMA-IR was mostly present in T2D patients ($P<0.05$) and not in controls ($P=0.06$) (see Figure 2). Regarding fasting glucose, the relationship remained significant also uniquely for T2D patients ($P<0.05$), and not in controls ($P=0.80$).

Discussion

The leading cause of T2D is thought to be an impaired β cell function [16] which depends on a complex interplay of genetic predisposition and environmental factors, such as obesity, inactivity and aging. In this sense, we aimed to compare, given a similar environment (defined as similar age and similar degree of obesity), which were the factors associated with the apparition of T2D. Therefore, we compared the metabolic and cytokine profile between 93 newly diagnosed T2D patients and 93 age- and BMI-matched controls. In addition, we also performed the first DNA methylation profiling of human peripheral blood covering the promoter of glucose-associated gene *GIPR* in T2D patients and controls.

T2D patients and controls were similar in age and BMI to control for any confounder effect of age and obesity on the results. Moreover, none of the T2D patients were on any pharmacological therapy for diabetes. Thus, no confounding effect of antidiabetic drugs or insulin therapy was possible, either. T2D patients had their clinical diagnosis of T2D recently and were in optimal glycemic control. Hence, no potential influence of hyperglycemia on the methylation pattern was possibe, or, if any, was low. Results showed that T2D patients were more insulin-resistant than controls, since they presented higher values of HOMA-IR. In concordance to this, T2D patients had a higher waist circumference as compared to controls. Large waist circumference is one component used for the diagnosis of the metabolic syndrome. Insulin resistance is associated with metabolic syndrome too [17]. Basically, in spite of the fact that T2D patients and controls had a similar grade of obesity, T2D patients presented a differential body fat distribution (particularly centralized obesity). This correlates with a differential adipokines secretion which might lead to a higher degree of insulin resistance in T2D patients. In contrast, and as expected, β cell function was already impaired in T2D patients as compared to controls (HOMA-B was significantly lower in T2D patients as compared to controls). These data illustrates the fact that impairment of β cell function is worse in

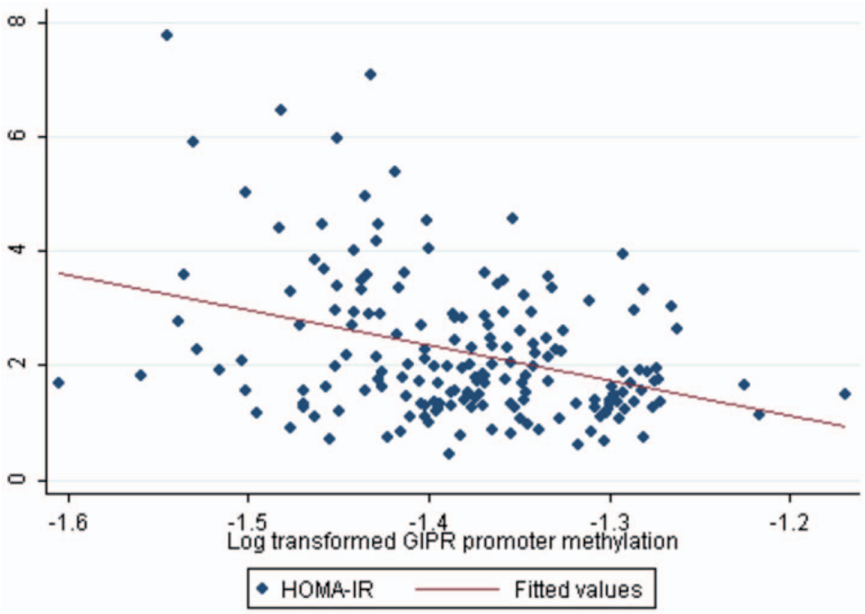

Figure 1. Correlation between average *GIPR* promoter methylation from peripheral blood DNA and insulin resistance. Log-transformed average *GIPR* promoter methylation is shown as the independent variable. HOMA-IR was used as a marker of insulin resistance. Spearman 's correlation r = −0.29, $P = 0.0001$. Adjusted $P < 0.01$ (diabetes status, age, BMI and gender).
doi:10.1371/journal.pone.0075474.g001

T2D patients compared to age- and BMI- matched controls. These results are in concordance with the existing literature [18]. In addition, we found that T2D patients had lower adiponectin levels in serum than controls. Epidemiological studies have shown that higher adiponectin levels in serum are associated with a lower risk of T2D_ENREF_19. Moreover, adiponectin has been proposed as a strong biochemical predictor of T2D [19]. Adiponectin is exclusively and abundantly expressed in white adipose tissue and has been shown to have insulin-sensitizing and anti-inflammatory properties [20]. In fact, in our study, we found that fasting adiponectin had a negative correlation with HOMA-IR (Spearman correlation coefficient r = −0.28, $P<0.0001$) and a positive correlation with HOMA-B (r = 0.19, $P<0.01$), which supports the insulin-sensitizing properties of adiponectin.

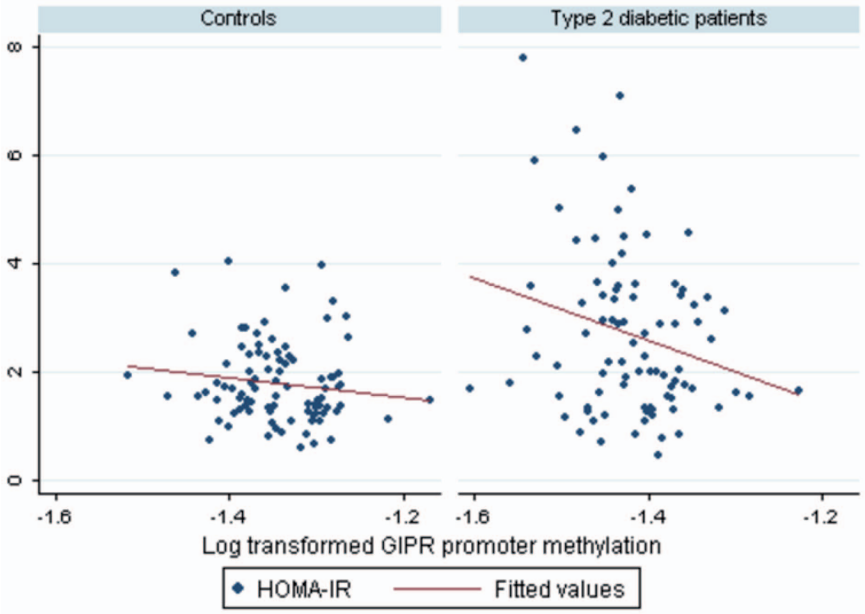

Figure 2. Correlation between average *GIPR* promoter methylation from peripheral blood DNA and insulin resistance, by subgroups (Type 2 diabetic patients and controls). Log-transformed average *GIPR* promoter methylation is shown as the independent variable. HOMA-IR was used as a marker of insulin resistance. $P = 0.06$, adjusted for age, BMI and gender in controls (n = 93). $P<0.05$, adjusted for age, BMI and gender in patients with Type 2 diabetes (n = 93).
doi:10.1371/journal.pone.0075474.g002

Table 2. Results of correlation analysis between *GIPR* promoter methylation and the listed dependent variables.

Outcome variable	Spearman's correlation	Unadjusted p-value	Adjusted p-value*	R^2 (%)†
Waist circumference	−0.26	**<0.01**	0.59. β = −5.2 (−24.4 to 13.9)	44.8
Fasting glucose	−0.50	**<0.0001**	**<0.05. β = −2.4 (−4.5 to −0.2)**	52.9
Fasting insulin	−0.12	0.10		
HOMA-IR	−0.29	**<0.001**	**<0.01. β = −4.6 (−7.5 to −1.8)**	22.7
HOMA-B	0.28	**<0.001**	0.53. β = −0.5 (−1.9 to 1.0)	39.4
Fasting leptin	−0·05	0·49		
Fasting adiponectin	0.23	**<0.01**	0.28. β = 5.1 (−4.1 to 14.2)	23.8
IL-1B	−0.02	0.75		
IL-8	−0.03	0.72		
IL-6	0.08	0.32		
IL-10	0.06	0.43		
IL-12	−0.22	**<0.01**	0.55. β = −37.7 (−161.6 to 86.1)	6.1
TNFα	0.03	0.65		

*Adjustment for age, BMI, sex and diabetes status by creating linear regression analyses between log-transformed GIPR promoter methylation and the dependent variables that presented a significant correlation in Spearman's analysis. Regression coefficients and corresponding 95% CIs are shown.
†R^2 reflects the variance (%) in outcome measures accounted for age, BMI, sex, diabetes status and GIPR promoter methylation.
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On the other hand, lower levels of the anti-inflammatory IL-10 were found in T2D patients, which is consistent with previous research that showed that low levels of IL 10 are associated with T2D [21]. IL-12 serum levels were almost double in T2D patients than in controls. A recent study showed that elevated serum IL-12 was present at the onset of T2D, and that further increases in IL-12 correlated with endothelial dysfunction and cardiovascular disease progression [22]. In addition, it has also been showed that IL-12 might have a role in β cell dysfunction [23]. Overall, the first part of our research demonstrate that T2D patients have an impaired β cell function and are more insulin resistant than age- and BMI-matched controls. These differences in β cell function and insulin resistance are related to differences in adipokines and inflammatory metabolites, which might be the underlying mechanisms that lead to overt T2D [24].

Next, we performed a DNA methylation profiling of *GIPR* promoter in DNA from peripheral blood and we sought for associations of methylation with blood-and T2D-based biomarkers. We found that *GIPR* promoter was hypomethylated in T2D patients as compared to controls. These results are consistent with a recent study which showed that hypomethylation in specific genomic regions in peripheral blood DNA was associated with T2D [11]. However, their analysis did not cover the genomic region we studied. To our knowledge, *GIPR* promoter methylation analysis in peripheral blood DNA between T2D patients and age- and BMI-matched controls has not been done before. There is great interest to perform methylation profiling in peripheral blood to find methylation disease-related associations since specific methylated regions could be used as potent biomarkers [25]. However, to understand how these methylated regions have a mechanistic role in the development of the disease of interest, the methylation analysis should focus in the target-tissues of the genes studied. *GIPR*, or gastric inhibitory polypeptide receptor, gene is expressed in various tissues, including β cells, adipose tissue, and brain [26]. It has been shown that *GIPR* expression is down-regulated in pancreatic tissue of T2D patients [27]. Here, we found that methylation of *GIPR* promoter in blood was negatively correlated with a surrogate marker of insulin resistance (HOMA-IR) and fasting glucose. In other words, decreased methylation in this promoter is associated with higher insulin resistance and higher fasting glucose. The subgroup analysis showed that this association was mostly relevant for T2D patients. The mechanisms underlying this association remain unknown and were not the purpose of the current research. On the other hand, methylation of *GIPR* promoter was not associated with HOMA-B. It has been shown that *GIPR* is involved in obesity and insulin resistance [28]. Recently, GIP was proposed as having a role in inflammation and insulin resistance by modulating the expression of osteopontin in adipose tissue [29]. Moreover, carriers of *GIPR* rs10423928 A-allele showed better insulin sensitivity [29]. The possible DNA methylation contribution to these effects has not been studied yet and warrants further study. Methylation patterns are thought to be tissue-specific [4,5], thus we might not extrapolate the methylation pattern found in blood to the methylation pattern present in adipose tissue. Further research is needed to define the role of methylation changes in *GIPR* promoter in adipose tissue and their potential impact on insulin resistance.

The strength of our research is that we have demonstrated that newly diagnosed and drug-naïve T2D patients have differences in specific hormones (adiponectin) and proinflammatory metabolites (especially IL 12) as compared to age- and BMI-matched controls. We also found that *GIPR* promoter was hypomethylated in T2D patients as compared to controls, as well as, new correlations between insulin resistance, fasting glucose and *GIPR* promoter methylation in DNA from peripheral blood. However, despite accounting for the major confounding factors (age, BMI, diabetes pharmacologic therapy), residual confounding and reverse causation remain possible. We cannot exclude a potential effect of the diet on methylation results in cases. However, there is not published data supporting that a low-carbohydrate diet would affect the methylation pattern of *GIPR* promoter in peripheral blood. We have already controlled for the potential effects of hyperglycemia and antidiabetic medication on the methylation values. A method for overcoming this issue, as proposed by Relton et al [30], is by applying a *"genetical epigenomics"* approach. In our case, this would mean to study the genetic variants that would be related to the methylation pattern, and then to verify whether the

correlation with methylation values and insulin resistance remains. However, this was out of scope of the present study.

In conclusion, our research showed that newly diagnosed and drug-naïve T2D patients have impaired β cell function and are more insulin resistant as compared to age- and BMI-matched controls. In addition, adiponectin was lower in T2D patients and correlated with β cell function. IL-12 levels in serum were almost double in T2D patients as compared to controls. The targeted epigenetic analysis in DNA from peripheral blood identified that *GIPR* promoter was hypomethylated in T2D patients as compared to controls. Hypomethylation of *GIPR* promoter correlated with higher fasting glucose and insulin resistance in T2D patients. Further research should unveil the potential role of these findings in the physiopathology of T2D.

Supporting Information

Table S1 Peripheral blood DNA methylation values (in %) for each CpG site analyzed in the *GIPR* promoter in type 2 diabetic patients and age- and BMI- matched controls*.
(DOCX)

Appendix S1 Quantitative DNA methylation analysis.
(DOCX)

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Author Contributions

Conceived and designed the experiments: SC RG. Performed the experiments: ER EF-R MP. Analyzed the data: SC BK. Contributed reagents/materials/analysis tools: ER EF-R MP. Wrote the paper: SC. Recruited patients: SC AS LG. Critical review and contribution to writing: FH AN.

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Table S1

Peripheral blood DNA methylation values (in %) for each CpG site analyzed in the *GIPR* promoter in type 2 diabetic patients and age- and BMI- matched controls*.

CpG site†	Position‡	Type 2 diabetic patients (n=93)	Controls (n=93)
CpG 1**	-2126	43.6±9.3	48.7±9.6
CpG 2***	-2097	63.8±9.7	72.9±8.0
CpG 3***	-2005	66.8±6.7	63.4±4.2
CpG 4	-1980	3.2±1.1	3.6±1.2
CpG 6***	-1842	2.5±0.7	4.3±2.1
CpG 7***	-1833	2.5±0.7	4.3±2.1
CpG 8**	-1781	90.6±7.2	94.3±7.1
CpG 9***	-1526	33.7±6.6	38.4±8.0
CpG 10***	-1517	33.7±6.6	38.4±8.0
CpG 17***	-1328	0.4±0.6	2.0±1.0
CpG 18	-1317	1.8±1.2	1.6±0.8
CpG 19	-1295	7.6±7.7	7.6±3.7
CpG 20	-1272	4.3±0.7	4.3±1.2

* Values shown are the mean ±SD. P values were calculated using the Mann-Whitney U test. Statistical significance was set at p<(0·05/13) using Bonferroni correction.
*** denotes P value lower than 0·0001.**denotes P value lower than 0·004 (0·05/13).

†CpG dinucleotides have been numbered relative to ATG.

‡CpG dinucleotide position has been determined according to the ATG position for the *GIPR* gene (ENSG00000010310).

Appendix S1:

Quantitative DNA methylation analysis

Primers used*

GIPR **CpGs 1-8 LEFT**	aggaagagagTGGTGTGTGTTTGGAATTTTAGTTA
GIPR **CpGs 1-8 RIGHT**	cagtaatacgactcactatagggagaaggctCTCACTCACAAATAAACAATCATCC
GIPR **CpGs 9-20 LEFT**	aggaagagagGTGGATGATTGTTTATTTGTGAGTG
GIPR **CpGs 9-20 RIGHT**	cagtaatacgactcactatagggagaaggctATCACTTACTCCTACAACCCCTACC

* LEFT: 10-mer tag sequence. RIGHT: T7 promoter tag with an 8 bp insert (for prevention of abortive cycling and constant 5' fragment for RNaseA reaction).
Capital letters indicate the actual sequence for the primers.

Methylation analysis.

Sequenom's MassARRAY platform was used to perform quantitative DNA methylation analysis. This system utilizes MALDI-TOF mass spectrometry in combination with RNA base-specific cleavage (MassCLEAVE). A detectable pattern is then analyzed for methylation status. PCR primers for the amplification of the promoter of the gene *GIPR* were designed using *Epidesigner* (Sequenom). When possible, amplicons were designed to cover CpG islands in the same region as the 5′ UTR. For each reverse primer, an additional T7 promoter tag for *in vivo* transcription was added, as well as a 10-mer tag on the forward primer to adjust for melting-temperature differences. The primers used appear in the above table.

PCR reactions were carried out in 5 μl total volume with 10 ng/ml bisulfite-treated DNA, 0.2 units *Taq*DNA polymerase (Sequenom), 1x supplied *Taq* buffer, and 200 mM PCR primers. Amplification was done as follows: preactivation of 95°C for 15 min, 45 cycles consisting of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s, finishing with a 72°C incubation for 4 min. Dephosphorylation of unincorporated dNTPs was performed by adding 1.7 ml of H_2O and 0.3 units of shrimp alkaline phosphatase (Sequenom), incubating at 37°C for 40 min and then at 85º C for 10 min, to deactivate the enzyme. The MassCLEAVE biochemistry was performed as follows: *in vivo* transcription and RNA cleavage was achieved by adding 2 μl of PCR product to 5 μl of transcription/cleavage reaction and incubating at 37°C for 3 h. The transcription/cleavage reaction contains 27 units of T7 RNA&DNA polymerase (Sequenom), 0.64x of T7 R&DNA polymerase buffer, 0.22 μl T Cleavage Mix (Sequenom), 3.14 mM DTT, 3.21 μl H_2O, and 0.09 mg/ml RNaseA (Sequenom). The reactions were additionally diluted with 20 ml H_2O and conditioned with 6 mg of CLEAN Resin (Sequenom) for optimal mass-spectra analysis.

5 DISCUSSION

The present thesis contains the results of two research papers that aimed to explore various aspects related with DNA methylation profiles in *GIPR* and *TCF7L2* genes in the context of type 2 diabetes. The study of methylation differences in two groups of subjects where the major difference was the presence of type 2 diabetes and the correlational study of methylation values with clinical and biochemical factors in these two groups have been the driving force of this work.

The idea of this thesis emerged from the initial observation that there was no other scientific paper investigating the pattern of DNA methylation in *GIPR* and *TCF7L2* genes comparing type 2 diabetic patients and non-diabetic subjects that were matched on age and body mass-index (BMI) with the diabetic patients. Further work of this thesis would be the understanding of the intimate mechanisms that lead to the methylation changes in the tissues where the genes have effects.

The papers presented in this thesis represent the result of a translational approach from basic science to clinical practice and have been performed in collaboration with the highly specialized teams of the Laboratory of Diabetes and Obesity, of the Department of Endocrinology and Nutrition, of the Primary Care Health Centre Les Corts and of the Biobank from the Hospital Clinic and Institute d'Investigacions Biomèdiques August Pi i Sunyer.

As in any scientific approach, each of the presented studies has its own limitations and the results presented in this work are open to debate. Below, I will discuss and go deeper into the major aspects and conclusions which emerge from the articles of this thesis.

Metabolic, hormonal and cytokine differences between recently diagnosed, drug-naïve patients with T2D and age- and BMI-matched controls.

Type 2 diabetic patients and controls were similar in age and BMI to control for any confounder effect of age and obesity on the results. Moreover, none of the type 2 diabetic patients were on any pharmacological therapy for diabetes. Thus, no confounding effect of antidiabetic drugs or insulin therapy was possible either. Type 2 diabetic patients received counselling about exercise and healthy diet in order to control their diabetes. This could explain why the % of physically inactive subjects was higher in the control group as compared to the type 2 diabetic patients. The majority of type 2 diabetic patients (67%) were on statins as compared to controls. This could explain the differences in mean total cholesterol between the two groups. Type 2 diabetic patients were in optimal glycemic control (mean glycated hemoglobin 5.8%) and had their clinical diagnosis of T2D recently. Hence, no potential influence of hyperglycemia on the methylation pattern was possible, or, if any, was low.

Results showed that type 2 diabetic patients were more insulin-resistant than controls, since they presented higher values of HOMA-IR. In concordance to this, type 2 diabetic patients had a higher waist circumference as compared to controls. Higher waist circumference is one component used for the diagnosis of the metabolic syndrome and previous research showed that it correlates with poorer glucose control in type 2 diabetic patients(170). Insulin resistance is associated with metabolic syndrome too(171). Basically, in spite of the fact that T2D patients and controls had a similar grade of obesity, T2D patients presented a differential body fat distribution (particularly centralized obesity). This correlates with a differential adipokine secretion which might lead to a higher degree of insulin resistance in T2D patients. In contrast, and as

expected, β-cell function was already impaired in type 2 diabetic patients as compared to controls (HOMA-B was significantly lower in type 2 diabetic patients as compared to controls). These data illustrates the fact that impairment of β-cell function is worse in type 2 diabetic patients as compared to age- and BMI- matched controls. These results are in concordance with the existing literature(172-174).

In addition, we found that T2D patients had lower adiponectin levels in serum than controls. Epidemiological studies have shown that higher adiponectin levels in serum are associated with a lower risk of T2D. Moreover, adiponectin has been proposed as a strong biochemical predictor of T2D(175). Adiponectin is exclusively and abundantly expressed in adipose tissue and has been shown to have insulin-sensitizing and anti-inflammatory properties(176). In fact, in our study, we found that fasting adiponectin presented a negative correlation with HOMA-IR (Spearman coefficient r = - 0.28, $P<0.001$) and a positive correlation with HOMA-B (r = 0.19, $P<0.01$), which supports the insulin-sensitizing properties of adiponectin. On the other hand, lower levels of the anti-inflammatory IL-10 were found in T2D patients, which is consistent with previous research that showed that low levels of IL-10 are associated with T2D(177). IL-12 serum levels were almost double in T2D patients than in controls. A recent study showed that elevated serum IL-12 was present at the onset of T2D, and that further increases in IL-12 correlated with endothelial dysfunction and cardiovascular disease progression(178). In addition, it has also been showed that IL-12 might have a role in β-cell dysfunction(179).

Overall, the first part of the present work demonstrates that T2D patients have an impaired β-cell function and are more insulin resistant than age and BMI-matched controls. These differences in β-cell function and insulin resistance are related to differences in adipokines and inflammatory metabolites, which might be the underlying

mechanisms that lead to overt T2D(180).

DNA methylation profiles in *GIPR* and *TCF7L2* promoters in T2D patients and age and BMI-matched non-diabetic subjects.

In this work, we report the methylation pattern of *TCF7L2* and *GIPR* promoters from peripheral blood DNA in drug-naïve type 2 diabetic patients and age- and BMI-matched controls. We found that several CpGs had significant differences between type 2 diabetic patients and controls, although overall the methylation patterns did not show a clear differential pattern related to T2D. These results are consistent with previous data of promoter methylation patterns from peripheral blood DNA where a global directional change in methylation levels that would affect all neighboring CpGs systematically and that would be characteristic of the disease has not been identified(181).

We found that *GIPR* promoter was hypomethylated in T2D patients as compared to controls. These results are consistent with a recent study which showed that hypomethylation in specific genomic regions in peripheral blood DNA was associated with T2D(155). In relation to *TCF7L2* promoter, methylation data was very disperse. Due to the high variability of the methylation values and the low correlation between all of them, we thought it was not accurate to use the mean promoter methylation as principal measure of methylation. Thus, we decided to study each CpG site individually (instead of using the mean of all the CpG sites). The absolute differences in methylation values between T2D patients and controls were not very substantial. However, when applying the statistical tests, both non-parametric tests and logistic regression models and after correcting for multiple testing using Q-values, we found that 13 out of 22 CpG

sites (59%) remained significantly different between T2D patients and controls in *TCF7L2* promoter.

On the other hand, a recent study found some T2D-related methylation patterns in peripheral blood DNA(155) but their analysis did not cover the genomic regions we studied. There is great interest to perform methylation profiling in peripheral blood to find methylation disease-related associations since specific methylated regions could be used as potent biomarkers(182). In cancer research, where the search for epigenetic biomarkers has started earlier than in metabolic disorders, promoter-methylation regions are being studied for their potential use as diagnostic, predictive and prognostic biomarkers(183). In this regard, methylation patterns detected in accessible tissues as blood are of special interest. For example, it is now available a commercial detection kit for early detection of colorectal cancer by analyzing *SEPT9* promoter methylation in plasma(183, 184). Furthermore, others have discovered that some methylated marks in plasma might predict clinical response to treatment(185). Regarding diabetes research, some progress has been done as well in terms of biomarker discovery. For example, in type 1 diabetes, it is now available a methodology for detecting a surrogate marker of β-cell death by identifying a differentiated circulating methylated region(186, 187). This novel approach will be useful for tracking the disease progression as well as the clinical response to treatment. In type 2 diabetes, there is still no known new biomarker which could detect subjects at risk for future development of the disease. Prospective studies including subjects with prediabetes that will be followed until they develop the disease are needed. In this sense, our research shows that differentially methylated regions exist between T2D patients and matched controls in accessible tissues such as peripheral blood in specific genomic regions. Further research is needed to corroborate these

findings in prediabetic subjects in order to possibly detect useful predictive or diagnostic epigenetic biomarkers of Type 2 Diabetes.

Correlation of DNA methylation values with blood- and T2D-related markers.

As mentioned above, it has been proposed that epigenetic mechanisms could be involved in the complex interplay between genes and the environment. DNA methylation is a reversible process that can be modulated by both stochastic and environmental stimuli. A previous study has shown that T2D-related methylation may be reflected in accessible tissues such as peripheral blood(155).

In our study, we found that methylation of specific CpG sites on *TCF7L2* promoter in blood was correlated with fasting glucose, total cholesterol and LDL-cholesterol. In line with our results, it has been recently shown that β-cells cultured with high-glucose-lipid medium presented aberrant DNA methylation in different loci, among which was *TCF7L2* gene promoter(188). Moreover, Hu et al showed that, while *TCF7L2* promoter was hypermethylated, *TCF7L2* mRNA expression increased, and, unexpectedly, the protein expression of *TCF7L2* was decreased in β cells(188). The mechanisms of this opposite regulation remain unknown, although it could be speculated that DNA methylation may affect the *TCF7L2* splice variants(189), i.e., the increase in mRNA levels could represent transcripts of *TCF7L2* which would encode less active isoforms(188).

With reference to *GIPR* gene, we speculated that it could be affected by alterations in DNA methylation in T2D patients, which could explain the dysregulation of GIP action in T2D patients. To our knowledge, *GIPR* promoter methylation analysis in peripheral blood DNA between T2D patients and age and BMI-matched controls has not been

done before. *GIPR*, or gastric inhibitory polypeptide receptor, gene is expressed in various tissues, including β-cells, adipose tissue, and brain(190). It has been shown that GIPR expression is downregulated in pancreatic tissue of T2D patients(191). Here, we found that methylation of *GIPR* promoter in blood was negatively correlated with a surrogate marker of insulin resistance (HOMA-IR) and fasting glucose. In other words, decreased methylation in this promoter is associated with higher insulin resistance and higher fasting glucose. The subgroup analysis showed that this association was mostly relevant for T2D patients. The mechanisms underlying this association remain unknown and were not the purpose of the current research. On the other hand, methylation of *GIPR* promoter was not associated with HOMA-B. It has been shown that *GIPR* is involved in obesity and insulin resistance(192). Recently, GIP was proposed as having a role in inflammation and insulin resistance by modulating the expression of osteopontin in adipose tissue(193). Moreover, carriers of *GIPR* rs10423928 A allele showed better insulin sensitivity(193). The possible DNA methylation contribution to these effects has not been studied yet and warrants further study.

Methylation patterns are thought to be tissue specific(154, 194, 195), thus we might not extrapolate the methylation patterns found in blood to those present in β-cells (referring to the case of *TCF7L2* gene) or adipose tissue (in relation to the results of *GIPR* gene). Further research is needed to define the role of methylation changes in *GIPR* and *TCF7L2* promoters in the relevant tissues for each gene and their potential impact on insulin resistance and Type 2 Diabetes. Nevertheless, the first methylome reference in human pancreatic islets has been just published(196). Dayeh et al performed a genome-wide DNA methylation analysis of human pancreatic islets from type 2 diabetic and non-diabetic donors(196). In this study, *TCF7L2* gene presented differential methylation values in diabetic pancreatic islets as compared to non-diabetic pancreatic islets. It

should be noted though that the region they studied in *TCF7L2* gene is further downstream (3') than the region we studied. In addition, no mention about *GIPR* gene was made(196).

Limitations and strengths of the work

One limitation of the present research is that we did not account for cellular heterogeneity in the analysis. Indeed, recently it has been shown that DNA methylation pattern may vary between blood cell types. In our study, we cannot distinguish DNA from specific cell types in the whole blood. Thus, we cannot exclude the possibility that the differential methylation level might reflect differences in the proportions of different cell types. However, at recruitment, there were no differences in blood cell counts amongst T2D patients and controls. It would have been good to include complete blood counts (CBCs) as covariates in the statistical analysis, as well as cell type proportions estimated by blood count. However, we did not have access to these data.

Another possible limitation could be that we did not validate the results by using a second methodology of DNA methylation analysis. However, the methylation analysis was performed following Sequenom's Epityper procedure and protocols, which include an internal quality control of the methylation data. Such control refers to the control of the signal/noise ratio, the deviation of the expected fragment size, presence of primer's dimers, guanine depurination control, etc. In addition, together with the samples and controls a 4-peak calibrator is included in order to calibrate the obtained spectra.

In relation to the bisulfite conversion, it was done for all the samples (all T2D patients and controls) together, with the same reactive preparation, and same operator. The methylation analysis was done during the same day for all the samples (T2D patients

and controls). Methylation data was generated in duplicate for each CpG. The analysis was done by the same operator during the same day in the same machine. For each run, a fully methylated positive control was included. Thus, any batch-effect would be of minimal effect.

In relation to data normalization, the MassArray R package supports assay design and data analysis for the mass-spectrometry-based Sequenom EpiTYPER assay(197). Poor-quality and non-valuable data for the quantitative methylation of each CpG unit measured by MALDI-TOF-MS were excluded. CpG units that yielded data in greater than 25% of the samples passed initial quality control. From these data, samples that yielded data for greater than 80% for all CpG units within an amplicon were met standard for inclusion in further analysis for that sample/amplicon pair. Outliers values were removed and recoded as missing values for the statistical analysis.

Overall, even though we used a single methylation analysis methodology, all precautions were made to account for possible methodological errors and, if any still may be present, it would be of minimal effect.

In addition, despite accounting for the major confounding factors (age, BMI, diabetes pharmacologic therapy), residual confounding and reverse causation remain possible. We cannot exclude a potential effect of the diet on methylation results in the T2D patients. However, there is not published data supporting that a low-carbohydrate diet would affect the methylation pattern of *GIPR* and *TCF7L2* promoters in whole blood DNA. We have already controlled for the potential effects of hyperglycemia and antidiabetic medication on the methylation values. A method for overcoming this issue, as proposed by Relton et al(198), is by applying a ‘‘*genetical epigenomics*’’ approach. In our case, this would mean to study the genetic variants that would be related to the

methylation pattern, and then to verify whether the correlation with methylation values and blood-parameters remains. However, this was out of scope of the present study.

The strength of our research is that we have demonstrated that newly diagnosed and drug-naïve T2D patients have differences in specific hormones (adiponectin) and proinflammatory metabolites (especially IL 12) as compared to age- and BMI-matched controls. In addition, we proved that type 2 diabetic patients have differences in concrete CpGs sites of *TCF7L2* and *GIPR* promoters as compared to age- and BMI-matched controls. In particular, we found that *GIPR* promoter was hypomethylated in T2D patients as compared to controls, as well as, new correlations between insulin resistance, fasting glucose and *GIPR* promoter methylation in DNA from peripheral blood. We also present novel correlations between fasting glucose, total cholesterol and LDL-cholesterol with DNA methylation in specific CpG sites of *TCF7L2* promoter in DNA from peripheral blood.

Overall, our research proves the presence of epigenetic alterations in patients with T2D as compared to age- and BMI-matched controls in genomic regions that have been previously linked to T2D and hyperglycemia, as *TCF7L2* and *GIPR* promoters. These novel results enlighten the current view of the association between epigenetic alterations and known genomic risk regions for T2D and open new streams of research on this topic.

6 CONCLUSIONS

1. β-cell function is already compromised in early stage of Type 2 Diabetes, in spite of the degree of obesity and aging, which correlates with a detrimental profile of insulin-sensitizing and anti-inflammatory factors, such as adiponectin, and a rise in pro-inflammatory factors, such as IL-12.

2. Type 2 Diabetes is associated with an altered epigenetic signature in target genomic regions such as *TCF7L2* and *GIPR* promoters in DNA from whole blood. This altered epigenetic signature correlates with specific blood-related parameters (glucose, HOMA-IR) specially in the diabetic patients. The precise functions of these epigenetic alterations in the target tissues were the genes affected have a role remain unknown.

3. Our findings add to the growing understanding of the interplay between epigenetic alterations and risk genes for Type 2 Diabetes such as *TCF7L2* and *GIPR* genes. Novel lines of research are opened aiming to unveil the potential role of these data in the physiopathology of the disease.

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8 SUMMARY IN SPANISH

Introducción

La diabetes mellitus tipo 2 es una enfermedad metabólica multifactorial que se desarrolla debido a una desregulación del metabolismo de la glucosa como consecuencia de defectos en la secreción de insulina, disminución de la sensibilidad a la insulina o una combinación de ambos. La prevalencia de la diabetes tipo 2 está aumentado en las últimas tres décadas y se prevé que siga aumentando en los próximos años hasta llegar a afectar a un considerable porcentaje de la población. Con referencia a los datos de España, recientemente se realizó un estudio poblacional (el estudio Di@betes) donde se vió que la prevalencia actual de la diabetes mellitus es del 14% y que la prevalencia del estado de "prediabetes" oscila entre el 2% y el 9%. Adicionalmente, se ha demostrado que el padecer de diabetes confiere una disminución en los índices de calidad de vida en comparación a sujetos no diabéticos. El incremento en la prevalencia de la enfermedad está íntimamente ligado al aumento en la tasa de obesidad. El sobrepeso y la obesidad son los factores de riesgo más importantes de la diabetes tipo 2. Sin embargo, no todos los obesos desarrollan la enfermedad así como también algunos diabéticos son delgados. Por otra parte, la diabetes tipo 2 tiene un gran componente genético, tal como lo han demostrado los estudios genéticos, empezando por estudios de asociación de genes candidatos hasta estudios poblacionales que interrogan todo el genoma (*"Genome-wide association studies" GWAS*). Globalmente se han identificado más de 60 regiones genómicas (*SNPs*) que confieren riesgo para desarrollar la enfermedad. Entre ellos, el *SNP* que comporta el mayor riesgo de diabetes tipo 2 es el que se encuentra en el gen *TCF7L2*. El gen *TCF7L2* codifica para un factor de transcripción del mismo nombre. Los mecanismos concretos por los que el polimorfismo en el gen confiere riesgo para la diabetes tipo 2 se desconocen todavía. Se cree que hay una alteración en la expresión del gen la cual provoca una disminución en

la secreción de insulina a nivel de la célula β pancreática. Por otro lado, recientemente se descubrió que un polimorfismo en el gen *GIPR* se asociaba con los niveles glucémicos postprandiales. El gen *GIPR* codifica para el receptor de GIP, o polipéptido inhibidor gástrico. GIP es un polipéptido sintetizado por las células K del duodeno y el intestino delgado. Se demostró implicado en la estimulación de la liberación de insulina en presencia de glucosa elevada (a nivel posptrandial). Posteriormente, el efecto insulinotrópico en los islotes de células β pancreáticas fue reconocido como la acción fisiológica principal de GIP. Junto con el péptido similar al glucagón-1 (GLP-1), GIP es en gran parte responsable de la secreción de insulina después de comer. En los pacientes con diabetes tipo 2 se ha visto una disminución en los niveles de expresión de *GIPR* asi como una supresión de la respuesta en la secreción de insulina después de la acción de GIP, con lo que se ha sugerido que alteraciones a nivel de *GIPR* podrían estar relacionadas con la patogenia de la diabetes tipo 2.

Sin embargo, gran parte del riesgo genético sigue sin explicarse por los datos encontrados hasta la fecha, concepto que se conoce como heredabilidad perdida ("*missing heritability*"). La epigenética, término que literalmente se define como "más allá de la genética", podría tener un papel importante en parte de la heredabilidad perdida de enfermedades complejas, como la diabetes tipo 2. La epigenética estudia los cambios heredables en la función génica que se producen sin un cambio en la secuencia del ADN. Hay varios mecanismos epigenéticos de regulación génica. Los más importantes son: la metilación del ADN, la modificación de las histonas, y el ARN no codificante. La metilación del ADN es la adición de un grupo metilo a la base citosina. Por lo general la metilación se da en mayor grado en las islas CpG (regiones con alta concentración de citosina y guanina) las cuales forman parte de la región promotora de los genes. La metilación del ADN es un regulador muy importante en la transcripción

de los genes. Se ha demostrado que la metilación aberrante del ADN está asociada con el silenciamiento no programado de los genes y los genes que tienen niveles muy altos de 5-metilcitosina en su región promotora son generalmente transcripcionalmente silenciados. La metilación del ADN es esencial en el desarrollo embrionario, y en las células somáticas. Los patrones de metilación del ADN son, en general, transmitidos a las células hijas con gran fidelidad. Los patrones aberrantes de metilación del ADN han sido asociados con un gran número de enfermedades del ser humano y se han encontrado de dos maneras distintas: hipermetilación e hipometilación, comparado con los estándares normales. La hipermetilación es una de las mayores modificaciones epigenéticas responsable de reprimir la transcripción vía la región promotora de los genes. La hipometilación también ha sido implicada en el desarrollo y progresión de enfermedados complejas a través de diferentes mecanismos. La metilación del ADN puede modificar se con cambios ambientales, tales como cambios en la dieta o por el stress, y puede ser reversible.

La diabetes tipo 2 es una enfermedad compleja con gran componente ambiental así como base genética. El enfoque actual es estudiar la interacción del ambiente con la susceptibilidad genética afín de mejorar la comprensión de la fisiopatología de la enfermedad. En éste sentido, las alteraciones epigenéticas cobran gran importancia. Estudios recientes han demostrado la presencia de alteraciones epigenéticas en varios tejidos: sangre, músculo, islotes pancreáticos. Adicionalmente, alteraciones en los patrones de metilación pueden conllevar a importantes efectos metabólicos. Por ejemplo, alteraciones epigenéticas pueden provocar una disminución de expresión de genes claves para la identidad de la célula β. Asimismo, también se ha propuesto que la metilación del ADN se asocia en parte a la memoria hiperglucémica, o, memoria metabólica, a través de mecanismos todavía desconocidos. Finalmente, se han detectado

a nivel de sangre periférica marcas epigenéticas que podrían identificar sujetos a riesgo posterior de padecer la enfermedad.

Hipótesis

En base a los conocimientos resumidos anteriormente, se plantea la hipótesis que los pacientes diabéticos tipo 2 se diferencian respecto a sujetos no diabéticos de misma edad e índice de masa corporal (IMC) en patrones de metilación específicos que podrían implicar cambios en la expresión génica en los tejidos diana implicados en la fisiopatología de la diabetes tipo 2.

En vista de la interacción entre el medio ambiente y la genética, y, que los patrones de metilación pueden cambiar en respuesta a los factores ambientales, se deduce que los patrones de metilación relacionados con la diabetes estarían presentes en los genes clave implicados en la fisiopatología de la diabetes tipo 2.

Entre los genes que puedan verse afectados por cambios en la metilación, pensamos que tanto los genes *TCF7L2* y *GIPR* pueden presentar patrones de metilación específicos en pacientes diabéticos tipo 2, en comparación con sujetos no diabéticos apareados por edad e IMC.

Como el patrón de metilación podría influir en la expresión génica, estudiamos la región promotora de los genes seleccionados.

Por último, como las marcas epigenéticas pueden ser detectadas a partir de tejidos fácilmente accesibles, se estudiaran las marcas de metilación en el ADN de la sangre total en los dos grupos de sujetos.

Para completar, con el fin de evitar cualquier efecto de confusión por parte de los fármacos antidiabéticos en los datos de metilación, se seleccionará un grupo de

pacientes diabéticos tipo 2 de reciente diagnóstico, que no estén bajo tratamiento farmacológico para la diabetes tipo 2.

Objetivos

1er Objetivo. Diseño de la población en estudio (casos y controles).

A. Selección e inclusión de pacientes diabéticos tipo 2 de reciente diagnóstico tratados sólo con dieta (casos) de un centro de atención primaria.

B. Selección e inclusión de sujetos no diabéticos apareados por edad e IMC (controles) de un centro de atención primaria.

C. Estudio comparativo del perfil metabólico, hormonal y de citoquinas en los dos grupos de estudio.

2do Objetivo. Estudio del patrón de metilación del ADN en el promotor del gen TCF7L2 en pacientes diabéticos tipo 2 (casos) y sujetos no diabéticos apareados por edad e IMC (controles), utilizando como fuente de ADN un tejido de fácil acceso (sangre periférica).

A. Estudio comparativo de los valores de metilación en el promotor del gen *TCF7L2* entre pacientes diabéticos tipo 2 (casos) y sujetos no diabéticos apareados por edad e IMC (controles) en el ADN de sangre periférica.

B. Estudio correlativo entre los valores de metilación del promotor del gen *TCF7L2* y parámetros clínicos y bioquímicos en pacientes diabéticos tipo 2 (casos) y sujetos no diabéticos apareados por edad y IMC (controles).

3er Objetivo. Estudio del patrón de metilación del ADN en el promotor del gen GIPR en pacientes diabéticos tipo 2 (casos) y sujetos no diabéticos apareados por edad e

IMC (controles), utilizando como fuente de ADN un tejido de fácil acceso (sangre periférica).

A. Estudio comparativo de los valores de metilación en el promotor del gen *GIPR* entre pacientes diabéticos tipo 2 (casos) y sujetos no diabéticos apareados por edad e IMC (controles) en el ADN de sangre entera.

B. Estudio correlativo entre los valores de metilación del promotor del gen *GIPR* y los parámetros clínicos y bioquímicos en pacientes diabéticos tipo 2 (casos) y sujetos no diabéticos apareados por edad e IMC (controles).

Resultados

Todos los sujetos incluidos en el estudio tenían sobrepeso y la media de edad era de 68 años. Había una similar proporción de hombres en los dos grupos. Los pacientes con diabetes tipo 2 presentaban un perímetro de cintura mayor que los controles (102.7 cm vs 97.9 cm, P=0.002). Los pacientes con diabetes tipo 2 realizaban más ejercicio físico que los controles (28% de sedentarismo en los pacientes con diabetes vs. 53.8% en los controles, P<0.001). La proporción de no fumadores era similar en los dos grupos.

Los pacientes con diabetes tipo 2 eran más insulino-resistentes que los controles apareados por edad e IMC (media de HOMA IR 2,6 en los diabéticos vs 1,8 en los controles, P<0,001) y tenían una función de célula β más pobre (media de HOMA B 75,7 en los diabéticos vs 113,6 en los controles, P<0,001). Los valores de adiponectina en ayunas fue inferior en los pacientes con diabetes tipo 2 en comparación con los controles (7,0 ± 3,8 μgr / ml vs 10,0 ± 4,2 μgr / mL, P<0,001). Los niveles de IL 12 en suero eran casi el doble en los pacientes con diabetes tipo 2 en comparación con los controles (52,8 ± 58,3 pg / ml vs 29,7 ± 37,4 pg / ml, P<0,001).

Los resultados mostraron que el 59% de las CpGs analizadas en el promotor de *TCF7L2* mostraron diferencias significativas entre los pacientes diabéticos tipo 2 y controles apareados por edad e IMC. Además, la glucosa en ayunas, HOMA-B, HOMA-IR, el colesterol total y el colesterol LDL correlacionaban con la metilación en CpGs específicas del promotor de *TCF7L2*. Después de ajustar por edad, índice de masa corporal, el género, la inactividad física, la circunferencia de la cintura, el tabaquismo y el estado de la diabetes, únicamente la glucosa en ayunas, el colesterol total y el colesterol LDL se mantuvieron significativos. Por otra parte, se encontró que el promotor de *GIPR* estaba hipometilado en los pacientes con diabetes tipo 2 en comparación con los controles. Además, HOMA-IR y la glucosa en ayunas presentaban una correlación negativa con la metilación del promotor de *GIPR*, especialmente en los pacientes con diabetes tipo 2.

Discusión

La primera parte del trabajo demuestra que los pacientes con diabetes tipo 2 de reciente diagnóstico tienen una función de las células β alterada y son más insulino-resistentes que los controles apareados por edad e IMC. Estas diferencias en la función de las células β y la resistencia a la insulina están relacionadas con diferencias en los perfiles de adipoquinas así como metabolitos inflamatorios, que podrían reflejar parte de los mecanismos subyacentes que conducen a la diabetes tipo 2 manifiesta.

Los resultados encontrados en relación al patrón de metilación de los dos promotores son consistentes con los datos previos sobre patrones de metilación de promotores en ADN de sangre periférica en los que no se identificó, hasta la fecha, un cambio de dirección global en los niveles de metilación que afectase a todos las CpGs sistemáticamente y que sería característica de la enfermedad.

Por otro lado, un estudio reciente encontró algunos patrones de metilación relacionados con la diabetes tipo 2 en ADN de sangre periférica, pero su análisis no cubrió las regiones genómicas que hemos estudiado. Hay un gran interés para realizar perfiles de metilación en sangre periférica para encontrar asociaciones de metilación relacionadas con la enfermedad ya que regiones metiladas específicas podrían ser utilizadas como biomarcadores potentes de diagnóstico o prognóstico. En éste sentido, los patrones de metilación detectados en tejidos accesibles como sangre periférica son de especial interés. En la diabetes tipo 2, todavía no se conoce un biomarcador que podría detectar sujetos con riesgo para desarrollar en el futuro la enfermedad. Se necesitan estudios prospectivos que incluyan sujetos con prediabetes que se seguirían hasta que desarrollasen la enfermedad. En éste sentido, nuestra investigación muestra que existen regiones diferencialmente metiladas en regiones específicas del genoma entre los pacientes con diabetes tipo 2 y controles apareados por edad e IMC en tejidos accesibles, como la sangre periférica. Se necesitan más investigaciones para corroborar estos hallazgos en pacientes prediabéticos y así poder detectar potenciales nuevos biomarcadores epigenéticos predictivos o de diagnóstico de la diabetes tipo 2.

En nuestro estudio, encontramos que la metilación de sitios CpGs específicas del promotor *TCF7L2* en sangre se correlaciona con la glucosa en ayunas, colesterol total y LDL-colesterol. En línea con nuestros resultados, se ha demostrado recientemente que la células β cultivadas con un medio alto en glucosa y lípidos presentan metilación aberrante del ADN en diferentes loci, entre los que se encuentra el promotor de *TCF7L2*.

Por otro lado, se encontró que la metilación del promotor de *GIPR* en la sangre se correlaciona negativamente con un marcador subrogado de la resistencia a la insulina (HOMA-IR) y con la glucosa en ayunas. En otras palabras, la disminución de la

metilación en este promotor se asocia con una mayor resistencia a la insulina y un aumento en la glucosa en ayunas. El análisis de subgrupos mostró que esta asociación era sobre todo relevante en los pacientes con diabetes. Los mecanismos que subyacen a esta asociación se desconocen y no eran el objetivo de la investigación actual.

Se cree que los patrones de metilación son específicos de tejido, por lo tanto no se pueden extrapolar los patrones de metilación encontrados a nivel de sangre periférica a los patrones presentes en células β (en referencia al caso del gen *TCF7L2*) o tejido adiposo (en relación con los resultados del gen *GIPR*). Por consiguiente, se abren nuevas vías de investigación para definir el papel de los cambios de metilación en los promotores de *GIPR* y *TCF7L2* en los tejidos diana de cada gen así como estudiar el impacto potencial en la resistencia a la insulina y la diabetes tipo 2.

La fuerza de nuestra investigación es que hemos demostrado la presencia de alteraciones epigenéticas en pacientes con diabetes tipo 2 en comparación con controles emparejados por edad e IMC en ciertas regiones del genoma que han sido previamente vinculados a la diabetes tipo 2 y a la hiperglucemia, como los genes *TCF7L2* y *GIPR*. Estos nuevos resultados aclaran la visión actual de la asociación entre las alteraciones epigenéticas y regiones genómicas de riesgo conocido para la diabetes tipo 2 y se abren nuevas líneas de investigación sobre este tema.

Conclusiones

1. La función de las células β está ya comprometida en la etapa temprana de la diabetes tipo 2, independientemente del grado de la obesidad y de la edad, la cual se correlaciona con un perfil perjudicial de factores que favorecen la sensibilidad a la insulina y factores anti-inflamatorios, tales como la adiponectina, así como a un aumento en los factores proinflamatorios, como la IL-12.

2. La diabetes tipo 2 se asocia con una marca circulante epigenética alterada en regiones genómicas diana tales como los promotores de *TCF7L2* y *GIPR* en el ADN de sangre periférica. Esta firma epigenética alterada se correlaciona con parámetros específicos en sangre (glucosa, HOMA-IR) especialmente en los pacientes diabéticos. Las funciones precisas de estas alteraciones epigenéticas en los tejidos diana de los genes afectados son desconocidos.

3. Nuestros resultados se suman a la creciente comprensión de la interacción entre las alteraciones epigenéticas y los genes susceptibles de riesgo para la diabetes tipo 2, tales como *TCF7L2* y *GIPR*. Con éste estudio, se abren nuevas líneas de investigación con el objetivo de conocer el papel potencial de éstos datos en la fisiopatología de la enfermedad.