INTRODUCTION

1. DIABETES MELLITUS

Diabetes mellitus defines a group of metabolic disorders characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both.

It is one of the most common metabolic syndromes, since there are 200 million diabetic individuals in the world; this creates a need to understand the etiology of the disease and the factors influencing its onset.

Several pathogenic processes are involved in the development of diabetes; these range from autoimmune destruction of the β-cells of the pancreas with consequent insulin deficiency to abnormalities that result in resistance to insulin action. Deficient action of insulin on target tissues and hyperglycemia are the basis of the abnormalities in carbohydrate, fat, and protein metabolism, causing diabetes'characteristic clinical features, micro and-macrovascular complications and increased risk of cardiovascular disease *(1)*.

The new classification system (American Diabetes Association 2004) identifies four types of diabetes mellitus: type 1, type 2, "other specific types" and gestational diabetes.

Type 1 diabetes mellitus (T1D) is characterized by β-cell destruction caused by an autoimmune process, usually leading to absolute insulin deficiency *(2, 3)*. This form of diabetes, which accounts for only 5–10% of all diabetes, is a juvenile-onset diabetes; it results from a cellular-mediated autoimmune destruction of the β-cells of the pancreas by CD4 and CD8 T cells and macrophages infiltrating the islets. In this case insulin therapy is required for survival, to prevent the development of ketoacidosis, coma and death.

Gestational diabetes mellitus (GD) is defined as any degree of glucose intolerance with onset or first recognition during pregnancy. It is a common condition affecting about 7% of all pregnancies; its detection is important because of associated maternal and fetal complications. Pregnancy is a diabetogenic condition itself (placental secretion of hormones, such as progesterone, cortisol, placental lactogen, prolactin, and growth hormone), characterized by insulin resistance with a compensatory increase in β-cell response and hyperinsulinemia. Insulin resistance usually begins in the second trimester and progresses throughout the remainder of the pregnancy; insulin sensitivity is reduced by as much as 80% *(4)*. The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus *(2, 5)* recognized an intermediate group of subjects whose glucose levels, although not meeting criteria for diabetes, are nevertheless too high to be considered normal. This category includes the impaired glucose tolerance (IGT) and the impaired fasting glucose (IFG).

Patients with IFG and/or IGT are now referred to as having "pre-diabetes" indicating the relatively high risk for development of diabetes in these patients. In the absence of pregnancy, IFG and IGT are not clinical entities in their own right but rather risk factors for future diabetes as well as cardiovascular disease. They can be observed as intermediate stages in any of the disease processes listed in *Table I*

Table I. *Etiologic classification of diabetes mellitus*

I. Type 1 diabetes (β-cell destruction, usually leading to absolute insulin deficiency)
- A. Immune mediated
- B. Idiopathic

II. Type 2 diabetes (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with insulin resistance)

III. Other specific types
- A. Genetic defects of _-cell function
  1. Chromosome 12, HNF-1_ (MODY3)
  2. Chromosome 7, glucokinase (MODY2)
  3. Chromosome 20, HNF-4_ (MODY1)
  4. Chromosome 13, insulin promoter factor-1 (IPF-1; MODY4)
  5. Chromosome 17, HNF-1_ (MODY5)
  6. Chromosome 2, *NeuroD1* (MODY6)
  7. Mitochondrial DNA
  8. Others
- B. Genetic defects in insulin action
  1. Type A insulin resistance
  2. Leprechaunism
  3. Rabson-Mendenhall syndrome
  4. Lipoatrophic diabetes
- C. Diseases of the exocrine pancreas
  1. Pancreatitis
  2. Trauma/pancreatectomy
  3. Neoplasia
  4. Cystic fibrosis
  5. Hemochromatosis
  6. Fibrocalculous pancreatopathy
- D. Endocrinopathies
  1. Acromegaly
  2. Cushing's syndrome
  3. Glucagonoma
  4. Pheochromocytoma
  5. Hyperthyroidism
  6. Somatostatinoma
  7. Aldosteronoma
- E. Drug- or chemical-induced
  1. Vacor
  2. Pentamidine
  3. Nicotinic acid
  4. Glucocorticoids
  5. Thyroid hormone
  6. Diazoxide
  7. β-adrenergic agonists
  8. Thiazides
  9. Dilantin
  10. α-Interferon
- F. Infections
  1. Congenital rubella
  2. Cytomegalovirus
- G. Uncommon forms of immune-mediated diabetes
  1. "Stiff-man" syndrome
  2. Anti–insulin receptor antibodies
- H. Other genetic syndromes sometimes associated with diabetes
  1. Down's syndrome
  2. Klinefelter's syndrome
  3. Turner's syndrome
  4. Wolfram's syndrome
  5. Friedreich's ataxia
  6. Huntington's chorea
  7. Laurence-Moon-Biedl syndrome
  8. Myotonic dystrophy
  9. Porphyria
  10. Prader-Willi syndrome

IV. Gestational diabetes mellitus (GDM)

Patients with any form of diabetes may require insulin

2. TYPE 2 DIABETES MELLITUS (T2D)

Type 2 Diabetes Mellitus (T2D) is a complex heterogeneous group of metabolic condition characterized by elevated levels of serum glucose; according to WHO, it is defined as resulting from a defect in both insulin secretion and in insulin sensitivity. β-cell dysfunction includes abnormalities in pulsatility and in kinetics of insulin secretion, quantitative and qualitative abnormalities of insulin, β-cell loss and its progression.

T2D exerts a huge toll in human suffering and economy. A recent evaluation using a computerized generic formal disease model revealed that excess global mortality due to diabetes in the year 2000 was equivalent to 5.2% of all deaths and diabetes is likely to be the fifth leading cause of death, similar in magnitude to numbers reported for HIV/AIDS *(6).* The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030, with India, China and USA being the top 3 countries estimated to have the highest numbers of people with diabetes *(7).*

2.a Etiology of T2D

The recent global epidemic of T2D almost certainly indicates the importance of environmental triggers over last several decades. In all over the world, the diabetes epidemic is due to the increase in prevalence of obesity, linked to "westernized" lifestyle, namely changes in nutritional habits, with increased intake of saturated fats, refined sugars and alcohol, and reduced intake of fibres, and at the same time, reduction in physical activity. The comparison between Pima Indians from Arizona and Pima Indians from a remote area in Mexico, and native Mexicans showed the major role of environmental factors compared to genetic factors in the occurrence of diabetes *(8).* The role of environment has also been demonstrated from many years

by urban–rural comparisons of diabetes prevalence, higher in the urban areas inside any ethnic group, in a lot of epidemiological studies all around the world.

Nonetheless, T2D is among many complex diseases for which a genetic contribution is well accepted. Identification of the genetic components of type 2 diabetes is one of the most important areas of diabetes research because elucidation of the diabetes genes will influence all efforts toward a mechanistic understanding of the disease, its complications, and its treatment, cure, and prevention.

Multiple lines of evidence support the view that genetic components plays an important role in the pathogenesis of T2D *(9)*.

- *The spectrum of T2D prevalence in different ethnic groups':* The prevalence of T2D varies widely among populations, from 1% in Chile Mapuche Indian, 2% among Caucasians in Europe to as high as 41% in the Nauru (Pacific Island) and 50% among Pima Indians in Arizona. Part of this observed ethnic variability can be attributed to non-genetic environmental and cultural factors; however, the observation that the disease prevalence varies substantially among ethnic groups that share a similar environment supports the idea that genetic factors contribute to disease predisposition *(9)*.

- *Familial aggregation:* Other than genes, families share environments, culture and habits, yet familial aggregation of the disease is another source of evidence for a genetic contribution to the disease. Evidence for a genetic role includes the nearly 4-fold increased risk for T2D in siblings of a diabetic proband compared with the general population, the odds ratio (OR) of 3.4–3.5 with only a single affected parent, and the increase in the OR to 6.1 if both parents are affected *(10)*.

- *Twin studies:* Multiple studies of twin concordance rates have been undertaken in T2D. Estimates for concordance rates have ranged from 0.29 to 1.00 in monozygotic (MZ) twins, while in dizygotic (DZ) twins the range was 0.10–0.43 *(11-14)*.

Concordance among both MZ and DZ twins increases with the duration of follow up period *(14)*. In spite of several caveats in twin studies, the high concordance in MZ twins and the 50% fall in DZ twins provides compelling evidence for a genetic component of T2D.

- *Heritability of intermediate phenotypes:* Insulin sensitivity and insulin secretion deteriorate in parallel in most human T2D. Both defects predicted subsequent T2D in several studies and both defects are shown to be present in nondiabetic but genetically identical co-twins of a diabetic proband *(15)*. Data from multiple laboratories support a genetic basis for measures of both insulin sensitivity and insulin secretion *(16-18)*.

The relations between genetic and environmental factors in the development of T2D may be complex. Environmental factors may be responsible for the initiation of β-cell damage or other metabolic abnormalities, while genes may regulate the rate of progression to overt diabetes; indeed, in some cases genetic factors may be necessary for environmental factors even to start processes leading to the development of the disease *(19)*.

2.b Pathophysiology of T2D

The early pathophysiology of T2D remains uncertain and controversial. Three key defects mark the onset of hyperglycemia in T2D: increased hepatic glucose production, diminished insulin secretion, and impaired insulin action *(20, 21)*. Unfortunately, at the time of hyperglycemia, glucose and possibly lipid toxicity obscure the primary effects. Prospective and cross-sectional analyses of euglycemic individuals at risk (relatives of T2D individuals) circumvent this dilemma, and suggest a key early and predictive role of reduced insulin sensitivity in T2D pathogenesis. Nonetheless, few individuals with genetic insulin resistance develop

diabetes, and 25% of nondiabetic individuals may have insulin sensitivity as low as that seen in T2D. The increase in hepatic glucose production was formerly viewed as a late event, occurring only at the onset of hyperglycemia and glucose intolerance. However, knock out murine models suggest a more primary role for hepatic insulin resistance.

3. Insulin

It is one of the most potent anabolic hormone; its major function is to counter the concerted action of a number of hyperglycemia-generating hormones and to maintain low blood glucose levels.

The molecule contains two peptide chains linked together by two disulfide bonds; specific protease activity cleaves the center third of the molecule, which dissociates as C peptide, leaving the amino terminal B peptide disulfide bonded to the carboxy terminal A peptide.

Insulin is synthesized as a preprohormone in the β-cells of the islets of Langerhans in response to increased circulating levels of glucose. Then, it is directly infused via the portal vein to the liver, where it exerts profound metabolic effects. These effects are the response of the activation of the insulin receptor which belongs to the class of cell surface receptors that exhibit intrinsic tyrosine kinase activity *(22)*. This receptor is a heterotetramer of 2 extracellular α-subunits disulfide bonded to 2 transmembrane β-subunits; it consists of extracellular ligand binding and intracellular tyrosine kinase domains. With respect to hepatic glucose homeostasis, the binding of insulin to the extracellular portion of the receptor activates its kinase activity resulting in autophosphorylation of specific intracellular tyrosine residues that lead to an increase in the storage of glucose with a concomitant decrease in hepatic glucose release to the circulation.

3.a Insulin resistance

It has emerged as an impaired biological response to insulin caused by reduced insulin-stimulated glucose uptake in skeletal muscle and by impaired suppression of endogenous glucose production, which are critical for maintaining normal glucose homeostasis. Initially, is present a compensatory increase in insulin secretion to maintain normal blood glucose levels and, if this is sufficient, blood glucose concentration increases in both the fasting (impaired fasting glucose) and postprandial (impaired glucose tolerance) states. The coexistence of insulin resistance and hyperinsulinaemia appears to contribute directly or indirectly to several disorders, such as typical forms of type 2 diabetes mellitus, dyslipidemia, hypertension, atherosclerosis and a pro-coagulant state *(23)*.

Numerous studies focused on dysregulation of insulin post-receptor signalling have established associations between molecular events and insulin resistance (glucose, glucosamine, fatty acids) and glycated proteins, which often contribute to insulin resistance by increasing Ser/Thr phosphorylation of IRS-1. TNF-α and IL-6 decrease insulin signalling by impairing the insulin-stimulated tyrosine phosphorylation of IRS molecules and by inducing the suppressors of cytokine signalling, SOCS-1 and -3, which have at least three different mechanisms of action: they compete with IRS-1 for association with insulin receptor, they inhibit Janus kinase, involved in insulin signalling, and they augment proteosomal IRS-1 degradation *(24)*.

High concentrations of circulating fatty acids also contribute to impair the induction of insulin-induced PI3K activation and inhibit insulin-mediated glucose metabolism by affecting a number of protein kinases in rats. Finally, glicated proteins, emerging as a consequence of hyperglycemia, were shown to diminish intracellular PI3K, PKB and GSK-3 activity, thus possibly contributing to insulin resistance *(25)*.

4. PED/PEA-15 protein

During a study using a differential display technique to identify genes whose expression was altered in type 2 diabetes, our group has demonstrated the involvement of a "new" gene named pea-15 (Phosphoprotein Enriched in Astrocytes), which has been re-named as PED (Phosphoprotein enriched in Diabetes).

Ped/pea-15 is a 15 kDa cytosolic protein widely expressed in different tissues and highly conserved among mammals *(26-28)*, whose gene maps on human chromosome 1q21-22 *(29)*.

Several studies in cultured cells and in rodent tissues have revealed that ped/pea-15 regulates multiple cellular functions by binding components of major intracellular transduction pathways.

Ped/pea-15 activates Extracellular Regulated Kinase 1/2 *(28, 30)*. It binds ERKs in the nucleus exporting it into the cytoplasm, preventing the entrance into the cell cycle caused by sustained phospho-ERK nuclear accumulation *(30, 31)*.

Ped/pea-15 also interacts with Akt and p90 ribosomal S6 kinase isozyme (RSK2), two key elements of the phosphoinositide 3-kinase (PI 3-K) and ERK signalling pathways whose activation is central to control of cell survival (*32, 33*).

In addition, ped/pea-15 exerts a wide anti-apoptotic action. It binds Fas associated death domain (FADD) and caspase-8 (FLICE) , thereby inhibiting FLICE activation by tumor necrosis factor-α and Fas-L, resulting in protection from cytokine-induced apoptosis *(34-36)*.

Ped/pea-15 inhibits p38 and JNK activation by stress inducing agents at a very upstream step in the stress-activated protein kinase activation cascade *(37)*.

This protein also binds to Omi/HtrA2, a serine protease and a mitochondrial protein, inhibiting apoptosis triggered by stress and physical agents *(38)*. Importantly, there

is evidence indicating that ped/pea-15 anti-apoptotic action has an important role in development and progression of certain cancers in humans as well as in rodents *(39-41)*.

Recent reports further revealed that ped/pea-15 binds to and enhances phospholipase D stability, resulting in increased intracellular levels of diacylglycerol *(42, 43)*. This effect dysregulates PKC signalling and generates resistance to insulin action on glucose transport in muscle and adipose cells and in tissues from transgenic mice overexpressing the ped/pea-15 gene *(43, 44)*. Inhibited glucose-regulated insulin secretion has also been evidenced in these animals contributing to their impaired glucose tolerance. The ped/pea-15 transgenic mice develop diabetes upon weight gain indicating an important interaction between obesity and the ped/pea-15 gene *(44)*.

4.a Role of PED-PEA-15 in insulin resistance

To investigate the role of ped/pea-15 gene in the insulin resistance, several studies have been performed *in vitro* and *in vivo*.

In vitro, ped/pea-15 cDNA has been stably transfected in L6 skeletal muscle and 3T3-L1 adipose cells to levels comparable to those occurring in type 2 diabetes. These cells overexpressing ped/pea-15 showed an impaired glucose uptake with a lack of further insulin-dependent uptake (and the use of an antisense oligonucleotide to inhibit endogenous ped/pea-15 expression in muscle cells and adipocytes significantly expanded insulin sensitivity of glucose uptake). Thus, ped/pea-15 may represent a physiological regulator of glucose transporter trafficking and glucose transport in the major insulin-responsive skeletal muscle and adipose tissues *(29)*.

In vivo, we reported that transgenic mice overexpressing ped/pea-15 to levels comparable to those occurring in human type 2 diabetes exhibit mildly elevated

random-fed blood glucose levels and become hyperglycaemic after glucose loading, indicating that increased expression of this gene is sufficient to impair glucose tolerance. Moreover, transgenic mice become diabetic when body weight increases after administration of high-fat diets, indicating that, *in vivo*, the overexpression of ped/pea-15 in conjunction with environmental modifiers may lead to diabetes. Based on the insulin tolerance tests, ped/pea-15 transgenic mice were less sensitive to insulin as compared with their nontransgenic littermates. Furthermore, transgenic mice were consistently hyperinsulinemic in the basal state and exhibited increased free fatty acid levels in blood: these findings indicate the presence of insulin resistance in ped/pea-15 mice. This abnormality has been associated with a decrease in insulin stimulated glucose uptake. The transgenic mice showed reduced insulin response to a glucose challenge, indicating that the overexpression of ped/pea-15 impairs insulin secretion in addition to insulin action *(44)*.

It has been demonstrated that both ped/pea-15 mRNA and protein levels were overexpressed in cultured skin fibroblasts from type 2 diabetics compared with non-diabetic individuals. Also skeletal muscle and adipose tissues, two major sites of insulin resistance in type 2 diabetes, showed the same behaviour. This overexpression was independent of obesity, suggesting it may contribute to the primary component of insulin-resistance in these subjects *(29)*.

Thus, these findings identify ped/pea-15 as a novel gene controlling insulin action and insulin secretion contributing, under appropriate environmental conditions, to genetic susceptibility to type 2 diabetes in humans.

AIM OF THE STUDY

Earlier findings from our group showed that ped/pea-15 is overexpressed in peripheral tissues from type 2 diabetics.

The aim of this work has been to investigate ped/pea-15 expression in a large population of individuals affected by type 2 diabetes and related phenotypes; to this end efforts have been devoted to the identification of ped/pea-15 in human blood cells which may represent a useful proxy system.

Finally, my purpose has been to understand if ped/pea-15 expression is associated with both environmental and genetic diabetes risk factors.

PATIENTS AND METHODS

Subjects

The subjects investigated in this study have been consecutively recruited in the outpatient facilities of the Department of Preventive Medical Sciences, the Metabolic Unit at the Federico II University of Naples Medical School and the Metabolic Unit at the University of Catanzaro Magna Graecia (offsprings from the European Network on Functional Genomics of Type 2 Diabetes - EUGENE2- study). Written informed consent was obtained from all participants. The study protocol has been approved by the Ethics Committee of the participating institutions and conducted in accordance to the principles of the Declaration of Helsinki.

The euglycemic individuals (n=150) were healthy subjects receiving no pharmacological treatment and were undergoing a routine health survey at the Department of Preventive Medical Sciences; the presence of type 2 diabetes-affected first-degree relatives (FDR) in the pedigrees was ascertained through a written questionaire and the medical history.

The type 2 diabetic patients (n=142) were recruited at the Metabolic Unit; of them, 19% were treated with diet alone, 48% with oral hypoglicemic agents, 30% with insulin and 3% with insulin and oral hypoglicemic agents. The mean diabetes duration in this group was 11 ± 9 years. The mean glycated hemoglobin (HbA_{1c}) level was 7 ± 1.48% of these diabetics showed no evidence of long-term complications; 24% had microvascular and 12% had macrovascular complications; 16% had both micro- and macrovascular complications. Anthropometric indexes (BMI was calculated by dividing the weight in kilograms by the square of the height in meters; waist circunference was measured midway between the lowest rib margin and the iliac crest to the nearest 0.5 cm) and detailed medical history (including information

on type 2 diabetes-affected relatives and smoking habit) were obtained from all of the participants. Blood pressure values were measured in the left arm of the supine patient, after 5 min of quiet rest, with a mercury sphigmomanometer. In each subject, whole-blood samples were drawn from an antecubital vein in the morning, after an overnight fast on two independent occasions. Plasma glucose, total and HDL cholesterol, and triglyceride and serum insulin levels were determined according to *(45, 46)*. Type 2 diabetes and impaired fasting glucose (IFG) diagnosis were established according to criteria described previously *(47)*. The clinical characterization of the T2D offsprings from the EUGENE2 cohort has been previously reported *(46, 48, 49)*.

For studies in matched groups (n=115) further individuals were recruited. Inclusion criteria in this arm of the study were as follows: age 52-58 years, BMI 26-32 kg/m^2 waist circumference 90-100 cm, systolic BP 120-140 mmHg, diastolic BP 70-85 mmHg, total cholesterol 4.9-5.4 mmol/l, HDL cholesterol 1.2-1.4 mmol/l, serum triacylglicerol 1.4-1.6 mmol/l. Fifty-six type 2 diabetics (mean disease duration 11±4 years, mean HbA_{1c} level 7.2±4.0%) and 59 healthy euglycaemic subjects (of which 34 had no family history of type 2 diabetes and 25 had at least one type 2 diabetes-affected FDR) were consecutively enrolled. Studies in euglycaemic offspring of type 2 diabetics were performed using the EUGENE2 cohort at the University of Catanzaro Magna Graecia *(46, 48, 49)*.

The OGTT and euglycaemic hyperinsulinemic clamp procedures have also been reported in *(46)*. Briefly, for clamp studies, insulin (Humulin, Eli Lilly & Co., Indianapolis, IN) was given as a prime continuous infusion to produce plasma insulin levels of about 420 pmol/liter. Thereafter, the insulin infusion rate was fixed at 40 mU/m^2/min. The blood glucose level was maintained constant throughout the study by infusing 20% glucose at varying rates according to blood glucose

measurements performed at 5-min intervals. Duration of the clamp was 120 minutes. Glucose disposal is expressed as milligrams per kilogram of lean body per mass per minute. Insulin secretion was estimated by the homeostasis model assessment of pancreatic β-cell function (HOMA-B) index according to *(50)* and the insulinogenic index as the difference between the 30- and 0-min OGTT plasma insulin values divided by the difference between the corresponding plasma glucose values (ΔI30/ΔG30) according to *(51)*.

Methods

White blood cells separation and tissue sampling. For peripheral blood leukocyte (PBL) separation, EDTA-treated whole-blood samples were first centrifuged at 300 g for 10 min and plasma removed. PBL were separated using a 6% dextran gradient in filtered phosphate buffered saline (PBS), pH 7.4 according to *(52)*, washed in PBS 3-times, counted and resuspended in 1 ml PBS for subsequent use. Neutrophils and other major leukocyte subpopulations were further separated as previously described *(52, 53)*. The purity of the different cell populations was confirmed by microscopic examination. Biopsies were obtained from subcutaneous adipose tissue from the anterior abdominal wall and skeletal muscle from rectus abdominis; they were obtained simultaneously from patients undergoing elective abdominal surgery for gall bladder disease. PBLs were collected at the same time as fat and muscle specimen. For this part of the study, the diabetic patients and the euglycemic subjects (with and without type 2 diabetic FDR in their pedigree) were consecutively recruited to achieve a 1:1:1 ratio between the three groups (total: 21 patients). Tissues were rinsed and dissected free of erythrocytes and connective tissues as previously described *(54)*.

Cell and tissue harvesting and Western blotting. PBL and tissue samples were solubilized at 4C in TAT buffer (50 mM HEPES, pH 7.5, 150 mmol NaCl, 10 mmol EDTA, 10 mmol $Na_4P_2O_7$, 2 mmol Na_3VO_4, 100 mmol NaF, 10% glycerol, 1% TRITON X-100) supplemented with 1mmol phenylmethanesulfonylfluoride and 10 μg/ml aprotinin according to *(54)*. Lysates were centrifuged at 500 x g for 20 min and supernatants were frozen at –20°C until used. For Western blotting, 50 μg of lysate proteins were heated at 100°C in Laemmli buffer *(55)*. Proteins were separated by 15% SDS-PAGE and then transferred to 0.45 mm Immobilon-P membranes (Millipore, Bedford, MA). Filters were probed with ped/pea-15 antibodies *(29)*, revealed by enhanced chemiluminescence and autoradiography, and ped/pea-15 bands quantitated by laser densitometry. Inter-assay variation was < 15%.

RNA samples and Real-time RT-PCR. RNA from white blood cells were immediately extracted following cell isolation using the QIAamp RNA Blood Mini Kit (Quiagen, Valencia, CA) according to the manufacturer's instructions. Ped/pea-15 gene expression was measured by real-time RT-PCR (SYBRGreen, iCycler IQ Multicolor Real-Time Detection System, Biorad, USA). The following primer pairs were designed using the Beacon Designer Software based on published gene sequences (GeneBank Accession Nos. AH008227 and M10277, respectively). Human hped1 (forward) 5'-GAGCGCTCAGCTCCAGAGG-3'; human hped3 (reverse) 5'-CAGGACGGCGGGAGATCT-3'; human β-actin (forward) 5'-TGCGTGACATTAAGGAGAAG-3'; human β-actin (reverse) 5'-GCTCGTAGCTCTTCTCCA-3'. Real-time RT-PCR was performed with a cDNA input of 50 ng total RNA with all combinations of primers. Data acquisition and analysis were authomatically performed by the iCycler IQ Multicolor Real-Time Detection System Optical System Software V.3.1 (Biorad, USA).

Statistics. Data are means ± SE. Comparisons between groups were tested by unpaired student's *t* test or the Mann-Whitney *U* test and among groups by one-way ANOVA. Mean values, after adjusting for covariates, were evaluated by ANCOVA. A p value <0.05 was considered significant. Analyses were performed by the statistical package SPSS version 12 (Chicago, IL).

RESULTS

To identify tissues where ped/pea-15 gene expression can be non invasively quantitated, we first analyzed human peripheral blood leukocyte (PBL) lysates by immunoblotting with specific ped/pea-15 antibodies. In samples from 21 subjects, these assays revealed expression of ped/pea-15 with no significant difference between the granulocyte, lymphocyte and monocyte cells (Fig.1).

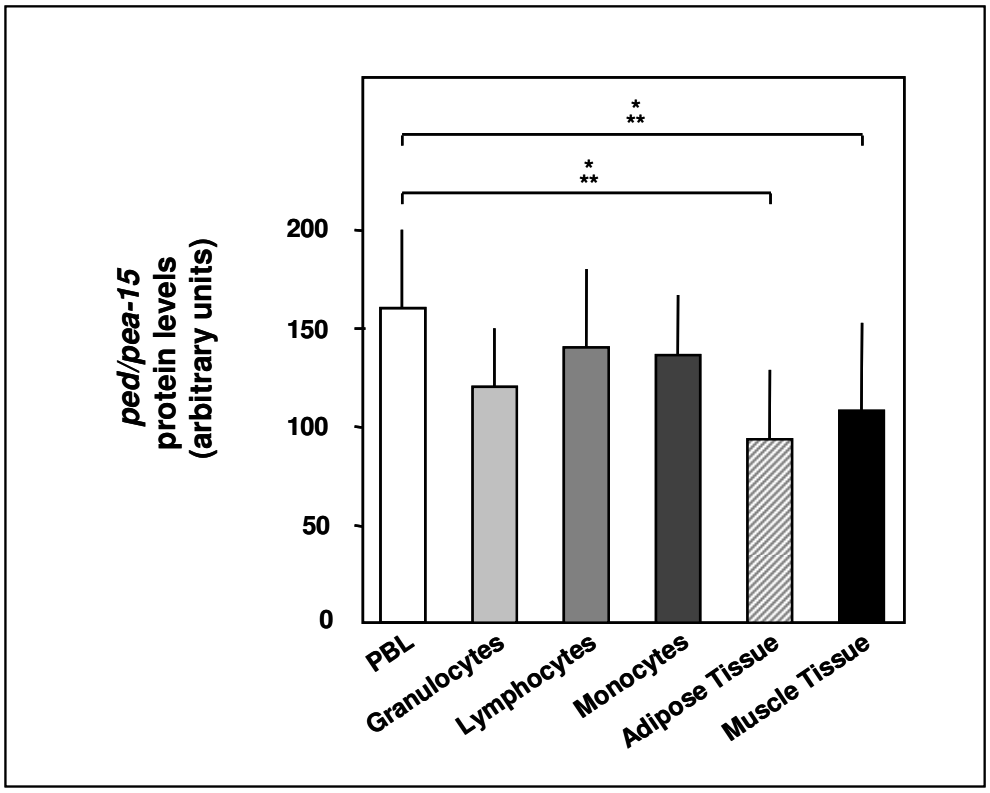

Fig.1 ***Expression of ped/pea-15 protein in human white blood cells.*** Peripheral blood leukocytes (PBL) and the relative granulocyte, lymphocyte and monocyte fractions were prepared from 21 individuals (T2D: euglycemic FDR of the T2D: to euglycemic control individuals = 1:1:1) as described under Research design and methods. Cells and tissues were solubilized and analyzed by Western blotting with ped/pea-15 antibodies as described under Research design and methods. Blots were revealed by ECL and autoradiography and ped/pea-15 levels quantitated by laser densitometry. Bars represent the means ± SE of 4 independent experiments.

On a per milligram protein basis, the expression of ped/pea-15 in these cells was 30% higher than that occurring in human skeletal muscle and adipose tissues ($p < 0.001$). Importantly, in these same subjects, a significant correlation existed between ped/pea-15 expression in the PBL and in fat as well as in skeletal muscle tissues ($p < 0.001$; Fig. 2, 3).

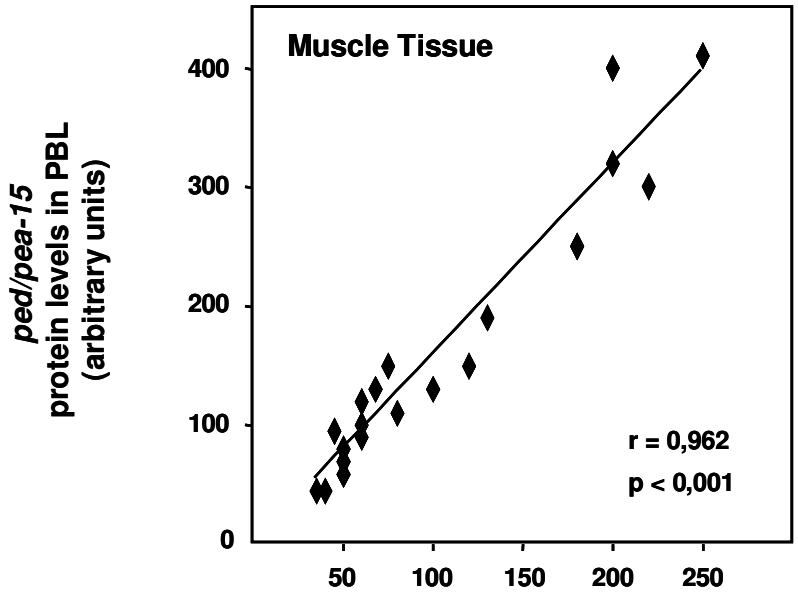

Fig.2 ***Expression of ped/pea-15 protein in human white blood cells and in muscle cells.*** Correlation between *ped/pea-15* expression in PBL and muscle was tested by linear regression analysis as described under Research design and methods.

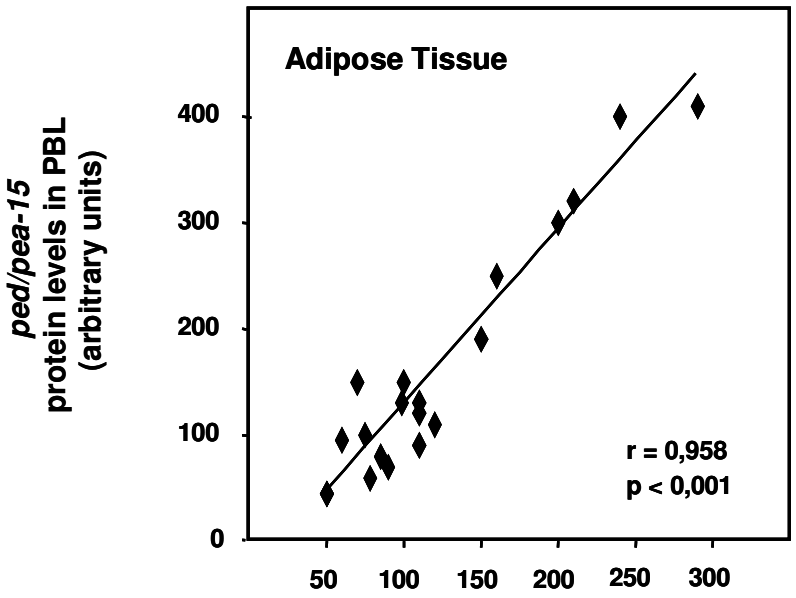

Fig.3 ***Expression of ped/pea-15 protein in human white blood cells and in adipose cells.*** Correlation between *ped/pea-15* expression in PBL and fat was tested by linear regression analysis as described under Research design and methods.

We then compared the expression of ped/pea-15 in PBL lysates from 30 consecutive euglycemic individuals with at least one type 2 diabetes (T2D)-affected relative (FDR; these subjects are known to be at increased risk of diabetes *(56, 57)*) and in 120 euglycemic subjects with no family history of disease. Their clinical characteristics are shown in Table II.

TABLE II. ***Clinical and biochemical features of the euglycemic and type 2 diabetic individuals***

	EuF- ***n* = 120**	**EuF+** ***n* = 30**	**T2D** ***n* = 142**
Gender (M/F)	84/36	14/16	90/52
Age (years)	49 ± 9	49 ± 6	58 ± 10**
Smokers (%)	27	20	21
BMI (kg/m^2)	28 ± 5	28 ± 3	29 ± 4
SBP (mmHg)	126 ± 15	128 ± 16	134 ± 21***
DBP (mmHg)	79 ± 9	81 ± 9	80 ± 9
Total serum cholesterol (mg/dl)	236 ± 45	223 ± 39	201 ± 36***
HDL cholesterol (mg/dl)	53 ± 14	55 ± 12	45 ± 16***
Serum triglycerides (mg/dl)	133 ± 62	115 ± 43	168 ± 144***
Fasting plasma glucose (mg/dl)	90 ± 7	90 ± 6	170 ± 55***

Data are the means ± SD. or %. BMI, body mass index; SBP systolic blood pressure; DBP diastolic blood pressure. **difference vs. EuF- significant at the $p < 0.01$ level; ***difference vs. EuF- significant at the $p < 0.001$ level.

A 2-fold increase in ped/pea-15 protein expression was found in the FDR ($p < 0.001$; Fig.4). This increased expression was of similar magnitude compared to that observed in an additional group of 142 T2D patients whose clinical features are also shown in Table II.

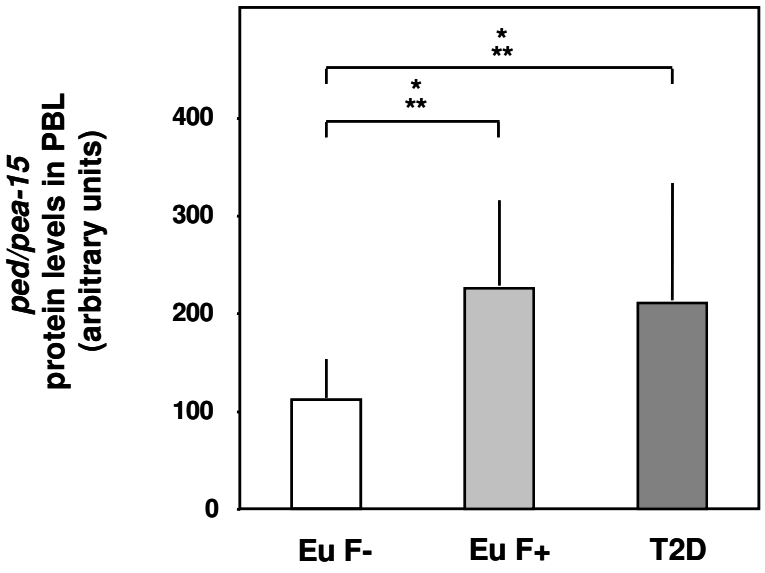

Fig.4 ***PBL expression of ped/pea-15 in euglycemic first-degree relatives of T2D.*** PBL from 30 euglycemic individuals with at least one T2D first-degree relative (EuF+) and 120 euglycemic subjects lacking family history of diabetes (EuF-) were consecutively collected. For comparison, PBL from 142 T2D patients were also analyzed. Cells were solubilized and ped/pea-15 protein levels were quantitated in the lysates. Bars represent the mean ± SE of 3 independent experiments, each in duplicate.

As in the case of the T2D patients, close to one third of the FDR subjects expressed ped/pea-15 protein amounts higher than 2 SDs from the mean of the euglycemic subjects lacking family history of diabetes (Fig.5).

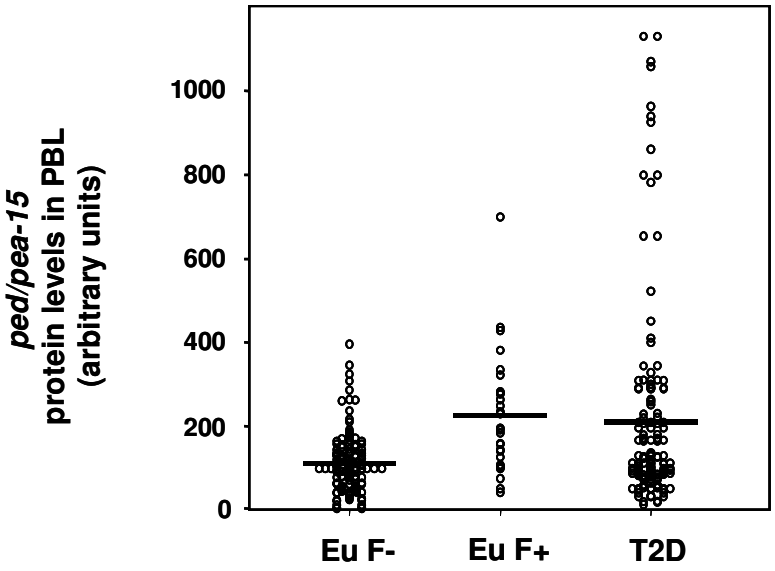

Fig.5 ***PBL expression of ped/pea-15 in euglycemic first-degree relatives of T2D.*** Expression levels (average of 3 determinations) of each individual subject.

The increased levels of ped/pea-15 protein detected in PBL from the FDR and the T2D subjects corresponded to a similarly sized increase in ped/pea-15 mRNA (Fig.6), indicating that the overexpression previously demonstrated in isolated fibroblasts, skeletal muscle and adipose tissues from T2D individuals *(29)* can be detected in PBL as well, and involves, at least in part, a transcriptional abnormality.

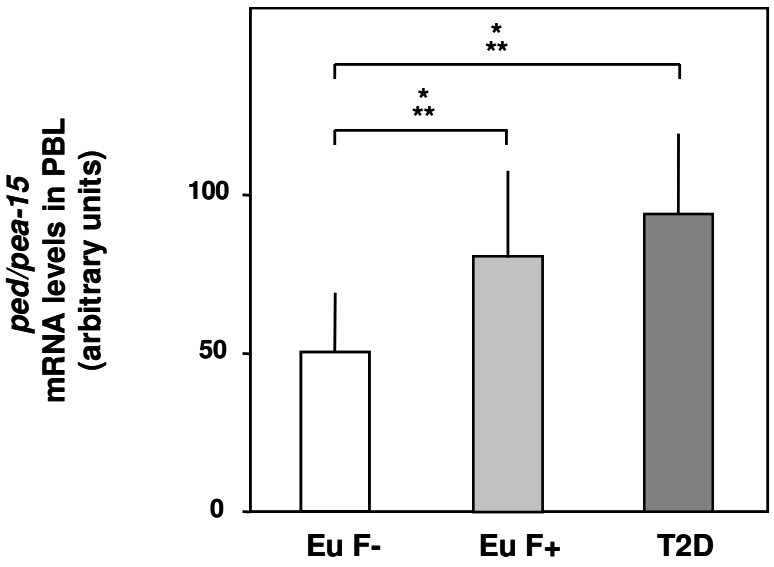

Fig.6 ***mRNA levels in PBL of euglycemic first-degree relatives of T2D.*** RNA was extracted and ped/pea-15 mRNA quantitated by real-time PCR as described under Research design and methods. Bars represent the mean ± SE of 3 independent experiments.

Same as in our previous report *(29)*, there was no difference in ped/pea-15 expression in the obese and lean individuals in the present study, whether euglycemic or diabetic. To address the effect of other major T2D risk factors, we have analyzed the expression of ped/pea-15 in 55 further individuals diagnosed impaired fasting glucose (IFG) according to *(47)*. Ten of these subjects had at least one T2D-affected FDR in their pedigree (IFG F+), while the others had no family history of disease (IFG F-). PBL levels of ped/pea-15 in subjects from this latter subgroup were found to be very comparable to those detected in euglycemic individuals lacking family history of T2D (Fig.7).

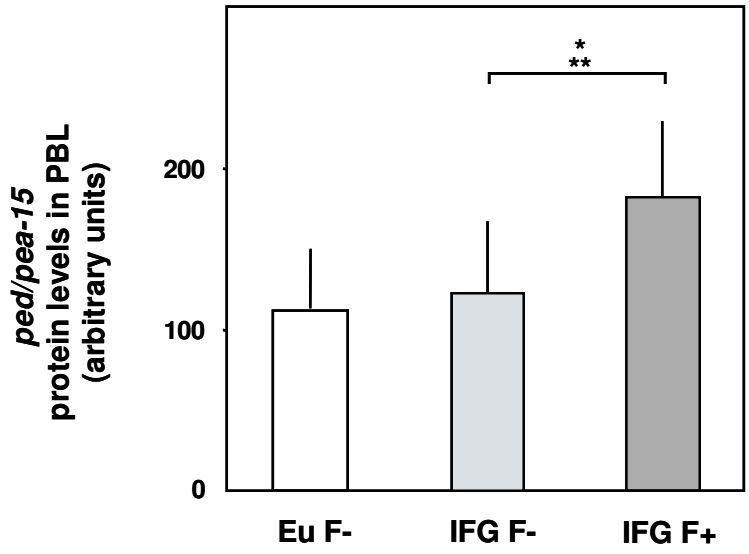

Fig.7 ***T2D-associated phenotypes and risk-factors related to ped/pea-15 expression.*** PBL were obtained from 55 consecutive individuals with IFG diagnosed according to (21). 10 of these subjects had at least one T2D-affected first-degree relative (IFGF+) while the others exhibited no family history of diabetes (45; IFGF-). Cells were solubilized and ped/pea-15 protein levels were quantitated in the lysates. For comparison, data obtained from PBL from the euglycemic subjects lacking family history of diabetes and shown in Fig.4 (EuF-) are also presented. Bars represent the mean ± SE of 3 independent experiments, each in duplicate.

As in the case of the euglycemic individuals however, those IFGs exhibiting at least one T2D-affected FDR expressed *ped/pea-15* to a level that was significantly higher than that detected in the IFGs with no T2D FDR ($p < 0.05$), further pointing to an important effect of the family history on *ped/pea-15* expression level in euglycemic and IFG humans.

In the euglycemic subjects (both the T2D FDRs and those with no T2D family history) and in the T2D patients described in Table I, there were no gender-related differences in *ped/pea-15* expression. Also, no significant correlation was found between the expression of *ped/pea-15* and the age, BMI, waist circumference, SBP and

DBP, and fasting cholesterol, triglyceride and glucose levels of the subjects, indicating no influence of these variables on *ped/pea-15* expression in humans. To examine this hypothesis further, we have compared *ped/pea-15* levels in euglycemic subjects lacking family history of diabetes and in T2D subjects upon matching for age, BMI, waist circumference, SBP and DBP and fasting HDL cholesterol and triglyceride levels (Table III).

TABLE III. ***Clinical and biochemical features of the matched euglycemic and T2D individuals***

	EuF- ***n* = 36**	**T2D** ***n* = 58**	***P***
Gender (M/F)	30/6	36/22	-
Age (years)	54 ± 5	56 ± 7	N.S.
Smokers (%)	25	24	N.S.
BMI (kg/m2)	29 ± 4	29 ± 4	N.S.
Waist circ. (cm)	93 ± 10	95 ± 8	N.S.
SBP (mmHg)	132 ± 13	132 ± 24	N.S.
DBP (mmHg)	82 ± 7	79 ± 10	N.S.
Total serum cholesterol (mg/dl)	208 ± 35	203 ± 32	N.S.
HDL cholesterol (mg/dl)	48 ± 10	48 ± 10	N.S.
Serum triglycerides (mg/dl)	128 ± 42	137 ± 56	N.S.
Fasting plasma glucose (mg/dl)	92 ± 5	171 ± 56	< 0.001

Data are the means ± SD. or %. BMI, body mass index; SBP systolic blood pressure; DBP diastolic blood pressure.

A 2-fold increase in *ped/pea-15* expression was again evidenced in the diabetics ($p < 0.01$; Fig.8), supporting ped/pea-15 independence of these variables in humans.

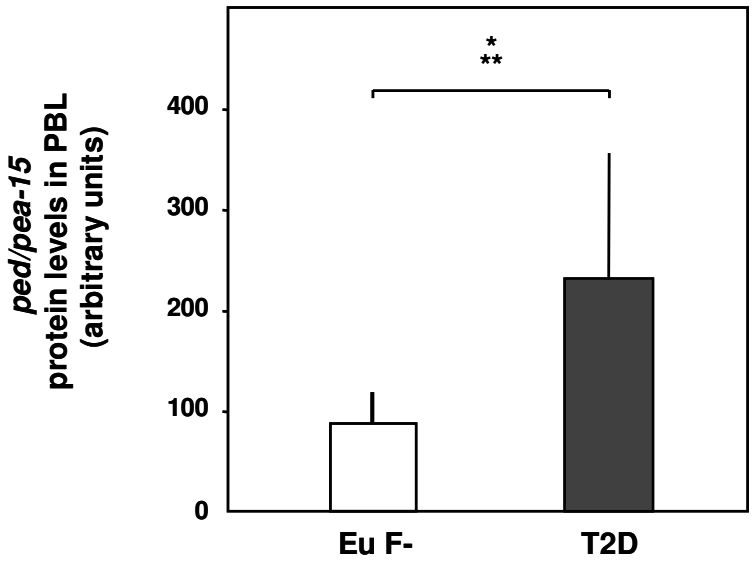

Fig.8 ***T2D-associated phenotypes and risk-factors related to ped/pea-15 expression.*** Two subgroups of the euglycemic subjects lacking family history of type 2 diabetes (36; EuF-) and the type 2 diabetics (58; T2D) were matched for age, BMI, waist circumference, SBP and DBP and fasting cholesterol and triglyceride levels as described under Research design and methods. PBL from these subjects were obtained and solubilized and ped/pea-15 protein levels quantitated. Bars represent the mean ± SE of 4 independent experiments, each in duplicate.

To address the functional significance of *ped/pea-15* overexpression in T2D FDR, we searched for association with diabetes-related phenotypes. We have therefore analyzed an additional group of 25 euglycemic offsprings of T2D-affected couples from the EUGENE2 cohort (20,22,23). PBL expression levels of ped/pea-15 in these offsprings were comparable to those of the other T2D FDRs investigated in this study (Table IV). The other clinical characteristics of these subjects are also shown in Table IV.

TABLE IV. *Clinical and biochemical features of the T2D offsprings*

	Mean ± SD
Number (male/female)	13/12
Age	30.1 ± 8.3
BMI (kg/m^2)	24.5 ± 4.1
Waist circumference (cm)	83.5 ± 12.2
Systolic blood pressure (mmHg)	112 ± 11
Dyastolic blood pressure (mmHg)	75± 6
Total cholesterol (mg/dl)	1841± 34
HDL cholesterol (mg/dl)	59 ± 13
Triglycerides (mg/dl)	92± 64
Fasting plasma glucose (mg/dl)	87 ± 10
2h glucose (mg/dl)	104 ± 25
Fasting plasma insulin	8 ± 3
Fat-free mass glucose disposal (mg x kg^{-1} x FFM x min^{-1})	12 ± 3
HOMA-IR	1.8 ± 0.9
HOMA-beta	174 ± 65
Insulinogenic index (Ins_{30}-Ins_0/$Gluc_{30}$-$Gluc_0$)	18 ± 12
Ped/pea-15 (arbitrary units)	257± 35

Data are the means ± SD. BMI, body mass index; SBP systolic blood pressure; DBP diastolic blood pressure.

A negative correlation was evidenced between the individual levels of ped/pea-15 in PBL and the insulin-stimulated glucose disposal by the fat-free mass, as determined by euglycemic hyperinsulinemic clamp (r = -557, p = 0.01; Fig.9). The correlation remained significant after correction for age, gender and BMI (r = -491, p = 0.02).

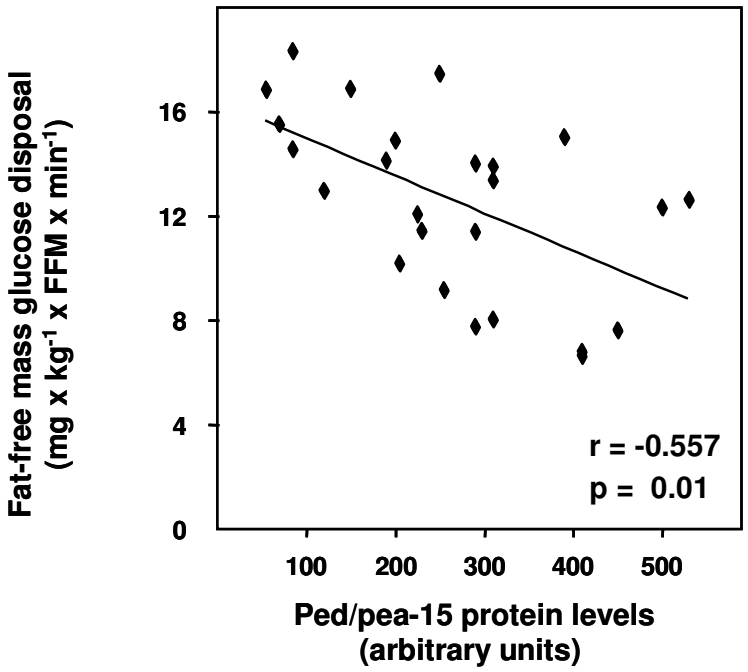

Fig.9 ***T2D-associated phenotypes and risk-factors related to ped/pea-15 expression.*** Ped/pea-15 protein levels were determined in PBL from 25 euglycemic offsprings of couples one of which member was affect by T2D. Glucose disposal was assessed by euglycemic hyperinsulinemic clamp as described under Research design and methods and corrected for the fat-free mass. Correlation between ped/pea-15 expression in PBL and glucose disposal was tested by linear regression analysis as described under Research design and methods.

As in the case of the other FDRs investigated in this study, ped/pea-15 levels did not correlate with age, BMI, waist circumference, SBP and DBP, and fasting cholesterol, triglyceride and glucose levels in these offsprings. Thus, the overexpression of ped/pea-15 may induce insulin-resistance in euglycemic offsprings of T2D-affected couples.

DISCUSSION AND CONCLUSION

Previous studies evidenced that ped/pea-15 is a multifunctional protein controlling a number of cellular functions including proliferation, apoptosis and insulin-regulated glucose transport *(29-38, 43, 44, 37)*. In transgenic mice fed high-fat diets, the overexpression of ped/pea-15 leads to diabetes *(44)*. Ped/pea-15 overexpression was also shown to occur in skeletal muscle and adipose tissues from type 2 diabetics (T2D), independent of obesity *(29)*. Whether this abnormality associates with T2D risk factors different from obesity in humans is unknown. Whether it contributes to specific phenotype(s) associated to human diabetes is also unclear. In the present work, we have addressed these questions in T2D related phenotypes, such as first-degree relatives (FDR) of T2D subjects. These individuals have a very high risk of T2D *(56, 57)* and develop different diabetes-related features years before diabetes onset and independent of the metabolic abnormalities associated to this disorder *(58-60)*. In this study it is shown that the ped/pea-15 gene is overexpressed in PBL from euglycaemic FDR of T2D individuals. As previously shown in the T2D, the overexpression in these FDR occurs both at the ped/pea-15 mRNA and the protein levels indicating it is caused, at least in part, by a transcriptional abnormality. Ped/pea-15 expression in the PBL closely correlates with that in fat and skeletal muscle tissues. Thus, PBL detection provides a convenient tool to investigate the role of ped/pea-15 overexpression during the progression toward T2D. In this study, the overexpression of ped/pea-15 was demonstrated in almost one third of the euglycaemic FDR and in a similar proportion of the T2D individuals compared to euglycaemic subjects with no family history of diabetes. It appears therefore that increased ped/pea-15 levels represents a common abnormality in both T2D and at risk individuals suggesting it might preceed diabetes onset in the latter.

In both T2D and their FDR, ped/pea-15 levels were independent of BMI, waist circumference, systolic and diastolic blood pressure, HDL cholesterol, triglyceride and glucose levels, indicating that expression of this gene is unaffected by the main variables associated to the metabolic syndrome in humans. Ped/pea-15 levels were also unrelated to gender, age as well as to reduced physical activity and smoke habit, two recognized risk factors for T2D and insulin-resistance.

As demonstrated in a previous study from our group *(61)* ped/pea-15 is overexpressed also in women affected by polycystic ovary syndrome (PCOS), an endocrinologic disease in which insulin secretion and action occur. Interestingly, the overexpression was of a similar magnitude to that evidenced in T2D subjects. Moreover, the univariate analysis showed no effect of BMI variation on ped/pea-15 expression levels (Fig.10); thus, similar to what happened to T2D patients, ped/pea-15 overexpression in subjects affected by PCOS was independent from body weight and other typical clinical features of metabolic syndrome.

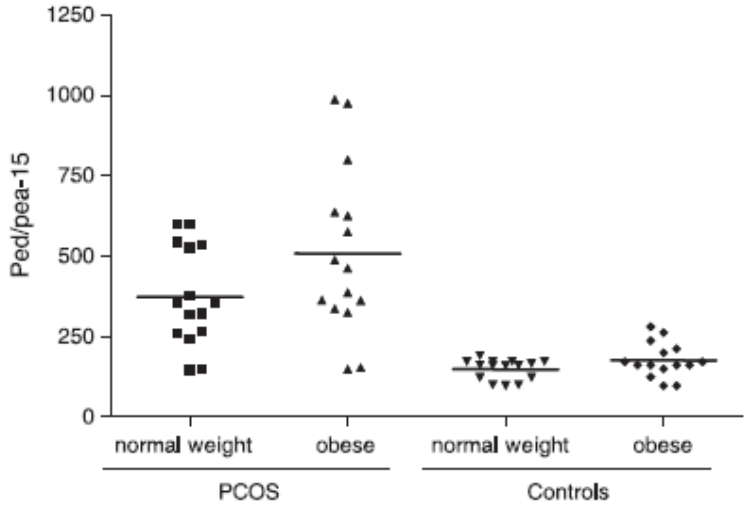

Fig. 10 Ped/pea-15 expression in PCOS population. The expression was significantly higher in both obese and normal-weight women with PCOS, than in obese and normal-weight controls ($p<0.001$). Conversely, there were no differences in Ped/pea-15 expression between obese and normal-

weight women within the two groups of PCOS and controls.

We report that, similar to those which are euglycemic, T2D first-degree relatives with IFG also overexpress ped/pea-15. In contrast, IFG subjects lacking family history of T2D do not, further indicating familiar clustering of this trait. The overexpression of the ped/pea-15 gene occurring in the T2D and their FDR may be genetically or environmentally determined, or determined by both factors. Indeed, previous studies *(29)* have evidenced that the overexpression persists in human skin fibroblasts after several generations in culture, suggesting that, at least in part, the occurrence of similarly high ped/pea-15 levels in the euglycaemic and IFG first-degree relatives of T2D and in the T2D is a genetically determined effect.

Similar data were observed in above mentioned study *(61)*, in which we found that family history for diabetes was one of the major determinants of ped/pea-15 overexpression in patients with PCOS; thus, further supporting the relevance of familial clustering of hereditable abnormalities in insulin secretion and action in women with PCOS.

At variance with these euglycaemic and IFG individuals, the presence of first-degree relatives affected by T2D in the pedigree elicited no effect on ped/pea-15 levels in patients with established diagnosis of T2D. Once deranged glucose tolerance enables overt diabetes to occur, factors secondary to the disease might elevate ped/pea-15 gene expression and/or protein levels even in those individuals in which the gene is not primarily overexpressed. Consistent with this possibility, recent studies by our own as well as other laboratories have shown that ped/pea-15 is highly regulated at the post-translational level *(26,27,32,38)*.

Previous studies in cultured cells and in transgenic mice have shown that overexpression of the ped/pea-15 gene determines resistance to insulin action and

impairs glucose-induced insulin secretion. In this study, whether ped/pea-15 overexpression associates to impaired insulin action and/or secretion has been further addressed in euglycemic offsprings of T2D-affected couples. In these individuals at risk of diabetes, insulin-resistance and reduced non-oxidative glucose metabolism appear years before the onset of hyperglycemia *(58, 59)*. In addition, they may also have β-cell dysfunction, as evidenced by decreases in insulin and amylin secretion in response to glucose stimulation *(60)*. However, ped/pea-15 levels were not correlated with any index of β-cell function in the offspring.

Based on euglycemic hyperinsulinemic clamp, a negative correlation was established between ped/pea-15 levels and the insulin-stimulated glucose disposal by the fat-free mass of the offsprings, suggesting that high levels of ped/pea-15 protein contribute to development of skeletal muscle resistance to insulin action in these individuals. In these same FDR, ped/pea-15 expression levels did not correlate with any index of β-cell function.

Ped/pea-15 levels were also weakly correlated with fasting plasma insulin in these FDR, but not in euglycaemic individuals lacking a family history of type 2 diabetes.

Ped/pea-15 might induce insulin resistance only in conjunction with other diabetes-related genes enriched in at-risk individuals. In this case, as previously demonstrated *(62)*, gene enrichment analysis *(63)* may help to unravel the significance of increased levels of ped/pea-15 in human diabetes.

Gene overexpression may impair glucose-triggered insulin secretion in transgenic mice and not in humans. This is unlikely, however, as transfection of a ped/pea-15 cDNA in human β-cell lines grossly impairs insulin response to glucose. Alternatively, in the type 2 diabetic FDR, the consequence of the high ped/pea-15 levels on insulin action might appear more precociously than those on β-cell function.

There is evidence that impaired glycogen synthesis is responsible for the early insulin-resistance in non-diabetic first-degree relatives of patients with type 2 diabetes *(58, 60)*, and an association has been noted between a polymorphism in the glycogen synthase gene and the presence of diabetes in a subgroup of patients with strong family history of type 2 diabetes, hypertension and marked insulin-resistance *(62)*. In transgenic mice, overexpression of the ped/pea-15 gene reduces insulin-stimulated glucose disposal mainly by impairing glucose transport *(44)* and glycogen synthesis. It is possible, therefore, that ped/pea-15 overexpression may contribute to reduced non-oxidative glucose metabolism in type 2 diabetes FDR as well.

In conclusion, in the present study, we have shown that ped/pea-15 overexpression represents a common abnormality in both T2D and their FDR and can be conveniently detected in PBL. In at risk individuals, this defect associates with the presence of T2D-affected FDR in the pedigrees and not with other major T2D risk factors, while diabetes-secondary abnormalities may contribute to increasing ped/pea-15 levels once overt diabetes has developed. In euglycemic FDR of T2D, high ped/pea-15 levels strongly correlate with resistance to insulin action in skeletal muscle suggesting that ped/pea-15 contributes to early appearance of insulin-resistance in these individuals.

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