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# The Enlarging List of Phenotypic Characteristics That Might Allow the Clinical Identification of Families at Risk for Type 1 Diabetes

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## 1. Introduction

Type 1 diabetes is a chronic metabolic disease whose aetiology and pathogenesis remain not completely understood. Current criteria for the diagnosis of diabetes are: 1) haemoglobin A1c  $\geq 6.5\%$  (assayed using a method that is certified by the National Glycohemoglobin Standardization Program, NGSP, and standardised or traceable to the Diabetes Control and Complications Trial, DCCT, reference assay), 2) fasting plasma glucose (FPG)  $\geq 126$  mg/dl, 3) 2-hour plasma glucose  $\geq 200$  mg/dl during an oral glucose tolerance test (OGTT, 75 g), 4) a random plasma glucose  $\geq 200$  mg/dl (American Diabetes Association, 2011). The classification of diabetes includes: type 1 diabetes, type 2 diabetes, other specific types of diabetes due to other causes, and gestational diabetes mellitus. Type 2 diabetes, which is usually associated with obesity and older age, results from insulin resistance and progressive failure of pancreatic beta-cell function. Type 1 diabetes, which has usually an abrupt onset in younger people, is an organ-specific autoimmune disease characterised by absolute insulin deficiency resulting from beta-cell destruction. However, autoimmunity may not be the primary cause: environmental triggers are believed to precipitate type 1 diabetes in genetically susceptible individuals (van Belle et al., 2011). The overall incidence of type 1 diabetes is increasing; the majority of the increase is observed in the youngest age group, which also appeared to be the heaviest (Evertsen et al., 2009). Indeed, the accelerator hypothesis (Wilkin, 2009) suggests that type 1 and type 2 diabetes are the same disorder of insulin resistance set against different genetic backgrounds. Three processes could variably accelerate the loss of beta cells through apoptosis: constitution, insulin resistance, and autoimmunity. None of these accelerators leads to diabetes without excess weight, which causes an increase in insulin resistance and, thus, the weakening of glucose control. In turn, the glucotoxicity accelerates beta-cell apoptosis directly and by inducing beta-cell immunogens and autoimmunity in genetically predisposed subjects. Insulinitis is commonly observed in recent-onset type 1 diabetes, but it does not uniformly affect all insulin-containing islets (differences in islet function?). It has been suggested that under increased insulin demand (puberty, adolescence, high sugar intake, etc.) a population of islets may be more prone to dysfunction or death, thereby attracting antigen presenting cells and

promoting insulinitis in susceptible individuals (Rowe et al., 2011). In a genome-wide association study, 41 distinct genomic locations provided evidence for association with type 1 diabetes in the meta-analysis (Barrett et al., 2009). The Type 1 Diabetes Genetics Consortium (T1DGC) has recruited families with at least two siblings who have type 1 diabetes in order to identify genes that determine an individual's risk of type 1 diabetes. T1DBase is the web-based resource focused on the genetics and genomics of type 1 diabetes susceptibility (<https://www.t1dgc.org>) that provides the updated table of human loci associated with type 1 diabetes (Table 1).

Chromosome	Gene of interest	Abbreviation
1p13.2	Protein tyrosine phosphatase, non-receptor type 22	PTPN22
1q31.2	Regulator of G-protein signalling 1	RGS1
2q12	Interleukin 18 receptor accessory protein	IL18RAP
2q24.2	Interferon induced with helicase C domain 1	IFIH1
2q33.2	Cytotoxic T-lymphocyte-associated protein 4	CTLA4
3p21.31	Chemokine (C-C motif) receptor 5	CCR5
4q27	Interleukin 2	IL2
5p13		
6p21.31 6p21.33	Major histocompatibility complexes	HLA-B, -A, -DRB1, -DQB1, -DPB1
6q15	similar to BTB and CNC homology 1, basic leucine zipper transcription factor 2	BACH2
6q23.3	similar to Tumor necrosis factor, $\alpha$ -induced protein 3	TNFAIP3
6q25.3	T-cell activation Rho GTPase-activating protein	TAGAP
10p15.1	Interleukin 2 receptor, $\alpha$	IL2RA
10p15.1	Protein kinase C, $\theta$	PRKCCQ
11p15.5	Insulin II	INS
12q13.2		
12q13.3	Kinesin family member 5A	KIF5A
12q24.12		
15q25.1		
16p13.3		
18p11.21	Protein tyrosine phosphatase, non-receptor type 2	PTPN2
18q22.2	CD226 antigen	CD226
21q22.3		
22q13.1		

(from: [http://t1dbase.org/page/PosterView/display/poster\\_id/386](http://t1dbase.org/page/PosterView/display/poster_id/386))

Table 1. Human loci associated with type 1 diabetes.

With regards to the causative environmental triggers that have been implicated in the pathogenesis of type 1 diabetes, they have been recently reviewed (van Belle et al., 2011; Vehik & Dabelea, 2011) and include particularly viral infections, gut microbial flora and other bacteria, early life feeding patterns, wheat proteins, and vitamin D.

## 2. Identifying individuals at risk for type 1 diabetes

In Europe, the number of adults with diabetes was expected to reach 55.2 million (8.5% of the adult population) in 2010; about 112,000 children and adolescents were estimated to have type 1 diabetes mellitus (<http://www.diabetesatlas.org/content/europe>).

Most diabetic cases are complex diseases resulting from interactions between genetic and environmental determinants in genetically predisposed individuals. Empirical evidence suggests a architecture of many genetic loci with many variants of small effect (Wray & Goddard, 2010). Genome-wide association studies have suggested that the majority of susceptible loci have small contributions to phenotypic variation and therefore there should be a large number of susceptibility loci involved in the genetic basis of complex diseases (consistent with the polygenic model). Moreover, the differentiation of sporadic and familial cases has implied that most complex diseases are genetically heterogeneous. Family history has a high positive predictive value, but a low negative predictive value. Yang et al. (2010) have shown that 1) the proportion of sporadic cases depends on disease prevalence and heritability of the underlying liability scale, and 2) a large proportion of sporadic cases is expected under the polygenic model due to the low prevalence rates of common complex genetic diseases. Thus, the causal mechanisms cannot be inferred from the observed proportion of sporadic cases alone. The prediction of disease risk to relatives from many risk loci or markers requires a model that combines the effects of these loci. The constrained multiplicative, Odds and Probit models fitted data on risk to relatives, but it is difficult to distinguish between them until genetic variants that explain the majority of the known genetic variance are identified (Wray & Goddard, 2010). Hence, genetic risk modelling to derive prediction of individual risk and risk to relatives are still difficult to reconcile.

In most individuals with autoimmune type 1 diabetes, beta cell destruction is a chronically progressive and very slow process that starts long before overt disease. During this “silent” phase, autoantibodies are produced and self-reactive activated lymphocytes infiltrate the islets of Langerhans (Rowe et al., 2011). Autoantibodies that target self-antigens in the insulin-secreting beta cells of the pancreas include: islet cell autoantibodies (ICA), insulinoma-associated antigen-2 antibodies (IA-2A), antibodies against the related antigen IA-2 beta (IA-2 $\beta$ ), insulin autoantibodies (IAA), autoantibodies to the 65kDa isoform of glutamic acid decarboxylase 65 (GADA), and the recently identified autoantibodies to the zinc transporter 8 (ZnT8A) (Table 2).

Islet autoantibodies are potent tools for the prediction of type 1 diabetes and are the basis for recruitment in prevention trials and immunointervention trials. In the general childhood population in Finland, one-time screening for GADA and IA-2A was capable of identifying about 60% of those individuals who will develop type 1 diabetes over the subsequent 27 years; both positive and negative seroconversions occurred over time reflecting a dynamic process of beta cell autoimmunity, but positivity for at least two diabetes-associated autoantibodies represented in most cases a point of no return (Knip et al., 2010). So far, however, the place of autoantibody-based risk assessment in routine clinical practice is limited because no proven therapeutic interventions is available for people at high risk of progression to type 1 diabetes. Until therapies modulating the disease process become available, the benefit to individual patients is questionable - awareness of risk is rather useless or even stressful - and diabetes antibody testing does not yet have a role in clinical care (Bingley, 2010). It is considered likely that islet-related autoantibodies are not directly pathogenic, whereas autoreactive CD4 and CD8 T cells mediate beta cell damage.

Therefore, standardised autoantibody screenings should be combined with the detection of autoreactive T cells. Unfortunately, none of the currently available T cell assays satisfies all the features of a good assay: small blood sample required, simplicity, specificity, low intra- and inter-assay variability (Fierabracci, 2011). Notwithstanding recent developments based on immunosorbent spot and immunoblotting techniques, the International Workshops of the Immunology Diabetes Society concluded that T cell results are still inconclusive and novel approaches are currently being investigated.

In conclusion, it may be that in the future combination screening predicts type 1 diabetes clinical onset, but actually genetic risk, serum autoantibody profiling and T cell assays are uneconomical when applied in the general population.

Autoantibodies against	Abbreviation	Method
38-kDa glycosylated islet cell membrane-associated protein	GLIMA	Immunoprecipitation
51-kDa aromatic-L-amino-acid decarboxylase	AADC	Immunoprecipitation
52-kDa rat insulinoma	52-kDa RIN	Immunoblot
Aminoacyl-tRNA synthetase	ARS	ELISA*
Carbonic anhydrase II	CA II	ELISA*
Carboxypeptidase H	CPHA	Radiobinding assay
Chymotrypsinogen-related pancreatic 30-kDa		Immunoblot analysis
DNA topoisomerase II	TopIIA	ELISA* and Western blot
Ganglioside GM2-1	GM2-1	Indirect immunoperoxidase technique
Gangliosides GM1, 2, 3, etc.		*ELISA
Glucose type-2 transporter	GLUT2	Western blot
Glutamic acid decarboxylase	GADA	Radiobinding assay, ELISA*
Heat shock proteins	HSP	*ELISA
Insulin	IAA	Radiobinding assay
Insulinoma-associated antigen-2	IA-2A	Radiobinding assay, ELISA*
Insulinoma-associated antigen 2 $\beta$	IA-2 $\beta$	Radiobinding assay
Islet cell	ICA	Indirect immunofluorescence
Islet cell surface	ICSA	Radiobinding assay
Proinsulin	PIAA	Radiobinding assay
Zinc transporter 8	ZnT8	Radiobinding assay

\* Enzyme linked immunosorbent assay

Table 2. List of islet autoantibodies detected in type 1 diabetes (modified from Winter & Schatz, 2011).

### 3. Phenotyping type 1 diabetes families

Translational research aims to integrate basic life science (genomics, transcriptomics, proteomics, and metabolomics) with insights gained from clinical experience to comprehensively study complex biological system and complex human diseases. Translation requires, among others, methods that relate molecular and cellular phenotypes

to clinical characteristics (Bebek et al., 2011). Indeed, the correlation between quantitative phenotypes and traits allows for a more efficient use of the genetic information; hence the importance of accurate family phenotyping studies. Unaffected family members can contribute as much to the analysis as individuals with the disease diagnosis. For example, the finding of cognitive deficits in individuals with schizophrenia and in the clinically unaffected relatives of these individuals suggested that these deficits are part of the innate underlying distinct differences that make some individuals vulnerable to schizophrenia. Examining these complementary biological phenotypes in genetic studies has been found to provide valuable information about the pathway that connects genotype to clinical disease (Almasy et al., 2008). Similarly, large-scale genetic fine mapping and genotype-phenotype associations implicated polymorphisms in the IL2RA region in type 1 diabetes: IL2RA type 1 diabetes susceptibility genotypes were associated with lower circulating levels of the biomarker, soluble IL-2RA (Lowe et al., 2007). However, despite the theoretical advantages of quantitative trait analysis and testing of multiple plausible domains, some matters have emerged since quantitative traits may not be the most relevant phenotypes to investigate in search for the genetic etiology of disease. Identifying the “best” phenotype for genetic studies needs to survey family members and examine coexisting features and familial segregation patterns. A focus on careful assessment of the most genetically relevant phenotypes has been recommended (Brzustowicz & Bassett, 2008).

Over the years, our research efforts have sought primarily to gain a comprehensive understanding of the common phenotypic elements that characterise families with a sporadic case of type 1 diabetes. Here we provide a research-based overview of these familial peculiarities that include multifaceted, easily detectable, clinical perturbations: physical (BMI), cardiovascular (blood pressure response to exercise and circadian blood pressure pattern), biochemical (fasting plasma glucose, HbA1c, lipids, homeostasis model assessment of insulin sensitivity, plasma markers of oxidative damage), cellular (cellular markers of oxidative damage, transplasma membrane electron transport systems, mitochondrial membrane potential), and immunological (lymphocyte subsets).

#### **4. Body weight in type 1 diabetes families**

According to epidemiological findings and the accelerator hypothesis, the prevalence of overweight in preadolescent children is increasing, it tracks into adulthood and may increase diabetes and cardiovascular disease risk in adulthood. The risk of childhood obesity seems to increase with exposure to diabetes or cigarette smoke in utero, high birth weight, rapid weight gain in infancy, and shorter breastfeeding duration. The Diabetes Autoimmunity Study in the Young (DAISY) examined longitudinally 1,718 children from birth that were at increased risk for type 1 diabetes (Lamb et al., 2010). Gender, diabetes exposure in utero, size for gestational age, weight gain in the first year of life, and total breastfeeding duration (inverse) showed significant association with higher childhood BMI. Mediation analysis suggested that 1) the protective effect of breastfeeding duration on childhood BMI was largely mediated by slower infant weight gain, and 2) the increased risk of higher childhood BMI associated with exposure to diabetes in utero was partially explained by greater birth size. Maternal obesity before pregnancy and weight gain during pregnancy significantly predicted increased risk of persistent multiple positivity for islet autoantibodies in offspring with high genetic susceptibility for type 1 diabetes (Rasmussen et al., 2009). A systematic review and meta-analysis (12 studies) indicated that high birth

weight and increased weight gain during the first year of life were associated with an increased risk of type 1 diabetes in later life (Harder et al., 2009).

Metabolic demand and insulin resistance have been suggested to be involved in the development of type 1 diabetes (Evertsen et al., 2009; Wilkin, 2009), but the evidence is not consistent across the studies. In 1650 prospectively followed children of mothers or fathers with type 1 diabetes (BABYDIAB cohort), islet autoantibodies-positive children were not insulin resistant (based on homeostasis model assessment of insulin resistance, HOMA-IR) and did not have increased BMI around and early after seroconversion (Winkler et al., 2009). In this study, of 777 children with HOMA-IR measurements, 84 developed islet antibodies during the study: analysis of HOMA-IR by age showed no significant difference between islet autoantibody-positive and islet autoantibody-negative children, with a tendency towards a lower HOMA-IR in the antibody-positive children compared with the antibody-negative children.

In a primary school health program in Pisa we screened 869 primary school children (448 M, 421 F, mean age  $118 \pm 5$  months): height, weight, four skinfolds, and four circumferences were measured; a family-reported questionnaire was used to determine family composition, history, and lifestyle (Giampietro et al., 2002). The percentages of children who could be considered overweight ( $BMI \geq 95^{\text{th}}$  percentile of age- and sex-specific National Health and Nutrition Examination Survey I, NHANES I, reference data) were boys, 10.0%, and girls, 9.3%. It emerged that offspring BMI was correlated with birth weight, parental BMI and scholarship level, children blood pressure, and hours per day spent in television viewing. Family history for diabetes was associated with higher BMI, skinfold thickness at the subscapular area (SSF), waist circumference, and upper thigh. Family history for hypertension was associated with higher SSF/skinfold thickness at the triceps area (TCF) ratio. We concluded that anthropometric and anamnestic data on child and family yield more accurate estimates of risk profile: fat distribution seems relevant for metabolic and cardiovascular disorders.

Since our initial investigations on type 1 diabetes families, we found that first degree relatives' BMI tended to be higher when compared with healthy control subjects who had no first-degree relative with type 1 diabetes, although the difference did not always reach statistical significance (Matteucci & Giampietro, 2000a; Matteucci et al. 2004a, 2004b; Matteucci et al. 2006). In recent years, on the contrary, the difference in BMI between unaffected siblings of type 1 diabetic probands and healthy control subjects has reached the statistical significance (Figure 1, Matteucci et al., 2010).

This finding probably reflects the trend toward increasing body weight and obesity in the general population, declining physical activity and unhealthy dietary habits that we have documented (Matteucci et al., 2004b, 2007, 2008). However, the emerging difference in BMI between unaffected relatives and control subjects suggests that additional factors are operative in type 1 diabetes families, which remain unknown. The single nucleotide polymorphism rs9939609 in the fat mass and obesity associated gene (FTO) region on chromosome 16q12, which increases the risk of childhood obesity and type 2 diabetes, did not alter susceptibility to type 1 diabetes (Field et al., 2007). Although increased early growth was associated with disease risk in various European populations, any role of infant feeding in this association remained unclear (EURODIAB Substudy 2 Study Group, 2002). Scientific evidences suggested associations of allelic variations in the Vitamin D receptor gene and phenotypes related to body weight, glucose homeostasis, diabetes and

its vascular complications (Reis et al., 2005). Whatever the case, our data in adult members of type 1 diabetes families highlight that the ‘familial’ predisposition to overweight remains throughout life.

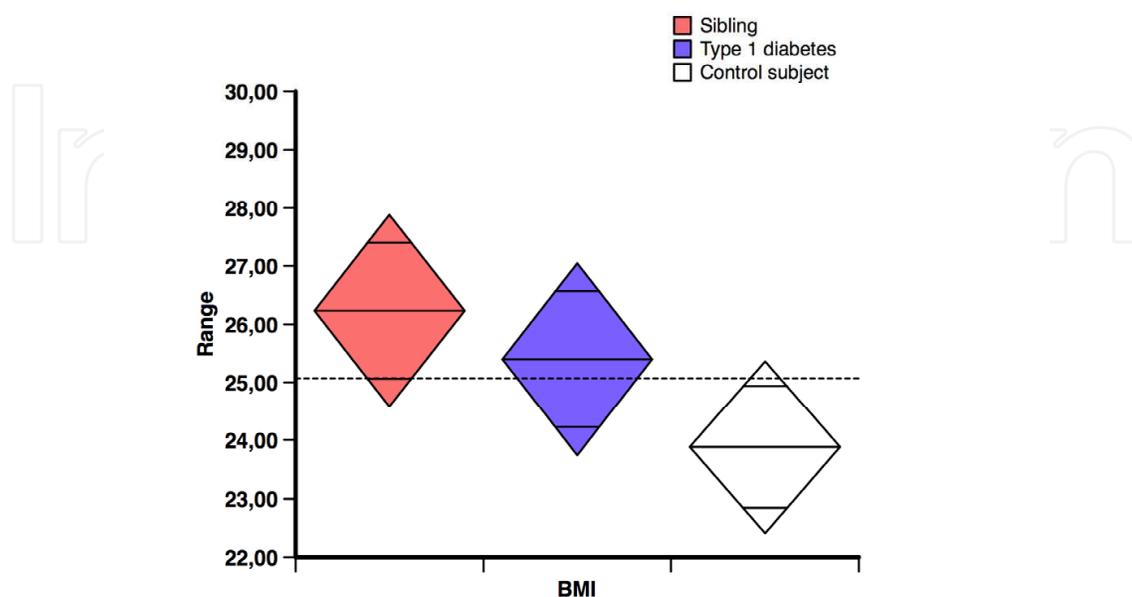


Fig. 1. Body mass index (BMI) in control subjects, type 1 diabetic patients and their siblings (Matteucci et al., 2010).

### 5. Familial cardiovascular abnormalities

Diabetes and hypertension are strongly associated although the role of glycaemia in promoting hypertension is a matter of debate (Invitti, 2003). HbA1c variability predicts not only incident microalbuminuria and progression of established renal disease but also cardiovascular disease events in patients with type 1 diabetes (Wadén et al., 2009). Moreover, HbA1c concentration predicts cardiovascular disease and all-cause mortality in adults without diabetes (Khaw et al., 2004). In healthy non-diabetic and non-hypertensive men, fasting plasma glucose is independently associated with blood pressure at rest and during exercise and development of elevated blood pressure after 7-years follow-up (Bjørnholt et al., 2003). Usually, in type 1 diabetes families, parental hypertension has been associated with diabetic nephropathy in adult and young offspring (Viberti et al., 1987; Marcovecchio et al., 2010), but the familial/hereditary factors that have an impact on diabetic nephropathy have not been so far identified. In a large homogeneous population from the Finnish Diabetic Nephropathy study, a cluster of parental hypertension, cardiovascular disease, cardiovascular mortality, and type 1 diabetes was associated with diabetic nephropathy in offspring with type 1 diabetes. It seemed that the more the traits clustered in family, the higher the risk for diabetes nephropathy (Thorn et al., 2007). In this regard it is noteworthy that enhanced sodium/lithium countertransport and sodium/hydrogen exchange had been suggested to predict diabetic nephropathy (Walker et al., 1990; Ng et al., 1990). However, we found evidence contradicting this favourite hypothesis. Indeed, our data demonstrated convincingly that sodium/hydrogen exchange activity was significantly higher in type 1 diabetes with no difference among the two groups

of diabetic patients with and without nephropathy. Moreover, enhanced sodium/hydrogen exchange activity was also a common feature of nondiabetic first-degree relatives of type 1 diabetic patients with no difference among the corresponding groups of relatives. The association between antiport activities of diabetic probands and their relatives suggested that the altered activity of the transporter was primarily determined by familial factors whose nature remained to be clarified (Matteucci & Giampietro, 2000b).

Generally, the observation of raised arterial blood pressure in relatives of type 1 diabetes patients was based on history, a single measurement of arterial blood pressure, or a 24-h ambulatory record; we were first to evaluate the response to ergometer exercise (Matteucci et al., 2006). Blood pressure response to exercise had been evaluated as a predictor of future hypertension and cardiovascular disease (Sharabi et al., 2001). Moreover, the heritability for resting blood pressure and blood pressure response to exercise was under investigation (An et al., 2000). We identified an abnormal blood pressure response to exercise testing not only in type 1 diabetic probands but also in asymptomatic normotensive non-diabetic relatives of type 1 diabetics, in which it was associated with indices of metabolic syndrome and oxidative damage. Furthermore, in healthy normotensive non-diabetic control subjects without family history of type 1 diabetes, strong associations were found 1) between resting systolic blood pressure and fasting plasma glucose as well as fasting plasma insulin levels, and 2) between systolic blood pressure response to exercise and HbA1c levels (Matteucci et al., 2006).

In a recent study, we performed 24-hour ambulatory blood pressure monitoring in type 1 diabetes families with the primary aim of investigating the circadian variability of blood pressure and the ambulatory arterial stiffness index in healthy siblings of type 1 diabetes patients vs healthy control subjects who had no first-degree relative with type 1 diabetes (Matteucci et al., 2010). Secondary aims of the study were to explore the influence of both cardiovascular autonomic function and erythrocyte electron transfer activity as oxidative marker on the ambulatory blood pressure profile. Indeed, human erythrocytes possess a transplasma ferricyanide reductase activity (measured as the erythrocyte velocity of ferricyanide reduction) that transfers reducing equivalents from intracellular reductants to extracellular oxidants (Matteucci & Giampietro, 2000c) and belongs to the ubiquitous transplasma membrane electron transport systems. Transplasma membrane electron transport activities have been related to the regulation of vital cellular processes and to the pathogenesis of various human disorders (Lane & Lawen, 2009) and exist also in endothelial cells where they have been suggested to regulate redox status and possibly atherogenesis through regulation of haeme oxygenase-1 expression (Lee et al., 2009).

We found that systolic blood pressure midline-estimating statistic of rhythm and pulse pressure were higher in type 1 diabetes patients and correlated positively with diabetes duration and the rate of oxidant-induced erythrocyte electron transfer to extracellular ferricyanide. Autonomic dysfunction was associated with diastolic blood pressure ephasia and increased ambulatory arterial stiffness index. Siblings had higher BMI (Figure 1), lower insulin sensitivity (Figure 2), larger systolic blood pressure amplitude (Figure 3), and higher ambulatory arterial stiffness index than controls. Daytime systolic blood pressure was positively, independently associated with BMI and erythrocyte electron transfer to extracellular ferricyanide. Among non-diabetic people, there was a significant correlation between ambulatory arterial stiffness index and fasting plasma glucose. We concluded that siblings of type 1 diabetes patients exhibited a cluster of sub-clinical metabolic abnormalities associated with consensual perturbations in blood pressure variability. Moreover, our

findings supported, in a clinical setting, the proposed role of transplasma membrane electron transport systems in vascular pathobiology.

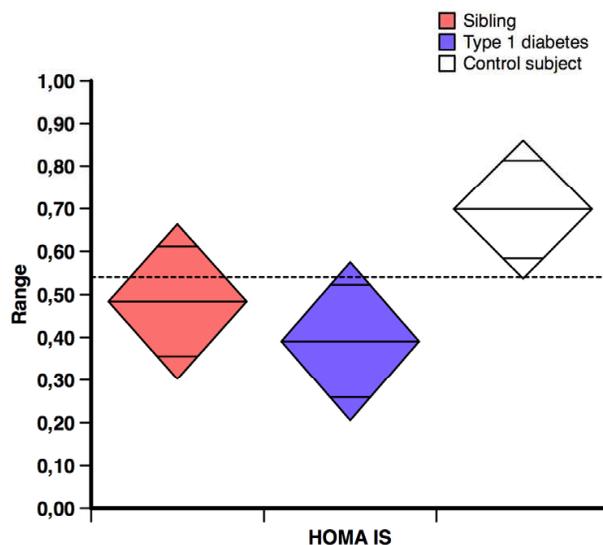


Fig. 2. Homeostasis model assessment of insulin sensitivity (HOMA-IS) in the same study groups (Matteucci et al., 2010).

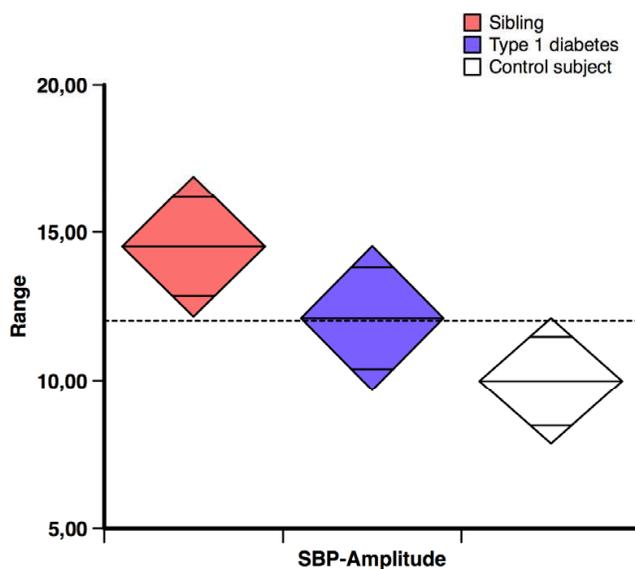


Fig. 3. Systolic blood pressure amplitude (SBP-Amplitude) in the same study groups (Matteucci et al., 2010).

## 6. Biochemical phenotype and redox balance in type 1 diabetes relatives

Our studies over the years have linked family history of type 1 diabetes (first-degree kinship) with multiple biochemical abnormalities. Since 2000 we documented metabolic perturbations in nondiabetic relatives: parents differed from age-matched control subjects in the higher plasma concentrations of glucose and Lipoprotein (a); their fibrinogen was borderline but did not reach any statistical significance; in turn, siblings of type 1 diabetes

patients differed from age-matched control subjects in the higher levels of Lipoprotein (a) (Matteucci et al., 2000a). In the same study, we investigated the redox status and antioxidant defences in these families.

The premises were the following:

- enhanced levels of free radicals found in diabetes mellitus and impaired glucose tolerance has long been assumed to be related to chronically elevated glucose levels (Baynes and Thorpe, 1999; Vijayalingam et al., 1996),
- oxidative stress was suggested to play a primary role in the pathogenesis of diabetes and its complications but Authors still discussed whether oxidation preceded the appearance of complications or it merely reflected their presence (Baynes and Thorpe, 1999).

We suggested an alternative hypothesis, i.e. that oxidative stress preceded diabetes mellitus. In the case, indirect evidence for increased oxidative stress could be also detectable in non-diabetic relatives of type 1 diabetic patients. In order to provide evidence of a familial imbalance between radical production and antioxidant defences, we investigated indices of glucose and lipid metabolism, markers of plasma and cell lipid peroxidation, a novel marker of oxidant-induced protein damage, and the effects of oxygen radicals on erythrocytes of patients with type 1 diabetes and their relatives. We measured blood creatinine, glucose, HbA1c, cholesterol, triglycerides, Lipoprotein (a), fibrinogen, malondialdehyde, and advanced oxidation protein products. Erythrocyte response to oxidative stress (3-h-incubation at 37°C with or without a radical generating system) was evaluated by measuring erythrocyte glutathione, erythrocyte malodialdehyde, and haemolysis. Plasma and erythrocyte malodialdehyde were found to be significantly elevated in diabetics and relatives than in controls. Basal erythrocyte glutathione was lower in diabetics and incubations of cells caused in diabetics a decrease in erythrocyte glutathione of lesser degree than in control subjects, while a significant increase in haemolysis. Among relatives, haemolysis was increased both at baseline and after incubation. Plasma malodialdehyde was associated with blood glucose, creatinine, and fibrinogen; basal erythrocyte malodialdehyde with plasma Lipoprotein (a), fibrinogen, and plasma malodialdehyde. Basal erythrocyte glutathione content correlated with serum glucose and erythrocyte malodialdehyde production.

In that occasion, we were pioneers of the research on redox balance in type 1 diabetes families. We presented first evidence that markers of lipoprotein metabolism (Lipoprotein (a)), oxidative stress (plasma and erythrocyte malodialdehyde), and cellular fragility (haemolysis) are abnormal in non diabetic relatives of type 1 diabetics supporting the view that familial elements even precede diabetes. It seemed reasonable that the same biologic markers considered major predictors of cardiovascular disease could also trace familial susceptibility to type 1 diabetes, just as they have been associated with the development of type 2 diabetes (Matteucci et al., 2000a).

Based on the finding of elevated circulating markers of lipid peroxidation and increased cellular fragility, we decided to complete and integrate our investigation with further biochemical measurements of possible first-chain initiating or stimulating factors in order to evaluate, in the same families, the contribution of extracellular antioxidants to the increased oxidative stress. We also aimed to understand the eventual relationship between oxidative stress and the abnormal sodium/hydrogen exchange activity previously observed

(Matteucci et al., 2001). We were unable to find out any abnormalities in circulating metal ions (such as iron, transferrin, ferritin, copper, and ceruloplasmin) or extracellular antioxidant defences (such as serum uric acid, albumin, bilirubin,) that could favour oxidative stress in non-diabetic relatives of type 1 patients. On the contrary, we confirmed our previous finding of a generalised increase in sodium/hydrogen exchange activity. The rate of amiloride-sensitive hydrogen efflux from erythrocytes was significantly associated with both erythrocyte glutathione content and some markers of radical-induced damage such as plasma advanced oxidation protein products and malondialdehyde, erythrocyte osmotic fragility, and erythrocyte malondialdehyde accumulation under oxidative stress. Hence, this additional study provided the first *in vivo* demonstration of a significant association between oxidative stress and sodium/hydrogen exchange upregulation. The familiarly overactive sodium/hydrogen exchange itself could be viewed as further evidence pointing to the presence in these families of a redox disequilibrium where oxidation seems to be prevailing.

Taken into account that:

- mitochondria are the cellular site of oxidation-reduction reactions and energy transfer processes; mitochondrial dysfunction is believed to play a role in the development of diabetes and its complications because of the active generation of free radicals (Maiese et al., 2007),
- a reactive oxygen species-mediated long-term 'memory' of hyperglycaemic stress has been reported in the mitochondria of endothelial cells (Ihnat et al., 2007), but impairment of mitochondrial function has been also observed in subjects with family history of type 2 diabetes before the onset of impaired glucose tolerance (Petersen et al., 2004),

in the last step of our research we measured the mitochondrial membrane potential in peripheral blood granulocytes from type 1 diabetic patients and their unaffected siblings using the mitochondrial indicator 5,5',6,6'-tetra chloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) in conjunction with flow cytometry (Matteucci et al., 2011). This was the first study to examine mitochondrial membrane potential of circulating leukocytes in type 1 diabetes families and to document consistent evidence for mitochondrial hyperpolarisation that was highest in type 1 diabetic patients and intermediate in their siblings. Fasting plasma glucose was the only correlate of leukocyte mitochondrial membrane potential. Confirming previous observations in type 1 diabetes families, siblings had fasting plasma glucose slightly higher than control subjects yet lower HbA1c levels. The combination of higher mean fasting plasma glucose, lower homeostasis model assessment of insulin sensitivity (HOMA-IS) and lower HbA1c levels suggested that siblings had both impaired basal glucose clearance rate and enhanced insulin-stimulated muscle glucose disposal.

We hypothesised that in type 1 diabetes families, radical-induced mitochondrial membrane potential oscillations may be synchronized toward polarized states. The positive association between mitochondrial membrane potential oscillations and fasting plasma glucose within the range from normal to dysglycemic conditions suggested that hyperglycaemic challenge implied increased glucose metabolism, enhanced oxidant formation and hyperpolarisation of the mitochondrial membrane.

It is noteworthy that succination of proteins, which is an irreversible chemical modification of cysteine by the Krebs cycle intermediate fumarate, is increased by hyperpolarisation of

the inner mitochondrial membrane and develops in concert with mitochondrial and oxidative stress in diabetes (Frizzell et al., 2011).

## 7. Immunological functions in type 1 diabetes families

Although type 1 diabetes is a T-cell-mediated autoimmune disease, until a few years ago relatively few studies have attempted to associate T-cell autoreactivity with disease progression, in comparison with efforts directed on monitoring autoantibodies, and those that have been performed were largely limited to CD4 T-cells (Roep, 2008). Currently, islet epitope-specific CD8 T cells are believed to have a pivotal role in the destruction process. Unfortunately, monitoring multiple epitope-specific CD8 T cell populations poses many technical problems. Recently, monitoring of CD8 T cells reactive to beta-cell-derived antigens has been performed using the combinatorial quantum dot technique, which has been validated using peripheral blood cells from recent-onset type 1 diabetic patients, their siblings, and control subjects (Velthuis et al., 2010). Moreover, during the progression of autoimmune diabetes, memory autoreactive regulatory CD8 T cells can be expanded that could effectively suppress the expansion of dominant and subdominant effectors (Khadra et al., 2010). Increasing evidence shows the significance of CD4 and CD8 regulatory T cells, expressing the marker CD25 or IL-2 receptor, in autoimmune disease models. On the contrary, very few study have dealt with the role of CD23 or low affinity IgE receptor. In 2004, given that abnormalities in redox balance clustered in type 1 diabetes families and the intracellular redox status seemed to modulate immune function, we aimed to investigate the relationship between oxidative stress and immunologic features. We measured oxidative markers, serum pro-inflammatory cytokines, soluble cytokine receptors, and subsets of peripheral blood lymphocytes (by varying combinations of CD4, CD8, CD23, and CD25) from type 1 patients, low-risk (i.e. without underlying islet autoimmunity) non-diabetic first-degree relatives of diabetic patients, and healthy subjects (Matteucci et al., 2004a). In these families, protein and lipid oxidation was confirmed from reduced sulfhydryl groups, increased advanced oxidation protein products, increased plasma and erythrocyte malondialdehyde. Relatives had decreased counts of monocytes, of cells coexpressing CD23 and CD25, and of CD25<sup>+</sup> cells in peripheral blood. Patients with type 1 diabetes had similar defects and, in addition, showed decreased counts of peripheral CD4<sup>+</sup>CD8<sup>+</sup> lymphocytes and increased serum levels of soluble receptors for IL-6 and IL-2. This was the first demonstration of leukocyte abnormalities in low-risk T1DM relatives, also presenting signs of oxidative stress. Moreover, our study reported first evidence that the oxidative stress observed in type 1 diabetes families was correlated to immunological hallmarks suggestive of different immunoregulatory mechanisms. A crucial question remained open: did the alteration in immune functions follow the altered intracellular redox status or vice versa?

More recently, we have characterised CD26 expression of T cell subsets in patients with type 1 diabetes because 1) high expression of CD26 among CD8<sup>+</sup> T cells has been suggested to be a marker of effective long-term memory T cell formation typical of acute resolved viral infections (Ibegbu et al., 2009), and 2) an increased risk of persistent viral infections, such as hepatitis C (HCV), was reported among diabetic patients (Lonardo et al., 2009).

No significant difference was seen in percentages or absolute numbers of CD4<sup>+</sup>CD26<sup>+</sup>, CD4<sup>+</sup>CD26<sup>-</sup>, CD8<sup>+</sup>CD26<sup>+</sup>, and CD8<sup>+</sup>CD26<sup>-</sup> between type 1 diabetes and control people.

However, the fluorescence intensity of CD26 expression on CD8<sup>+</sup> lymphocytes revealed a significant decrease in type 1 diabetic patients compared with control subjects. Mean fluorescence of CD8<sup>+</sup>CD26<sup>+</sup> cells was inversely correlated with the absolute number of CD4<sup>+</sup>CD26<sup>-</sup> cells (Matteucci et al., 2010). We interpreted the finding (low expression of CD26 among CD8<sup>+</sup> T cells in type 1 diabetes) as indicating a defect in successfully developed long-term memory CD8<sup>+</sup> T cells or in CD8<sup>+</sup> T cells activation, even though the negative association with the number of CD4<sup>+</sup>CD26<sup>-</sup> T cell does not support a recent activation of peripheral T cells. We intend to continue research in this field in consideration of the immunomodulating role of the multifunctional CD26 (Ohnuma et al., 2011).

### 8. Concluding remarks

Today, there is a great need to integrate molecular biology with whole organ physiology. Findings from molecular and cellular studies must be brought back to intact organ systems without losing the physiological context (Königshoff et al., 2011). This is especially true in the field of metabolic diseases where the study of individual proteins and signalling pathways in detail may not be easily translated to the intact organism. Taken into account the enlarging list of phenotypic characteristics that might allow the early clinical identification of families possibly at risk for sporadic cases of type 1 diabetes, many questions await an answer. We suggest the two main (in our opinion) issues.

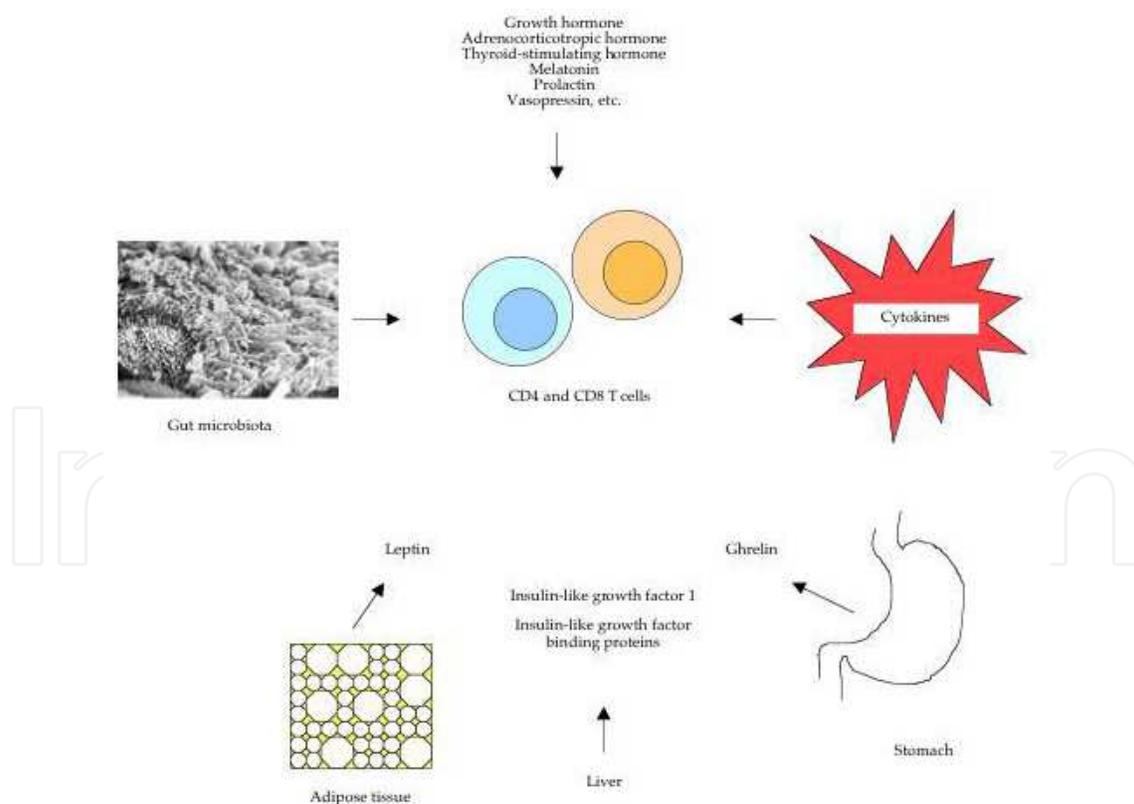


Fig. 4. Some of the potential mechanisms linking metabolic syndrome and T cell maintenance.

First question: may insulin-resistance be the common denominator of the observed familial peculiarities? And therefore, second question: could an early correction of one/some of

these common clinical abnormalities modify the natural history of the disease and thence its epidemiology? The data above summarised suggest to consider also alternative ways beyond the traditional immuno-based interventions so far extensively investigated in the field of type 1 diabetes. There is increasing attention to the role of metabolic syndrome and immune responses as well as to the relation between the immune and neuroendocrine systems (Figure 4). The adipocyte-derived proinflammatory hormone leptin can affect the survival and proliferation of autoreactive CD4 T cells (Matarese et al., 2008; Galgani et al., 2010). Immune and neuroendocrine systems have bidirectional communications (Kelley et al., 2007; Berczi et al., 2009). Growth hormone and ghrelin are expressed in immune cells, which in turn bear receptors for these hormones (Hattori, 2009). Leptin, ghrelin, insulin-like growth factor 1, insulin-like growth factor binding protein 3, and cytokines regulate both thymopoiesis and maintenance of T cells. Therefore, elucidation of metabolic syndrome, T cell metabolism, hormones, and microbiota may lead to new insights into the maintenance of proper immune responses (Hsu & Mountz, 2010).

At the present state of knowledge and given the current diabetes epidemic, it would seem reasonable that proper, more realistic, public health interventions (by general and family practitioners) are designed that address general issues such as feeding, lifestyle, overweight, 'borderline' blood pressure, impaired fasting glucose, etc. These health interventions, beyond the conventional boundaries that have for so long limited the visual field, might have a favourable cost-benefit ratio.

## 9. References

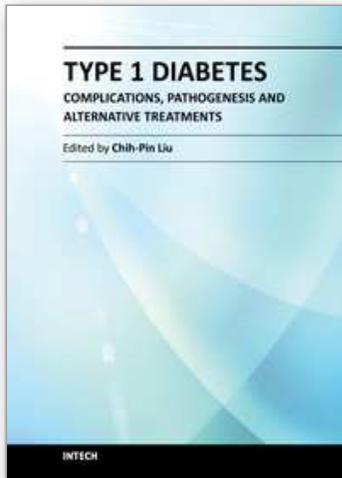
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## **Type 1 Diabetes - Complications, Pathogenesis, and Alternative Treatments**

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This book is intended as an overview of recent progress in type 1 diabetes research worldwide, with a focus on different research areas relevant to this disease. These include: diabetes mellitus and complications, psychological aspects of diabetes, perspectives of diabetes pathogenesis, identification and monitoring of diabetes mellitus, and alternative treatments for diabetes. In preparing this book, leading investigators from several countries in these five different categories were invited to contribute a chapter to this book. We have striven for a coherent presentation of concepts based on experiments and observation from the authors own research and from existing published reports. Therefore, the materials presented in this book are expected to be up to date in each research area. While there is no doubt that this book may have omitted some important findings in diabetes field, we hope the information included in this book will be useful for both basic science and clinical investigators. We also hope that diabetes patients and their family will benefit from reading the chapters in this book.

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