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“Meta-immunological profiling of children with type 1 diabetes identifies new biomarkers to monitor disease progression”

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This dissertation is based upon the following publication:

- Galgani M, Nugnes R, Bruzzese D, Perna F, De Rosa V, Procaccini C, Mozzillo E, Cilio CM, Lernmark A, Larsson HE, La Cava A, Franzese A, Matarese G. Meta-immunological profiling of children with type 1 diabetes identifies new biomarkers to monitor disease progression. Diabetes. 2013 Feb 8.

Other publications (scientific production) during PhD course:

- Mozzillo E, Raia V, Fattorusso V, Falco M, Sepe A, De Gregorio F, Nugnes R, Valerio G, Franzese A. Glucose derangements in very young children with cystic fibrosis and pancreatic insufficiency. Diabetes Care. 2012 Nov;35(11):e78.

- Camarca ME, Mozzillo E, Nugnes R, Zito E, Falco M, Fattorusso V, Mobilia S, Buono P, Valerio G, Troncone R, Franzese A. Celiac disease in type 1 diabetes mellitus. Ital J Pediatr. 2012 Mar 26;38:10.

- Franzese A, Mozzillo E, Nugnes R, Falco M, Fattorusso V. "Type 1 Diabetes Mellitus and Co-Morbidities", Type 1 Diabetes Complications. Edited by Wagner- Intech 2011, pp 85-108. ISBN: 978-953-307-788-8.

These publications, not included in the present dissertation, are attached as PDF-file at the end of the manuscript.

ABSTRACT

Type 1 diabetes (T1D) is an autoimmune disease characterized by the destruction of insulin-secreting pancreatic β-cells in genetically susceptible individuals, whose incidence is increasing worldwide. So far, our ability to curb this pathogenic immune response is limited by the poor understanding of the exact nature and kinetics of the immunological mechanisms leading to T1D. Indeed, triggers of islet autoimmunity and the precise mechanisms responsible for the progressive β-cell failure are not completely understood. Several scientific evidence supports a crucial role for the environment, which is also suggested by the rapid increase of incidence of this autoimmune disease. The search for a single cause of T1D has been vain, more probably there are a number of different factors that may contribute either to the initiating of the disease process or precipitating the manifest disease in subjects on their way to get diabetes. High living standard, good parent education, few siblings, pronounced hygiene, are all factors with possible important effects on immune system and autoimmune risk. In addition, the recent escalation of obesity in affluent countries has been suggested to contribute to the increased incidence of T1D, as a precipitating factor. Understanding the link between metabolism and immune tolerance could lead to the identification of new markers for the monitoring of disease onset and progression. We studied several immune cell subsets and factors with high metabolic impact as markers associated with disease progression in type 1 diabetes patients at onset, at 12 and at 24 months after diagnosis, in high-risk subjects and in non-diabetic controls. A multiple correlation matrix among different parameters was evaluated statistically and assessed visually on two-dimensional graphs. The meta-immunological profile changed significantly among the three studied groups and in patients over time, and a specific signature that associated with worsening disease was identified. Finally, markers to predict residual β-cell function up to one year after diagnosis were identified in a multivariate logistic regression model. This latter model, measuring age, body mass index (BMI), fasting C-peptide (C-pep), number of circulating $CD3^{+}CD16^{+}CD56^{+}$ cells and percentage of $CD1c^{+}CD19^{-}CD14^{-}CD303^{-}$ type 1 myeloid dendritic cells (mDC1s) at disease onset had a significant predictive value. The identification of a specific meta-immunological profile associated to disease status may contribute to understand the basis of diabetes and its progression, and provide novel biomarkers that could help clinicians in disease monitoring and in the choose of the most appropriate therapeutic approach according to disease status and aggressiveness.

1. Background

1.1 Epidemiology of type 1 diabetes: urgency for prevention

Type 1 Diabetes (T1D) is an autoimmune disease characterized by a T cell-mediated specific destruction of insulin-secreting pancreatic β-cells in genetically susceptible individuals.
T1D is one of the most common and serious chronic diseases in children. Incidence varies greatly between different countries, within countries, and between different ethnic populations. Annual incidence rates for childhood T1D show the highest incidence of 64 per 100,000/year in Finland (*Harjutsalo et al. 2008*). Incidence in Italy is characterized by a large variability, from geographical areas with very high levels of incidence, as in Sardegna (36.9 per 100,000/year), to areas with intermediate levels of incidence, as peninsular areas (8.4 per 100,000/year). Moreover, data from the "Registry for Type 1 Diabetes Mellitus in Italy (RIDI)" Study Group report significant higher incidence rate in boys than in girls in Sardegna (particularly in the age-group 10-14 years), while few differences between sexes have been observed in peninsular regions. Finally, it is interesting to observe that T1D incidence has showed wide variations not only between Sardegna and mainland Italy, but also among the peninsular regions. Indeed, incidence rates of 11.2 in North Italy, 9.3 in Central Italy, and 6.2 in South Italy have been reported (*Carle et al. 2004***).** A seasonal variation in the presentation of new cases is well described, with the peak being in the winter months.
Over the past 60 years, the incidence of T1D worldwide has been increasing by 3-5% per year (Figure 1), doubling approximately every 20 years.
A recent report from Finland, which has the highest incidence in the world, suggested that the incidence is also increasing faster than before (*Harjutsalo et al. 2008*). The rising incidence of T1D has been accompanied by a disproportionately greater increase in children under the age of 5 years (*DIAMOND Project Group 2006; Vehik et al. 2007; Patterson et al. 2012*). A recently published review reported an overall annual increase of 3.9% in the incidence of childhood T1D in Europe and predicted that the incidence rate in children aged < 5 years will double between 2005 and 2020 (*Patterson et al. 2009*).

Figure 1. T1D incidence has doubled every 20 years. Data for Finland are from the Finnish National Public Health Institute (Harjutsalo V.); data for Sweden are from the Swedish Childhood Diabetes Registry; data for Germany are a compilation of two reports; data for Colorado are from the Colorado IDDM Registry, the Barbara Davis Center for Childhood Diabetes, and SEARCH for Diabetes in Youth.

The rising incidence and earlier age of onset of pediatric T1D increase the urgency for its prevention and for interventions able to preserve functional β-cell mass.
The goals of a successful prevention approach in T1D should be cessation of β-cell destruction, reversal of autoimmunity and preservation of surviving β-cells in order to allow any natural regenerative potential to be realized. In addition, prevention interventions should realize these goals with a minimal degree of interference with the general functions of the immune system in order to avoid severe disturbances of immune surveillance mechanisms leading to intolerable side effects.
To this aim, biomarkers to stage and predict progression of the disease are needed and a more detailed understanding of disease mechanisms is required in order to efficiently curb islet autoimmunity and ultimately design appropriate cures.

1.2 Natural history of type 1 diabetes: genetic and environmental factors influencing the development of islet autoimmunity

The model of the natural history of T1D suggests stages that commence with a genetic susceptibility, autoimmunity without clinical disease, and finally clinical diabetes (Figure 2) (*Eisenbarth 1986*).

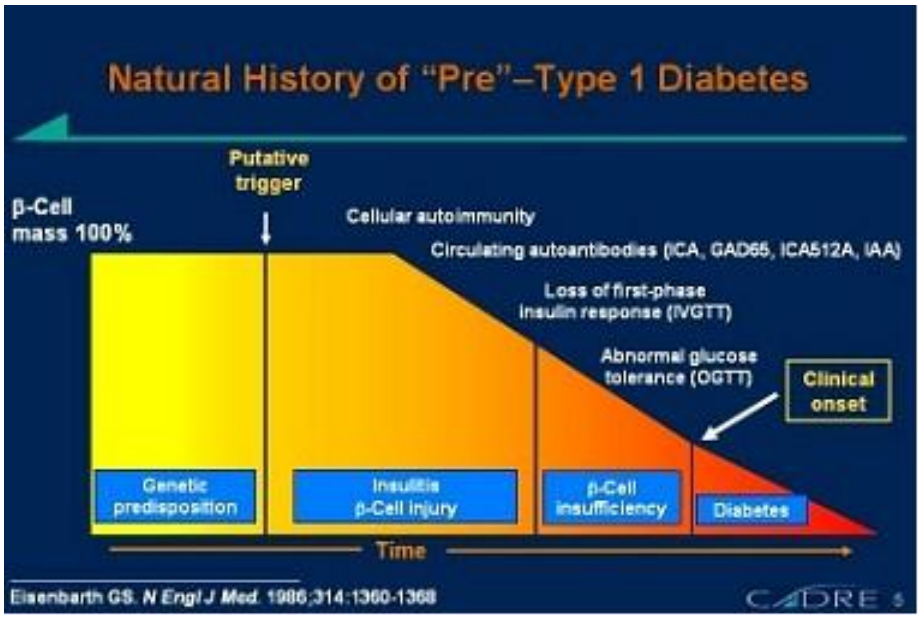


Figure 2. Natural history of type 1 diabetes (from Eisenbarth GS, 1986).

Genetic susceptibility to autoimmune T1D is well documented and associated with multiple genetic loci. The human leukocyte antigen (HLA) class II region on the short arm of chromosome 6 confers approximately half of the genetic risk of T1D. In addition, several non-HLA susceptibility genes have also been identified, mostly supporting a dominant role for an immune-mediated pathogenesis (*Steck et al. 2005*). Among HLA susceptibility genes, markers conferring increased risk include: HLA DRB1*03 - DQA1*0501 - DQB1* 0201 and HLA DRB1*04 - DQA1*0301 - DQB1* 0302 (*Erlich et al. 2008*). Of note, several recent studies have shown that there has been a decrease over time in the proportion of the high-risk HLA genotype, with an increase in low- to moderate-risk genotypes among type 1 diabetes patients in Finland (*Hermann et al. 2003*), UK (*Gillespie et al. 2004*), Colorado (*Vehik et al. 2008*), Australia (*Fourlanos et al. 2008*) and in Sweden (*Resic-Lindehammer et al. 2008*).

The presence of a genetic susceptibility, however, is not a sufficient condition for the development of islet autoimmunity and type 1 diabetes. Several environmental factors have been suggested to act as a trigger for autoimmunity and influence progression to clinical diabetes, in genetic susceptible individuals (Figure 3). Furthermore, the rapid increase in T1D incidence observed in the latest decades, together with the spreading to children bearing moderate- to low-risk HLA genotypes, strongly suggests an increased environmental pressure and a powerful influence of the environment interacting with the genetic background. The increased incidence in the very youngest children strongly suggests a role for early environmental exposures, as well.

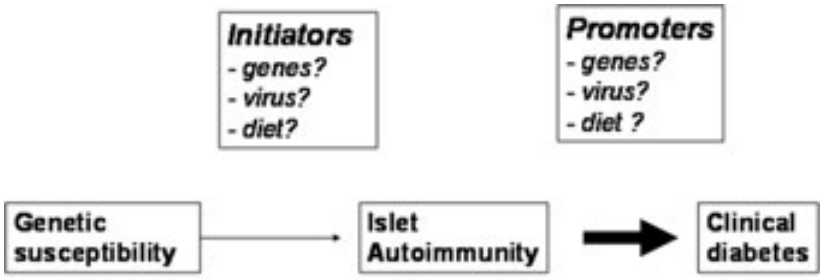


Figure 3. Environmental triggers influence the development of islet autoimmunity and progression to clinical diabetes

The development of an autoimmune process against Langerhans islets is characterized by the appearance of islet autoantibodies, which can be measured in the serum of these subjects. Islet autoantibodies identified so far include autoantibodies against insulin (IAAs), the 65-kDa isoform of GAD (GADA), the protein tyrosine phosphatase-related molecule IA-2 (IA-2A), and more recently autoantibodies against the pancreatic β-cell specific protein, zinc transporter 8 (ZnT8). Once islet autoantibodies have developed, the progression to overt diabetes in autoantibody-positive individuals is determined by the age of antibody appearance and by the magnitude of the autoimmunity, in turn related to the age of the subject (*Achenbach et al. 2005*). The period preceding the clinical onset of T1D, called "pre-diabetes", is usually asymptomatic or characterized by subtle metabolic disturbances. When most of the β-cell mass is lost, clinical signs of chronic hyperglycemia become evident and consequently patients need frequent blood glucose testing and daily insulin replacement therapy.
Over the last 20 years, several groups have carried out prospective studies from birth (BABYDIAB, Finnish DIPP, DAISY, TEDDY) that investigated the

development of islet autoimmunity and diabetes (*Hummel and Ziegler 2011; Ziegler and Bonifacio 2012; Kimpimaki et al. 2001; Stene et al. 2004; Snell-Bergeon et al. 2012; TEDDY Study Group 2008*). Such studies have shown that islet autoimmunity usually occurs very early in life. Indeed, the majority of children developing T1D in early childhood (< 10 years of age) have the first signs of islet autoimmunity by 2 years of age (*Ziegler and Bonifacio 2012*). Children who develop autoantibodies within the first 2 years of life are those who most often develop multiple islet autoantibodies and progress to type 1 diabetes in childhood. Autoantibodies do not exclusively develop before age 2 years, but children who develop autoantibodies later have a slower progression to multiple autoantibodies and type 1 diabetes (*Hummel et al. 2004*).

Environmental factors that have been proposed to contribute to islet autoimmunity and T1D risk include exposures taking place during pregnancy, infancy, childhood, and beyond. Several gestational factors are considered to increase the risk for T1D in childhood, although there is a gap in understanding to what extent gestational factors may trigger islet autoimmunity or merely increase T1D susceptibility in the offspring. Potential gestational risk factors include exposure to rubella during pregnancy (*Menser et al. 1978*), mother's enterovirus infections during pregnancy (*Hyoty et al. 1995*), and high birth weight for gestational age (*Stene et al. 2004*).

Candidate risk factors operating during infancy include those related to exposure to infectious agents, improved hygiene and mucosal exposure to dietary constituents. Enteroviruses, particularly coxsackie B viruses, are currently considered as the prime candidate among infectious agents by nature of their tropism for β-cells, possible molecular mimicry, and early and more recent reports of their presence in β-cells of patients with T1D (*Yeung et al. 2011; Atkinson et al. 1994; Dotta et al. 2007; Yoon et al. 1979*).

Seemingly in contrast to the infectious hypotheses is the notion that improved hygiene is responsible for upward trends in T1D incidence (*Gale 2002*). Few studies have examined the relationship between hygiene and development of islet autoimmunity. Related to hygiene is a potential role of vaccinations in the development of islet autoantibodies or progression to T1D. Some have suggested that vaccinations increase T1D risk, but well-designed studies have found no evidence for this (*Graves et al. 1999; De Stefano et al. 2001*). The temporal relationship of vaccinations to the development of islet autoantibodies or T1D has never been examined. Prospective analyses of children from 3 months of age through the entire period of mandatory or voluntary vaccinations are needed to establish effects of vaccinations on islet autoimmunity and progression to T1D (*TEDDY Study Group 2008*).

Several studies are also exploring the hypothesis that alterations in the intestinal microbiota in early childhood could increase susceptibility to the development of T1D (*Hara et al. 2013; Hummel and Ziegler 2011; Norris et al. 2003*).

Overall, several environmental factors seem to modify the risk of islet autoimmunity and T1D and have been considered to trigger autoimmune responses against pancreatic β-cells, eventually leading to β-cell destruction. The heterogeneity of T1D progression and clinical manifestations is likely a reflection of this complex multifactorial pathogenesis. Among environmental factors, several evidence points to infectious agents, components of early childhood diet and gut microbioma. However, besides the "classical" triggers (infections, diet, gut), growing evidence points at inflammation and metabolic changes as significant cofactors in T1D pathogenesis. These could even precede the triggering of islet autoimmunity, and may promote chronic immune dysregulation through effects on both innate and adaptive immune responses. Inflammation and insulin resistance are emerging as additional cofactors, which might be interrelated with the other known environmental factors**.** Secular trends in increased BMI and sedentary life style have been thought to contribute to the increased spreading of autoimmune type 1 diabetes (*Knip et al. 2008*). Obesity, insulin-resistance, or both have been suggested as important risk factors for type 1 diabetes (*Hypponen et al. 2000; Rosenbloom 2003; Wilkin 2008*).

There are several possible mechanisms to explain the increased risk of type 1 diabetes with increasing BMI in children. Fat cells secrete adipokines and cytokines, which may affect not only β-cell function, but also either increase the risk for or accelerate islet autoimmunity by influencing deleterious or protective local immune responses occurring in the pancreas. Furthermore, overweight increases insulin-resistance and the combination between islet autoimmunity and a demand for more insulin with increasing BMI may lead to an exhaustion of β-cells.

Overall, environmental triggers that contribute to the initiation and progression of pancreatic β-cell destruction still remain largely unknown. However, it is well documented that the "pre-diabetic" phase can last from months to years before the manifestation of clinical symptoms. In addition, at the time of clinical onset, the remaining portion of β-cell mass, although small (about 15-20% of the original β-cell mass), still has noticeable benefits. For these reasons, many efforts are being reserving to the identification of new immune therapies able to prevent the clinical onset in subjects with islet autoantibodies and/or preserve residual β-cell function in patients newly diagnosed with type 1 diabetes. Clinical immune interventions thus far have been mostly ineffective in delaying progression to overt diabetes in relatives at increased risk, or in reducing further loss of insulin secretion and producing lasting remission in patients with new-onset diabetes. This limited success may reflect our incomplete understanding of key pathogenic mechanisms,

the lack of truly robust biomarkers of both disease activity and β-cell destruction and the inability to assess the relative contributions of various pathogenic mechanisms at various time points during the course of the natural history of T1D. One of the most important current limitation to T1D immunotherapy is the lack of suitable biomarkers of the successive phases of disease.

Finally, the process of β-cell destruction is believed to be non-linear (*von Herrath et al. 2007*). In addition, 43-56% of children with T1D experience a honeymoon-phase, characterized by a temporary amelioration of β-cell function, which can last up to 24 months or even longer (*Buyukgebiz et al. 2001*). Both non-linear β-cell destruction and the honeymoon-phase suggest that the nature of the immune response towards β-cells potentially changes over time, yet the exact kinetics for these processes are unknown. It is also possible, that in humans β-cell loss is a relapsing syndrome, in which possible subsiding immune attack periods separate the infiltration of different lobes of the pancreas. Hence, immunotherapeutic approaches should ideally be tailored to the status of an individual's disease, specifically his/her β-cell mass. However, one of the current limitations to such an approach is the lack of biomarkers available to assess the existing β-cell mass and immunoreactivities to β-cell antigens precisely, this being particularly important before exposing very young patients and/or pre-diabetic (clinically healthy) individuals to reagents developed to interfere with their immune system.

1.3 Pathogenesis of type 1 diabetes: immune mechanisms underlying the selective destruction of pancreatic β-cells

Insulin-secreting β-cells are localized in the islets of Langerhans, scattered throughout the exocrine portion of the pancreas. Methodological options in studying immune responses leading to human T1D are extremely limited by the localization of the pancreas deep inside the abdominal cavity. Therefore, tissue sampling from T1D subjects is currently restricted to peripheral blood. As a consequence, one of the current limitation to the comprehension of T1D pathogenesis is undoubtedly that our hypotheses are usually built on the premise that cell populations found in the blood at least to some extent mirror the ones that infiltrate the islets.

Studies in animal models, particularly in non-obese diabetic (NOD) mice, have identified roles for several different immune cell types in β-cell destruction. $CD4^+$ and $CD8^+$ T cells, as well as macrophages, have been shown to have a role in β-cell death. However, other cell types are present in the pancreatic infiltrate and in the pancreatic draining lymph node, where the initial presentation of islet antigen by dendritic cells (DCs) to islet antigen-specific T cells occurs. These cells include B cells, Natural Killer (NK) cells and NKT cells, as well as DC subsets, and they could also contribute to β-cell death. This strongly suggests that there is substantial crosstalk between the immune cells that are involved in pathogenesis and those involved in immune regulation (*Lehuen et al. 2010*). Moreover, pro-inflammatory cytokines secreted by local antigen-presenting cells (APCs), NK cells, and T-cells contribute to further recruitment and activation of immune cells, consequently enforcing this autoimmune assault on the β-cells.

An overview of the disease mechanisms that are thought to be involved at various stages of human T1D development is provided by the Figure 4.

In response to an environmental trigger, β-cells produce inflammatory mediators such as interferon (IFN)-alpha and up-regulate their surface expression of MHC class I. Under such conditions of stress, β-cell antigens are released and taken up by antigen-presenting cells, such as dendritic cells and macrophages. The APCs migrate to the pancreatic draining lymph nodes and present β-cell auto-antigens to both Th1 cells and T-regs, with a critical role for TNF-alpha in generation of T-regs. In normal individuals, T-reg cells inhibit the action of Th1 cells via cytokines such as IL-10. In susceptible individuals, however, a detrimental Th1 response and interferon (IFN)-gamma production is initiated, leading to B cell activation and autoantibody production. Together with direct recognition and killing of β-cells by cytotoxic T lymphocytes (CTLs), these effector mechanisms ultimately lead to β-cell death and insulin deficiency (*Coppieters et al. 2011*).

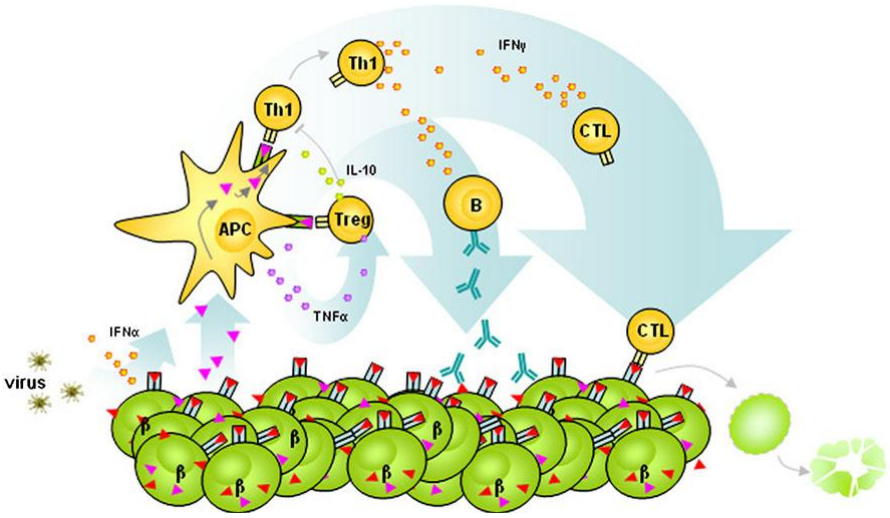


Figure 4. Overview of the disease mechanisms involved in the pathogenesis of T1D.

It is hypothesized that, during the pre-diabetic phase, a variable interplay between destructive and regulatory immune responses can occur and ultimately the destruction overtakes any attempts of the β-cells make to regenerate or proliferate.

1.4 Lymphocytes and type 1 diabetes

There is considerable evidence that T cells have an important role in the development and progression of T1D in both humans and animal models.
Studies in NOD mice have shown that T1D development depends on both $CD4^+$ and $CD8^+$ T cells (*Phillips et al. 2009*). There are several ways in which T cell-mediated β-cell death might occur. Indeed, $CD8^+$ T cells could kill pancreatic β-cells through MHC class I-mediated cytotoxicity, and both $CD4^+$ and $CD8^+$ T cells produce cytokines, such as interferon-γ (IFN-γ), that induce expression of the death receptor FAS and chemokine production by β-cells. Activation of FAS by FAS ligand (FAS-L)-expressing activated T cells could initiate β-cell apoptosis. Chemokine production by β-cells results in further recruitment of mononuclear cells to the site, thereby enhancing inflammation (*Eizirik et al. 2009*). In addition, IFN-γ can activate macrophages and induce increased pro-inflammatory cytokine production, including interleukin-1β (IL-1β) and tumor necrosis factor (TNF). β-cells express high levels of IL-1 receptor and seem to be more sensitive to IL-1β-induced apoptosis than other endocrine cells in the islet. This crosstalk between T cells and macrophages undoubtedly exacerbates the immune-mediated stress on β-cells and contributes to their destruction.
Although T cells have a pathological role in T1D development, there is also evidence supporting a role for T cells in the prevention of β-cell destruction. Indeed, islet antigen-specific T cells can differentiate into either pathogenic effector T cells (diabetogenic T cells) or protective T-reg cells. T-regs seem to play an important role in preventing the onset of autoimmune disease, including T1D (*Wildin and Freitas 2005*).
In conclusion, T cells are clearly pivotal for T1D development. However, there are also data suggesting an involvement of other cell types such as B cells. Indeed, It has been hypothesized a role for B cells as antigen-presenting cells that maintain islet antigen-specific T cell activity (*Lehuen et al. 2010*).

1.5 Innate immune cells in type 1 diabetes

Although the importance of T cells in T1D pathogenesis has been largely elucidated, there is increasing evidence that innate immune cells have crucial roles in T1D pathogenesis. Macrophages, DCs and NK cells are required for the development of T1D in various mouse models and have been detected in the pancreas of patients with T1D (*Uno et al. 2007*). Several studies support a pathogenic role for macrophages in both the initiation and destruction phases of T1D. Recruitment of macrophages to islets is mediated by the secretion of chemokines by $CD4^+$ T cells and pancreatic β-cells. Macrophages recruited to the pancreas produce IL-1β and TNF that can cause β-cell death (*Yang 2008*). NK cells are also involved in T1D pathogenesis. Several reports have described a correlation between the frequency and/or activation of NK cells with the destructiveness of the pancreatic infiltrate. NK cells are both cytotoxic and producers of cytokines, particularly IFN-γ, so that they could contribute directly and indirectly to the destruction of β-cells (*Dotta et al. 2007; Alba et al. 2008*).
However innate immune cells have shown an ambivalent behavior as they can also be involved in protection against the disease. In particular, dendritic cells are an heterogeneous group of innate effectors that serve two general functions in controlling T-cell immunity. First, they are professional antigen-presenting cells that process and present antigens and are responsible for the activation of naive T cells. Second, they can critically influence the characteristics of the T-cell responses through the secretion of cytokines that condition the extracellular milieu and determine the nature of the T-cell response. Indeed, through the production of pro-inflammatory or suppressive cytokines, they can define the milieu in which islet antigen-specific T cells are activated, determining the type of immune response that ensues and whether a deleterious or protective local immune response occurs in the pancreas. These opposing functions, in inflammatory responses against microbs and in immune-tolerance to self-antigens, are governed by the maturation status and types of cytokines secreted by dendritic cells**.** Under non-inflammatory or homeostatic conditions, the majority of dendritic cells are found in an immature state. Immature dendritic cells presenting self-antigens fail to both stimulate T-cells and secrete cytokines. Under certain conditions, immature dendritic cells presenting self-antigens can be tolerogenic by inducing either a state of unresponsiveness or apoptosis in the autoreactive T-cells (*Wallet et al. 2005*). Presumably, in normal conditions, immunogenic DCs provide active host defenses, while tolerogenic DCs guard against autoimmunity and control established immune reactions. In autoimmune diseases, there is strong evidence to support the idea that tolerance is overridden by the development of immunogenic DCs that favor autoimmune responses.

Three types of dendritic cells can be isolated from in human blood: $CD1c^+$ myeloid dendritic cells (mDC1s), $CD141^+$ myeloid dendritic cells (mDC2s), and $CD303^+$ plasmacytoid dendritic cells (pDCs).

Myeloid dendritic cells are considered as a sort of ”sentinels” of immunity, ideally positioned throughout the body gateways and equipped with unique properties to transport antigens from the periphery to lymphoid tissues. They are professional antigen-presenting cells transmitting incoming infectious signals to T cells, the key players of adaptive immunity. However, besides this essential immunostimulatory function, these cells may act as pivotal players in the peripheral tolerance network by active induction of T cells with immunosuppressive functions and regulation of T effector cell activity (*Steinbrink et al. 2009*). In the context of autoimmune diabetes, mDCs could capture self-antigens released after β-cell death in the pancreatic islets and present them to islet antigen-specific T cells in the pancreatic lymph nodes where the diabetogenic response is initiated (*Marleau et al 2008*).

By contrast, pDCs have a decreased capacity to take up, process and present soluble antigens. They are rather known for their capacity to detect viral RNA or DNA through TLR7 and TLR9 and to respond by secreting large amounts of type I IFNs, Il-12 and pro-inflammatory chemokines (*Lande and Gilliet 2010*). A putative role for pDCs in the development of T1D is supported by observations in both human and mouse models that type I IFNs, which are usually produced by this cell type, can in some situations induce or enhance T1D.

Several studies have investigated mDCs and pDCs compartments and functions in diabetic individuals, and controversy exists whether numerical and/or functional disturbances of circulating DCs are associated with the disease. Indeed, there have been apparently discrepant data regarding the frequency of DCs in the blood of patients with T1D, which may reflect differences in the time when the blood samples were taken. Interestingly, pDCs from early-diagnosed patients were shown to present immune complexes to T cells more efficiently than mDCs, suggesting a possible detrimental role of pDCs in T1D onset (*Peng et al. 2003; Vuckovic et al. 2007; Chen et al. 2008; Allen et al. 2009*).

1.6 Leptin as an immune-endocrine mediator

Leptin is an hormone, mainly secreted by the adipose tissue, reflecting the amount of energy stored. It regulates the balance between food intake and energy expenditure, signaling to the brain the changes in stored energy. In addition, it has been shown to play an important role in the regulation of neuroendocrine function and energy homeostasis and other energy-demanding physiological processes, such as reproduction, hemopoiesis, and angiogenesis (*Matarese et al. 2005*).
Although the most important role of leptin is to regulate body weight through the inhibition of food intake and stimulation of energy expenditure by increased thermogenesis, recent evidence has indicated that leptin also play a key role in the regulation of the immune system (*La Cava and Matarese 2004*). Indeed, leptin act also as a cytokine and has structural similarity to other cytokines, such as IL-6, IL-12, granulocyte colony-stimulating factor (G-CSF).
Leptin actions are mediated by the leptin receptor, a membrane-spanning glycoprotein, that belongs to the class I cytokine receptor family (including receptors for IL-6, IL-12), and exists in at least six alternatively spliced forms with cytoplasmic domains of different length. Short forms of the leptin receptor are expressed by several non-immune tissues and seem to mediate the transport and degradation of leptin. The long form is expressed by the hypothalamus in areas that are responsible for the secretion of neuropeptides and neurotransmitters that regulate appetite and body weight, but is also expressed by immune cells, supporting a role for leptin in immune system. In addition, a soluble form of leptin receptor, present in the peripheral blood, represents the main leptin binding activity in human blood and modulates the bioavailability of free leptin.
Leptin affects both innate and adaptive immunity (Figure 5). In innate immunity, leptin seems to promote activation of and phagocytosis by monocytes/macrophages and their secretion of leukotriene B4 (LTB4), cyclooxygenase 2 (COX2), nitric oxide and pro-inflammatory cytokines. Moreover, leptin can induce chemotaxis of neutrophils and the release of oxygen radicals. Finally, leptin also affects NK cell development and activation both in vitro and in vivo.
The effects of leptin on adaptive immune responses have been extensively investigated on human $CD4^+$ T cells. Leptin has different effects on proliferation and cytokine production by human naive ($CD45RA^+$) and memory ($CD45RO^+$) $CD4^+$ T cells, both of which express leptin receptor. In particular, leptin promotes proliferation and IL-2 secretion by naive T cells, whereas it minimally affects the proliferation of memory cells, on which it promotes the switch towards Th1-cell responses. This process is then sustained by an autocrine loop of leptin secretion by Th1 cells. Furthermore, leptin increases the expression of adhesion molecules

by $CD4^+$ T cells, which could then be responsible for the induction of clustering, activation and migration of immune cells to sites of inflammation.

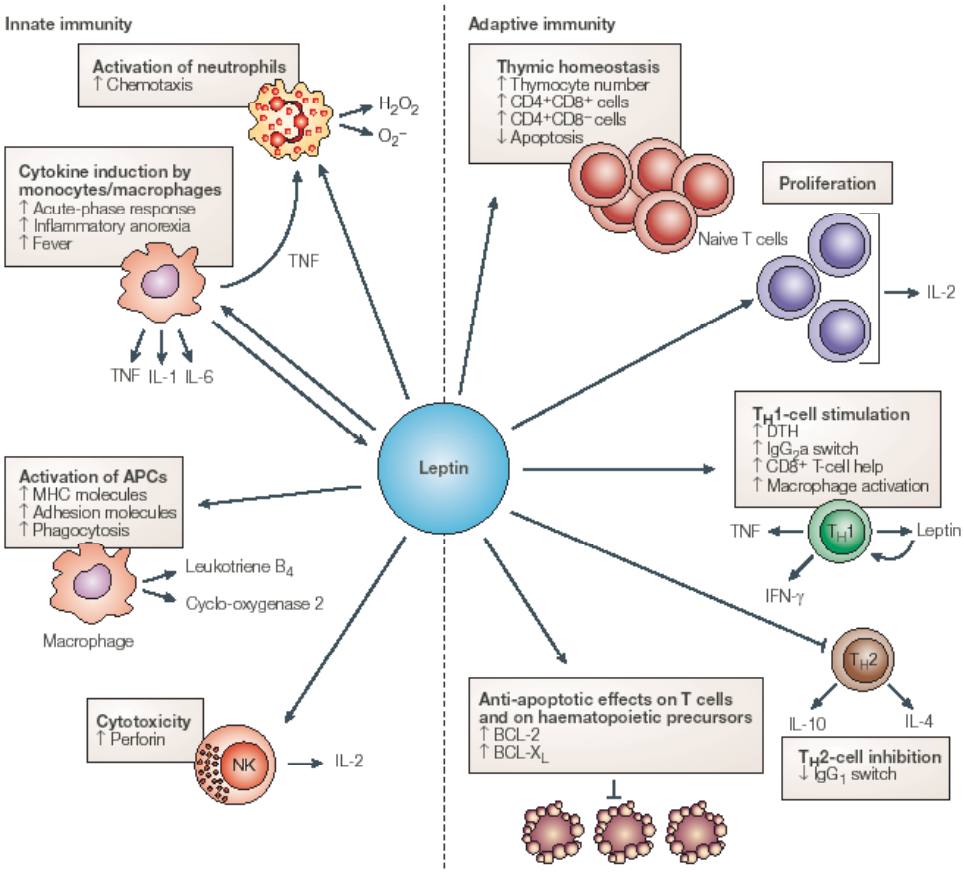


Figure 5: Schematic representation of the effects of leptin on both innate and adaptive immunity.

Overall, leptin is an adipocyte-derived hormone/cytokine that links nutritional status with neuroendocrine and immune functions. Research on leptin has provided important insights into the intricate network that links nutrition, metabolism and immune homeostasis.

Altered serum levels of leptin have been reported in several chronic inflammatory conditions, such as pelvic endometriosis, nonalcoholic hepatitis, chronic pulmonary inflammation, IBD, inflammatory nephritis, Behcet's syndrome, Graves' disease, T1D and rheumatoid arthritis (*La Cava and Matarese 2004*). Moreover, it is common to find an increase of leptin concentrations in the early phases of autoimmune diseases and before relapses (*La Cava and Matarese 2004*).

Of note, sampling and disease staging must be carefully evaluated when interpreting the results of these studies.

The role in autoimmunity could be mediated by leptin produced by adipocytes present in the perilymphonodal adipose tissue and in the lymph nodes. In this context, leptin could promote the differentiation of T helper 1 (Th1) cells, the activation of monocytes/macrophages and the secretion of cytokines of the acute-phase response and oxygen radicals. Autoantigens could be presented to Th0 cells that express the long form of the leptin receptor. Leptin could then affect the priming of autoreactive T cells towards Th1-type pro-inflammatory responses. Paracrine effects of Th1 cells that produce leptin after activation with antigen could then sustain an autocrine loop of proliferation and T-cell survival.

The role of leptin in autoimmune diabetes has mainly been investigated in animal models. Serum levels of leptin increases in non-obese diabetic (NOD) mice before the onset of hyperglycemia and diabetes. In addition, intraperitoneal injection of leptin accelerates autoimmune destruction of insulin-producing β-cells in NOD mice (*Matarese et al. 2002*). Leptin has pro-inflammatory properties and could aggravate the development of Th1-dependent autoimmune diseases.

Several evidence suggests the influence of metabolic factors on the development of autoimmunity and type 1 diabetes. Indeed, in the past decades we have witnessed a dramatic obesity epidemic, affecting not only adults but also pediatric population. Several studies report a dramatic increase in the prevalence of childhood overweight and obesity in recent decades (*Wang and Lobstein 2006; de Onis et al. 2010*). Such raising trend has been paralleled by a similarly dramatic increase in the incidence of type 1 diabetes. This increased incidence appears not to be uniformly distributed: T1D associated with high-risk HLA alleles has remained stable, whereas that associated with low-risk genotypes has increased significantly and a tendency to associate with obesity and insulin resistance has been observed (*Carlsson et al. 2012*). In addition, cohort studies have shown that children who develop type 1 diabetes were heavier in the first year of life as compared to their peers who remain free of disease, and that an increasing body mass index (BMI) strongly correlated with an earlier disease presentation (*Hyppönen et al. 1999; Betts et al. 2005*). Also, insulin-resistance related to overweight not only often preceded clinical onset, but was also the strongest predictor of type 1 diabetes, aside from HLA genotype (*Fourlanos et al. 2008; Wilkin TJ 2008*).

In conclusion, increasing evidence is revealing the importance of several molecules, including leptin, operating at the interface between metabolism and immune responses (*Odegaard and Chawla 2012*). However, the intimate connection between molecules that influence body weight and the network of molecules, cells and pathways that finely tune the immune response makes these

studies complex. There is therefore still much to learn about the interaction between metabolism and immune responses.

2. Aim of the study

The development of type 1 diabetes involves a complex interaction between pancreatic β-cells and both arms of the innate and adaptive immune system, which undoubtedly changes during the progression of the disease. In addition, several evidence is suggesting an important role for molecules operating at the interface between metabolism and immune responses in the development and progression of autoimmune diseases, such as type 1 diabetes. Meta-immunology has been developing over the last 10 years, and it can be used in the study of type 1 diabetes, i.e. to link metabolism with immunity including immune tolerance. Altered functions, an imbalance of immune cell subsets, or both, and metabolic/inflammatory molecules may contribute to the initiation and progression of the β-cell autoimmune responses resulting in the clinical onset of the disease.
In this study, we aimed at analyzing the meta-immunologic profile of newly diagnosed type 1 diabetes children, high-risk children and non-diabetic controls, and comparing the three studied groups in order to identify immune and/or metabolic markers specific of different stages of disease progression.
Most of the studies that have examined meta-immunologic parameters in type 1 diabetes had discrepant results, and did not yield definitive conclusions (*Peng et al. 2003; Vuckovic et al. 2007; Chen et al. 2008; Allen et al. 2009*). Moreover, they typically involved relatively small numbers of subjects and analyzed single (or a few) parameters (*Schloot et al. 2007; Pfleger et al. 2008*). More important, no study so far has examined meta-immunologic parameters for the prediction of residual β-cell function over time, e.g. via the monitoring of metabolic and immunologic functions characterizing type 1 diabetes.
Therefore, we then examined all immune and metabolic parameters in order to find one or more immune/metabolic marker able to predict, since the time of disease onset, the residual β-cell function over time.
Our approach tried to provide a way for discriminating, at disease diagnosis, children who will maintain a good β-cell function from those losing their function up to one year from diagnosis.

During my PhD course, I also collaborated to other scientific studies in the field of pediatric diabetes. Such studies resulted in publications that are attached as PDF-file at the end of the present manuscript.

3. Research, design and methods

3.1 Subjects

Children (n = 114, Table 1) receiving the diagnosis of type 1 diabetes were recruited after glycemic stabilization on exogenous insulin, achieved in 5 days. Diabetes was defined according to the Global IDF/ISPAD Guidelines for Diabetes in Childhood and Adolescence, and included symptoms of diabetes in addition to casual plasma glucose concentration ≥11.1 mmol/l (200 mg/dl), or fasting plasma glucose ≥7.0 mmol/l (≥126 mg/dl), or 2 hour post-load glucose ≥ 11.1 mmol/l (≥200 mg/dl) during an Oral Glucose Tolerance Test (OGTT), and Glycated Haemoglobin (HbA1c) ≥6.5 (*IDF/ISPAD 2011 Global Guideline for Diabetes in Childhood and Adolescence*). Among the 114 diabetic children, 40 had ketoacidosis at disease onset and 25 had at least another autoimmune disorder besides autoimmune diabetes, more frequently celiac disease (n = 12) or autoimmune thyroiditis (n = 13).

Table 1 . Baseline characteristics of controls, high-risk subjects and diabetic patients at disease onset.

Baseline characteristics of studied groups	Controls	High-risk subjects	Diabetics at onset
Number of subjects	34	29	114
Age (years)	10.42 ± 4.18 (1.08-17.58)	6.46 ± 2.57 (4-15.08)	9.09 ± 3.76 (2-17.50)
Sex (M/F)	17/17	15/14	66/48
Body Mass Index (kg/m^2)	20.78 ± 5.23 (14.44-33.97)	16.42 ± 1.49 (13.70-19.30)	18.62 ± 4.46 (11.90-35.71)
Body Mass Index SD Score (BMI-SDS)	0.69 ± 1.47 [(-3.35) - (+2.90)]	0.35 ± 1.02 [(-1.43) - (+1.98)]	0.26 ± 1.34 [(-4.62) - (+2.59)]
Glycated hemoglobine (%)	-	4.4 ± 0.28 (3.60-4.80)	11.01 ± 2.56 (5.3-18.10)
Insulin dose (units/kg of body weight/24h)	-	-	0.55 ± 0,31 (0-1.26)
Basal C-peptide (ng/ml)	1.72 ± 1.05 (0.03-5.03)	0.23 ± 0.11 (0.12-0.47)	0.68 ± 0.48 (0.1-2.77)
Ketoacidosis at diagnosis	-	-	40/114
Other autoimmune diseases	-	-	25/114 (13 AIT†; 12 CD††)

Data are expressed as mean ± SD (Range); Body mass index (BMI) is the weight in kilograms divided by the square of the height in meters. † AIT: Autoimmune Thyroiditis; †† CD: Celiac Disease.

Thirty-four healthy controls (n = 34, Table 1) were selected on the basis of the following criteria: fasting blood glucose of <5.5 mmol/L (<100 mg/dl), negative personal and familial history of autoimmune disorders and negativity for islet autoantibodies at the 99th percentile. Both diabetic children and control subjects were recruited in Department of Pediatrics at the University of Naples Federico II. A local Ethical Committee approved the study and parents gave informed consent. The third group designed as "high-risk" subjects included 29 individuals recruited

from baseline of the Diabetes Prevention - Immune Tolerance study at the Skåne University Hospital, Malmö, Sweden. Children carrying HLA susceptibility alleles for type 1 diabetes were defined as high-risk based on the presence of GAD65 autoantibodies (GADA) and at least one more islets-specific autoantibodies (IA-2A, ZnT8A or IAA). The Lund Regional Ethics Board approved the study and informed consent was obtained from all subjects or their parents in accordance to the Declaration of Helsinki. There was no significant difference among the three study groups concerning to gender and BMI Standard Deviation Score (BMI-SDS). High-risk subjects were younger and consequently with lower BMI than controls and diabetics at onset. Characteristics of the study subjects are summarized in Table 1.

3.2 Study design

At the beginning of the study, children from each group were analyzed for a wide range of immune (Table 2) and metabolic parameters (Table 3). Diabetic children were followed for a period of twelve (n = 60) to twenty-four (n = 30) months after disease onset and were then analyzed for the same parameters measured at the diagnosis. In addition, patients were dichotomized into two different groups on the basis of residual fasting C-peptide (C-pep) evaluated one year later upon disease diagnosis. Specifically, diabetic children with C-pep <0.5 ng/ml were designed as "severe disease patients", while patients with C-pep levels >0.5 ng/ml were defined "mild disease patients".
A multivariate logistic regression analysis was performed to identify, at the time of diagnosis, biological parameters predictive of a maintained pancreatic function 12 months later. To exclude the possible influence of glucose levels on metabolic parameters, blood was drawn when glicemic values were into the range of 80-180 mg/dl (4.4–10 mmol/L). The actual glucose levels at the time of draw were not considered in the analysis.

3.3 Flow cytometry

Immune cell profiling of all subjects of the three studied groups was done at the time of blood drawing. Prior to flow cytometry for lymphocyte subsetting, whole blood cells were analysed with a clinical-grade hematocytometer to determine absolute lymphocyte numbers in each sample. For the controls and type 1 diabetes patients, 100 µl of blood was incubated 30' at room temperature with the specific antibodies combinations. Red blood cells were lysed using BD FACS lysing Solution 2 (BD Bioscience) for 10' and samples subsequently washed and

resuspended in 300 µl phosphate buffered saline (PBS). Flow cytometry was carried on cells gated on $CD45^+$ - Side Scatter (SSC). Immunophenotypic analysis was performed with an EPICS XL flow cytometer (Beckman Coulter, Milan, Italy) using the Beckman Coulter software program XL System II. Triple combinations of different human mAbs, e.g., FITC- and phycoerythrin (PE)-anti-CD3, PE- and PC-5-anti-CD4, PC5-anti-CD8, PE-anti-CD16, PC5-anti-CD19, PE-anti-CD25, FITC-anti-CD45, and PE-anti-CD56 (all from Coulter Immunotech, Marseille, France) were used to identify different cell populations. For high-risk subjects, whole blood samples were stained with the following monoclonal anti-human antibodies in various combinations for flow cytometry: fluorescein isothiocyanate (FITC)-conjugated CD3; phycoerythrin (PE)-conjugated CD19 and CD8; peridinin chlorophyll protein (PerCP)-conjugated CD4; CD16 and CD56 (PE) (all from BD Bioscience, Becton, Dickinson, Franklin Lakes, NJ, USA). Briefly, 100 µl of blood was used for each staining, and samples were incubated 20-30 min. at room temperature. Red blood cells were lysed using BD FACS lysing Solution 2 and samples were washed and resuspended in 300 µl in FACS buffer for flow cytometry. Lymphocytes cell analysis was done on gated $CD45^+$ - Side Scatter (SSC) cells with a FACSCalibur (BD, Bioscience, NJ, USA). Data were analysed using CellQuest software (Becton Dickinson). No differences in the analysis of three studied groups can be ascribed to procedures employed, since the same clones of monoclonal antibodies and similar experimental conditions were used in the flow cytometry setting.
Circulating myeloid (mDCs) and plasmacytoid (pDCs) dendritic cells were evaluated using the Blood Dendritic Cells Enumeration Kit (Miltenyi Biotec, Germany). Specifically, mDC1s, mDC2s and pDCs were identified based on the expression of CD1c, CD141 and CD303 markers, after exclusion in the analysis of the $CD19^+$ and $CD14^+$ cells. The samples were analyzed by a CyAn flow cytometer and Summit software (Instrumentation Laboratory, Massachusetts, USA). Cell numbers are expressed as percent of a given cell population multiplied by number of lymphocytes/100, except for the number of DCs for which the percentage is referred to white blood cells (WBCs).

3.4 Laboratory tests

For controls and high-risk subjects, a 4 ml blood sample was obtained at the time of recruitment. For patients, a 4 ml blood sample was obtained at diabetes onset and after 12 and at 24 months. An aliquot from each blood sample was used to perform immune cell profiling by flow cytometry, and the remaining part of the sample was used for serum-based assays. Sera were centrifuged and kept at –80 °C until use. Fasting C-peptide levels were measured in duplicate serum samples, at

the same time in all samples, using a commercial ELISA kit (Millipore Corporation, Billerica, MA, USA). Results for each assay were validated and a high-level and a low-level control sample were included.
Circulating leptin (Lep) and soluble leptin receptor (sLepR) were determined in duplicate serum samples using human Leptin and human Leptin sR Immunoassays, respectively (R&D System, Inc. Minneapolis, MN, USA).
Soluble CD40L (sCD40L), soluble ICAM-1 (sICAM-1), monocyte chemoattractant protein-1 (MCP-1), myeloperoxidase (MPO), osteoprotegerin (OPG), resistin and soluble TNF-R (sTNF-R) were analyzed using the bead-based analyte detection system Human Obesity 9plex kit (Bender MedSystem Inc, Burlingame, CA, USA) in duplicate serum samples.
Plasma glucose levels were measured using enzymatic hexokinase method. HbA1c was measured by HPLC (HLC-723 G7 TOSOH, Bioscience, Tokyo, Japan). Islet autoantibodies (GADA, IA-2A and IAA) were measured by commercial ELISA (Pantec, Torino, Italy).

3.5 Statistical Analysis

Normally distributed continuous variables are reported as mean ± standard deviation; median (min - max) was used to describe not-normally distributed continuous variables; categorical variables are reported as number of occurrences and percentages. For all study variables, comparison among controls, high-risk subjects and patients was based on the non-parametric Kruskal-Wallis procedure followed by the Mann-Whitney U test with Bonferroni correction. Paired comparisons between patients at baseline and after 12 and 24 months from diagnosis were carried out using the Friedman test, followed by Wilcoxon signed-rank test with Bonferroni correction. Spearman correlation coefficient was computed to investigate the biological correlations between the different variables.
Due to the large number of the variables examined and to control the family-wise error rate at level $\alpha = 0.05$, significance of Kruskall-Wallis, Friedman and Spearman correlation p values were judged by using the adaptive Bonferroni procedure (*Guo et al. 2010; Guo 2009*).
A multivariable logistic regression model was fitted to predict the residual C-peptide secretion (dichotomized as 1 if ≤ 0.5 ng/ml and 0 if > 0.5 ng/ml) after 12 months from the diagnosis on the basis of patients' baseline measurements. The following two steps model strategy was adopted. All variables reported in Table 2 and Table 3 were tested in univariate analysis. Only the variables showing a univariate association with the outcome ($p < 0.25$) were included in the subset of candidate predictors. Afterwards, a backward elimination procedure (with a probability for removal equal to 0.10) was applied to identify those variables

independently associated with the residual C-peptide. In this second step, the model was further adjusted for the following covariates measured at diagnosis: age, BMI and fasting C-peptide secretion (dichotomized as 1 if ≤0.5 ng/ml and 0 if >0.5 ng/ml). This full model, including variables independently associated with residual C-peptide, was compared with a base model including only the adjusting covariates (i.e. age, BMI and C-peptide secretion measured at diagnosis). Prognostic validity of the fitted models was evaluated by ROC curve analysis and measured using the area under the ROC curve (AUC). Comparison among different AUC ‘s was carried out using the non-parametric approach (*De Long et al. 1988*). For all analyses, we used two-sided tests, with p values <0.05 denoting statistical significance (unless otherwise specified). Modelling and statistical analyses were carried out using R version 2.12.1 and SPSS software version 16 for Mac (SPSS Inc, Chicago, IL, USA).

4. Results

4.1 Meta-immunological profiling of type 1 diabetes patients at onset, high-risk subjects and controls

To depict the immunological profile of type 1 diabetes at onset, we analyzed several immune cell populations in the peripheral blood of children at onset in comparison with high-risk subjects and controls (Table 2).

Table 2. Absolute number and percentages of circulating immune cells in controls, high-risk subjects and diabetic patients at disease onset (percentages in brackets).

	Controls (n = 34)	High-risk subjects (n = 29)	Diabetics at onset (n = 114)	*p*
$CD3^+$	1725.47 ± 602.82 (71.47 ± 8.53)	1642.54 ± 534.49 (73.32 ± 4.63)	1869.11 ± 715.81 (71.73 ± 6.99)	ns ns
$CD4^+$	925.38 ± 335.92 (38.56 ± 7.46)	889.86 ± 318.08 (39.34 ± 3.99)	1067.04 ± 440.6 (41.04 ± 6.9)	ns ns
$CD8^+$	613.71 ± 265.58 (25.41 ± 6.59)	622.51 ± 216.31 (27.87 ± 4.5)	604.26 ± 267.31 (23.11 ± 5.39) (§)	ns < 0.001
$CD3\text{-}CD16^+CD56^+$	252.53 ± 197.33 (10.18 ± 7.16)	156.98 ± 112.01 (6.91 ± 3.58)	233.23 ± 184.46 (8.78 ± 4.88)	ns ns
$CD19^+$	369.85 ± 204.36 (14.76 ± 4.85)	374.38 ± 134.16 (16.94 ± 3.65)	439.19 ± 335.26 (15.76 ± 6.49)	ns ns
$CD4^+CD8^+$	19.77 ± 17.04 (0.91 ± 0.79)	nd	16.37 ± 18.16 (0.65 ± 0.64)	ns ns
$CD3^+CD45RA^+$	1186.47 ± 544.78 (47.74 ± 10.37)	nd	1282.39 ± 602.56 (48.21 ± 9.13)	ns ns
$CD3^+CD45RO^+$	559.17 ± 205 (23.74 ± 8.9)	nd	605.77 ± 328.04 (23.43 ± 8.4)	ns ns
$CD4^+CD28^+$	816.41 ± 298.13 (33.91 ± 6.62)	nd	930.32 ± 403.47 (35.74 ± 7.53)	ns ns
$CD4^+DR+$	28.35 ± 23.53 (1.15 ± 0.86)	nd	28.4 ± 27.14 (1.09 ± 1.01)	ns ns
$CD4^+CD25^+$	46.48 ± 65.65 (1.71 ± 1.38)	nd	41.78 ± 39.16 (1.69 ± 1.46)	ns ns
$CD4^+CD45RA^+$	620.47 ± 315.72 (25.06 ± 8.04)	nd	762.07 ± 754.65 (26.13 ± 7.95)	ns ns
$CD4^+CD45RO^+$	304.76 ± 123.43 (13.5 ± 6.37)	nd	362.86 ± 147.2 (14.96 ± 5.81)	< 0.05 ns
$CD8^+DR$	19.35 ± 24.01 (0.82 ± 0.94)	nd	18.66 ± 19.25 (0.74 ± 0.73)	ns ns
$CD8^+CD11b^+$	78.82 ± 89.87 (3.24 ± 3.45)	nd	83.94 ± 115.94 (2.86 ± 2.81)	ns ns
$CD3^+CD16^+56^+$	63.85 ± 55.67 (2.88 ± 2.37)	nd	63.8 ± 153.14 (2.24 ± 2.75)	ns ns
$CD3^-CD8^+$	82.62 ± 55.86 (3.35 ± 2.1)	nd	103.87 ± 101.92 (3.85 ± 2.36)	ns ns
mDC1s (*)	30.55 ± 21.14 (0.53 ± 0.35)	nd	37.56 ± 29.97 (0.58 ± 0.33)	ns ns
mDC2s (**)	1.15 ± 1.09 (0.02 ± 0.02)	nd	1.49 ± 1.75 (0.02 ± 0.02)	ns ns
pDCs (***)	21.88 ± 14.12 (0.39 ± 0.26)	nd	33.14 ± 26.63 (0.54 ± 0.4)	< 0.05 < 0.05

Data are expressed as mean ± SD; * type 1 myeloid dendritic cells (mDC1s): $CD1c^+CD19^-CD14^-CD303^-$ cells; ** type 2 myeloid dendritic cells (mDC2s): $CD141^+CD1c^-CD19^-CD14^-CD303^-$ cells; *** plasmacytoid dendritic cells (pDCs): $CD303^+CD1c^-CD19^-CD14^-$ cells; § statistical significance between diabetic patients at onset and high-risk subjects ($p < 0.0001$); nd = not determined, ns = not significant.
Absolute cell numbers and percentages (in brackets) of circulating immune cells in the three studied groups expressed as mean ± SD. The absolute number per mm^3 of all cell populations was calculated as follows: percent of a given cell population multiplied by the number of lymphocytes/100, except for the number of dendritic cells for which the percentage was referred to white blood cells (WBCs).

We found that patients at onset had a significantly lower percentage of circulating $CD8^+$ T cells as compared to high-risk subjects (p = 0.001, after Bonferroni correction) but not compared to healthy controls. Furthermore, diabetic children also showed a significant higher number of $CD4^+$ T cells with a memory phenotype ($CD4^+CD45RO^+$) as compared to the controls (p = 0.03, after Bonferroni correction). Finally, patients had a significantly higher percentage (p = 0.03, after Bonferroni correction) and total number (p = 0.01, after Bonferroni correction) of $CD303^+CD1c^-CD19^-CD14^-$ plasmacytoid dendritic cells (pDCs) compared to healthy subjects (Table 2).

To characterize the meta-immunological profile of type 1 diabetes, several indicators of inflammatory and metabolic activities (leptin, sLepR, MCP-1, sCD40L, MPO, sICAM-1, resistin, OPG and sTNF-R) were analyzed (Table 3 and Figure 6). Diabetic children at onset had lower levels of circulating leptin (p <0.001, after Bonferroni correction) and higher concentrations of circulating sLepR compared to both high-risk (p <0.001, after Bonferroni correction) and control subjects (p <0.001, after Bonferroni correction). High-risk subjects showed intermediate levels of sLepR between controls and patients (p <0.001, after Bonferroni correction) (Table 3 and Figure 6).

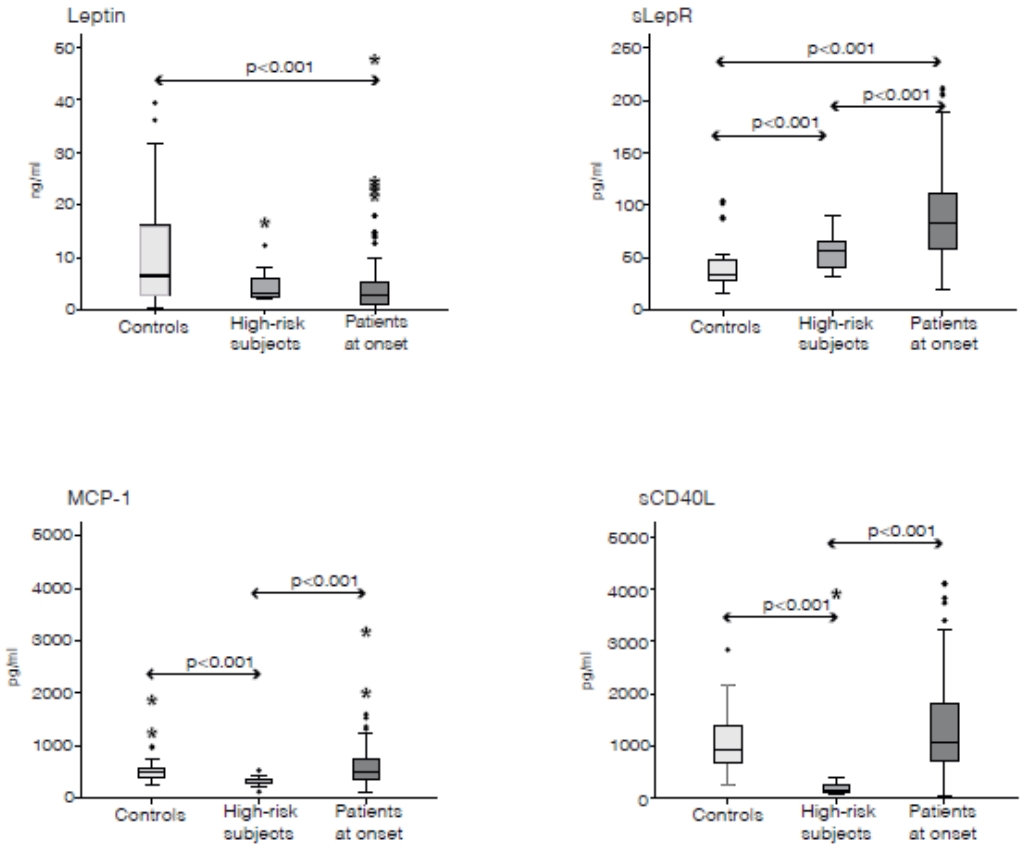
We also observed that circulating levels of the pro-inflammatory chemokine MCP-1 were significantly higher in children affected by type 1 diabetes than in high-risk subjects (p <0.001, after Bonferroni correction) and significantly lower in high-risk subjects as compared to controls (p <0.001, after Bonferroni correction). The same trend was observed for the circulating plasma levels of sCD40L (Table 3 and Figure 6).

Table 3. Serum levels of inflammatory/metabolic circulating factors in controls, high-risk subjects and diabetic patients at disease onset.

	Controls (n = 34)	High-risk subjects (n = 29)	Diabetic patients at onset (n = 114)	*p*
Leptin (ng/ml)	6.41 (0.32-39.45)	2.99 (2.12-16.57)	2.71 (0-47.69) (^)	<0.0001
sLepR (pg/ml)	33.32 (16.6-102.1)	55.8 (32.1-89.9) (†)	83.05 (19.2-210) (^) (§)	<0.0001
MCP-1 (pg/ml)	485.07 (257.41-1863.94)	300.98 (118.43-526.15) (†)	507.27 (119.35-14513.37) (§)	<0.0001
sCD40L (pg/ml)	938.49 (269-4159.8)	170.82 (79.2-3917.5) (†)	1076.57 (64-4125.7) (§)	<0.0001
Resistin (ng/ml)	6289.6 (2990.7-43816.8)	4899.5 (1804.3-8019.7)	5571.3 (377.3-40032)	ns
MPO (ng/ml)	135.6 (27.61-300.02)	91.45 (29.42-323.91)	69.22 (8.01-351.06)	ns
sICAM-1 (µg/ml)	467.07 (255.03-1503.42)	454.37 (208.29-694.9)	465.26 (58.28-2447.41)	ns
sTNFr (ng/ml)	0.66 (0.23-1.5)	0.64 (0.33-3.42)	0.67 (0.08-5.08)	ns
OPG (pg/ml)	16.45 (0-120)	23.44 (0-130)	48.5 (0.38-143.42)	ns

Data are reported as median and range; ^ Statistical significance between diabetic patients at onset and controls (p < 0.0001); § Statistical significance between diabetic patients at onset and high-risk subjects (p < 0.0001); † Statistical significance between high-risk subjects and controls (p < 0.0001); ns = not significant.

Figure 6. Meta-immunological profiling in patients at onset, in high-risk subjects, and in controls

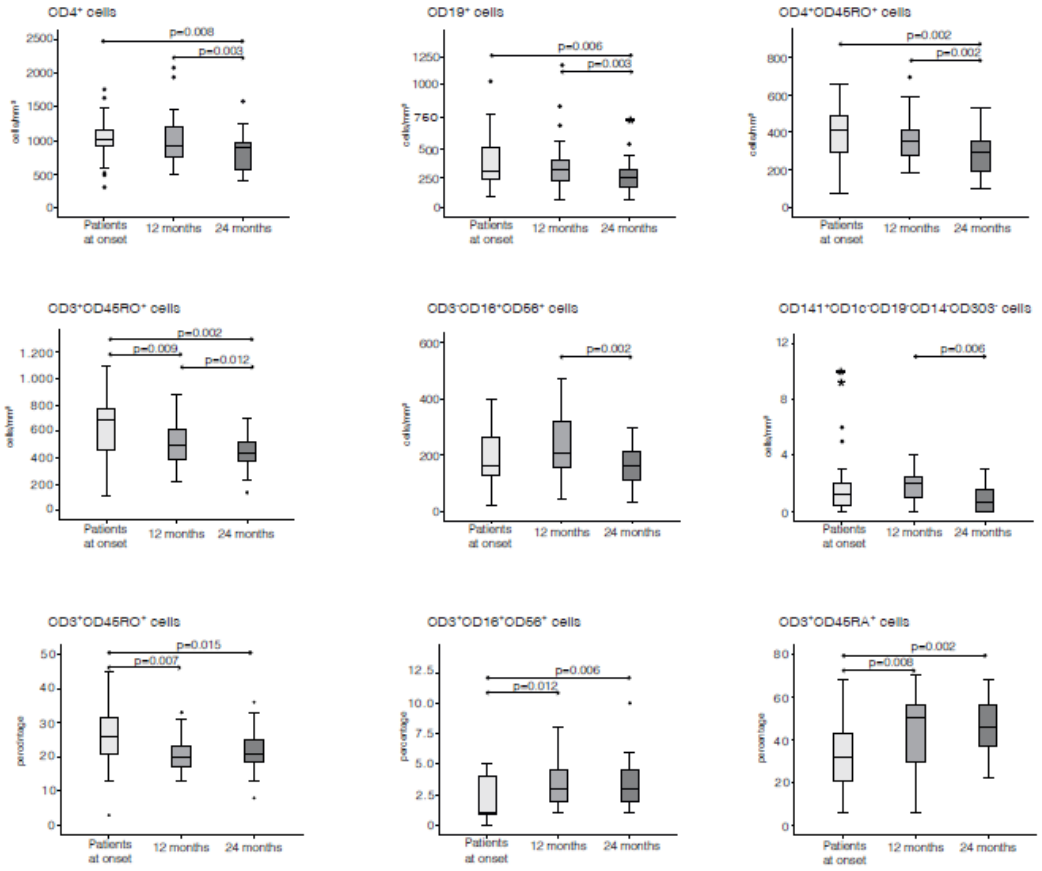


Box plots show the distribution of circulating level of leptin, sLepR, MCP-1 and sCD40L in type 1 diabetes at onset, high-risk subjects and controls. Dots represent outliers values, i.e. data points below Q1 - 1.5xIQR or above Q3 + 1.5xIQR while asterisks represent extreme values, i.e. data points below Q1 – 3xIQR or above Q3 + 3xIQR. Q1=25th percentile; Q3=75th percentile; IQR (Interquartile Range) = Q3-Q1. Data are shown as median (min-max). Patients had lower serum levels of Lep ($p < 0.001$) and higher serum levels of sLepR ($p < 0.001$) than controls. High-risk subjects had intermediate levels of sLepR between children with type 1 diabetes and controls. Both patients and controls had higher levels of MCP-1 than high-risk subjects; the same was observed for sCD40L levels.

4.2 Meta-immunological follow-up profiling of type 1 diabetes patients

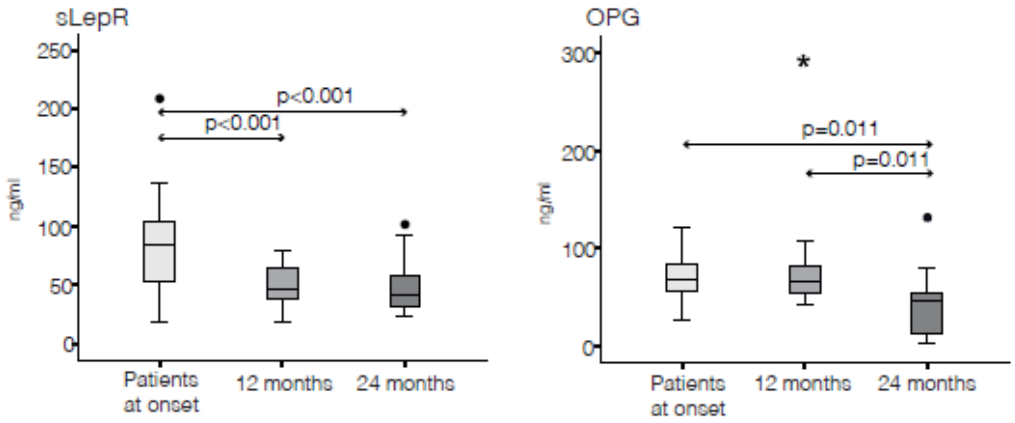
Sera from patients were also analyzed at follow-up, namely at 12 and 24 months after disease diagnosis. It was found that during disease progression there was a significant progressive reduction in the number of $CD4^+$ T cells, with lowest values at 24 months after diagnosis (Figure 7). The same trend was observed for the absolute number of circulating $CD19^+$ B cells. In addition, during disease progression, there was a progressive reduction in the absolute number of circulating T cells with a memory phenotype ($CD4^+CD45RO^+$ or $CD3^+CD45RO^+$). Conversely, the percentage of naïve T cells ($CD3^+CD45RA^+$) was lower at disease onset as compared to values observed at later time points. In addition, there were higher absolute numbers of NK cells ($p = 0.002$, after Bonferroni correction) and $CD141^+CD1c^-CD19^-CD14^-CD303^-$ type 2 myeloid dendritic cells (mDC2s) at 12 months compared with those observed at 24 months after disease onset ($p = 0.006$, after Bonferroni correction). The $CD3^+CD16^+CD56^+$ cells subset was lower at onset as compared to later time points. In serum, a progressive decline of circulating sLepR levels at 12 and at 24 months ($p < 0.001$ for both, after Bonferroni correction) and a progressive reduction of circulating OPG were observed from disease onset thereafter (Figure 8).

Figure 7. Immunological profiling in the follow-up of T1D patients



Children affected by type 1 diabetes were studied at diagnosis, 12 and 24 months after disease onset. Box plots show the distribution of $CD4^+$ T cells, $CD19^+$ B cells, $CD4^+$ $CD45RO^+$ T cells, $CD3^+CD45RO^+$ T cells, $CD3^-CD16^+CD56^+$ cells, $CD141^+$ cells and percentages of $CD3^+CD45RO^+$ T cells, $CD3^+CD16^+CD56^+$ cells, and $CD3^+CD45RA^+$ T cells in patients at onset and after 12 and 24 months.

Figure 8. Metabolic profiling in the follow-up of T1D patients



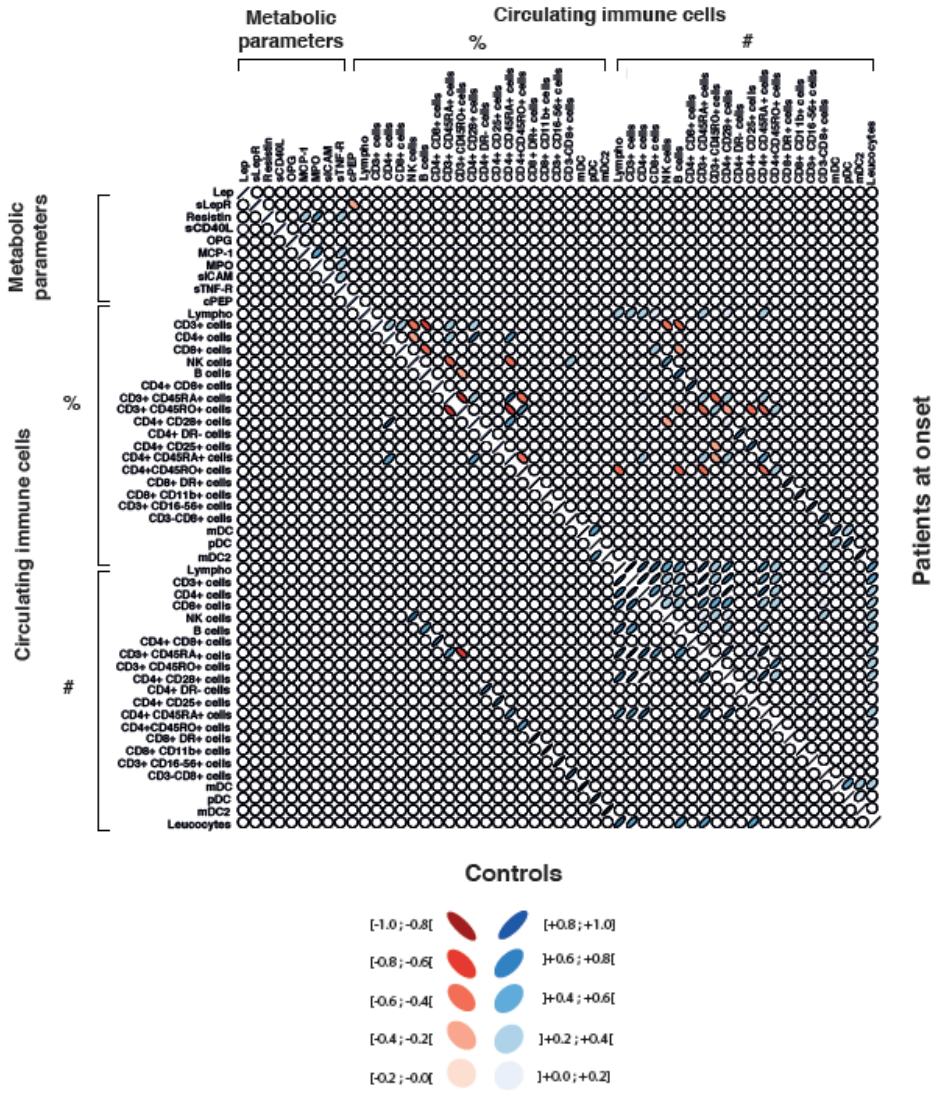
Children affected by type 1 diabetes were studied at diagnosis, 12 and 24 months after disease onset. Box plots show the distribution of circulating levels of sLepR and OPG, in diabetic children at onset, 12 and 24 months later. Patients had very high circulating levels of sLepR at disease onset. Serum levels of sLepR showed a trend towards reduction over time, with lower serum levels after 24 months from disease onset ($p < 0.001$). Serum levels of OPG pregressively decreased over time ($p = 0.011$).

4.3 Multiple correlations among meta-immunological markers

We performed multiple correlation analyses in diabetic patients at onset, high-risk individuals, and controls. In the group affected by type 1 diabetes, sLepR inversely correlated with circulating levels of fasting C-pep ($r = -0.39$, $p < 0.001$), which reflects β-cell function. This correlation was not present in controls or in high-risk subjects (Figure 9 and data not shown). Moreover, in patients at onset the circulating levels of resistin positively correlated with those of sTNFr ($r = 0.47$, $p < 0.001$), MCP-1 (0.52 with $p < 0.001$) and MPO ($r = 0.71$, $p < 0.001$). Circulating MCP-1 positively correlated with MPO and sTNFr levels ($r = 0.62$, $p < 0.001$ and $r = 0.52$ with $p < 0.001$, respectively). All these correlations were not present in controls and in high-risk subjects (Figure 9 and data not shown).

In terms of immune cell subsets, several correlations were only found in patients and not in controls and high-risk subjects. In particular only in the group of patients, the percentage of $CD3^+$ cells inversely correlated with the percentage of NK ($r = -0.41$, $p < 0.001$) and B cells ($r = -0.65$, $p < 0.001$). Moreover, the NK cells inversely correlated with both $CD3^+CD45RA^+$ ($r = -0.51$, $p < 0.001$) and $CD4^+CD45RA^+$ ($r = -0.46$, $p < 0.001$) naïve T cells (Figure 9).

Figure 9. Multiple biological correlations in type 1 diabetes

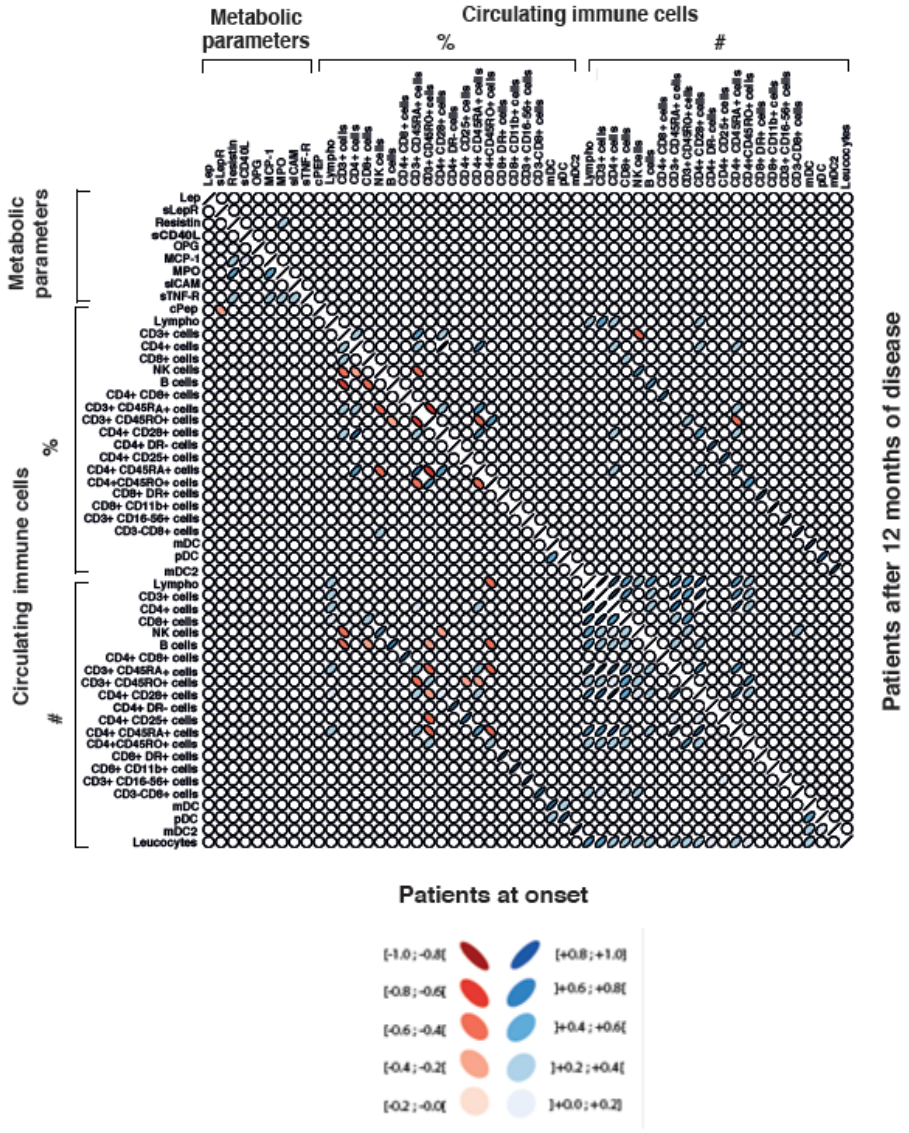


Two-dimensional graphical representation of the Spearman ρ non-parametric correlation matrix among the studied variables. The presence of a significant correlation between two variables is expressed by means of a red (negative correlation) or blue (positive correlation) ellipse, while an empty circle refers to a non-significant correlation. The color intensity and thickness of each ellipse are proportional to the correlation value (see graphic legend for numeric values). Correlation analysis, performed on all parameters analyzed, revealed a distinctive immune/metabolic profile for controls and new onset diabetic patients. A negative correlation between sLepR and β-cell residual fasting C-pep secretion (r= -0.39, p <0.001) was observed in individuals affected by type 1 diabetes but not in controls.

4.4 One year follow-up

Meta-immunological correlations in diabetic patients at onset and 12 months post diagnosis were also studied. Significant differences were observed as compared to the same patients at diabetes onset (Figure 10). For example, the inverse correlation between sLepR and fasting C-peptide observed at disease onset, was lost after 12 months. A series of positive correlations at diagnosis, such as resistin vs MCP-1 ($r = 0.52$, $p < 0.001$) and MPO ($r = 0.70$, $p < 0.001$) as well as MCP-1 vs MPO ($r = 0.22$, $p < 0.01$) were observed. Only the correlation between resistin and MPO was maintained over time ($r = 0.51$, $p < 0.001$) (Figure 10). At the cellular level, the correlations among percentage $CD3^+$ T cells vs NK and B cells observed at diagnosis was lost after 12 months, whereas the percentage $CD4^+CD45RO^+$ T cells inversely correlated with that of $CD3^+CD45RA^+$ T cells and $CD4^+CD45RA^+$ T cells at disease onset. These correlations were not maintained one year later (Figure 10).

Figure 10. Multiple biological correlations in type 1 diabetes children over time



Two-dimensional graphical representation of the Spearman ρ non-parametric correlation matrix among the study variables. The presence of a significant correlation between two variables is expressed by means of a red (negative correlation) or blue (positive correlation) ellipse while an empty circle refers to a non-significant correlation. The color intensity and the thickness of each ellipse are proportional to the correlation value (see graphic legend for numeric values). Correlation analysis, performed on all parameters analyzed, revealed a distinctive immune/metabolic profile for children with type 1 diabetes over time. The inverse correlation between sLepR and fasting C-pep, observed at disease onset, was lost 12 months later.

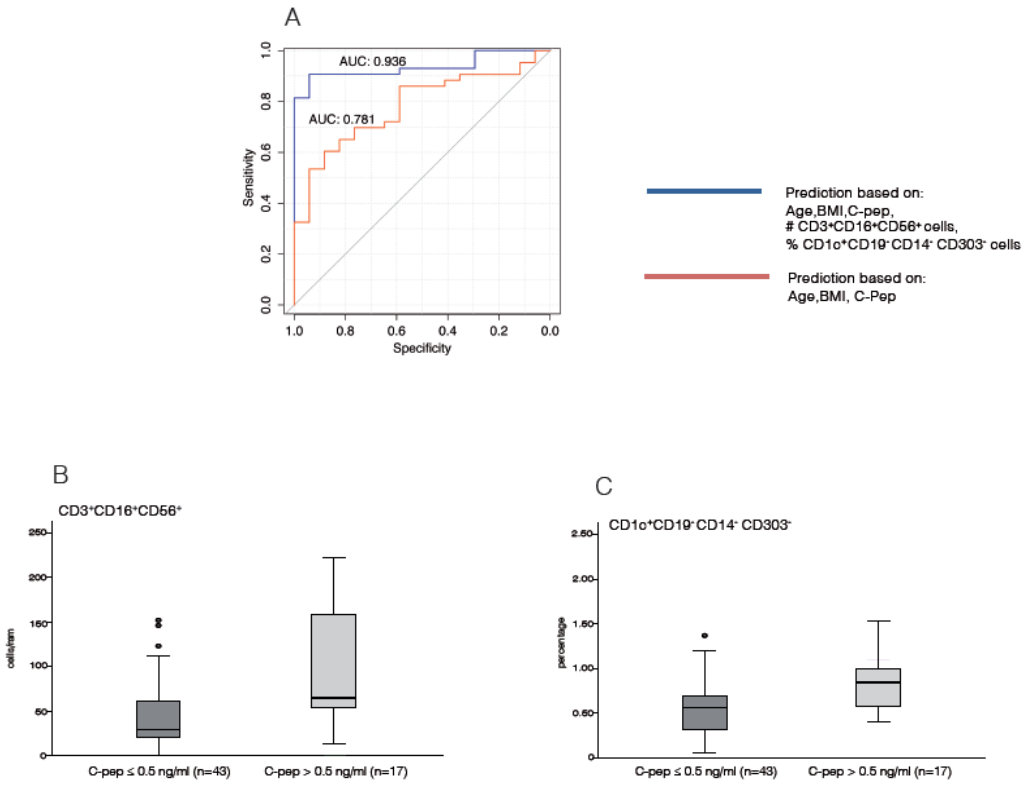
4.5 A novel predictive tool of residual ß-cell function over time

The possibility to predict residual C-peptide levels after disease onset and the identification of biomarkers assessing therapeutic efficacy is a major goal in type 1 diabetes monitoring and prognosis. Multivariable logistic regression analysis showed that the number of $CD3^+CD16^+CD56^+$ T cells and the percentage of $CD1c^+CD19^-CD14^-CD303^-$ type 1 myeloid dendritic cells (mDC1s) at disease diagnosis were independent predictors of low (≤0.5) residual C-peptide secretion after 12 months from diagnosis, after adjustment for age and BMI as covariates measured at diagnosis. The data in Table 4 report the regression coefficient, the corresponding O.R. and 95% C.I. for the model and for a reduced base model including only the adjusting factors. In addition, a linear regression model confirmed the predictive role of $CD3^+CD16^+CD56^+$ T cells and $CD1c^+CD19^-CD14^-CD303^-$ type 1 myeloid dendritic cells (mDC1s) independently of age, BMI and C-peptide values at onset (data not shown). Figure 11 shows the ROC curves associated to the base model and to the full model. The AUC of the full model was 0.936 ($p < 0.001$) and, compared to the AUC of the base model (0.781, $p < 0.001$), was significantly higher ($p = 0.005$). Box plots in Figure 11 indicate that low $CD3^+CD16^+CD56^+$ T cells and low mDC1s at disease onset associated with a reduced β-cell activity (fasting C-pep ≤0.5 ng/dl) in type 1 diabetes one year later.
To characterize further the patients with different residual β-cell function, we performed a correlation analysis of "severe disease patients" (fasting C-pep ≤0.5 ng/dl) and "mild disease patients" (fasting C-pep >0.5 ng/dl) at disease onset and one year later. We observed that in patients with a worse pancreatic function (fasting C-pep ≤0.5 ng/dl), resistin positively correlated with the levels of C-pep, MCP-1 and sTNF-R. In addition, in this group there was also a positive correlation between MCP-1 and sTNF-R. These correlations were not observed in patients with residual β-cell function (fasting C-pep >0.5 ng/dl). An inverse correlation between the percentage of naïve T cells ($CD4^+CD45RA^+$) and memory T cells ($CD4^+CD45RO^+$) was observed in severe-disease patients, but not in mild-disease patients. Many of the correlations observed at diabetes onset were not present 12 months after disease onset. $CD3^+$ positively correlated with $CD4^+$ (and $CD4^+CD28^+$) in severe-disease patients, but not in mild-disease patients. Only in mild-disease patients did we observe an inverse correlation between $CD3^+CD45RA^+$ and $CD3^+CD45RO^+$ (Figure 12).

Table 4. Estimated regression coefficients and adjusted odds ratio (with their 95% CI) for predictors of residual C-pep secretion in the base and in the full logistic regression model.

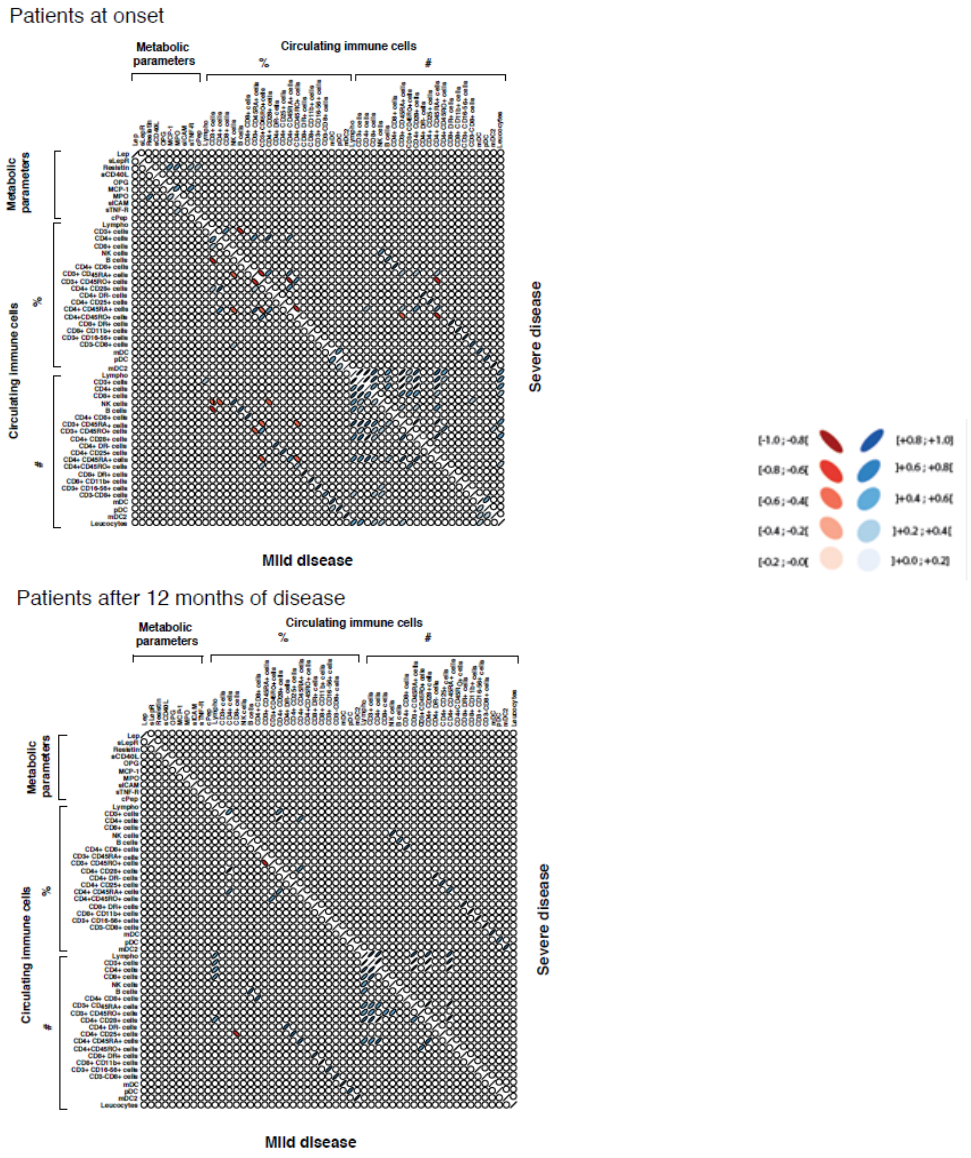
	Base Model			Full Model		
	Estimated Coefficient	O.R. (95% C.I.)	*p*	Estimated Coefficient	O.R. (95% C.I.)	*p*
Age at diagnosis	- 0.175	0.84 (0.69 - 1.03)	0.091	- 0.376	0.69 (0.51 - 0.93)	0.015
BMI at diagnosis	0.064	1.07 (0.92 - 1.24)	0.406	0.119	1.13 (0.92 - 1.39)	0.262
C-pep secretion at diagnosis ≤ 0.5	2.259	9.57 (1.83 - 50.11)	0.008	2.390	10.91 (1.81 - 67.00)	0.010
% of $CD1c^+CD19^-CD14^-CD303^-$ cells	-	-		- 3. 471	0.03 (0.01 - 0.59)	0.021
# of $CD3^+CD16^+CD56^+$ cells	-	-		- 0.020	0.98 (0.96 - 0.99)	0.021

Figure 11. Prediction analysis



A) Receiving operating characteristics (ROC) curves of the model-based prognostic scores for residual ß-cell functioning. The base model included only age, BMI and fasting C-pep secretion measured at disease onset, while the full model added the number of $CD3^+CD16^+CD56^+$ T cells and the percentages of mDCs to the base model.
B) Dot plot showing that high numbers of $CD3^+CD16^+CD56^+$ T cells at onset associated with a reduced β-cell activity one year later. Low numbers were associated to a residual β-cell function.
C) Dot plot showing that the high percentage of mDCs at onset was associated to a reduced β-cell activity one year later. Conversely, low numbers of these cells associated with residual β-cell function.

Figure 12. Distinctive correlation profiles of patients affected by type 1 diabetes according to disease severity, at onset and 12 months later



Graphical representation of the Spearman ρ non parametric correlation matrix among the study variables. The presence of a significant correlation between two variables is expressed by means of a red (negative correlation) or blue (positive correlation) ellipse while an empty circle refers to a non-significant correlation. The colour intensity and thickness of each ellipse are proportional to the correlation value (see graphic legend for numeric values). The correlation profile between patients with mild and severe disease at onset and 12 months later is shown.

5. Discussion

The comprehension of the pathogenesis of type 1 diabetes has improved considerably in recent years, yet there is a lack of predictive markers of risk for disease progression and pancreatic β-cell loss. Such markers could be very useful to monitor the disease, to evaluate therapeutic efficacy and to ameliorate prognosis.

The meta-immunological profiling described here for type 1 diabetes at onset and during progression identified 3 circulating immune cell populations and 4 metabolic/inflammatory markers as significantly different among diabetic patients at onset, in high-risk subjects and in controls. $CD8^+$ T lymphocytes were significantly higher in high-risk subjects as compared to patients according with previous report (*Pinkse et al. 2005*), a finding that is reminiscent of the known increased proliferation of this cell subset during insulitis, before the onset of hyperglycemia and overt diabetes. Higher numbers of memory T cells in patients at onset as compared to controls were also found. Interestingly, in the 24-months follow-up there was a progressive decline in the number of memory T cells in diabetic patients, in accordance with the findings that GAD65-specific T cells in diabetic patients bear a memory phenotype whereas in healthy individuals they are naïve T cells (*Oling et al. 2012*). We hypothesize that the progressive decrease of memory T cells might be secondary to a gradual exhaustion of islet-specific T cell clones after initial epitope spreading. Moreover, plasmacytoid dendritic cells were higher in children with type 1 diabetes at onset as compared to healthy children. This aspect is interesting because in presence of β-cell-specific autoantibodies, pDCs process and present islet autoantigens to $CD4^+$ T-cells to amplify and maintain T-cell responses favouring epitope spreading (*Allen et al. 2009*).

Previous investigations studied the frequency of this cell subset with discrepant results, showing either a reduced pDCs number in the blood of patients as compared to healthy controls (*Chen et al. 2008; Vuckovic et al. 2007; Hinkmann et al. 2008*) or an increased frequency of pDCs at diagnosis of type 1 diabetes (*Allen et al. 2009*). These conflicting results could depend on the procedures for the analysis, the timing of sampling, and the size of the studied population. Our study is the first one analyzing pDCs longitudinally for 24 months on a large cohort of patients following disease diagnosis.

Metabolically, the leptin/sLepR axis was altered in newly diagnosed diabetic children as compared to high-risk subjects and controls. Leptin profoundly affects metabolism and immune functions (*La Cava and Matarese 2004*), and promotes the development of type 1 diabetes in NOD mice (*Naito et al. 2011*). On the contrary, it has been reported that leptin improves insulin-deficient type 1 diabetes, reverting catabolism through the suppression of hyperglucagonemia, resembling

the anabolic action of insulin monotherapy, and normalizing HbA1c (*Wang et al. 2010*).

Soluble leptin receptor (sLepR), the main leptin-binding protein in human blood, can modulate leptin effects on target organs by influencing leptin action in two ways: 1) by inhibiting the binding of leptin to its membrane receptors; 2) by increasing the availability of circulating leptin, delaying its clearance. We found that children affected by type 1 diabetes at diagnosis had lower circulating leptin and higher sLepR levels as compared to high-risk subjects and controls, which is consistent with the report of elevated serum sLepR in diabetic children with metabolic decompensation (*Kratzsch et al. 2006*). Low levels of leptin in subjects with type 1 diabetes could be partially explained by the high amount of sLepR in the same patients. sLepR could mask leptin epitopes and hamper detection of this adipokine. The behaviour of sLepR levels during diabetes progression suggests a potential of sLepR as an early novel marker of type 1 diabetes. This is linked to the finding that, during diabetes progression, sLepR levels decreased over time but still remained higher than in healthy controls. In addition, sLepR circulating levels inversely correlated with β-cells function, measured as levels of circulating fasting C-pep, at disease onset. This finding was not confirmed in high-risk and control groups (although the lack of significance could be attributed to the reduced power due to smaller sample size for those groups). A better understanding of the mechanisms controlling the leptin/sLepR axis may lead to speculate on the possibility to use leptin as a substitute for or a combination therapy with insulin in type 1 diabetes.

The importance of our study relies upon the current lack of markers able to predict diabetes progression and severity in type 1 diabetes. Such markers could help clinicians in the choose of most appropriate therapeutic intervention, in disease monitoring, and in the evaluation of therapeutic efficacy, ultimately improving prognosis. Since type 1 diabetes is a complex multifactor disease with strong genetic component and significant environmental influences, a broad immunological assessment and multifactor algorithm to pick patterns likely needed to dissect the complex pathogenesis and identify key patterns of disease progression. By using a multivariate logistic regression analysis adjusted for age, BMI and the value of fasting C-peptide at disease onset, we identified here two specific immune cell populations, both measured at disease onset, that were capable to predict C-peptide secretion as a surrogates measure of β-cell mass in humans with type 1 diabetes. Indeed, the number of $CD3^{+}CD16^{+}CD56^{+}$ T cells and the percentage of mDC1s were independent predictors of residual C-peptide secretion 12 months after diagnosis.

Our prognostic score of the fitted model reached an AUC that was significantly higher than the AUC of a prognostic base model that included only age, BMI and fasting C-peptide measured at disease onset ($p < 0.001$). We also provided a wide

range of biological correlates of disease as compared to healthy and disease-progressing individuals. The biological correlations between meta-immunological parameters in subjects with mild and severe type 1 diabetes indicated that in mild-disease patients the T cells negatively correlated with NK and B cells, both correlations not detectable in subjects with severe disease. An advantage of the current study is the multi-parametric analysis on a large, well characterized cohort of newly diagnosed patients with type 1 diabetes followed prospectively and longitudinally for 24 months. As such, this is the first comprehensive study associating β-cell secretion capability with circulating immune cell subsets. The number and percentage of mDC1s and $CD3^{+}CD16^{+}CD56^{+}$ T cells were prognostic factors of pancreatic insulin secretion up to one year after disease onset. Finally, our approach could also be of valuable help to understand the molecular basis of slow versus fast progressors to disease and to monitor high-risk subjects towards the development of β-cell failure in conjunction with tests measuring the acute insulin response to glucose (ie. AIR-glucose) which could reflect very early an ongoing immune attack against insulin producing β-cells.

In conclusion, our experimental model could represent a novel tool to monitor the staging of type 1 diabetes patients and may be useful, together with previously known parameters (i.e. glycated haemoglobin, insulin requirement), to assess disease progression and also contribute to define the most appropriate therapeutic approach according with the aggressiveness of the disease at onset. Our results could lead to the discovery and/or validation of biomarkers that represent early events in the disease as well as those that may serve as surrogate read-outs or endpoints for efficacy of specific interventions.

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List of abbreviations used:

T1D: type 1 diabetes
BMI: body mass index
C-pep: C-peptide
DCs: dendritic cells
mDC1s: type 1 myeloid dendritic cells
mDC2s: type 2 myeloid dendritic cells
pDCs: plasmacytoid dendritic cells
HLA: human leukocyte antigen
HbA1c: glycated heamoglobin
IAAs: autoantibodies against insulin
GADA: autoantibodies against the 65-kDa isoform of GAD
IA-2A: autoantibodies against the protein tyrosine phosphatase-related molecule IA-2
ZnT8: autoantibodies against the pancreatic β-cell specific protein, zinc transporter 8
T-regs: T-regulatory cells
NOD mice: non-obese diabetic mice
NK cells: Natural Killer cells
NKT cells: Natural Killer-T cells
APCs: antigen-presenting cells
CTLs: cytotoxic T lymphocytes
Lep: leptin
sLepR: soluble leptin receptor
sCD40L: soluble CD40L
sICAM-1: soluble ICAM-1
MCP-1: monocyte chemoattractant protein-1
MPO: myeloperoxidase
OPG: osteoprotegerin
sTNF-R: soluble TNF-R

At the end of this phase of my life, most of all, I want to thank the two most important people who led to what I am today: my mother and my father. They are two extraordinary people and, for me, an example of dedication, generosity and love.

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This work is dedicated to the memory of Mario Diana.

Rosa

OBSERVATIONS

Glucose Derangements in Very Young Children With Cystic Fibrosis and Pancreatic Insufficiency

Cystic fibrosis–related diabetes (CFRD) is considered the most common comorbidity in patients affected by cystic fibrosis (CF), with a prevalence increasing with age (1). Recently, more attention has been turned to other less severe glucose metabolism derangements (GMD), since prediabetes may be related to increased morbidity (1), and early treatment may improve the clinical course in patients with CF (2). According to recent guidelines released by the Cystic Fibrosis Foundation, the American Diabetes Association, and the Pediatric Endocrine Society, the oral glucose tolerance test (OGTT) is recommended yearly in patients with CF over 10 years of age (3). Some authors recommend annual OGTT after the age of 6 years in CF patients with pancreatic insufficiency (4).

In order to compare the prevalence of GMD in CF patients with pancreatic insufficiency by age, OGTT was performed in all CF patients >2 years of age, excluding those with pancreatic sufficiency in regular follow-up at the CF Care Center of Federico II University in Naples in 2011. The study population was represented by 157 patients: 84 male, 73 female; mean age 10.5 ± 3.95 years (range 2.4–18.0); forced expiratory volume in the 1st second 88 ± 28 (range 28–180; $n = 113$); 5 subjects were excluded because of noncompliance to OGTT. Therefore, 152 patients were effectively studied. The study was approved by the local ethics committee of the University Federico II of Naples. GMD were classified into three categories: CFRD (glycemia ≥11.1 mmol/L at time 120 min [T120']), impaired glucose tolerance (IGT, glycemia ≥7.7 mmol/L at T120'), and indeterminate glucose tolerance (INDET, glycemia ≥11.1 mmol/L at T30' and/or T60' and/or T90' of OGTT but <7.7 mmol/L at T120'). Prevalence of GMD was compared among three age groups: between 2.4 and 5.9 years ($n = 24$), between 6 and 9.9 years ($n = 42$), and ≥10 years ($n = 86$). Among patients aged <6 years, 2 were CFRD, 4 were IGT, and 2 were INDET (GMD 33.3%); among patients aged 6–9.9 years, 1 was CFRD, 7 were IGT, and 2 were INDET (GMD 23.8%); and among patients aged ≥10 years, 7 were CFRD, 22 were IGT, and 9 were INDET (GMD 44.2%); $P = 0.025$ between groups aged 6–9.9 years and ≥10 years.

Our results confirm the high prevalence of GMD in CF patients with pancreatic insufficiency between 6 and 10 years (4) and provide new information on the presence of a consistent number of GMDs even in patients <6 years of age, therefore we suggest that the screening of GMDs may be indicated from the youngest age at least in those with pancreatic insufficiency (4,5). It is questionable if OGTT is the most appropriate screening method in the youngest age. Further longitudinal studies are needed to evaluate the prognostic role of very early diagnosis of GMD in CF.

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REVIEW Open Access

Celiac disease in type 1 diabetes mellitus

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Abstract

Celiac Disease (CD) occurs in patients with Type 1 Diabetes (T1D) ranging the prevalence of 4.4-11.1% versus 0.5% of the general population. The mechanism of association of these two diseases involves a shared genetic background: HLA genotype DR3-DQ2 and DR4-DQ8 are strongly associated with T1D, DR3-DQ2 with CD. The classical severe presentation of CD rarely occurs in T1D patients, but more often patients have few/mild symptoms of CD or are completely asymptomatic (silent CD). In fact diagnosis of CD is regularly performed by means of the screening in T1D patients. The effects of gluten-free diet (GFD) on the growth and T1D metabolic control in CD/T1D patient are controversial. Regarding of the GFD composition, there is a debate on the higher glycaemic index of gluten-free foods respect to gluten-containing foods; furthermore GFD could be poorer of fibers and richer of fat. The adherence to GFD by children with CD-T1D has been reported generally below 50%, lower respect to the 73% of CD patients, a lower compliance being more frequent among asymptomatic patients. The more severe problems of GFD adherence usually occur during adolescence when in GFD non compliant subjects the lowest quality of life is reported. A psychological and educational support should be provided for these patients.

Keywords: Diabetes, Celiac disease, Genetic background, HLA, Dietetic compliance, Glycaemic index, Gluten free diet, Quality of life

Introduction

Type 1 Diabetes Mellitus (T1D) is frequently associated to other autoimmune conditions. These conditions can severely affect clinical management of the disease, especially in paediatric age.

The most frequent are autoimmune thyroid disease (AIT), celiac disease (CD), Addison's disease (AD) and vitiligo. These diseases are associated with organ-specific autoantibodies: AIT with thyroid peroxidase (TPO) and thyroglobulin autoantibodies (TG), CD with endomysial (EMA) and transglutaminase (TTG) autoantibodies, and AD with adrenal autoantibodies. Using these autoantibodies, organ-specific autoimmunity may be often detected before the development of clinical disease, in order to prevent significant morbidity related to unrecognized disease [1]. These diseases are very often clustered in the same individual and a shared genetic background probably explains this association [2].

Genetics

The majority of autoimmune endocrine diseases, including T1D, are inherited as complex genetic traits. Multiple genetic and environmental factors interact with each other to confer susceptibility to these disorders. Genetic risk factors associated with T1D, ATD, CD and AD include HLA genes and non-HLA genes.

HLA DR4 and DR3 are strongly associated with T1D and approximately 30-50% of patients are DR3/DR4 heterozygotes. The DR3/DR4 genotype confers the highest diabetes risk with a synergic mode of action, followed by DR4 and DR3 homozygosity, respectively. The HLA-DQ (particularly DQ 2 and DQ8) locus has been found to be the most important determinant of diabetes susceptibility. Approximately 90% of individuals with T1D have either DQ2 or DQ8, compared to 40% of the general population [3]. So, the highest-risk human leukocyte antigen (HLA) genotype for T1D is DR3-DQ2, DR4-DQ8.

DR3-DQ2 shows a strong association with CD; homozygosity for DR3-DQ2 in a population with T1D carries a 33% risk for the presence of TTG autoantibodies [4].

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Non-HLA genes are also involved in the predisposition to T1D and other autoimmune diseases, such as MIC-A, PTPN22, CTLA-4 [1].

Epidemiology

Traditional studies, both in children and adults, have shown that CD occurs in patients with T1D with a prevalence that varies from 4.4 to 11.1% compared with 0.5% of the general population (Table 1 for references from) [5-14]. The mean age at diagnosis of classical CD is commonly around 2-3 years, while the mean age at diagnosis of T1D is 7-8 years. The age at onset of T1D is younger in patients with the double disease than in those with only T1D [15]. The risk of CD is negatively and independently associated with age at onset of diabetes, with an higher risk being seen in children age < 4 years than in those age > 9 years [16]. In patients with T1D, diabetes is usually diagnosed first, CD precedes diabetes onset only in 10-25% [16,17], while generally CD diagnosis in T1D patients occurs, trough the screening performed at diabetes onset, in 70-80% of patients with a median age > 8 years. Some authors hypothized that in genetically susceptible patients one disease could predispose to another. Particularly, it has been suggested that untreated (latent or silent) CD could be an immunological trigger and induce diabetes and/or thyroid disorders due to gluten as a driving antigen [18]. In accordance with this, the prevalence of autoimmune disorders in CD is closely related to age at diagnosis or, in other words, to the duration of exposure to gluten [19] and thyroid-related antibodies tend to disappear during twelve months of gluten-free diet, like CD-related antibodies [20]. However, at present, it is unknown whether treatment of CD reduces the likelihood of developing autoimmune disorders, or changes their natural history and actually others found no correlation between duration of gluten exposure in adult CD and risk of autoimmune disorders [21].

CD clinical symptoms

The most severe CD-related symptoms are generally related to gastrointestinal malabsorption and include malnutrition, failure to thrive, diarrhea, anorexia, constipation, vomiting, abdominal distension, and pain. These features are more common in children younger than three years of age. Non-gastrointestinal symptoms of CD include short stature, pubertal delay, fatigue, vitamin deficiencies, and iron deficiency anemia and are more commonly observed in older children. The gastrointestinal presentation of CD rarely occurs in T1D patients (< 10%), but many patients with CD and T1D are either asymptomatic (silent CD) or present only mild symptoms [17,20,22]. Furthermore, the wide spectrum of CD include also subjects with positive celiac-related antibodies without diagnostic small-bowel mucosal villous atrophy. This condition is defined as potential celiac disease (pot-CD) [23-25]. Data from the majority of childhood diabetes care centers in Italy showed that prevalence of pot-CD patients in this population (higher in females than males) is 12.2%, while the prevalence of pot-CD in the CD control population is 8.4% and only few of them present CD-related symptoms [26].

CD-screening

Diagnosis of CD is regularly due to screening protocols which are widely recommended and performed. Actually diagnosis is commonly performed by means of TTG IgA (confirmed by EMA) or TTG-IgG if IgA-deficiency is present. Screening has to performed at followed times: 1) at the time of diabetes onset, 2) yearly in the first 4 years of follow up, 3) each 2 years in the successive 6 years of follow up [27,28]. In the presence of CD-related antibodies positivity it is mandatory to perform bowel biopsy to confirm diagnosis of CD, even if in very recent guide-lines of ESPGHAN Society [29] it is proposed that in evident CD-cases it is possible to avoid biopsy (4 main criteria).

Table 1 Prevalence of CD in patients with T1D in recent literature (2004-2011)

Reference	Country	N.	Age (yr)	Screening	Prevalence (%)
Cerutti et al. 2004	Italy	4322	11.8 ± 4.2	AGA + EMA	6.8
Contreas et al. 2004	North Italy	357	Children	EMA	7
Sanchez et al. 2005	Germany	281	Children	AGA + EMA	6.4
Araujo et al. 2006	Brasil	354	Children	TG	10.5
Goh et al. 2007	UK	113	Children	EMA + TG + AGA	4.4
Larsson et al. 2008	Sweden	300	< 20	EMA	10
Karavanaki et al. 2009	Greece	144	12.3 ± 4.6	TG	4.8
Djuric et al. 2010	Serbia	121	Mean 10.8	TG	5.79
Bhadada et al. 2011	India	189	10.81 ± 7.3	TG	11.1
Gabriel S et al. 2011	Romania	119	11 ± 4	TG	9.2

CD-treatment

GFD should be proposed actually only in patients with mucosal atrophy.

In patients with overt CD, identifying and treating CD with gluten free diet (GFD) surely confer benefit in reducing/resolving malabsorption, infertility, osteoporosis, poor nutrition, impaired growth and long-term malignancy risks and mortality rates [30-32]. Similarly, children with T1D and symptomatic CD benefit from GFD [33] and also metabolic control of diabetes could be ameliorated [34].

On the contrary, in symptom-free patients weight gain and bone mineral density (BMD) changes have been non-univocally described as benefit [35-37]. The different viewpoints highlight the need of a prolonged follow up in patients affected by T1D and asymptomatic CD to clarify the role of GFD. Some authors argument that GFD in asymptomatic CD-T1D patients should be opportunely proposed but not excessively stressed [38,39].

Finally, no definite consensus exists among experts about to treat by GFD pot-CD patients, in whom recently it has been suggested that GFD could be a benefit [40]. Concerning to the natural history of patients whit pot-CD, a recent study shows that 30% of these patients develops overt CD in a three years follow-up and underlines the necessity of re-testing [41]. However no data are available about the follow-up of patients with T1D and pot-CD.

Surprisingly, intestinal inflammation has been described also in T1D patients without CD-related antibodies and structurally normal intestinal mucosa [42]. According to this, our group has observed a gluten-related inflammation either in rectal either in small bowel mucosa of children with T1D [43,44]. It can be speculated that gluten could be an optimal candidate to stimulate an abnormal innate immune reaction in intestinal mucosa due to its pro-inflammatory characteristics. It remains a crucial issue to establish if the extended intestinal inflammation in T1D is gluten-dependent and whether it precedes the occurrence of the disease.

Bone impairment: a hidden threat

In patients with only T1D it is possible to demonstrate impairment of bone metabolism and structure, specially in relationship with duration and/or poor control of diabetes [45]. Furthermore CD also have been underlined as cause of bone impairment. Clinical observation indicates that clustering of three autoimmune diseases (T1D, CD and generally thyroiditis) significantly increases the occurrence of osteopenia (37.5%). It is possible that bone impairment might be considered not only a complication due to endocrine or nutritional mechanisms, but also a consequence of an immunoregulatory imbalance [46]. In fact osteoclasts are now considered as the innate immune cells in the bone, since they are able to produce and respond to cytokines and chemokines. Bone remodelling involves complex interactions between osteoclasts and other cells in bone microenvironment (marrow stromal cells, osteoblasts, macrophages, T-lymphocytes and marrow cells) [47]. Several cytokines, like the cytokine receptor activator of NFkB ligand (RANKL) and the macrophage colony stimulating factor (M-CSF), can promote osteoclast formation and activity. Also osteoprotegerin (OPG), a circulating secretory glycoprotein, could have a role in bone remodelling in children with T1D because it could promote differentiation, fusion, survival, activation and apoptosis of the osteoblasts. Alterations or abnormalities of the RANKL/OPG system have been implicated in different metabolic bone diseases characterized by increased osteoclast differentiation and activation, and by enhanced bone resorption [48].

In patients affected by both T1D and CD, the risk of developing osteopenia is also influenced by the compliance to GFD. In fact, osteopenia occurs more frequently in patients with diabetes and CD with poor compliance to GFD [49]. Recent observations indeed indicated an imbalance of cytokines relevant to bone metabolism in untreated celiac patients' sera and the direct effect of these sera on in vitro bone cell activity. In particular the RANKL/osteoprotegerin (OPG) ratio was increased in patients not on gluten-free diet [46].

In conclusion osteopenia seems to be a new occult problem in CD patients, in T1D patients and in patients with two or three immunological diseases, depending also on GFD.

GFD composition

Diet is a fundamental part of the treatment in both T1D and CD. However GFD composition could present some problems for diabetic people (Table 2).

The glycemic index (GI) provides an indirect measure of the ability of a food to raise blood glucose.GI is retained a direct index of absorption of carbohydrates, being: "the area under curve of blood glucose after eating a food containing a determined quantity of carbohydrate". White bread (GI = 100) is usually compared as reference value. In normal subjects ingestion of foods with high GI leads to a rapid blood glucose increase causing a marked insulin response. In diabetes, diet containing food with high GI is

Table 2 Variations of HbA1c, BMI gain and height velocity after GFD in children with T1D-CD

Reference	HbA1c	BMI gain	Heigh velocity
Westman et al.	unchanged	Unchanged	unchanged
Saukkonen et al.	unchanged	Increased	unchanged
Amin et al.	reduced	Increased	unchanged
Saadah et al.	unchanged	Increased	unchanged
Valletta et al.	unchanged	Unchanged	unchanged

considered inopportune because in condition of insulin deficiency (T1D) or insulin inefficacy (type 2 diabetes) the normal insulin response is not obtainable; traditionally the common diet of the diabetes people consists principally in foods with low GI. In 2002 American Society for Clinical Nutrition published an international table, revised by an older published of the sixties, which shows the GI of most common foods, evaluated in comparison to glucose and to white bread [50], (Table 3) where gluten-free products have higher GI-foods than similar products gluten containing. In Paker's study [51] six types of gluten-free foods are compared with white bread containing gluten. These foods were eaten from 11 adult patients with type 2 diabetes and blood glucose was measured after eating. Results showed no difference about GI among gluten-free foods and those containing gluten. On the contrary, Berti et al. show a higher blood glucose curve for gluten-free foods, although with similar insulin curves and with contradictory results between in vivo and in vitro analysis. (Table 4), [52]). Specific studies both in healthy patients and in type 1 and type 2 diabetic patients should be necessary, particularly in pediatric age.

In addition, gluten-free foods are prepared using corn flour, rice and wheat, where the percentage of fiber, carbohydrate, fats and micronutrients isn't completely known. Scarce contrasting data generally describe in gluten free foods composition few proteins, more fat and few fibers than gluten containing foods. (from SCHAR website and Ministry of Agriculture website, Tables 5 and 6). In addition in the review of Kupper [53] GFD seems to can be the cause of a multiple nutrients deficiency, especially of vitamin B, vitamin D, calcium, magnesium, iron, zinc, but sources of his information are not well documented. Finally Berti et al. [52] reported higher amount of fats in gluten-free bread then those with gluten, but the same amount of fibers (Table 7).

Compliance/adherence to GFD and quality of life (QOL)

Adherence to GFD among T1D-CD patients, in our experience, is generally good in patients who experienced clear clinical symptoms of CD, but is scarce among patients with few symptoms or asymptomatic. In Table 8 a summary of the data of literature is presented, but authors did not specify whether patients had experienced symptoms; data of our group are also presented

Table 3 GI of some gluten-free foods, compared to glucose and white bread

	GI glucose = 100	GI bread = 100
Gluten-free multigrain bread	79 ± 13	113
Gluten-free white bread	76 ± 5	108 ± 7
Gluten-free fiber enriched bread	73 ± 4	104 ± 5

Table 4 GI of gluten-free foods evaluated in vivo, compared to white bread (= 100)

Food	GI
Gluten-free bread	230
Gluten-free pasta	255
Quinoa	186

[36]. In contrast with T1D population, dietary compliance in CD patients (without T1D), seems to be higher: approximately 73% of patients followed the diet strictly [54]. Probably for a patient with T1D, already engaged in coping day by day a complex chronic disease, the addition of a second "limiting" condition, is a remarkable discomfort [55]. Consequently, in the case of double diagnosis (T1D + CD), it is very difficult to manage patients who did not experienced CD symptoms.

Studies on the compliance/adherence to GFD in non diabetic people showed that, in relation to the social life, children usually have a better compliance to GFD than adults [56]. In a follow-up of 10 years in the Netherlands conducted on children from 2 to 4 years who received CD diagnosis by screening, authors described a general improvement of health without deterioration in QOL [57]. In concordance Kolsteren showed that the QOL of celiac children is quite similar to that of other children [58]. Usually the difficulty with the diet occurs when the patient became adolescent, because she/he needs to feel equal to peers, especially when she/he decides to go out to eat and more acutely she/he feels limits imposed by GFD. According to Wagner et al. [59] celiac adolescents non compliant with GFD reported a lower general QOL, more physical problems, a higher burden of illness, more family troubles, and more problems in leisure time than adolescents who are compliant with GFD. No differences between compliant patients with CD and adolescents without any chronic condition were found in all QOL aspects.

It is also important remark that the balance between GFD adherence and daily life is difficult to achieve for the child/adolescent who is also affected by an other chronic disease such as T1D. The need to coordinate insulin therapy with proper nutrition and a healthy lifestyle, in order to maintain adequate metabolic control, is already a considerable effort for the young T1D-patient and families [60]. Rebellions are frequent especially in adolescents, who are already feeling diabetes as very "invasive" for all the aspects of daily life and who receive a further "restriction" constituted by the GFD. Consequently there it could be the risk, especially if this proposal is not properly, to elicit a response of complete rebellion which will endanger not only the adherence to the GFD [36,54,55], but also the entire management of T1D, causing a sharp deterioration of general compliance and increasing the risk of severe acute complications (recurrent ketoacidosis, unawareness

Table 5 Nutritional composition of gluten free and containing gluten foods

Products	PROT (g)	CHO (g)	SUGARS (g)	FATS (g)	FIBER (g)	KCALS
Flour 00	11	77.3	1.7	0.7	2.2	340
Gluten-free flour	1.2	86.3	1.5	0.8	4.5	357
Crackers containing gluten	9.4	80.1	2.5	10	2.8	428
Gluten-free crackers	5.2	74.7	5.2	12.7	2.9	434
Cereal flakes containing gluten	6.6	87.4	10.4	0.8	3.8	361
Gluten-free cereal flakes	8	80	5.3	16	5	361
Rusks containing gluten	11.3	82.3	2.2	8.2	3.5	408
Gluten-free rusks	4.8	82.9	5.4	1.4	3.6	425
Pasta containing gluten	10.9	79.1	4.2	2.5	2.7	353
Gluten-free pasta	9	73.7	0.4	7.9	2.2	353
Biscuits	6.6	84.8	18.5	14.3	2.6	416
Gluten-free biscuits	2.7	79.9	16.1		0.8	459

Table 6 Portion size, macronutrient and micronutrient composition of test meal.

	White bread	GF biscuits	GF white Unsliced bread	GF fibre Sliced bread	GF white Sliced bread	GF fibre Unsliced bread	GF pasta
Serving (g)	107	73	101	119	101	119	64
Energy (kcals)	232	335	221	225	221	236	230
Protein (g)	8.1	2.55	3.03	3.57	3.03	3.57	5.05
Carbohydrate (g)	50	50	50	50	50	50	50
(g sugars)	3.21	17.5	4.54	5.36	4.50	5.35	0.61
Fat (g)	1.39	13.87	1.01	1.19	1.01	2.38	1.02
(g satured)	0.32	4.38	0.50	0.60	0.50	1.19	0.32
Fibre (g)	1.61	2.92	1.01	5.95	1.01	7.7	0.96
Sodium (g)	0.56	0.37	0.51	0.47	0.51	0.35	0.05

GF = gluten-free

hypoglycemia). In addition it is possible to think that this further limit could be a trigger also of eating disorders in adolescent patients, being eating disorders not rare and previously reported in diabetes. Regarding QOL, Sud et al. [61] in children with T1D-CD showed that the double diagnosis appears to have a minimal impact on QOL, even if patients' parents reported a very important difficulty on management. It is interesting that not significant differences in QOL were observed with regard to age at CD diagnosis and duration, or on the basis of adherence with a GFD. Furthermore parents of T1D-CD children did express greater concern about their child's social functioning.

Conclusions

Prevalence of CD among children with T1D is significantly higher than in non diabetic children. In a large proportion CD is asymptomatic or characterized by modest/atypical symptoms. Periodic screening of CD auto-antibodies is mandatory. CD diagnosis requires the biopsy confirmation and it is necessary to prescribe GFD in the presence of mucosa impairment. Concerning the clinical benefits of GFD in T1D-CD patients, data are contrasting, except in severely symptomatic patients.

Osteopenia seems to be a new occult problem in CD patients, in T1D patients and in patients with two or three immunological diseases, depending also on GFD.

Table 7 Weight of meal and nutrient composition of 50 g available carbohydrate portions of the foods studied as served.

Tests foods	Weight of meal (g)	Protein (g)	Water (g)	Carbohydrate (g)	Fat (g)	Total dietary fibers (g)
Bread	100	9.4	31.4	50	3.6	2.8
GF Bread	125	5.7	38.5	50	9.7	3.9
Gf Pasta	156	3.2	61.2	50	0.6	2.1
Quinoa	320	3.4	75.9	50	2.0	2.8

GF = gluten-free

Table 8 Adherence to the GFD in children with T1D-CD

Reference	Country	Prevalence (%)
Acerini et al.	United Kingdom	Partial
Westman et al.	Australia	30
Valerio et al.	Italy	59
Saadah et al.	Australia	25

It is unclear whether GFD composition could present any disadvantages regarding of glycemic index, fibers, percentage of fat and micro-nutrients. Data are not univocal on this point.

Communication of the need of GFD in patients with T1D-CD is particularly delicate, especially in adolescents where it is possible to trigger rebellion behaviors. The coexistence of these two diseases in the same patient requires care by clinicians and probably a specific psychological approach.

Abbreviations

AIT: Autoimmune thyroid disease; AD: Addison's disease; BMD: Bone mineral density; CD: Celiac disease; EMA: Endomysial autoantibodies; GFD: Gluten-free diet; HbA1c: Glycated hemoglobin; HLA: Human leukocyte antigen; pot-CD: Potential celiac disease; QOL: Quality of life; T1D: Type 1 diabetes; TG: Thyroglobulin autoantibodies; TPO: Peroxidase autoantibodies; TTG: Transglutaminase autoantibodies

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MEC (MD), EM (MD), PB (MD): have been involved in drafting the manuscript, except "Composition diet", "Compliance/adherence to GFD and quality of life" and "Genetics". EZ (Psy. D): has been involved in drafting "Compliance/adherence to GFD and quality of life". MF (MD), VF(MD): acquisition of data. SM (Dietitian): has been involved in drafting "Composition diet". RN (MD): has been involved in drafting "Genetics". GV (MD), RT (MD): have rivisited critically the manuscript. AF (MD): conception and design of the manuscript

Competing interests

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4

Type 1 Diabetes Mellitus and Co-Morbidities

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1. Introduction

Co-morbid conditions are relatively frequent in Type 1 Diabetes Mellitus (T1DM). They can severely affect clinical management of the disease, especially in pediatric age.
Furthermore, these conditions could present very interesting ethiopatogenetic mechanisms.

2. Associated autoimmune conditions

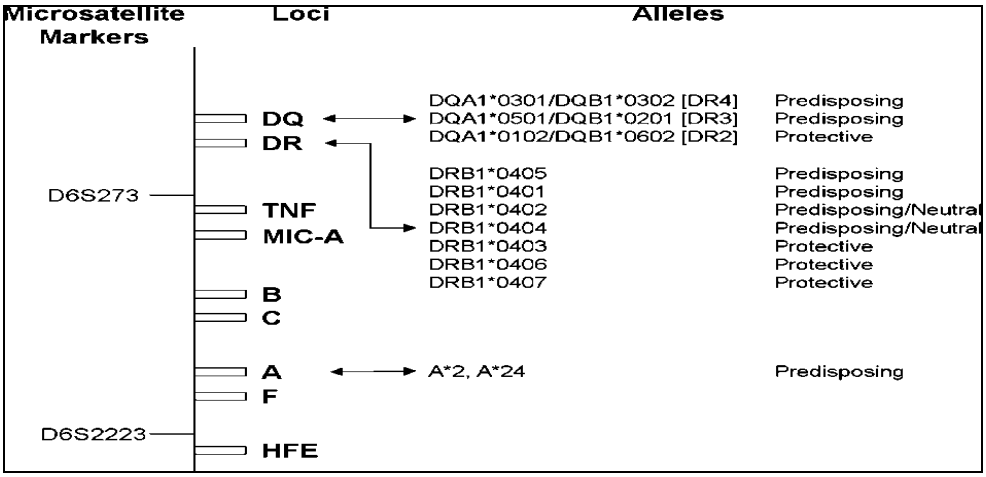
2.1 Genetic associations

Patients with type 1 diabetes (T1D) have an increased risk of other autoimmune conditions, such as autoimmune thyroid disease (AIT), celiac disease (CD), Addison's disease (AD) and vitiligo. These diseases are associated with organ-specific autoantibodies: AIT with thyroid peroxidase (TPO) and thyroglobulin autoantibodies (TG), CD with endomysial (EMA) and transglutaminase (TTG) autoantibodies, and AD with adrenal autoantibodies. Using these autoantibodies, organ-specific autoimmunity may be often detected before the development of clinical disease, in order to prevent significant morbidity related to unrecognized disease (Barker, 2006). The probable mechanism of these associations involves a shared genetic background (Myśliwiec et al., 2008; Smyth et al., 2008).
The majority of autoimmune endocrinopathies, including T1D, are inherited as complex genetic traits. Multiple genetic and environmental factors interact with each other to confer susceptibility to these disorders. Genetic risk factors associated with T1D, ATD, CD and AD include HLA genes and non-HLA genes.

2.1.1 HLA genes

The major histocompatibility complex (MHC) has been extensively studied in these diseases. HLA molecules are highly polymorphic and multiple different peptides can be presented to T cells by these molecules. In general it appears that the alleles associated with autoimmunity are not abnormal, but functional variants, that aid in determining specific targets of autoimmunity. The leading hypothesis is that these molecules contribute to determine risk through the peptides they bind and present to T-lymphocytes, either by influencing thymic selection, or peripheral antigen presentation. (Ide & Eisenbarth, 2003).
HLA DR4 and DR3 are strongly associated with T1D and approximately 30-50% of patients are DR3/DR4 heterozygotes. The DR3/DR4 genotype confers the highest diabetes risk with a synergistic mode of action, followed by DR4 and DR3 homozygosity, respectively. The

HLA-DQ (particularly DQ 2 and DQ8) locus has been found to be the most important determinant of diabetes susceptibility. Approximately 90% of individuals with T1D have either DQ2 or DQ8, compared to 40% of the general population (Ide & Eisenbarth, 2003). So, the highest-risk human leukocyte antigen (HLA) genotype for T1D is DR3-DQ2, DR4-DQ8.
DR3-DQ2 shows a strong association with CD; homozygosity for DR3-DQ2 in a population with T1D carries a 33% risk for the presence of TTG autoantibodies (Bao et al., 1999). Moreover, in families with multiple members affected with T1D and AIT, DR3-DQ2 has been linked with AIT and T1D (Levin et al, 2004). AD has been associated with the presence of a rare subtype of DR3-DQ2, DR4-DQ8 in which the DR4 subtype is DRB1*0404. This subtype is found in less than 1% of the general population compared with 30% of the population with AD (Barker et al., 2005; Myhre et al., 2002; Yu et al., 1999). A schematic representation of the HLA region and its association with T1D is shown in the Figure 1.



(from Pugliese A. and Eisenbarth G.S., Chapter 7, Type 1 Diabetes: Molecular, Cellular, and Clinical Immunology, www.barbaradaviscenter.org)

Fig. 1. The HLA Region and T1D susceptibility. Schematic representation of the HLA region showing microsatellite markers, loci, and alleles associated with T1D susceptibility. Distances between loci are grossly approximated.

2.1.2 Non-HLA genes

Non-HLA genes are also involved in the predisposition to T1D and other autoimmune diseases, such as MIC-A, PTPN22, CTLA-4 (Barker, 2006).
Polymorphisms of MIC-A (MHC I-related gene A) have been associated with T1D, CD and AD. This gene encodes for a protein that is expressed in the thymus and interacts with the receptor NKG2D, which is important for thymic maturation of T cells (Hue et al., 2003). It is hypothesized that the loss of this interaction is a way in which immunological tolerance may be lost. NKG2D also regulates the priming of human naïve CD8+ T cells, providing an alternative explanation for associations with autoimmune diseases (Maasho et al., 2005).
The PTPN22 gene is expressed in T cells and encodes lymphoid tyrosine phosphatase (LYP). LYP appears to be important in the signal cascade downstream from the T-cell receptor. A

specific polymorphism, changing an arginine to tryptophan at position 620, has been associated with T1D (Bottini et al., 2004; Smyth et al., 2004) and also other autoimmune disorders, such as rheumatoid arthritis, systemic lupus erythematosus, Graves' disease and weakly with AD. The association with many autoimmune diseases suggests that this gene may be playing a role in susceptibility to autoimmunity in general.
Another non-HLA gene associated with T1D which has a generic role in susceptibility to autoimmunity is CTLA-4 (Cytotoxic T lymphocyte-associated antigen-4) (Vaidya & Pearce, 2004). CTLA-4 gene is an important susceptibility locus for autoimmune endocrinopathies and other autoimmune disorders, including T1D (Ueda et al., 2003). The CTLA-4 gene, which is located on chromosome 2, encodes a costimulatory molecule that is expressed on the surface of activated T cells. It plays a critical role in the T-cell response to antigen presentation, binding costimulatory molecules and inhibiting T-cell activation. (Vaidya & Pearce, 2004). The inhibitory effect of CTLA-4 on T-cell activation has led the investigations into its role in different human autoimmune disorders. Polymorphisms within the CTLA-4 gene have been linked to AIT (Vaidya et al., 1999). CTLA-4 has also been linked to AD and more strongly to subjects affected by AD in association with T1D and AIT compared with AD alone (Vaidya et al., 2000). CTLA-4 has been associated with a wide range of other autoimmune disorders, including primary biliary cirrhosis, multiple sclerosis, CD and rheumatoid arthritis. These observations have suggested that CTLA-4 is a general autoimmune locus, and that the susceptibility polymorphisms within the gene may lead to general defects in the immune regulation, while other tissue-specific (e.g. insulin gene polymorphisms) or antigen-specific (e.g. MHC) genetic factors and environmental factors determine the involvement of particular target organs (Vaidya & Pearce, 2004).

Gene	Associated diseases
MIC-A	T1D, CD, AD
PTPN22	AIT, AD
CTLA-4	T1D, AIT

Table 1. Non-HLA genes associated with T1D and other autoimmune diseases

2.2 Type 1 diabetes and celiac disease

2.2.1 Prevalence and age at starting

Traditional studies, both in children and adults, have shown that CD occurs in patients with T1D with a prevalence that varies from 1,5 to 10 % compared with 0.5 % of the general population (Cronin & Shanahan, 2007; Vaarala, 2000). The mean age at diagnosis of classical CD is commonly around 2-3 years, while the mean age at diagnosis of DM1 is 7-8 years. The age at onset of T1D is younger in patients with the double disease than in those with only T1D (Kaspers et al., 2004). The risk of CD is negatively and independently associated with age at onset of diabetes, with an higher risk being seen in children age < 4 years than in those age > 9 years (Cerutti et al., 2004). In patients with T1D, diabetes is usually diagnosed first, CD precedes diabetes onset only in 10-25% (Cerutti et al., 2004; Valerio et al., 2002), while generally CD diagnosis in T1D patients occurs, trough the screening performed at diabetes onset, in 70-80% of patients with a median age >8 years. Some authors hypothized that in genetically susceptible patients one disease could predispose to another. Particularly, it has been suggested that untreated (latent or silent) CD could be an immunological trigger and induce diabetes and/or thyroid disorders due to gluten as a driving antigen (Pococco &

Ventura, 1995). In accordance with this, the prevalence of autoimmune disorders in CD is closely related to age at diagnosis or, in other words, to the duration of exposure to gluten (Ventura et al., 1999) and thyroid-related antibodies tend to disappear during twelve months of gluten-free diet, like CD-related antibodies (Ventura et al., 2000). However, at present, it is unknown whether treatment of CD reduces the likelihood of developing autoimmune disorders, or changes their natural history and actually others found no correlation between duration of gluten exposure in adult CD and risk of autoimmune disorders (Viljamaa et al., 2005).

2.2.2 Clinical features and follow up

The classic presentation of CD describes symptoms related to gastrointestinal malabsorption and includes malnutrition, failure to thrive, diarrhea, anorexia, constipation, vomiting, abdominal distension, and pain. This predominance of gastrointestinal symptoms is more common in children younger than three years of age. Non-gastrointestinal or atypical symptoms of CD include short stature, pubertal delay, fatigue, vitamin deficiencies, and iron deficiency anemia and are more commonly observed in older children. The classical presentation of CD can occur in T1D patients, but many patients with CD and T1D are either asymptomatic (silent CD) or present with only mild symptoms (Holmes, 2001a; Ventura et al., 2000). Diagnosis of CD is regularly performed because screening protocols are universally recommended and performed. In patients with overt CD, identifying and treating CD with gluten free diet (GFD) surely confer benefit in reducing complications such as malabsorption, infertility, osteoporosis, poor nutrition, impaired growth and reducing long-term malignancy risks and mortality rates (Collin et al., 2002; Freemark & Levitsky, 2003; Rubio-Tapia et al., 2009), while no evidence exists on long-term morbidity in silent CD. Similarly, children with T1D with evidence of symptomatic CD benefit from GFD (Hansen et al., 2006; Saadah et al., 2004); in symptom-free cases the demonstrated benefit is limited to weight gain and bone mineral density (BMD) changes.(Artz et al., 2008; Rami et al., 2005; Simmons et al., 2007). Recently a 2-year prospective follow up study has provided additional evidence that only in some of the children with T1D and few classical symptoms of CD, identified by screening as being TG+ present, the demonstrated benefit of GFD is limited to weight gain and BMD changes (Simmons et al., 2011); moreover, other authors have reported an improved glycemic control in GFD-compliant celiac patients (Sanchez-Albisua et al., 2005). On the contrary, silent untreated CD has no obvious effect on metabolic control in T1D patients, but could negatively influence weight gain (Rami et al., 2005). In any case, the adherence to GFD by children with T1D has been reported generally below 50% (Acerini et al., 1998; Crone et al., 2003; Hansen et al., 2006; Saadah et al., 2004, Westman et al., 1999). The different viewpoints highlight the need of a long follow up of patients affected by T1D and asymptomatic CD to clarify the role of a GFD. Actually some authors argument against the need to stress GFD in nonsymptomatic T1D patients (Franzese et al., 2007; Van Koppen et al., 2009). However, the wide spectrum of CD include also subjects with positive celiac-related antibodies without diagnostic small-bowel mucosal villous atrophy. This condition is defined as potential celiac disease (pot-CD) (Holmes, 2001b; Paparo et al., 2005; Troncone et al., 1996). Some authors described that the prevalence of pot-CD among patients with T1D recruited from the majority of childhood diabetes care centers in Italy is 12.2 %, with an higher prevalence of females. The prevalence of pot-CD in the CD control population is 8.4 % (Franzese et al., 2011). Case reports and small follow-up studies indicated that only few pot-CD patients may suffer from CD-related symptoms

before the development of villous atrophy (Troncone et al., 1996). No definite consensus exists among experts about to treat pot-CD patients with GFD. No data are available on the natural history of these patients in the long term, nor on the risks they are exposed if left on normal gluten-containing diet, while a recent paper provided evidence that pot-CD children may benefit from GFD treatment (Kurppa et al., 2010).
Other studies have shown intestinal inflammation also in T1D patients without CD-related antibodies and structurally normal intestinal mucosa (Westerholm-Ormio et al., 2003). According to this, our group has observed a gluten-related inflammation either in rectal either in small bowel mucosa of children with T1D (Maglio et al,. 2009; Troncone et al., 2003). It can be speculated that gluten could be an optimal candidate to stimulate an abnormal innate immune reaction in intestinal mucosa due to its pro-inflammatory characteristics. It remains a crucial issue to estabilish to what the extented intestinal inflammation in T1D is gluten-dependent and whether it precedes the occurrence of the disease.

2.3 Type 1 diabetes and autoimmune thyroid disease

2.3.1 Prevalence and age at starting

Antithyroid antibodies have been shown to occur during the first years of diabetes in 11-16.9% of individuals with T1D (Kordonouri et al., 2002). Long-term follow up suggests that as much as 30 % of patients with T1D develop AIT (Umpierrez et al., 2003). The range of prevalence of AIT in patients with T1D is unusually wide (3.4-50%) (Burek et al., 1990; Radetti et al., 1995). Thyroid antibodies are observed more frequently in girls than in boys, often emerging along during pubertal maturation (Kordonouri et al., 2005).

2.3.2 Clinical features and follow-up

Hyperthyroidism is less common than hypothyroidism in association with T1D (Umpierrez et al., 2003), but still more common than in the general population. It may be due to Grave's disease or the hyperthyroid phase of Hashimoto's thyroiditis. The presence of abnormal thyroid function related to AIT in the population with T1D has the potential to affect growth, weight gain, diabetes control, menstrual regularity, and overall well-being. In particular clinical features of hypothyroidism may include the presence of a painless goitre, increased weight gain, retarded growth, tiredness, lethargy, cold intolerance and bradycardia while diabetic control may not be significantly affected. Clinical features of hyperthyroidism may include unexplained difficulty in maintaining glycaemic control, weight loss without loss of appetite, agitation, tachycardia, tremor, heat intolerance, thyroid enlargement or characteristic eye signs. The treatment of hypothyroidism is based on replacement with oral L-thyroxine (T4) sufficient to normalise TSH levels and usually this allows regression of the goitre if present. The treatment of hyperthyroidism is based on the use of carbimazole and beta-adrenergic blocking drugs, if necessary.
There are studies showing worse diabetes control in patients with a second autoimmunity, including AIT and CD (Franzese et al., 2000; Iafusco et al., 1998). The factors responsible for the worsened control have not been completely elucidated. Thyroid dysfunction could be responsible of variations in absorption of carbohydrates and increased insulin resistance. There are studies showing similar diabetes control in patients with and without a second autoimmunity, in these studies thyroid autoimmunity does not lead to worsening of diabetic metabolic control in children with T1D (Kordonouri et al., 2002; Rami et al., 2005; Sumnik et al., 2006). The thyroid status is not different between diabetic patients with and

without CD: children with both T1D and CD do not have an increased risk of AIT development compared to diabetic patients without CD (Sumnik et al., 2006).

2.4 Type 1 diabetes, Addison disease and polyglandular syndromes

2.4.1 Prevalence and age at starting

Addison's disease (AD) affects approximately 1 in 10,000 of the general population. The autoimmune process resulting in AD can be identified by the detection of autoantibodies against the adrenal cortex (Anderson et al., 1957; Lovas & Husebye, 2002). Up 2 % of patients with T1D have antiadrenal autoantibodies (De Block et al.; 2001, Falorni et al., 1997; Peterson et al., 1997).

AD is occasionally associated with T1D in the Autoimmune Polyglandular Syndromes (APS I and II). APS I, also known as autoimmune polyendocrinopathy candidiasis ectodermal dysplasia (APECED), is a rare polyendocrine autoimmune disease caused by mutations of the autoimmune regulator gene (AIRE) on chromosome 21q22.3 (Aaltonen et al., 1994; Ahonen et al., 1990), which is characterized by the association of mucocutaneous candidiasis, adrenal insufficiency, and/or hypoparathyroidism. Follow-up of subjects with this disorder has revealed that many organ systems may be involved in the autoimmune process including the pancreatic β cell. Approximately 20% of subjects with APS-I develop T1D (Barker, 2006). APS II is more common in adults, but is also observed in children in association with autoimmune thyroiditis (Dittmar & Kahaly, 2003). Other less common disorders observed in APSII include Addison's disease, hypogonadism, vitiligo, alopecia, pernicious anemia and myasthenia gravis. Another rare disorder associated with T1D in early childhood is the Immunodysregulation Polyendocrinopathy X-linked Syndrome (IPEX), which is characterized also by severe enteropathy and autoimmune symptoms due to a clear genetic defect (FOX-P3) (Chatila et al., 2000). FOX-P3 is expressed in CD4+CD25+ regulatory T cells; mutations result in the inability to generate these regulatory T cells resulting in multiorgan autoimmunity (Barker, 2006).

2.4.2 Clinical features and follow-up

The condition of AD is suspected by the clinical picture of frequent hypoglycaemia, unexplained decrease in insulin requirements, increased skin pigmentation, lassitude, weight loss, hyponatraemia and hyperkalaemia. The diagnosis is based on the demonstration of a low cortisol, especially in response to ACTH test. Treatment with a glucocorticoid is urgent and life-threatening. In some cases the therapy has to be supplemented with a mineralocorticoid. In asymptomatic children with positive adrenal antibodies, detected on routine screening, a rising ACTH level suggests a failing adrenal cortex and the development of primary adrenal insufficiency (Kordonouri et al., 2009). There are no current recommendations for screening of adrenal autoimmunity.

2.5 Type 1 diabetes and vitiligo

Vitiligo is an acquired pigmentary disorder characterized by a loss of melanocytes resulting in white spots or leukoderma. The association of vitiligo with other autoimmune disorders, including thyroid disease, adrenal insufficiency, gonadal dysfunction, polyendocrine failure, diabetes mellitus, pernicious anemia, myasthenia gravis and alopecia areata, has been well documented (Bystryn, 1997; Handa & Dogra, 2003). This condition is present in about 6% of diabetic children (Hanas et al., 2009). Spontaneous re-pigmentation is rare and

not usually cosmetically acceptable. Treatment is difficult and multiple therapies have been tried with little success . (Ho et al., 2011)

2.6 Type 1 diabetes and collagenopathies

2.6.1 Rheumatoid arthritis

The tendency of autoimmune diseases to aggregate is well known as clusters of autoimmune diseases within families and individuals. Analysis of susceptible genetic loci for the distinct autoimmune disease shows considerable overlap that suggests the possibility of shared pathways in their pathogenesis. Reports on the clustering of T1D, AIT, CD and rheumatoid arthritis (RA) in the same patient are very scarce. The major genetic predisposition to RA is contributed by variants of the class II HLA gene, HLA DRB1. In exploring the overlap between T1D, CD and RA, there is strong evidence that variation within the TAGAP gene is associated with all three autoimmune diseases. Relatively little is known about the TAGAP gene, which encodes a protein transiently expressed in activated T cells, suggesting that it may have a role in immune regulation. So the TAGAP gene, previously associated with both T1D and CD, is also associated with RA susceptibility. Interestingly a number of loci appear to be specific to one of the three diseases currently studied suggesting that they may play a role in determining the particular autoimmune phenotype at presentation (Eyre et al., 2010). The majority of the published case reports are girls. The predominance of females among the affected individuals may reflect that certain genes play role in the pathogenesis as gender-specific factors or the penetrance of multiple risk genes are enhanced in females. In most reported patients, diabetes is diagnosed first, thyroid autoimmunity and juvenile rheumatoid arthritis develop after a period of several months to years. (Nagy et al., 2010; Pignata et al., 2000; Valerio et al., 2000).

2.6.2 Sclerodermia, systemic lupus erythematosus

The association of T1D with Systemic Lupus Erythematosus (SLE) and Sclerodermia is rare but reported in literature (Inuo et al., 2009, Zeglaoui et al., 2010). Some authors found a significant association between DQ2 allele and the presence of anti-SSA antibodies, while others described an association between CD and the presence of A1B8DR3 haplotype, which seems to be frequent in SLE and in Sclerodermia (Black et al., 1983; Mark, 2000; Sollid & Thorsby, 1993). In human, the CTLA-4 and PD-1 genes significantly contributed to the development of various autoimmune diseases in different genetic backgrounds (Inuo et al., 2009).). It has been suggest the involvement of CTLA-4 and PD-1 (inhibitor receptors of CD28) to the development of T1D, SLE or other autoimmune diseases.

Juvenile sclerodermia is present in 3% of sclerodermia cases, SLE in children is present in 9% of cases of SLE; one case of a 15 years girl with CD and SLE and Sclerodermia has been reported (Zeglaoui et al., 2010).

2.7 Screening for associated autoimmune disorders

Since Type 1 Diabetes is associated with the presence of additional autoimmune disease, such as AIT, CD and AD, which are associated with the production of organ-specific antibodies, it is possible to screen patients with T1D by means of these ones. However, only a subset of the subjects with organ-specific antibodies develops clinical disease. The frequency of screening and follow up of patients with positive antibodies remain controversial. The current American Diabetes Association (ADA) recommendations are to

screen for CD-associated antibodies at diagnosis of T1D and in presence of symptoms. The International Society of Pediatric Adolescent Diabetes (ISPAD) recommends to screen for CD at the time of diagnosis, annually for the first five years and every second year thereafter. More frequent assessment is indicated if the clinical situation suggests the possibility of CD or the child has a first-degree relative with CD. Respect to the screening for thyroid disease, current recommendations from the ADA are for screening TSH after stabilization at onset of diabetes, with symptoms of hypo- or hyperthyroidism, and every 1–2 yr thereafter. ISPAD recommends to screen by circulating TSH and antibodies at the diagnosis of T1D and, thereafter, every second year in asymptomatic individuals without goitre or in the absence of thyroid autoantibodies. More frequent assessment is indicated otherwise, subjects with positive TPO autoantibodies and normal thyroid function are screened on a more frequent basis (every 6 months to 1 yr). There are no current recommendations for screening of adrenal autoimmunity (Barker, 2006). Authors observed that the prevalence of adrenal antibodies in diabetic patients with thyroid antibodies compared with those without thyroid antibodies is increased (5,1 vs 0,6%) (Riley et al., 1981). It is possible conclude that routine screening for AD in children with T1D is not warranted unless there is a strong clinical suspicion or family history of AD (Marks et al., 2003)

Celiac disease	Transglutaminase antibodies	Yearly
Thyroiditis	TSH, FT4, thyroid antibodies	Yearly
Addison disease	Cortisolemia, adrenal antibodies	Screening if AD in family
Collagenopathies	Specific auto-antibodies	No screening

Table 2. Autoimmune diseases associated with T1D, recommended systems and frequency of the screening

3. Associated non-autoimmune conditions

3.1 Type 1 diabetes and growth

Type 1 diabetes and other chronic diseases are well known to adversely affect linear growth and pubertal development, this can include a wide spectrum of different conditions, from poor gain of weight to Mauriac Syndrome (MS); MS classically involves hepatomegaly, growth impairment, and Cushingoid features in poorly controlled diabetic patients. Although MS, the most important expression of growth alteration due to severe insulin deficiency in diabetic patients, is now rare, impaired growth in children with T1D is still reported. This is particularly true in patients with poor metabolic control (Chiarelli et al., 2004; Franzese et al., 2001). Some studies report that poorly controlled patients show a decrease in height standard deviation score over the next few years, while better controlled patients maintain their height advantage (Gunczler & Lanes, 1999; Holl et al., 1998).

Longitudinal bone growth is a complex phenomenon involving a multitude of regulatory mechanisms strongly influenced by growth hormone (GH) (Chiarelli et al., 2004) and by the interaction between insulin-like growth factors (IGF-I and IGF-II), that circulate bounded to specific insulin-like growth factor binding proteins (IGFBPs). IGFBP-3, the major circulating binding protein during post-natal life, is GH-dependent. Insulin is an important regulator of this complex. In fact, adequate insulin secretion and normal portal insulin concentrations are

needed to support normal serum concentrations of IGFs and IGFBPs and indirectly to promote growth. Poor gain of height and weight, hepatomegaly, non alcoholic steatosis hepatis (NASH) and late pubertal development might be seen in children with persistently poorly controlled diabetes. Similar to healthy adolescents, the pubertal growth spurt represents the most critical phase for linear growth and final height in children with T1D. The pubertal phase is characteristically associated with reduction in insulin sensitivity, which is known to be more severe in patients with T1D, and might negatively influence growth and height gain (Chiarelli et al., 2004). Although the chronological age at onset of puberty and the duration of the pubertal growth spurt is not significantly different between subjects with T1D and healthy adolescents, several studies have shown a blunted pubertal growth spurt which seems to be associated with a reduced peak of height velocity SDS (Vanelli et al., 1992). Although loss of height from the onset of diabetes has been widely reported, an impaired final height has not been reported in children with T1D. In fact, while some studies, especially those performed in the pre-intensive insulin therapy era, showed an impaired final height in children with diabetes (Penfold et al., 1995), more recent studies show a normal or only slightly reduced final height (Salerno et al., 1997).

The Diabetes Control and Complications Trial (DCCT) and other studies have reported increased weight gain as a side effect of intensive insulin therapy with improved metabolic control (DCCT Research Group, 1993). As obesity is a modifiable cardiovascular risk factor, careful monitoring and management of weight gain should be emphasised in diabetes care. Girls seem to be more at risk of overweight and as well of eating disorders.

Monitoring of growth and development and the use of percentile charts is a crucial element in the care of children and adolescents with diabetes. Improvements in diabetes care and management and especially newer insulin schedules based on multiple daily injections or insulin pumps have led to a reduction in diabetic complications and seem to ameliorate growth in children with T1D. Start an intensive insulin regimen since the onset of diabetes might prevent the induction of abnormalities of the GH–IGF-I–IGFBP-3 axis potentially achieving near-normal portal insulin concentrations and thereby leading to normal IGF-I and IGFBP-3 levels and physiological growth in children and adolescents with T1D.

3.2 Type 1 diabetes and eating disorders

Eating disorders (EDs) are a significant health problem for many children and adolescents with T1D similar to that observed in other high risk groups, such as competitive athletes, models and ballet dancers. EDs and subclinical disordered eating behaviors (DEBs) have been described in adolescents with T1D with a higher prevalence than in a non-diabetic population. The start of insulin treatment and the need to comply with dietary recommendations both lead to weight gain, which in turn leads to body dissatisfaction and a drive for thinness. Since the dietary restraint usually requires ignoring internal cues of hunger and satiety, it has been suggested that it may be a triggering factor in the development of cycles of binge eating and purging. The concurrence of T1D and EDs can greatly increase morbidity and mortality. In diabetic subjects, EDs are associated with insulin omission for weight loss and impaired metabolic control. On the contrary, in a five year longitudinal study, the expected relationship between ED and poor metabolic control was not evident, although there was a trend for higher haemoglobin A1c in individuals with an EDs (Colton et al., 2007). This offers hope that early interventions might prevent the worsening metabolic control that is often associated with EDs. In addition subclinical DEBs

among youth with T1D have been associated with increased risk of poor metabolic control and increased prevalence of microvascular complications such as retinopathy and nephropathy (Rydall et al., 1997). Some studies have examined the prevalence of EDs and DEBs in youth with T1D. Prevalence rates vary considerably from study to study possibly due to differences in sample, screening tools, and data collection methods. In a multi-site, cross sectional case-control study, the prevalence of ED meeting DSM-IV diagnostic criteria was about 10% and that of their sub-threshold variants about 14%: both were about twice as common in adolescent females with T1D than in their non-diabetic peers. (Jones et al., 2000). However there are also rare cases in childhood (Franzese et al., 2002a).

3.2.1 Management

Nutritional treatment is one of the main difficulties in managing diabetes in the young. Diabetes clinicians should be aware of the potential warning signs in an adolescent with diabetes as well as assessment and treatment options for eating disorders with concomitant T1D. Clinical approaches should focuse on normalizing eating behaviour and enhancing self-esteem based on personal attributes unrelated to weight and eating, with a low threshold for referral for specialized EDs services (Colton et al., 2007). A multidisciplinary team, composed by clinicians, psychologist/psychiatric, dietitian/nutrition therapist, especially one with a background in EDs, is opportune to identify and treat unhealthy EDs and DEBs in T1D. Treatment for adolescents with T1D should include both diabetes management treatment and mental health treatment. The diabetes team and the mental health team have separate responsibilities but work collaboratively to address disordered eating in patients with T1D. Treatment begins with emphasis on nutritional rehabilitation, weight restoration, and adequate diabetes control. Psychotherapy should begin immediately for the patient and family (S.D. Kelly et al., 2005).

3.3 Necrobiosis lipoidica diabeticorum

Necrobiosis lipoidica diabeticorum (NBL) is an infrequent skin affection in pediatric age. The etiology is not clearly understood. The reported prevalence in children varies from 0.06% to 10% (De Silva et al., 1999). The female/male ratio is 3:1(Hammami et al., 2008). The average age of onset is 30–40 years. In the past, it has been described as a complication of diabetes and associated with microvascular complications (W.F. Kelly et al., 1993), but NBL has been observed also at the beginning of diabetes. NBL typically appears on the anterior lower legs. The lesions are usually bilateral and are characterized by well circumscribed yellow brown inflammatory plaques with raised borders and an atrophic center. Ulceration occurs in up to 35% of cases and is notoriously difficult to treat (Elmholdt et al., 2008). This complication negatively affects quality of life and implies a greater risk for secondary infection. Although NBL is usually observed in diabetic patients, there is some controversy regarding the degree of this association and it has been hypothesized that the strength of this association may have been overestimated in the past. Some authors have studied the effect of glucose control on NBL and found no correlation with glycosylated hemoglobin A1c levels (Dandona et al., 1981), while others found an association with a poor glucose control (Cohen et al., 1996).

3.3.1 Management

There is currently no standardized effective treatment of NBL. A wide variety of treatments have been used over the years in adults. These include: topical, systemic or intra-lesional

steroids, aspirin, cyclosporin, mycophenolate, becaplermin, excision and grafting, laser surgery, hyperbaric oxygen, topical granulocytemacrophage colony-stimulating factor and photochemotherapy with topical PUVA (Hanas et al., 2009). A recent study suggests the use of TNF inhibitors in selected patients for treatment of NBL (ulcerative forms) unresponsive to prior conventional therapies (Suárez-Amor et al., 2010). NBL in children can be hard to manage and may be associated with a long-term risk of malignant transformation to squamous cell carcinoma. Systemic therapies, such as corticosteroids and azathioprine are immunosuppressive and immunomodulatory and could facilitate malignant transformation (Beattie et al., 2006). Therefore, although NBL is not clearly related to poor metabolic control, we believe that the diabetic control may also be useful. Effective primary prevention strategies and new treatment options are needed to adequately control the disease and its progression.

3.4 Osteopenia

Children and adolescents with T1D can show several impairment of bone metabolism and structure, resulting in a higher risk of decreased bone mass and its related complications later in life. Consequently an assessment of quality of the bone through non-invasive methods (phalangeal ultrasonography) seems to be opportune in the care of diabetic patients, specially the ones with clusters of autoimmune diseases to define a possible involvement of the bone (Lombardi et al.,2010).

Bone impairment in multiple autoimmune diseases might be considered not only a complication due to endocrine or nutritional mechanisms, but also a consequence of an immunoregulatory imbalance.

3.4.1 Metabolic causes

Alterations of bone mineral density (BMD) are especially observed when diabetes is associated with CD and/or AIT. Bone loss, described in patients with T1D, AIT or CD is usually viewed as a complication of these diseases and is related to duration of diabetes and quality of metabolic control. The exact mechanisms accounting for bone loss in these diseases have been variably explained by metabolic derangements due to the impaired hormonal function in T1D or AIT (McCabe, 2007), or calcium malabsorption and secondary hyperparathyroidism in untreated CD patients (Selby et al., 1999). Alterations of homeostatic mechanisms might explain an imbalance of osteoclast activity leading to osteopenia (Lombardi et al.,2010; Wu et al., 2008).

3.4.2 Immune causes

Bone remodeling involves complex interactions between osteoclasts and other cells in their microenvironment (marrow stromal cells, osteoblasts, macrophages, T-lymphocytes and marrow cells) (Kollet et al., 2007; Teitelbaum, 2007). Besides their role in calcium mobilization from bone and initiation of bone remodeling, osteoclasts are now considered as the innate immune cells in the bone, since they are able to produce and respond to cytokines and chemokines. Some authors found altered levels of plasma Osteoprotegerin (OPG) in children with T1D. Osteoprotegerin is a circulating secretory glycoprotein and is a member of the tumor necrosis factor receptor (TNFR) family. It works as a decoy receptor for the cytokine receptor activator of NFkB ligand (RANKL). RANKL and OPG are a key agonist/antagonist cytokine system: RANKL increases the pool of active osteoclasts thus

increasing bone resorption, whereas OPG, which neutralizes RANKL, has the opposite effect. Alterations or abnormalities of the RANKL/OPG system have been implicated in different metabolic bone diseases characterized by increased osteoclast differentiation and activation, and by enhanced bone resorption (Galluzzi et al., 2005). Therefore, bone could be an additional target of immune dysregulation.
Cytotoxic T lymphocyte-associated antigen-4 (CTLA4), a well-known susceptibility gene for autoimmune disorders, might also represent a possible link between immune system and bone. In animal studies CTLA4 expressed on T regulatory (Treg) cells impairs osteoclast formation (Zaiss et al., 2007). Therefore the failure of Treg cell function in clustering of multiple autoimmune diseases could represent a mechanism to explain both the occurrence of poly-reactive autoimmune processes and the increase of bone resorption in the same individuals.
In patients affected by both T1D and CD, the risk of developing osteopenia is probably influenced by the compliance to gluten-free diet. Osteopenia occurs more frequently in patients with diabetes and CD with poor compliance to GFD. Interestingly, recent observations indicate also an imbalance of cytokines relevant to bone metabolism in untreated celiac patients' sera and the direct effect of these sera on in vitro bone cell activity. In particular the RANKL/osteoprotegerin (OPG) ratio was increased in patients not on gluten-free diet. Actually, the only presence of a second disease, either AIT or CD, do not seems to increase the frequency of osteopenia, provided a good compliance to GFD in CD patients, while the association of three autoimmune diseases significantly increases the occurrence of osteopenia (37.5%). In addition, poor compliance to GFD of CD patients could increase the occurrence of osteopenia more in patients with three autoimmune diseases (80%) than in those with two autoimmune diseases (18.8%) (Valerio et al., 2008).

3.5 Gastropathy

Gastrointestinal motility disorders are found in a consistent proportion of children with T1D and are associated with significant morbidity: they are usually associated with dyspeptic symptoms, such as nausea, vomiting, fullness and epigastric discomfort, and could be an important cause of morbidity in diabetic patients. Gastroparesis has been shown to be significantly correlated with a poor metabolic control in a population of T1D children with gastric electrical abnormalities. (Cucchiara et al., 1998). Furthermore it is conceivable that delayed gastric emptying may cause a mismatch between the onset of insulin action and the delivery of nutrients into the small intestine (Rayner et al., 2001). Diabetic children with unexplained poor glycemic control should be investigated for abnormalities in gastric motility (Shen & Soffer 2000). On the other hand, hyperglycaemia itself can affect the neuromuscular mechanisms regulating gastrointestinal motility and delay the gastric emptying process (Jebbink et al., 1994). Therefore, it is of great importance to try to reverse abnormalities of gastric motility and improve gastric emptying in patients with T1D and gastroparesis by the use of domperidone in children with T1D. (Franzese et al., 2002b).

3.6 Type 1 diabetes and limited joint mobility

Type 1 diabetes can be associated with other less common disabling conditions of locomotor system: Dupuytren's contracture, stiff hand, carpal tunnel syndrome, and limited joint mobility (LJM). Limited joint mobility is one of the earliest clinically apparent long-term complications of T1D in childhood and adolescence, characterized by a bilateral painless

contracture of the finger joints and large joints, associated with tight waxy skin. Changes begin in the metacarpophalangeal and proximal interphalangeal joints of the fifth finger and extend radially with involvement of the distal interphalangeal joints as well. Involvement of larger joints includes particularly the wrist and elbow, but also ankles and cervical and thoracolumbar spine (Komatsu et al., 2004). The limitation is only mildly disabling even when severe. With rare exception, LJM appears after the age of 10 years. The prevalence of LJM in T1D, evaluated in several studies ranges from 9 to 58% in paediatric and adult patients (Lindsay et al., 2005).

The biochemical basis of LJM may be a consequence of changes in the connective tissue, probably due to alterations in the structural macromolecules of the extracellular matrix. The hyperglycaemia can alterate the glycation of protein with the formation of advanced glycation end products (AGEs), which resist to protein degradation and consequently increase thickness of basal membranes in the periarticular tissues (Shimbargger, 1987). Development of LJM is related to both age and diabetes duration (Cagliero et al., 2002), while others showed that it can be compromised also in a precocious age and with a short duration of diabetes (Komatsu et al., 2004). Of note, fluorescence of skin collagen, which reflects the accumulation of stable AGEs, increases linearly with age, but with abnormal rapidity in T1D and in correlation with the presence of retinopathy, nephropathy and neuropathy (Monnier et al., 1986).

Some authors have showed that there is a clear link between upper limb musculoskeletal abnormalities and poor metabolic control (Ramchurn et al., 2009). It has been observed a reduction in frequency of LJM between the mid-70s and mid-90s in children, most likely due to the improved glucose control during this era (Infante et al., 2001; Lindsay et al., 2005).

3.7 Type 1 diabetes and oedema

Insulin oedema is a well-recognized and extremely rare complication of insulin therapy. It was found to occur equally in both sexes in adults, but a clear female predominance was noted in younger ages. The condition is self-limiting, but a progression to overt cardiac failure and development of pleural effusion has been reported. (Chelliah & Burge, 2004).

The pathophysiology remains vague. Intensive fluid resuscitation in an insulin-deficient catabolic state may lead to extravasation of fluid to the subcutaneous tissue, resulting in peripheral oedema. This may be exacerbated by the increased capillary permeability associated with chronic hyperglycemia. Renal tubular sodium reabsorption is enhanced by insulin therapy via stimulating the Na+/K+-ATPase as well as the expression of Na+/H+ exchanger 3 in the proximal tubule. Transient inappropriate hyperaldosteronism has also been suggested to contribute to the fluid retention (Bas et al., 2010). Loss of albumin from the circulation due to increased transcapillary leakage probably contributed to the formation of oedema and the decreased serum albumin, but was not severe enough to account for the magnitude of oedema (Wheatly & Edwards 1985). Cases with normal serum albumin have also been reported.

Clinically, insulin oedema may present with a spectrum of severity until to frank anasarca. Pleural effusions have uncommonly been reported, although some of these patients were elderly and may have had pre-existing cardiac disease. Rarely, the oedema extended from peripheral tissues to serosal cavities with ascites and cardiac failure (Bas et al., 2010). Fluid and salt restriction should be implemented and this may be all that is necessary. Diuretic

therapy may be indicated in more severe decompensated cases. Administration of an aldosterone antagonist such as spironolactone may be considered from a pathophysiological point of view in the presence of inappropriate hyperaldosteronism (Kalambokis et al., 2004). In most instances no specific therapy is needed and spontaneous recovery is noted.

Impaired growth	Poor metabolic control	Monitoring of growth and physical development using growth charts
Eating disorders	Dietary restriction	Ameliorating of nutritional assistance
Necrobiosis lipoidica diabeticorum	Parallel dermopathy	Routine clinical examination of the skin
Osteopenia	Probably even present, but worsened by poor metabolic control/comorbidity	Eventually controlled by Bone ultrasonography/ DEXA
Gastropathy	Poor metabolic control	Investigating of dyspeptic symptoms
Limited joint mobility	Parallel condition	Routine clinical examination of the joint mobility
Oedema	Unknown	Clinical examination

Table 3. Non autoimmune associated conditions to Type 1 diabetes, causes and detection

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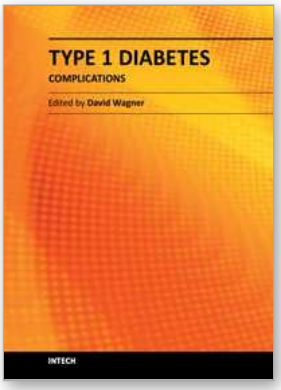

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This book is a compilation of reviews about the complication of Type 1 Diabetes. T1D is a classic autoimmune disease. Genetic factors are clearly determinant but cannot explain the rapid, even overwhelming expanse of this disease. Understanding etiology and pathogenesis of this disease is essential. The complications associated with T1D cover a range of clinical obstacles. A number of experts in the field have covered a range of topics for consideration that are applicable to researcher and clinician alike. This book provides apt descriptions of cutting edge technologies and applications in the ever going search for treatments and cure for diabetes.

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