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Chapter 8

Environmental Factors and Type 1 Diabetes Mellitus in Pediatric Age Group

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1. Introduction

Type 1 diabetes mellitus (T1DM) is the most common endocrinopathy in pediatric age group, due to an autoimmune process characterized by a selective destruction of insulin producing pancreatic β-cells progressing over different stages [1]. T1DM develops in genetically susceptible subjects by activation of so far uncharacterized environmental factors that trigger an inflammatory process with infiltration of pancreatic islets and subsequent loss of β-cells. Despite the growing incidence of T1DM, the causative mechanisms are not completely defined up to now, and the identification of factors triggering the immune process represents a challenge for clinical immunologists, with practical, diagnostic and therapeutic implications [2,3]. The clinical onset of T1DM is preceded by an asymptomatic period characterized on pathology grounds by insulitis, i.e. an infiltration of the pancreatic islet of Langerhans by CD4+, CD8+ T lymphocytes (both Th1 and Th2 subsets), B lymphocytes, macrophages and dendritic cells. T lymphocytes can differentiate into 2 major subsets: Th1, producing IL-2 and IFN-γ, and Th2, secreting mainly IL-4. All these cells produce cytokines which can be directly cytotoxic to β-cells or play an indirect role on β-cell destruction influencing some cells of the immune system, then resulting in either acceleration or arrest of the immune attack [4]. Worldwide T1DM incidence has grown more than two to three fold during the last decades, particularly in Finland, where T1DM incidence has increased from 12 to 63 cases per 100,00 [5]. A raising incidence has also been reported in Italy, where Sardinia Region shows an incidence rate similar to Finland, therefore is called "Hot Spot" [6,7]. Interestingly, this rise of incidence was not followed by a parallel increased frequency of the major risk genes [8]. T1DM can be defined as a polygenic disease, and the genes mainly involved include

Major Histocompatibility Complex (MHC) class II (DR and DQ) on chromosome 6, responsible for 40% of genetic risk, and insulin gene located on chromosome 11. Moreover thanks to whole genome screening techniques more than 15 loci have been identified. In particular, an allele of the gene for a negative regulator of T-cell activation, i.e. Cytotoxic T Lymphocyte Antigen 4 (CTLA-4), on chromosome 2q33, and a variant of PTNP22 gene encoding LYP (a suppressor of T cell activation) and ILrRA gene are considered as other important susceptibility loci [8]. Recently, the prevalence of MHC class II genes seems to be decreasing [9]. Moreover, studies in identical twins showed a concordance rate ranging from 27 to 61%, otherwise lower in non-identical twins (3.8-12%) [10]. Despite the growing incidence of T1DM, the causative mechanisms are not completely defined up to now. The paradigm of autoimmune dysregulation has not offered a clear explanation for its raising incidence.

The reported discrepancy between higher incidence of T1DM without concomitant shift in the frequency of susceptibility genes, suggests that environmental factors play a key role in the development of the autoimmune process leading to clinical onset of the disease [11]. Moreover the shift to younger age at T1DM clinical onset is caused by environmental risk factors accelerating the on-going β-cell destructive process up to clinical disease even in children with lower levels of genetic risk otherwise exposed to such factor [12-14].

The high T1DM incidence is a phenomenon of the 20th century, even if the disease has been described already in antiquity. This increasing incidence and its difference among neighboring regions strengthens the role of multiple environmental factors in the pathogenesis of T1DM. In the present chapter the main environmental factors involved in T1DM pathogenesis according to the most relevant scientific evidence will be considered. The main topics are: perinatal and socioeconomic factors, hygiene hypothesis, dietary components both in mother and in children, gut permeability, infectious agents, vaccinations, obesity and Accelerator Hypothesis, epigenetic.

2. Perinatal factors

Environmental risk factors combined with genetic susceptibility are thought to contribute to the development of autoimmune destruction of pancreatic β-cells. The rapid increasing incidence of T1DM, especially in the youngest age group [15], cannot be explained by genetic factors. It has been postulated that gestational or perinatal events could trigger T1DM.

2.1. Infections

It has been reported that certain infections during pregnancy contribute to an increased risk of T1DM in the offspring. The first report of a link between infection and diabetes was the exposure to rubella in intrauterine life. Studies showed that about 20% of children born with congenital rubella develop T1DM during infancy [16,17]. Other reports describe an increased risk of T1DM if the mother has had an enterovirus infection during pregnancy [18,19]. Anyway these studies are not confirmed by all investigators [20,21] and whether en-

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terovirus infection during the first trimester of pregnancy is associated with increased risk for T1DM in the offspring remains controversial up to now [21]. Not only congenital infections are associated with the risk of T1DM, but also perinatal infections are discussed as protective factors or triggers of the disease [22]. Certain studies reported that two infections in the first year of life seem to be protective against T1DM, while neonatal respiratory diseases are associated with a increased risk of disease [22].

2.2. In utero and postnatal dietary exposure

To explain the growing incidence in T1DM within the first year of life, it has been hypothesized that certain dietary nutrients could be protective for islet autoimmunity. Maternal intake of vitamin D is significantly associated with a decreased risk of islet autoimmunity in offspring, independent from HLA genotype, family history of T1DM, presence of gestational diabetes mellitus and ethnicity (adjusted HR=0.37; 95% CI 0.17-0.78). Instead, vitamin D intake via supplements, ω-3 fatty acid and ω-6 fatty acids intake during pregnancy are not associated with appearance of islet autoimmunity in offspring [23]. There is also an increased interest in nutritional factors in the first months of life as risk factors for T1DM. Some authors reported that children exposed to cereals between 0 and 3 months of life were more likely to develop islet cell auto-antibodies compared to those who were exposed during the fourth through sixth month [24]. Another study showed that ingestion of gluten-containing foods before 3 months of age was associated with increased islet cell autoimmunity compared to children who received only breast milk until 3 months of life. Then other studies showed that a high intake of cow's milk could have a protective effect [25]. On the other hand, some authors claim that milk protein carries an increased risk of T1DM [26,27]. It is also been reported a correlation between a high intake of nitrosamines, nitrites and nitrates and T1DM [25,28][Table 1].

Protective effect	Vitamin D intake	HR*	CI**
		0.49;95%	(0.17-0.78)
Increased risk	Inadequate prenatal care	0.53;95%	(0.40-0.71)
	Medicaid insurance	0.67;95%	(0.58-0.77)
	Unmarried mother	0.79;95%	(0.69-0.91)
	Mother's age ≥ 25 yrs	1.28;95%	(1.13-1.45)
	Mother's BMI ≥ 30 kg/m^2	1.29;95%	(1.01-1.64)
	Mother's age ≥ 35 yrs	1,32;95%	(1.01-1.64)

HR*: Hazard Ratio; CI**: Confidence Interval

Table 1. Maternal factors and T1DM risk.

2.3. Birth-weight

An association between birth weight and risk for T1DM has been postulated. A meta-analysis study of 12.807 cases of T1DM found an increased risk in children heavier at birth: children with birth weight from 3,5 to 4 Kg showed an increased risk of 6% (OR 1.06; 95% CI 1.01-1.11) (p=0.02) and children with birth weight over 4 Kg have an increased risk of 10% (OR 1.10; 95% CI 1.04-1.19) (p=0.003), compared to children weighing 3 to 3,5 Kg at birth [29]. Several studies support this link [30], while others did not find any association with T1DM [31].

2.4. Caesarean section

Another controversial question is the role of caesarean section. A meta-analysis study of 9.938 cases reported a 20% increase in the risk of childhood-onset T1DM (adjusted OR 1.19, 95% CI 1.04-1.36, p=0.01) [32], while other authors did not find any association between caesarean delivery and risk for T1DM [33].

2.5. Other perinatal factors

It is also been investigated the association between blood incompatibility and risk for T1DM: ABO incompatibility was related to an increased risk for the disease in some studies [34], while others found an association just only with Rhesus immunization [33].

A report have shown that also neonatal jaundice of unknown cause confers an increased risk for T1DM [34].

Another topic discussed is about the stress events. Some authors found an increased risk of T1DM in children diagnosed between 5 to 9 years of age who experienced stress events [25], while others showed that stressful events during the first two years of life increased the risk of the disease, probably by affecting the autoimmune pathogenetic process [35]. Finally, some investigators have reported a decreased risk for T1DM in children of prenatal smokers [36,37].

3. Social factors

Other factors such as maternal age may contribute to increase the risk for T1DM. It is been observed an increased incidence of disease in children born to older mother [25,34,38,39]. These data are confirmed by a population-based case-control study in Washington State on children younger than 19 years from 1987 to 2005, an increased OR in children of mothers older than 25 years (age 25-34 HR=1.28; 95% CI 1.13-1.45; age≥35 HR=1.32; 95% CI 1.10-1.58) has been reported [31]. Risk for T1DM is also been related with maternal weight: mother with a BMI of 30 Kg/m^2 or higher had an increased ORs for the disease (BMI≥30: OR 1.29; CI 1.01-1.64). Pregnancy-related factors also include birth order: the first-born child has the highest risk for T1DM and the risk decreases with number of children born [38,40]. Several

studies have found an inverse association between increasing number of siblings and risk of T1DM [31,37,41,42]. An inverse correlation has also been observed with lower economic status or care access, such as unmarried mother (OR 0.79; 95% CI 0.69-0.91), inadequate prenatal care (OR 0.53; 95% CI 0.40-0.71), or Medicaid Insurance (OR 0.67; 95% CI 0.58-0.77) [31]. Another widely discussed topic is tobacco exposure, as influencing immune system, and represents a risk factors for T1DM. It's been questioned if the decrease of passive smoking in children may be a predisposing factor for the increasing incidence of T1DM, in according with the hygiene hypothesis. To clarify this aspect, ABIS, a population-based prospective long term cohort study, revealed no difference in prevalence of immunological markers (GAD and IA-2 antibodies) between tobacco smoke-exposed and non-exposed children [43].

3.1. Hygiene hypothesis

Recently, attention has been focused on lifestyle changes as a major factor in the rise of T1DM frequency, as well as other immune or allergic diseases [44]. Improved hygiene and living conditions decreased the frequency of childhood infections, leading to a modulation of the developing immune system and increasing risk for autoimmune and allergic diseases such as T1DM and asthma [45]. This theory, called "Hygiene Hypothesis", finds its roots in the 1870 when Charles Harris Blackley noticed that aristocrats and city dwellers were more likely to get hay fever than farmers [46]. One century later, in 1966, Leibowitz and colleagues noted that in Israel the incidence of multiple sclerosis (MS) was positively related to levels of sanitation [47]. More recently, Correale et al. showed that patients with multiple sclerosis who become infected with helminths have a strikingly reduced rate of disease progression [48]. However, the term "Hygiene Hypothesis" was proposed in 1989 by Strachan, who noted that hay fever was less frequent in families with many siblings [49].

In accordance to hygiene hypothesis, several studies report the lowest incidence of T1DM in areas with poorest hygiene condition [50,31]. These data are supported by the experiments in non-obese diabetic (NOD) mice (mice that spontaneously develop a condition resembling T1DM) and in BB rats, in which caesarean delivery and isolated living conditions increased the incidence of diabetes from 40% to 80%. In humans, several studies reported a significant inverse correlation between the incidence of T1DM and certain socioeconomic index (unemployment, lack of a car, crowded housing conditions, and living in rental housing rather than purchased property) [50,51]. In the people living in Washington state from 1987 to 2005, D'Angeli and colleagues found a negative association between T1DM and some indicators of lower economic status or care access, such as an unmarried mother (OR 0.79%; 95% CI 0.69-0.91), inadequate prenatal care (OR 0.53%; 95% CI 0.40-0.71), or Medical insurance (OR, 0.67; 95% CI 0.58-0.77) [31]. Young children with older brothers and sisters and sharing the bedroom, as well as those who attended a day-care centre during the first six months of life showed a lower incidence of T1DM later in life than children who did not attend a day-care centre and who had no older siblings [52].

3.1.1. Hygiene hypothesis and autoimmunity

A topic discussion of our day is whether the reduced exposure to certain infections, as result of improving socioeconomic conditions, may be responsible for the increased incidence in diabetes and other autoimmune conditions such as systemic lupus erythematosous and multiple sclerosis [45,53-55]. As regards the rise in the disease in Western Europe and the USA during the twentieth century strikingly correlates with the decline of helminths infections, particularly E. vermicularis [56]. Experimental studies showed in Non-Obese Diabetic (NOD) mice, infected with mycobacterium or helminthes, a reduced frequency of T1DM [54,57-58]. Moreover, infection of 4-5 week-old NOD mice with Schistosoma mansoni or injection of soluble eggs (SEA) seems to prevent diabetes clinical onset. One possible explanation is that helminths antigens are able to induce either IL-10 production by dendritic cells and activation of Natural Killer T cells (NKTs) and Regulatory T cells (TRegs). Considering the role of IL-10 in delaying or inhibiting the host immune response and limiting tissue pathology [59-61], exogenous administration of IL-10 inhibits the development of diabetes in NOD mice [62]. Moreover, some bacterial infections can inhibit diabetes development in NOD mice. In mice infected with S. typhimurium the protective mechanism could be the key role of dendritic cells in modulating the trafficking of diabetogenic T cells to the pancreas [63]. Another way by which bacteria and viruses could protect against autoimmune disorders is related to Toll-Like Receptor (TLRs). In fact, when TLRs bind bacterial ligands, stimulate mononuclear cells to produce several cytokines, which down-regulate the autoimmune response. Wen and colleagues showed that Specific-Pathogen Free (SPF) NOD mice are protected from the disease when knocked-out from the MyD88 gene, encoding an adaptor for multiple TLRs [64]. Modification of the immune system in knocked out MyD88 seriously impairs the interactions between the immune system and microbiota. Due to these positive results after treatments with a mycobacterium extract [65], helminthiases treatment and probiotics [66,67] in patients with atopic dermatitis and multiple sclerosis, have recently been reported [68,69]. Instead, vaccination with bacille Calmette-Guèrin produced negative results in patient with T1DM [70,71].

Nowadays a topic discussion is about the role of gut bacteria in the control of autoimmune diseases. In fact changes in the composition of the gut flora influence the development of autoimmune and allergic diseases. It has been observed that the use of lactobacilli, derived from the gut, decreases the incidence of diabetes in NOD mice [72]. More recently, Takiishi et al. showed that treatment of NOD mice with Lactococcus lactis, a common and food-grade commensally bacterium genetically modified, which is able to secrete IL-10 and human pro-insulin auto-antigen, can revert autoimmune diabetes in newly diagnosed NOD mice, by increasing frequency of TRegs [73]. Dan Litman's group showed that a single commensally bacteria, i.e. segmented filamentous bacteria (SFB), is able to drive the appearance of CD4+ T helper cells producing interleukin 17 (IL-17) and IL-22 (Th17 cells) in the lamina propria, thereby influencing the microbiota equilibrium [74]. On the other hand, colonization of germ-free mice with a defined intestinal flora resulted in Treg generation, expansion and activation in the lamina propria [75]. Based on these encouraging results in animal models, the use of probiotics to delay or prevent T1DM in humans has become an area of inter-

http://dx.doi.org/10.5772/53267

est. The PRODIA study, currently ongoing in Finland, is investigating whether, the use of probiotics during the first 6 months of life decreases the clinical onset of T1DM in children with genetic susceptibility [76].

4. Dietary components

4.1. Feeding and risk of T1DM

The T1DM is a chronic disease characterized by a preclinical phase in which environmental exposure, such as food, can contribute to the development of the autoimmune process of pancreatic β-cells destruction. Recent studies have focused upon the role of breastfeeding, introduction of cow's milk, wheat/cereals/gluten, vitamin D and E, ω-3 fatty acids [77]. Some studies suggest that already during pregnancy, low maternal consumption of vegetables may influence the future of the unborn [78,79].

4.2. The influence of breastfeeding

The influence of breastfeeding on the development of diabetes remains a controversial issue; for some it seems to have a protective role, for others, a predisposing role, for others no effect [80]. Gerstein conducted in 1993 a meta-analysis of retrospective case-control studies showing that breast-feeding for short periods (<3 months) is associated with the development of T1DM, with an odds ratio (OR) of 1.43 [81]. A Finnish study has shown that early introduction of cow milk-based formula was associated with an increased risk of β-cell autoimmunity in genetically predisposed children, but the duration of breastfeeding was not associated with an increased risk of autoimmunity in children with first-degree relatives with T1DM in Germany, Australia and USA. The risk of diabetes seems to be higher in patients with first-degree relatives with T1DM, and this risk is increased in carriers of HLA genotype [82-84]. The positive correlation between short duration of breastfeeding and the development of diabetes has been studied in non-diabetic children at the age of 5 years, evaluating the presence of circulating antibodies predictive of the disease [Auto-Antibodies to Insulin (IAA), Glutamic Acid Decarboxylase Antibodies (GADA) and Protein Tyrosine Phosphatase-like (IA-2A)]. This study demonstrates the long-term increased risk of developing T1DM with the early introduction of formula milk. A protective role of breast milk which, for the presence of cytokines and growth factors, promote the maturation of the intestinal mucosa and the development of the immune system has been suggested [85]. Conflicting results can be explained by observing the many differences in feeding practices between the different countries. There is variation between countries and cultures in the proportion of babies first introduced to milk-based formula and there are differences in the kind of complementary food that infants who are not first exposed to milk-based formula [85].

4.3. Introduction of gluten

It has been known as T1DM is connected with other autoimmune diseases, such as thyroiditis or celiac disease. Two prospective studies in USA and Germany showed a high risk for

the development of β-cells auto-immunity when gluten's introduction happens before the fourth month rather than after the seventh; moreover this risk is similar when gluten ingestion starts before the third month [24,86]. Several studies were aimed to explain the etiology of this phenomenon. Simpson et al. compared the levels of antibodies to a wheat storage globulin homologue of Glo-3A, which is a non-gluten component of the wheat protein matrix. They have shown that in children with islet auto-immunity, the antibody titer was directly linked to the early introduction of gluten, and inversely to breastfeeding duration [87]. Not all authors agree with this association; a prospective analysis from the DIPP study did not show a correlation between early or late introduction of gluten and subsequent development of pancreatic β-cells autoimmunity [88]. Mojibian et al. hypothesized that the passage of gliadin (a polypeptide of the wheat) through the intestinal epithelial barrier may trigger an inflammatory response, and then an autoimmune disease, in genetically predisposed individuals. The passage of protein molecules is facilitated by inflammation produced by intestinal infections. The location of an uncovering receptor for Coxsackie and Adenovirus at the level of tight junctions may explain the development of T1DM. The bowel inflammation and T-cells activation by gluten could activate and potentiate β-cell auto-immunity, like viral infections [89]. Recently a study in NOD mice demonstrated that there is a statistically significant protection from diabetes in mice that received gluten-free diet [90].

4.4. Vitamin D and E

Some studies have shown an increased risk of developing diabetes in children with low intake of vitamin D. An European case-control study has quantified the reduction in risk with an OR of 0.67 (95% CI 0.53-0.86) in children supplemented with vitamin D [Table 2]. Also a Finnish study showed a protective role of vitamin D, with an OR equal to 0.12 (95% CI 0.03-0.51), comparing children who received regular doses of 2000 IU/day rather than 400 IU/day, and an OR of 3 (95% CI 1.0-9.0) comparing children with an irregular supplementation rather no supplementation with vitamin D [91,92]. Simpson et al. followed from 1993 to 2011 2,664 children at increased risk of T1DM, monitoring the intake of vitamin D and blood levels of 25(OH)D. They have shown that vitamin supplementation is not associated with an increased protection from autoimmune phenomena [93]. Vitamin D deficiency predisposes individuals to type 1 and type 2 diabetes, and receptors for its activated form 1α25-dihydroxyvitamin D3 have been identified in β-cells and immune cells. In some populations, T1DM is associated with certain polymorphisms within the vitamin D receptor gene. In studies in non-obese diabetic mice, pharmacological doses of 1α25-dihydroxyvitamin D3, or its structural analogues, have been shown to delay the onset of diabetes, mainly through immune modulation [94]. Human studies reported that vitamin D is able to modulate the immune response by suppressing pro-inflammatory cytokines and promoting the secretion of anti-inflammatory ones [23]. Therefore it seems appropriate the supplementation with vitamin D in countries with an increased risk of deficiency, especially if T1DM incidence is high. Other authors emphasized the important role of vitamin E for its antioxidant function; Vitamin E ameliorates oxidative stress in T1DM patients and improves antioxidant defense system [95].

European study	Vitamin D intake	HR	CI
		0.67;95%	(0.53-0.86)
Finnish study	Vitamin D intake (2000UI/d)	0.12;95%	(0.03-0.90)
	Vitamin D intake (400UI/d)	3.00;95%	(1.0-9.0)

Table 2. Child's diet and T1DM risk: protective effect with Vitamin D supplementation

4.5. ω-3 fatty acids and other factors

An observational study in children at high risk of T1DM reported that ω-3 fatty acid intake is not associated with progression to overt disease; however the protective influence of ω-3 fatty acids remains controversial. On the other hand, ω-6 fatty acids seem to exert an opposite role. It has been argued that use of cod liver oil in the first year of life reduces the risk of the disease. The case-control study DAISY [Diabetes AutoImmunity Study in the Young] demonstrates that use of ω-3 fatty acids, between 1 and 6 years, exerts a risk reduction with an hazard ratio of 0.45 [96,97]. The immunomodulatory role of ω-3 fatty acids is quite similar to the role exerted by Vitamin D. Conversely, ω-6 fatty acids like arachidonic acid promote the pro-inflammatory cytokine prostaglandin E_2 with subsequent development of β-cell autoimmunity in genetically predisposed subjects [23]. Recently, an interesting case-control study of 298 Italian children aged 0-15 years (145 affected by T1DM) showed a significant association, dose-response, between frequency of T1DM and meat consumption. The association proposed by Benson et al. between T1DM and daily consumption of water containing nitrates, nitrites and nitrosamines is intriguing [98,99].

5. Gut permeability

In the recent years a topic discussion is about the link between T1DM and gut. The role of gut as a regulator of T1DM was first suggested in animal studies. Changes affecting the gut immune system modulated the incidence of diabetes. In particular structural changes, such as a decreased expression of tight junctions (TJ) proteins claudin-1 and occludin, together with increased gut permeability were noted in the intestinal morphology of Bio-Breeding (BB) rats, compared with Wistar rats [100,101]. These data are supported by the observations that early onset of autoimmune diabetes in BB-rats was associated with high gut permeability [102] and in NOD-mouse increased intestinal permeability precedes the clinical onset of T1DM [103]. In humans, studies showed that gut permeability, measured by the lactulose-mannitol test, is increased in T1DM patients [104,105] and can precede clinical onset [106]. These results are supported by the discovery of high serum zonulin concentrations, a novel member of tight-junction protein that correlates with increased ratios in sugar permeability testing, in patients with T1DM [105] and in subjects at risk of T1DM i.e. β-cell autoantibody-positive individuals [106]. Based on these findings, Wats et al. showed that the administration of zonulin antagonist reduced the cumulative incidence of T1DM in diabetic-prone rats [107]. It has also been hypothesized that changes in the normal flora may contribute to the

development of T1DM by affecting intestinal permeability. Duodenal administration of Lactobacillus plantarum increased the expression of epithelial TJ proteins occluding and Zo-1 in the biopsies obtained by human volunteers [108]. Moreover, antibiotic treatment that impairs intestinal bacteria, protects from autoimmune diabetes in BB-rat model [109]. In DP-rats (Diabetes-prone rats), the onset of T1DM could be delayed by the administration after weaning of Lactobacillus johnsonii, isolated from DR-rats (Diabetes Resistant Bio-Breed rats) [110]. The composition of intestinal microbiota may not only affect permeability but may also have immune-modulating effects. Recent studies suggest for intestinal microbiota an important regulator role of Th17 immunity in the gut [74]. It has been reported that Lactobacillus johnsonii enhances Th17 differentiation of T cells upon TCR stimulation [112]. The up-regulation of IL-17 immunity in the mucosal surface has been shown to activate an antimicrobial response together with mucosal repair mechanisms and support of the gut barrier [111]. Also virus, such as rotavirus and enterovirus act as promoters of the diabetogenic gut environment with high intestinal permeability, enhanced immune activation, and via the gut-pancreas link, causing activation of β-cell autoimmunity in pancreatic lymph nodes [112]. It is also discussed the role of antiviral cytokines that damage barrier function [113] or the direct effect of virus, as suggested for Rotavirus and Coxsackie viruses [114,115]. The increased gut permeability in T1DM patients may be due to the uptake of dietary antigens causing improper immune activation and intestinal inflammation. Studies suggested that early exposure to dietary wheat may trigger β-cell auto-immunity in children at genetic risk [24,86]. In vitro, gliadin-stimulation of small intestinal biopsies taken from patients with T1DM, caused increase in T-cells and their activation markers, i.e. CD-25 and ICAM-1, promoting intestinal inflammation [116]. Gliadin may also induce an increase in intestinal permeability and zonulin released by binding to the chemokine receptor CXCR3 expressed by epithelial cells and T cells [117]. It has been noted that dietary prevention of diabetes in NOD-mice with a gluten-free diet was associated with a decrease in the number of ceacal bacteria [118]. In humans, epidemiological studies suggest that the short breastfeeding time and early feeding of cow milk (CM) proteins in the infancy increase the risk of diabetes [119]. This may be due to the lack of breastfeeding role of support epithelial and immunological maturation of gut, such as the gut closure [120] and the IgA system [121]. It has been hypothesized that CM may contain diabetogenic factors, such as immunogenic bovine insulin, that could trigger insulin-specific immunity in the gut and, in the context of impaired oral tolerance, contribute to expansion of this immune response against β cells [122]. Weaning to a hydrolyzed casein formula decreased the gut permeability [102] and led to lower expression of IFN-γ [123] in islet infiltrating lymphocytes of BB-rats, resulting in a 50% reduction in the development of autoimmune diabetes [102]. In humans, recent results of the TRIGR pilot study, have showed that weaning to hydrolyzed casein decreased the risk of β-cell autoimmunity by 40% in the infants at genetic risk [124]. In the FINDIA pilot study, the use of bovine-insulin-free whey-based formula, during the first 6 months of life, decreased the appearance of β-cell auto-antibodies by 3 age [125].

http://dx.doi.org/10.5772/53267

6. Infections

6.1. Background

Several studies in humans and animal models have supported the hypothesis that infectious agents, in particular some viruses, can be considered as one among the environmental agents able to elicit or enhance the autoimmune response characterizing T1DM [44]. On the other hand viral infections could exert a protective role against auto-immunity [126]. This opposite scenario might be explained by the type of infecting virus, the immune status of the host and the timing of infection [127]. A possible explanation could be the significant changes in human living standards (i.e. sewage treatment, availability of microbiologically pure water) during the last century, followed by reduced repeated exposure to fecal-oral transmitted agents particularly early in life.

The major obstacle in clinical research is represented by the limited availability human samples. In fact the pancreas is very difficult to access, and routine biopsy aimed to study the role of viruses in the target organ cannot be proposed, since the majority of newly-diagnosed patients are children.

However five lines of evidence link virus to T1DM [128]:

1. Some viruses are able to destroy β-cells and cause mononuclear infiltration
2. Experimental animal models report development of T1DM in mice infected with different strains of Picornaviruses
3. Some viral infections in humans have been followed by T1DM (i.e. congenital rubella)
4. Direct isolation of viruses from humans or animals with T1DM has been documented
5. Virus DNA or RNA are able to initiate antiviral immune response which cross-reacts with insulin or other components within or on the surface of β-cells.

6.2. Viruses and β-cells

Viruses can directly damage β-cells or induce a strong cellular immune response leading to progressive lack of insulin and development of clinical signs and symptoms of the disease. Besides direct cytotoxic effect, other mechanisms involved in β-cell destruction are molecular mimicry and bystander activation [129].

The hypothesis that viral infections are capable of triggering islet auto-reactivity has been proven by several evidences both in humans and in animal models. The host immune response to viruses consists of the secretion of interferon-γ, acting as initiator of inflammation. In the pancreas interferon-γ up-regulates MHC class I molecules on β-cells, making them vulnerable to autoimmune attack [130]. Up-regulation of MHC class I molecules is followed by lymphocytic infiltration in β-cells, as reported also in humans [131]. Moreover viral particles or even isolate live virus have been detected in pancreas from patients deceased at clinical onset of T1DM.

Another evidence strengthening the association between viruses and T1DM is the identification of 4 protective genetic variations of IFIH1 gene, responsible for interferon production after viral infection [132]. Individuals with IFIH1 predisposing alleles have higher IFIH1 levels, while individuals with protective alleles have lower IFIH1 levels. After a HEV infections, the predisposed group showed increased stimulating capacity of dendritic cell, with production of pro-inflammatory cytokines and development of T1DM. The opposite scenario has been reported in the protected group.

The key role of viruses as trigger of autoimmune response may result from molecular similarities between viral antigens and host cell auto-antigens, otherwise defined as "Molecular Mimicry". These similarities are responsible for a break of the immune tolerance to endogenous auto-antigens. In particular, analogies between an epitope of Coxsackie B virus (P2-C 35-43) and an epitope of GAD 65 auto-antigen (GAD 65 258-266) has been reported also in humans [133]. Molecular mimicry is able to enhance or accelerate autoimmune process, however it does not start auto-immunity.

Another link between viruses and auto-immunity is the so called "Bystander Activation". Pre-existing auto-reactive T-cell precursors, activated by viral infections, become auto-aggressive and induce the autoimmune response. Bystander activation has been reported in animal model infected by Coxsackie B4 virus who later develop T1DM [134]. Molecular mimicry and bystander activation are not mutually exclusive.

The direct viral infection and lysis of β-cells has been reported in the so-called "Fulminant Diabetes" (FD). FD accounts for about 20% of diabetes mellitus in Japan and is characterized by extremely rapid and severe destruction of pancreatic β-cells in absence of insulitis, but with high titers of anti-enterovirus IgA, compatible with recurrent HEV infections [135].

Several viruses have been linked to T1DM, i.e Coxsackie, Mumps, Rubella, Cytomegalovirus, Retroviruses and Rotaviruses [136-139], otherwise several evidences link enteroviruses, in particular Coxsackie B4 virus to T1DM [140].

6.3. Coxsackie viruses and T1DM

Human EnteroViruses (HEV) [141] are small, non-enveloped viruses (30 nm), characterized by an icosahedric capsid consisting of 60 capsomers; one capsomer comprises 4 structural proteins (VP1, VP2, VP3, VP4). HEV belong to the Picornaviridiae family and 5 different species are recognized: Poliovirus and HEV A, B, C, D. Enteroviruses are ubiquitous and transmitted by faecal-oral route, and characterized by a great genetic variability and consequent broad spectrum of tissue tropism and pathological effects. HEV infections are usually asymptomatic or characterized by fever, malaise, sometimes respiratory involvement or cutaneous Rash. More severe diseases such as meningitis, encephalitis and pericarditis have been reported.

Six different serotypes characterize Coxsackie virus B (CVB 1-6); the B4 serotype is defined "diabetogenic" [142]. Affected patients harbor enterovirus RNA homologous to that of Coxsackie B4 in peripheral blood mononuclear cells [143], and in small intestine samples, suggesting a persistent enterovirus infection [144].

http://dx.doi.org/10.5772/53267

Recently direct evidence of Coxsackie B4 enterovirus infection in human β-cells with reduced insulin secretion and islet inflammation mediated by natural killer cells has been provided [145-147].

6.4. Viruses: foes or friends?

It has been reported a protective role of viral infection in the development of T1DM. Studies in animal models report a protective effect of enterovirus infections when contracted precociously, before weaning, which disappears if the infection occurs thereafter [148]. A virus with protective effect exerts a inflammatory profile very different if compared to diabetogenic one, with opposite consequences on autoimmune reaction. The kind of virus, its β-cell affinity, and the timing of infection play a crucial role in T1DM occurrence. In fact proliferation virus-induced auto-reactive T cells after recurrent infections with protective viruses determine protection from β-cell autoimmune destruction with deviation of the auto-inflammatory response, a trafficking of auto-reactive T cells and a stimulation of Treg cells [127].

7. Vaccines and risk of T1DM

The role of vaccine in the development of T1DM has been matter of debate. In fact there is a temporal association between increased incidence of the disease after improvement of living conditions and reduction of infectious diseases in childhood, thanks to the widespread use of vaccines. Moreover, some vaccines prevent or induce T1DM in animal models. Furthermore, it has been postulated that only early vaccinations (i.e. within the first month of life) could prevent T1DM [149]. The same author reported a clusters of cases of T1DM 2-4 years post-immunization with pertussis, MMR, and BCG vaccine, but it remains to define the link between the haemophilus-vaccine and T1DM [150]. On the other hand, a large epidemiological study on all children born in Denmark from 1990 and 2000, for whom correct information about vaccine schedule and clinical diagnosis of T1DM 2 to 4 years after vaccination, revealed no significant association between vaccines and development of T1DM. Moreover, no evidence of any clustering of cases after vaccination with any kind of vaccine [151]. This nationwide cohort, together to the prospective and independent ascertainment of vaccination history and the time of T1DM diagnosis overcame the risk of selection bias and recall bias [151]. De Stefano et al., in a case-control study, didn't support an association between any of the recommended childhood vaccines and increased risk of T1DM [152]. Similar results have been reported in a retrospective cohort study in active components of US Military between 2002-2008 [153]. Another retrospective cohort study in Sweden examining the risk of autoimmune and neurological disorders in people vaccinated against pandemic influenza A demonstrated no changes in the frequency of several autoimmune diseases, including T1DM [154].

The possibility that vaccination may increase the risk of T1DM has been evaluated in a few epidemiologic studies. Classen has provided the only evidence of a possible increased risk,

but the nature of the evidence is strictly ecological, involving comparisons between countries or between different time periods in the same country. Such comparisons, however, may be influenced by many factors unrelated to vaccination, i.e. genetic predisposition. Moreover, similar ecological analyses did not found significant correlations between diabetes and BCG, pertussis, and mumps vaccine.

Recently, in Japan a case of fulminant T1DM has been reported after influenza vaccination [155]. On the other hand the absence of autoimmunity in this form of diabetes is recognized. The role of vaccinations in T1DM deserves attention. Even if vaccinations are not triggers of autoimmune process leading to overt diabetes, it is otherwise possible that in genetically predisposed subjects vaccine exposure could anticipate the clinical symptoms and therefore being associated to T1DM.

8. Obesity as environmental factor

In the past decades a worldwide rising incidence of the disease has been reported [157], with a significant trend toward earlier age at diagnosis than previously observed [158]. This shift to a younger age at T1DM diagnosis could be explained by exposure to higher doses of several environmental factors, like viral infections, polluted air, and more recently, sedentary lifestyle [159-160]. In particular, physical inactivity results in obesity, whose incidence within pediatric age is dramatically rising [156,160,161]. In younger children obesity-induced insulin resistance exerts in metabolic β-cells up-regulation, accelerating their loss through glucotoxicity, and can potentially bring forward the earlier age of diabetes clinical onset, according to the so-called Accelerator Hypothesis [162].

8.1. Accelerator hypothesis

The Accelerator Hypothesis, firstly postulated by Wilkins, argues that diabetes mellitus is a unique disorder of insulin resistance set against different genetic backgrounds, rather than two distinct diseases (type 1 and type 2), and focuses on the tempo of β-cell loss [162]. Therefore the concept of tempo might explain the commonality between type 1 and type 2 diabetes, which are distinguished only by the rate of β-cell loss and by the specific accelerator involved [163]. Three main accelerators play a pathogenetic role: the first is the intrinsic potential for β-cell apoptosis, a necessary but insufficient step in the development of diabetes. The second accelerator is insulin resistance secondary to obesity, and represents the link between type 1 and type 2 diabetes. Insulin resistance increases insulin secretory demands on β-cells and may trigger damage in these metabolically up-regulated cells by increasing antigen presentation. Insulin resistance is characterized by a decreased ability of insulin to stimulate the use of glucose by the muscle and adipose tissue, where the suppression of lipase controlled by insulin is impaired [164]. The consequent excessive supply of free fatty acids further affects glucose transportation in the skeletal muscles, and inhibits insulin activity [165]. In the liver, insulin resistance leads to increased hepatic glucose production, initially compensated by increased insulin secretion. If the process persists, glucotoxicity can

http://dx.doi.org/10.5772/53267

occur, leading to chronic hyperglycemia and clinical diabetes [166]. The third accelerator is genetic susceptibility, predisposing to β-cell autoimmunity [167]. Several studies support the role of the Accelerator Hypothesis, showing that BMI increasing and precocious weight gain are inversely related to age at diagnosis of T1DM [168-173]. Noteworthy, other reports don't agree with the primary pathogenic role of obesity [174,175]. Recently another study in a large cohort of patients from the Mediterranean area makes this theory controversial and unproven up to now [176].

In our previous report in a limited cohort of 174 Italian patients from Genoa (northern Italy) we demonstrated that obesity is not a common finding in younger children at T1DM diagnosis [177].

In particular, the obesogenic environment, i.e. sedentary lifestyle, which promotes insulin resistance and other metabolic consequences deserves attention.

On the other hand, some studies don't support the role of Accelerator Hypothesis. In fact, data from UK compared BMI at T1DM diagnosis with age at diagnosis in South Asian and white children and did not find significant differences. The authors concluded that BMI could be too crude as indicator of insulin resistance, and that other specific indicators should be considered [178].

In a large cohort of Mediterranean patients diagnosed with T1DM between 1990 and 1994 BMI-SDS has not significantly increased. In addition a positive association between BMI-SDS and age at diagnosis has been also reported [176].

It is plausible that Accelerator Hypothesis does or not does become manifest because of the genetic background and environmental factors, including the prevalence of overweight and obesity.

All studies include children BMI to define obesity; however, this measurement seems to be a too crude measure of insulin resistance, as well as of percentage fat mass and its distribution and for the critical variable of cardiovascular fitness, which is the major determinant of insulin sensitivity.

9. Epigenetic

The study of epigenetic in the pathogenesis of autoimmune diseases represents a new challenge and a fascinating field for clinicians and researchers, particularly as regards T1DM. It is recognized that genetic background is only one aspect in T1DM pathogenesis, and the role of environment, gender and aging deserves equally attention. In fact genetic background is responsible for susceptibility or protection from clinical onset of the disease. Moreover, genome wide association studies discovered significant associations underlying immune tolerance breakdown only in a relatively small group of patients, leading to the concept of "Missing Heritability" [179]. Furthermore the low concordance rate of T1DM in monozygotic twins reinforces the concept that external additional factors play a crucial role, and the

link between genetic susceptibility and environment as trigger of auto-immunity can be represented by epigenetic [180].

In contrast to genetic alterations, epigenetic changes determine and/or perpetuate an heritable change in gene expression without a change in DNA sequence. Epigenetic mechanisms are involved in eukaryotic gene regulation through modification in chromatin structure in part packaging DNA, in part as modulating gene expression. Epigenome can be defined as a cell specific and stable pattern of gene expression determined by epigenetic mechanisms. Epigenetic mechanisms are involved in cell type development and function, since they are able to determine stable gene expression or repression. Another important feature of epigenetic mechanisms consists of determining metabolic plasticity to cells, with subsequent adaptation to environmental modifications [181].

The main epigenetic abnormalities include DNA methylation and histone modifications, leading to spatial and temporal changes in gene regulation. Studies in identical twins showed that the appearance of epigenetic differences increase with age and the most significant epigenetic differences have been occurred in those twins who spent less time together [182].

As regards T1DM pathogenesis, epigenetic role is by modulating lymphocyte maturation and cytokine expression, both involved in the development of autoimmune attack to β-cells [183]. In particular T-helper lymphocyte differentiation is under epigenetic control [184]. Another mechanism by which epigenetic modifications play a role in T1DM pathogenesis is by influencing β-cell development and repair. In fact glucose and insulin regulate methylation process which takes place in the cell via elevated homocysteine and homocysteine re-methylation, with a concomitant reduced capacity to remove homocysteine by means of transulfuration processes [185]. Homocysteine can be re-methylated to form methionine. The maintenance of methylation patterns in DNA and histone are linked to cellular methyl group metabolism, which is influenced by nutritional intake of folate [185]. Maternal nutrition state can influence newborn metabolic phenotype through epigenetic modifications. In fact the relationship between nutritional status and epigenetic is crucial during embryogenesis, intrauterine life and perinatal period, influencing offspring's pancreas vascularisation and development [186]. Furthermore Dutch people exposed to famine during intrauterine life in the years of the Second World War experienced higher frequency of type 2 diabetes and cardiovascular risk in adulthood [187]. As regards a direct epigenetic involvement in T1DM pathogenesis few data are available. On the other hand a possible contribution is represented by food intake, for methyl donors (i.e. methionine and choline) and cofactors (i.e. folic acid and vitamin B12) which are important for DNA and histone methylation.

10. Conclusions

Even if diabetes mellitus is a condition described in the ancient Egypt, no specific etiologic factor has been defined up to now. Fascinating case reports and large multicenter studies demonstrated the complexity of pathogenetic events characterizing autoimmune diseases.

http://dx.doi.org/10.5772/53267

Several environmental factors, old and new, play a crucial role in the development of T1DM, being as protective as dangerous, and their interplay with genetic susceptibility can explain the difficulty to find a single causative agent [188].

On the other hand the study of environmental factors increases the knowledge of natural history of the disease, and allows the recognition and knowledge of those protective agents which can delay the clinical onset of the disease and represent the basis for primary prevention programs.

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