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Clinical genetics of Type 1 Diabetes Genetic correlates of early growth and disease progression

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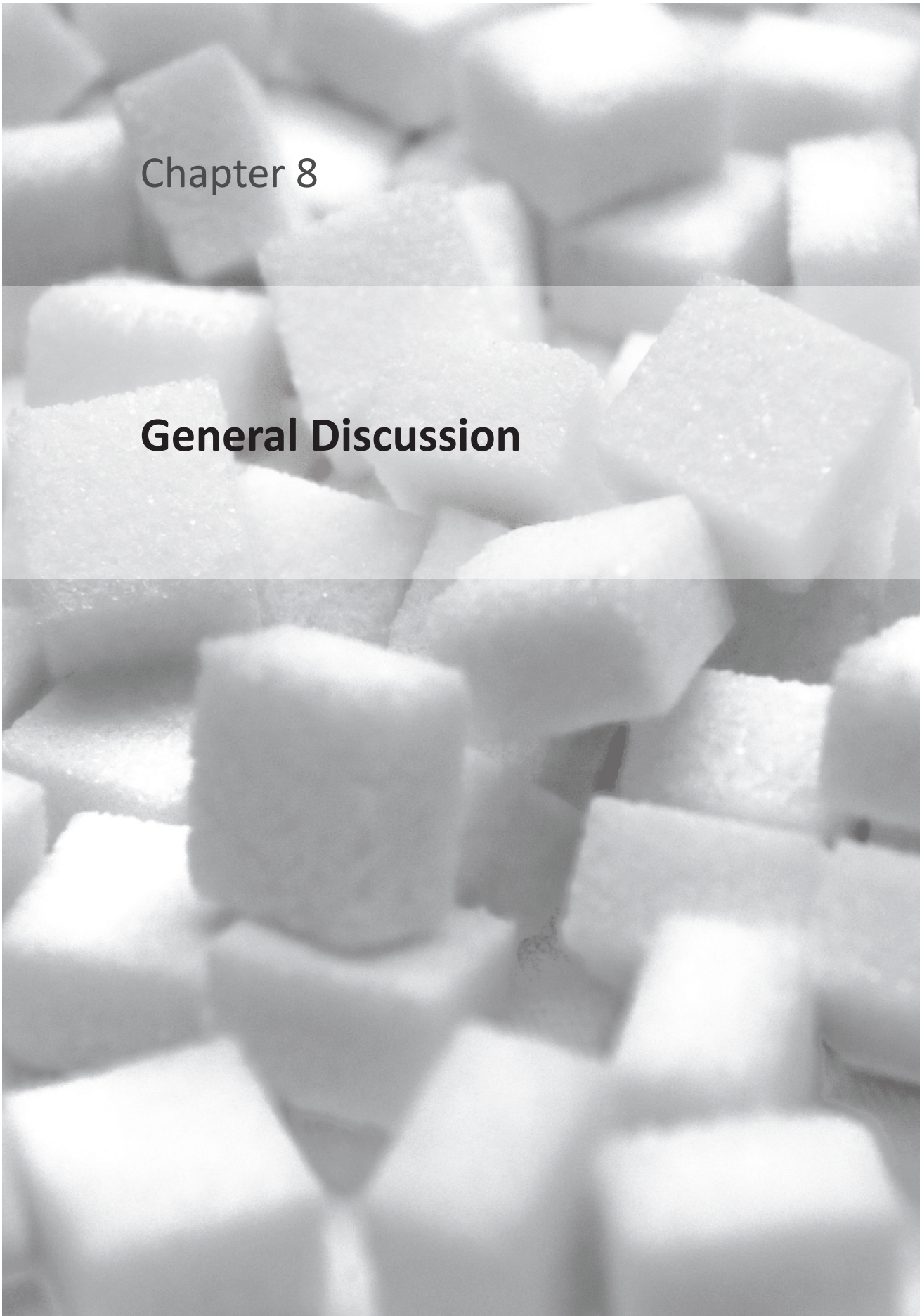

Chapter 8

General Discussion

The immune system of an individual normally has the ability to ignore "self" while mounting an immune response to non-self. Tolerance, postulated more than 50 years ago, is the process in which an individual neutralizes or eliminates auto-reactivity, i.e. does not develop an immune response against self antigens. Failure or breakdown of tolerance, driven by genetics and environment, causes auto-immunity. The precise genesis of tolerance is still unknown. Diseases are often the result of a complex interaction between the genetic profile and the environment, typified as being multifactorial. Type 1 diabetes is a prototypic example of this, in which three components are essential in disease pathogenesis, namely: i) genetic predisposition; ii) environmental factors; and iii) auto immunity, i.e. loss of tolerance. The challenge for research today is to further unravel the missing pieces in knowledge concerning each of these, with the ultimate goal to optimize treatment or, better still, cure this chronic metabolic disease. In an attempt to close the gap, this thesis addresses some of the lacunas still existing in the current knowledge of the aetiopathogenesis of T1D. **Box 1** gives an overview of current hypotheses aiming to explain the rise of T1D. The goal of the present thesis is to examine some of the gaps of these various hypotheses.

Several years ago, a network project was initiated aiming at identifying the molecular mechanisms of candidate risk genes for T1D through their function in pathogenesis. In this context, we studied phenomena of T1D with clinical relevance in great detail and aimed to find genetic correlates for these phenomena. This thesis provides validation and extension of several clinically relevant gene polymorphisms with functional consequences in relation to growth (INS-VNTR, IGF1) [**chapter 2-4**], disease progression and remission (IFN-γ, IL-10) [**chapter 5-6**], adiponectin, IL-1ra (Pfleger, Mortensen et al. 2008), islet autoimmunity (INS VNTR) [**chapter 3**], and environment (Lewis) [**chapter 7**]. In this

Box 1 ***Hypotheses aiming to capture and explain aetiopathogenic factors and rise of T1D***	**Support by this thesis**
Accelerator hypothesis (Wilkin 2001) Insulin resistance is central. This insulin resistance is caused by excess weight gain which may accelerate beta cell apoptosis in individuals at genetic risk	yes
Overload hypothesis (Dahlquist 2006) This is an extension of Wilkin's view, suggesting that the high insulin demand on the beta cell results from overfeeding , and that the resulting accelerated growth makes the beta cells vulnerable to autoimmune attack and apoptosis	yes
Beta cell stress hypothesis (Ludvigsson 2006) This hypothesis tries to unify 'all` the hypotheses by encompassing all known and potential players (increased hygiene/ increased growth/ infections/feeding patterns etc), while suggesting that increased beta cell stress stimulates or sometimes even initiates the autoimmune islet disease.	largely
Hygiene Hypothesis (Bach 2002) This suggests the existence of an inverse relationship between allergic and auto-immune diseases and infections during early childhood	possibly

The hypotheses put forward in Box 1 represent merely mechanistic paradigms not amenable to clinical experiments. A major drawback is the fact that there is no distinction between prenatal and postnatal factors involved, e.g. in terms of accelerated growth. New techniques like high resolution 3D ultrasound do allow accurate assessment of prenatal growth, whilst foetal DNA can be acquired from maternal blood specimens for early selective typing purposes (Lo, Corbetta et al. 1997; Yagel, Cohen et al. 2009)

thesis we tried to better characterize earlier findings of accelerated growth prior to T1D onset and we aimed at finding genetic correlates for this phenomenon. We further aimed to characterize, genetically and immunologically, the honeymoon phase, i.e. the period of clinical remission soon after diagnosis, following initial insulin treatment of newly diagnosed T1D. This period is of utmost interest in view of therapeutic opportunities, and might enable us to predict important features of disease progression. We finally addressed a long existing debate regarding a possible association between T1D and a blood group antigen, proposing an underlying mechanism for this association.

Bridging the gaps

Early growth

Comparable to islet auto-immunity, growth patterns are also known to deviate early in life, long before clinical disease onset. In 1975, early growth, defined as weight increase in the first year of life, was found to be higher in prediabetic children as compared to unrelated matched controls (Baum, Ounsted et al. 1975). Unfortunately, this group did not study or comment on whether growth differences were confined to the first year of life. Since then, several groups have confirmed the phenomenon of increased growth prior to juvenile T1D onset, although the vast majority of these studies did not report whether the differences between prediabetic children and the reference population lasted until clinical disease manifestation or were time-restricted. (Blom, Persson et al. 1992; Johansson, Samuelsson et al. 1994; Bruining 2000; 2002; Betts, Mulligan et al. 2005; Larsson, Hansson et al. 2008; Ljungkrantz, Ludvigsson et al. 2008). Furthermore, growth was not consistently defined as increase in length/height SDS and weight SDS in the various studies.

The relatively long latency time between determining early events and clinical disease onset, as expressed by the increasingly acknowledged concept of perinatal programming, makes it a challenge to study early risk factors for T1D. This rather aged concept of perinatal programming dates back as early as 1809 with Lamarc, as reviewed elsewhere (Dubos, Savage et al. 1966; Plagemann 2005). It acquired different names over time, and hypothesizes that very early in

Box 2
Perinatal Programming

Perinatal programming explores the evidence linking the role of early life events, i.e. deviations from the normal developmental patterns, to long-term physical and psychological health outcomes. The way subtle early-life influences can produce long-term functional and structural changes was recently reviewed by Gluckman PD et al. (Gluckman, Hanson et al. 2008), who expressed the hope that this may lead to development of preventive and interventional strategies. Indeed mounting evidence points to early life events influencing later disease development, like cancer and metabolic disease. This rather old concept is probably best known as Barkers "foetal origin hypothesis". This hypothesis proposes disorders to originate through developmental plasticity, in which malnutrition during early periods (foetal, infancy, early childhood) permanently changes the structure and function of the body (Barker 2007). This phenomenon is called programming. We propose, as earlier suggested in wider context by Dorner (Dorner and Plagemann 1994), that this also applies to the development towards type 1 diabetes which can also be characterized by a latency period between environmental trigger(s) and clinical disease onset.

life, environmental changes, be it nutritional, hormonal, or metabolic, may cause predisposition to certain diseases later in life [**box 2**].

This thesis attempts to address this gap in current knowledge, by establishing a new and larger growth cohort from the same background population as described in the introduction, to validate our earlier findings and study early growth in more detail [**Chapter 2**]. The unique design of this cohort also allowed us to correct for confounding family effects (Maraganore 2005), too often not dealt with in the several case-control studies published on this subject. This study design conveys the advantage of avoiding problems of population stratification, potentially leading to spurious associations with non-causal genes, although at the cost of reduced statistical efficiency due to genetic overmatching. Contrary to most other studies on this subject and despite the retrospective nature of this cohort, we had a relatively high number of measurements of biometric parameters, which permitted us to include both length and weight measurements in our growth model. A drawback of our study design is that one cannot exclude the possibility that the non-affected sibling may at some time in life contract the disease. Additionally, temporal trends such as maternal smoking during pregnancy or excessive maternal weight gain during pregnancy, factors known to influence offspring's growth, may have occurred. Furthermore, given its retrospective design, not all subjects had data available on size-for-gestational-age measures, an important and preferred parameter to be included in prospective studies performing risk assessment in this context [as rightfully pointed out by Algert, McElduff et al. 2009].

We confirmed earlier findings by our group and others of accelerated growth in prediabetic children. Remarkably though, we found this early accelerated growth to be limited to the first year of life in prediabetic children as compared to their non-affected siblings. We suggest that increased growth beyond the first year of life can also be attributed to familial growth patterns rather than predisposition to T1D per se. Indeed, we revealed accelerated growth in siblings of patients compared to the general population, which still differs from the diabetic proband in the first year of life.

Given the global rise in incidence of T1D, especially in the very young (Weets, De Leeuw et al. 2002), we were also interested whether age of T1D manifestation was correlated with increased weight, as argued and found by several research groups, supporting the earlier mentioned accelerator hypothesis [**Box 1**]. Indeed, we confirmed that those with earlier disease onset tended to be heavier, indicating the importance of ill-understood aspects of very early life to be paramount to the development of T1D, whilst the hypotheses which have been proposed in light of the rise in incidence of T1D, are not contradicted by the findings of this thesis [**Box 1**].

Contrary to some large cohort studies, we did not find differences in birth weight between cases and controls (Dahlquist, Patterson et al. 1999; Stene, Magnus et al. 2001; Cardwell, Carson et al. 2005; Dahlquist, Pundziute-Lycka et al. 2005). While it may be tempting to argue that this may likely be a power issue, we believe that the finding of a lack of association with birth weight and later T1D is explained by our study design. Given our family-based design, we suggest that the lack of birth weight differences is a reflection of the common background of the cases and ('nested')

controls, though divergences in weight between proband (prediabetic child) and sibling are found to manifest as early as of 3-6 months of age. With this premise we seemingly challenge two recent meta-analyses arguing the contrary (Harder, Roepke et al. 2009; Cardwell, Stene et al. 2010), although the authors of these two meta-analyses pointed out that high birth weight confers a relatively small risk of type 1 diabetes. Given our family-based design, in contrast to the population based cohorts included in the reviews, this small risk may well disappear. We therefore believe that the issue as to whether increased birth weight is indeed a risk factor for type 1 diabetes remains open for debate. Of course one cannot disregard the importance of a possible influence of population and disease heterogeneity in all of this. Furthermore, one could consider birth weight a proxy for intra uterine growth, which in case of T1D may well be increased even earlier than we found [**Chapter 2**], i.e. already intrauterine as previously suggested (Dahlquist, Bennich et al. 1996). This birth weight issue may be prospectively explored in detail *in utero* with novel ultrasound equipment, especially since it is conceivable that episodes of enhanced growth are exchanged with episodes of stunted growth resulting in various birth weights, lengths and head circumferences. Such follow up studies can be accompanied by the prospective collection of maternal data, like weight increase during pregnancy, which lacks in earlier studies.

Role of Genetics in early growth

In our quest to find genetic correlates for the observed and confirmed increased growth preceding clinical disease, we selected two variants of the insulin [INS] and insulin-like growth factor 1 [IGF1] gene. Both IGF-I and insulin are known to be important in early growth and T1D (Hill and Hogg 1989; Jones and Clemmons 1995; Laron 2001; Eerligh, Roep et al. 2004; Ong, Langkamp et al. 2009).

Polymorphisms in the region encoding ***IGF1*** have been variably reported to be associated with birth (Arends, Johnston et al. 2002; Day, King et al. 2002; Frayling, Hattersley et al. 2002; Landmann, Geller et al. 2006; Geelhoed, Mook-Kanamori et al. 2008; Vella, Bouatia-Naji et al. 2008). Our group previously studied an IGF1 microsatellite in 206 families with T1D and its interaction with the polymorphism near the insulin (INS) gene variable number of tandem repeats. A protective effect exhibited by the 194 bp allele was demonstrated (Eerligh, Roep et al. 2004).

Additionally, polymorphisms in the variable number of repeats of the gene promoter of the ***insulin gene*** located on chromosome 11 have been widely studied in the context of features of growth. Similarly to the IGF1 gene variants, conflicting data is present with regard to the association of the INS-VNTR variants and several growth features, like birth size or postnatal growth (Dunger, Ong et al. 1998; Bennett, Sovio et al. 2004; Mitchell, Hattersley et al. 2004; Ong, Petry et al. 2004; Heude, Petry et al. 2006; Landmann, Geller et al. 2006; Vu-Hong, Durand et al. 2006; Mook-Kanamori, Geelhoed et al. 2007; Lindsay, Hanson et al. 2003; Osada, Seki et al. 2007). In our study, non-carriers of the (protective) allele IGF-I*194 [wild type] correlated with accelerated growth in the first year of life in both patients and their healthy siblings. In contrast, no significant difference in growth was found for the ["T1D protective"] INS-VNTR*III variant in patients, indicating that disease susceptibility of this variant

is not conveyed through an effect on accelerated growth.

Considering the conflicting findings of the association between features of growth and the two studied gene variants as reported in literature, we decided to study these variants in another cohort which showed early increased growth. Hence, we were fortunate to have at our proposal data from the Project On Preterm and Small-for-gestational age infants cohort [**POPS**] of 250 infants born very premature i.e. a gestational age below 32 weeks, of which growth -and several biometric and laboratory- parameters had been collected earlier (Verloove-Vanhorick, Verwey et al. 1986).

The POPS cohort of very premature subjects is known to show catch-up growth during the first years of life. This catch-up growth is comparable to the early enhanced growth seen in later type 1 diabetic children of our family cohort. The POPS cohort allowed the study of the association between gene variants and accelerated growth, independent of the possible influence of pre-T1D, whilst exact data on size-for-gestational-age measures for all subjects were available. Although non-significant, a trend was observed between the wild type carriership of the IGF1 variant and length SDS. No correlation was found between several aspects of growth and INS-VNTR. However, functionality for the IGF1 variant was observed: carriers of the wild type IGF1 allele manifested a smaller head circumference from birth until the age of 5 years, whilst reduced head circumference was associated with a lower IQ at young adult age. This concurs with the general idea that head growth is generally preserved over somatic growth in conditions inhibiting foetal growth, resulting in small-for-gestational infants.

These findings, combined with our findings in **chapter 3** and those of others (Landmann, Geller et al. 2006), imply that the effect of the IGF1 variant on early growth is independent of T1D. The POPS cohort underscores that accelerated infant growth per se does not predispose to development of T1D. Additional factors, such as genetic predisposition and environmental factors are required for loss of immunological tolerance and development of insulin deficiency.

Proposed mechanism for accelerated growth prior to juvenile onset T1D

The challenge is to establish the biological mechanism behind early accelerated growth preceding juvenile onset T1D. Based on the findings in this thesis [**chapter 2 and 3**] and in conjunction with comparable clinical and experimental studies, we propose the following model as depicted in figure 1.

The proposed model reflects the combined role played by insulin and IGF-I. These important components of the early growth axis are held responsible for accelerated growth, contributing to T1D development.

The best example of insulin's role as a growth factor comes from the observation that ill-regulated diabetic mothers give birth to "big babies". Moreover, perinatal hyperinsulinism, induced by gestational hyperglycaemia or neonatal overfeeding, has previously been postulated as being a risk factor for both types of diabetes (Dorner and Plagemann 1994). Although overfeeding may well be associated with increased T1D risk (Dahlquist 2006), we believe that the increased risk for T1D

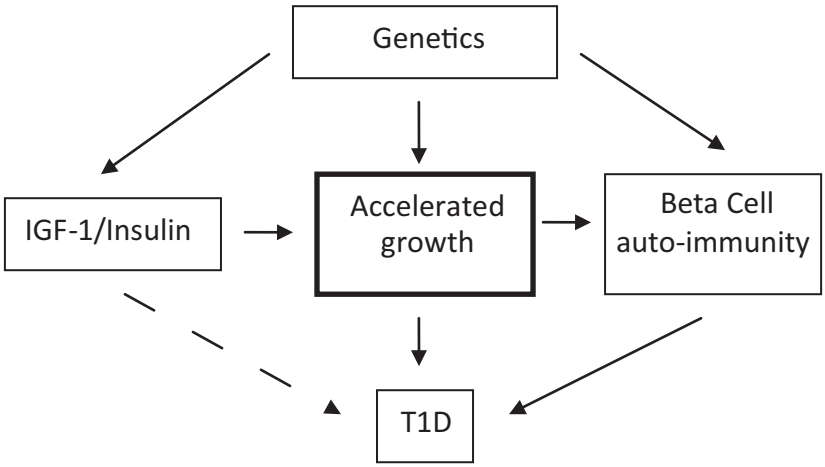

Figure 1 | The role of Accelerated growth prior to clinical type 1 disease onset in a genetically predisposed subject. During a critical phase early in life auto-immunity is enhanced by accelerated growth, fulfilling the role of a catalyst. Insulin, IGF-1 and their genetic counterparts, are key ingredients for the ongoing beta cell destructive auto-immune process, leading to clinical diabetes.

posed by accelerated growth is independent of overfeeding, as also indicated by other investigators (Hypponen, Kenward et al. 1999; Pundziute-Lycka, Persson et al. 2004; Dahlquist 2006).

As mentioned earlier, conflicting data exists regarding the INS-VNTR gene variants and different features of growth. There seems to exist a complex relationship between these variants and growth features. For example, it has been found that children with the INS-VNTR gene variant which predisposes to T1D, secrete insulin more rapidly with increasing BMI than subjects with the wild type variant (Dos Santos, Fallin et al. 2004; Heude, Petry et al. 2006). In this thesis we found that the studied INS-VNTR variant only correlated positively with first year growth in the unaffected siblings.

Periods of growth are normally associated with increased insulin demands and resistance. The latter, although widely known in type 2 diabetes, is now increasingly being recognized as an important feature in T1D [**box 3**]. Both increased insulin demand and resistance results in increased work load for the beta cells, adding to beta cell stress. This supports the findings of several studies reporting beta cells to be more vulnerable to auto-immune attack when displaying higher metabolic activity (Nerup, Mandrup-Poulsen et al. 1988; Palmer, Helqvist et al. 1989; Roep, Arden et al. 1990; Bjork, Kampe et al. 1992; Skowera, Ellis et al. 2008). Additionally, hyperinsulinemia per se, i.e. exposing the immune system to more insulin which can be regarded as an antigen, can stimulate the immune system.

Earlier studies in a prospective cohort of infants born small for gestational age (SGA) showed that

'catch-up' growth during the first year is correlated with insulin sensitivity and secretion (Soto, Bazaes et al. 2003). The normal, more marked increase in weight rather than in length that occurs in the first year of life, may result in an increase of fat mass rather than lean tissue. This pattern was also elucidated in our study. Cytokines released from fat tissue may also influence the immune system, enhancing both insulin resistance and beta cell auto-immunity (Grimble 2002; Matarese, Sanna et al. 2002; Tilg and Moschen 2006).

In our previous work (Bruining 2000) we found that an early increased growth pattern in prediabetic children correlated with the presence of IA-2 antibodies. This finding suggests that early growth is associated with an accelerated rate in pancreatic destruction by these auto-antibodies. In support of this concept, Couper et al. found that the appearance of auto- immunity positively correlated with accelerated growth in subjects at increased risk for T1D (Couper, Beresford et al. 2009).

The paracrine and autocrine levels of IGF-I are responsible for growth in the late gestational period. Approximately six months after birth, IGF-I production and growth become growth hormone dependent. In this thesis [**chapter 3**] we found that in prediabetic children an IGF1 variant only correlated with accelerated growth in the first year of life. Previously, circulating IGF-I levels infants have been demonstrated to be associated with early postnatal catch up growth (Ong, Kratzsch et al. 2002). Interestingly, IGF-I levels also correlated with body composition at the age of 5 years.

Based on our findings [**chapter 2 and 3**] we infer that variations in the IGF1 gene, influencing IGF-I serum levels, contribute to T1D via accelerated growth. In addition to its important role in early growth, IGF-I is also important for glucose homeostasis and insulin sensitivity (Clemmons 2006). These combined roles of IGF-I are found in puberty, a period of increased growth and insulin resistance (Moran, Jacobs et al. 1999). We hypothesize that an increase of autocrine and paracrine IGF-I levels in combination with insulin resistance is responsible for the early accelerated growth preceding type 1 diabetes. Our hypothesis intrinsically implicates that IGF-I levels vary during the early stages of life. These IGF-I levels are at least partly influenced by IGF1 gene polymorphisms. While it appears that IGF-I levels are linked to insulin secretion, the exact mechanism behind this remains obscure. Besides its role in growth, direct effects on the pancreatic beta cells have been proposed for IGF-I, as shown in comparative biology models (George, Ayuso et al. 2002; Ong, Petry et al. 2004).

The central part in our model [**figure 1**] portrays accelerated growth which we compared with catch-up growth as observed in infants born SGA in the POPS study. This catch-up growth is a phenomenon widely studied in man as well as in animal models and may hold an important key in the understanding of later disease development. Whether the occurrence of accelerated growth is indeed part of a set of crossroads where essential components of this thesis like IGF-I, insulin and cytokines besides critical organ development [brain/intestine/pancreas/etc.], nutrition and (epi)genetics merge, could be a challenging forum for future research.

Box 3
Insulin resistance (IR) in type 1 diabetes[1]

IR is a long known concept in Type 2 diabetes and is more and more acknowledged to also be of importance in T1D. IR basically applies in situations when the amount of produced insulin is not sufficient to maintain normal blood glucose levels. The euglycaemic hyperinsulinaemic clamp test is considered the 'gold-standard' test although several methods are known to assess IR. T1D is often diagnosed during episodes associated with insulin resistance as ***increased growth*** (puberty) and illness. One of the mechanisms postulated for the ***honeymoon phase*** is a temporary resolution of IR. Furthermore, IR is also shown to be of importance later in T1D, as it is shown to be an independent risk factor for vascular disease complications in T1D. Additionally, IR plays a central role in the ***accelerator hypothesis***. So IR in T1D should be considered an important feature of the pre-, peri- and postclinical disease manifestation.

1 (DeFronzo, Hendler et al. 1982; Leslie, Taylor et al. 1997; Wilkin, Ludvigsson et al. 2004; Kilpatrick, Rigby et al. 2007; Pang and Narendran 2008)

Disease progression, the honeymoon phase

The fascinating but poorly understood period of clinical remission after clinical manifestation of hyperglycaemia and initiation of insulin replacement therapy, long known to clinicians, was officially documented in 1940 by Jackson et al., when they described a reduction in insulin dose after initial treatment with insulin in a group of patients (Jackson, Boyd et al. 1940). This apparent natural remission period in the course of T1D has been under renewed interest since it presents an attractive phase to study potential therapeutic (in particular immune) intervention, aiming at halting or reversing ongoing disease. Immunological parameters determining disease progression or prediction are scarce. In light of this knowledge gap, some years ago we started a collaborative effort to study parameters of the immune process around the time of the honeymoon phase.

An important starting point for this study was the Th1/Th2 paradigm, postulating that naïve T cells differentiate into Th1 or Th2 T cells after their "cross talk" with antigen presenting cells. This Thl/Th2 dichotomy leads to either Th1 T cells, mainly producing Interleukin-2 (IL-2), interferon-γ (IFN-γ) and tumour necrosis factor-β (TNF-β), or Th2 T cells characterized by production of IL-4, 5, 13 and IL-10. The Th1 phenotype is associated with a beta cell destructing immune response due to breakdown of tolerance, whilst the Th2 leads to an immune response protective against beta cell damage (Eisenbarth 1986; Romagnani 1994). Subsequent research has led to the discovery of a population of hitherto undiscovered T cells playing a pivotal role in the induction of tolerance (Moore, de Waal Malefyt et al. 2001). These T cells are now known as regulatory T cells and are believed to be critical players in protecting beta cell function as they are tolerogenic, secreting the immune suppressive cytokine IL-10, as opposed to the pro-inflammatory auto-reactive islets cell destructive T cells (Arif, Tree et al. 2004). Hence, we now prefer to speak of the "Th1/ Treg paradigm".

The results collected initially in this project showed that the combination of several cytokines and chemokines allows one to accurately predict islet antibody positivity in individual patients, suggesting a close association of islet antibody status with systemic immune regulation in T1D

(Hanifi-Moghaddam, Schloot et al. 2003). These findings were extended to the remission phase although preliminary results did not show a clear association between T1D remission and systemic levels of a broad range of immune mediators. This corroborates the findings of others, reporting no remission associated changes of auto antibody levels (Peig, Gomis et al. 1989; Petersen, Dyrberg et al. 1994). However, it appeared that low concentrations of IFN-γ and IL-10 at baseline were independently associated with later clinical improvement, suggesting an immunological contribution to remission. These findings were published in a later phase (Schloot, Hanifi-Moghaddam et al. 2007).

Based on these earlier findings showing that a) clinical remission was not accompanied by changes in mediator levels except for a moderate decrease of IL-10 plasma levels in subjects showing no remission and b) that baseline levels were associated with a later clinical course in that low concentration of IFN-γ and IL-10 were observed in subjects who remitted, we aimed to further define correlates of disease progression. The role of cytokine profiles (plasma levels) and their corresponding, potentially functional, polymorphic genotypes was taken into consideration [**chapter 5**]. We examined whether these parameters are associated with (partial) remission in newly diagnosed T1D patients, participating in an international multi-centre case control study [Hvidøre].

Remarkably, serum IFN-γ concentrations predicted to be high by genotype, proved to be low in remitting patients [**chapter 5**]. We argued that dysregulation in the disease process may overrule genetic predisposition. Individuals with genotypes expected to produce high levels of IFN-γ concentrations, were less frequently observed in remitters. Nevertheless, these genotypes were predictive of remission if serum IFN-γ levels were low in subjects recently diagnosed with T1D.

These findings were extended by adding a surrogate marker for beta cell function, namely the mixed meal stimulated tolerance test. We studied several international cohorts [among which were the Diab Marker & Hvidøre cohorts], [**chapter 6**] in which the relationship of disease progression in T1D, determined by preserved beta-cell function in the first 6 months after diagnosis, with serum IL-10 and IFN-γ cytokines and their genetic counterparts were assessed. IL-10 serum levels showed an inverse relation with age and C-peptide. Increased serum IFN-γ concentrations correlated with rapid disease progression. The gene polymorphisms did not differ between the progression pattern groups, again implying that disease mechanisms take precedence over genetic predisposition.

In conclusion, remission appears partially immune-mediated and involves regulation of IFN-γ transcription whilst a functional genetic polymorphism of IFN-γ was found to be associated with disease progression and natural remission. It may thus prove of value to determine IFN-γ and IL-10 plasma levels and their corresponding genotype in the characterization of disease progression after clinical disease onset. Since there were no obvious centre differences, one could argue that the widely seen geographic variation in T1D incidence should not be sought in the immunological basis of clinical remission.

While debating prospectively the patho-physiological mechanisms of feeding practices leading to clinical T1D, an old discussion reopened over the relevance of the minor blood group antigen

Lewis. This blood group was suggested to be associated with T1D. In an observational study we confirmed an association between the prevalence of Lewis blood group phenotype and T1D, with a female preponderance (**chapter 7**). The Lewis antigens are soluble molecules, which at birth are fully present in secretions, bodily fluids and epithelial cells of the GI tract. After birth they become passively adsorbed to the red cell. There are evident biochemical structural similarities between the Lewis and ABO blood group system, while the secretory status of both Lewis and ABO are influenced by the same gene (Morgan and Watkins 2000)**.** Mucosal surfaces express substantial amounts of these Lewis carbohydrate blood group antigens which can mediate binding of micro organisms (Boren, Falk et al. 1993; Henry 2001).

One can speculate on the possible ramifications of our findings of a lower prevalence of the Lewis negative phenotype in T1D. Antibodies against Lewis antigens may cross-react against: (i) the local intestinal micro environment; or (ii) against other (self-) epitopes like beta cells or specific food components presented in the intestine. Thereafter a local inflammatory response influencing intestinal permeability may occur. This mechanism has been implicated in diseases originating in early life (Liu, Li et al. 2005).

Regardless of disease mechanism, the ultimate consequence of these intestinal events is loss of tolerance to self-antigens, giving rise to beta cell auto-immunity. Indeed, the long neglected role of the intestine in the process of autoimmunity is now increasingly being acknowledged, also in regard to type 1 diabetes (Vaarala, Atkinson et al. 2008).

With respect to the association between Lewis phenotype and T1D our views are in line with the "hygiene hypothesis" (Strachan 1989). In this hypothesis, vigorously debated in the literature, it is assumed that autoimmune and allergic diseases increased in the developed countries on account of the protective loss of acquiring infectious diseases and the simultaneous decrease in incidence of these infections (Bach 2002). However, direct i.e. prospective and substantiated evidence is inconclusive and may prove hard to obtain, especially since infection appears to be a two edged sword also appearing to protect against T1D (Goldberg and Krause 2009).

Closing remarks

The data presented in this thesis has strengthened the notion that early events are pivotal in T1D pathogenesis as exemplified by early growth. The role of environmental factors in the aetiopathogenesis is also compelling given the increased incidence of T1D in a relatively short time window. This is strengthened by the diminishing role for the HLA in disease predisposition, in particular in case of clinical onset of T1D in adults (Gillespie, Bain et al. 2004). Although our findings are sound, prospective studies examining the role for environmental factors in the aetiopathogenesis of T1D, comparable to earlier initiatives, are still imperative (2007; 2008).

Research initiatives including subjects not following normal background growth patterns are warranted since these will allow us to study subjects who, given the concept of perinatal programming, may be considered prone for later disease (**Box 2**). Future research in the context of T1D, should prospectively study the additional value of

including early growth pattern in the prediction of T1D. These early growth patterns may prove of value in disease prediction, and may point to therapeutic strategies influencing the pace of disease onset and progression.

Based on the data presented in this thesis, we disqualify the view that overfeeding *causes* early accelerated growth. We favour the possibility that early feeding practices exert their pivotal role in disease pathogenesis by causing a skewed immune response early in life. Alternatively, and in line with the "perinatal programming" concept (**Box 2**), it cannot be excluded that the neuro-endocrine axis is "re-programmed" in such a way as to affect the offspring with impaired satiety at a critical time point, resulting in overfeeding and increased growth. Given our obesogenic environment, possibly leading to the increase in T1D incidence, it would be of value to study the potential role of the several hormones and relevant genetic parameters (like Growth Hormone and IGF-I and their binding proteins and receptors, Leptin, Ghrelin, LRP-8 and Tubs) in the pathway to T1D. Regrettably, most studies investigating these early phases and events focus on type 2 diabetes.

In this thesis the role of genes is equivocal. For increased growth prior to disease onset genetics are deemed imperative, while for disease progression, it was of lesser importance as disease mechanisms appeared to overrule genetics. Given logistic constraints, apart from the genetic complexity causing the Lewis phenotype, this was not explored in depth. The axis of nutrition, intestinal flora and disease predisposition is increasingly recognized as important and worthy of further investigation.
The relatively new field of epigenetics, defined as a heritable change in gene expression not involving a DNA sequence change, may possibly shed more light on the aetiopathogenesis of T1D, especially since the role of environmental factors remains a difficult one to untangle.

The greater part of type 1 diabetes patients are adults and represent a relatively heterogeneous population. They are already of particular interest for potential interventional therapies and suited to study genetic and immunological parameters determining disease progression/acceleration.

As summarized in **Box 4,** in this thesis we have shown that accelerated growth in the first year, rather than birth weight, is a predisposing factor to T1D, in a newly collected family based type 1 diabetes cohort. The studied IGF1 gene variant correlated with this early increased growth but not with T1D. Conversely the INS-VNTR gene variant, a primary correlate of T1D disease, is associated with T1D susceptibility rather than with early growth. Furthermore, genetic and functional correlates for disease progression were identified showing that remission is partially immune mediated, whilst cytokine profiling and genetic typing may prove valuable in disease progression kinetics, though disease mechanisms seemed to surmount genetics. Finally, we challenge those who propound the gastro-intestinal tract as merely the human sewage system, considering the vast amount of data implicating this in immune mediated disease pathogenesis (Groschwitz and Hogan 2009).

Food for thought

The pancreatic gland containing the key target of the auto immune process in type 1 diabetes is an integral part of the gastro-intestinal tract, an

increasingly recognized environment containing the largest part of the immune system. As such, it is conceivably involved in the development of clinical disease. In this light, how should the data presented in this thesis be valued?

This thesis addressed several unresolved issues in T1D disease mechanisms with regard to growth, environment, pancreatic autoimmunity and disease progression, all in relation to genetic background **[Box 4**]. For instance, our findings on Lewis blood groups in relation with T1D may point to the influence of gastro-intestinal flora and mucosal immunity contributing to disease. While gene/environment studies often have limited relevance for disease prediction, the major current environmental correlate of disease studied in this thesis virtually precludes primary disease prevention, given their risk predisposition of very early events (first year of life, or perhaps even prenatal). Nonetheless, this thesis may prove relevant in the context of disease intervention. For instance, early growth patterns were neglected in randomisation for early intervention studies, such as infant feeding (2007), intranasal or oral insulin (Gale, Bingley et al. 2004; Skyler, Krischer et al. 2005; Skyler, Weinstock et al. 2005) or gluten-free diet (Hummel, Bonifacio et al. 2002).

It would be interesting in ongoing or future clinical studies like the Trial to Reduce Insulin dependent diabetes in the Genetically at Risk [**TRIGR**] (2007), to also study the relationship between early growth patterns and disease risk and progression. Furthermore, in prospective studies, one should aim to concisely collect all data known to interfere with -or pose as- potential risk factors for T1D (and progression) of all subjects. Only then will these factors associated with T1D allow us to establish and study in greater depth their role and impact on the pathogenesis and progression of T1D. Ideally, these studies should be carried out in subjects included from birth or even from pregnancy onwards. Further, such studies will also highlight discrepancies, as observed in this thesis, between suspected genetic influence on e.g. cytokine profiles(s), and actual levels throughout disease manifestation.

Lastly, we stressed the critical role of the reference population with a family based preference, appreciative of disease and of population heterogeneity. Nested controls also take into account potential confounding environmental factors such as life style and feeding patterns and habits.

Box 4
Main Contributions of this thesis

- Collection of a sizable family based T1D growth cohort
- Increased early growth rather than birth weight is a risk factor for T1D
- Accelerated growth prior to T1D is limited to early life i.e. the 1st year of life
- In contrast to the INS-VNTR variant, the IGF1 gene variant is indirectly associated with T1D via early increased growth
- Remission is at least partially immune-mediated
- IFN-γ level combined with its corresponding genotypes can be valuable in predicting (partial) clinical remission at T1D clinical onset
- T1D disease mechanism appear to overrule genetic influences in disease progression

Our findings on early infant growth, associations with inheritance and plausible maternal influences point to the need for studies *in utero*, such as intrauterine measurements of growth (Yagel, Cohen et al. 2009) defining even earlier growth patterns. This will allow discrimination between prenatal i.e. exposure to exogenous factors from the mother, and postnatal factors.

Finally it is still unknown whether environmental or genetic correlates, as defined in this thesis, contribute to variation in disease progression after clinical manifestation.

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