Thesis

Persistence of proinsulin secretion in type 1 diabetes – what is really measured?

Submitted by

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Statutory Declaration

I declare on my honor that I have written this thesis independently and without assistance, I have not used other than the specified sources, and parts taken from other sources, verbatim or in substance, have been identified as such.

Graz, March 8th, 2021 Veronika Anoptchenko eh

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List of abbreviations

Aab, Aabs	Autoantibody, autoantibodies
ADA	American Diabetes Association
AID	Automated insulin delivery system
BM-HSCs	Bone marrow-derived hematopoietic stem cells
BMI	Body mass index
BM-MSCs	Multipotent mesenchymal stromal cells derived from bone marrow
CP	C-peptide
CPE	Carboxypeptidase E
CSII	Continuous subcutaneous insulin infusion
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CVD	Cardiovascular disease
DDP-4 inhibitor	Dipeptidyl peptidase 4 inhibitor
DKA	Diabetic ketoacidosis
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked Immunosorbent Assay
ESCs	Embryonic stem-cells
FDR, SDR	First-degree relative, second-degree relative
FIASP	Faster-acting insulin aspart
FPG	Fasting plasma glucose test
GAD(A)	Glutamic acid decarboxylase (autoantibody/autoantibodies)
GIRO	Graz Diabetes Registry for Biomarker Research
GLP1-RA	Glucagon-like-peptide 1 receptor agonist
GvHD	Graft-versus-host-disease
HbA1c	Glycated hemoglobin 1c
HLA	Human leukocyte antigen
IA-2(A)	Insulinoma-associated-antigen 2 (autoantibody/autoantibodies)
IAA	Insulin autoantibody/autoantibodies

ICA	Islet cell autoantibody/autoantibodies
IL2RA	Interleukin-2-receptor subunit alpha
iPSCs	Induced pluripotent stem cells
LCM analysis	Laser capture microdissection analysis
mAb	Monoclonal antibody
MDI	Multiple daily injections
MHC	Major histocompatibility complex
NOD mouse	Non-obese diabetic mouse
NPH insulin	Neutral Protamine Hagedorn insulin
OGTT	Oral glucose tolerance test
PC1/3, 2	Prohormone convertase 1/3, 2
PCR	Polymerase chain reaction
PI	Proinsulin
PTM	Post-translational-modifications
PTPN22	Protein Tyrosine Phosphatase Non-Receptor Type 22
RAA	Rapid-acting insulin analogs
RER	Rough endoplasmic reticulum
RIA	Radioimmunoassay
SAP	Sensor-augmented pump therapy
SGLT2 inhibitor	Sodium-glucose cotransporter 2
SU	Sulfonylureas
ß-cell	Beta-cell
T1DM	Diabetes Mellitus Type I, autoimmune subtype
T2DM	Diabetes Mellitus Type II
TNF	Tumor necrosis factor
TUDCA	Tauroursodeoxycholic acid
TZD	Thiazolidinedione
US	United States
ZnT8	Zinc transporter 8

List of figures and tables

German Abstract

Hintergrund: Diabetes Mellitus Typ I ist eine Autoimmunerkrankung, die durch einen absoluten Insulinmangel und daraus resultierender Hyperglykämie gekennzeichnet ist. Dabei führt das Zusammenspiel von genetischen Faktoren, Umweltfaktoren und einer Fehlregulation des Immunsystems zur Zerstörung der insulin-produzierenden ß-Zellen in den Langerhans-Inseln des Pankreas. Zur klinischen Manifestation des Typ I Diabetes kommt es, wenn ca. 80% der ß-Zellen zerstört sind, was meist im Kindes- oder Jugendalter eintritt. Neue Erkenntnisse liefern zunehmende Hinweise auf eine persistierende, obgleich minimale C-Peptid-Produktion, selbst nach langjährigem Krankheitsprozess. Dies könnte auf erhaltene, funktionierende ß-Zellen hinweisen. Überraschenderweise wurde in einer kürzlich durchgeführten Studie von Sims et al. publiziert, dass auch Proinsulin, das Vorgängermolekül von Insulin, bei ca. 90% der Studienpopulation mit langjährigem Typ I Diabetes nachgewiesen werden konnte, selbst bei Probanden mit nicht messbaren C-Peptid-Konzentrationen. Dies könnte darauf hindeuten, dass ein gewisser Anteil der ß-Zellen auch nach langjähriger Erkrankung noch in der Lage sein könnte, Proinsulin zu produzieren, und somit eine persistierende Insulinproduktion in bestimmtem Umfang noch stattfindet. Diese Erkenntnisse könnten das Verständnis über die Pathophysiologie des Typ I Diabetes verändern und neue Therapieoptionen bei Typ I Diabetes ermöglichen. Deshalb war es das Ziel unserer Studie, diese Ergebnisse mittels zwei verschiedenen Proinsulin Assays zu validieren und zu überprüfen, ob der hohe Prozentsatz an Proinsulin-Positivität, der von Sims et al. angegeben wurde, reproduziert werden kann oder die Ergebnisse auf unspezifische Assays zurückzuführen sind.

Methoden: Es wurden 69 Studienteilnehmern (28 Frauen, 41 Männer) mit Typ I Diabetes und einer mittleren Erkrankungsdauer von 19.9±14.3 Jahren aus dem Grazer Diabetesregister für Biomarkerforschung in die Studie eingeschlossen. Dabei kamen zwei verschiedene Assays zum Einsatz: ein Radioimmunoassay (Katalog-Nummer HPI-15K, Millipore, Burlington, MA, USA) und ein Sandwich ELISA (Mercodia, Uppsala, Schweden). Die Proinsulinkonzentrationen wurden mit beiden Methoden gemessen und die Ergebnisse anschließend nach Krankheitsdauer kategorisiert. Außerdem wurden auch die C-Peptid-Konzentrationen gemessen und gemäß der Diabetesdauer in

drei Gruppen eingeteilt. Zusätzlich wurden die Proinsulinkonzentrationen auch in der C-Peptid-negativen Gruppe gemessen.

Ergebnisse: Mit dem Radioimmunoassay (Sensitivität von 3.1 pmol/L) wurden 60.3% der Probanden als Proinsulin-positiv klassifiziert, während mit der ELISA Methode (Sensitivität von 0.5pmol/L) nur 31.9% in die Kategorie „Proinsulin-positiv“ fielen. In der C-Peptid-negativen Kohorte, die durch ein C-Peptid unter 0.08nmol/L definiert war, wurden 54.4% mittels RIA als Proinsulin-positiv eingestuft, während es mit der ELISA nur 20.7% waren. Dabei nahmen die gemessenen Proinsulinlevel bei beiden Messmethoden mit zunehmender Krankheitsdauer ab.

Diskussion: Unter Berücksichtigung der beträchtlichen Unterschiede der gemessenen Proinsulin-Positivität mit beiden Methoden, ist die Signifikanz der Proinsulin Messwerte in Frage zu stellen. Weitere Untersuchungen sind notwendig, um die Frage zu beantworten, was mit welcher Methode tatsächlich gemessen wird. Es sollte auch die Möglichkeit von Interaktionen mit Antikörpern in Betracht gezogen werden, beispielsweise mit Glutamat-Antikörpern oder Inselzell-Antikörpern.

Abstract

Background and aim: Type 1 diabetes is an autoimmune disease that is characterized by specific immune destruction of the insulin-producing ß-cells. There is increasing evidence supporting the notion that in individuals with long-standing T1DM, a sustained or minimal C-peptide production can be found(2). Interestingly, researchers recently claimed that proinsulin, a precursor molecule of insulin, is detectable in up to 90% of individuals with long-standing type 1 diabetes, despite C-peptide levels below the limit of detection(3). In this study, a radioimmunoassay with high sensitivity and specificity was used. It could be hypothesized that incomplete insulin production still occurs and the capacity to secrete proinsulin seems to persist in those subjects. This could enable potential new treatment options. We aimed to validate those findings shown by Sims et al. by using two different assays and to investigate whether this high percentage of proinsulin-positive subjects is due to unspecific assays or if this finding can be reproduced in a large cohort of patients with type 1 diabetes.

Methods: We analyzed proinsulin levels of 69 subjects (28 females) with T1DM, a mean age of 39.1 ± 16.0 years and a mean diabetes duration of 19.9 ± 14.3 years, from the Graz Diabetes Registry for Biomarker Research using two different proinsulin assays: a radioimmunoassay (RIA, catalog number HPI-15 K, Millipore, Burlington, MA, USA) and a sandwich enzyme-linked immunosorbent assay (ELISA, Mercodia, Uppsala, Sweden).

Results: Using the RIA method, 60.3% of subjects were proinsulin-positive (with a sensitivity of 3.1 pmol/L), whereas the ELISA classified only 31.9% as proinsulin-positive (with a sensitivity of 0.5 pmol/L). In the C-peptide negative group, defined as fasting C-peptide below 0.08 nmol/L(4), the RIA measurement classified 54,4% as proinsulin-positive, whereas only 20.7% were proinsulin-positive according to ELISA measurement. Overall, proinsulin levels measured by both assays decreased with increasing diabetes duration.

Discussion: Taking into account this considerable difference between the proinsulin-positivity rates observed with the two assays, the significance of these proinsulin results remains to be elucidated. Further research is needed to clarify what is really measured with which assay in this cohort, among other things, the possibility of the interference

of autoimmune antibodies such as glutamic acid decarboxylase antibodies or islet cell antibodies with the proinsulin assay in subjects with type 1 diabetes needs to be acknowledged.

1 Introduction: Type 1 Diabetes Mellitus

Type 1 diabetes mellitus, also known as autoimmune diabetes, is a chronic disease that is characterized by hyperglycemia due to cellular-mediated autoimmune destruction of pancreatic insulin-producing ß-cells leading to insulin deficiency(1). Typically, the symptomatic onset occurs during childhood and adolescence, even though symptoms can present much later in adulthood(5). Although the precise pathophysiological and etiological mechanisms of T1DM remain to be determined, T1DM is considered to develop as a consequence of a complex interplay between genetic factors, widely unknown environmental factors, and the immune system(6), and autoreactive T-cells are thought to play a crucial role as the primary mediators responsible for the destruction of the ß-cells. Disease progression can be divided into three stages (figure 1), starting with the detection of autoantibodies in stage 1, over dysglycemia in stage 2, to overt hyperglycemia representing stage 3(1). Besides, some authors consider long-standing disease as stage 4.

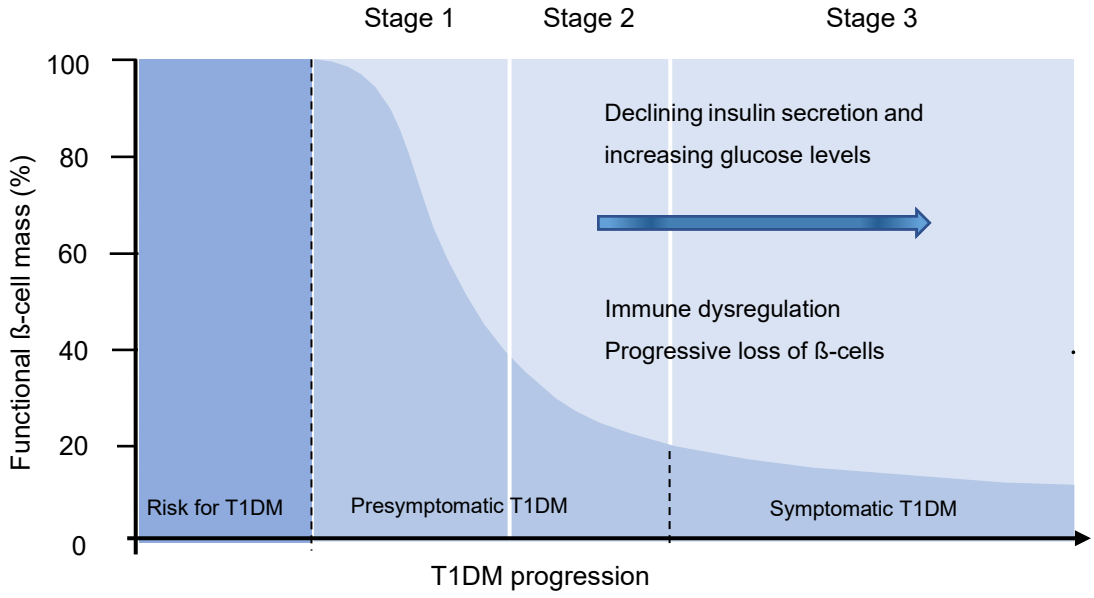

Figure 1: Natural history of T1DM, adapted from (1)

The majority of patients (70-90%) has autoimmune T1DM, also known as type 1A, where the formation of AAbs against ß-cells results in destruction of the same, meaning

that T1DM is diagnosed after a pre-symptomatic interval of varying length during which the ß-cell mass is reduced to that extent that blood glucose homeostasis can no longer be maintained(7). A smaller subset of patients shows no immune responses or AAbs, thus, the cause of ß-cell destruction is unknown(1). Therefore, this subtype is called idiopathic type 1 diabetes, or type 1B, and is strongly inherited but not HLA associated(1). Even though this type affects only a minority of patients with T1DM, most of those who fall into this category have Asian or African genetic background(8). Here, the focus lies on type 1A, which for simplicity will be referred to as T1DM.

Today, a cure for T1DM still remains elusive, and even though new therapeutic strategies are available, such as hybrid closed-loop systems and continuous glucose monitoring, patients are still dependent on a lifelong insulin substitution. Furthermore, despite the fact that better glycemic control is reached today, helping to reduce the incidence of macrovascular and microvascular complications, most patients with T1DM still experience these complications(1). Although knowledge about specific disease characteristics such as genetics, epidemiology, immune- and ß-cell phenotypes and pathophysiology has progressively increased over the past decade, the etiology of T1DM(6) is still not understood to the fullest, and major research effort is needed in the future to achieve early diagnosis, prevent ß-cell destruction and improve treatment options for T1DM(1).

1.1 Epidemiology

According to the International Diabetes Federation, 9.3% of the adult population (in the age group 20-79 years) – about 463 million people worldwide - are currently living with diabetes, 10-15% of them have T1DM(9). This number is expected to increase to 578 million (10.2%) in 2030 and 700 million (10.9%) in 2045(10). In Europe, the number of adults (in the same age group) living with diabetes is estimated to be around 59 million in 2019, around 66 million in 2030, and about 68 million in 2045(10), with 10-15% accounting for T1DM. For Austria, the numbers are as follows (for the age group 20-79 years): in 2019, around 641.000 people were living with diabetes, this number is expected to increase to 716.000 in 2030 and 747.000 in 2045, again, 10-15% account for T1DM(10). Worldwide, the number of children aged 0-19 years living with T1DM in

2020 is estimated to be over one million(11), for Europe, the prevalence in children in 2019 is 296.000(12), and in Austria, about 3000 children aged 0-19 years are living with T1DM in 2019(10).

Worldwide, both the incidence and prevalence of T1DM are increasing, although with global variations(13), driven by rapid urbanization and striking changes towards a sedentary lifestyle(14). Several factors are considered to be related to the increasing diabetes incidence seen during the past decades, including certain lifestyle habits related to wealth, since T1DM is associated with estimates of general wealth, such as gross domestic product(15). Almost 130.000 children are diagnosed each year worldwide(11), while the number of new cases of T1DM per year in Europe for children under 20 years of age was predicted to be around 31.000 in 2019(10) and about 300 in Austria. The lifetime risk now surpasses 1% in North America and Europe(16).

The incidence also varies between countries, and a north-south-divide can be observed, with the highest incidence rates in Scandinavian countries(1), e.g. in Finland, an incidence rate of >40 cases per 100.000 people can be observed(17). By contrast, T1DM is rare in Asian countries such as China, Korea, and Japan(1) and also uncommon in India and Venezuela, where an incidence rate of 0.1 cases per 100.000 people can be found(5). These differences in incidence may be due to genetic susceptibility as the prevalence of HLA genetic risk factors differs among populations(1). However, it has to be noted that genetic factors alone cannot fully explain these regional differences in incidence rates, implying lifestyle factors such as hygiene and childhood infections(1), as well as environmental influences such as infant and adolescent diets, vitamin D, viruses, and decreased gut microbiome diversity(6). The key role of environmental and lifestyle factors in terms of T1DM etiology is further supported by the fact that the incidence is also increasing in genetically low-risk individuals and that genetically similar populations, that are separated by socioeconomic borders, have great differences in the incidence of T1DM(6).

Additionally, a possible involvement of other environmental factors is reported, such as the role of seasonality regarding the month of diagnosis and the month of birth of children suffering from T1DM, with more cases diagnosed in autumn and winter in certain

geographical regions(18), and an increased likelihood of T1DM in certain regions in children being born in spring(19).

Saeedi et al.(9) presented that allocation to a specific World Bank Income group was associated with diabetes prevalence: in 2019, diabetes had a higher prevalence among high-income countries (10.4%) compared to low-income countries (4.0%).

Although T1DM is the predominant form of diabetes in children(1), it is often diagnosed much later, approximately one-fourth of people with T1DM are diagnosed as adults(13), even though the classification of the disease is often challenging in adults, as T1DM is often mistaken for T2DM in this group and up to 10% of adults initially classified as having T2DM, are found to have antibodies related to T1DM(5).

1.2 Biosynthesis of insulin and physiology of the ß-cell

Insulin is an anabolic peptide-hormone secreted by the ß-cells in the Islets of Langerhans with a molecular weight of 5.8kDa(20), consisting of 51 amino acids. The insulin molecule consists of two polypeptide chains, named A and B chains, with 21 amino acids and 30 amino acids, respectively, which are linked by two disulfide bonds(20). These two polypeptide chains are not generated separately but rather created by the specific processing of PI, a larger precursor(21). The primary function of insulin is to regulate glucose homeostasis. Besides, insulin plays a significant role in regulating other cellular procedures, such as DNA and RNA synthesis, protein and fat synthesis, cell growth, and differentiation(22).

Insulin exists primarily as a monomer, its circulating and biologically active form, at low concentrations(20). It forms dimers at micromolar concentrations and aggregates to hexamers - the storage form of insulin - at high concentrations and the presence of zinc ions(20). Upon secretion into circulation, the hexamers dissociate to generate bioactive insulin monomers(20).

The biosynthesis of Insulin is a multi-step process that takes place in several organelles of the ß-cells in the pancreatic islets of Langerhans. Insulin biosynthesis occurs via two intermediates(23). Initially, insulin is translated as preproinsulin and subsequently pro-

cessed to PI(20). PI is then processed to insulin and CP and stored in secretory granules until released on demand(20). In the PI molecule, the CP represents the connection between the A and B chains of insulin(21).

More precisely, in humans, a single insulin gene called INS exists, which is located on chromosome 11(24) and encodes a single-chain pre-prohormone, the preproinsulin(20). Preproinsulin is synthesized in the rough endoplasmic reticulum of the ß-cell and is then transferred to the RER lumen, where the signal peptide is separated by a signal peptidase, resulting in PI(20). Subsequently, within the cisternae of the RER, PI is brought into its native tertiary structure, the direct precursor of insulin, by rapid folding and formation of three disulfide bonds between the A and B domain(22, 24) that are essential for stability and bioactivity. After that, PI is translocated in small vesicles to the Golgi apparatus and packaged into secretory granules(24). There, PI is converted into insulin and CP(20-22, 24). In the granules of the Golgi apparatus, a decline of pH from 6.5 to about 5.0-5.5 can be noticed, leading to augmented activity of the prohormone convertase PHC1/3 and PHC2, and carboxypeptidase E (CPE), which cleave the CP and detach residual C-terminal basic amino acids from the resulting peptide chains, yielding equimolar amounts of mature insulin and CP and leaving only minimal a concentration of PI in the mature secretory granules(21). CP remains intact while stored in the ß-cells and is secreted in equimolar amounts with insulin, its original derivation(21), along with small amounts (2-3%) of PI(22). This process of insulin biosynthesis takes place in less than two hours and is efficient, as only a small amount remains as PI within the secretory granules(24).

The most potent stimulator of insulin secretion is glucose, while other agents like free fatty acids and amino acids can lead to amplified glucose-induced insulin secretion(20). Besides, other hormones and chemical substances are capable of regulating insulin biosynthesis and secretion(20), including glucagon, glucagon-like peptide, cholecystokinin, and gastric inhibitory peptide, while other substances like catecholamines are able to inhibit insulin secretion(22). However, the amount of insulin secretion induced by glucose is much higher than the stimulus of insulin secretion by protein or fat. For example, in humans, oral glucose intake results in an increase of plasma insulin levels to 250-300pmol/L in 30 min from basal rates, by contrast, ingestion of fat or protein in

similar quantities will heighten plasma insulin levels to 50 and 60 pmol/L(20). In healthy individuals, the amount of released insulin is exact to perfection to the metabolic demand, which is possible due to sensing of changes in plasma glucose levels by the ß-cells and their response through the release of the required amounts of insulin(20).
Plasma insulin levels are mostly the product of ß-cell mass and their functional status. Therefore, deficiency in either ß-cell mass or function can lead to insulin deficiency, resulting in hyperglycemia and diabetes.

1.3 Etiopathogenesis of T1DM

T1DM is considered to result from a complex interplay between a genetic predisposition, widely unknown environmental factors, the immune system, and stochastic events, but the specific immunologic, genetic, and physiologic events that lead to the manifestation of the disease and its progression have yet to be unraveled(6). Finally, T1DM culminates in the destruction of insulin-producing ß-cells.
As mentioned earlier, the disease shows two subtypes: type 1A, which is the common, immune-mediated form, and type 1B, a rare form where the cause of ß-cell destruction is unknown(1). Here, the focus lies on type 1A. It represents the result of an organ-specific immune-mediated destruction of insulin-producing ß-cells in the islets of Langerhans(25), although the exact etiology of this disease is still largely obscure. These ß-cells are capable of sensing blood glucose levels very accurately and release insulin in an exact amount to keep physiologic glucose levels within a relatively narrow range(26). Once destroyed, patients with T1DM are no longer able to maintain glucose homeostasis, which can result in both acute conditions like diabetic ketoacidosis or severe hypoglycemia, as well as in secondary complications, including micro- and macrovascular diseases(26). Therefore, one characteristic of T1DM is the need for life-long insulin treatment.
The pathogenesis of T1DM is considered to be a continuum that can be classified into three stages: stage 1 is indicated by the presence of at least two AAbs, but normoglycemia and is pre-symptomatic, while stage 2 shows two or more AAbs, but impaired blood glucose and is pre-symptomatic. Stage 3 represents the onset of symptomatic disease and clinical diagnosis(1).

Current data provides information that although the majority of individuals with long-standing T1DM have only few residual ß-cells, there is proof for ß-cell regeneration(5), and the historical dogma that T1DM is a condition characterized by the complete loss of insulin-producing cells is challenged today. Thus, the Eisenbarth model developed in 1984, although still used, does not assess the complexity and heterogeneity of T1DM(6) and evidence for residual ß-cell function, assessed by preserved CP secretion, is seen in many individuals, even with long-standing disease(2, 27, 28).

At diagnosis, a reduced ß-cell mass and function is detected, and with improvement of hyperglycemia, these ß-cells may experience a partial recuperation of insulin secretory function, resulting in a “honeymoon period” after diagnosis with minimal or no need for exogenous insulin(6). With longer disease duration, many of these residual ß-cells are lost. However, individuals with long-standing disease can show signs of the presence of residual ß-cells decades after diagnosis(2), marked by persistent CP secretion. Thus, despite declining ß-cell quantity and function over time, this decrease does not lead to a total loss of all ß-cells(27, 28), which is noteworthy because persistent CP secretion is associated with reduced hypoglycemic events and microvascular complications(29, 30).

1.3.1 Disease pathogenesis and autoimmune-mediated processes

Much of our knowledge about the pathogenesis of T1DM is derived from animal models, such as the non-obese diabetic (NOD) mouse model, as well as from autopsy studies of pancreata of individuals with T1DM. Studies using the NOD mouse model have provided important insights into the immunological pathways and the pathophysiological and environmental impact on disease(26).

The pathogenesis develops as a consequence of a complex interplay between the ß-cells and innate and adaptive immunity(6). The NOD mouse model demonstrated that T1DM develops as a result of a breakdown of immune regulation, leading to the expansion of autoreactive CD4+ and CD8+ T-cells, AAb-producing B-lymphocytes, and activation of the innate immune system, that participates in the destruction of the insulin-producing ß-cells(26). T1DM can be considered the end stage of a consecutive series of deteriorated homeostatic checkpoints for selection and activation of immunity(31),

and the autoimmune responses in T1DM are determined by a balance between pathogenic and regulatory T-lymphocytes(26). These observations are also applicable for the pathogenesis in human T1DM.

Evidence that T1DM is an autoimmune disease with autoreactive CD8+ T-cell playing a key role is derived from autopsy studies of pancreata from T1DM patients, who had died shortly after diagnosis(32). The majority of subjects had significant lymphocytic infiltration within insulitis lesions, with predominance of CD8* T-cells, followed by macrophages (CD68+), CD4+ T-cells, and B-lymphocytes (CD20+)(33), while plasma cells (CD138+), regulatory T-cells, and NK-cells were rare in these lesions(34). The predominance of CD8+ T-cells, along with up-regulation of MHC class I by islet cells, implies the key role of cytotoxic T-cells in ß-cell destruction(32). Further evidence for the crucial role of T-lymphocytes as the primary mediators of disease pathogenesis is supported by the recognition of autoreactive T-lymphocytes in the circulation of subjects diagnosed with T1DM, as well as in individuals at risk for T1DM, before disease onset, and the fact that immunosuppressive therapies that specifically target T-lymphocytes are able to slow down disease progression(20). In this regard, it is presumed that defects in negative selection of T-cells in the thymus play a key role in genetic predisposition towards T1DM(26). The important role T-cells play in the pathogenesis of T1DM is furthermore underpinned by the fact that NOD mice that are genetically athymic or T-lymphopenic or were thymectomized at birth do not progress to T1DM(35). In support of the notion that B-cells do also play an essential role in the pathogenesis of T1DM is the observation that NOD mice who are absent of B-cells do not develop insulitis or diabetes(32). Thus, T1DM can be considered a T-cell mediated, B-cell facilitated disease(36).

The assumption that T1DM can be considered the end stage of a consecutive series of deficient homeostatic checkpoints for selection and activation of immunity(31) is underpinned by the fact that immune tolerance to self is usually maintained through a series of checkpoints in both the thymus and periphery. Hence, in T1DM, this balance between potentially pathogenic self-reactive immune cells and the regulatory mechanisms that keep them under control is destroyed, resulting in autoimmunity and loss of tolerance to ß-cells(32). It is suggested that ß-cell autoantigens may be generated by

posttranslational modifications leading to “foreign” ß-cell proteins, which are not present during selection in the thymus and therefore result in AAb production(37). Since proteins with tissue-restricted or peripheral expression, such as these newly generated “foreign” proteins, are supposed to be unavailable for presentation in the thymus, it has been suggested that the only way to achieve tolerance to such proteins is through mechanisms of peripheral tolerance(32).

The immunologic process of ß-cell destruction is assumed to be initiated when an as yet unknown immunologic event occurs in individuals with genetic risk factors and launches a chronic low-grade immunologic process that involves infiltration of innate immune cells into the pancreatic islets(26). There, ß-cell peptides are presented by antigen-presenting cells, precisely by macrophages and dendritic cells, which then immigrate to the pancreatic lymph nodes in order to interact with autoreactive CD4+ T-cells through MHC, which subsequently lead to the mobilization of autoreactive CD8+ T-cells via interleukin signaling(6). Latter then return to the islet and eradicate ß-cells that express self-antigens through MHC class I(6). This destruction of ß-cells is further amplified by proinflammatory cytokines and ROS, which are set free from innate immune cells (macrophages, natural killer cells, neutrophils)(6). Moreover, ß-cell death leads to the release of intracellular antigens, resulting in the mobilization of additional autoreactive T-cells, leading to the augmentation of the initial autoimmune response(38). This process is further exacerbated by defects in regulatory T-lymphocytes, which are not able to successfully suppress autoimmunity(6). In the pancreatic lymph node, activated T-cells further lead to the stimulation of AAbs production against ß-cells by B-lymphocytes, these AAbs can later be found in the circulation and serve as biomarkers(6). This immunologic attack specifically targets insulin-producing ß-cells, while the alpha-cells, delta-cells, and pancreatic-polypeptide-cells remain unharmed as evidence for immune specificity(26).

In the period near clinical presentation, ß-cell killing is constantly increased. In addition to ß-cell death, the disease progression is implied by reversible changes in the kinetics of insulin secretion(39), indicating that there are changing chronic cellular stressors during advances in disease development.

1.3.2 Etiology of T1DM

1.3.2.1 Genetic determinants

T1DM is considered a polygenic disease, where a small number of genes account for large effects on disease development and progression, such as HLA, and a large number of genes account for small effects(32). Today, over 50 loci are known to contribute to T1DM genetic risk(40, 41).

The HLA region

An important role in the pathogenesis of T1DM is attributed to MHC class I and II molecules, which are thought to account for both positive and negative selection of autoreactive T-cells(32). Observations to assess an association between genetic HLA markers and T1DM were already performed since the 1970s through approaches in DNA-based genotyping technologies, international collaborations, and genome-wide association studies(42). More than 40 non-HLA regions have been related to T1DM(43). However, the HLA region remains, without doubt, the most significant contributor to genetic susceptibility to T1DM, as it accounts for 30-50% of genetic T1DM risk, even though the explicit processes by which HLA and other affiliated loci contribute to T1DM susceptibility still need to be elucidated(42). The remaining genetic risk is attributed to several diverse genes, all having a small individual effect on genetic susceptibility(43). It is thought that autoimmunity develops due to an altered balance between tolerance induction and immune responsiveness, which is influenced by the presence of class I and class II molecules and their presentation of tissue-specific antigenic peptides(32). HLA plays a major role in antigen presentation and controls immune responsiveness to one or more islet cell antigens, either in the thymus or in the periphery, or both(32, 44). It is assumed that in genetically susceptible subjects, the class II molecules are not able to present self-peptides in an effective way, thus resulting in an inadequate negative selection of autoreactive T-cells(32).

Certain combinations of alleles or haplotypes of the DRB1, DQA1, and DQB1 genes influence the individual disease risk, ranging from highly susceptible to highly protective combinations(45). Specific genotypic combinations such as DRB1*0301-DQA1*0501-

DQB1*0201 (DQ2) /DRB1*0401-DQA1*0301-DQB1*0302 (DQ8) heterozygotes, referred to as DR3 /DR4(32, 42), which are common among North Americans and Europeans, represent the DR/DQ haplotypes associated with the highest genetic risk for T1DM in the western world(42, 46). Besides accounting for the greatest observed susceptibility(7, 47), these two haplotypes simultaneously represent the most significant risk factors for the expression of ß-cell-targeting AAbs(1). Consequently, HLA-associated risk factors are likely to increase T1DM risk through their predisposition to develop ß-cell targeting AAbs(1).

Importantly, as the frequency of HLA haplotypes and genotypes related to T1DM differs among populations, so do their predisposing or protective effects(25).

Even though 40% of the general population in the US inherit either the HLA-DR3 or -DR4 allele, at least one of these alleles is found in 95% of individuals with T1DM(43). Moreover, about 40% of T1DM patients are heterozygous for HLA-DR3 and DR4, while less than 3% of the general population show this combination(43). Children who carry the highest-risk genotype DR3/4 have a 1 in 15 to 1 in 25 risk to develop T1DM, compared to a risk of 1 in 300 for the general population(43, 48). This risk is significantly increased if the child has a sibling with diabetes and the same haplotype(43). Moreover, Steck et al. suggested that children who show the HLA risk genotypes DR3/4-DQ8 or DR4/DR4 and have a relative with T1DM, have risk for expressing islet AAbs during childhood that exceeds 20%, while children with the same genotype but a negative family history have a 1 in 20 risk(43). Present approaches for prediction of T1DM make use of the major genetic risk factors presented earlier, in particular by genotyping for HLA-DR and HLA-DQ loci in combination with family history and screening for AAbs directed against the ß-cells(49), to identify people at risk for T1DM. Although in more than 85% of individuals with T1DM, a positive family history for the disease is absent(43), in the remaining 15%, high family aggregation can be found, and first-degree relatives have a higher risk of developing T1DM than does the general population, with a mean disease prevalence of 6% in siblings compared to 0.4% in the remaining population(43). Within families, disease susceptibility depends on the degree of genetic identity with the relative who has T1DM, and the highest risk is observed in identical

twins(50). Consistent with these findings, Dabelea et al.(51) reported that approximately 400 in every 100.000 US children will be born into a family affected with T1DM, and these children will have a disease risk that surpasses 5%, vs. 0.4% disease risk in the remaining children. This risk can be further classified based on which family member is affected with T1DM (2-3% for children with an affected mother vs. 6-9% if the father has T1DM)(52). It can be said that siblings of patients with T1DM are at a 15-fold increased risk for T1DM than the remaining population, which highlights the important role of genetic factors in disease susceptibility(43). Moreover, if one of these children will have two affected first-degree relatives, the risk will rise to 20%(31). Besides, siblings with the DR3/4 haplotype who have the ident both HLA haplotypes like their diabetic DR3/4 sibling have a higher disease risk than when only one or no haplotype is corresponding(50). If a child shows DR3/DR4 heterozygosity, it is at increased risk for T1DM compared to DR3/DR3 and DR4/DR4 homozygosity(42).

By contrast, the HLA-DQ locus also harbors T1DM protective alleles(47). Certain HLA haplotypes provide strong protection from T1DM, like DRB1*1501 and DQA1*0102-DQB1*0602(53), which confers resistance to T1DM, even when islet AAbs are present(43). Approximately 1% of new-onset patients with AAbs carry DQB1*0602, whereas 20% of the general population have this allele(43). The significance of protective effects certain alleles such as HLA DQB1*0602 offer, becomes obvious when the risk for T1DM in a child with family history and these protective alleles is reduced to 1% of the risk in a child with a similar family history but the absence of this allele(31).

HLA class I is also linked to diabetes risk and is considered to have an influence on age of onset and rates of ß-cell destruction(54). Besides, for some HLA class I loci, both susceptible and protective alleles were found, such as for HLA-B (as B*39 is susceptible and B*38 is protective) and for HLA-A (A*24 susceptible and A*11 protective)(55, 56).

Although the genetic complexity that underlies this metabolic disorder of T1DM is not yet unraveled, much progress has been made during the last decades in order to get a better understanding of the pathogenesis and genetic background(5). Despite these complexities mentioned above, knowledge about underlying genetic determinants that

modify the risk of developing T1DM provides great opportunities for prediction and prevention, risk stratification, and selection of therapeutic targets to improve disease management. The major opportunity of HLA genetics lies in screening for prevention trials, as certain alleles are used to identify individuals at high risk for disease and for the exclusion of subjects with resistance alleles.

The non-HLA region

The highest risk DR/DQ haplotypes for T1DM are DR 3 and DR4, which, among other additional genes within the HLA region, are the major genetic risk factors for T1DM(7, 43), followed by genes outside the HLA region, which also confer to disease risk but with a smaller contribution than that of HLA(7).

Currently, over 50 non-HLA regions of the genome are known that harbor T1DM susceptibility genes and thus have a remarkable effect on the risk for T1DM(46).

Candidate gene studies identified the following four non-HLA T1DM risk loci: INS, CTLA4, PTPN22, and IL2RA(57-60). After HLA, polymorphisms of the INS and PTPN22 genes confer most to diabetes risk but with a smaller effect(43). Current data indicate that these risk loci for T1DM affect the immune system on multiple levels, such as at the establishment of the immune repertoire, cell function, or controlling of cellular responses leading to autoimmunity(7).

Autoantibodies and their genetic basis

AAbs against islet antigens are the hallmark of disease development. One of the most important changes in T1DM risk status takes place when AAbs against ß-cells evolve, which rarely happens prior to 6 months of age(31). These islet-specific AAbs often appear months to years before clinical onset and are prevalent for varying durations after onset(38). So far, AAbs to different islet antigen groups have been identified: insulin or proinsulin, islet cell cytoplasm, GAD, IA-2, and ZnT8, which are adequate for the purposes of prediction for T1DM in children and young adults, while being useful, but unsatisfactory for sensitive prediction of T1DM in adults(31). Although they are not considered the cause of the disease, they are used as a serologic proof of ongoing autoimmune attack, as about 90-95% of newly diagnosed subjects express at least one

positive AAb(38, 61). The number of present islet AAbs in a patient contributes to risk information: while the presence of AAbs to just one of these four antigen groups alone is related to only a minimal rise in risk, both in individuals with and without a family history of T1DM, the disease risk is remarkably increased when islet AAbs to two or more of the antigen groups are present in a child(31). Furthermore, the earlier AAbs appear, the faster T1DM develops(31). With the combined measurement of ZnT8A, GADA, IA-2A, and IAA, it is possible to increase detection rates for autoimmunity to 98% at disease onset(62), showing the immense value of islet-specific AAbs in terms of disease prediction(32). At diagnosis, 70-80% of Caucasian children with T1DM present with ICA, with falling frequency as the disease progresses(63). GADA are measurable in 70-80% of individuals with new-onset T1DM and in prediabetics before clinical onset(32), but in contrast to ICA, GADA show persistence over several years into the disease process, and this is why GADA testing should be preferentially performed prior to ICA testing in adults where LADA is assumed(38). By contrast, IA-2A is less commonly found at T1DM onset than ICA or GADA(64). IAA are found in 40-50% of children with new-onset T1DM(65). IAA are usually the first islet AAbs to appear in the disease course and a strong indicator of disease progression, as a high titer of IAA at younger ages is related to a more aggressive disease course(32). Besides, the age of T1DM diagnosis was remarkably correlated with the age of development of the first AAb and IAA levels (32). Testing for IAA should be performed before the initiation of insulin treatment because measurements of IAA are no longer valid once the patient received insulin injections for >10 days, since exogenous insulin can elicit insulin antibody responses that seem to be identical with AAb production(38). ZnT8 AAbs were found in 60%-80% of newly diagnosed T1DM and in up to 30% of patients suffering other autoimmune disorders related to T1DM, compared to <2% of healthy controls and <3% of patients with T2DM(62). In addition, 26% of individuals, who were negative for the other AAbs, expressed AAb to ZnT8(62).

T1DM is seen as a continuum of 3 stages, from pre-symptomatic appearance of AAbs to apparent clinical disease, while there is no typical progression from the presence of islet AAbs to overt diabetes, which can be a matter of weeks to decades, some islet AAb-positive individuals can sustain a low level of insulin production for years(31).

An analysis of the DIPP study and the Finnish pediatric diabetes register participants confirmed that DR-DQ haplotypes are strong predictors for the first AAb to appear(66), and in AAb-positive TEDDY participants, specific HLA haplotypes were linked to the primary AAb observed at seroconversion: IAA usually is the first AAb to appear in children who carry the HLA-DR4-DQ8 haplotype(1), while children who develop GADA first usually carry the HLA-DR3-DQ2 haplotype(1). ZnT8A is associated with DR13-DQB1*0604 haplotype(67).

1.3.2.2 Environmental factors

Finally, environmental factors are considered to have a substantial effect on disease development and progression. Notwithstanding some rare monogenic forms of T1DM(8), the common form is thought to result from interactions of multiple genetic and environmental factors. The latter factors are thought to play an increasingly important role in disease pathogenesis, and the discordance rate for disease incidence in identical twins strongly reinforces this observation(38). Interestingly, the concordance rate for T1DM in monozygotic twins is less than 100%(68), it is estimated to average 50%, compared to 6%-10% in dizygotic twins(43), which is comparable to the concordance rate in nontwin siblings(43). An essential role in diabetes etiology due to environmental factors is ascribed to the “hygiene hypothesis,” which postulates that the increase in incidence of T1DM is most pronounced in industrialized societies where exposure to parasites is reduced(26). Other environmental determinants include the composition of gut microbiota, vitamin D, omega-3 fatty acids, environmental stress, and toxins(69). Besides, a potential role for viruses in disease pathogenesis is discussed(69). In patients with recently diagnosed T1DM, antibodies to Coxsackie viruses were found more often than in controls(70). A recent meta-analysis(71) proposed a statistically significant association between enteroviral infections and diabetes-related autoimmunity or clinical apparent T1DM. It has to be mentioned that most of these studies performed analysis on peripheral whole blood, thus, solid proof of a viral infection within the pancreas was absent(72).

However, it still has to be elucidated to what extent environmental factors affect autoimmunity. Do they lead to the development of islet AAbs in subjects at genetic risk, cause a faster disease progression in subjects with apparent AAbs, or both(73)?
Nevertheless, wide gaps in knowledge about T1DM etiology still do exist(74). Most of the information on underlying genetic risk factors of T1DM was collected from investigations of pediatric-onset T1DM in European-ancestry populations, while the most substantial increase in T1DM incidence is observed in non-European ancestry groups, which show partially congruent but also different genetic associations with T1DM(74). Besides, as mentioned earlier, certain HLA haplotypes and genotypes associated with T1DM vary among populations, and so does their susceptibility(25). Genetic risk prediction will play a key role in facilitating early intervention in T1DM by identifying individuals at high risk for the disease. The present challenge is to unite the wealth of knowledge about T1DM genetics and make it useful for diagnosis, risk assessment, and clinical routine(25).

1.4 Diagnosis

For diagnosis of T1DM, several diagnostic serum markers can be taken into account, such as glucose-related biomarkers, AAbs against ß-cell autoantigens, and the serum CP levels.

1.4.1 Glucose-related biomarkers

These include a fasting blood glucose higher than 126mg/dl, any blood glucose of 200mg/dl or higher with symptoms of hyperglycemia, or an abnormal 2h oral glucose tolerance test (OGTT)(8). When symptoms are absent, dysglycemia must be verifiable on two different occasions(8). Diabetes can also be diagnosed based on glycated hemoglobin concentrations (HbA1c) of 6.5% or higher(8). Latter test represents an indicator of the average blood glucose levels of the past 2 to 3 months(75). Thus, it is a reflection of chronic hyperglycemia, and although this test may not be as sensitive as fasting plasma glucose (FPG) and the OGTT when it comes to diabetes diagnosis(6), it shows some advantages compared to FPG and OGTT, that are greater convenience, greater preanalytical stability, and less day-to-day disturbance during stress, diet or

illness(8). Nevertheless, all these biomarkers still rely on the consequences of hyperglycemia, such as the resulting high blood glucose or glycated hemoglobin. Therefore, we still experience a lack of effective serum biomarkers that reflect ß-cell mass or function and ß-cell stress and thus could serve as predictors of the disease progression(76), enabling to detect T1DM development at a very early stage before symptomatic onset.

1.4.2 Autoantibodies

AAbs against ß-cell autoantigens have shown great potential in distinguishing T1DM from T2DM and other subtypes based on their presence in patients at risk. So far, the following commonly tested AAb markers are used to diagnose T1DM: autoantibodies to islet-cell cytoplasm (ICA), glutamic acid decarboxylase autoantibodies (GADA), insulinoma-associated protein 2 autoantibodies (IA-2A), insulin autoantibodies (IAA), and zinc transporter 8 autoantibodies (ZnT8A)(61). With the combined measurement of these AAbs, it is possible to raise detection rates for autoimmunity to 98% at T1DM onset(62). So far, measurements of GADA and IA-2A are recommended for the initial diagnosis of T1DM(77). If negative, individuals should be tested for ICA, and in children, testing for IAA should be performed(77). The following positivity rates for islet-antigen specific AAbs at clinical diagnosis of T1DM for individuals from the general population have been demonstrated: ICA were found in 70-80% of individuals, IAA in 60% of children, IA2 in 60% of individuals, and GADA in 70-80% of individuals (77). ZnT8A can be detected in the prediabetic period, are seen to persist in individuals with long-standing T1DM, and were detectable in 63% of individuals at clinical diagnosis of T1DM(78). At least 1 AAb is present in >95% of individuals with T1DM upon clinical diagnosis(38). It has to be noted that these AAbs alone are insufficient to detect all new-onset T1DM cases since there remains a subset of individuals who do not show the above-listed AAbs upon diagnosis, including patients with type 1B, where the destruction of ß-cells is not immune-mediated.

1.4.3 Serum C-peptide levels

Stimulated serum CP levels, as a surrogate for insulin - since both insulin and CP are released in equimolar amounts from mature granules - have been considered a persistent and sensitive indicator of ß-cell function and useful to differentiate autoimmune-diabetes from other diabetes subtypes(79). However, CP measurement may not be a reliable diagnostic marker with the increasing obesity epidemic.

After diagnosis, the capacity to maintain residual ß-cell function, as determined by the level of CP, is variable regarding the number of individuals who still show signs of residual CP production despite long-standing T1DM(2). In this regard, disease heterogeneity is a significant aspect. Even though endogenous ß-cell mass and function decreases in long-standing disease, it does not result in a total loss of all ß-cells(6, 27, 28). This is a significant observation because in the Diabetes Control and Complications Trial, persistent CP secretion >0.2 nmol/L was related to a reduced incidence of microvascular complications and hypoglycemia(6). A more recent DCCT report indicates that even lower levels of CP are associated with fewer diabetes complications(29, 30). Besides, residual CP secretion after T1DM diagnosis could further raise the chances of a treatment response to future interventions aiming at rescuing or augmenting residual ß-cell mass(6).

It has to be mentioned that the American Diabetes Association (ADA) and the National Institute for Health and Care Excellence guidelines advise against routine testing of CP or AAbs to diagnose T1DM(80). However, these markers could be helpful for diagnostic purposes in clinically questionable cases(8, 81).

1.4.4 Correct classification of diabetes subtypes

Correct disease classification is essential in order to assign the most effective preventive, diagnostic, and treatment strategies for each patient. However, absent AAbs do not allow the complete exclusion of T1DM. Classification is a prerequisite for correct therapy, but some individuals cannot be clearly classified to T1DM or T2DM at the time of diagnosis(8).

Children with T1DM usually present with symptoms such as polyuria, polydipsia, weight loss, and fatigue, which can occur several days to a few weeks before to diagnosis,

and about a third shows symptoms of diabetic ketoacidosis (DKA) at diagnosis(82). Unfortunately, this number is increasing(82), highlighting the urgent need for early diagnosis and better diagnostic criteria. Stage 3 diabetes becomes apparent when typical biochemical parameters such as hyperglycemia, glucosuria, ketonemia, and ketonuria develop. However, adults can have a more variable disease onset and might not show the classic symptoms seen in children(6). Thus, especially among adults, the correct classification of T1DM or other subtypes of diabetes can be challenging. It is assumed that approximately 5-15% of adults initially being diagnosed with T2DM, do actually express AAbs against islet cells and have T1DM(5). Although the CP level is an important marker for endogenous insulin deficiency, a specific clinical feature to perfectly distinguish T1DM from T2DM remains elusive. Other factors that could impede correct diagnosis of T1DM include the problem of obesity since observational studies show increasing incidence of overweight and obesity among children and adolescents with T1DM(83), contributing to difficulties in disease recognition(5). Thus, obesity should not prevent from T1DM diagnosis. To distinguish between T1DM, T2DM, monogenic diabetes, and other forms of diabetes, a combinatory approach that is able to assess risk factors for T1DM such as genetic susceptibility, family history, patient characteristics like age and body mass index (BMI), and biomarkers including an islet AAb panel is needed(6, 8).

1.4.5 Predictive biomarkers

The pathogenesis of T1DM represents a continuum that can be categorized into three stages: stage 1 is marked by the presence of at least 2 AAbs, but normoglycemia and is pre-symptomatic, while stage 2 and 3 are defined by the presence of AAbs and metabolic abnormalities, reaching from impaired blood glucose at stage 2 to symptomatic onset at stage 3(6). As mentioned earlier, there are multiple well-established serum biomarkers for the diagnosis of T1DM, including a combination of glucose, glycated molecules, CP, and AAbs(76). However, they often indicate a late stage of the disease when about 90% of the ß-cells have already been destroyed(76). Therefore, it is imperative to have specific markers that can predict the disease course and its progression in order to identify individuals at risk for T1DM and enable interventions to slow or stop

immune-mediated ß-cell loss before most of ß-cell mass has been lost at the time of diagnosis(76). The pursuit of novel predictive biomarkers should be the focus of further investigation.

Several efforts have been made in order to reach successful disease prediction. Today, we have four main categories of biomarkers that are either already in use or the focus of research to enable disease prediction: genetic markers, AAbs, risk scores, and novel biomarkers(76, 78).

1.4.5.1 Genetic markers

Although most of T1DM cases are absent of family history for the disease, FDR of an affected family member have a 15-fold increased risk for T1DM(84). In this notion, HLA genes are responsible for about half of the genetic risk. The remaining risk is comprised of non-HLA genes and other genes outside the HLA region, as presented earlier. Therefore, the aim is to detect those individuals at high risk for the disease through genetic screening, combined with other biomarkers that will be discussed below, since the positive predictive value of HLA testing alone is low, as the majority of subjects with high-risk genotypes will not progress to T1DM(85). Therefore, even those individuals who show high-risk genotypes will need follow-up testing for AAbs(70).

1.4.5.2 Autoantibodies

Besides having great diagnostic utility in T1DM, islet targeting AAbs are also considered the current gold standard for the prediction of disease development(76), as an asymptomatic period that can be determined by serum islet AAbs precedes the symptomatic onset of T1DM.

Most subjects with a single AAb do not develop T1DM(86), whereas the presence of two or more AAbs in children is related to a significantly increased disease risk(31), and the majority of patients with two or more islet-targeting AAbs will most commonly progress to symptomatic disease(86, 87). The risk of progression to the symptomatic stage is not only linked to the number of AAbs expressed by an individual but also to the age

of seroconversion of the first AAb, as well as the AAb type, affinity, and titer(86). Overall, the number of islet AAbs is a stronger predictor of the disease than any specific combination of islet AAbs(77).

Although the exact role of islet AAbs in disease pathogenesis still remains unclear, islet AAbs are unlikely to cause T1DM and are rather a reflection of disease progression or a secondary response(76). Among all discovered AAbs, ICA, GADA, IAA, IA-2A, and ZnT8A remain the most sensitive and specific for disease prediction(76). IAA are usually the first AAbs to appear, and together with GADA, the most common islet AAbs in childhood(88). GADA are the characteristic emblem of adult-onset T1DM(88). Only a small subset of children shows IA-2A or ZnT8A as their first AAb, and they all persist through diagnosis(76). The detection of IA-2 or ZnT8 AAbs is associated with more rapid progression to clinical overt T1DM than the absence of both(89).

Despite the apparent utility of AAbs in T1DM prediction, combined with information about genetic susceptibility, screening for AAbs shows several limitations. First, AAb screening is only recommended for first-degree relatives (FDR) in the setting of clinical trials, although 85% of people who develop T1DM lack a family history of disease, or for individuals from the general population who show T1DM related genetic risk factors(8). Second, only a proportion of AAb-positive subjects will develop clinical diabetes(90). Finally, AAbs are not helpful as biomarkers to assess therapeutic outcomes(90).

Various studies suggest that the assessment of islet AAbs in individuals with high genetic risk for T1DM will recognize those who are likely to progress to overt T1DM(8). Such a testing strategy, together with education about symptoms and close follow-up, could prove successful in order to achieve earlier identification of T1DM onset(8). Indeed, the risk of T1DM grows as the number of relevant AAbs increases(31), as mentioned earlier.

Also, the age at which AAbs appear should be taken into consideration as a predictive marker, since the risk of developing islet AAbs in a 1-year-old child is significantly increased compared to the risk of a 5-year-old child(88). Progression from a single to two or more AAbs is seen more frequently in children younger than five years of age and

occurs less commonly after four years of initial seroconversion(86). The age also affects the progression rate to hyperglycemia: if a child shows seroconversion to islet AAb positivity by age 1, disease progression will happen faster than with seroconversion at five years of age(88). By contrast, age barely affects the risk of progression to clinical diabetes in children with multiple islet AAbs(88). It is noteworthy that biomarkers change with age and predicting T1DM in adults is distinct from disease prediction in children, as older patients are less likely to present with the same biomarkers found in children(88). That is mainly because genes have a smaller impact on the disease, as in individuals with disease onset after 20 years of age, the high-risk HLA DR and DQ haplotypes are less common(88). Second, subjects with adult-onset T1DM show less islet AAbs than those subjects in whom T1DM develops during childhood, and in many individuals with adult-onset T1DM, only GADA is present(88). Therefore, the smaller number of islet AAbs diminishes the ability of disease prediction or diagnosis in adult-onset T1DM.

1.4.5.3 Risk score quantification

None of the AAbs associated with T1DM are currently able to provide high enough specificity or are accessible in high enough throughput methods to be suitable for routine population screening(78). Therefore, risk scores based on other clinical criteria allow us to identify individuals in the prediabetic phase in a more specific way, thereby complementing the aforementioned AAb screening(78). One of them is “The Development, Validation, and Utility of the Diabetes Prevention Trial-Type 1 Risk Score” (DPTRS), a risk score based on data from the DPT-1, assessed for disease prediction in FDR- and SDR- ICA-positive relatives of individuals with T1DM(91). This score utilizes several parameters, such as age, BMI, fasting CP levels, total glucose, and total CP levels obtained from a 2-hour OGTT(78). Similar risk scores are available, but it was not yet possible to demonstrate their predictive utility in the general population(78). Even though diabetes risk scores are able to predict probability of T1DM onset to a certain extent, they are not suitable to assess the severity of ongoing autoimmune process or ß-cell destruction.(78) The future goal will be to implement similar risk scores

for use in the general population and identify those individuals at risk for T1DM before clinical onset.

1.4.5.4 Potential novel biomarkers

There is ongoing research for novel biomarkers. Here, the focus lies on three main categories of novel biomarkers: protein biomarkers, nucleic acid biomarkers, and metabolomic biomarkers.

Protein biomarkers: serum protein biomarkers, PTMs, Cytokines, PI:C

Despite being highly abundant in plasma, the serum protein biomarkers lack specificity to insulin-producing ß-cells(76). Besides, several studies showed inconsistency and were not able to provide promising protein biomarkers. Thus, further research in this field is needed. Likewise, research towards assessing cytokines as biomarkers still needs to be continued, as the lack of organ specificity is a potential problem since serum cytokines can change in response to damage and inflammation in all types of tissues and in many immune-related diseases(76). Also, the research regarding post-translational modifications (PTMs) as biomarkers for disease prediction is still in its infancy(76).

A possible bright spot in biomarker research could outline the ratio of PI to CP (which is a surrogate for insulin). It was demonstrated that an elevation in this ratio is indicative of ß-cell dysfunction and assumed to be a reflection of alterations in insulin protein folding and processing(92). Increased PI:CP ratio is seen to precede T1DM onset in several studies, especially in younger children(92, 93) and therefore has potential as a diagnostic biomarker, as it is able to represent the direct functional ability of the pancreatic ß-cells and thus is capable of quantifying the severity of ß-cell dysfunction during the preclinical stages of T1DM. Furthermore, PI:CP ratios at diagnosis are seen to be inversely correlated with the length of the honeymoon period(93). Future studies investigating PI:CP ratios, in combination with other biomarkers, are necessary for the evaluation of their utility in predicting T1DM onset during the pre-symptomatic phase(76). Finally, the evaluation of this ratio may also permit supervision of disease evolution after diagnosis.

Nucleic acid biomarkers

Circulating unmethylated insulin DNA has generated great interest as a biomarker target for early identification of ß-cell death in T1DM(94, 95). This is due to the fact that certain cytosine-guanine sites in the insulin gene are specifically unmethylated in ß-cells and methylated in most other tissues(76). As T1DM progresses over time, fragments of the characteristic unmethylated insulin DNA, which is thought to originate from damaged pancreatic ß-cells(88), are released into the bloodstream and become measurable(76). Thus, the amount of unmethylated insulin DNA in the circulation may be a reflection of the degree of ß-cell death(76). In line with these findings, Sims et al.(96) postulated that so far, most studies investigating circulating DNA biomarkers of ß-cell death have proposed that certain genes that are inhibited show proof of cytosine methylation, by contrast, genes that are expressed lack this alteration. Therefore, the ß-cell-specific gene encoding preproinsulin (INS) has been the target of interest regarding this topic, and studies considered the absence of cytosine methylation of this gene to be a typical feature of ß-cells(96). By the application of different PCR methods that target distinct cytosine residues in the INS gene, multiple studies have been able to measure elevated levels of unmethylated INS DNA in the circulation of NOD mice just before T1DM onset(96). These observations are in agreement with the notion that dying ß-cells raise levels of circulating unmethylated INS DNA and were subsequently confirmed in subjects prior to or with recent onset of T1DM(94, 96). Consistent with these observations, Herold et al.(94) have shown that the ratio of unmethylated/methylated insulin DNA was persistently and significantly higher in subjects with new-onset T1DM and subjects at high risk for T1DM, compared to healthy controls(97). In addition, other ß-cell-enriched genes, like the glucokinase gene or islet amyloid polypeptide, have also been the focus of research(96), but their clinical relevance remains uncertain. While the sensitivity of these DNA-based biomarkers appears to be high due to the fact that they are detected by using sensitive PCR techniques, their specificity remains a relevant restriction, as some tissues display proof of unmethylated INS DNA, even though at lower levels in proportion to methylated INS DNA levels(<20%)(96). Nevertheless, when considering the difference in mass between the very low ß-cells mass and other tissues in the body, it seems to be a possible option that an unmethylated INS signal

could originate from one of these other tissues(96). However, the utility of circulating DNA markers in the prediction of T1DM still needs further evaluation, as in theory, these markers must sooner or later be depleted with the advancing loss of ß-cells, which possibly limits their utility as soon as ß-cell mass is low, thus, other markers of ß-cell death could be helpful in supplementing nucleic acid biomarkers in their predictive potential(88).

Metabolomics biomarkers

Glucose is a helpful and straightforward metabolic biomarker that has great utility as a diagnostic biomarker of diabetes, as mentioned above. However, it is not specific to T1DM, and its predictive potential is limited since impaired glucose metabolism is supposed to reflect a late pre-symptomatic stage in disease pathogenesis, often right before progression to overt T1DM. A few amino acids and lipid metabolites are assumed to be associated with T1DM(76). A significant role in inflammation and insulin signaling was assigned to sphingolipids, which have been evaluated for biomarker utility(76). In addition, decreased levels of phosphatidylcholine at birth, reduced triglycerides and antioxidant ether phospholipids throughout follow-up, and increased proinflammatory lysophosphatidylcholine have been found various months before seroconversion to AAb positivity(98).

With further research, metabolomics biomarkers may have the potential to contribute to disease prediction.

Although it is possible to identify children at risk for the disease through a combination of AAb screening, genetic risk susceptibility, and other parameters, the challenge of having a diagnosis but no treatment option remains, as no therapy is yet able to halt the progression to clinical T1DM(99). At this point, the following question may arise: why then diagnose pre-symptomatic T1DM? Because it is the first important step towards effective treatment of the disease. In addition, with further research, novel targets for prevention will be identified. Moreover, there is evidence that therapeutic interventions in pre-symptomatic T1DM may be more promising of success than when T1DM becomes clinically overt because individuals in the pre-symptomatic stage do probably

show a greater ß-cell function and thus may be more responsive to immunotherapeutic treatment strategies(99). That is why we need to find a widely applicable screening method in order to identify individuals at risk for T1DM before symptomatic onset and bring it into clinical routine(99). Besides, another advantage early diagnosis of T1DM provides is the reduction of events associated with metabolic decompensation, such as DKA(99), which is linked to increased mortality, worse metabolic control, and adverse neurocognitive outcomes(86). Early diagnosis is associated with a better metabolic outcome, is easier to manage with decreased hypoglycemic episodes, and the preservation of CP secretion is linked to a reduced risk of progression to complications such as retinopathy and nephropathy. All in all, early diagnosis is able to reduce mortality and ameliorates long-term outcomes(86).

Another challenge related to early diagnosis remains: implementing general population screening into clinical routine. So far, in most studies, FDR and SDR of individuals with T1D, who are approximately at 10-20-fold increased T1DM risk compared with the general population, have been undergoing screening for the presence of islet AAbs in different studies, like the DPT-1 and the pathway to prevention natural history trial of TrialNet(100). The ability of this approach to risk screening is limited by the fact that only about 15% of newly diagnosed subjects with T1D show a positive family history of disease(100). Thus, it is not able to assess the majority of susceptible patients, and therefore, most of individuals at risk are omitted(100). A different strategy to improve the detection of at-risk individuals is to screen for genetic risk and subsequently assess these subjects for onset of islet AAbs during follow-up, which has been realized in the TEDDY natural history study(101) and the DAISY study(102). In order to assess at-risk children beyond high-risk HLA genotypes, a general population should be screened for islet AAbs. The Fr1da project(100, 103), which was the first such general population-based screening, was launched in 2015 in Bavaria, offered screening for multiple islet AAbs at well-child visits at 3 and 4 years of age for up to 200 000 children from the general population and could statue an example for possible future approaches of general population screening.

However, the increasing incidence of T1DM worldwide, the fact that about 80% - 90% of ß-cell mass is considered to be lost at the moment of clinical diagnosis, the resulting

acute and chronic disease complications, as well as the practical and emotional problems individuals with T1DM are confronted with, and the possible economic burden associated with the disease, imply the urgent demand for more efficient early disease prediction and diagnosis. All in all, biomarkers play a crucial role in achieving these goals in the future.

1.5 Associations and complications

1.5.1 Associations and comorbidities

In addition to diabetes-related complications, it is important to be aware of common comorbidities related to T1DM that can complicate disease management. Diabetes comorbidities are conditions that occur more frequently in patients with diabetes than in age-matched individuals without diabetes(104).

1.5.1.1 Autoimmune diseases

Individuals with T1DM are at higher risk to develop other autoimmune diseases that may affect several organs and tissues resulting in a non-organ-specific autoimmune disease, e.g. rheumatoid arthritis, or may be organ-specific autoimmune diseases, whereby thyroid disease, celiac disease, and gastric autoimmunity including pernicious anemia are the most common specific autoimmune disease associated with T1DM(105). These associated autoimmune conditions may occur isolated or as part of an autoimmune polyglandular syndrome type 1, 2, or 3, which are characterized by autoimmunity against more than one endocrine organ(106). Nederstigt et al. (105) found these autoimmune diseases to be associated with T1DM with the following prevalence: autoimmune-thyroid disease with hypothyroidism was prevalent in 9.8% of patients (with a prevalence ranging from 0.6 to 44% depending on the age of the study population and diabetes duration), celiac disease in 4,7%, gastric autoimmunity including pernicious anemia in 4.3%, and vitiligo in 2.4% of patients. Compared to the general population, the prevalence among T1DM patients is twice as high for hypothyroidism, 4.5 to 9-fold higher for celiac disease, twenty times as high for pernicious anemia, and about 6-fold higher for vitiligo, respectively(105). Routine screening for thyroid disorder

should be performed in all patients with T1DM soon after diagnosis and regularly thereafter, considering its high prevalence among T1DM patients(104). Screening for celiac disease should be performed in adults with T1DM who show typical symptoms or signs of this disease and in children soon after T1DM diagnosis and should be repeated after 2 and 5 years(104, 107). Evaluation of vitamin B12 levels should be performed in subjects with T1DM and unexplained anemia or peripheral neuropathy(104). Specific HLA haplotypes were associated with the autoimmune diseases named above. For example, the presence of HLA DQA1*0301, DQB1*0301, and DQB1*0201 haplotypes is associated with the development of hyperthyroidism(106), while the presence of the HLA haplotype DQA1*0501-DQB1*0301 in patients with T1DM is related to an elevated risk of gastric autoimmunity. Furthermore, it has been suggested that in patients with T1DM, the HLA haplotype DQA1*0501-DQB1*0201 predisposes for celiac disease(106). Other associated diseases include autoimmune hepatitis, Addison disease, dermatomyositis, and myasthenia gravis(104). Early diagnosis and treatment of co-occurring autoimmune disorders can lead to improved lipid and glycemic control(106).

1.5.1.2 Cognitive impairment

Patients with diabetes have a significantly increased risk of developing cognitive impairment and dementia(104). The increasing diabetes prevalence and the decreasing age at diabetes diagnosis indicate cognitive dysfunction associated with diabetes will presumably increase likewise(108). The direct effects of changes in glucose metabolism and diabetes-related complications, in particular retinopathy, are major contributors to the development of cognitive dysfunction(108). Recurrent hypoglycemia, which leads to cerebral adaptation resulting in impaired hypoglycemia awareness, is responsible for inconstant blood glucose levels and therefore may add to cognitive dysfunction(108). However, the latter relationship between hypoglycemia and cognitive decline seems to be controversial(109). T1DM influences the effectiveness of a subgroup of cognitive areas like intelligence, attention, psychomotor speed, cognitive flexibility, and visual perception, and mental slowing is considered to be the principal cognitive deficit linked to T1DM(108). Unlike in T2DM, learning and memory are affected only rarely in T1DM(108). Regarding the severity of cognitive deficits, age of diabetes onset seems

to play an essential role in developing cognitive dysfunction, as children's brains are considered to be increasingly susceptible to the effects of diabetes, and children, who present with overt T1DM before the age of 7, have a higher risk of serious cognitive deficits than those who are diagnosed with T1DM at an older age(108).

1.5.1.3 Epilepsy

Some studies(110-113) proposed that epilepsy, and specifically idiopathic generalized epilepsy, was frequently associated with T1DM, with GADA representing the most noteworthy link between epilepsy and T1DM(114). However, this association is disputable, as other studies(115, 116) could not confirm this correlation, and data on the strength of this association are conflicting, as multiple methodological issues restrict the interpretation of the findings. For example, in some cases, it was not possible to differentiate epileptic seizures from seizures secondary to metabolic disorders such as hypoglycemia(112, 115).

1.5.1.4 Fractures

In T1DM, the age-specific hip fracture risk is remarkably heightened, with a relative risk of 6.3 in both sexes(117). Moreover, T1DM is associated with osteoporosis(104).

1.5.1.5 Psychological issues

Although a lot of progress in disease management has been made during the past decades, psychosocial factors, including complex environmental, social, behavioral, and emotional factors, have a significant impact on T1DM patients' life and play an important role in disease care and health status(118). Therefore, special attention must be paid to psychological well-being, as this is a prerequisite for achieving treatment goals and obtaining quality of life. People with diabetes are at increased risk for mental health issues, with diabetes distress, depression, anxiety, and disordered eating being among the most common mental health issues related to T1DM(118). Obtaining quality of life and a mental well-being can often be challenging for people affected by diabetes, as it represents a chronic disease with treatment complexities and inherits increased risk for short- and long-term complications. Besides, it results in drastic incursions in

social, emotional, and physical life(118). Thus, the mental status of patients living with diabetes should be considered regularly. Diabetes distress, which comes along with significant negative psychologic reactions associated with emotional burdens regarding the management of this chronic disease, is seen frequently among T1DM patients who are struggling with the constant behavioral demands related to medication dosing and frequency, the need for frequent blood glucose monitoring, food related issues, eating patterns and physical activity(118). Another common psychological disorder associated with diabetes is anxiety disorder, characterized by worries and anxiety regarding diabetes complications, insulin administration, as well as fear of hypo- or hyperglycemia(118). The lifetime prevalence of generalized anxiety disorder in people with either T1DM or T2DM is assumed to be 19.5%(119). Moreover, specialists should also be aware of the increased risk for depression in patients with diabetes. Therefore, the psychological impact of such a challenging disease like diabetes must not be underestimated, and the immense impact of diabetes on quality of life should be considered. Many opportunities for psychosocial screening for mental health problems related to diabetes exist at diabetes diagnosis, during management visits, during hospitalization, when a change in disease course or treatment occurs, or at new-onset of complications(118).

1.5.2 Disease complications

Complications can be classified into two major categories: short-term and long-term complications. Latter can be further subdivided into macrovascular and microvascular complications, including nephropathy, retinopathy, and neuropathy(82).

Despite the fact that improved glycemic control could be reached over the past decades, individuals with T1DM still experience increased morbidity and mortality compared to the non-diabetic population, owing to acute and chronic complications. Although T1DM does not lead to immediate death nowadays - which was not the case before insulin was available - the risk of death in patients with T1DM is increased compared to subjects without diabetes, mainly due to cardiovascular causes, leading to a loss of life expectancy of about 12 years compared to those without diabetes(120), or even more, when diagnosed before ten years of age(121). This implements that further

efforts need to be made in terms of early detection and prevention of diabetes complications.

1.5.2.1 Acute complications

Acute complications include DKA and hypoglycemia. DKA is usually seen in new-onset T1DM, at insulin omission, and at increased levels of stress-associated counterregulatory mechanisms, e.g. during infection(122). Although disease management and diagnosis improved over the past decades, about one-third of children still present with DKA at diabetes diagnosis(8), implying the need for early diagnosis and improved disease management. Another acute condition associated with T1DM is hypoglycemia, which can often occur due to inadequate or excessive insulin administration or exercise(38). Especially in children with T1DM, a strong association between exercise and hypoglycemic events can be seen, both during training and afterward. Additionally, nocturnal hypoglycemia is often seen after exercise(123). Severe hypoglycemic events with the need for treatment assistance happen at rates of 16-20 per 100 person-years, those hypoglycemic events resulting in loss of consciousness or seizure at a rate of 2-8 per 100 person-years(6). Repetitive hypoglycemia can lead to hypoglycemia unawareness, which can subsequently result in severe hypoglycemic events, starting a vicious circle(6). Even though significant technological advancements have been achieved during the last years, methods of glucose monitoring and insulin replacement still need further improvement, as the risk of hypoglycemia is still present and limits ideal treatment of the disease(82).

1.5.2.2 Chronic complications

The importance of preventing hyperglycemia cannot be emphasized enough, as the deteriorating effects on the vascular system are the primary cause of morbidity and mortality associated with diabetes(124). Overall, the devastating consequences of hyperglycemia can be classified either as macrovascular complications, which are coronary artery disease, peripheral arterial disease, and stroke, or microvascular complications, including diabetic nephropathy, retinopathy, and neuropathy(124).

Cardiovascular disease

Atherosclerotic cardiovascular disease includes coronary heart disease, cerebrovascular disease, or peripheral arterial disease and is the leading cause of death in patients with diabetes(125). In comparison to the general population, macrovascular complications in T1DM develop much earlier, show a more diffuse and accelerated progression, and have a higher mortality(126).

Hypertension significantly increases the risk for both CVD and microvascular complications(127). Moreover, dyslipidemia and impaired platelet function are further risk factors for cardiovascular disease (CVD)(128). The EDIC study, which followed patients with T1DM for long-term complications, confirmed that intensive diabetes treatment was able to decrease the risk of a cardiovascular disease event by 42%, also, the risk of nonfatal myocardial infarction, stroke, or death from cardiovascular disease could significantly be reduced by 57% compared to conventional treatment(129). Diabetes is considered an independent risk factor for CVD in adults, as it contributes to a two- to fourfold increased incidence of CVD(82). Epidemiological studies have recognized important targets for risk reduction for CVD in T1DM patients, which are hypertension, proteinuria, obesity, the level of HbA1c, lipid levels, and smoking(130). Although CVD events are not likely to affect children, even in the presence of T1DM, it is known that adolescents with T1DM may already show subclinical CVD abnormalities as early as within ten years after diagnosis(130).

Microvascular complications

Although microvascular complications are rare in prepubertal children and in individuals with diabetes duration of less than two years, they may develop after puberty or after a diabetes duration of 5-10 years(82). Thus, early detection and prevention are of great importance. There are three major manifestations of microvascular disease: nephropathy, retinopathy, and neuropathy. Hyperglycemia is the primary risk factor for microvascular disease, thus, slower progression to microvascular disease can be achieved by intensive diabetes management, resulting in lower levels of HbA1c(124). In this regard, HbA1c is an excellent indicator of long-term glycemic control and very useful for the prediction of diabetes complications(1).

Diabetic nephropathy

Screening for nephropathy should be performed at least once a year by assessing urinary albumin and the eGFR in patients with diabetes duration of five years and longer (in children at puberty or older than ten years of age, with diabetes duration of five years and more) (107, 131). Early detection of diabetic nephropathy is highly important since it is considered the leading cause of end-stage renal disease(124). Proteinuria is present in 15-40% of patients with T1DM(132), and according to the European Diabetes Prospective Complications Study, microalbuminuria occurs at an incidence of 12% over a period of seven years among individuals with T1DM(124). Comparable to other microvascular complications, the risk of developing nephropathy is strongly associated with glycemic control(124).

Diabetic retinopathy

Diabetic retinopathy is prevalent in the majority of T1DM patients after a long disease duration and is presumably the most frequent microvascular complication(124). Both diabetes duration and the severity of hyperglycemia are significant risk factors associated with the incidence and development of retinopathy(124). It is a highly specific complication affecting the vascular system of both T1DM and T2DM, and it is considered the leading cause of new cases of blindness among adults aged 20-74 years in developed countries(126). Besides, other eye conditions, like cataracts and glaucoma, are seen to develop earlier and more frequently in people with diabetes(126). Additional risk factors for retinopathy besides diabetes duration and chronic hyperglycemia include diabetic nephropathy and hypertension(126). In patients with T1DM, screening for diabetic retinopathy should be performed within five years after diabetes diagnosis, in children after 11 years of age or during puberty and should include an initial dilated and comprehensive eye examination, which should be repeated every two to four years or more frequently in adults(107, 131).

Diabetic neuropathy

According to the ADA, diabetic neuropathy represents a heterogeneous group of disorders and is a diagnosis of exclusion(131). Peripheral sensorimotor neuropathy and

autonomic neuropathy are the most common neuropathies in T1DM(126). The risk of developing neuropathy is directly associated with both duration and severity of hyperglycemia, just as with other microvascular complications(124). Screening for neuropathy is recommended to be performed five years after diabetes diagnosis in adults with T1DM and equally in children with T1DM, starting at puberty or after ten years of age, and should be repeated yearly thereafter(107, 131).

Before the discovery of insulin about a decade ago, T1DM was a fatal disease. Nowadays, although not causing immediate death, individuals with T1DM still experience short-term and long-term complications that result in a loss of life expectancy. Because diabetes complications are causative for increased morbidity and mortality in patients with T1DM and diminish the quality of life of patients affected, major effort is needed to reach early detection and prevention of diabetes complications. To prevent these complications, several risk factors, most of all, hyperglycemia and hypertension, must be treated. Practice recommendations include the assessment of individual glycemic targets, which should be achieved through optimal insulin therapy(127). Routine blood pressure measurement and evaluation of individualized blood pressure targets should be performed(127). Likewise, lipid testing should be performed at least annually. Antiplatelet therapy as secondary prevention of CVD is indicated for those individuals with diabetes who have a history of CVD(127). Finally, smoking cessation plays a vital role in the prevention of CVD(126).
Progress in medical technologies has led to an improvement in disease management and has therefore increased life expectancy. However, a consequence of the latter is an increased risk for the development of long-term diabetes complications.

1.6 Therapy

With the discovery of insulin almost a century ago by Frederick Banting and Charles Best, T1DM shifted from a once terminal to a manageable disease nowadays. The management of T1DM has subsequently changed over the past 25 years, and intensive insulin therapy became the standard of care following the publication of the hallmark DCCT in 1993(133). Despite new technologies available today, including high-tech

blood glucose monitoring systems and automated insulin delivery systems (AID), which have undoubtedly benefited those living with diabetes, a cure still remains elusive, and individuals with T1DM still experience labile blood glucose levels and long-term complications, since exogenous insulin does not always provide optimal metabolic regulation. One could statue that diabetes can be described as a global epidemic that is palliated but not cured by exogenous insulin interventions.

After diagnosis, the primary therapeutic goal is to preserve insulin secretion, as it is associated with less incidence of microvascular complications and less severe hypoglycemic events(29). These goals can be achieved by intensive insulin therapy. Although endogenous insulin secretion is usually low, there are differences in children and adults, as children lose the ability of endogenous insulin production at a higher rate than adults(5).

1.6.1 Therapeutic targets

According to the ADA, an HbA1c target of less than 7% for adults and for the majority of pediatric patients is appropriate(75, 107). Lower HbA1c levels (<6.5%) may be tolerable in certain individuals if this target can be reached safely without severe hypoglycemia or adverse effects(75). However, these targets should be individualized based on many factors, such as comorbidities, patient capability, and available resources, as well as other circumstances like pregnancy(122), with lower targets being often recommended during pregnancy or for women anticipating pregnancy(6). Less strict targets might be rational for individuals with hypoglycemia unawareness, a history of severe hypoglycemia, severe diabetes-related complications, and reduced life expectancy, as well as for older and multimorbid patients(75). In adults, pre-prandial blood glucose targets are 80-130mg/dl and the peak post-prandial glucose target is <180mg/dl(75). In addition, in order to achieve an ideal outcome, multidisciplinary care and education should be provided by an interdisciplinary team consisting of diabetes educators, nurses, nutritionists, physicians, dieticians, exercise physiologists, social workers, and psychologists(6). The DCCT demonstrated the advantages of intensive (mean HbA1c about 7%) over standard (mean HbA1c about 9%) glycemic control in T1DM subjects: intensive control was able to reduce the development of microvascular complications

by 50-76%(133), and its follow up in EDIC study was able to prove the persistence of these benefits over two decades, despite diminishing differences between treatment groups regarding glycemic control(134, 135). Similar results were observed in children and adolescents, with lower HbA1c resulting in a lower risk of microvascular and macrovascular complications(12, 136).

1.6.2 Insulin therapy

Insulin preparations have experienced significant improvements since the discovery of insulin a century ago, from purified animal insulins over human insulins to insulin analogs and biosimilars(137). Insulin analogs enable improved correspondence between insulin action profiles and glucose excursions, and although insulin remains the cornerstone of disease management, over the last decades, new rapid-acting and long-acting insulin analogs have been elaborated(138). They show distinct but more advantageous pharmacokinetics compared to recombinant human insulins(137), thus, insulin analogs have mainly replaced recombinant human insulin. In addition, improvements in insulin delivery methods and glucose monitoring have substantially ameliorated glycemic control and patient survival(139). Nevertheless, daily insulin injections do not cure T1MD and are often limited by hypoglycemic episodes(139). The treatment goal is to achieve insulin regimens that mimic physiological insulin release in order to maintain glucose levels as close to normal through intensive insulin therapy(137) while avoiding hypoglycemia.

1.6.2.1 Insulin analogs

In individuals with T1DM, treatment with insulin analogs is associated with less hypoglycemia(140), lower HbA1c levels, and less weight gain compared to treatment with human insulin(138). Long-acting insulin analogs such as insulin detemir, insulin glargine - the first long-acting analog - and insulin degludec, show durations of action of 20-24 hours, 24 hours, and 24-42 hours, respectively(1). They display a longer duration of action and flatter, more constant activity profiles than NHP insulin(138). Insulin glargine does not show any pronounced peak, unlike NPH, and is usually given once daily, while insulin detemir usually requires twice-daily injections and shows a lower

within-subject variability than glargine(141). Insulin degludec shows a prolonged, stable release with an ultra-long and flat glucose-lowering profile and provides a four times less within-subject variability compared to insulin glargine(141). Thus, a flexible three-times-weekly administration that can be given at any time of the day is sufficient(141), and this flexibility does not diminish its effectiveness of safety(142). The newer long-acting insulin analogs insulin degludec and glargine U300 have demonstrated a reduced risk of nocturnal hypoglycemia and are very flexible regarding the time of administration(137), with duration of action over 42 hours and 32 hours, respectively(137). Overall, long-acting insulin analogs show substantial improvements in variability in their insulin action profiles, which is associated with a significant reduction in the risk of nocturnal hypoglycemia(137). Long-acting insulin analogs are also approved for use in children with different age minimums(137).

Short-acting insulin analogs include insulin aspart, insulin lispro, and insulin glulisine, which all have alike onset of action of about 15 minutes, show a peak effect within 1-2 hours and a duration of action of 4 hours(1), with a quicker onset and shorter duration of action compared to regular human insulin(138), therefore offering an insulin-action profile that more suitable to manage the glucose excursion after a meal than human regular insulin(137). The rapid-acting insulin analogs (RAA) allow more flexibility in mealtime administration which also has an impact on the improvement of quality of life, as they allow injections closer to meals and are thus less disruptive in daily life(137), and could especially be advantageous for certain patient groups with difficulties foreseeing food intake, e.g. toddlers or fussy eaters. All rapid-acting insulin analogs are admitted for use in children, but with varying minimum age limits, as well as for use in continuous subcutaneous insulin infusion (CSII)(137).

Lately, two new insulins with improved rapid action profiles have been presented: inhaled human insulin and FIASP. Inhaled insulin with a rapid peak and shortened duration was found to cause less hypoglycemia and weight gain, compared to RAA and faster-acting insulin aspart (FIASP)(138). FIASP is able to reduce prandial excursions better than RAA(138) and can reduce glucose levels to a greater extent than insulin aspart(137). Patients who could profit most from the use of FIASP are users of CSII(137). Moreover, the use of FIASP could offer an advantage for the development

of high-speed and fully automated closed-loop systems(143). Other forms of insulin, like premixed insulins and regular human insulin, are available but less physiological and therefore rarely used(137). Ultra-rapid inhaled insulin is also attainable, but little interest for its routine use exists because of its fixed dosing, problems with consistent delivery, high costs, and the need for assessing pulmonary function(6). Research in this field is continuing in order to develop even longer-lasting insulin preparations, or novel technologies such as antibody-linked insulins, to create insulin preparations that are administered only once a week(137). Besides, efforts are made to develop “smart” insulins(143), which would be discharged from depots under the skin at high glucose levels, such as a new glucose-responsive insulin delivery system that utilizes the cell membrane of erythrocytes(143), and alternative insulin administration methods.

The advent of insulin analogs enabled intensive insulin therapy without interfering too much with daily life activities and paved the way to achieve tight glycemic control with decreased risk of hypoglycemic events(137). Nevertheless, insulin therapy in T1DM continues to be challenging, as the risk of hypoglycemia and weight gain will continue to be an inevitable part of T1DM treatment until the optimal insulin preparation, which will be able to fully imitate physiological insulin secretion, is found(137).

1.6.2.2 Insulin administration and glucose monitoring

To achieve optimal glycemic control, the best therapeutic option is basal-bolus insulin therapy, which can either be administered via multiple daily injections or via CSII, e.g. insulin pumps(141). In general, 50% of the daily insulin dose is required as basal and 50% as prandial, while the total daily insulin dose can be calculated based on weight, usually ranging from 0.4 to 1.0 units/kg/day(138). It is important to consider that higher doses are required during puberty, pregnancy, and illness(138). Sometimes, especially in newly diagnosed individuals, fewer insulin doses may be needed due to temporary remission after initiation of insulin administration(141), known as the “honeymoon period.” When it comes to selecting glycemic targets, the long-term health benefits of lower HbA1c levels should be weighed against the risk of hypoglycemia and the concomitant developmental burdens such as neurological vulnerability(107).

When using multiple daily injections (MDI), basal insulin is given in the form of long- or intermediate-acting insulin analogs, while meal-related glucose demand is regulated by bolus injections of rapid-acting insulin analogs (141). Insulin can be administered via syringes, pens, or rarely used, but available, inhaled insulin(144). “Smart” pens can be programmed to determine insulin doses and offer downloadable data reports(144).

Insulin pump therapy replaces the need for frequent injections and is able to imitate physiologic insulin release by administration of a 24-hour preselected and adjustable basal rate of rapid-acting insulin analogs, accompanied by patient-administrated boluses for mealtime-related glucose excursions(141). Moreover, pump therapy permits easier adaptation of insulin to specific physiologic needs, such as reduced temporary basal rates during exercise(141). Potential adverse events include pump malfunction, which could result in DKA, and catheter-site infection(144). Some studies showed improved glycemic control with CSII, with more patients reaching targeted and lower HbA1c levels, while the risk for severe hypoglycemic events is reduced(144). However, whether CSII or MDI is the better therapeutic option is debated since outcomes reported in studies varied substantially and must be evaluated individually.

In recent years, significant improvements regarding insulin preparations and delivery systems have been achieved, also, advancements to enhance glycemic control have been made, in particular with real-time continuous glucose monitoring(5). It was reported that real-time continuous glucose monitoring could improve glycemic control with lower HbA1c and lower rates of hypoglycemic events in adults and could therefore be advantageous for certain individuals with T1DM(144). The use of CMG has been shown to improve diabetes management and supplements insulin pump therapy in the form of sensor-augmented pump therapy (SAP)(145). Among other trials, Bergenstal et al.(146) compared the efficacy of SAP and MDI and was able to show significant HbA1c reductions when using SAP. While SAP uses each device separately, integration of both systems was realized in closed-loop systems, which is made up of three components: the insulin pump, a continuous glucose sensor, and an algorithm that regulates insulin delivery(144). In such manner, insulin delivery can be suspended and – in addition - increased or decreased depending on the sensor glucose levels(144).

Moreover, such systems were seen to reduce the risk of exercise-related hypoglycemia, are considered safe, and show better glycemic control compared to SAP(138, 144). Closed-loop systems can be classified into insulin-only and bi-hormonal control systems(145). Insulin-only closed-loop systems are able to achieve glucose targets by insulin delivery alone, by contrast, the latter system makes use of both insulin and glucagon to reach glycemic targets(145). Unlike with a “fully” closed-loop system, where user input to the control algorithm for meal-related administration of insulin is not required, a “hybrid” closed-loop system still requires manual mealtime announcement(145). The first commercially available hybrid closed-loop system for use in patients seven years and older in Europe was the Medtronic MiniMed® 670G pump, a single hormone hybrid closed-loop system, which enables low-glucose insulin-suspend and predictive low-glucose insulin-suspend, but also increases insulin delivery in response to hyperglycemia or predicted hyperglycemia(147). Major limitations of AID are due to insulin kinetics, as both the slow onset of action when insulin is administered subcutaneously and the continued action for several hours are still unsolved problems(147). Although the algorithm could detect food intake, the onset of action of insulin would be too slow to prevent postprandial hyperglycemia, and over-delivery of insulin in order to reduce hyperglycemia will at the same time lead to increased risk of postprandial hypoglycemia(147). Therefore, faster-acting insulin and alternative delivery sites might be a prerequisite for the successful use of fully closed-loop systems. Other improvements being examined for AID systems include additional physiological inputs into algorithms, such as heart rate and indicators of physical activity(147). Although the optimal solution would be a cure for T1DM, in terms of replacing damaged ß-cells with healthy ones, the artificial pancreas might constitute a ‘bridge’ until that cure is developed(145).

The use of technology in the treatment of T1DM should be individualized on the basis of patient’s needs, self-management capabilities, skill level, motivation, and availability of devices since there is no universal approach for every patient, and although technology can help in managing blood glucose and lifestyle, not everyone profits from new technology(144).

1.6.3 Non-insulin treatment

Frandsen and colleagues have recently published an expert opinion on non-insulin pharmacological therapies for the treatment of T1DM, unless otherwise specified, for information in the following paragraphs, see(148): Despite being the gold standard of T1DM therapy, intensified insulin therapy increases the risk of hypoglycemia, and although better insulin products are available today and treatment regimens improved, blood glucose variability continues to be challenging in terms of disease management. Several hurdles need to be overcome in order to achieve treatment targets in individuals with T1DM. Thus, efforts have been made to assess the benefit of non-insulin pharmacological therapies in combination with insulin therapy in order to achieve optimal treatment, which should ensure lower HbA1c levels without heightening the risk for hypoglycemic events, lead to weight loss in overweight persons and decrease the incidence of cardiovascular disease. These include medication used in the treatment of T2DM, such as pramlintide, GLP1-RA, DDP-4 inhibitors, metformin, TZD, SU, and SGLT2 inhibitors.

Amylin, a 37-amino-acid peptide, which is co-secreted (1:100) with insulin in the ß-cells, reduces food intake and indirectly inhibits glucagon secretion. The amylin analog, pramlintide, shows equivalent effects in humans. Pramlintide and glucagon-like peptide-1 receptor agonists (GLP-1RA) lead to satiety, suppress glucagon secretion, and hamper gastric emptying rate, as well as enhance glucose-induced insulin secretion and induce weight loss. Besides, GLP-1 receptor agonists cause higher reductions in HbA1c in combination with insulin therapy compared to insulin alone. A positive effect on body weight was also observed with SGLT2 inhibitors but with an increased risk for ketoacidosis. Sodium-glucose cotransporter-2 (SGLT2) inhibitors decrease renal glucose reabsorption, leading to urinary glucose excretion, which results in weight loss. As this process is insulin-independent, a prominent effect on glucose control can be suggested in T1DM. Dipeptidyl peptidase-4 (DPP-4) inhibitors cause a two-to-three-fold rise of endogenous GLP-1 levels, enhance glucose-dependent insulin secretion and glucagon inhibition, whereas they do not show any effect on bodyweight. Metformin leads to the inhibition of hepatic glucose production and causes a minimal weight loss,

while it does not improve HbA1c in T1DM patients. Thiazolidinediones (TZD) are primarily able to ameliorate insulin sensitivity, which is related to weight gain in many cases, whereas the mechanism of action of sulfonylureas (SU) is to augment insulin secretion by the stimulation of pancreatic ß-cells, thus, their clinical effects in patients with long-standing T1DM are absent.

Pramlintide is the only non-insulin pharmacological therapy approved for treatment in adults with T1DM as an adjunct to prandial insulin(138). Studies show variable reductions of HbA1c levels and body weight with the addition of pramlintide to insulin therapy. It is usually given four times daily prior to main meals. Nevertheless, only a minority of patients are being treated with pramlintide. The major hurdle to a routine use of this agent is that its administration demands additional 3-4 subcutaneous injections per day, which has proven too inconvenient for most patients with T1DM(149). In addition, the increased psychological burden that comes along with an additional treatment supplementing their diabetes treatment, the risk of non-compliance due to a more complicated regimen, and the additional financial burden should be thoughtfully weighed against possible, widely unpredictable benefits(149).

Overall, investigations regarding non-insulin therapeutic agents are not very promising, and success regarding glycemic control and reduced insulin requirements without increasing the frequency of adverse events is still absent. That is why the use of non-insulin drugs adjunct to insulin therapy is currently not recommended for the general population of patients with T1DM.

1.6.4 Physical activity

Physical activity should be included in diabetes management due to health benefits, as it improves blood glucose control and insulin sensitivity, reduces cardiovascular mortality, and contributes to weight loss(118). According to the ADA(118), for children and adolescents with T1DM, it is recommended to perform moderate to vigorous-intensity activity of at least 60min/day and muscle-strengthening and bone-strengthening activities at least three days per week, while for most adults, 150min or more of moderate- to vigorous-intensity activity per week are recommended. Despite these benefits physical activity offers for patients with T1DM, care needs to be taken when recommending

exercise for patients with T1DM, as each patient with T1DM shows a variable blood glucose response to exercise(118), bearing the risk of both hypoglycemia and hyperglycemia.

1.6.5 Prevention

In 2015, the Juvenile Diabetes Research Foundation, the Endocrine Society, and the ADA proposed the adoption of a new staging classification, which enabled diagnosis of T1DM before the development of symptomatic hyperglycemia. Since then, several efforts have been made in order to investigate new therapeutic methods to prevent disease onset. Primary prevention studies, performed in individuals at high genetic risk but without islet AAbs (before stage 1)(5), have focused on dietary modifications early in infancy. The TRIGR(150), FINDIA(151), and BABYDIET(152) studies all investigated the effect of the absence of certain proteins on disease prevention, including bovine insulin and gluten. The TRIGR study(150) compared outcomes of two cohorts of infants with a FDR, with one receiving hydrolyzed (hypoallergenic) infant formula and the other receiving conventional formula, whenever breast milk was not available during the first 6-8 months of life. First results showed that infants in the hydrolyzed formula arm were less likely to develop islet AAbs(150). However, subsequent post-intervention results did not find a difference between these two cohorts in terms of AAb prevalence(153). The FINDIA trial(151) reported less progression to islet AAb after three years of follow-up when bovine insulin was removed from infant formula (compared with normal cow's milk formula). However, there was no long-term follow-up. Unfortunately, the BABYDIET trial did not show any significant differences in diabetes risk with modulating gluten exposure(152).

Secondary prevention trials are performed in subjects with multiple islet AAbs but no overt hyperglycemia (stage1). Preclinical studies suggested that in individuals with a high genetic risk for T1DM, cellular stressors might cause the release of ß-cell antigens to which no immune tolerance has been achieved. Thus, immune recognition of these new antigens, or even cellular stress itself, would lead to the destruction of ß-cells. Therefore, it was proposed that ß-cell rest, induced by the administration of insulin, could prevent ß-cell loss. Hence, the DPT-1 investigated the effects of parenteral and

oral insulin - based on the individual risk score - in individuals with positive AAbs who had a FDR or SDR with T1DM in terms of secondary prevention, but could not find a delay in disease progression, neither in the parenteral insulin arm nor in the oral insulin arm(154). In regard to this observation, the Type 1 Diabetes TrialNet Network conducted a trial(155) with low-dose oral insulin in a second cohort of individuals with AAb profiles similar to those of the DPT-1 subgroup, but neither could this trial show a successful outcome. Other agents investigated for purposes of secondary prevention, including nicotinamide (ENDIT trial) and intranasal insulin (DIPP), were not able to delay or prevent diabetes onset(154). In the INIT-I study, intranasal insulin was administered to AAb positive individuals; however, this trial similarly had no influence on T1DM onset, as intranasal insulin did neither prevent nor accelerate T1DM(156). The INIT-II study, launched in 2006, is evaluating whether intranasal insulin is able to delay or prevent clinical onset of T1DM in AAb positive individuals (NCT00336674).

The time of onset and delay of T1DM diagnosis can have a very striking impact on morbidity and mortality since the loss of life expectancy is greatest in those who are diagnosed as children, emphasizing the benefits of delayed onset to younger individuals. Therefore, prevention is a key therapeutic goal.

It is obvious that greater effort is needed in terms of primary and secondary prevention trials. First, earlier therapeutic intervention may have a positive impact on immune processes, and second, greater preserved β-cell mass may be beneficial for therapeutic success(157). However, such primary and secondary prevention strategies rely on effective case finding: for primary prevention, genetic screening at birth is needed, and those with risk genes need to be included for trials(157). For secondary prevention, either following individuals with high genetic risk from birth until the development of AAbs, or screening for AAbs among high-risk subjects, such as FDR of individuals with T1DM, is needed (157).

1.6.6 Immunotherapy

In addition to prevent disease onset, much effort has been made towards reversing ß-cell destruction and inducing the formation of immune tolerance against ß-cells. In this regard, research on immunotherapies has been conducted since the 1980s, focusing

on the reestablishment of tolerance, T-cell or B-cell inhibition, induction of regulatory T-cells, and the suppression of innate immunity(158). First attempts in order to achieve these goals were made with several immunosuppressive agents, including single-agent trials with cyclosporine, azathioprine, monoclonal anti-CD3 antibodies, rituximab, and abatacept, that started an attempt to maintain ß-cell mass in symptomatic patients(1). About three decades ago, one of the first clinical trials for immunological interventions tested cyclosporine A, an inhibitor of T-cell activation. Although studies showed that cyclosporine was able to alter the disease course and decreased the need for exogenous insulin for over one year, it was limited by adverse effects, and the therapeutic effect waned after withdrawal(36, 159). The adverse effects of antigen non-specific interventions, as well as the failure of permanent remission of disease with any agent tested, increased interest in antigen-specific interventions, which might be preferable over broad immune-suppressive agents. Hence, in order to achieve these goals, the GAD65 immunization trial was launched, which involved the provision of self-antigens. Unfortunately, it was unsuccessful(160). In 2007, an autologous hematopoietic stem-cell transplantation together with high-dose immunosuppression was conducted and demonstrated that CP production and insulin independence could be achieved in the majority of subjects (161). However, this effect diminished over time. More than three decades ago, the efficacy of cyclosporine in reducing the need for exogenous insulin was reported; ever since, immunomodulatory therapies have failed to meet the expectations and were not able to induce long-term clinical success(139). Overall, while some strategies have shown moderate benefits, true durable remission remains elusive. Several immunotherapeutic approaches are being tested that can be classified into two major categories: antigen-dependent strategies and antigen-independent strategies.

1.6.6.1 Antigen-specific approaches

Recurrent early exposure to an antigen may promote immune tolerance to that antigen(162). This approach has also been assessed for success in both pre-symptomatic and in new-onset T1DM(162).

Autoantigen-specific approaches were tested in prevention studies due to the safety and possible fewer striking effects and were performed with autoantigens, such as insulin(163), in order to achieve immune tolerance to these compounds(164). It is noteworthy that the outcome of such approaches depends on several factors, including the molecular form, dose, and way of administration of the autoantigen(165). One important antigen-based trial was the DPT-1, which was conducted to evaluate whether insulin is able to prevent diabetes onset in high-risk relatives with positive ICAs and consisted of two arms, the parenteral and the oral insulin arm(154). However, both strategies were unsuccessful in T1DM prevention, but some effects were noticed in the subgroup with high IAA in the oral insulin arm(154). Recently, a trial was carried out to evaluate whether GAD-based immunization could preserve insulin production in individuals with recent-onset of T1DM, but results showed that this immunization did not affect the course of insulin secretion for one year(166). Antigen-specific therapies were considered the most promising approach in the field of immunotherapy for many years, aiming only to inactivate pathogenic autoreactive T-cells and other processes involved in ß-cell death, while the remainder of the immune system would be left unperturbed, but it seems that their effect, if any, may be restricted to the early phase of T1DM, or to the pre-diabetic period(163). Nevertheless, due to the lack of adverse effects, this field is very attractive for future investigation(163). However, antigen-based immunotherapy should consider the high degree of diversity in specific T-cell response against ß-cells among T1DM individuals; thus, it seems very challenging to build up tolerance in a sufficient number of self-reactive T-cell clones residing in the islet, which are responsible for disease progression(167). The most promising strategy may be the combination of different approaches affecting different mechanisms.

1.6.6.2 Antigen-independent approaches

Antibody-based therapies

Immuno-regulatory-based approaches use exogenous agents like anti-CD3 antibodies that inhibit T-cell function and thus prevent ß-cell destruction. Teplizumab and otelixizumab are such agents and were able to preserve CP release in NOD mice but not in

clinical phase III trials(168). Herold et al.(169) studied the effect of anti-CD3 monoclonal antibodies in patients with T1DM and found out that this treatment seemed to halt insulin production decay for at least one year when administered early after diagnosis. In another anti-CD3-based trial (DEFEND-1), the anti-CD3 mAb otelixizumab helped to preserve CP levels and thus ß-cell function in individuals with initial residual ß-cell function at or above the 50th percentile for a period of at least 18 months(170). Also, another study, which enrolled 763 patients, demonstrated that teplizumab prevented ß-cell loss and was able to preserve CP levels(171). A phase III trial (DEFEND-2) was launched to finally confirm the striking effects of anti-CD3 mAb treatment earlier studies have shown, but instead, the trial was unsuccessful as CP levels did not differ from baseline(172). It was unclear whether an earlier intervention would be more successful, as all the earlier mentioned studies were performed in individuals with clinical overt T1DM. Therefore, a recent trial of teplizumab in relatives of patients with T1DM, with high risk for T1DM at stage 2, was performed and represented a turning point of the streak of disappointing study results(173). The primary endpoint was the time to diagnosis of T1DM, which was diagnosed in 43% of the teplizumab group and in 72% of the placebo group, and at the end of the trial, the percentage of subjects without T1DM in the teplizumab group (57%) was twice as high than in the placebo group (28%) and the median time to diabetes was delayed from 2 to 4 years(173). The effects of teplizumab were related to the absence of AAbs to ZnT8, the absence of HLA-DR3, the presence of HLA-DR4, and CP responses to oral glucose that were below the median for all participants(174). However, together with data from preclinical studies, these findings suggest that an active immune response is required for teplizumab to be most effective.

Another possible therapeutic strategy was tested in B-cell-depleting studies. One example is the trial carried out by Pescovitz et al.(175), where rituximab, an anti-CD20 mAb, was tested in patients with T1DM in order to deplete B-cells. Rituximab was able to preserve ß-cell mass, lower HbA1c levels and increase CP levels, but rituximab failed to revert T1DM, and a two-year follow-up reported that it was not able to alter antibody production(176). However, monotherapy with depleting agents may not be the correct approach, and combinatory strategies may be required.

Activation of regulatory T-cells

Since the number of regulatory T-cells has shown to be reduced in T1DM, several trials have started the attempt to restore functional regulatory T-cells by isolating regulatory T-cells from peripheral blood of patients with T1DM, followed by in vitro expansion, and transferring them back into the donor, and phase I clinical trials are examining autoimmune responses(177). In this regard, Bluestone et al.(177) found a population of regulatory T-cells that were still in circulation after one year post transfer, and no decrease of CP levels and no worsening in HbA1c over one year post transfer could be detected. Overall, these data indicates that therapeutic strategies targeting regulatory T-cells could help to preserve ß-cell mass in T1DM(158). However, despite enormous efforts to develop an immune-modulatory treatment for T1DM, none of these therapies are currently available to patients.

Suppression of innate autoimmunity and inflammation

Since inflammation is considered a condition predisposing ß-cell destruction, many trials were carried out to investigate anti-inflammatory strategies. Gottlieb et al.(178) tested mycophenolate mofetil alone and in combination with daclizumab, but neither of them had an effect on CP loss. Soble et al.(179) reported that cyclosporin A, together with methotrexate, was able to induce disease remission. Canakinumab, an antibody against interleukin-1ß, and anakinra, an interleukin-1ß inhibitor, were also tested for effects on ß-cell destruction but failed to show any significant prevention of ß-cell loss(180). Another approach was to target inflammation and innate immunity. An inhibitor of TNF-a, Etanercept, was able to improve HbA1c and CP levels in participants with recent-onset T1DM(181). Imatinib, an inhibitor of protein tyrosine kinase, does also show anti-inflammatory effects, including reduced production of TNF-a. NOD mice models demonstrated that imatinib was able to protect ß-cells against apoptosis(182, 183) and was currently tested in a phase II study in recent-onset T1DM (NCT01781975).

Although immunotherapy may be able to modify the disease course, it has not yet led to durable remission. Thus, a chronic or intermittent therapy should be evaluated(85). Further, combination therapies could be a successful approach, as well as intervention

in early disease stages(162). Most single-agent studies have targeted recent-onset diabetes when a considerable mass of ß-cells has already been lost(158). To address this problem, several preventive therapies are currently being evaluated in individuals at high-risk and positive AAbs(158). Immunotherapy aims to selectively suppress pathogenic proinflammatory events and ß-cell-specific T-cell reactivity without altering acquired immunity.

Future perspectives envision multiple therapeutic options and treatment in all disease stages with new approaches in order to personalize treatment and to assess treatment response using specific biomarkers(162). However, even though multiple therapies were able to show effective short-term CP preservation, no therapy was able to establish a long-lasting effect on autoimmune destruction of ß-cells(162). Another remaining challenge is the introduction of immunotherapy to pediatric populations(85). Generally, ethical guidelines and regulatory agencies require proof of both the efficacy and safety of certain therapies in adults before approving these trials in children(85). Nevertheless, such an approach could result in the dismissal of many therapies considered ineffective, when they in fact could have been successful in younger participants due to a different disease course and distinct therapeutic response seen in children(85).

Moreover, the lack of accessibility of insulin-producing ß-cells constitutes a difficulty regarding the evaluation of therapeutic success, implying a need for biomarkers to monitor therapeutic response(184). Stage 1 and 2 of disease can provide a window for intervention, where immunotherapy should display the greatest effect(184). Thus, the recruitment of patients at stage 3, as in most trials, may constitute a specific obstacle that has limited the potential of therapeutic success(184).

Overall, the named results from clinical trials have illustrated that the effect of immunotherapy in T1DM is limited and short-term, which could lead to the misconception that immunotherapy is not effective in T1DM. But the aim for the future is to identify the “right” therapy, at the “right” time, for the “right” patient(184) and remains a challenge for forthcoming investigations.

1.6.6.3 Pancreas and Islet Transplantation

Although improvements in insulin delivery methods and glucose monitoring have substantially ameliorated glycemic control and patient survival, daily insulin injections do not constitute a cure and often cause hypoglycemic episodes(139). So far, pancreas or islet transplantation represents the most promising approach to cure T1DM(139). Unfortunately, many limitations are present, such as donor scarcity, adverse effects of lifelong immunosuppression, including an increased risk for infections and tumors, innate and alloimmune graft rejection, as well as recurrence of autoimmunity(185). Due to the risk related to chronic immunosuppression and other limitations named above, these treatment options are recommended only for individuals with recurrent hypoglycemic episodes, including hypoglycemia unawareness(164, 186), or renal failure secondary to T1DM, in latter case, simultaneous pancreas-kidney transplantation should be performed(164). However, pancreas transplantation is associated with a considerable risk for surgical complications(187). In this regard, islet transplantation represents a less invasive alternative, whereby the isolation of islets enables ectopic engraftment of insulin and glucagon secreting cells(187). Islet transplantation initially started in 1989 but was not efficient until the introduction of the “Edmonton protocol” in 2000 by Shapiro et al.(188), who announced a modified immunosuppressive regimen for improved outcomes. This was a landmark in islet transplantation, as it enabled insulin independence one year post transplantation in all patients tested(188). Since then, steady advances regarding islet purification and cell mass preservation could be achieved, leading to long-term outcomes mirroring those of whole-organ pancreas transplantation(186). As indicated earlier, allogeneic islet transplantation alone is an established alternative for T1DM patients suffering severe hypoglycemia, impaired hypoglycemia awareness, or excessive glycemic liability where technological approaches did not succeed to reach metabolic control(187). Besides, the choice between islet and pancreas transplantation should be made based on the patient’s age, BMI, renal status, and cardiopulmonary status(187). Unlike a pancreas transplant, islets are not attainable for biopsy. Thus, rejection is indicated mainly by a decline of CP levels(187). In addition to insulin independence, islet transplantation has substantial benefits regarding quality of life and overall metabolic homeostasis(187). However, major challenges are still present, as

many islets are instantly lost after intraportal infusion(164). Another concern is the possible absence of an islet-host connection owing to avascularized islets, contributing to post-transplant graft loss(164, 186). Despite the fact that revascularization occurs in islet transplants, this process is slow and inefficient(164). Therefore, multiple islet transplants, often from 2 to 3 donors, are required to treat one patient, contributing to the problem of donor scarcity(164). This, taken together with graft loss and adverse effects of immunosuppression, are the major challenges regarding transplantation to cure T1DM. Therefore, novel therapies to address these problems are highly desirable(164). One solution to these therapeutic challenges, precisely for graft loss and the need for immunosuppression, is the approach of bioengineered encapsulation devices that protect the transplanted graft from immune response. Therefore, several groups are developing macro- or microencapsulation devices(164), which are capable of eradicating systemic immunosuppression by physical isolation of transplanted cells from the host's immune responses(186). To assess the problem of donor scarcity, new therapeutic approaches using human stem cells to generate insulin-producing cells are being examined, which could provide a potentially unlimited cell source for islet transplantation(187). Another alternative could be Xenotransplantation of porcine islets into T1DM patients, which are compatible for transplantation into human recipients(164). They can be obtained from pathogen-free isolated pigs and encapsulated in microcapsules to anticipate disease transmission and rejection(164). Clinical trials have been initiated to test its safety and efficacy in T1DM patients, and in New Zealand and Argentina, non-immunosuppressed T1DM individuals received transplants of encapsulated neonatal porcine islets(186). However, they showed only minimal function(186). Another interesting approach to consider is the possibility of extrahepatic transplant sites: the liver has become the implantation site of choice for islet transplant, as it remains the only microenvironment that is able to commonly achieve insulin independence(186). The need for new transplant sites comes along with possible new sources of islet cells, such as stem cell-derived ß-cells and xenogenic islet sources. Thus, an engraftment site that enables visualization, biopsy, and retrieval may become obligatory. Attractive options may be the omentum, as it has a rich blood supply, portal drainage and is accessible for minimal surgery(186). Islets could also be transplanted into the gastric submucosal

space(186). Several immune-privileged sites have also been evaluated, including the brain, the thymus, and the anterior chamber of the eye, with their utility lying in the avoidance of chronic immunosuppression(186). In addition, intramuscular and subcutaneous islet transplantation are being investigated, as they enable easy access to the graft(186). Overall, the optimal site for ß-cell replacement remains elusive, but for the time being, the liver remains the choice for islet transplantation(186).

1.6.6.4 Stem Cell Therapy

Despite successful outcomes related to islet transplantation and immunomodulatory therapies, the demand for an effective ß-cell replacement approach in order to cure T1DM is still present(139). In this regard, stem cell therapy may provide a promising strategy to overcome the earlier mentioned challenges related to islet transplantation(139). First, stem cells are a possible source for a supply of self-replenishing insulin-producing cells. Second, their immunomodulatory characteristics may be useful in preventing, arresting, or reversing autoimmunity(139). Possible sources could be embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), bone marrow-derived hematopoietic stem cells (BM-HSCs) and multipotent mesenchymal stromal cells derived from bone marrow (BM-MSCs), umbilical cord blood-, adipose tissue- and pancreas-derived multipotent precursor cells, as well as pancreatic ß-cell progenitors and facultative ß-cell progenitors from spleen, liver, and the endometrium(189). ß-cell replacement and regenerative strategies comprise, among others, the reproduction of pre-existing ß-cells, neogenesis from ductal and non-ß-cell progenitors, trans-differentiation of fully differentiated acinar cells, trans-determination of liver progenitor cells and the directed differentiation of stem cells/ß-cell progenitors(139).

Although ESCs offer tremendous therapeutic potential as a cell therapy to cure T1DM, several concerns remain to be solved, including ethical considerations, the oncogenic potential, and the need to achieve total elimination of undifferentiated cells(139). IPSCs may also constitute a feasible nonembryonic source for the generation of ß-cells; nevertheless, their clinical utility is limited by several aspects, such as the incomplete maturation of differentiated cells and their oncogenic potential(139). Also, the risk that iPSCs could be a target for autoimmune response still exists(139). BM-HSCs and BM-

MSCs are the most intensively studied adult stem cells so far, and clinical trials were able to provide proof of exogenous insulin independence after nonmyeloablative, autologous HSC transplantation in new-onset T1DM(190). Even though autologous HSCs do not seem to differentiate into insulin-producing cells themselves, they may help to preserve residual ß-cells and support increases in ß-cell mass by providing neovascularization, diminishing apoptosis, and stimulating proliferation(139). This questionable potential to directly transform in vivo into ß-cells, as well as the lack of success beyond the early-onset of the disease and the possibility of life-threatening adverse effects, limit their widespread clinical use(139). However, allogenic HSC transplants, besides carrying the risk of GvHD, may cause more prolonged remission because both infusion of autoreactive lymphocytes is avoided and, in addition, a graft-versus-autoimmunity effect can occur, that may lead to the eradication of autoreactive recipient lymphocytes by donor lymphocytes(139). Although BMSCs have enormous therapeutic potential and have achieved a satisfactory rate of remission, limitations such as the absence of a standardized protocol to generate these SCs, the problem of poor engraftment and only moderate differentiation under in vivo conditions, as well as their oncogenic potential and the risk of unwanted cytokine release still do exist and diminish their clinical utility(139).

In conclusion, it is likely that a combinatorial approach that takes advantage of targeting different arms of the immune system will be most effective and most promising in order to find a permanent cure for T1DM(139). Defining a population that will respond to treatment is essential, as there is no one-size-fits-all approach. Therefore, adequate biomarkers are needed not only to identify a window for treatment opportunity but also to monitor treatment response.

1.7 Background

Questions concerning insulin biosynthesis and secretion were not resolved to the fullest until the discovery of PI, the precursor of insulin, in 1967(21). PI, a 9kDa protein, consists of both the A and B chain of insulin, connected by the CP. PI displays a full agonist of insulin, even though it only has about 3-5% of its biological activity(22). PI is synthe-

sized in the rough endoplasmic reticulum of the ß-cells by cleavage of the signal sequence of a prohormone, preproinsulin(21). The conversion of PI to insulin occurs in secretory granules of the Golgi apparatus, where PI is cleaved to insulin and CP via PHC1/3, PHC2, and CPE. In healthy individuals, PI is secreted along with CP and insulin in minimal (2-3%) amounts(22). Serum PI levels have been found to be elevated in relation to CP, assessed by an increased PI:CP ratio, both in individuals at high risk for T1DM, as well as in new-onset T1DM(92, 191). Elevated PI:CP ratio is thought to display ß-cell dysfunction and endoplasmic reticulum stress and therefore represents a useful biomarker to predict diabetes onset in the pre-symptomatic phase(92). Classic natural history models - like the Eisenbarth model established in 1984 - implement that T1DM is a condition of near-absolute ß-cell loss by the time of diagnosis. However, more recently, this long-standing opinion has been challenged, as endogenous insulin secretion, represented by serum CP levels, turned out to persist(192) in a significant proportion -up to 80%- of individuals with long-standing disease, even though decreasing with time from diagnosis (2, 27, 28, 193). In parallel, analysis of pancreata from organ donors with T1DM revealed residual insulin-containing ß-cells many years after diagnosis(194, 195). These observations are not new but have experienced revived attention recently, partially owing to improvements in sensitivity of CP assays. Due to the fact that CP is derived from PI, the observation that PI can be detected in subjects where CP is measurable is not unexpected(192). Interestingly, two studies by Sims et al.(3) and Steenkamp et al. (196) recently reported that PI is measurable in individuals with long-standing T1DM, despite the absence of measurable CP levels. Thus, the purpose of our study was to determine whether PI is measurable in patients with long-standing T1DM, with or without residual CP secretion, by using two different methods.

2 Material and Methods

We analyzed data of 69 subjects from the Graz Diabetes Registry for Biomarker Research using two different methods, a sandwich enzyme-linked immunosorbent assay and a radioimmunoassay.

2.1 Graz Diabetes Registry for Biomarker Research

Obesity and diabetes are the major challenges of the 21st-century health system, inheriting increased risk for various complications, such as cardiovascular diseases or cancer. With increasing incidence worldwide, there is an urgent need to identify biomarkers that help us predict these diseases in order to detect individuals at high risk for complications and to implement prevention strategies. For instance, despite having more than eight different groups of drugs available today for the management of T2DM, objective, evidence-based criteria on what the ideal therapy for each patient is, are still absent. Thus, identifying adequate biomarkers is not only the basis for disease prevention but also for therapeutic management. Unfortunately, we experience a deficit of prospective, systematic clinical data needed to evaluate the clinical utility of biobank samples. In order to address this lack of clinical data regarding biomarker research, the Graz Diabetes Registry for Biomarker Research (GIRO) was started in November 2015. The GIRO aims to establish a representative cohort of patients to validate new biomarker candidates regarding their clinical significance. Therefore, patients from the Graz outpatient diabetes clinic with different metabolic diseases such as diabetes, obesity, or lipid metabolism abnormalities are recruited and followed-up for ten years. The annual follow-up consists of data evaluation on biomarker samples and health status.

The inclusion criteria are the following:

- 18 years of age or older
- signed study-specific informed consent
- signed biobank informed consent
- one of the following diseases:
    - diabetes type 1
    - diabetes type 2
    - diabetes type 3
    - maturity-onset diabetes of the young (MODY)
    - late-onset diabetes of the adult (LADA)
    - obesity with a BMI >= 30kg/m^2
    - abnormalities of lipid metabolism

Patients fulfilling the named criteria are recruited at the outpatient diabetes clinic during routine visits at the clinic, after willing to sign the study-specific informed consent and the biobank informed consent for acquisition, storage, and further use of scientific samples by the biobank, as they are both prerequisites for inclusion into the study. Further, a baseline visit is performed, which includes a questionnaire about pre-existing illness, risk factors for metabolic diseases, and current medication, as well as a blood sampling for biomarker research. The follow-up visits are carried out annually within the context of the patients' routine control visits in the outpatient diabetes clinic, including another questionnaire and blood sampling for biomarker research. The annual follow-up could alternatively be performed via phone or email if the patient did not show up for the routine visit.

The aim is to include 2000 patients, with recruitment lasting until December 2020. By the end of March 2020, a total of 1860 individuals have been recruited, with the following patient characteristics: 39% T2DM, 35% T1DM, 9% obesity, 13% abnormalities in lipid metabolism, and 4% other rare subtypes of diabetes. The study is supposed to end after 15 years of duration in December 2030 with a recruitment time of 5 years and a follow-up of 10 years.

2.2 Proinsulin Assays

PI is a precursor molecule of insulin, which is the principal hormone regulating glucose metabolism. As previously reported, PI is synthesized in the pancreatic ß-cells in the Islets of Langerhans and is subsequently processed to form CP and insulin. We used two different methods for the detection of PI: a radioimmunoassay and a sandwich enzyme-linked immunosorbent assay.

2.2.1 Radioimmunoassay

RIA was first reported in 1960 for the measurement of endogenous plasma insulin by Solomon Berson and Rosalyn Yalow in New York.

The underlying principle of RIA is the competition between labeled and unlabeled antigen for specific antibody sites, creating antigen-antibody complexes(197-199): radioimmunoassay is performed by incubating a known quantity of radioactively labeled

tracer antigen with a constant dilution of antiserum containing a known amount of antibody for that antigen, resulting in the formation of specific antigen-antibody-complexes, whereby the concentration of antigen-binding sites on the antibody is limited(199). For example, only 50% of the entire tracer concentration can be bound by antibody(199). Subsequently, a sample containing an unknown quantity of the same but unlabeled antigen is added in order to initiate a competition between the unlabeled antigen in the serum and the labeled tracer for a constant number of antibody binding sites(199). Thus, as the amount of the unlabeled antigen will increase, the quantity of tracer bound to the antibody will decrease(199). This can be measured after the separation of antibody-bound from free tracer and measurement of one or the other, or both fractions, by assessing the radioactivity with a gamma counter. A standard curve is established with rising concentrations of standard unlabeled antigen, and from this curve, the quantity of antigen in unknown samples can be measured(199). For the following paragraphs, see(199):

We used the EMD Millipore Human Proinsulin RIA Kit (Burlington, USA) for quantitative determination of proinsulin in plasma, serum, and other biological media, which shows the following characteristics: this assay has no cross-reaction, neither with Human Insulin (<0.1%), nor with Human C-Peptide (0.1%), and therefore suits to measure “true” Proinsulin levels with a sensitivity of 3.1pmol/L.

The EMD Millipore Human Proinsulin assay uses 125I-labeled human proinsulin as tracer antigen and a constant dilution of human proinsulin antiserum to assess the level of proinsulin in serum/plasma by the double antibody/PEG technique. Each kit is sufficient to run 250 tubes and consists of the following reagents: an assay buffer, human proinsulin antibodies, 125I-human proinsulin, human proinsulin label hydrating buffer, human proinsulin standards, a matrix solution, quality controls 1 and 2, and a precipitating reagent.

After preparation of the standard, the assay set-up is performed by adding buffer, the standards, quality controls, and the samples to the tubes, as well as the matrix solution-HPI and human proinsulin antibodies, followed by incubation for 48 hours at room temperature. Subsequently, the hydrated 125I-Proinsulin tracer is added and incubated for 22 hours at room temperature. After several final steps, where the precipitating reagent

is added, after incubation for 20 minutes at 4°C and centrifugation for 25 minutes, each tube is counted in a gamma counter for 1 minute, and the level of proinsulin in unknown samples is counted through automated data reduction procedures.

Regarding specimen collection, it is important that a maximum of 200 µL per assay tube of serum or plasma is used, as the limit of sensitivity for the Human Proinsulin assay is 3.055 pM when using a 200 µL sample size. Smaller sample volumes could be used when proinsulin concentrations are expected to be elevated or with a limited sample size. Additional assay buffer must be added to balance a possible difference so that the volume is equivalent to 200 µL.

The calculation of results is realized by computerized calculation and by manual calculation. The automated calculation can be performed by most gamma counters having data reduction capabilities. Each laboratory should establish its own expected range of values. The following results may serve as a reference until own values are available: normal fasting range of 7.9 ± 1.5 pM.

The Specificity was the following: Intact Human Proinsulin 100%, Des 31,32 Human Proinsulin 95%, Des 64, 65 Human Proinsulin <0.1%, Human Insulin <0.1%, and Human C-Peptide <0.1%.

For Quality Control, specimens must be run with each standard curve to evaluate assay performance. For this purpose, two levels of controls are given.

2.2.2 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay is analogous to RIA, except having a distinct label, which is an enzyme in ELISA rather than a radioisotope in RIA(200). The basic principles of RIA will be presented hereafter, see(200, 201): the enzyme-linked immunosorbent assay was introduced in 1971 by Peter Perlmann and Eva Engvall at Stockholm University in Sweden. ELISA is a commonly performed analytical assay used for detection and quantification of substances such as proteins, antibodies, and hormones. The general principle is to quantify an antigen on a solid surface by utilizing a specific antibody with a covalently coupled enzyme. The first step is the immobilization of a sample containing an unknown amount of antigen on a solid surface either via adsorption or specifically through a so-called capture antibody in a Sandwich-ELISA.

After immobilization of the antigen, the detection antibody is added, resulting in the formation of an antigen-antibody-complex. This detection antibody can be directly conjugated to an enzyme (referred to as direct ELISA) or can itself be detected by a labeled secondary detection antibody (indirect ELISA). In between the single steps of this procedure, washing steps are used in order to remove unbound reagents. After the final washing step, the enzyme's substrate is added in order to develop the plate, and a visible signal is generated, which assesses the concentration of antigen in the sample and is measured by a spectrophotometer. The measured signal then indicates the concentration of the analyte in the sample. Even though this enzyme/chromogenic substrate system is a common principle, many other techniques exist(200).

For the following paragraphs, see(202):

We used the Mercodia Proinsulin ELISA (Uppsala, Sweden) for quantitative determination of proinsulin, which is calibrated against the International Reference Reagent for human proinsulin, IRR 84/611 and has a sensitivity of 0.5pmol/L. It is based on the sandwich technique. During first incubation at room temperature (18-25°C) for 1 hour, proinsulin in the sample reacts with capture antibodies bound to the microtitration well. After washing, anti-proinsulin detection antibodies, which are conjugated with the enzyme peroxidase, are added, and after the second incubation for one hour at room temperature and a washing step, unbound enzyme-labeled antibody is removed. Finally, the substrate, 3,3', 5,5'-tetramethylbenzidine, is added, leading to a reaction with the bound conjugate, and incubated for 15 minutes at room temperature. The reaction is ended by adding the stop solution 0.5M H_2SO_4 to give a colorimetric endpoint that is read at 450nm spectrophotometrically to calculate the results and read again after 30 minutes. The calculation of results is realized by computerized calculation and by manual calculation.

Good practice demands the establishment of an own expected range of values for each laboratory. The following results may represent a reference until own values are available: fasting levels of 112 tested, apparently healthy individuals, yielded a mean of 10pmol/L, a median of 7 pmol/L, and a range corresponding to the central 95% of the observations of 3.3-28 pmol/L. The Mercodia ELISA shows the following performance characteristics: the detection limit is 0.5 pmol/L calculated as two standard deviations

above the Calibrator 0. Samples with a concentration of up to 80.000 pmol/L can be measured without giving falsely low results. The specificity of the Mercodia ELISA was determined through the following cross-reactions: insulin <0.03%, C-peptide <0.006%, Proinsulin Des (64-65) 84%, Proinsulin Split (65-66) 90%, Proinsulin Des (31-32) 95%, Proinsulin Split (32-33) 95%.)

3 Results

3.1 Patient characteristics

For our measurements, we recruited 69 subjects (41 males, 28 females) from the Graz Diabetes Registry for Biomarker Research, who showed the following characteristics (table 1): diagnosis of T1DM, a mean age of 39.1 ± 16.0 years, a mean diabetes duration of 19.9 ± 14.3 years, an average systolic blood pressure of 128 ± 15mmHg, an average diastolic blood pressure of 82 ± 9mmHg, 18% presented with albuminuria, 16% with retinopathy, 13% with polyneuropathy and 36% with hyperlipidemia. The mean BMI was 25 ± 4kg/m^2, the mean abdominal girth was 88 ± 12cm, and the mean hip size101+-10cm.

Study population	
Females	28
Mean age in years (±SD)	39.1±16
Diabetes duration in years (±SD) <10 years 10-20 years >20 years	19±14 26% 38% 36%
Blood pressure syst. (mmHg)	128±15
Blood pressure diast. (mmHg)	82±9
Albuminuria (%)	18
Retinopathy (%)	16

Table 1: Patient characteristics

3.2 C-Peptide levels

84% of subjects were classified as CP-negative (table 2), with fasting CP levels below 0.08 nmol/L(4). Subsequently, 16% of subjects were defined as CP-positive. In line with data presented by Sims(3), we considered three groups of fasting CP values (table 2): the majority (69% of all subjects) had CP levels below the limit of detection (<0.017nmol/L), while 22% of subjects had low CP values (0.017-0.20 nmol/L) and only 9% of individuals showed significant endogenous insulin secretion, represented by CP values >0.20nmol/L(203).

C-peptide (nmol/L)	% of the study population
<0.017	69%
0.017-0.20	22%
>0.20	9%
<0.08	84%

Table 2: C-peptide levels

In line with the data reported by Sims et al.(3), CP levels were inversely correlated with diabetes duration, in the sense that they slowly decreased with increasing disease duration (table 3). We found a decrease from 0.37 ± 0.74 nmol/L at disease duration less than 10 years, over 0.18 ± 0.34 nmol/L at diabetes duration of 10-20 years, to 0.01 ± 0.02 nmol/L in subjects who had T1DM for over 20 years.

Diabetes duration	C-peptide (nmol/L)
<10 years	0.37 ± 0.74
10-20 years	0.18 ± 0.34
>20 years	0.01 ± 0.02

Table 3: C-peptide levels based on diabetes duration

3.3 Proinsulin levels

Table 4 shows the PI results: according to RIA measurements, 60.3% of patients were classified as PI-positive (with a sensitivity of 3.1 pmol/L), whereas only 31.9% were PI-positive when using the ELISA method (with a sensitivity of 0.5 pmol/L). In the CP-negative group, 54.4% were considered as PI-positive with RIA measurement, while only 20.7% were PI-positive according to ELISA measurements.

	RIA	ELISA
Proinsulin-positive, n (%)	41 (60)	22 (32)
Proinsulin-positive with C-peptide <0.08 nmol/L (%)	54	21

Table 4: Proinsulin levels

Moreover, PI levels measured with both assays were classified according to diabetes duration (<10 years, 10-20 years, and >20 years) (table 5). Interestingly, although also PI levels seem to decrease with increasing duration of disease, there were some discrepancies between the two methods: the ELISA showed a steady decrease in PI levels, from 4.8 ± 9.1 pmol/L at diabetes duration <10 years, over 0.34 ± 0.59 pmol/L at diabetes duration of 10-20 years, to 0.1 ± 0.26 pmol/L in subjects who had diabetes for over 20 years. By contrast, the PI levels measured by the RIA method appear to decline too, with levels of 4.7 ± 5 pmol/L, before rising again to 6.2 ± 6.5 pmol/L with a diabetes duration of >20 years.

Diabetes duration	RIA (pmol/L)	ELISA (pmol/L)
<10 years	9.2 ± 8.8	4.8 ± 9.1
10-20 years	4.7 ± 4.9	0.34 ± 0.59
>20 years	6.2 ± 6.5	0.1 ± 0.26

Table 5: Proinsulin levels based on diabetes duration

4 Discussion

4.1 C-peptide results

In line with our and Sims' results, the study carried out by Oram et al.(28) reported that with increasing disease duration, absolute CP levels were seen to decrease. However, most patients with long-duration T1DM maintain secretion of very low levels of endogenous insulin, as evidenced by increasing CP levels after meal stimulation, representing functionally responsive ß-cells. This response of very low-level CP to a mixed meal is a meaningful observation because it is strongly supportive of the assumption that the residual ß-cells are functional and make it very unlikely that low CP levels are the result of analytical noise(28).

These findings indicate that ß-cells are either escaping immune attack or experience regeneration(28), which challenges the classical notion according to T1DM models like the Eisenbarth model, that at the time of diagnosis, nearly all insulin-producing cells are destroyed. Precisely, 73% of patients with T1DM and a disease duration of more than five years had detectable CP concentrations(28). The Joslin medalists study (with a disease duration >50 years) showed similar results: 67% of medalists had CP levels above the detection limit(2). Also, Davis et al.(27) reported that residual CP secretion is preserved in almost one out of three individuals after three or more years after disease onset.

The CP assays Steenkamp(196) and Sims(3) used to determine their CP-positive subjects were able to detect CP down to 17pM, which is far below what is considered significant. However, the question remains whether such low CP concentrations, even if above detection limit, have any clinical relevance, as significant endogenous insulin secretion is considered to be maintained at CP levels >0.2nmol/L, the cut-off for clinically meaningful CP in individuals with T1DM(203). In particular, the same could be true for PI concentrations, raising the question if the reported PI levels are clinically relevant and whether they are responding to physiological stimuli. Nevertheless, the cut-off level for clinical meaningful PI levels is yet unknown and awaits further investigation. Sullivan et al.(204) suggested 6.0pmol/L as a cutoff for relevant PI levels, based on stimulated PI levels from healthy controls.

In summary, even though CP-levels are seen to decrease with time from diagnosis, they indicate the presence of residual functioning ß-cells. Supporting this assumption, PI is measurable in early years after disease onset. Nonetheless, it is not expected that PI is also secreted in remarkable amounts beyond the early years after disease onset.

4.2 Proinsulin results

As mentioned above, our findings suggest that in individuals, who secrete detectable amounts of CP, PI is measurable as well, which is not surprising. The actual surprising result is that in a subset of individuals with long-standing disease, where no CP secretion was detectable, PI could still be measured, suggesting a discordance between PI production and conversion to CP and insulin in some individuals with established T1DM. Sims et al.(3) confirmed this assumption, as they found a correlation between CP and PI levels only in the group with high residual CP.

In line with our findings, Sims(3) and Steenkamp(196) demonstrated preserved PI secretion in CP-negative subjects as well, although with different PI-positivity rates: Sims et al. reported the persistence of measurable serum PI in most individuals (95.9%) with long-standing T1DM, this finding was also observed in individuals who had undetectable serum CP (89.9%), while Steenkamp only found 16% of CP-negative subjects of the same exchange cohort to secrete PI. Interestingly, in contrast to our findings, serum PI levels measured by Sims et al. continued to remain relatively stable over four years of follow-up, despite advancing loss of CP.

However, explanations for these findings are yet to be unraveled, and numerous questions remain unanswered, like the question of why certain patients with T1DM without measurable CP maintain the ability to secrete PI and show remarkable endogenous insulin secretion many years after disease onset?

These findings could be explained by the presence of residual but dysfunctional ß-cells, which can set up PI production but are devoid of the ability to further process PI to mature insulin and CP, resulting in increased PI and undetectable CP levels. The elevated levels of PI and elevated non-fasting PI:CP ratios in subjects with T1DM compared to healthy controls mentioned earlier are supportive of this assumption. In indi-

viduals without diabetes, CP concentrations are remarkably higher than PI, approximately by a ratio of 1:100 (mol/mol)(196). In addition, Oram et al.(205) reported that at the tissue level, a notably reduced insulin content and abnormal hormone processing could be observed, also, PI was found to cumulate in the pancreas and the periphery. These findings confirm the earlier mentioned hypothesis that there are populations of ß-cells that are not able to fully process PI into mature insulin and CP(205).

Representing a possible reason for disturbed prohormone processing, Sims et al.(206) postulated that under conditions of ß-cell stress, when the whole-body insulin demand surpasses ß-cell secretory capacity, the procedure of PI processing can become insufficient. As a consequence, increased expression of PI relative to insulin and CP has been shown to occur in islets from donors with AAb positivity and long-standing T1DM(207, 208), explaining the increased PI:C ratio in the circulation(209) and increased levels of PI despite low or absent CP. Hence, the assessment of CP secretion alone could underestimate the ability of ß-cells to set up insulin production, while increased PI secretion may give insight into specific disease pathology(3). In congruence with these observations made by Sims et al., Steenkamp et al.(196) reported that the elevation of the serum PI: CP ratio displays ß-cell stress, in such a manner that increasing secretory demand on a “stressed ß-cell” results in incomplete PI processing, leading to the release of latter. Thus, the persistence of PI secretion in patients with T1D may be due to diminished immune destruction or regeneration of ß-cells, with some regenerated but dysfunctional ß-cells being only capable of producing PI but lack the ability to secrete mature insulin and CP(196).

Interestingly, Wasserfall et al.(208) recently observed diminished levels of prohormone convertase PHC1 mRNA and silencing of INS promoter compared to controls, consequently leading to incomplete transcription of the INS gene and incomplete prohormone processing in T1DM individuals. These observations could explain the findings of disrupted prohormone processing in T1DM patients named above. Notably, PHC and CPE activities do also play an important role in amyloid polypeptide processing(210), a ß-cell co-secretory molecule, and in congruence with the CP data, levels of islet amyloid polypeptide were not detectable in most T1D pancreata relative to healthy controls(208). Thus, the presence of PI, together with the low levels of insulin and with the

near absence of CP and islet amyloid polypeptide, is further supportive of the hypothesis that the processing machinery for PI into insulin and CP may be failed due to the previously mentioned findings that prohormone convertase enzyme mRNA levels are reduced in T1DM. In congruence with these findings, Sims et al.(207) evaluated PI processing enzyme mRNA levels by laser capture microdissection (LCM) and mass spectrometry analysis and demonstrated reductions in the PI processing enzymes PHC1/3 and CPE in T1DM donors. Furthermore, in order to confirm the hypothesis mentioned above, that reductions in PI processing enzyme expression are related to islet inflammatory stress, Sims et al.(207) additionally determined the expression of CPE, PC1/3, and PC2 in human islets from ten non-diabetic donors treated with 24 hours of a cytokine cocktail. They found out that cytokine treatment was related to notable reductions in mRNA expression levels for each of the processing enzymes(207), supporting the notion that inflammatory stress leads to reduced prohormone processing in T1DM. Previous studies have also reported increased PI release and diminished levels of PC1/3 and PC2 in human islets treated with inflammatory cytokines(211), leading to the assumption that agents targeting PI processing may have therapeutic benefit in some individuals with T1DM. In line with these data, it was reported that the administration of a protein-folding chaperone, TUDCA, to NOD mice diminished islet ER stress, increased insulin secretion, and reduced T1DM incidence(212). These observations raise the question, if therapies aimed at restoration of the PI processing pathway, e.g. the administration of protein-folding chaperones, could be successful in human T1DM, too. To answer this question, further research is needed.

However, one could oppose that the deprivation of ß-cell mass itself could result in an absolute reduction of PI processing enzymes, but Sims et al.(207) conducted LCM analysis only on islets that contained insulin - implying residual ß-cell mass - and found reductions in enzyme expression mentioned above. Additionally, the finding that cytokine stress directly reduces the expression of mRNAs encoding prohormone-processing enzymes reinforces the assumption of a processing defect that is not due to the loss of ß-cell mass(207).

In conclusion, some individuals with long-duration T1DM preserve the ability to secrete PI, even in the absence of CP(3). These findings presented above emphasize the possible role of defective prohormone processing in T1DM and constitute an explanation for the deficiency of CP (and insulin) in subjects who are otherwise PI-positive(207). Even if the question of why certain individuals with long-duration T1DM maintain the ability to secrete PI without detectable CP might be answered by further research, there is a need to investigate how long insulin production is preserved, whether ß-cell function is fully maintained, and what personal or disease features predict or contribute to residual ß-cell function.

4.3 Critical reflection and outlook

Our study is one of the few that have analyzed PI secretion in long-standing T1DM and therefore is of great importance in offering further insight into pathophysiological processes of T1DM and paving the way for new therapeutic strategies. Notwithstanding its value, our study does have some limitations: first, the relatively small sample size of 69 subjects and the fact that we did not have a control cohort. Moreover, we did not perform follow-up measurements. Furthermore, our samples were collected in a fasting manner, but no comparison to mixed meal tolerance test (MMTT) was performed.
Two other important studies already named above that have investigated the same question are those carried out by Sims(3) and Steenkamp(196). Even though their investigations focused on the same analyte, PI, the results are not easy to compare due to multiple factors, some of which are shown in the following table (table 6):

	Our study	Sims et al.	Steenkamp et al.
Disease duration in years	0-61, average 19	>3	>3
Sample collection	fasting	MMT and fasting	Non-fasting
Number of subjects	69	319	80
PI assay, detection limit (pmol/L)	RIA Millipore HPI-15K 3.1	RIA Millipore HPI-15K 3.1	ALPCO Stellux 0.14

Follow-up	none	4 years	none
CP cut-off (nmol/L)	0.08	0.017	0.017

Table 6: Comparison of different studies

One parameter that hampers comparability between different studies is the variable cut-off value used for the classification of CP-negative subjects, in whom PI was still detectable. In particular, the cut-off we used to define undetectable CP levels was 0.08nmol/L, with values >0.08nmol/L representing CP-positivity, while Sims et al. and Steenkamp et al. used 0.017nmol/L as a cut-off for the classification of CP-positivity or negativity. Besides, other factors restricting comparability of the results are differences in cohort selection criteria, methodological differences in sample collection and storage, as well as different measuring methods themselves. In this regard, the results of PI measurements by Sims(3) and Steenkamp(196) illustrate the difficulties in comparison due to methodological differences and different measuring methods: while Sims et al. reported that 89.9% of subjects had detectable PI levels despite undetectable CP levels, Steenkamp et al. only found 16% to be PI-positive. This discrepancy is supposed to originate from differences in the analytical sensitivity of the assays used for PI detection, as both study groups recruited probands from the same T1DM Exchange cohort. This problem of variability of results depending on the assay used was also pointed out by Jones et al.(4): numerous commercially available CP assays are utilized worldwide, and they all show remarkable variations of precision(213), limiting comparison between different studies. The ideal and equal standardization of measurement methods between different laboratories has yet to be reached,(4) implying that CP results measured by distinct methods, and sometimes by distinct laboratories using the same methods, inherit the difficulty of limited comparability (213, 214). The same is supposed to be true for the PI assays.

Interestingly, Steenkamp et al. suggested - after having performed extensive assay validation on Millipore's RIA, ALPCO's ultrasensitive Stellux ELISA, and Mercodia's ELISA - that Millipore's RIA, which was used by Sims and our study group for PI measurement, constantly overrated total PI. This could represent a possible explanation for

the high PI levels measured by Sims et al. with this radioimmunoassay and could furthermore account for the different rates of PI-positivity measured by the two assays in our study, too, as the PI-positivity rates measured by Millipore's RIA were significantly higher than those measured by Mercodia's ELISA, which performed best according to Steenkamps' validation analysis. Taking into account this observed wide range of PI-positivity dependent on the assay used in our study (54.4% with RIA, while only 20.7% with ELISA), the question, what is really measured, must be answered, since this variability of results can not solely be ascribed to different measurement methods.

Regarding the question of what is really measured, other findings reported by Sims et al. and our study group must be considered, too: although our and Sims' study group used the same assay to determine PI concentrations, the RIA Millipore HPI-15K with a sensitivity of 3.1pmol/L, our results did still differ, as Sims et al. found 89.9% in the CP-negative group to retain the ability of PI secretion, while our RIA measurement classified 54.4% as PI-positive. This variability of PI levels measured with the same assay implies differences in clinical cohorts, as well as methodological differences. Nevertheless, it remains unclear whether these factors alone are able to explain the wide range of results (89.9% vs. 54.4%) to the fullest. Therefore, it is necessary to clarify what is really measured with which assay. Sims(215) postulated that such quantitative immunoassay performance differences between different assays are not unexpected owing to antibody interactions with different epitopes on native antigens and differences regarding assay calibration. Moreover, their study group also performed validation experiments like specificity analysis and testing for cross-reactivity with human insulin, with human CP, with PI in the context of insulin antibodies, and with PI in individuals after pancreatectomy requiring exogenous insulin analogs. In addition, they performed quantitative testing for reproducibility with ten samples from individuals with undetectable CP but detectable PI using mass spectrometry in order to verify that the measured analyte in their samples was truly PI.

In summary, the comparability between these three studies – Sims', Steenkamp's, and our study - is restricted, although they all measure the same analyte, PI. Therefore, there is a need to identify and validate optimal PI measurement methods. Furthermore, it is necessary to reproduce the observed findings in additional clinical cohorts.

We need to consider the possibility that there is certain interference of autoimmune antibodies such as GADA or ICA with PI. Therefore, in a follow-up project, we will undertake further measurements, including targeted mass spectrometry analysis, to investigate what is really measured with those two assays used in our study and to establish a reference method that is independent of the antigen-antibody reaction.

5 Conclusion

In conclusion, the findings mentioned above demonstrate that PI secretion persists in some individuals with long-standing disease, even when no CP is detectable. This could be due to a malfunctioning PI processing machinery, with aggravations owing to islet inflammatory stress. These findings bear the tantalizing question of whether the residual PI-producing cells could potentially be accessible to therapeutic strategies aimed at restoring defect prohormone processing. Nonetheless, the question “what is really measured?” still awaits elucidation, and further research in this field is required.

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