Chapter 3

Early- Versus Late-Onset Type 1 Diabetes: Two Different Pathophysiological Subtypes with Implications for Therapy

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Additional information is available at the end of the chapter

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Abstract

Insulin, as measured by C-peptide, is produced for decades after onset of type 1 diabetes, and even very low levels of C-peptide have clinical significance. In this chapter we show that two distinct pathophysiological subtypes of type 1 diabetic subjects can be distinguished. Early-onset diabetic subjects (≤20 years) have rapid loss of C-peptide, whereas late-onset diabetic subjects (>20 years) have slower C-peptide declines over decades. Early-onset diabetics have significantly lower levels of persistent autoreactive CD8+ T cells than do late-onset diabetic subjects. In late-onset disease, robust production of autoreactive T-cells occurs even in the absence of C-peptide. Metabolomics analysis reveals frequent differences between the two subtypes of subjects in the levels of amino acids, carbohydrates, cofactors, lipids, peptides, and xenobiotics. There are statistically significant differences related to protective islet functions, islet health, development, blood sugar control, and regulation of exocrine pancreas function. Taken together these findings suggest that pancreas pathobiology, as well as durability of abnormal T-cell response should be considered in immune targeting treatments. Therapies aimed at immune defects alone are likely to work best in late-onset diabetics. Therapies aimed at islet cell preservation in early-onset diabetic subjects likely have greater efficacy if administered shortly after disease onset.

Keywords: type 1 diabetes, C-peptide, metabolomics, autoreactive T-cells, therapy, type 1 diabetic subtypes, early-onset diabetes, late-onset diabetes, metabolites

1. Introduction

This chapter summarizes the evidence supporting two distinct pathophysiological subtypes of type 1 diabetes (T1D) based on age of onset (AOO). The characterization of these two subtypes—early- versus late-onset—traces back to the finding that β-islet cells of the pancreas are still functional decades after disease onset. When first published in 2012 [1], this finding ran against conventional wisdom that all beta cells die within 2 years of onset and that type 1 diabetics usually have an absolute deficiency of insulin [2]. Instead, low levels of C-peptide, which is co-secreted with insulin and thus serves as the best measure of endogenous insulin secretion, persist for decades after disease onset. Confirmation of the study by Wang and colleagues came from several studies [3–8], showing that 80% of people with long-standing diabetes have low but detectable levels of C-peptide upon stimulation with oral glucose [9]. These studies were made possible by new ultrasensitive C-peptide assays with detection limits of 1.5–5.0 pmol/L. Older C-peptide assay typically only detected to 40–50 pmol/L. The low levels of C-peptide were indicative of intact β-islet cell function according to assays showing that C-peptide levels rose in response to hyperglycemia or a mixed-meal stimulus [1, 4, 5]. Intact β-islet cell function in long-standing disease suggests that β-islet cells are either regenerating or evading immune attack.

What's more is that these low levels of C-peptide or any persistent C-peptide have clinical significance. They are associated with fewer diabetic complications (e.g., nephropathy, neuropathy, foot ulcers, and retinopathy), better metabolic control via HbA1c, and prevention of hypoglycemia [7, 10–12]. The finding of C-peptide persistence and its clinical significance paved the way for an examination of the pathophysiological differences between early- and late-onset type 1 diabetes.

2. Rate of C-peptide decline differs between early- and late-onset diabetes

Early-onset diabetics have a rapid loss of residual C-peptide, whereas late-onset diabetics have a slower rate of C-peptide decline, which occurs over decades [1, 6, 8]. Here, we confirm this finding with one of the largest patient samples to date of fasting C-peptides.

Cross-sectional data from 1958 long-term type 1 diabetics, who were recruited to the Massachusetts General Hospital, show the gradual decades-long decline in fasting C-peptide secretion from the pancreatic islets (**Figure 1A**). The rate of decline in this group varied according to age of onset, with early-onset diabetics (n = 1063) showing a rapid decline and late-onset (n = 895) showing a slow decline (**Figure 1B**, left and right panels). The difference in C-peptide secretion between early-onset and late-onset subjects is statistically significant ($p < 0.001$). Detecting low fasting C-peptide levels during the decades-long decay is possible

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because of the improved detection limit with ultrasensitive ELISA[1] of only 1.5 pmol/L, which is significantly lower than older C-peptide assays with detection limits in the 40–50 pmol/L range [1]. The remainder of the chapter delves into the pathophysiological basis of this finding, using an assay for autoreactive CD8+ T lymphocytes (hereinafter referred to as autoreactive T-cells) and using metabolomics, the study of small molecules from intermediate metabolism.

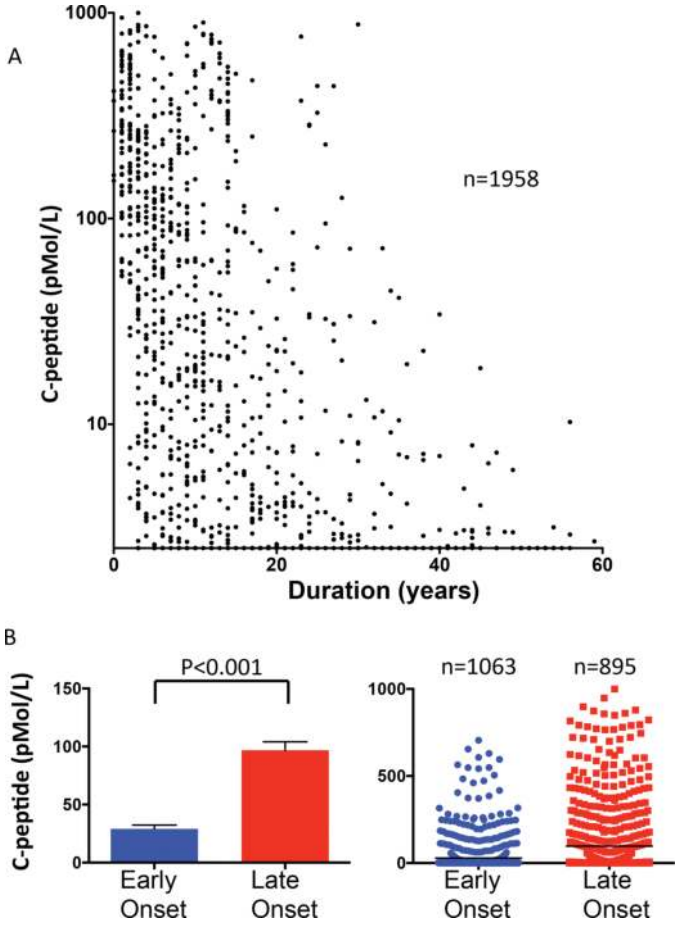

Figure 1. The decades-long persistence of C-peptide in patients with type 1 diabetes (n = 1958) and the more rapid fall in C-peptide levels with younger age of onset. (A) There is a gradual, decade-long decline in C-peptide detectable with an ultrasensitive assay. (B) The decline in C-peptide levels is related to the age of onset of the disease. Data are stratified by early- (left, blue) or late-onset diabetes (right, red). p Values were calculated using a Mann-Whitney U test (Wilcoxon rank-sum test), and the data are represented as mean ± SEM $p < 0.001$. The left panel depicts the large difference in mean C-peptide, whereas the right panel shows the same data according to individual data points.

[1] Cat. No 10-1141-01, Mercodia AB (Uppsala, Sweden). The assay was calibrated against the International Reference Reagent for C-peptide (IRR C-peptide 84/510; a WHO standard) and listed with the US Food and Drug Administration as Class I IVD device.

3. Levels of autoreactive T-cells differ between early- and late-onset diabetes

Here, we provide evidence that early-onset diabetics (≤20 years of age) have a significantly lower level of autoreactive T-cells than do late-onset diabetics (>20 years of age). We studied autoreactive T-cells in a subset (n = 178) of our sample (n = 1958) using a peptide-major histocompatibility complex class I (pMHC-I) multimer technique (fluorochrome-conjugated and peptide-loaded major histocompatibility complex class I multimers) in conjunction with flow cytometry. Two diabetes-specific peptides for autoreactive T-cell detection were used, i.e., peptide sequences from epitopes of pancreatic beta cells, islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) or insulin B chain (InsB) (tetramers were purchased from Beckman Coulter, Fullerton, CA, whereas dextramers were a generous gift from Immudex, Copenhagen, Denmark). For the IGRP peptide, pMHC-I was loaded with the peptide sequence for amino acids 228–236 (LNIDLLWSV). For the InsB peptide, pMHC-I was loaded with the peptide sequence corresponding to amino acids 10–18 (HLVEALYLV). For background fluorescence of T-cells, a matched negative (neg) HLA class I structure was loaded with an irrelevant peptide. The peptide sequence of the negative controls is kept proprietary by the companies but does not occur on mammalian cells (Beckman Coulter, Immudex). Using these methods, type 1 diabetics for decades after diagnosis have detectable levels of autoreactive T-cells measured with peptide-major histocompatibility complex class I (pMHC-I) multimers (**Figure 2**).

Type 1 diabetics were cross-sectionally studied for the presence or absence of autoreactive T-cells for decades after disease onset. The patients all had established type 1 diabetes (**Table 1**). Subjects had mean age of onset of 27.9 ± 1.6 years and a mean duration of diabetes of 14.9 ± 1.4 years. Typically, autoreactive T-cell detection studies are performed shortly after disease onset. However, because we wanted to know the association between prolonged C-peptide secretion and autoreactive T-cells, this study spanned decades of diabetes duration with simultaneous monitoring of C-peptide. Autoreactive T-cells were defined as samples staining with either IGRP or InsB MHC class I multimers. The data show that some long-standing diabetics have persistence of autoreactive T-cells decades after disease onset.

The type 1 diabetic subjects studied for autoreactive T-cells with pMHC-I multimers were divided into two groups, based on their status of early onset (n = 131, shown in blue) or late onset (n = 47), shown in red (**Figures 3** and **4**, **Table 1**). For each group, we determined the disease duration versus the serum C-peptide levels (**Figure 3A**). For each group, we also determined the presence of autoreactive T-cells versus disease duration (**Figure 4**). We once again observed that the decay of C-peptide secretion is faster in the early-onset group as compared to the late-onset group. For most subjects with early-onset, the C-peptide became undetectable within 10 years, whereas in the late-onset subjects, C-peptide lingered for decades (**Figure 3A**). The difference in C-peptide between the early-onset and late-onset was highly significant (C-peptide for early-onset 33.9 ± 5.4 pmol/L versus C-peptide for late-onset 98.6 ± 13.5 pmol/L (mean ± SEM; one-tailed Mann-Whitney U test $p < 0.0001$)).

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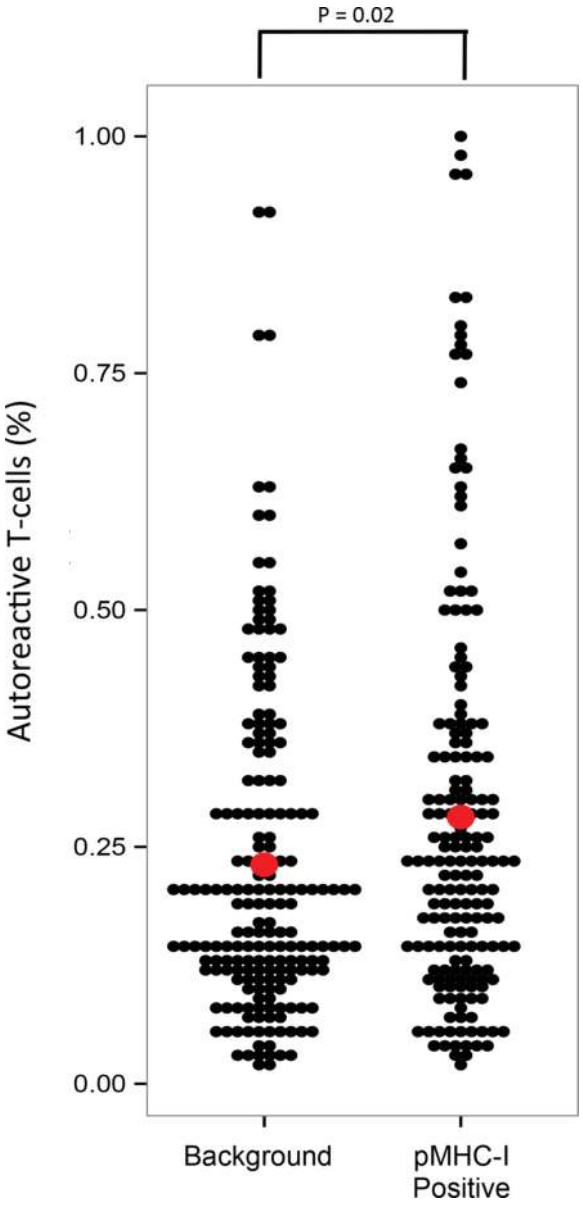

Figure 2. Type 1 diabetics for decades after diagnosis have detectable levels of autoreactive T-cells measured with peptide-major histocompatibility complex I (pMHC-I) multimers. Both insulin B and IGRP pMHC-I multimers were able to detect autoreactive CD8 T-cells of long-term diabetics. $n = 192$ samples for negative controls and $n = 178$ for samples stained for autoreactive cells. The means are represented by the large red dots. The background of 0.24% positive background fluorescence was used as the lower limits of detectability for the presence of pMHC-I multimers. Mann-Whitney U test shows a significant difference at $p = 0.02$.

	All	Early	Late
n	178	47	131
AOO (years)	27.9 ± 1.6	10.7 ± 1.2	34.7 ± 1.1
Duration (years)	14.9 ± 1.4	22.0 ± 2.6	11.3 ± 1.3
% Female	42.0%	46.2%	40.5%
C-peptide (pmol/L)	47.6 ± 14.3	3.8 ± 1.3	78.8 ± 18.9
Gad65 Ab (U/mL)	93.0 ± 12.0	84.7 ± 26.5	95.5 ± 13.5

All values are mean ± SEM.

Table 1. Clinical characteristics for early- and late-onset T1D.

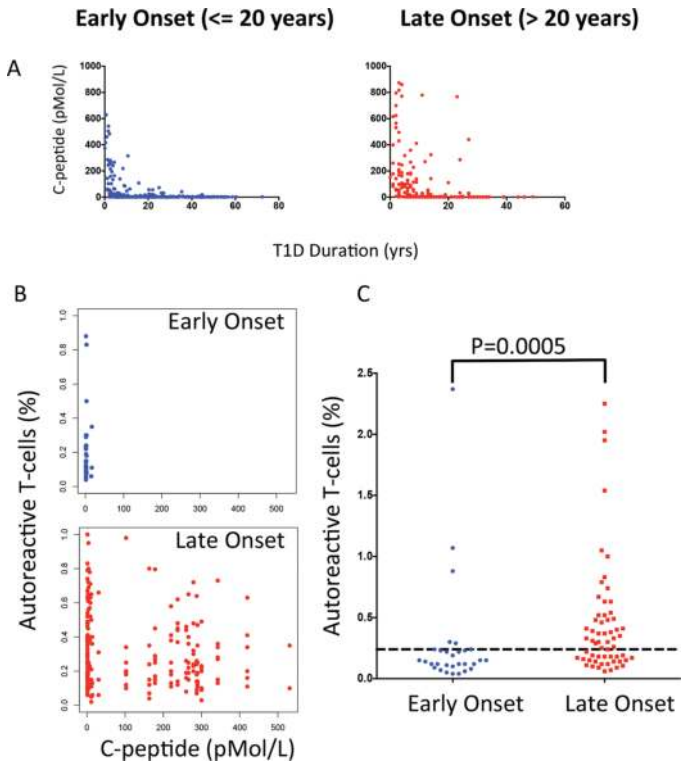

Figure 3. Early- and late-onset type 1 diabetics not only vary in the persistence of C-peptide but also dramatically vary by the presence or absence of autoreactive T-cells. (A) For this data set of early-onset diabetics (n = 47) and late-onset diabetics (n = 131), C-peptide decay continues to show prolonged presence exclusively in late-onset diabetes. (B) Late-onset diabetics have persistence of C-peptide and also the presence of abundant autoreactive T-cells. Early-onset diabetics have neither the persistence of C-peptide nor the presence of autoreactive cells. (C) At undetectable C-peptide levels in an ultrasensitive assay almost no autoreactive T-cells are detectable in early-onset subjects, but abundant autoreactive T-cells are present in late-onset diabetics. Mann-Whitney U test, p = 0.0005.

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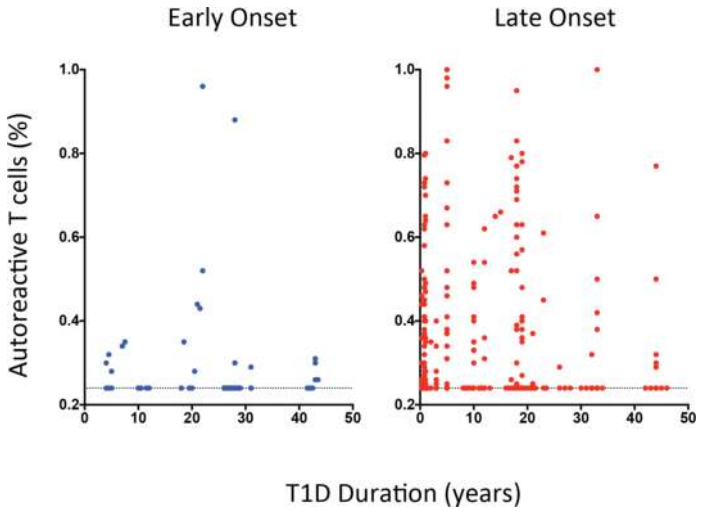

Figure 4. Relationship of diabetes disease duration and presence of autoreactive T-cells; comparison of early- and late-onset diabetes.

We then determined the levels of autoreactive T-cells in the early- and late-onset diabetic subjects using the pMHC-I multimer technique (**Figure 3B** and **C**). The early-onset group had little or no detectable C-peptide and little to no autoreactive T-cells. In contrast, the late-onset group frequently had large amounts of C-peptide coupled with large numbers of autoreactive T-cells. At first glance this seemed to make sense since the presence of islet activity in the pancreas is expected to be associated with the persistence of active CD8 autoimmunity. Also when we studied early- and late-onset subject pMHC-I autoreactive T-cells compared to disease duration, the early-onset diabetics had no relationship with duration and the presence or absence of T-cells. In contrast, late-onset diabetics had a decades-long decay in the presence of autoreactive T-cells (**Figure 4**).

But when early- and late-onset diabetics were compared when neither group had any detectable C-peptide, the late-onset diabetic subjects still continued to exhibit the abundant presence of autoreactive T-cells (**Figure 3B** and **C**, $p = 0.0005$). In marked contrast, when the early-onset diabetics were without detectable C-peptide, even when using an ultrasensitive C-peptide assay, almost no autoreactive T-cells were found in the early-onset cohorts. What are the possible explanations?

Our favored explanation is that autoreactive T-cells play a much more dominant role in disease etiology in late-onset than in early-onset disease. The massive abundance of detectable autoreactive T-cells in late-onset diabetics even without detectable C-peptide may reflect greater regenerative abilities of the pancreas, causing autoreactive T-cells to remain in the circulation. A trivial second explanation is that the two types of autoreactive T-cells measured, i.e., InsB and IGRP autoreactive T-cells, represent the dominant autoreactive epitopes in late-onset disease, but not in early-onset disease. Although C-peptide can now be detected to 1.5

pmol/L, this assay might still not be sensitive enough, and the T-cell assay is more sensitive for residual pancreatic activity, and that would also be an explanation for long-term autoreactive T-cells in late-onset diabetic subjects.

Why such non-detectable levels of autoreactive T-cells in early-onset diabetics? Primary pancreas defects unrelated to autoreactive T-cells may contribute to more rapid pancreatic islet failure in early-onset diabetics. The role of autoreactive T-cells may be less dominant in early-onset cases. Or as above, these early-onset diabetic subjects truly have no C-peptide and levels far below the sensitive assay levels of new C-peptide assays of 1.5 pmol/L.

4. Metabolomics differ between early- and late-onset diabetes

We studied metabolites in the serum of early-onset versus late-onset diabetics that might shed light on disease pathophysiology. Metabolomics was performed on two independently collected subject serum sets, referred to as serum set 1 and serum set 2, in order to verify findings. The subjects were two matched sets with 25 early- and 25 late-onset diabetic subjects with HbA1Cs in the same range. A total of 100 frozen serum samples were analyzed. The samples were sent for metabolic profiling to Metabolon (Durham, NC). Samples were extracted and prepared for analysis on Metabolon's integrated discovery platform that was based on a combination of gas and liquid chromatography techniques coupled with mass spectrometry for detection and identification. Metabolon's platform has met with considerable success in, among many studies, the identification of biomarkers of insulin resistance in subjects that are at risk of developing type 2 diabetes [13].

The clinical and biochemical characteristics of each serum set are shown in **Table 2**. The patient selections for each sample set were limited to subjects with disease duration of less than 25 years and a current age of less than 50 years to exclude kidney disease and other confounding factors in metabolism. We then statistically compared the data from early- versus late-onset diabetics and report only on those metabolites that were significantly different across both screens.

	AOO (years)	Age (years)	Duration (years)	C-peptide (pmol/mL)	HbA1C (%)	Female (%)
Serum set 1						
Early-onset	9.8 ± 0.7	23.3 ± 1.5	9.5 ± 1.2	40.5 ± 18.5	8.2 ± 0.4	32.0
Late-onset	35.8 ± 2.0	48.6 ± 2.3	8.4 ± 1.0	117.5 ± 43.5	7.2 ± 0.2	44.0
Serum set 2						
Early-onset	10.2 ± 0.7	16.8 ± 0.9	5.7 ± 0.9	87.9 ± 29.8	7.5 ± 0.2	36.0
Late-onset	32.8 ± 2.5	41.5 ± 2.3	7.7 ± 1.1	152.5 ± 50.6	6.7 ± 0.2	44.0

All values are mean ± SEM.

Table 2. Clinical characteristics of early- and late-onset diabetics for metabolomic assays .

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Table 3 lists 30 metabolites that display statistically significant differences between early-onset and late-onset type 1 diabetes from both serum set 1 and serum set 2. These metabolites are found in diverse super pathway families, including amino acids, carbohydrates, cofactors and vitamins, lipids, peptides, and xenobiotics. **Table 3** also lists the subpathways. **Figure 5** graphs the same data according to the fold differences and also the direction, i.e., early- < late-onset or early- > late-onset. The data show that the fold differences were extremely similar for serum set 1 subjects and serum set 2 subjects and always in the same direction. The data trends were remarkably reproducible given that these were independently collected human samples.

Metabolite	Super pathway	Subpathway	*p* Serum set 1	*p* Serum set 2
Creatinine	Amino acid	Creatine metabolism	0.0206	0.034
N-acetyl-3-methylhistidine	Amino acid	Histidine metabolism	0.0018	0.0441
3-methylglutaconate	Amino acid	Leucine, isoleucine, and valine metabolism	0.0049	0.0216
N2-acetyllysine	Amino acid	Lysine metabolism	0.0429	0.0334
N-acetylphenylalanine	Amino acid	Phenylalanine and tyrosine metabolism	0.0092	0.0345
Spermidine	Amino acid	Polyamine metabolism	0.0458	0.0224
N-acetyltryptophan	Amino acid	Tryptophan metabolism	0.001	0.036
Pro-hydroxy-pro	Amino acid	Urea cycle: arginine and proline metabolism	1.06E−05	2.81E−09
Trans-4-hydroxyproline	Amino acid	Urea cycle: arginine and proline metabolism	0.001	3.00E−04
Glucuronate	Carbohydrate	Amino sugar metabolism	0.025	3.34E−08
Sucrose	Carbohydrate	Disaccharides and oligosaccharides	0.007	4.00E−04
Fructose	Carbohydrate	Fructose, mannose, and galactose metabolism	0.020	0.003
Sorbitol	Carbohydrate	Fructose, mannose, and galactose metabolism	0.001	0.001
Glucose	Carbohydrate	Glycolysis, gluconeogenesis, and pyruvate metabolism	0.003	0.044
Ascorbate (vitamin C)	Cofactors and vitamins	Ascorbate and aldarate metabolism	0.018	0.012
Trigonelline (N′-methylnicotinate)	Cofactors and vitamins	Nicotinate and nicotinamide metabolism	0.005	1.40E−06
Alpha-tocopherol (vitamin E)	Cofactors and vitamins	Tocopherol metabolism	0.001	0.002
Pyridoxate (vitamin B6)	Cofactors and	Vitamin B6 metabolism	0.002	0.014

Metabolite	Super pathway	Subpathway	*p* Serum set 1	*p* Serum set 2
	vitamins			
CMPF	Lipid	Fatty acid, dicarboxylate	0.000	0.001
Eicosapentaenoate (EPA)	Lipid	Polyunsaturated fatty acid (n3 and n6)	2.00E−04	0.002
Hyocholate	Lipid	Secondary bile acid metabolism	0.001	0.023
Taurodeoxycholate	Lipid	Secondary bile acid metabolism	0.011	0.047
Etiocholanolone glucuronide	Lipid	Steroid	0.021	0.002
Cholesterol	Lipid	Sterol	0.014	0.009
Leucylglycine	Peptide	Dipeptide	0.003	0.012
Valylglycine	Peptide	Dipeptide	0.001	2.75E−07
1,3-dimethylurate	Xenobiotics	Xanthine metabolism	0.010	0.004
1,3,7-trimethylurate	Xenobiotics	Xanthine metabolism	0.020	0.001
1,7-dimethylurate	Xenobiotics	Xanthine metabolism	0.024	0.009
Paraxanthine	Xenobiotics	Xanthine metabolism	0.032	0.007

Table 3. Early- versus late-onset T1D: metabolites with significant differences.

A subset of 7 out of 30 metabolites is important and is involved in pancreatic function (**Table 4**). In all cases, the pancreas-related metabolites were statistically different between early-onset and late-onset diabetic subjects.

Subjects with early- versus late-onset diabetes have varying levels of acetyllysine. Developing and proliferating insulin-secreting β-islet cells have augmented acetyltransferase activity related to growth and insulin secretion often through beta-cell–specific transcription factors BETA2 and PDX-1. High lysine deacetylase activity would be expected to increase the removal of acetyl groups from proteins. In our data the early-onset diabetics had lower levels of acetyllysine. Lysine deacetylase inhibitors protect β-islet cells by increasing the acetylation of proteins. In support of our observations, treatment data from the nonobese diabetic (NOD) mouse, an animal model of type 1 diabetes, shows that lysine deacetylase inhibition protects β-islet cells by increased acetylation of proteins [14]. Indeed, if lysine deacetylase inhibitors are brought forward to humans, their impact might be greater in early-onset diabetic subjects by conferring a presumed β-islet cell protective factor and restoring acetyllysine levels to higher levels.

Eicosapentaenoate (EPA), a metabolite of the polyunsaturated fatty acid metabolic pathway, was lower in early-onset type 1 diabetes and high in late-onset diabetics even exceeding the levels of control populations. Several studies support the concept that EPA improves insulin secretion in pancreatic islets [15, 16]. Since we found that EPA is lower in early-onset diabetes compared to late-onset diabetics, this again supports a more rapid disease progression in such individuals. It also suggests late-onset diabetic subjects could make EPA as a protective factor.

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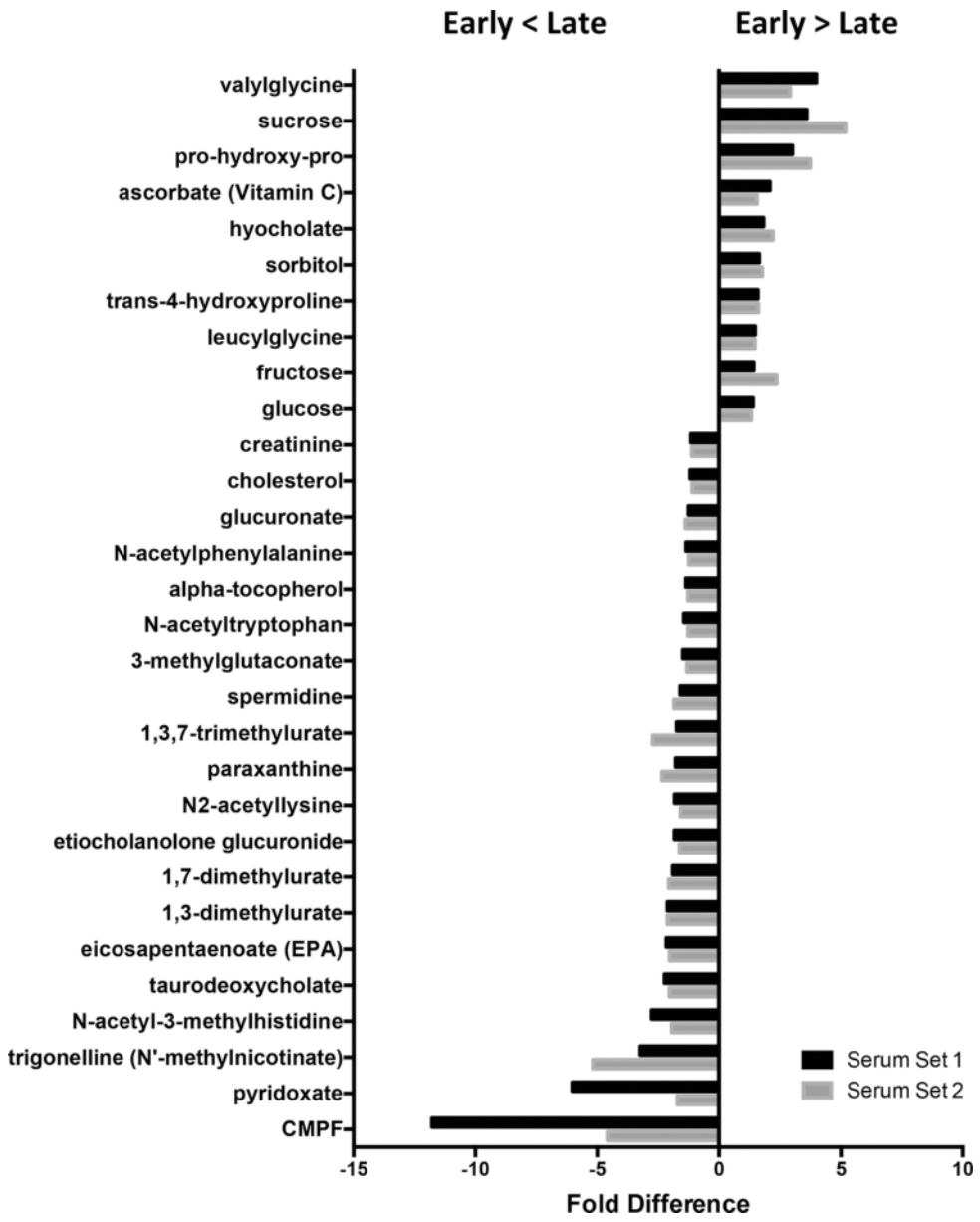

Figure 5. The over- and under-expression of metabolites associated with early- and late-onset diabetes. Shown is the ratio of metabolites in early- versus late-onset. For clarity, ratios between 0 and 1 were inverted (1/ratio) and made negative. Data are presented as rank order based on the magnitude of the statistically significant metabolic trends. Both sample sets demonstrate the tight reproducibility in the direction of the trends and also in the reproducibility of the magnitude of the derangements between early- and late-onset type 1 diabetics.

Metabolite	Pathway	Ratio early/late (average of both screens)	Association with diabetes	References
N2-acetyllysine	Lysine metabolism	0.596	Diabetes induces lysine acetylation of intermediary metabolism enzymes in the kidney	Kosanam et al. [14]
Spermidine	Polyamine metabolism	0.588	Spermidine may decrease ER stress in pancreatic beta cells and may reduce apoptosis via activating AMPK-dependent autophagy pathway. Spermidine content is decreased in islets of old obese ob/ob mice. Polyamines such as spermidine increase the stability of insulin mRNA and are necessary for the maintenance of normal insulin and protein biosynthesis in islets	Tirupathi Pichiah et al [36]; Sjoholm et al. [23]; Welsh [21]; Welsh [22]
N-acetyltryptophan	Tryptophan metabolism	0.742	Suppresses rise in blood sugar and preserves insulin secretion in type 2; related to activity of intestinal bacteria	Wikoff et al. [17]
Glucuronate	Amino sugar metabolism	0.761	Elevated in diabetes. Glucuronate pathway is overactive in diabetes	Winegrad and Burden [25]
CMPF	Fatty acid, dicarboxylate metabolism	0.152	Elevated in diabetes. Causes beta-cell dysfunction	Nolan [26]; Prentice et al. [27]
Eicosapentaenoate (EPA)	Polyunsaturated fatty acid metabolism	0.480	Improves insulin secretion in pancreatic islets	Kato et al. [15]; Shimano et al. [16]
Taurodeoxycholate	Secondary bile acid metabolism	0.470	Involved in regulation of exocrine pancreas function	Riepl et al. [18]

Table 4. Early- versus late-onset diabetics: islet-, insulin-, and pancreas-related metabolites with significant differences.

N-acetyltryptophan was also observed to be lower in early-onset diabetics. N-acetyltryptophan suppresses rises in blood sugars and preserves insulin secretion in type 1 diabetes [17]. Abundant n-acetyltryptophan also can be produced by select gut bacteria and modulates

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expression of proinflammatory genes and increases expression of anti-inflammatory genes possibly affecting the etiology of both early- and late-onset diabetes [17].

Taurodeoxycholate, a metabolite related to secondary bile acid metabolism, was more depressed in early-onset than late-onset diabetes. Previous evidence shows this metabolite is involved in the regulation of exocrine pancreas function [18]. Exocrine pancreatic dysfunction in type 1 diabetes is linked to a decrease in pancreatic volume, an observation of past reports [19]. Therefore, our findings again point to varying contributions of pancreas defects to T1D in early-onset subjects compared to late-onset diabetics.

Spermidine, a member of the polyamine metabolism, was decreased in early-onset diabetics and elevated in late-onset subjects. Past reports suggest type 1 diabetic subjects have reduced spermidine levels, and now our data suggests this deficiency is even more pronounced in early-onset diabetes [20]. Spermidine may decrease endoplasmic reticulum (ER) stress in pancreatic islet beta cells, is decreased in islets of obese-hyperglycemic ob/ob mice, and is also reported to reduce apoptosis via AMP-activated protein kinase (AMPK)-dependent autophagy pathways [21–24]. A deficiency in spermidine might accelerate disease onset.

Glucuronate, which was also lower in early-onset diabetes, is involved in amino sugar metabolism. Some reports characterize this metabolite as related to diabetic hyperglycemia [25]. Again this points to altered and varying levels between early- and late-onset subjects.

Lastly, the metabolite 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) was high in late-onset diabetic subjects and very low in early-onset diabetics and control subjects had intermediate levels. CMPF is associated with pancreatic function. This metabolite causes β-islet cell dysfunction when elevated [26, 27]; CMPF is part of the fatty acid and decarboxylase metabolism pathway. Here, the elevation in CMPF is restricted to late-onset disease compared to early-onset disease, where the levels are actually suppressed in early-onset diabetics compared to control populations (data not shown). This pancreas-related metabolite was the exception between early- and late-onset subjects since in all other six cases the early-onset subject demonstrated the more pronounced defect.

Is there evidence to support the hypothesis that intrinsic pancreas defects unrelated to the immune system contribute to a broader etiologic basis of diabetes, perhaps related to the target organ? Developmental biology has long been ignored in the etiology of autoimmune diseases [28, 29]. In the NOD mouse, there is both support for direct T-cell-driven autoimmunity and secondary organ failure from intrinsic developmental defects. Using NOD-scid mice without an immune system, NOD structural defects related to organogenesis can be observed in the pancreas and salivary glands from birth onward and in other organs. The structurally defective organs share a common developmentally related origin: Hox11-expressing progenitor cells during fetal development [29]. Hox11-derived organs such as the cochlea/inner ear are related to Hox11 developmental biology, as are the sensory portions of the tongue. NOD mice fail to form an inner ear and are born deaf; they also have malformed sensory end organs of the tongue [28]. The human evidence provided here by metabolomics offers additional support for the possibility that intrinsic pancreas developmental defects could contribute, at least in

early-onset human diabetes, to more rapid end-organ failure as has been shown in the NOD mouse.

In conclusion, there is supporting evidence from both autoimmune mice and humans that primary, not secondary, additional pancreas dysfunction in diabetes could be driven by developmental biology defects and that associated organs can also fail even without a lymphocyte influence.

5. Implications for therapy

What are the therapeutic implications of the pathophysiological differences underlying early- and late-onset diabetes? For early-onset diabetics, their rapid deficiency in autoreactive T-cells suggests that they are unlikely to respond to immunotherapy aimed at abolishing the autoimmune response if the therapy is started very long after the disease onset. Conversely, for late-onset diabetics, immunotherapies are likely to be effective longer because the patients still have a vigorous autoimmune response and in many cases pancreatic insulin secretion that could be reserved.

The first hint that immunotherapies may be preferentially effective for late-onset diabetes came from a phase I, proof-of-concept clinical trial, published by our laboratory in 2012. It was found that the immunotherapy bacillus Calmette-Guérin (BCG) transiently dampens autoimmunity by selective targeting for death autoreactive T-cells and inducing the beneficial T-regulatory T-cells. With BCG, patients with long-standing disease (mean duration of 15.3 years) fleetingly produced higher levels of C-peptide, showed increases in dead autoreactive T-cells, and produced more T-regulatory cells (Tregs) that suppress autoimmunity. When all weekly blood sera were analyzed by an ultrasensitive assay at the end of the trial, all BCG recipients (as well as placebo controls) were found to have low yet detectable C-peptide secretion.

BCG's apparent efficacy in type 1 diabetes stems from its induction of endogenous tumor necrosis factor (TNF)-α [30], which, in turn, selectively kills CD8+, but not CD4+, autoreactive T-cells in type 1 diabetics and other autoimmune diseases [31]. TNF is also known to expand T-regulatory T-cells as well. TNF itself is not a suitable exogenous immunotherapy because of its high toxicity, which is largely due to widespread expression of the TNF receptor 1 [32]. The TNF inducer BCG is a safer alternative to direct administration of TNF.

BCG is also advantageous because of its FDA approval, low cost, and its 100-year track record of safety as a tuberculosis vaccine and 40-year track record of safety as a treatment for bladder cancer. The BCG trial was among the first immunotherapy clinical trials to include long-standing diabetic subjects, as most if not all of immunotherapy trials over the past 20 years have excluded all but new-onset patients under the now erroneous assumption that β-islets are not salvageable.

A phase II clinical trial of BCG is now in progress, using more frequent doses, a larger group of subjects ($n = 150$), and a 5-year follow-up. The primary objective of this randomized, placebo-controlled double-blind trial is to determine the dose and timing of BCG administration

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necessary to trigger a significant improvement in HbA1c values. One of the inclusion criteria is that subjects have fasting or stimulated C-peptide levels between 5 and 200 pmol/L, because these levels are indicative of remaining pancreatic islet function. With the additional criteria that these subjects also must be adults (greater than age 18), the majority of the subjects will also be late-onset subjects.

Two other recent immunotherapy clinical trials in type 1 diabetes, using abatacept and teplizumab, have had disappointing results [33, 34]. One reason postulated to be behind the lackluster results was the heterogeneity of type 1 diabetics, including age of onset [35]. We agree that early- versus late-onset might have been one important source of disease heterogeneity that hampered the trials' success. The results of our BCG trial and the data we have presented here on C-peptide persistence and age of onset suggest that immunotherapy clinical trials no longer exclude all but new-onset cases.

In conclusion, the data that we report here reveal that early- and late-onset diabetics differ in terms of C-peptide production, autoreactive T-cell levels, and metabolites. Taken together, the data suggests that therapies aimed at immune defects are likely to have greater efficacy in late-onset diabetes, whereas therapies aimed at immune defects and β-islet preservation are likely to have greater efficacy in early-onset diabetes.

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Abbreviations

pMHC-I multimers peptide-major histocompatibility complex class I multimers

AOO age of onset

ER endoplasmic reticulum

T1D type 1 diabetes

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