

Biomarkers of β -Cell Stress and Death in Type 1 Diabetes

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Abstract The hallmark of type 1 diabetes (T1D) is a decline in functional β -cell mass arising as a result of autoimmunity. Immunomodulatory interventions at disease onset have resulted in partial stabilization of β -cell function, but full recovery of insulin secretion has remained elusive. Revised efforts have focused on disease prevention through interventions administered at earlier disease stages. To support this paradigm, there is a parallel effort ongoing to identify circulating biomarkers that have the potential to identify stress and death of the islet β -cells. Whereas no definitive biomarker(s) have been fully validated, several approaches hold promise that T1D can be reliably identified in the pre-symptomatic phase, such that either β -cell preservation or immunomodulatory agents might be employed in at-risk populations. This review summarizes the most promising protein- and nucleic acid-based

biomarkers discovered to date and reviews the context in which they have been studied.

Keywords Type 1 diabetes · miRNA · Cell-free insulin DNA · Biomarkers · Unmethylated insulin DNA · Proinsulin · Proinsulin/C-peptide ratio

Introduction

Type 1 diabetes (T1D) is characterized by the immune-mediated destruction of the insulin producing pancreatic β -cells. T1D arises from a break in immune tolerance and infiltration of auto-reactive T cells that target the β -cells, leading to loss of β -cell function and mass and a lifelong requirement for exogenous insulin [1]. To preserve β -cell function, a number of immunomodulatory drugs have been tested around the time of T1D clinical diagnosis. A handful of drugs, including anti-CD3, anti-CD20, CTLA4-Ig, and alefacept have led to a modest preservation of β -cell function [2–6]. However, true remission from T1D, as defined by insulin independence, remains elusive. These outcomes could be explained by the low proliferative and regenerative capacity of human β -cells [7, 8], combined with the possibility that interventions were initiated too late in disease, occurring after the time in which intrinsic stress pathways in β -cells have been so robustly activated that even modulation of autoimmunity could not prevent β -cell decline, or treating autoimmunity with the depleting/immunomodulatory agents mentioned above does not address the coexisting β -cell dysfunction. This may be an equally important pathogenic abnormality that should be also targeted therapeutically. In recent years, data characterizing intrinsic β -cell stress in T1D suggest that processes such as β -cell calcium dyshomeostasis, misfolded protein accumulation, oxidative stress, and endoplasmic reticulum stress become activated

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early during the evolution of T1D and act to either initiate immune activation through formation of neoantigens or serve to augment autoimmune-mediated β -cell death and dysfunction [9–11]. Whereas these pathways may serve as points of therapeutic intervention, focused methods to identify biomarkers that reflect an authentic signature of β -cell stress and/or death may also improve our ability to optimally time immunomodulatory and other forms of interventions in T1D. In such a paradigm, a valid signature of β -cell stress could serve as a signal to initiate immunomodulatory therapy in the pre-T1D diabetic period. Thus, interventions in the newly defined stages 1–2 of the pre-symptomatic phase of T1D [12] offer the promise of more effective preservation of β -cell mass. However, a challenge in the field of β -cell biomarkers is the fact that β -cells represent a very small fraction ($\leq 1\%$) [13] of total pancreatic mass and an even smaller fraction of total body cellular mass. Historically, detection of biomarkers that emanate from the β -cell has been largely outside of the technical capacity of most laboratories. However, with the advent of next-generation sequencing technologies and more sensitive/specific nucleic amplification, mass spectroscopic, and antibody-based methodologies, both the identification and detection of promising biomarkers that are β -cell specific/selective is becoming a reality. Here, we review the status of select protein and nucleic acid biomarkers that provide insight into the health of the β -cell and have the potential, alone or in combination, to identify individuals with T1D who may benefit from therapies aimed at preservation of insulin secretion.

Proinsulin/C-peptide Ratio as a Circulating Biomarker of β -Cell Stress in T1D

The islet β -cell is a highly secretory cell that is singularly tasked with the heavy biosynthetic burden of insulin production. Efficient protein translation and processing requires a robust endoplasmic reticulum (ER). Under conditions of T1D autoimmunity and inflammatory stress, insulin demand may exceed the ability of the β -cell ER to process newly translated proteins, leading to accumulation of unfolded proteins. If this process continues without resolution, it will lead to activation of ER stress and pro-apoptotic signaling pathways [14]. A growing number of preclinical studies suggest that β -cell ER stress contributes to the pathogenesis of T1D, prior to the onset of hyperglycemia [15–17]. In human T1D, immunostaining of islets from donors with T1D revealed abnormal expression of ER stress markers, including C/EBP homologous protein (CHOP), activating transcription factor 6 (ATF6), and spliced X-box binding protein 1 [15, 18].

Insulin biosynthesis begins with the production of preproinsulin at ribosomes. Preproinsulin is then converted to proinsulin within the ER by cleavage of the N-terminal

signal peptide. Further processing and cleavage occurs in the ER, Golgi network, and secretory granules before release of mature insulin and C-peptide into the circulation [19]. A hallmark of β -cell ER dysfunction is the accumulation and secretion of inadequately processed proinsulin molecules [14]. Therefore, β -cell ER stress may be detectable in a minimally invasive manner via measurement of the ratio of circulating proinsulin relative to insulin or C-peptide [16]. Using proinsulin/C-peptide (PI/C) ratios as a non-invasive marker of β -cell dysfunction could provide a means to identify individuals at risk of developing T1D prior to the onset of massive β -cell destruction. Evaluation of this ratio may also provide a means to monitor disease evolution after diagnosis [20]. In considering studies that examined PI/C ratios (see Table 1), it is important to recognize that ELISAs for proinsulin and C-peptide have evolved considerably over the last several years. Current assays exhibit significantly greater sensitivity and specificity.

A series of studies in Northern Europe have examined PI/C ratios in family members at risk for T1D. Fasting PI/C ratios were inversely correlated to first phase insulin response during an intravenous glucose tolerance test performed on 23 antibody-positive Finnish siblings of persons with T1D [25]. Among siblings followed longitudinally, 7/9 who progressed to develop T1D had an increase in PI/C ratio during the 6 months prior to diagnosis [39]. In the Belgian Diabetes Registry, autoantibody-positive first-degree relatives with random PI/C ratios above the 66th percentile had an increased 5-year risk of T1D development [26]. Sims et al. recently reported that within the TrialNet Pathway to Prevention cohort, an international cohort of autoantibody-positive family members of individuals with T1D, fasting PI/C ratios were significantly increased ~12 months prior to onset of T1D [27••]. Elevations were most pronounced in children ≤ 10 years, where median PI/C ratios were 3-fold higher than those who did not progress to T1D. However, even among the entire group, increased PI/C ratios were associated with increased odds of progression to T1D, after correction for age and BMI [27••].

Several groups have identified on average a 3–10-fold elevation in PI/C ratios at T1D diagnosis compared to controls [21–23]. Although C-peptide levels are typically increased in the months after diagnosis, this period has still been largely associated with a concomitant increase in proinsulin levels, with varying patterns in PI/C ratios during this partial remission or honeymoon period [21–24]. PI/C ratios at diagnosis are inversely correlated with length of the honeymoon period [21]. Treatment of new-onset subjects with cyclosporine reduced PI/C ratios, with an increased percentage of subjects experiencing a non-insulin requiring honeymoon [22]. Data regarding PI/C ratios in longer-standing T1D is more limited. Among 73 Swedish pediatric subjects with T1D for >2 years, fasting proinsulin and C-peptide levels were highly correlated. Notably in that study, proinsulin was still detected in 27.8% of

Table 1 Potential biomarkers of β -cell stress and death in type 1 diabetes

Potential biomarker	Data from ex vivo or preclinical T1D models	Human data
Proinsulin/C-peptide (PI/C) Ratio	Proinsulin/insulin ratios were increased in pre-diabetic and diabetic NOD mice [16].	1. PI/C ratios were increased in recent-onset T1D and reductions in the PI/C ratio were associated with T1D remission [21–24]. 2. PI/C ratios were increased in autoantibody-positive individuals who progressed to T1D [25, 26, 27••]. 3. Elevated PI/C ratios were found to be predictive of T1D onset [27••].
miR-375	Increased in plasma of streptozotocin-treated and pre-diabetic NOD mice [28].	Elevated in sera after allogeneic and autologous islet transplantation [29••].
miR-21, miR-34a, and miR-146a	Increased in human islets treated with proinflammatory cytokines and in islets of pre-diabetic NOD mice [30].	
miR-152, miR-30a-5p, miR-181a, miR-24, miR-148a, miR-210, miR-27a, miR-29a, miR-26a, miR-27b, miR-25, and miR-200a		Increased in sera of humans with recent-onset T1D [31].
miR-34a-5p, miR-21-3p, miR155-5p, miR-1290, miR663b, miR10b-3p	Increased in human islets infected with Coxsackievirus B5 [32].	
Unmethylated <i>INS</i> DNA		1. Increased in humans undergoing allogeneic islet transplantation [33, 34••]. 2. Increased in recent-onset T1D [34••, 35, 36••]. 3. Increased in high-risk autoantibody-positive individuals prior to the onset of T1D [37••].
Unmethylated <i>IAPP</i> DNA	Increased in sera of pre-diabetic and diabetic NOD mice [38].	Increased in recent-onset T1D [38].

subjects with a negative fasting C-peptide [40], suggesting either complete failure to process proinsulin in these individuals or significant differences in sensitivity of the assays for proinsulin and C-peptide.

Intriguingly, proinsulin/C-peptide ratios also appear to be increased in lower risk family members compared to controls with no family history of type 1 diabetes. Several cohorts have identified elevations in fasting proinsulin despite similar fasting C-peptide/insulin or blood glucose levels in siblings of subjects with type 1 diabetes, independent of high-risk HLA status [41–43]. Increased fasting proinsulin levels have also been reported in parents and children of individuals with type 1 diabetes [43]. Small-scale studies have shown elevations in fasting proinsulin, despite similar C-peptide values, in unaffected monozygotic twin siblings. Elevated proinsulin levels were independent of islet cell antibody positivity and were present >10 years after the sibling's diabetes diagnosis [44, 45].

These findings could represent an inherited predisposition to β -cell dysfunction that could theoretically contribute to type 1 diabetes pathogenesis in affected families. Alternatively, increased ratios could reflect a history of autoimmune β -cell injury that did not progress to frank diabetes. Interestingly, analysis of random proinsulin/C-peptide ratios in cord blood of newborn siblings of affected patients showed no difference

compared to controls [46]. These findings may point to later timing of an inherited β -cell defect versus physiologic differences in newborn proinsulin processing that masks familial disparities. Notwithstanding these questions, PI/C ratios remain likely to be an informative and useful component of T1D risk prediction algorithms, as well as an important biomarker of β -cell dysfunction in T1D treatment trials. Moreover, additional studies are needed to understand better the molecular mechanisms underlying these abnormalities.

Non-coding RNAs as Circulating Biomarkers of β -Cell Stress in T1D

Non-coding RNAs comprise a large group of RNA molecules that function post-transcriptionally to regulate a host of cellular activities ranging from differentiation and proliferation to apoptosis. They include classes of RNAs referred to as microRNAs (miRNAs), long non-coding RNAs (lncRNAs), circular RNAs (circRNAs), small nucleolar RNAs (snoRNAs), and stable intronic sequence RNAs (sisRNAs) among others. The best characterized class of non-coding RNAs with respect to β -cell function and biomarker development is the miRNAs. MicroRNAs are small RNA molecules of 21–23 nucleotides that post-transcriptionally regulate gene expression through

either the inhibition of mRNA translation or reduction of mRNA stability [47, 48]. In unusual cases, miRNAs can also positively regulate gene expression [49, 50]. In humans, over 2500 miRNAs have been cataloged [51], and it is estimated that collectively these miRNAs regulate approximately 60 % of protein-coding genes [52]. Whereas miRNAs are generated intracellularly and largely function in a cell-autonomous fashion, in recent years it has been appreciated that they can be selectively secreted extracellularly in microvesicles or exosomes and thereby transmit their regulatory functions to other cell types [53–55]. MicroRNAs have accordingly been regarded as potential circulating biomarkers that reflect activities of their cells of origin. In the context of diabetes, an emerging literature suggests that specific miRNAs or groups of miRNAs could serve as biomarkers of disease progression or diabetic complications [56–61]. The systemic stability of miRNAs relative to other RNA species allows for their measurement in a variety of biological fluids, including blood, urine, saliva, cerebrospinal fluid, milk, seminal fluid, and amniotic fluid, making them prime targets for biomarker discovery [62].

Because miRNAs are expressed broadly in a variety of tissues, none to date can be considered as exclusive to β -cells. However, several miRNAs have been proposed as biomarkers in individuals with T1D (see Table 1 for a summary). In-depth sequencing of human islets and isolated β -cells revealed >25 miRNAs that show relative enrichment in β -cells [63]. One of the most abundant miRNAs in β -cells is miR-375. MicroRNA-375 was identified early in studies of the mouse-derived β -cell line MIN6, where it was demonstrated to inhibit glucose-stimulated insulin owing to effects on vesicle exocytosis [64]. Using knockout mouse models, Poy et al. showed that miR-375 was required for the maintenance of normal β - and α -cell mass during mouse pancreas development [65]. The relative abundance and functional role of this miRNA naturally led to studies of its utility to serve as a biomarker of β -cell stress and/or mass. Erenner et al. [28] showed in mice that streptozotocin (STZ) treatment, which selectively destroys β -cells, results in increases in circulating miR-375 levels, and that the NOD mouse model of T1D exhibited elevations in miR-375 during the weeks prior to development of frank hyperglycemia. In the islet transplantation setting in humans where acute β -cell death is observed, Kanak et al. [29••] showed that circulating miR-375 levels were elevated immediately post-transplantation and remained elevated up to 7 days later. In the T1D setting, C-peptide-negative individuals exhibited higher circulating miR-375 levels compared to individuals with more substantial β -cell mass, such as non-diabetic controls, individuals with T2D, and individuals with maturity onset diabetes of the young (MODY) [66••]. These findings suggest that steady-state circulating levels of miR-375 are more likely a reflection of dying β -cells rather than prevailing β -cell mass.

To identify other miRNAs that contribute to the pathogenesis of T1D and might serve as biomarkers of β -cell stress, Roggli et al. [30] performed global microarray profiling of human islets treated with a cocktail of proinflammatory cytokines and demonstrated increases in miR-21, miR-34a, and miR-146a. Notably, these same miRNAs were elevated in the islets of pre-diabetic NOD mice, and blockade of these miRNAs using anti-miRs prevented the loss in insulin secretion from MIN6 β -cells exposed to proinflammatory cytokines [30]. Using a different stress paradigm of T1D, Kim and colleagues recently demonstrated increases in miR-34a-5p, miR-21-3p, miR155-5p, miR-1290, miR663b, and miR10b-3p in human islets infected with Coxsackievirus B5, and bioinformatics analysis identified 57 candidate type 1 diabetes risk genes predicted to be direct targets of Coxsackievirus B5 responsive miRNAs [32].

These findings suggested a pathogenic role of certain miRs in β -cell dysfunction in T1D, but still did not address whether these and other miRs might serve as circulating biomarkers reflecting β -cell stress. To address this possibility, Nielsen et al. [31] undertook a comprehensive sequencing analysis using serum of subjects with new-onset T1D. Relative to control subjects after adjustment for age and gender, 12 upregulated miRNAs were identified in the serum of new-onset T1D subjects, including miR-152, miR-30a-5p, miR-181a, miR-24, miR-148a, miR-210, miR-27a, miR-29a, miR-26a, miR-27b, miR-25, and miR-200a [31]. Other studies have identified additional miRNAs associated with T1D, emphasizing the correlation between miRNAs and T1D diagnosis and/or control [67, 68]. Notably, whereas some of the miRNAs have been shown to be enriched in human β -cells (e.g., miR21, miR24, miR29a, miR375) [63], many are not, suggesting that they may not be a reflection of endogenous β -cell stress. Nevertheless, miRNAs represent a potentially promising class of circulating nucleic acids that could serve as biomarkers of individuals at high-risk for T1D (Table 1), and future studies in high-risk populations are warranted.

Data on the role and potential as biomarkers of other non-coding RNAs are limited. An emerging class with biomarker potential is the lncRNAs. LncRNAs are defined as non-coding RNAs of >200 nucleotides in length. Although the mechanisms of lncRNAs is not well understood, like miRNAs, lncRNAs appear to function in a diversity of cellular processes, such as development and differentiation, proliferation, and apoptosis (for a recent review of lncRNAs in autoimmune diseases, see [69]). According to the NONCODE database [70], more than 140,000 lncRNA transcripts (encoded by more than 90,000 genes) have been identified in humans. As such, the potential for these RNAs to serve as biomarkers of disease susceptibility and development is gaining traction. Based on genome-wide association studies, several lncRNAs have been associated with T1D susceptibility [71–74]. To date, however, studies are limited with respect to the

expression and function of lncRNAs in the β -cell. A recent study in mouse β -cell-derived cell lines and NOD mouse islets showed that lncRNAs 1-4 are strongly upregulated in response to proinflammatory cytokines and disease progression, respectively [75]. Carter et al. [76] showed that in humans, the lncRNA GAS5 was negatively correlated with the prevalence of type 2 diabetes in a Veteran's Administration cohort. Thus, further studies on the potential for lncRNAs and other non-coding RNAs to serve as circulating biomarkers of β -cell stress is warranted.

Differentially Methylated DNA as a Biomarker of β -Cell Stress and Death

The presence of circulating, cell-free nucleic acids derived from dying cells has been recognized for over 60 years, and more recently the detection and quantitation of such nucleic acids has been used to detect apoptosis/necrosis and/or turnover of their cells of origin (for a review, see [77]). One particularly promising approach in biomarker development has been the detection of differentially methylated DNA fragments. DNA methylation at CpG sites occurs via the action of DNA methyltransferases in a chemical reaction involving cytosine and the cofactor S-adenosyl-L-methionine. In general, methylation patterns at a given gene tend to be inversely correlated with activity of the gene. Thus, genes that are predominantly methylated at CpG dinucleotides are highly expressed, whereas genes that are predominantly unmethylated at these same dinucleotides tend to be quiescent. DNA methylation is therefore a key epigenetic component in many stably repressed loci (such as the inactivated X chromosome) throughout the genome and in specific cell types [78]. Just as importantly, dynamic changes to DNA methylation frequently account for malignant transformation of many cell types as a result of activation or inactivation of tumor-promoting or repressing genes/loci [79]. It was this link between DNA methylation status and tumor formation that led investigators to consider the possibility that circulating differentially methylated DNA fragments arising from transformed cells could serve as a biomarker for cancer [80].

The islet β -cell expresses multiple genes in a nearly exclusive manner, which accordingly have the potential to be regulated by selective DNA methylation. Perhaps the most abundant and exclusive gene is the one encoding preproinsulin (*INS* in humans and *Ins1* and *Ins2* in mice). Relative to multiple other cell types, the human and mouse genes have been shown to be hypomethylated in islets and β -cells at selective CpG sites in the promoter and coding regions [33, 35, 81], and human *INS* expression is inversely correlated with methylation at many of these sites in human islets [82]. As with cancer cells, the concept that cell-free, hypomethylated *INS* (or mouse *Ins1/Ins2*) appearing in the circulation might reflect

an increase in β -cell death or turnover was first tested in early studies of Akirav et al. [35] using a SYBR Green dye-based methylation-specific PCR assay against a CpG site appearing in the coding region of the *Ins1* gene (at position +177 relative to the transcriptional start site). This study heralded the first generation of methylation-specific PCR assays in the T1D field, and involved a nested PCR approach, wherein isolated DNA from serum or plasma was subjected to bisulfite conversion, followed by a methylation insensitive PCR amplification step, manual extraction of the PCR product from a gel, followed by a methylation-specific PCR second step. The study by Akirav et al. [35] revealed that increases in circulating unmethylated mouse *Ins1* DNA (relative to the methylated counterpart) were detectable following STZ treatment of mice and in NOD mice prior to the onset of diabetes. Since the description of this first study, a subsequent study by Husseiny et al. [83] described the use of a similar SYBR Green-based assay for the mouse *Ins2* gene [83]. Other studies applied the assay to the analysis of human serum/plasma, showing that unmethylated *INS* (relative to methylated *INS* at the same CpG site) was increased at the time of T1D diagnosis [84] and following allogeneic islet transplantation [33]. Collectively, these studies provided the first evidence that β -cell-derived, unmethylated *INS* DNA correlates with the occurrence of clinical states where β -cell death is known to occur (see Table 1 for a summary of these studies).

The first generation assays were largely limited by the nature of SYBR Green-based methodologies, which included potential off-target annealing of methylation-specific primers, limited sensitivity that frequently required nested PCR strategies, and the requirement to perform separate PCRs for methylated and unmethylated target sequences. Fisher et al. [85] introduced a dual probe-based strategy (TaqMan), which allowed for the multiplex detection of both unmethylated and methylated *Ins2* DNA fragments (at position -182 bp relative to the transcriptional start site) in sera of mouse models of T1D, with low background signals and without the need for nested PCR. Use of this dual probe-based technology signaled the development of second generation assays that engage droplet digital PCR (ddPCR), a technique that involves a microfluidics approach to generate thousands of droplets that undergo thermal cycling and utilizes Poisson statistics to obtain absolute copy numbers of DNA fragments [36•, 86]. Using ddPCR and a probe that simultaneously detects two methylation-sensitive sites of the human *INS* gene (at positions +396 and +399 relative to the transcriptional start site), Herold and colleagues [37•] showed that individuals at risk for T1D (autoantibody-positive individuals) who later progressed to T1D in the TrialNet Pathway to Prevention study collectively (averaged over several visits) had a statistically higher ratio of unmethylated/methylated *INS* DNA compared to healthy control subjects.

In a separate study utilizing ddPCR, Fisher et al. [36••] developed an assay targeting a single methylation-sensitive site at position -69 bp relative to the *INS* transcriptional start site, and showed that pediatric subjects with new-onset T1D (within 48 h of diagnosis) had higher absolute levels of both methylated and unmethylated insulin DNA compared to age-, gender-, and weight-matched non-diabetic controls. At 8 weeks post-diagnosis, the same individuals exhibited reductions in unmethylated *INS* DNA (to levels comparable to controls), but had persistent elevations in the methylated *INS* DNA levels. At 1 year post-diagnosis, both *INS* DNA species were at levels comparable to controls. These findings point to an important distinction between the ddPCR assays of Herold et al. [37••] and Fisher et al. [36••]. The former group uses ratios of unmethylated/methylated *INS* DNA levels, the latter examined absolute levels of each species separately. Whereas the unmethylated *INS* DNA is thought to arise predominantly from dying β -cells, the source of the elevated methylated *INS* remains unclear. Notably, Yang et al. [82] demonstrated that islets from subjects with type 2 diabetes showed greater methylation at several CpG sites compared to islets from control subjects, suggesting that β -cells might exhibit increasing methylation of the *INS* gene with progression of disease states. In agreement with this observation, a recent report by the Herold group [87•] showed that the methylation status of the *Ins1/Ins2* gene in the β -cell becomes more methylated with disease progression in the NOD mouse.

Because some non- β -cell types show low, but measurable frequency of unmethylated CpG sites in the *INS* gene [34••, 81], the aforementioned studies cannot rule out the possibility that the signals corresponding to unmethylated *INS* DNA might be arising (in part) from non- β -cells. To address this concern, the studies of Lehmann-Werman et al. [34••] used next-generation sequencing methods to simultaneously detect methylation at six distinct CpG sites in the *INS* promoter to achieve very high specificity (since all six sites are simultaneously unmethylated in fewer than 0.01 % of DNA molecules arising from non- β -cells). Using this approach, these authors showed that they were able to distinguish subjects with recent-onset T1D from healthy controls in a small cohort with 100 % sensitivity and specificity. As noted above, however, further studies will be needed in larger cohorts to know whether dynamic methylation at CpG sites in the *INS* gene with disease progression might limit the sensitivity of this sequencing-based approach.

To date, most studies employing differentially methylated DNA-based biomarker assays have focused on the *INS* gene, primarily due to the specific expression of the gene in β -cells. The potential for other β -cell-specific genes (e.g., *IAPP*) was recently demonstrated [38]. With emerging data on whole genome methylation sequencing, it should be possible to

identify new, perhaps more specific, methylated and unmethylated CpGs in β -cells in an unbiased manner without consideration of gene expression patterns.

Conclusions and Future Directions

The identification of robust panels of β -cell-specific biomarkers is a field still in its infancy. Nevertheless, the observations on PI/C ratios and unmethylated *INS* DNA in at-risk cohorts [27••, 37••] are suggestive of the potential for these and other biomarkers to identify populations of subjects who are more likely to develop T1D. Hurdles that remain include (a) persistent issues regarding sensitivity and specificity of biomarkers that reflect both the need to identify very low-level β -cell stress/death and to ensure that the signals are emanating from β -cells and not other uninvolved cell types, and (b) miniaturization and standardization of assays such that minimal blood volumes are required and that assays can be performed in multiple laboratories, (c) biomarker half-life considerations as β stress/death may be cyclical during stages 1 and 2, and (d) careful consideration of the heterogeneity of T1D with the acknowledgement that important differences in disease progression are driven by age and underlying genetic risk. Some of these hurdles, particularly specificity, can be overcome by utilizing a panel of biomarkers that collectively can be used to derive a score for β -cell health. Additionally, given the growth of “omics” data, the possibility that other as yet unidentified proteins (e.g., proteins emanating from alternatively spliced mRNAs) and molecular species (e.g., lipid and metabolite species) uniquely emanating from β -cells during the process of T1D development has yet to be explored.

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Compliance with Ethical Standards

Conflict of Interest Raghavendra G. Mirmira, Emily K. Sims, Farooq Syed, and Carmella Evans-Molina declare that they have no conflict of interest.

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- Of major importance

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