

Multiplexing DNA methylation markers to detect circulating cell-free DNA derived from human pancreatic β cells

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It has been proposed that unmethylated insulin promoter fragments in plasma derive exclusively from β cells, reflect their recent demise, and can be used to assess β cell damage in type 1 diabetes. Herein we describe an ultrasensitive assay for detection of a β cell-specific DNA methylation signature, by simultaneous assessment of 6 DNA methylation markers, that identifies β cell DNA in mixtures containing as little as 0.03% β cell DNA (less than 1 β cell genome equivalent). Based on this assay, plasma from nondiabetic individuals ($N = 218$, aged 4–78 years) contained on average only 1 β cell genome equivalent/mL. As expected, cell-free DNA (cfDNA) from β cells was significantly elevated in islet transplant recipients shortly after transplantation. We also detected β cell cfDNA in a patient with KATP congenital hyperinsulinism, in which substantial β cell turnover is thought to occur. Strikingly, in contrast to previous reports, we observed no elevation of β cell-derived cfDNA in autoantibody-positive subjects at risk for type 1 diabetes ($N = 32$), individuals with recent-onset type 1 diabetes (<4 months, $N = 92$), or those with long-standing disease (>4 months, $N = 38$). We discuss the utility of sensitive β cell cfDNA analysis and potential explanations for the lack of a β cell cfDNA signal in type 1 diabetes.

Conflict of interest: DN, RS, BG, and YD filed patents (year 2019, application number 62/828,587) on cfDNA analysis technology and β cell-specific markers.

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Introduction

Pancreatic β cells are essential for the maintenance of glucose homeostasis, with their destruction, dysfunction, or hyperfunction all serving as hallmarks of several pathologies. In type 1 diabetes, β cells are destroyed via T cell-mediated autoimmunity (reviewed in refs. 1–3). In type 2 diabetes, β cell failure results from a combination of cellular dysfunction, insufficient β cell mass, and β cell death (reviewed in ref. 4). In monogenic congenital hyperinsulinism (CHI), a mutation in 1 of 11 genes — including *GCK*, *SCHAD*, *GLUDI*, or *ABCC8* (encoding the SUR1 subunit of the KATP channel) — leads to excessive insulin secretion from β cells (reviewed in ref. 5). In insulinomas, malignant transformation leads to uncontrolled expansion of β cells that may result in life-threatening hypoglycemia and metastases (6).

Despite the clinical importance of these diseases, major gaps remain in our understanding of human β cell turnover dynamics. This is due, in large part, to a lack of biomarkers capable of assessing β cell mass

and dynamics. Specifically, routine pancreas biopsies are not ethically feasible due to concerns of safety (7). Furthermore, β cells cannot yet be imaged in vivo, and there are no validated blood markers that can report on the number of β cells or their rate of death. In fact, much of our knowledge regarding human β cell dynamics is based on a limited number of samples from biopsies (8), autopsies, and organ donor tissue banks (9) or extrapolation from animal studies (10). A major unmet need for β cell biomarkers is particularly evident in the setting of type 1 diabetes. The presence of circulating autoantibodies against β cell autoantigens is predictive of type 1 diabetes risk, but it is not currently possible to predict the rate of β cell decline or timing of disease onset (11, 12).

Liquid biopsies to detect circulating cell-free DNA (cfDNA) are emerging as an important diagnostic tool in cancer, prenatal testing, and transplantation medicine, based on analysis of cfDNA fragments that are released from dying cells into blood (13). In a landmark study, Akirav et al. proposed that the insulin gene methylation pattern (believed to be unmethylated in β cells but methylated in other tissues) can be used to detect circulating cfDNA derived from β cells, allowing inference of β cell death (14). Using this approach, several studies have subsequently reported the presence of β cell–derived cfDNA in persons at increased risk for type 1 diabetes (15), patients recently diagnosed with the disease (16–18), and islet cell transplant recipients (18–22). In more recent studies, we have expanded the scope of the method to examine methylation markers of additional cell types, in order to infer cell death in conditions such as cancer and myocardial infarction (18, 22–24). However, the detection of unmethylated insulin cfDNA in patients with type 1 diabetes and those at risk for the disease has been inconsistent. This led us to develop a potentially novel method of cfDNA analysis, with greater specificity and sensitivity.

Herein we describe an ultrasensitive assay to simultaneously measure multiple DNA methylation markers and its application for the robust identification of β cell DNA. We determined assay performance, including reexamination of the insulin gene promoter specificity, and measured β cell–derived cfDNA in plasma samples from healthy subjects over a wide age range, as well as from individuals with pathologies known to involve β cell death.

Results

Developing a multiplex assay for DNA methylation markers. The plasma of healthy individuals contains approximately 1000 genome equivalents (GEq) of total cfDNA per milliliter, rendering the detection of rare cfDNA populations challenging (25). Bisulfite treatment deaminates unmethylated cytosines to uracils while leaving methylated cytosines intact, allowing for the detection of methylation patterns; however, bisulfite destroys approximately 80% of DNA molecules. Hence, when starting with 1 mL of plasma, a cell type contributing 1% to the total circulating cfDNA will be present at approximately 2 GEq after bisulfite conversion, a level that may not be detected consistently by PCR for any given locus. The sensitivity of cfDNA assays can theoretically be increased by using a larger volume of plasma or by simultaneously assessing multiple markers for the cell type of interest, an approach that has shown promise in the context of early cancer detection (26).

To efficiently amplify and sequence multiple targets from bisulfite-treated human cfDNA, we developed a potentially novel 2-step PCR protocol. The method is based on a first step amplifying multiple targets using primers that contain short adapters and a second step that uses these adapters to add Illumina sequencing adapters and barcodes (see Methods, Figure 1A, and Supplemental Figure 1; supplemental material available online with this article; <https://doi.org/10.1172/jci.insight.136579DS1>). Using hepatocyte-specific methylation markers as a proof of principle, we demonstrated this protocol to facilitate simultaneous balanced amplification of up to 30 targets in a single PCR reaction (Supplemental Figure 2A). We next developed a β cell–specific methylation marker cocktail based on predicted tissue-specific patterns of methylation, spread over multiple loci (genomic sequences adjacent to the genes coding for *Fbxl19*, *Mtg1*, *Leng8*, *Zc3h3*, as well as the sense and antisense strands of the insulin gene fragment). Multiplex amplification and sequencing of 5 of these loci from cfDNA in a single PCR reaction gave products equivalent quantitatively to the products of reactions using just 1 target per tube. In these control experiments, the multiplex approach used 20% of the starting material needed for testing of each individual marker separately (Supplemental Figure 2B).

Multiplex assay allows for highly specific and sensitive detection of β cell DNA. To validate the specificity of the β cell–specific marker cocktail, we amplified and sequenced bisulfite-treated genomic DNA from a panel of tissues. In contrast with our tissue-specific methylome atlas (22), which scored the methylation status of individual

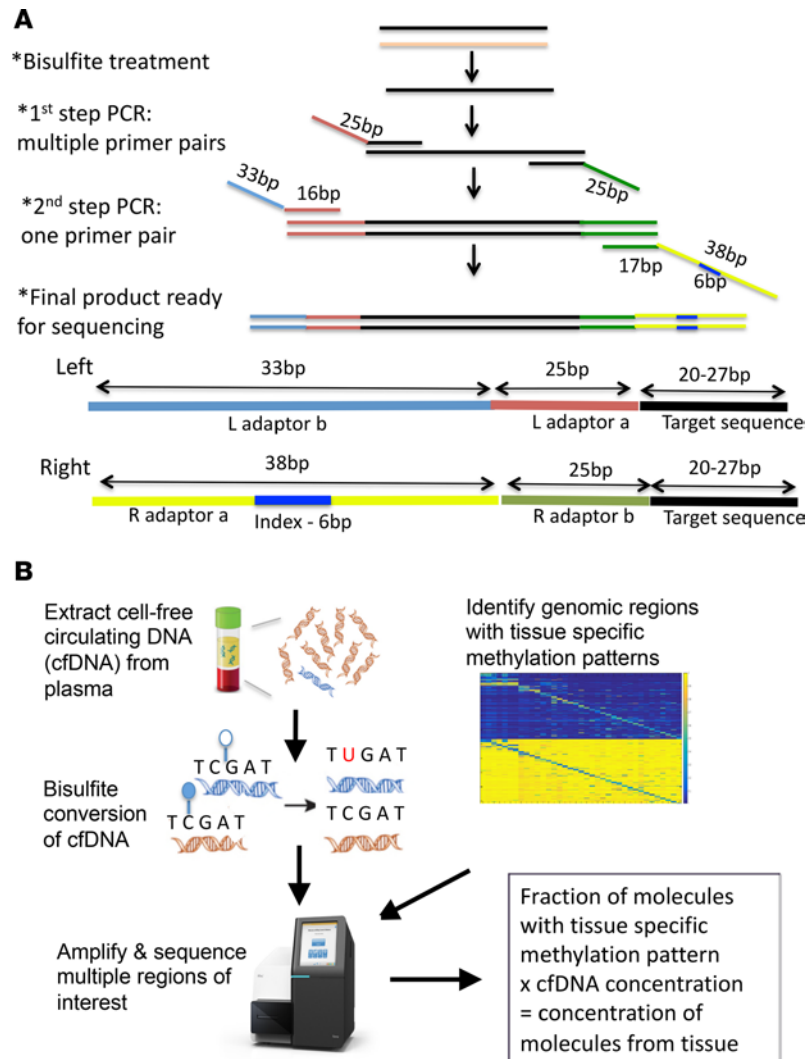


Figure 1. Procedure for analysis of β cell-specific methylation markers. (A) Procedure for multiplex amplification of methylation markers. Following bisulfite treatment, loci of interest were amplified using specific primers containing 5' 25-mer adapters. In a second PCR step, the adapters were extended to include the full adapter sequence required for Illumina sequencing, as well as a 6-bp index barcode allowing us to run multiple products simultaneously. Up to 30 distinct loci can be amplified using this 2-step PCR approach. (B) Schematic of workflow. First, cfDNA was extracted from rapidly spun plasma and treated with bisulfite to convert unmethylated cytosines to uracils. Methylation-independent PCR was used to amplify marker loci. Markers that are methylated or unmethylated only in a tissue of interest were selected based on comparative methylome analysis using an extensive matrix of tissue- and cell type-specific methylomes. The products of amplification were sequenced thousands of times on the MiSeq/NextSeq platform to determine the fraction of molecules in plasma that carry the tissue-specific methylation pattern. When the fraction was multiplied by the total concentration of cfDNA in a sample, the concentration of cfDNA molecules derived from a specific tissue was obtained.

CpG sites from Illumina methylation arrays, this analysis reported the methylation status of all CpG sites in the amplified loci. As we previously demonstrated (18), scoring only molecules that are unmethylated in all CpGs greatly increases assay specificity by reducing noise level in unrelated tissues. All 6 markers were completely unmethylated in more than 70% of sorted β cells (Figure 2A), while occasional methylation at 1–2 CpGs per molecule was detected from the remaining 30% of β cells (data not shown). There were no fully unmethylated molecules in other tissues examined, with 2 exceptions: (a) insulin and a sequence adjacent to the *Leng8* gene were unmethylated in 30%–40% of pancreatic acinar cells (and, accordingly, in unsorted human pancreas), and (b) insulin was unmethylated in 11% of DNA molecules from intestinal epithelial cells (Figure 2A).

Prompted by these unexpected findings, we performed a systematic analysis of the methylation pattern along the insulin gene and identified several areas that were unmethylated in a fraction of cells in the exocrine

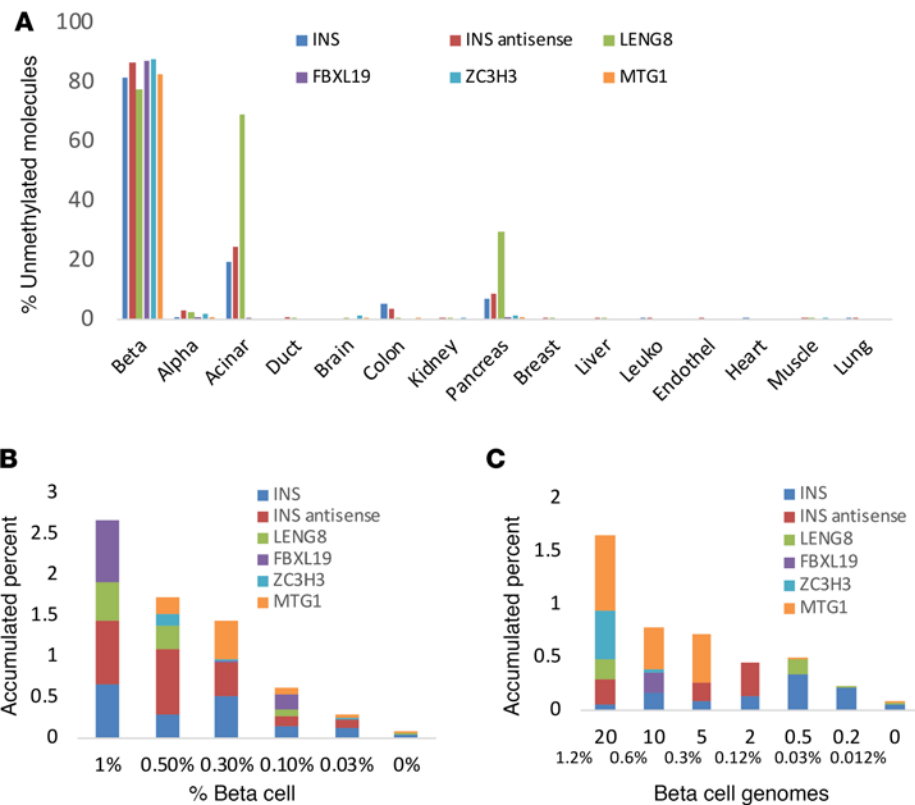


Figure 2. Specificity and sensitivity of beta cell methylation markers. (A) Tissue specificity of 6 methylation markers of human beta cells identified using comparative methylome analysis. Note that markers near the insulin and Leng8 genes are unmethylated in a proportion of pancreatic acinar cells and that insulin is unmethylated in approximately 10% of DNA molecules in the intestine. **(B)** Sensitivity of a 6-marker beta cell panel in identifying beta cell DNA embedded in blood DNA, based on fraction of beta cell genomes. Six beta cell markers (the 6 described in A including the insulin antisense, having the same specificity as insulin but representing independent molecules) were amplified and sequenced in mixtures of blood DNA and DNA from sorted primary beta cells in the indicated proportions. All samples included 60 pg beta cell DNA (10 GEq), mixed with 6 ng to 180 ng of leukocyte DNA. **(C)** Sensitivity of the 6-marker beta cell panel based on absolute number of beta cell molecules. The indicated numbers of beta cell genomes were mixed into 10 ng of blood DNA.

pancreas, as well as a region that was unmethylated exclusively in beta cells (1058–1222 bp downstream to the *INS* transcription start site, Supplemental Figure 3). In addition, we verified that the insulin promoter was unmethylated in a fraction of intestinal cells within human intestinal samples obtained from normal control subjects, colorectal cancer biopsies, and even cfDNA obtained from patients with colorectal cancer (Supplemental Figure 3). These findings cast doubt on the reliability and precision of *INS* DNA alone as a circulating methylation marker of beta cell–derived cfDNA and suggest the existence of previously unappreciated epigenetic heterogeneity in the exocrine pancreas and intestine. Nonetheless, these data support the use of a multiplex assay, combining insulin and additional beta cell–specific markers, to detect beta cell cfDNA.

To determine assay sensitivity, we prepared mixtures of leukocyte and beta cell–derived DNA in known proportions. Beta cell DNA was robustly identified when 10 GEq of beta cell DNA (60 pg) comprised 1%, 0.5%, 0.3%, 0.1% and 0.03% of the mixture, equivalent to the sensitivity of gold standards in the field of cancer liquid biopsies (27) (Figure 2B). Similar findings were obtained when different absolute amounts of beta cell DNA were included in the mixtures. Even when only 0.2 beta cell GEq was present, at least 1 of the markers in the cocktail provided a signal distinct from the baseline level of pure leukocyte DNA (Figure 2C). We conclude that the new multiplex assay for beta cell DNA enhances sensitivity and specificity compared with methods based on methylation of the insulin gene alone.

Low levels of beta cell cfDNA throughout the life span of healthy individuals. We next isolated cfDNA from 2–4 mL plasma (representing approximately 400–800 GEq following bisulfite treatment) from each of 121 healthy subjects aged 0–78 years and measured beta cell–derived cfDNA using our 6-marker multiplex assay. Figure 3A shows the level of signal for each marker from each sample evaluated. Consistent with the

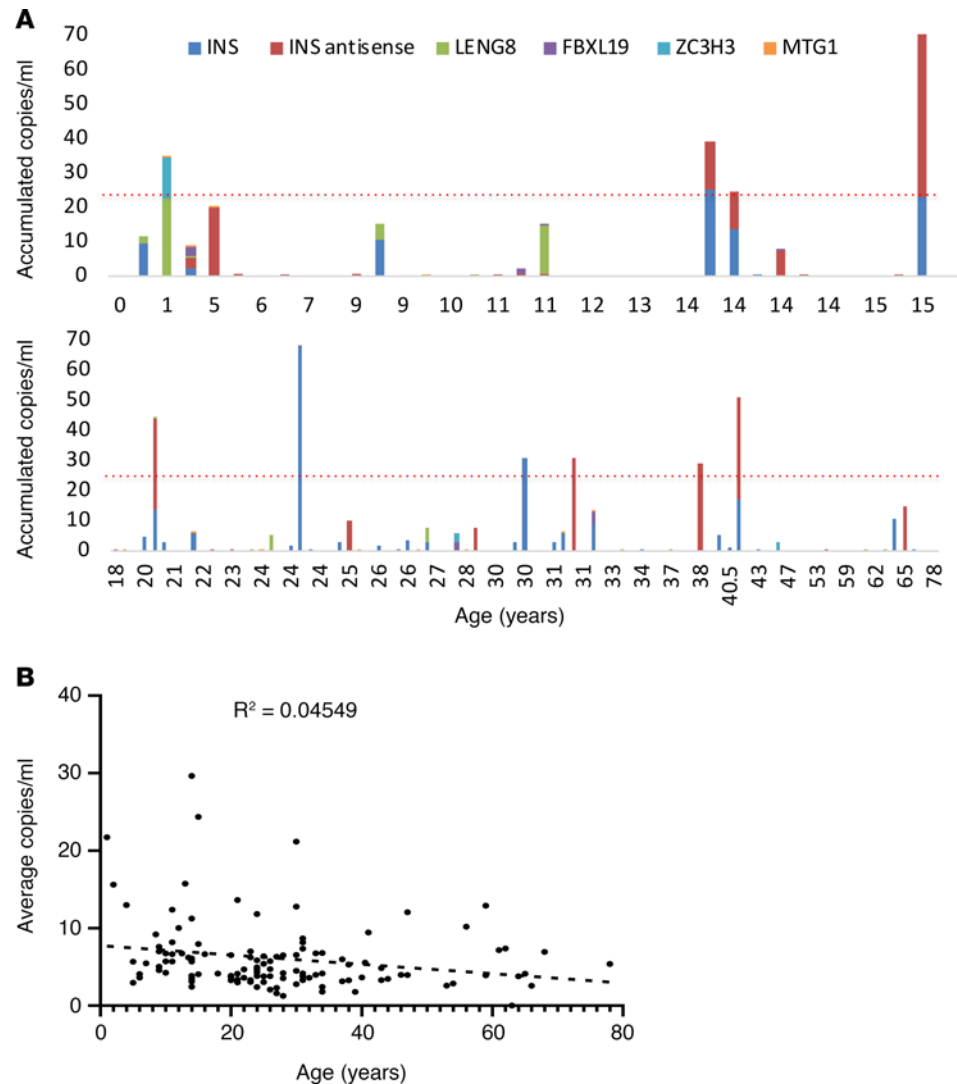


Figure 3. Baseline levels of β cell-derived cfDNA in healthy individuals of different ages. (A) Accumulated levels of 6 β cell markers in plasma samples from individuals at the indicated ages. Local young healthy controls, $n = 36$; local adult healthy controls, $n = 85$. Dotted red line, average (of cumulative values of all 6 markers) + 2SD of all healthy controls. (B) Levels of β cell cfDNA in healthy individuals, calculated as the average of the 6 markers per individual, presented as a function of donor age in years.

quiescent nature of β cells during postnatal life (28), β cell-derived cfDNA was extremely rare in plasma from healthy subjects. In most positive samples, only 1 or 2 of the 6 markers were detected. To assess the concentration of β cell GEq from cfDNA, we calculated the average signal from all 6 markers tested and found that healthy individuals had on average just 1 β cell-derived GEq/mL plasma. We further binned subjects into 3 age groups — children (4–10 years; $n = 16$), adolescents (11–18 years; $n = 20$), and adults (18–78 years; $n = 85$) — and determined control subjects to have comparable minimal levels of β cell cfDNA in plasma throughout the life span (Figure 3B).

Elevated levels of β cell cfDNA immediately after islet transplantation. We and others have shown that the plasma of patients transplanted with cadaveric or autotransplanted islets contains massive amounts of β cell-derived cfDNA in the hours that follow islet infusion, based on assays measuring unmethylated insulin DNA alone (18, 20, 21), as well as upon deconvolution of the entire plasma methylome (22). As expected, the β cell methylation markers were significantly elevated in the plasma from 10 islet transplant recipients, 1 hour posttransplant, as compared with healthy controls (Figure 4 and Supplemental Table 2), further validating performance of the multiplex assay in detecting β cell-derived cfDNA. The presence of β cell-derived cfDNA in the islet transplant setting is interpreted to reflect β cell death, with signal representing DNA from

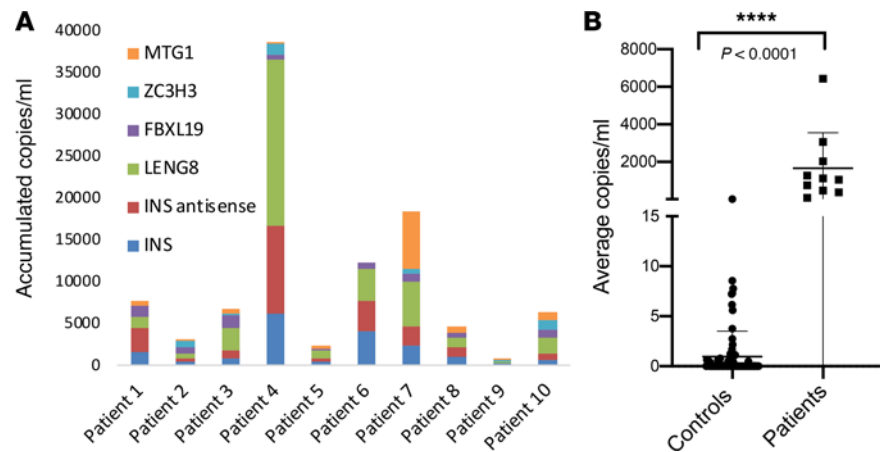


Figure 4. Levels of β cell-derived cfDNA in islet transplant recipients. Islet transplant recipients were sampled about 1 hour after transplantation. **(A)** Levels of individual markers, demonstrating identification of all 6 markers in most samples. **(B)** Average levels of β cell-derived cfDNA in islet transplant recipients versus healthy controls ($n = 85$) ($P < 0.0001$, Mann-Whitney U test).

β cells that died before, during, and after transplantation. There was no correlation between the number of islets transplanted and the concentration of total or β cell–derived cfDNA (Supplemental Table 2).

No evidence for elevated β cell cfDNA in patients with type 1 diabetes. To evaluate β cell–derived cfDNA as a potential biomarker of type 1 diabetes, we applied the multiplex PCR assay to plasma from 156 patients with type 1 diabetes and 186 age-matched control subjects, collected from 6 medical centers around the globe: University of Florida (Gainesville, Florida, USA), Benaroya Research Institute (Seattle, Washington, USA), The Queen Silvia Children’s Hospital (Gothenburg, Sweden), Shaare Zedek Medical Center (Jerusalem, Israel), Hadassah Medical Center (Jerusalem, Israel), and Schneider Children’s Hospital (Petach Tikva, Israel) (Table 1). Coded plasma samples were processed at the local collection centers and then shipped to The Hebrew University of Jerusalem for blinded analysis of β cell–derived cfDNA. Figure 5, Supplemental Figures 4–8, and Supplemental Table 2 present the data analyzed, collectively and per individual center.

Within each cohort, healthy controls had total cfDNA concentrations (Supplemental Figure 4) similar to control samples collected at The Hebrew University (Supplemental Figure 4, control local), suggesting that collection and processing occurred consistently across centers. Moreover, for each cohort examined, total cfDNA levels were comparable among recent-onset type 1 diabetes patients and matched controls (Supplemental Figure 4). Across all control cohorts, β cell cfDNA levels were similar (Figure 5A). Surprisingly, subjects with type 1 diabetes exhibited β cell cfDNA signals comparable to healthy controls when examined either by center (Figure 5A) or collectively (Figure 5B) for those with either more recently diagnosed (disease duration from diagnosis < 4 months) or established type 1 diabetes (disease duration from diagnosis > 4 months) (Figure 5C and Supplemental Figure 9). Hypothesizing that β cell death may peak before clinical diagnosis, we evaluated cfDNA from first-degree relatives of type 1 diabetes patients with 1 or more islet AAbs, i.e., pre–stage 1 or stage 1–2 type 1 diabetes (11). However, β cell–derived cfDNA levels were comparable across subjects positive for a single AAb or multiple AAbs, as well as control and recent-onset type 1 diabetes subjects. There were no differences between males and females (Figure 5, D and E; and Supplemental Table 2). Finally, we tested the possibility that β cell killing occurs in sharp peaks following the onset of type 1 diabetes. We analyzed plasma samples from patients in monthly intervals during the first year postdiagnosis, but none of the time points showed a signal above control onset (Figure 5F).

Examining samples from subjects with type 1 diabetes individually, 3 stood out. A 12-year-old boy (Schneider cohort) with disease duration of 43 months had 120 β cell GEq/mL, with 5 of the 6 markers being detected (Supplemental Figure 5). This patient had an unremarkable course of disease and glycemic control. Unfortunately, no additional samples were available from him. In addition, 2 samples from 2 individual patients at the Shaare Zedek Medical Center cohort, taken at diagnosis or 1 month later, had strong signals; however, signals came from only 1 locus for each sample, suggesting that they represented

Table 1. Patient characteristics at each medical center

Donor characteristics	No. of samples collected	Average age (range)	Sex
US cohort (University of Florida and Benaroya Research Institute)			
Control	30	10.1 (3–16)	11F 19M
Single AAb ⁺	4	9.8 (3–17)	1F 3M
Multiple AAb ⁺	28	19.2 (5–58)	12F 16M
New-onset T1D	3	25.4 (4–43)	1F 2M
Established T1D	2	23.0 (6–44)	2M
Swedish cohort			
Control	9	12 (8–16)	5F 4M
New-onset T1D	24	14 (8–18)	11F 13M
Established T1D	7	12 (9–14)	2F 5M
Shaare Zedek Medical Center cohort			
Control	32	10 (4–17)	11F 13M
New-onset T1D	61	11 (3–17)	31F 30M
Established T1D	15	12 (3–17)	9F 23M
Schneider Children's Medical Center			
Control	26	38 (12–50)	12F 14M
New-onset T1D	4	13 (11–15)	2F 2M
Established T1D	14	16 (9–37)	9F 5M
Hadassah-Hebrew University Medical Center cohort			
Pediatric controls	36	10 (0–16)	11F 22M
Adult controls	85	34 (18–78)	49F 36M
University of Alberta			
Islet transplant recipients	10		6F 4M

AAb, autoantibody.

PCR artifacts (Supplemental Figure 6). Beyond these, the next strongest signals were obtained from healthy controls (Figure 5A). Hence, the current multiplex assay does not detect elevated levels of β cell–derived cfDNA in donors with, or at risk for, type 1 diabetes.

β Cell cfDNA in a patient with CHI. Evidence from rare surgical specimens of children with CHI suggests that, in addition to hypersecretion of insulin, the disease involves an increased rate of β cell turnover. Histological sections of pancreases resected from patients with CHI due to inactivating mutations in genes encoding the β cell KATP channel show increased numbers of both proliferating and apoptotic β cells when compared with age-matched controls (29). This finding is consistent with the clinical observation of progressive improvement in hypoglycemia with the development of diabetes in some patients with CHI after conservative (nonsurgical) treatment (30). The mechanism of this clinical progression is not known because there are no biomarkers of human CHI dynamics, and liquid biopsies have not been reported.

We obtained 5 longitudinal plasma samples from a child with CHI spanning 21 months (age 9 months to 2.5 years). This child carried a de novo (i.e., both parents were negative) missense dominant ABCC8 mutation, c.4453G>A, causing a change in the protein p.Gly1485Arg (p.G1485R). This particular mutation has previously been reported (31). Importantly, and of relevance to our findings in this case, dominantly acting ABCC8 mutations have been reported to confer risk of diabetes in adulthood (32, 33). Strikingly, all 5 samples showed a β cell cfDNA signal (348–6.8 GEq/mL), derived from multiple markers, that was clearly above baseline levels recorded in 23 age-matched controls from the same clinic (Figure 6). A later sample taken at age 3.5 years was negative (data not shown). These findings support the ability of the multiplex cocktail to detect nonmalignant β cell death occurring in the native pancreas environment with various combinations of the 6 β cell markers driving the signal detected in each sample, pointing to the added sensitivity provided with the inclusion of multiple methylation markers. Beyond serving as validation for our methodology, these findings suggest that liquid biopsies can provide insights into CHI disease progression.

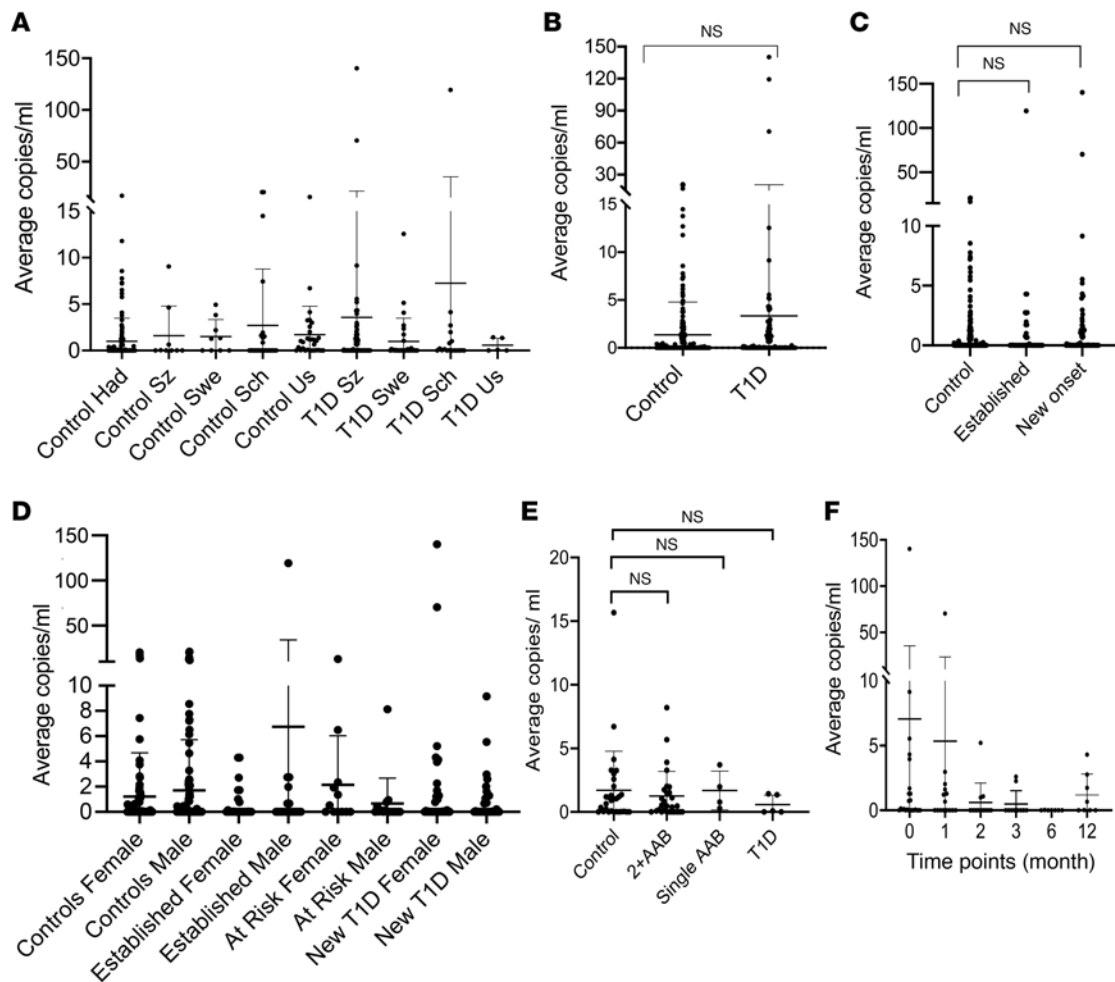


Figure 5. Levels of β cell-derived cfDNA in patients with type 1 diabetes. (A) Levels of β cell-derived cfDNA (average of 6 markers) in patients with recently diagnosed type 1 diabetes and healthy age-matched donors from 4 independent cohorts. Sz, Shaare Zedek Medical Center (32 controls, 72 patients); Swe, The Queen Silvia Children's Hospital (9 controls, 31 patients); Sch, Schneider Children's Hospital (26 controls, 18 patients); Us, University of Florida and Benaroya Research Institute (30 controls, 5 patients). No significant differences found between the groups ($P = 0.0984$, Kruskal-Wallis test). (B) Average levels of cfDNA from β cells in all cohorts. There were 218 controls (those shown in panel A) and 130 patients. $P = 0.8486$, Mann-Whitney test. (C) Levels of β cell-derived cfDNA in 218 healthy individuals, in 38 long-standing type 1 diabetes patients, and in 92 recently diagnosed patients. ($P = 0.7412$, Kruskal-Wallis test). (D) Average levels of β cell-derived cfDNA in healthy individuals (79 females and 29 males), in long-standing type 1 diabetes patients (25 females and 19 males), in donors with 2 AABs (12 females and 14 males), and in recently diagnosed patients (33 females and 32 males) from all cohorts ($P = 0.9892$, Kruskal-Wallis test). (E) Levels of β cell-derived cfDNA in healthy individuals ($n = 30$), donors with 2 or more AABs ($n = 28$), donors with 1 AAB ($n = 4$), and patients with type 1 diabetes ($n = 5$) from the US cohort ($P = 0.5829$, Kruskal-Wallis test). (F) Levels of β cell-derived cfDNA in type 1 diabetes patients from the Shaare Zedek Medical Center cohort, sampled from diagnosis to 12 months later (at diagnosis, $n = 24$; at 1 month after diagnosis, $n = 15$; 2 months, $n = 11$; 3 months, $n = 8$; 6 months, $n = 9$; 12 months, $n = 9$). No significant differences found between the time points ($P = 0.2176$, Kruskal-Wallis test).

Discussion

We report here the development of a sensitive method to detect methylation markers in plasma, based on multiplex amplification and sequencing of 6 independent DNA loci carrying tissue-specific methylation blocks. While we report its application for the study of β cell cfDNA, the method can be used for detection of any collection of methylation markers. Indeed, we validated the assay using a multiplexed panel of hepatocyte methylation markers before developing our β cell marker panel. Our data suggest that up to 30 independent markers can be readily amplified and analyzed using the procedure described here, and further adjustments will likely increase this number further. Given the potential to identify multiple methylation markers for each cell type of interest, this approach may provide the sensitivity needed for cfDNA-based early detection of pathologies in multiple disease settings, as has been shown in cancer (26).

Our extensive atlas of human tissue methylomes (22) revealed dozens of loci in the genome that have β cell-specific methylation patterns. In addition to INS and INS antisense markers, the 4 novel loci selected for analysis in this work proved to be specifically unmethylated in β cells in validation experiments,

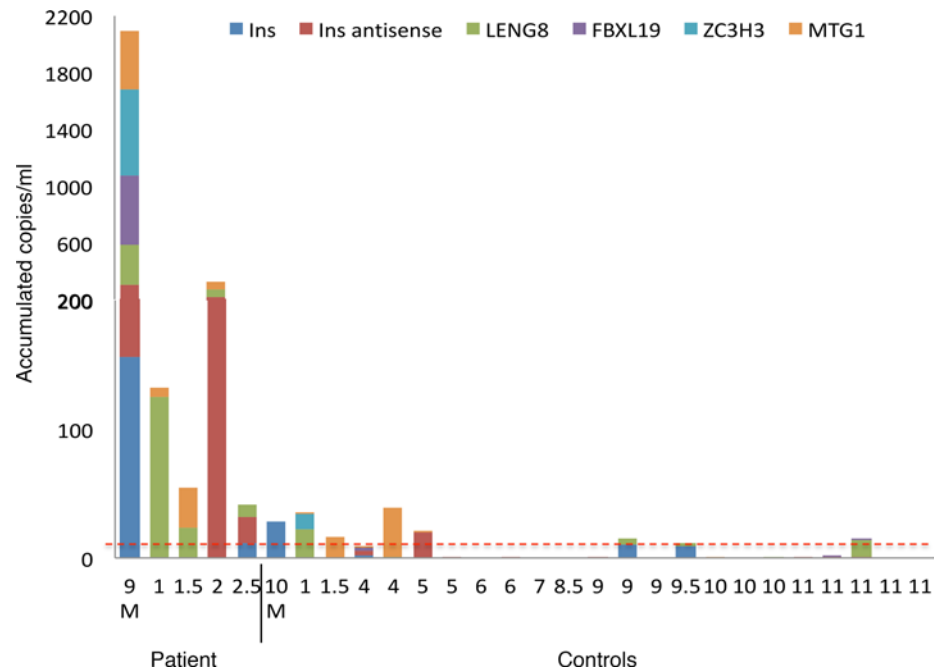


Figure 6. Measurement of β cell-derived cfDNA in a child with CHI. The patient was sampled at 5 times (age 9 months to 2.5 years) and compared with 22 age-matched controls. Dotted red line, average + 2SD of signal from healthy controls.

and certainly, many more candidates could be developed to further enhance assay sensitivity. Notably, we report here that the fragment of the insulin gene used as a β cell-specific methylation marker in all previous cfDNA studies has a more complex pattern of methylation. The presence of unmethylated insulin promoter fragments in pancreatic acinar cells and intestinal epithelial cells necessitates caution in the interpretation of cfDNA signals based on this marker alone and may explain some of the discrepancies between our findings and previous reports, which have reported a β cell cfDNA signal in type 1 diabetes compared with controls (14–18). Beyond utility as a cfDNA marker, the insulin methylation pattern in acinar tissue raises interesting biological questions: Which acinar cells carry an unmethylated insulin gene? Does this phenomenon reflect unappreciated epigenetic heterogeneity of acinar cells or even potential β cell dedifferentiation or regeneration? Are these cells more prone to endocrine reprogramming?

The presence of unmethylated insulin gene in a subset of intestinal epithelial cells is also interesting. The size of the population (10%) suggests that these are unlikely enteroendocrine cells, which comprise only 1% of the intestinal tissue. The detection of unmethylated insulin DNA in tumor biopsies and plasma samples from patients with colon cancer suggests that intestinal cells carrying unmethylated *INS* could be the origins of some colon cancers (34). Further studies involving single-cell methylation analysis are needed to address these questions. It is important to note that multiple CpG sites exist in the insulin locus, and some downstream elements do show higher β cell specificity (Supplemental Figure 3), though these have not been used for cfDNA analysis thus far.

A prime motivation of this work was to develop a method for early detection of β cell death in type 1 diabetes. We validated our multiplex PCR assay in samples collected from recent recipients of islet transplants, in which high levels of β cell death were expected and observed. However, our use of this novel methodology, which is theoretically 6 times more sensitive than previous approaches and certainly more specific, did not reveal elevated β cell cfDNA in the large cohort studied, including samples from patients with recent-onset or established type 1 diabetes and AAb-positive children at risk for type 1 diabetes. This is in contrast with previous reports, including our own, suggesting elevated levels of unmethylated *INS* cfDNA in patients with recent-onset type 1 diabetes (14, 15, 18). The presence of unmethylated *INS* in abundant non- β cells, including at least the exocrine pancreas and the intestine, may provide a partial explanation for these discrepancies (i.e., compromised assay specificity). In addition, at the time of our previous smaller study (18), we were less aware of confounders affecting cfDNA assays, such as (a) differences between plasma and serum, with the latter now considered less appropriate for cfDNA work;

(b) the importance of rapid centrifugation of blood when preparing plasma; and (c) the need to obtain samples from patients and controls at the same site, using exactly the same procedure of blood draw and plasma isolation.

To attempt to explain the lack of a cfDNA β cell death marker in the peripheral blood, we present the following assumptions about type 1 diabetes and cfDNA dynamics in a hypothetical 5-year-old child with new-onset type 1 diabetes: (a) the child has 500 million β cells (half the number of adult β cells), and by the time of diagnosis 50% have died (i.e., 250 million cells); (b) β cell death has taken place steadily over 1000 days; (c) all dying β cells shed their DNA to blood; (d) the half-life of cfDNA is 30 minutes; and (e) a 5-year-old child weighing 20 kg has 800 mL plasma. Under these assumptions, 250,000 β cells would be killed every day, or approximately 5000 cells per 30 minutes; hence, plasma should contain 6 β cell GEq/mL, which should be detectable by the current multiplex assay. However, this certainly must be tested empirically by enhancing assay sensitivity further while preserving specificity.

We believe that the potential benefit of a cfDNA test for type 1 diabetes is too high to drop the approach because of the current inability to identify a signal. We therefore consider here several potential reasons for the lack of β cell cfDNA signal in type 1 diabetes, measured by our current multiplex PCR method, which can be tested in future studies. First, the assay may not be sensitive enough to detect a signal from slow and steady β cell destruction in type 1 diabetes. This could be tested by further enhancing assay sensitivity via multiplexing as many as 100 β cell methylation markers. Second, the destruction of β cells might not be slow and steady. If type 1 diabetes is a remitting-relapsing disease as has been suggested (35), one would expect to see bursts of high β cell cfDNA and long periods with no signal. Our study was not designed to address this possibility. Longitudinal plasma samples may need to be studied beginning early in the course of disease with high temporal resolution. Third, the destruction of β cells might be less extensive than previously thought. While it is clear that β cell autoimmunity drives type 1 diabetes, and that β cells are nearly undetected in long-standing type 1 diabetes (9), it remains possible that at least some β cell loss in the early stages of type 1 diabetes may involve altered identity or function (i.e., loss of insulin, reviewed in ref. 36), similar to what has been proposed in type 2 diabetes (37). This concept should be tested, in mouse models using lineage tracing and in human type 1 diabetes via innovative approaches, potentially with methylome analysis serving as a surrogate lineage tracer. Fourth, the methylation pattern of β cells may be altered in type 1 diabetes, as has been suggested for the insulin gene in murine models of the disease (38, 39). While this warrants further investigation, we find it is unlikely that multiple β cell methylation markers consistently undergo *de novo* methylation in autoimmune diabetes, particularly in the absence of significant β cell proliferation. Fifth, autoimmune β cell death may not release amplifiable cfDNA; rather, most material from killed β cells may be taken up by phagocytes without ever reaching systemic circulation. The fact that we can detect β cell cfDNA in the transplant and CHI settings and our previous demonstration of brain cfDNA in patients with multiple sclerosis (18) suggest such a scenario is unlikely during the autoimmune destruction of β cells. Nonetheless, this idea is certainly consistent with our findings and should be tested experimentally to provide a better understanding of the biology and potential utility of β cell–derived cfDNA in type 1 diabetes.

The presence of β cell–derived cfDNA in longitudinal samples of an individual with CHI is also interesting beyond the demonstration that β cell death in the pancreas can generate a detectable cfDNA signal. Tools to monitor CHI are currently limited, and noninvasive measurement of β cell dynamics could be a helpful addition to clinical assessment. Our findings also add to the small but important body of evidence that CHI involves enhanced postnatal β cell proliferation and death (29, 40, 41).

Type 1 diabetes involves alterations in tissue dynamics beyond the destruction of β cells, which might be amenable for cfDNA methylation analysis. The observation that the pancreas is significantly smaller in patients with type 1 diabetes and subjects with AAb-positive pre–type 1 diabetes (42–45) suggests the involvement of the exocrine pancreas in the pathogenesis of type 1 diabetes. Methylation markers to evaluate exocrine pancreas loss exist and have been shown to be present in the plasma of patients with pancreatic cancer or pancreatitis (18). Hence, a study using our multiplex PCR approach to measure exocrine pancreas cfDNA in plasma from subjects with type 1 diabetes is warranted. In addition, immune processes taking place in the pancreas may not be reflected in altered cell counts in systemic circulation; however, cfDNA from specific immune cell populations reflecting altered turnover might reach peripheral blood, providing a sensitive readout for underlying inflammatory and autoimmune processes. Altogether, our novel multiplex cfDNA methylation assay offers a multitude of potential uses in various disease states. It provides sensitive and specific measurements to detect β cell death in the transplant and CHI settings but does not reveal elevated β cell cfDNA in type 1 diabetes.

Methods

Subject enrollment. Subject characteristics are presented in Table 1. The experimental workflow is presented in Figure 1B.

In the US cohort, normal controls were defined as persons with no reported autoimmune disease in their family ($N = 30$). New-onset type 1 diabetes was defined as 100 days or less from diagnosis ($N = 3$). Established type 1 diabetes was defined after 100 days of diagnosis ($N = 2$). AAb controls were autoantibody-negative relatives of people with type 1 diabetes or controls from the community. AAb⁺ individuals were participants in Type 1 Diabetes TrialNet's Pathway to Prevention Study (46), collected under an approved ancillary study. Single AAb⁺ individuals were persons who were tested and confirmed positive for 1 type 1 diabetes AAb ($N = 4$). Multiple AAb⁺ were individuals who were tested and confirmed positive for 2 or more type 1 diabetes AAbs ($N = 28$). The participants in this at-risk data set were tested as part of the TrialNet Pathway to Prevention screening study, which tests for all 5 known type 1 diabetes-related AAbs, including islet cell antibodies (ICA), antibodies to glutamic acid decarboxylase (GAD-65), insulin autoantibodies (IAA), zinc-transporter 8 autoantibodies (ZnT8A), and tyrosine phosphatase autoantibodies (ICA512/IA-2A). Age- and gender-matched normal controls were tested for only 3 autoantibodies: GAD-65, IAA, and ICA512/IA-2A autoantibodies.

The Swedish cohort included 31 children diagnosed with type 1 diabetes at The Queen Silvia Children's Hospital in Gothenburg and 9 nondiabetic control children. New-onset type 1 diabetes ($n = 24$) was defined as within 1 week of diagnosis. Established type 1 diabetes ($n = 7$) was within 2 to 6 months after diagnosis.

In the cohort from Shaare Zedek Medical Center, new-onset type 1 diabetes was defined as 4 months or less from diagnosis ($N = 61$). Established type 1 diabetes was defined as 5 months to 12 months from diagnosis ($N = 15$). The cohort included 32 healthy controls.

In the cohort from Schneider Children's Medical Center, new-onset type 1 diabetes was defined as 4 months or less from diagnosis ($N = 4$). Established type 1 diabetes was defined as more than 5 months from diagnosis ($N = 14$). The cohort included 26 healthy controls.

In the Hadassah Medical Center cohort, controls (newborn to 16 years of age) were patients arriving at the pediatric emergency medicine center with nonabdominal, nonfebrile issues (usually minor orthopedic trauma); children undergoing elective surgery; or children undergoing endocrine testing, mainly for short stature or delayed/precocious puberty before injection of stimulants ($N = 36$). Adult healthy controls were 18–78 years old ($N = 85$).

Plasma samples from patients with type 1 diabetes receiving a first pancreatic islet transplantation were collected at the University of Alberta.

Sample collection and processing. Blood samples were collected by routine vein puncture in 10-mL EDTA Vacutainer tubes or Streck blood collection tubes and stored at room temperature for up to 4 hours or 5 days, respectively. Tubes were centrifuged at 1500 *g* for 10 minutes at 4°C (EDTA tubes) or at room temperature (Streck tubes). The supernatant was transferred to a fresh 15-mL conical tube without disturbing the cellular layer and centrifuged again for 10 minutes at 3000 *g*. The supernatant was collected and stored at –80°C, before shipping in dry ice to Hebrew University for processing.

cfDNA was extracted from 2–4 mL of plasma, depending on availability, using the QIA Symphony liquid handling robot (QIAGEN). Concentration of cfDNA was determined using Qubit double-strand molecular probes kit (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions.

Islets from cadaveric donors were used to isolate β cells, acinar cells, and duct epithelial cells as previously described (18, 47, 48). Genomic DNA from other tissues was purchased as previously described (23, 24).

DNA derived from all samples was treated with bisulfite using EZ DNA Methylation-Gold (Zymo Research), according to the manufacturer's instructions, and eluted in 20 μ L elution buffer.

Selection of β cell markers. Methylation candidate biomarkers specific for β cells were selected using comparative methylome analysis, based on publicly available data sets (22), to identify loci having more than 5 CpG sites within 150 bp, with an average methylation value for a specific cytosine (present on Illumina 450K arrays) of less than 0.4 in the β cells, greater than 0.9 in leukocytes, and greater than 0.8 in over 90% of tissues. Indeed, from our previously described atlas of human tissue-specific methylomes (22), we identified approximately 200 CpG sites that are unmethylated in β cells and methylated in all other major tissues, as well as approximately 30 CpG sites that are methylated in β cells and unmethylated elsewhere. We selected 4 of these sites (i.e., Fbx119, Mtg1, Leng8, and Zc3h3), which were methylated in the highest number of non- β cell tissues, and designed primers to amplify approximately 100-bp fragments surrounding them using

a novel multiplex 2-step PCR amplification method (see below and Supplemental Table 1). In addition, we prepared primers to amplify the previously described fragment of the insulin gene (18), separately targeting the sense and antisense strands of the insulin fragment, because each strand can serve as an independent marker in bisulfite-treated DNA, potentially increasing assay sensitivity (23) (Supplemental Table 1).

PCR. To efficiently amplify and sequence multiple targets from bisulfite-treated cfDNA, we developed a 2-step multiplex PCR protocol (Figure 1A and Supplemental Figure 1). In the first step, up to 30 primer pairs were used in 1 PCR reaction to amplify regions of interest from bisulfite-treated DNA, independent of methylation status. Primers were 18–30 bp with primer melting temperature ranging from 58°C to 62°C. To maximize amplification efficiency and minimize primer interference, the primers were designed with 25-bp adapters comprising Illumina TruSeq Universal Adapters without index tags. Primers are listed in Supplemental Figure 1 and Supplemental Table 1. All primers were mixed in the same reaction tube. For each sample, the PCR reaction was prepared using the QIAGEN Multiplex PCR Kit according to the manufacturer's instructions with 10 μ L of bisulfite-treated cfDNA. Reaction conditions for the first round of PCR were 95°C for 15 minutes; followed by 30 cycles of 95°C for 30 seconds, 57°C for 3 minutes, and 72°C for 1.5 minutes; followed by 10 minutes at 68°C.

In the second PCR step, the products of the first PCR reaction were treated with Exonuclease I (Thermo Fisher Scientific) for primer removal according to manufacturer instructions. Purified PCR products were amplified using 1 unique TruSeq Universal Adapter primer pair per sample to add a unique index barcode to enable sample pooling for multiplex Illumina sequencing (Figure 1A). The PCR reaction was prepared using 2x PCRBIOS HS Taq Mix Red (PCR Biosystems) according to manufacturer instructions. Reaction conditions for the second round of PCR were 95°C for 2 minutes; followed by 15 cycles of 95°C for 30 seconds, 59°C for 1.5 minutes, and 72°C for 30 seconds; followed by 10 minutes at 72°C. The PCR products were then pooled, run on 3% agarose gels with ethidium bromide staining, and extracted by ZymoClean Gel DNA Recovery Kit (Zymo Research).

Next-generation sequencing. Pooled PCR products were subjected to multiplex next-generation sequencing using the MiSeq Reagent Kit v2 (Illumina) or the NextSeq 500/550 v2 Reagent Kit (Illumina). Sequenced reads were separated by barcode, aligned to the target sequence, and analyzed using custom scripts written and implemented in R. Reads were quality filtered based on Illumina quality scores. Reads were identified as having at least 80% similarity to the target sequences and containing all the expected CpGs. CpGs were considered methylated if “CG” was read and unmethylated if “TG” was read. Proper bisulfite conversion was assessed by analyzing methylation of non-CpG cytosines. We then determined the fraction of molecules in which all CpG sites were unmethylated. The fraction obtained was multiplied by the concentration of cfDNA measured in each sample, to obtain the concentration of tissue-specific cfDNA from each donor. The data for each plasma sample, including patient and sample information, value in copies/mL for each β cell marker, average value for all markers, and total concentration of cfDNA, are detailed in Supplemental Table 2.

Calculation of GEq/mL plasma. To obtain absolute levels of β cell cfDNA (GEq/mL), we multiplied the fraction of β cell-derived molecules by the total concentration of cfDNA present in the sample, as measured by Qubit. It was assumed that a haploid human genome contains 3×10^9 bp of DNA and that the mass of a single bp of double-stranded DNA is 650 Da. Because a Dalton is equivalent to 1.67×10^{-24} g, it follows that the mass of a haploid genome is 3.3 pg, and as such, the concentration of cfDNA could be converted from units of ng/mL to haploid GEq/mL by multiplying by a factor of 303.

Statistics. To assess the significance of differences between groups, we used a 2-tailed Mann-Whitney *U* test. For multiple-group associations, the Kruskal-Wallis test was performed. We calculated all confidence intervals at the 95% level, and *P* value was considered significant when less than 0.05.

Study approval. This study was conducted according to protocols approved by the institutional review boards at each study site: Hadassah-Hebrew University Medical Center, Jerusalem, Israel; Sahlgrenska University Hospital, Gothenburg, Sweden; Uppsala University Hospital, Uppsala, Sweden; Shaare Zedek Medical Center, Jerusalem, Israel; University of Alberta, Edmonton, Canada; Benaroya Research Institute, Seattle, USA; and University of Florida, Gainesville, USA.

Procedures were performed in accordance with the Declaration of Helsinki. Blood and tissue samples were obtained from donors who provided written informed consent. Case report forms were filled out by donors detailing underlying diseases. When using material from deceased organ donors, those with legal authority were consented.

Author contributions

DG, OK, CG, BG, DAS, RS, and YD conceived the study. DN, JM, SP, DC, and OF investigated. DG, AZ, TO, OS, FS, GF, FLK, DA, SH, CS, CG, JH, and AMJS provided resources. AP, MAA, BG, DAS, RS, and YD wrote the manuscript.

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