Identification of tissue-specific cell death using methylation patterns of circulating DNA

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Minimally invasive detection of cell death could prove an invaluable resource in many physiologic and pathologic situations. Cell-free circulating DNA (cfDNA) released from dying cells is emerging as a diagnostic tool for monitoring cancer dynamics and graft failure. However, existing methods rely on differences in DNA sequences in source tissues, so that cell death cannot be identified in tissues with a normal genome. We developed a method of detecting tissue-specific cell death in humans based on tissue-specific methylation patterns in cfDNA. We interrogated tissue-specific methylome databases to identify cell type-specific DNA methylation signatures and developed a method to detect these signatures in mixed DNA samples. We isolated cfDNA from plasma or serum of donors, treated the cfDNA with bisulfite, PCR-amplified the cfDNA, and sequenced it to quantify cfDNA carrying the methylation markers of the cell type of interest. Pancreatic β-cell DNA was identified in the circulation of patients with recently diagnosed type-1 diabetes and islet-graft recipients; oligodendrocyte DNA was identified in patients with relapsing multiple sclerosis; neuronal/glial DNA was identified in patients after traumatic brain injury or cardiac arrest; and exocrine pancreas DNA was identified in patients with pancreatic cancer or pancreatitis. This proof-of-concept study demonstrates that the tissue origins of cfDNA and thus the rate of death of specific cell types can be determined in humans. The approach can be adapted to identify cfDNA derived from any cell type in the body, offering a minimally invasive window for diagnosing and monitoring a broad spectrum of human pathologies as well as providing a better understanding of normal tissue dynamics.

circulating DNA | diagnosis | methylation

Cell death plays a critical role in the pathogenesis and therapy of a large number of diseases affecting every organ system in the body. Examples range from pancreatic β-cell death in type 1 diabetes (T1D) to neuronal death in neurodegenerative disease and after traumatic brain injury (TBI). In most cases, cell death can be measured only by its functional consequence: hyperglycemia or cognitive dysfunction in the examples given. These measures typically lack sensitivity, represent late phases of disease, and, in the case of T1D and neurologic disease, are often measurable only after potential therapeutic windows have been closed.

It has been known for decades that plasma and serum contain small fragments of cell-free circulating DNA (cfDNA) derived from dead cells (on average, 5,000 genome equivalents/mL) (1). Although the mechanisms underlying the release and rapid clearance of cfDNA (2) remain obscure, the phenomenon is currently being exploited for a variety of applications with clinical relevance. The recognition that fragments of fetal DNA travel briefly in maternal circulation has opened the way for next-generation sequencing (NGS)-based prenatal testing to identify fetal trisomies and other genetic aberrations, potentially replacing invasive and potentially dangerous procedures such as amniocentesis (3–5). In cancer biology, tumors are known to release DNA (including tumor-specific somatic mutations) into the circulation, allowing tumor dynamics and genomic evolution to be monitored by liquid biopsies (6–8). In addition, cfDNA has been used to detect graft cell death after

Significance

We describe a blood test for detection of cell death in specific tissues based on two principles: (*i*) dying cells release fragmented DNA to the circulation, and (*ii*) each cell type has a unique DNA methylation pattern. We have identified tissue-specific DNA methylation markers and developed a method for sensitive detection of these markers in plasma or serum. We demonstrate the utility of the method for identification of pancreatic β-cell death in type 1 diabetes, oligodendrocyte death in relapsing multiple sclerosis, brain cell death in patients after traumatic or ischemic brain damage, and exocrine pancreas cell death in pancreatic cancer or pancreatitis. The approach allows minimally invasive monitoring of tissue dynamics in humans in multiple physiological and pathological conditions.

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kidney, liver, or heart transplantation, based on SNPs that distinguish the DNA of the donor from that of the recipient (9–11). In all these cases, genetic differences exist between the DNA sequence of the tissue of interest (fetus, tumor, or graft) and that of the host, providing the basis for highly specific assays.

Blood levels of cfDNA increase in many conditions, such as TBI (12), cardiovascular disease (13), sepsis (14), and intensive exercise (15). However, in these cases, the cfDNA sequence is identical to that of genomic DNA, making it impossible to use sequence variation to identify the source of the elevated cfDNA and thus greatly compromising the utility of cfDNA as a diagnostic or prognostic tool. For example, the cfDNA could originate from parenchymal cells of the injured tissue but also from dying inflammatory cells.

Despite having an identical nucleotide sequence, the DNA of each cell type in the body carries unique methylation marks correlating with its gene-expression profile, and these marks represent a fundamental aspect of tissue identity. Methylation patterns are unique to each cell type, are conserved among cells of the same type in the same individual and among individuals, and are highly stable under physiologic or pathologic conditions (16). Therefore, it is possible to use the DNA methylation pattern of cfDNA to determine its tissue of origin and hence to infer cell death in the source organ.

DNA methylation in cfDNA has been studied extensively but usually in the context of epigenetic aberrations unique to the specific pathology, such as abnormal methylation in promoters of tumor-suppressor genes, which may lead to insights into cancer biology (17–20). By contrast, our approach seeks to identify, in plasma or serum, the normal, stable methylation signature of a specific tissue as a sensitive biomarker of cell death. Therefore the approach can be applied to pathologies that retain a stable genome and epigenome.

A classic example of tissue-specific DNA methylation is provided by the insulin gene (*INS*) promoter, which is unmethylated in insulin-producing pancreatic β cells and is methylated elsewhere. Recent studies have identified unmethylated *INS* promoter DNA in the circulation of patients with recently diagnosed T1D and in islet-graft recipients, likely reflecting both autoimmune and alloimmune destruction of β cells (21–25). However, published data suggest that the analytic approaches used were not sufficiently specific to differentiate robustly between β-cell– and non–β-cell–derived cfDNA. We sought to optimize the method and generalize the approach toward proof of the concept that tissue-specific methylation patterns in cfDNA can be used to detect tissue cell death with a high level of specificity and sensitivity in multiple human pathologies. Here we demonstrate the detection of cell death in different pathologies of the pancreas and the brain.

Results

Identification of Tissue-Specific Methylation Markers. We started by identifying tissue-specific DNA methylation markers distinguishing individual tissues or cell types from other tissues. Particular attention was given to markers that differ between the tissue of interest and hematopoietic cells, which contribute the majority of cfDNA in healthy individuals. We analyzed publicly available (The Cancer Genome Atlas and Gene Expression Omnibus) and locally generated methylomes to identify individual CpG dinucleotides with differential methylation patterns, i.e., unmethylated in the tissue of interest but methylated elsewhere (*SI Materials and Methods* and the schematic of the procedure in Fig. S1).

The Illumina Infinium HumanMethylation450 BeadChip array provides information on the methylation status of individual CpG dinucleotides. The discriminatory power of any single CpG is limited, because it can be randomly methylated or unmethylated in a small fraction of molecules from tissues where it typically is unmethylated or methylated, respectively. To increase the signal-to-noise ratio of the assay, we exploited the regional nature of DNA methylation. We defined an "expanded window" of four to nine CpG sites adjacent to the original CpG marker site, reasoning that accidental methylation or demethylation of multiple adjacent cytosines in the same molecule is unlikely. We obtained DNA from different human tissues and treated it with bisulfite to convert unmethylated cytosines to uracils. We then PCR-amplified short fragments containing the signature CpG site and multiple adjacent CpGs. Amplicons were designed to maximize the number of potentially informative CpG sites (to improve specificity in the detection of tissue-specific patterns) while minimizing overall fragment size (to improve the sensitivity of the assay, given that the average size of cfDNA fragments is 165 bp). We sequenced multiple molecules from the PCR product using Illumina MiSeq to assess the fraction of molecules with tissue-specific methylation patterns. In some cases, as an alternative approach to the comparisons between Illumina methylome arrays, we selected and validated tissue-specific markers based on promoters of known tissue-specific genes (Fig. S1). As shown in the examples below, scoring for DNA molecules in which multiple adjacent CpG sites share the same tissue-specific methylation pattern gave a much greater discriminatory power between the tissue of interest and other tissues compared with the information content of individual CpG sites.

Thus, we have defined short sequences of DNA containing four to nine CpG sites whose combined methylation status constitutes an epigenetic signature unique to a tissue of interest relative to blood cells and other tissues.

Presence of Unmethylated *INS* Promoter in the Circulation of T1D Patients. To detect cfDNA derived from β cells, we used the *INS* promoter as a β-cell–specific methylation marker. Previous studies seeking to identify DNA derived from β cells in peripheral blood samples have used methylation-specific PCR based on the methylation status of two or three CpG dinucleotides in the *INS* promoter (22). However, the *INS* promoter contains additional CpG sites in close proximity, which can be used to improve the distinction between DNA of β cells and other tissues (Fig. 1*A*). To test this concept, we amplified a 160-bp fragment of the *INS* promoter from bisulfite-treated DNA obtained from multiple tissues and sequenced the product to determine the methylation status of each CpG in each tissue. As shown in Fig. 1*B*, each individual CpG was unmethylated in 90–95% of the DNA molecules from human β cells and in 5–15% of the DNA molecules from other tissues. However, when we assessed six sites in combination, we found that six fully unmethylated sites were present in roughly 80% of the DNA molecules from β cells but in less than 0.01% of the molecules from any other tissue, thus giving an extremely high specificity for β cells.

To determine the sensitivity and linearity of the assay, we spiked human β-cell DNA into human lymphocyte DNA in different proportions and determined the frequency of unmethylated *INS* promoter DNA. The measured methylation signal was in excellent correlation with the input material, and β-cell DNA could be detected even when diluted 1:1,000 in lymphocyte DNA (Fig. 1*C*).

We then used this information to look for β-cell–derived cfDNA in the circulation. Plasma DNA from healthy volunteers and T1D patients was treated with bisulfite, PCR amplified, and sequenced to determine the fraction of molecules containing fully unmethylated *INS* promoter DNA. The fraction obtained was multiplied by the concentration of cfDNA measured in each sample to obtain the concentration of β-cell–derived DNA circulating in the blood of each patient (Fig. S1).

The cfDNA of healthy volunteers ($n = 31$) had an extremely low frequency of fully unmethylated *INS* promoter molecules (i.e., with all six CpGs unmethylated); less than 0.12% of circulating fragments had this sequence. When multiplied by the total amount of cfDNA in each individual, we found that less than 0.06 ng cfDNA/mL plasma was derived from β cells (equivalent to 10 genomes/mL), consistent with a very low rate of β-cell turnover in healthy adults (Fig. 1*D*). Plasma from all T1D patients

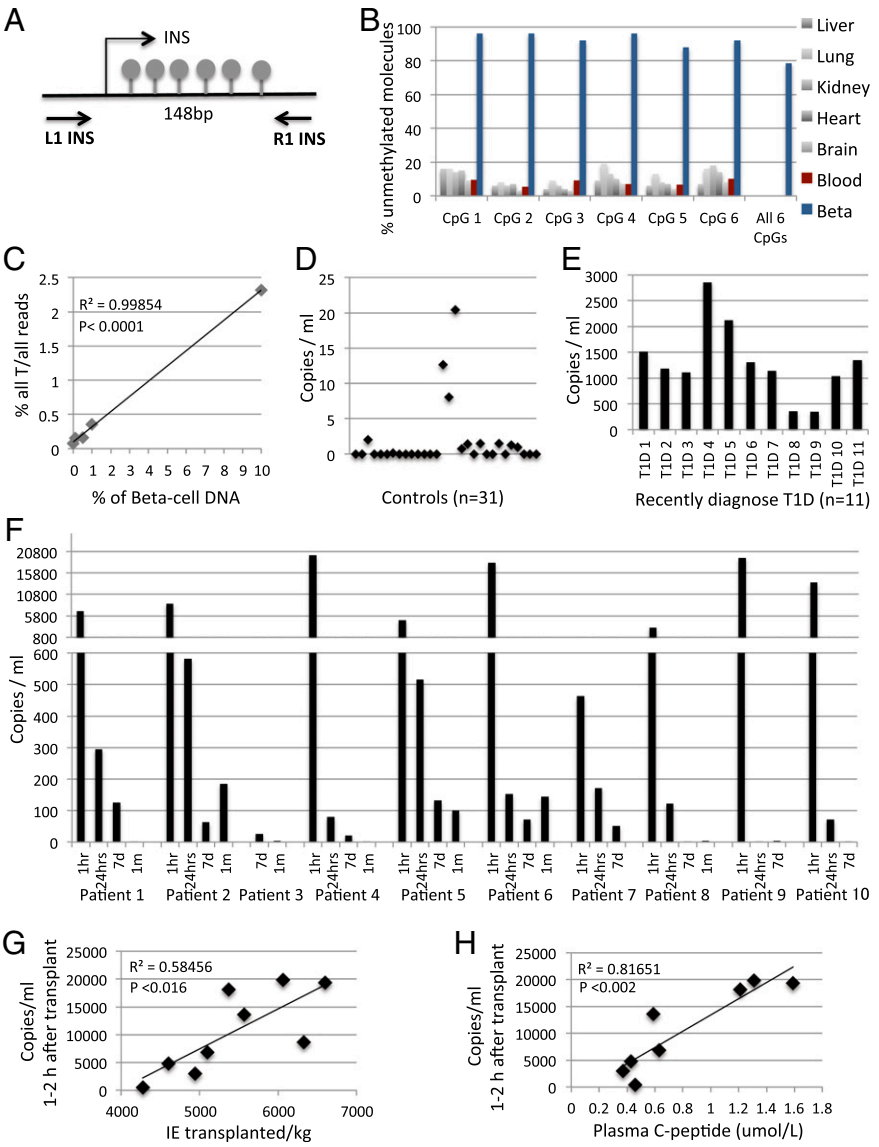

Fig. 1. β-Cell–derived DNA in the circulation of T1D patients. (*A*) Structure of the *INS* promoter fragment used as a marker. Lollipops represent CpG sites; arrows mark positions of PCR primers. (*B*) Methylation status of individual CpG sites in the *INS* promoter in multiple tissues. The graph shows the percentage of unmethylated molecules in DNA from each tissue. The set of columns on the far right describes the percentage of molecules in which all six CpG sites are unmethylated, demonstrating the increase in signal-to-noise ratio afforded by interrogating all six CpGs simultaneously. (*C*) Spike in experiment. Human β-cell DNA was mixed with human lymphocyte DNA in the indicated proportions (0.1% to 10%), and the percentage of fully unmethylated *INS* promoters (in which all six CpG sites were converted by bisulfite to T) was determined. (*D*) β-Cell–derived DNA in the plasma of healthy controls. The fraction of fully unmethylated *INS* promoter DNA molecules (reflective of the fraction of β-cell–derived cfDNA) (Table S1) was multiplied by the absolute level of cfDNA measured in each individual. This value (in nanograms per milliliter) was multiplied by 330 to obtain the number of copies of β-cell–derived *INS*/mL plasma. (*E*) β-Cell–derived DNA in the plasma of recently diagnosed T1D patients. Mann–Whitney test for controls vs. patients, $P < 0.0001$. (*F*) β-Cell–derived DNA in the plasma of long-time T1D patients sampled at the indicated time points after intrahepatic islet transplantation. (*G*) Correlation between the number of transplanted islets (IE, islet equivalents; each islet contains ∼1,000 β cells) per kilogram and β-cell copies/mL 1–2 h after transplantation. $n = 9$ patients. (*H*) Correlation between plasma c-peptide levels and unmethylated *INS* promoter cfDNA 1–2 h after islet transplantation. $n = 8$ patients.

sampled 2–16 wk after diagnosis ($n = 11$) showed a clear signal of unmethylated *INS* promoter DNA in cfDNA, (350–2,900 copies of unmethylated *INS* promoter DNA/mL of plasma, equivalent to 175–1,450 β-cell genomes/mL), indicating ongoing β-cell death (Fig. 1*E*). The fraction of cfDNA derived from β-cells ranged from 0 to 0.1% in controls and from 1.9–5.5% in patients (Table S1).

To confirm that the combined methylation pattern of multiple CpG sites at the *INS* promoter was necessary to detect β-cell–derived DNA in the circulation, we examined the methylation status of each individual CpG in the plasma of healthy individuals and of persons with recently diagnosed T1D. Each individual CpG did not have a different pattern in the plasma of healthy controls or of T1D patients (unmethylated in ∼15% of cfDNA molecules), but collectively the six CpG sites yielded a clear signal in the plasma of T1D patients that was absent in healthy controls (Fig. S2).

We also studied plasma samples taken from patients with long-standing T1D who had been transplanted with cadaveric allogeneic islets and treated with immune suppressants (26). As shown in Fig. 1*F*, the plasma of all patients ($n = 10$) had a high signal (unmethylated *INS* promoter DNA) 1–2 h after transplantation, which declined dramatically in the hours and days that followed.

The extensive loss of grafted β cells immediately after transplantation is consistent with a previous imaging study of a transplanted patient (27). The levels of β-cell cfDNA shortly after transplantation were in good correlation with the amount of transplanted islets, presumably because transplanted islets contained a fraction of dead or dying cells (Fig. 1*G*). In most patients, signals above background were clearly detected at 7 d and even 1 mo after transplantation. Given the short half-life of cfDNA (2), the observed signals (up to 200 copies/mL plasma) may reflect significant ongoing loss of β cells despite immune suppression. More work will be required to determine if cfDNA levels predict long-term transplant outcome.

The current standard assay to assess β-cell damage in the transplant setting is plasma c-peptide, which reflects both physiologic insulin secretion and insulin released from damaged or dead β cells. In the hours immediately after transplantation, when patients are maintained in normoglycemic status by exogenous insulin, circulating c-peptide reflects β-cell damage. Indeed, c-peptide levels and unmethylated *INS* promoter cfDNA were in excellent correlation in the plasma of patients 1–2 h after transplantation (Fig. 1*H*), supporting the validity of β-cell cfDNA as a marker of acute β-cell death. These results demonstrate that our NGS-based method represents a highly sensitive and specific assay for the detection of cfDNA derived from specific tissues. With respect to T1D, signal analysis achieved complete separation between healthy controls and recently diagnosed patients; this result contrasts favorably with previous reports, which demonstrated a significant signal overlap between healthy controls and diabetic patients (21–24). Thus, it may be possible to use this assay to reliably identify β-cell death before clinical diagnosis (24) as

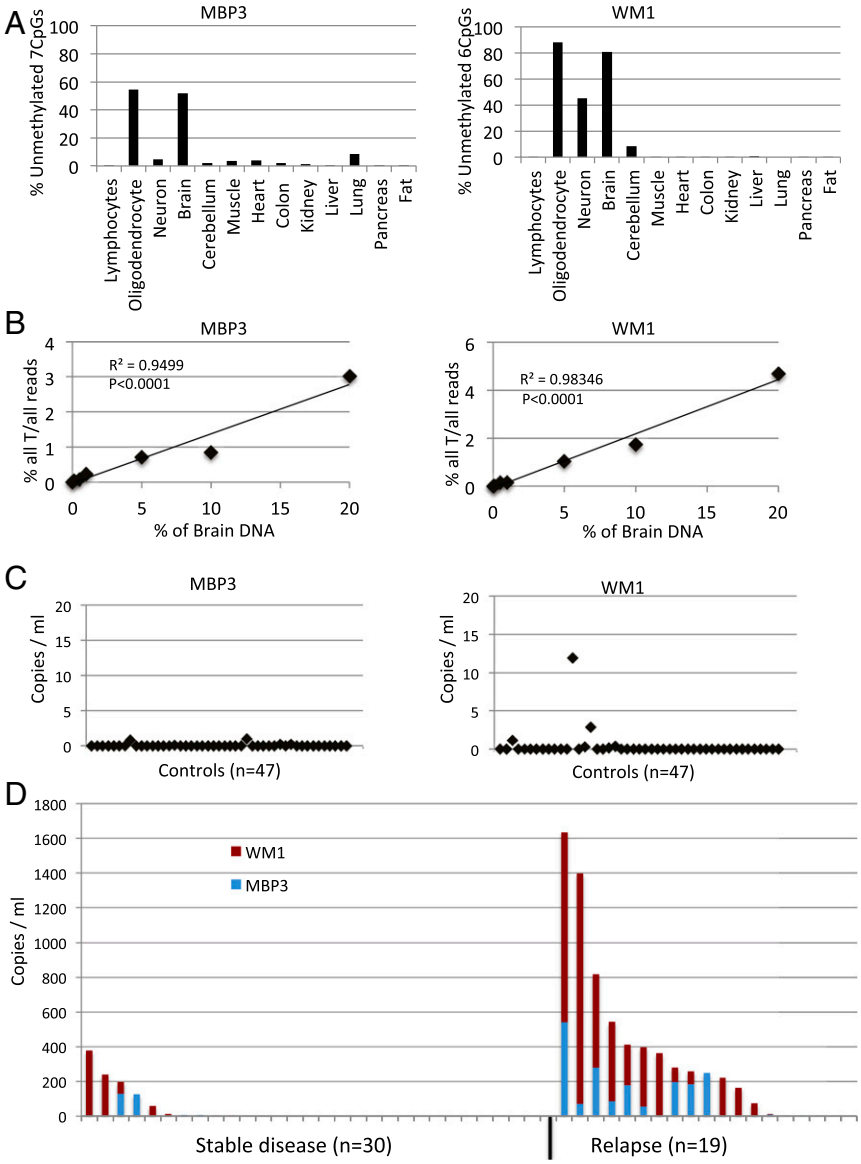

Fig. 2. Identification of oligodendrocyte-derived cfDNA in MS. (*A*) Methylation status of *MBP3* and *WM1* in DNA from multiple tissues and from sorted human neurons and oligodendrocytes (see also Figs. S3 and S4). (*B*) Spike-in experiments. Brain DNA was mixed with lymphocyte DNA, and lack of methylation of oligodendrocyte markers was used to estimate the fraction of oligodendrocyte DNA in the mixtures. Note that the measured frequency is lower than the input frequency, likely because input (brain DNA in this case) is a mixture of DNA from glial and other cell types. (*C*) Oligodendrocyte-derived DNA in the serum or plasma of healthy individuals, derived from the fraction of oligodendrocyte DNA (Table S1) and the total amount of cfDNA. (*D*) Oligodendrocyte-derived DNA in the serum of remitting and relapsing MS/NMO patients. The graph shows the cumulative values of unmethylated *MBP3* and *WM1* in each sample. Controls vs. stable disease, $P = 0.6$; controls vs. relapsing disease, $P < 0.0001$; stable vs. relapsing disease, $P < 0.0001$; controls vs. all patients, $P = 0.021$.

well as in additional settings of interest, such as monitoring the efficacy of immune suppression used to prevent rejection of transplanted β cells.

Identification of Oligodendrocyte-Derived cfDNA in Multiple Sclerosis. Noninvasive detection of brain-cell death is particularly challenging. To test the hypothesis that brain-specific methylation patterns can be used to identify brain-derived cfDNA, we looked for evidence of oligodendrocyte DNA circulating in the blood of patients with multiple sclerosis (MS) and neuromyelitis optica (NMO). Both are autoimmune diseases in which oligodendrocyte and astrocyte cell death occurs and in which the blood–brain barrier is disrupted (28, 29). We analyzed the published methylome of normal human white matter (30) and identified clusters of adjacent CpG sites in the 3′ UTR of myelin basic protein (termed here *MBP3*) and around an unannotated locus (CG10809560 in the Illumina array, herein *WM1* for white matter 1) that were unmethylated selectively in sorted human oligodendrocytes (Fig. 2*A*). As with the *INS* promoter, individual CpGs in these clusters had a moderate signal-to-noise ratio, but combining all CpGs at the *MBP3* and *WM1* loci greatly increased the discrimination between oligodendrocyte DNA and DNA from other sources, including lymphocytes (Figs. S3 and S4). Thus, DNA fragments from the *MBP3* or *WM1* loci, when unmethylated in all CpG sites, can serve as an exclusive marker of oligodendrocytes.

We then spiked human brain DNA into human lymphocyte DNA in different proportions and found excellent correlation between measured methylation signal and the input material, so that oligodendrocyte DNA was detected even when diluted 1:1,000 in lymphocyte DNA (Fig. 2*B*). We then tested if these oligodendrocyte-derived fragments can be found in plasma or serum. Healthy individuals (n = 47) had negligible levels of unmethylated *MBP3* or *WM1* in their circulation, suggesting minimal basal turnover of oligodendrocytes (Fig. 2*C*). Strikingly, during disease exacerbation (a relapse documented both clinically and using brain MRI close to the time of sampling), most MS patients (14/19) had unmethylated cfDNA of *MBP3*, *WM1*, or both in their serum (Fig. 2*D*). Stable MS patients (n = 30) had a minimal or absent signal in serum (Fig. 2*D*). This observation is consistent with the notion that short-lived unmethylated *MBP3* or *WM1* cfDNA reflects acute oligodendrocyte cell death. Initial analysis did not reveal clinical correlates to the lack of signal in some relapsing patients. No correlation was observed between the signal and age, sex, Expanded Disability Status Scale (EDSS), or disease duration.

These results indicate that acute oligodendrocyte death can be detected by increased levels of fully unmethylated cfDNA fragments from the *MBP3* or *WM1* loci, presenting an opportunity for diagnosis and monitoring of demyelinating diseases.

Identification of Brain-Derived cfDNA After Acute Brain Damage. To obtain a more generally applicable marker of brain injury, we scanned the Illumina arrays for loci whose methylation status distinguished brain DNA from other tissues. A cluster of nine CpG sites around locus CG09787504 (here termed "*Brain1*") was fully unmethylated in 70% of DNA from various sources of brain tissue (enriched for either neurons or glia) and in <5% of DNA molecules from other tissues (likely reflecting DNA from peripheral neurons present in these tissues). Importantly, <0.03% of molecules in lymphocytes were unmethylated, giving a >2,000-fold difference in methylation of this locus between brain and lymphocytes (Fig. 3*A* and Fig. S5). Spike-in experiments showed that human cortex DNA can be detected even when diluted 1:1,000 into human lymphocyte DNA and that the signal recorded correlates perfectly with the level of input material (Fig. 3*B*).

Healthy individuals (n = 47) had extremely low levels of fully unmethylated *Brain1* in serum or plasma (Fig. 3*C*). This low baseline signal may reflect minimal physiological neuronal turnover or an alternative mechanism for the clearance of DNA from dying brain cells. We then examined serum samples from patients in two scenarios, cardiac arrest and TBI; both situations involve neuronal injury in combination with disruption of the blood–brain barrier.

Following cardiac arrest, patients with documented ischemic brain damage sampled at multiple time points (n = 10 patients, 60 samples) (31) all had high levels of unmethylated *Brain1* in serum (Fig. 3*D*). Similarly, patients hospitalized in an intensive care unit after severe TBI (n = 15 patients, 102 samples) had elevated unmethylated *Brain1* in serum (Fig. 3*E*). Both sets of results are consistent with cfDNA fragments derived from dead brain cells (neurons or glia) in these patients. The amount and temporal patterns of brain-derived cfDNA varied between patients with cardiac arrest and those with TBI. In the group of patients with cardiac arrest, the strongest signals were observed at the first time point, shortly after resuscitation, with a decline in subsequent days in most patients. In the group of patients with TBI, a more delayed rise in brain-derived cfDNA was observed. Initial analysis did not reveal a correlation between brain cfDNA and levels of the brain damage biomarker NFL. Larger studies may be required to identify correlations between brain cfDNA and clinical data.

These findings indicate that, based on unique methylation markers, brain-specific DNA as well as oligodendrocyte-specific DNA can be identified in the circulation of patients with neuroinflammatory, traumatic, and ischemic brain pathologies and used to report quantitatively on the rate of brain cell death.

Identification of Exocrine Pancreas-Derived cfDNA in Pancreatic Cancer and Pancreatitis. Finally, we tested whether the approach can be used to detect cfDNA in the context of cancer. Although tumors have extensive methylation changes compared with normal tissue, the majority of tissue-specific methylation sites remain intact in tumors (32). Thus, cell death in tumors should release cfDNA carrying the normal methylation pattern of the source tissue. Pancreatic ductal adenocarcinoma is thought to originate from either acinar or duct cells in the exocrine pancreas. We used antibodies to FACS-purify duct and acinar cells from cadaveric human material and obtained their methylomes using Illumina 450k arrays. Analysis of these data revealed multiple CpGs that were unmethylated in the exocrine pancreas and methylated in most other tissues, including the endocrine pancreas. We selected two sites for further analysis and identified clusters of adjacent CpGs that could be used as markers for the exocrine pancreas, distinguishing acinar and ductal cells from other cell types (Fig. 4*A* and Figs. S6 and S7). Spike-in experiments showed that human pancreas DNA can be detected even when diluted 1:1,000 into human lymphocyte DNA and that the signal recorded correlates perfectly with the level of input material (Fig. 4*B*). Healthy subjects (n = 47) had very low levels of unmethylated exocrine pancreas markers in their cfDNA (both plasma and serum), consistent with a low turnover of this tissue (Fig. 4*C*). Nearly half the patients with pancreatic cancer (20/42) displayed exocrine pancreas-derived cfDNA above background level in plasma or serum (Fig. 4*D*). There was a trend toward a stronger signal in patients with advanced disease, and these patients were more likely to show a signal above background level. Nevertheless, some patients (11/29) with stage 1 and 2 (localized) disease had a clear signal, suggesting this method has the potential to identify cell death in pancreatic cancer at a resectable stage.

The majority of pancreatic cancers carry somatic mutations in the *KRAS* (Kirsten rat sarcoma) gene, and these mutations can be detected in blood to monitor tumor cell death (although the presence of mutant *KRAS* per se does not reveal the tissue origin of mutant DNA) (33, 34). To compare the performance of our methylation-based assay with the detection of mutant *KRAS*, we

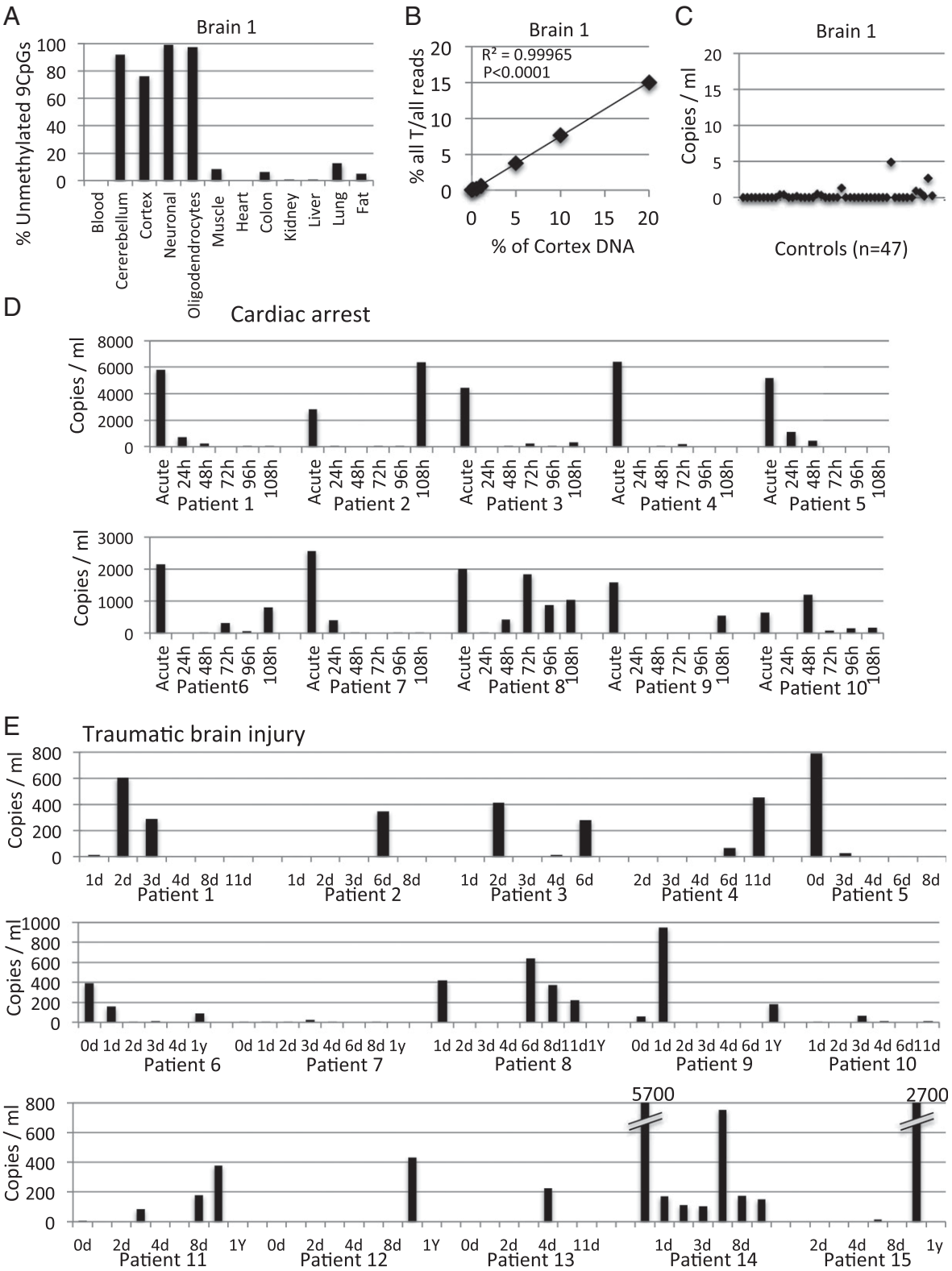

Fig. 3. Identification of brain-derived cfDNA after brain damage. (*A*) Methylation status of CpG sites at the CG09787504 locus (*Brain1*) in multiple tissues, as determined by deep sequencing. Bars represent the percentage of molecules in which all nine CpGs of the locus are unmethylated. (*B*) Spike-in experiment. Cortex DNA was mixed with lymphocyte DNA, and lack of methylation of *Brain1* was used to estimate the fraction of brain DNA in the mixtures. (*C*) Brain-derived DNA in the serum or plasma of 47 healthy volunteers, derived from the fraction of fully unmethylated *Brain1* molecules (Table S1) and the amount of cfDNA in each individual. (*D*) Brain-derived DNA in the serum of 10 patients after cardiac arrest. Each patient was sampled immediately after resuscitation ("acute") and at subsequent time points. Healthy controls vs. patients (all time points), $P < 0.0001$. (*E*) Brain-derived DNA in the serum of 15 patients after TBI, sampled at different days after admission to a neurotrauma unit. Healthy controls vs. patients (all time points), $P < 0.005$.

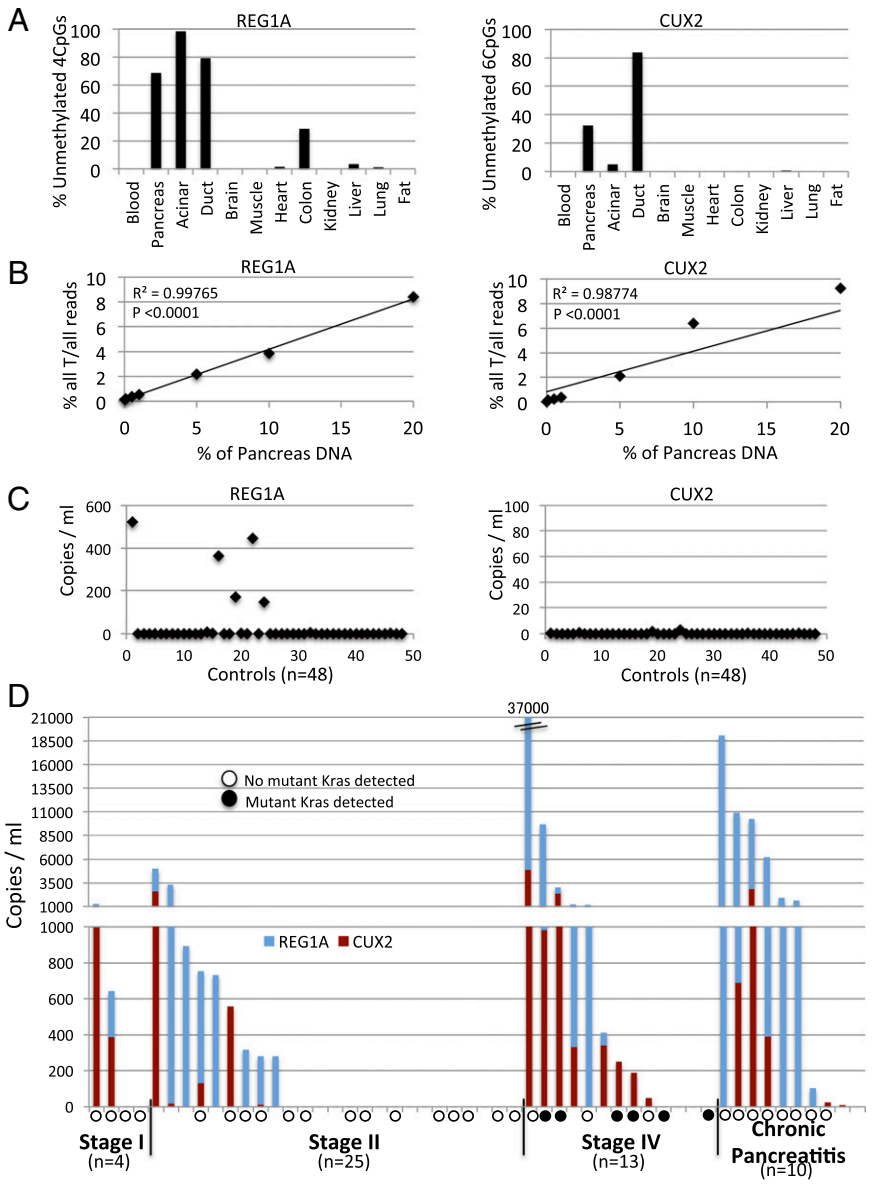

Fig. 4. Identification of exocrine pancreas-derived cfDNA in patients with pancreatic cancer or pancreatitis. (*A*) Methylation status of CpG clusters in the *CUX2* and *REG1A* loci in multiple tissues. *CUX2* appears to be unmethylated selectively in ducts, whereas *REG1A* is unmethylated in both ducts and acinar cells and also in ~30% of colon cells. (*B*) Spike-in experiments. Pancreas DNA was mixed with lymphocyte DNA, and unmethylated *REG1A* and *CUX2* were used to estimate the fraction of exocrine pancreas DNA in the mixtures. (*C*) Levels of unmethylated *CUX2* and *REG1A* DNA fragments in plasma or serum of healthy individuals, derived from the fraction of exocrine pancreas cfDNA (Table S1) and the concentration of cfDNA. (*D*) Levels of unmethylated exocrine pancreas markers in the circulation of patients with pancreatic cancer or chronic pancreatitis. The graph shows the intensity of the signal from each marker for each patient, after reducing the background (the highest signal seen among healthy controls: 520 and 2.9 copies/mL for *REG1A* and *CUX2* respectively; see *C*). Controls vs. all cancer patients, $P < 0.0001$; controls vs. localized cancer, $P < 0.0001$; controls vs. metastatic disease, $P < 0.0001$; localized vs. metastatic cancer, $P = 0.047$; controls vs. pancreatitis, $P < 0.0001$. Circles under the graph mark cfDNA samples that were tested for *KRAS* mutations. Filled circles indicate a mutation in codon 12 or 13 of *KRAS* was detected; empty circles indicate a *KRAS* mutation was not detected.

used sequencing to quantify codon 12/13 *KRAS* mutations in cfDNA of patients diagnosed with pancreatic cancer. We detected mutant *KRAS* in 22% of the samples examined ($n = 27$), all among patients with stage 4 disease; by contrast, 48% of samples showed a methylation signal. Overall, there was 59% agreement between the two tests (Fig. 4*D*). More work will be needed to compare the sensitivity of *KRAS* mutation detection in cfDNA and pancreas-derived cfDNA by methylation.

To test further the hypothesis that cell death leads to increases in tissue-specific cfDNA irrespective of etiology, we examined the serum of patients with chronic pancreatitis. Indeed, 7 of 10 patients had elevated pancreas-derived cfDNA levels (Fig. 4*D*). No mutant *KRAS* was found in the serum of patients with pancreatitis (Fig. 4*D*). We note that patients with pancreatitis had a clearer signal with a marker that was unmethylated in both acinar and ductal cells (regenerating islet-derived 1 alpha; *REG1A*), whereas patients with pancreatic cancer had a stronger signal with a marker that was preferentially unmethylated in ductal cells (cut-like homeobox 2; *CUX2*), perhaps reflecting the different epigenetic identities of dying cells in the two pathologies. In summary, cfDNA carrying the methylation patterns of the exocrine pancreas is present in the blood of patients with pancreatic cancer and pancreatitis, reflecting death of exocrine cells in these conditions.

Discussion

The dynamics of cell death in vivo remain largely inaccessible to noninvasive investigation and diagnosis. The assays presented herein rely on two well-established principles in biology, namely, that dying cells release cfDNA and that each tissue has a unique DNA methylation pattern. Combining these principles allowed us to identify the tissue origins of cfDNA and hence to assess the rate of cell death in tissues of interest. A unique feature of the method is the ability to detect cfDNA derived from tissues with normal genomes that are not accessible to mutation-based or genomic variant-based cfDNA analysis. As a proof of principle we assessed six tissue-specific methylation markers in >600 plasma/serum samples from >200 individuals and demonstrated the presence of tissue-specific cfDNA in patients with T1D (β-cell DNA), MS (oligodendrocyte DNA), brain damage caused by TBI or cardiac arrest (neuron or glia DNA), and pancreatic cancer or pancreatitis (exocrine pancreas DNA). In all cases, a considerable proportion of patients had tissue-specific cfDNA at levels far above baseline. This proof-of-principle study did not allow a full assessment of the diagnostic performance of our assay, but there is a reason to believe that measurements of cfDNA methylation-based cell death can contribute to clinical practice. In islet-graft recipients, β-cell cfDNA correlated well with acute levels of c-peptide, the standard assay to assess acute graft cell loss; in a chronic setting, cfDNA is expected to be more informative than c-peptide as a measure of β-cell death because c-peptide reflects a combination of legitimate insulin secretion and pathologic β-cell damage. In MS, currently available biomarkers include MRI to detect brain lesions and oligoclonal bands in the CSF, indicative of nonspecific neuroinflammation. Further studies will be required to compare glial cfDNA and these markers in terms of specificity, sensitivity, and ability to determine disease relapse or activity. In the context of brain damage, there is an unmet clinical need for biomarkers to diagnose concussions and to predict outcome in all forms of TBI. The results of our study encourage larger studies to determine the utility of this biomarker. In pancreatic cancer, there currently are no clinically validated biomarkers for early diagnosis. Although the detection of circulating tumor DNA can provide important information, it does not reveal the tissue of origin of the cancer. Our approach offers the ability to identify cell death and track tumor dynamics using universal tissue-specific markers without prior knowledge of patient-specific somatic mutations. This ability provides a tremendous advantage over existing methods. However, our method does not provide information about the pathology that led to cell death. We envision the combination of a screen for oncogenic mutations in cfDNA (which suggests the presence of a growing tumor but does not reveal its tissue of origin) with a search for the tissue origins of cfDNA in the same patients to detect the tissue source of mutant cfDNA and infer the presence of a tumor in a specific organ.

Previously published cfDNA assays that detect β-cell death in T1D were limited by high background signal in healthy control subjects (21, 22, 24, 25). We interpreted this signal as being caused by chance demethylation of CpGs in cfDNA from non–β-cell tissues. To address this issue, we turned to another well-described biologic principle: that in many situations, relevant methylation changes are regional, with the general pattern demonstrated by a number of adjacent CpGs providing a more robust biologic effect than any single CpG alone. By interrogating four to nine adjacent CpGs simultaneously (while keeping amplicons as short as possible, to allow amplification from cfDNA fragments), we showed that background signal can be reduced to nearly zero, thus greatly enhancing specificity (Figs. S8 and S9). At the same time, this strategy retains sufficient sensitivity to detect the presence of as little as 0.1–0.5% of DNA from the tissue of interest.

For β-cells the known uniqueness of insulin expression directed us and others to study the insulin-regulatory regions, but the selection of target loci for other cell types was not trivial. We took a genome-wide approach to identify marker genomic loci, and these provided excellent discrimination between the methylation patterns in white matter, brain cells, or exocrine pancreas as opposed to all other tissues tested. In general, the resolution of the assay should increase with the use of more methylation markers for each tissue tested.

Our current protocol searches for cfDNA derived from a specific tissue of interest based on one or more unique methylation marks. We present data on six markers in four different cell types, but the method can be used to identify cell-type–specific makers in any tissue of the body. While this paper was being prepared for submission, Sun et al. (35) reported a different approach for mapping the tissue origins of plasma DNA using genome-wide bisulfite sequencing. Although both approaches rely on tissue-specific methylation patterns, they differ substantially. The Sun study requires deep and expensive sequencing (currently >$1,000 per sample), which may not be practical for routine diagnostics; our approach is based on targeted sequencing of specific markers (~$10 per sample). In addition, the Sun study demonstrated the tissue origins of cfDNA in conditions in which the source tissue differs genetically from the host (pregnancy, transplantation, and cancer), whereas we show the origins of cfDNA—and infer cell death—in pathologies in which no alternative to methylation exists, e.g., β-cell death in diabetes and brain cell death in MS and head trauma.

In summary, we present a method for the detection of cell death in specific tissues, based on tissue-specific methylation patterns in circulating DNA. The approach may have multiple applications, including assessment of tissue damage after injury, evaluation of both targeted and off-target (toxicity) cell death in response to therapy, and early diagnosis of diseases such as T1D, neurodegenerative disease, and cancer. However, its use will require a deeper understanding of the rules that govern cfDNA dynamics and of the baseline distribution of tissue-specific cfDNA in different individuals across a variety of physiological conditions. In the long run, we envision a new type of blood test aimed at the sensitive detection of tissue damage without a priori suspicion of disease in a specific organ. We believe that such a tool will have broad utility in diagnostic medicine and in the study of human biology.

Materials and Methods

Patients. All clinical studies were approved by the relevant local ethics committees. The study in cardiac arrest patients was approved by the Ethics Committee at Uppsala University. The study in patients with TBI was approved by the Ethics Committee at University of Gothenburg, Sweden. Studies in patients with pancreatic cancer and pancreatitis were approved by the Ethics Committees of the Hebrew University-Hadassah Medical Center of Jerusalem, Sheba Medical Center, Israel; and the Department of Surgery, Philipps University of Marburg, Germany. The study in patients with T1D was approved by the Ethics Committee at the University of Florida. The study with islet-graft recipients was approved by the Ethics Committee at the University of Alberta, Canada. The study with MS and NMO patients was approved by the Ethics Committee at the Hadassah Medical Center, Jerusalem. Informed consent was obtained from all subjects or from their legal guardians before blood sampling. Fully de-identified samples were shipped to the Hebrew University Medical School for analysis. See *SI Materials and Methods* for detailed information about patient selection.

Biomarkers. Tissue-specific methylation biomarkers were selected after a comparison of extensive genome-wide DNA methylation datasets generated using Illumina Infinium HumanMethylation450k BeadChip array. See *SI Materials and Methods* for a detailed explanation of the datasets used and the method for selecting tissue-specific methylation markers. To detect mutant *KRAS* in plasma, bisulfite-treated cfDNA was PCR amplified using primers flanking codons 12 and 13 of the *KRAS* gene and specific for bisulfite-treated DNA. PCR products were sequenced on an Illumina MiSeq machine.

Sample Preparation and DNA Processing. Blood samples were collected in plasma-preparation tubes or serum-separator tubes and were centrifuged for 10 min at 3,320 × *g* within 2 h after collection. The experiments with T1D patients were performed using plasma. The MS, TBI, cardiac arrest, and pancreatitis studies were performed using serum because of the limited availability of archived material. Pancreatic ductal adenoma carcinoma samples were both serum and plasma, with no consistent differences detected between the sources. Control samples were matched to patient samples in volume and method of preparation. In general, we found that both plasma and serum allowed effective detection of methylation patterns in cfDNA; however we prefer the use of plasma given the reduced levels of DNA derived from lysed blood cells (36). Cell-free DNA was extracted from 0.2–1 mL of serum or plasma using the QIAquick kit (Qiagen) and was treated with bisulfite (Zymo Research). DNA concentration was measured using Qbit single-strand molecular probes (Invitrogen). Bisulfite-treated DNA was PCR amplified, using primers (*SI Materials and Methods*) specific for bisulfite-treated DNA but independent of methylation status at monitored CpG sites. Primers were bar-coded, allowing the mixing of samples from different individuals when sequencing products using MiSeq (Illumina). Sequencing was performed on PCR products using MiSeq Reagent Kit v2 (MiSeq, Illumina method). Sequenced reads were separated by barcode, aligned to the target sequence, and analyzed using custom scripts written and implemented in Matlab (Dataset S1). Reads were quality filtered based on Illumina quality scores. Reads were identified by having at least 80% similarity to target sequences and containing all the expected CpGs in the sequence. CpGs were considered methylated if "CG" was read and were considered unmethylated if "TG" was read. Efficiency of bisulfite conversion was assessed by analyzing the methylation of non-CpG cytosines.

Statistical Analysis. To assess the significance of differences between groups, we used a two-tailed Mann–Whitney test based on values of unmethylated tissue-specific DNA in each patient.

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