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

Roni Lehmann-Werman[1]*, Daniel Neiman[1]*, Hai Zemmour[1]*, Joshua Moss[1], Judith Magenheim[1], Adi Vaknin-Dembinsky[2], Kaj Blennow[3], Henrik Zetterberg[3,4], Kirsty Spalding[5], Desmond Schatz[6], Carla Greenbaum[7], Shmuel Ben-Sasson[1], Gideon Zamir[8], Aharon Razin[1], Howard Cedar[1], AM James Shapiro[9], Ben Glaser[10], Ruth Shemer[1], Yuval Dor[1]

[1]Department of Developmental Biology and Cancer Research, The Institute for Medical Research Israel-Canada, The Hebrew Uniersity-Hadassah Medical School, Jerusalem 91120, Israel

[3]Clinical Neurochemistry Laboratory, Institute of Neuroscience and Physiology, Department of Psychiatry and Neurochemistry, The Sahlgrenska Academy, University of Gothenburg, S-431 80 Mölndal, Sweden

[4]UCL Institute of Neurology, Queen Squar, London WC1N 3BG, United Kingdom

[5]Department of Cell and Molecular Biology, Karolinska Institute, Berzelius väg 35, Stockholm 171-77, Sweden

[6]Division of Endocrinology, University of Florida College of Medicine, Gainesville, FL, USA

[7]Benaroya Research Institute, Seattle, WA 98101

[9]Department of Surgery and the Clinical Islet Transplant Program, University of Alberta, Edmonton, Alberta, Canada

[2]Department of Neurology, [8]Department of Experimental Surgery, and [10]Endocrinology and Metabolism Service, Hadassah-Hebrew University Medical Center, Jerusalem 91120, Israel

* these authors contributed equally to this work

Correspondence: Yuval Dor, email yuvald@ekmd.huji.ac.il

Summary

Cell-free circulating DNA (cfDNA) released from dying cells is emerging as an important tool for the diagnosis of disease. Non-invasive prenatal testing (NIPT) detects fetal chromosomal aberrations using cfDNA in maternal blood, and information on somatic mutations in cancer can be used to monitor circulating tumor DNA as a marker of tumor dynamics. However, the utility of cfDNA is limited in conditions where injured tissues have a normal genome or mutations are not known. We present a method to identify the tissue origins of cfDNA, based on conserved tissue-specific methylation patterns that are retained in circulating DNA fragments. We used methylation markers of specific cell types to identify elevations in pancreatic beta-cell DNA in the circulation of recently diagnosed type 1 diabetes (T1D) patients and in islet graft recipients, brain DNA in the circulation of patients after traumatic brain injury or cardiac arrest, and oligodendrocyte DNA in the circulation of patients with multiple sclerosis during relapse. The approach offers a minimally-invasive window into the monitoring and diagnosis of human diseases involving cell death, as well as normal human tissue dynamics.

It has been known for decades that the plasma contains small fragments of cfDNA derived from dead cells (on average 5000 genome equivalents per ml) (1). While the mechanisms underlying the release and clearance of cfDNA remain obscure, the phenomenon is rapidly gaining applications. The recognition that fragments of fetal DNA travel briefly in maternal circulation has opened the way to next generation sequencing (NGS)-based prenatal testing to identify fetal trisomies and other genetic aberrations, potentially replacing amniocentesis (2-4). In cancer biology, tumors are known to release DNA (carrying tumor-specific somatic mutations) to the circulation, providing means for liquid biopsies to monitor tumor dynamics and evolution (5-7). cfDNA has also been used to detect graft cell death after kidney, liver or heart transplantation, based on single nucleotide polymorphisms (SNPs) distinguishing the DNA of donor from that of recipient (8-10). In all these cases, genetic differences exist between the DNA sequence of the tissue of interest (fetus, tumor or graft) and the host, providing the basis for highly specific assays.
The levels of cell-free circulating DNA are known to increase in multiple additional conditions such as traumatic brain injury (11), cardiovascular disease (12), sepsis (13) and intensive exercise (14). However in these cases, the source of elevated cfDNA is not known (for example, it could originate from parenchymal cells of the injured tissue but also from dying inflammatory cells), which greatly reduces the utility of cfDNA as a diagnostic tool.

Despite having an identical sequence, the DNA of each cell type in the body does carry unique epigenetic marks. In particular, DNA methylation is a fundamental aspect of tissue identity that is unique to each cell type, conserved among cells of the same type in the same individual and between individuals, and is highly stable under physiologic or pathologic conditions (15). Therefore, it should be possible to use the DNA methylation pattern of cfDNA to determine its tissue of origin and hence to infer cell death in the source tissue.
Theoretically, such an approach could identify the rates of cell death in a tissue of interest, taking into account the total amount of cfDNA, the fraction derived from a tissue of interest, and the estimated half life of cfDNA (15-120 minutes) (16, 17). Note that since the approach relies on normal, stable markers of cell identity, it cannot identify the nature of the pathology (e.g. distinguishing cfDNA derived from dead tumor cells or dead wild type cells due to trauma or inflammation in the same tissue).

One classic example of tissue-specific DNA methylation is provided by the insulin gene promoter, which is unmethylated in insulin-producing pancreatic beta-cells and methylated elsewhere. Recent studies have identified unmethylated insulin promoter DNA in the circulation of newly diagnosed T1D patients, reflective of autoimmune destruction of beta-cells, and in islet graft recipients (18-20). We sought to generalize the approach towards a proof of concept that tissue-specific methylation patterns in cfDNA can be used to detect tissue-specific cell death in multiple human pathologies.

Identification of tissue-specific methylation markers

We started by establishing tissue-specific DNA methylation markers, distinguishing individual tissues or cell types from other tissues. Particular attention was given to markers that differ between a tissue of interest and blood, which is thought to be the

main contributor of cfDNA and hence is a major potential source of noise in the system. We analyzed publicly available methylomes (mostly Illumina 450k array data from The Cancer Genome Atlas [TCGA]) to identify individual CpG dinucleotides with tissue-specific methylation patterns (i.e. unmethylated or methylated specifically in one tissue and methylated or unmethylated elsewhere) (see schematic of procedure, Supplemental figure S1).

The Illumina arrays provide information on the methylation status of individual CpG dinucleotides. The discriminatory power of each site is limited, since it can be randomly methylated or unmethylated in a small fraction of molecules from tissues where it is supposed to be methylated or unmethylated, respectively. For example, if a specific CpG site is methylated in 95% of beta-cells and 5% of blood cells, it will be difficult to identify beta-cell DNA embedded in blood DNA in a frequency smaller than 5%. To increase the signal to noise ratio of the assay we took advantage of the regional nature of DNA methylation. We defined an "expanded window" of 5-9 CpG sites adjacent to the original CpG marker site, reasoning that the chances are small for accidental methylation or demethylation of multiple adjacent cytosines in the same molecule. To determine the status of methylation of these expanded windows we obtained DNA from different human tissues, and treated with bisulfite to convert unmethylated cytosines to uracils. We then PCR-amplified short fragments containing the signature CpG site and multiple adjacent CpGs, and sequenced multiple molecules from the PCR product using Illumina MiSeq. As an alternative approach to the comparisons between Illumina methylome arrays, in some cases we selected and validated tissue specific markers based on promoters of tissue specific genes (which might have been absent from the Illumina arrays) (Supplemental Figure 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 dramatically higher 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 6-9 CpG sites, whose combined methylation status is typical to a tissue of interest relative to blood and other tissues.

Unmethylated insulin gene promoter in the circulation of T1D patients

To detect cfDNA derived from beta-cells, we used the insulin gene promoter as a beta-cell specific methylation marker. Previous studies have performed methylation-specific PCR, based on the methylation status of 2 CpG dinucleotides in the insulin promoter, to identify DNA derived from beta-cells (19). However, the insulin promoter contains additional CpG sites in the same area, which theoretically can be used to better distinguish DNA of beta-cells and other tissues (Figure 1A). To test this idea, we amplified a 160bp fragment of the insulin gene 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 Figure 1B, each CpG was unmethylated in 90-95% of the DNA molecules from human beta-cells, and in 5-15% of the DNA molecules from other tissues. However when we combined this information and calculated the fraction of DNA molecules in which all 6 CpG sites were unmethylated, the difference between beta cells and all other tissues became dramatically larger: while ~80% of DNA molecules from beta-cell were fully unmethylated, <0.01% of the molecules from any other tissue were fully

unmethylated. Thus a stretch of 6 adjacent unmethylated CpG sites in the insulin gene promoter distinguishes beta-cells from other tissues with a signal to noise ratio close to 10,000:1. We next used this information to look for beta-cell derived DNA in the circulation of T1D patients. Plasma DNA from patients was treated with bisulfite, PCR amplified and sequenced to determine the fraction of molecules containing fully unmethylated insulin promoter DNA. The fraction obtained was multiplied by the concentration of cfDNA measured in each sample, to obtain a value of beta-cell derived DNA (ng/ml) circulating in the blood of each patient (Supplemental Figure S1).

The cfDNA of healthy volunteers had an extremely low frequency of fully unmethylated insulin gene promoter molecules (<xxx% of circulating insulin promoter fragments). When multiplied by the total amount of cfDNA in each individual (Supplemental Figure S1), this suggested <0.01 ng/ml circulating DNA derived from beta-cells, consistent with a low background from non beta-cells, and a very low rate of beta-cell turnover in healthy adults (Figure 1C). T1D patients, sampled multiple times between 3 and 18 months after diagnosis, showed a clear signal of unmethylated insulin promoter DNA in cfDNA, indicative of ongoing autoimmune destruction of beta-cells (Figure 1D). Not surprisingly, the levels of beta-cell-derived DNA greatly varied between patients, although we could not relate this variation to the levels of c-peptide or other recorded clinical parameters. Interestingly, while all patients showed evidence of abnormally high unmethylated insulin promoter DNA in the circulation, all had multiple measurements that were completely negative (that is, no unmethylated insulin promoter cfDNA above healthy baseline). The reason for the observed fluctuations of unmethyalted insulin in cfDNA remains to be elucidated. A fascinating possibility is that this is a reflection of the hypothesized remitting / relapsing nature of T1D (21). One feature of the data that did correlate with the clinical course of disease was that in several patients, the signal of unmethylated insulin appeared to decline eventually to baseline levels, likely reflecting loss of the vast majority of beta-cells. Note that at this time, all patients still had measurable c-peptide levels, indicative of remaining functional beta-cells. It will be important to determine if absence of beta-cell-derived cfDNA in c-peptide+ patients reflects tolerance of surviving beta-cells to immune destruction, or continued beta-cell killing at levels below the sensitivity limits of the assay.

We also examined the circulation of long-time T1D patients that were transplanted with cadaveric allogeneic islets and treated with immune suppressants (22). As shown in Figure 1E, the plasma of all patients had a high signal (unmethylated insulin DNA) 1-2 hours after transplantation, which dramatically declined in the hours and days that followed. The extensive loss of grafted beta-cells immediately after transplantation, potentially resulting from acute ischemia, is consistent with a previous imaging study of a transplanted patient (23). Note that in most patients, the signals detected at 7 days and even 1 month after transplantation were still clearly above background, suggesting continuous, low level killing of beta-cell despite immune suppression.

To test if the combined methylation pattern of multiple CpG sites at the insulin gene promoter was necessary to detect beta-cell derived DNA in circulation, we examined the methylation status of each individual CpG in the plasma of healthy individuals and recently diagnosed T1D patients. Each individual CpG did not have a different pattern in the plasma of healthy controls and T1D patients (unmethylated in ~15% of cfDNA molecules). However combining the 6 CpG sites gave a clear signal in the plasma of T1D patients that was absent in healthy controls (Supplemental Figure S2).

These results support the utility of the NGS-based method for the detection of cfDNA derived from specific tissues. Specifically for T1D, they raise interesting questions about the dynamics of the disease which will be addressed elsewhere.

Identification of oligodendrocyte-derived cfDNA in Multiple Sclerosis

Non-invasive detection of brain cell death is particularly challenging. In theory, 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), autoimmune diseases in which myelin-producing oligodendrocytes in the white matter are destroyed. We have analysed the published methylome of normal human white matter (24), and identified clusters of adjacent CpG sites in the promoter of Myelin Basic Protein 3 (*MBP3*) and around an un-annotated locus (CG10809560 in the Illumina array, which we termed WM1 for White Matter 1) which were unmethylated selectively in oligodendrocytes (Figure 2A). As with the insulin gene promoter, individual CpGs in these clusters showed a moderate signal to noise ratio: they were unmethylated in 60-85% of DNA molecules derived from sources rich for oligodendrocytes (glial preps, white matter and whole brain), and in 2-20% of DNA from other tissues (Supplemental Figures S3, S4). Combining all CpGs at the MBP3 and WM1 loci greatly increased the difference between DNA enriched for oligodendrocytes and DNA from other sources including blood (Supplemental Figures S3, S4). Thus DNA from the MBP3 or WM1 loci unmethylated in all adjacent CpG sites can serve as a marker of oligodendrocytes. Healthy individuals (n=12) had negligible levels of unmethylated MBP3 or WM1 in plasma, suggesting minimal basal turnover of oligodendrocytes (Figure 2B). Most remitting MS or NMO patients (n=11) had no or very low signal. However most patients during a relapse (documented using MRI close to the time of sampling, n=21) had in their plasma unmethylated DNA of either MBP3, WM1 or both (Figure 2B). This observation is consistent with the idea that short-lived circulating unmethylated MBP3 or WM1 DNA reflects acute oligodendrocyte cell death. Initial analysis did not reveal clinical correlates to the lack of signal in some relapsing patients.
These results indicate that acute autoimmune destruction of oligodendrocytes can be detected as increased circulating levels of fully unmethylated DNA fragment from the MBP3 or WM1 loci. Additional methylation markers of oligodendrocyte can be developed to further increase the specificity and sensitivity of the assay.

Identification of brain-derived cfDNA after acute brain damage

To obtain a more general marker for brain injury, we scanned the Illumina arrays for loci whose methylation status distinguished brain DNA from other tissues. A cluster of 9 CpG sites around locus CG0978 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 of peripheral neurons present in these tissues). Importantly, <0.03% of molecules in blood were unmethylated, providing a >2000 fold difference in methylation between brain and blood (Figure 3A and Supplemental Figure S5).
Healthy individuals had extremely low levels of fully unmethylated GC0978 in the plasma (Figure 3B). This low baseline may reflect either neuronal turnover below our sensitivity limit or an alternate mechanism of clearance of DNA from dead brain cells. We then examined plasma samples from patients in two settings of brain

damage, both known to involve neuronal injury in combination with disruption of the blood-brain barrier. Strikingly, patients (n=10) sampled at multiple time points after cardiac arrest with documented ischemic brain damage (25) showed high levels of unmethylated CG0978 in plasma (Figure 3C). Similarly, patients (n=5) hospitalized in a neurotrauma unit after suffering head injuries had elevated unmethylated CG0978 in plasma (Figure 3D). Both sets of results are consistent with circulating DNA fragments derived from dead brain cells (neurons and/or glia) in these patients. The amount and temporal patterns of brain-derived cfDNA varied between patients. In the group with cardiac arrest, the strongest signals were observed in the first time point, shortly after resuscitation, with a decline in subsequent days in most patients. In the group of patients with head trauma, a more delayed pattern of brain-derived cfDNA was observed.

The absolute levels of cfDNA were elevated to a similar extent in both groups of patients compared with healthy controls, consistent with previous reports on cfDNA after traumatic brain injury (11) (Supplemental Figure S6). Interestingly, the levels of brain-derived cfDNA were much higher in patients with cardiac arrest, suggesting that the trauma patients had other, non-brain sources of extensive cell death (supplemental Figure S6).

In summary, brain-specific DNA as well as oligodendrocyte-specific DNA can be identified in the circulation of patients with neuroinflammatory, traumatic and ischemic brain pathologies, based on unique methylation markers. More extensive studies are underway to identify correlations between brain-specific cfDNA and clinical observations.

Discussion

The process of cell death in vivo remains largely inaccessible to non-invasive investigation and diagnosis. The approach proposed here relies 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 allows 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 analysis of cfDNA. As a proof of principle we demonstrated the presence of tissue-specific cfDNA in patients with T1D (beta-cell DNA), brain damage due to head injury or cardiac arrest (neuron/glia DNA), and multiple sclerosis (oligodendrocyte DNA). In all cases, a proportion of patients showed tissue-specific cfDNA at levels far above baseline. It remains to be seen why the measured levels of tissue-specific DNA in each pathology fluctuate so dramatically in all patients examined. This pattern may represent unrecognized oscillatory dynamics of cell death (e.g. fluctuations of autoimmune destruction of cells in T1D and MS), or alternatively a variation in the process by which DNA is released to, or cleared from the circulation. While the phenomenon calls for in depth investigation to understand the underlying biology, for practical purposes it may mean that cfDNA-based diagnosis will have to rely on samples obtained at multiple time points for each patient.

Our current protocol searches for cfDNA derived from a specific tissue of interest based on one or few methylation markers of this tissue. This approach may have multiple applications, for example in the assessment of tissue damage after injury, in

the evaluation of cell death in response to therapy, and in the early diagnosis of diseases. 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. While such a tool can find a broad utility in diagnostic medicine, it will require first a deeper understanding of the rules that govern cfDNA dynamics, and baseline distribution of tissue-specific cfDNA in many individuals over many physiological conditions.

Acknowledgements

Supported by grants from the Juvenile Diabetes Research Foundation, JDRF, Beta cell biology consortium, and the Sir Zalman Cowen Universities Fund.

References

1. Schwarzenbach H, Hoon DS, & Pantel K (2011) Cell-free nucleic acids as biomarkers in cancer patients. *Nature reviews. Cancer* 11(6):426-437.
2. Bianchi DW*, et al.* (2014) DNA sequencing versus standard prenatal aneuploidy screening. *The New England journal of medicine* 370(9):799-808.
3. Lo YM*, et al.* (1997) Presence of fetal DNA in maternal plasma and serum. *Lancet* 350(9076):485-487.
4. Fan HC*, et al.* (2012) Non-invasive prenatal measurement of the fetal genome. *Nature* 487(7407):320-324.
5. Diaz LA, Jr. & Bardelli A (2014) Liquid biopsies: genotyping circulating tumor DNA. *J Clin Oncol* 32(6):579-586.
6. Dawson SJ*, et al.* (2013) Analysis of circulating tumor DNA to monitor metastatic breast cancer. *The New England journal of medicine* 368(13):1199-1209.
7. Murtaza M*, et al.* (2013) Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. *Nature* 497(7447):108-112.
8. Snyder TM, Khush KK, Valantine HA, & Quake SR (2011) Universal noninvasive detection of solid organ transplant rejection. *Proc Natl Acad Sci U S A* 108(15):6229-6234.
9. Beck J*, et al.* (2013) Digital droplet PCR for rapid quantification of donor DNA in the circulation of transplant recipients as a potential universal biomarker of graft injury. *Clinical chemistry* 59(12):1732-1741.
10. Sigdel TK*, et al.* (2013) A rapid noninvasive assay for the detection of renal transplant injury. *Transplantation* 96(1):97-101.
11. Campello Yurgel V*, et al.* (2007) Role of plasma DNA as a predictive marker of fatal outcome following severe head injury in males. *Journal of neurotrauma* 24(7):1172-1181.
12. Antonatos D, Patsilinakos S, Spanodimos S, Korkonikitas P, & Tsigas D (2006) Cell-free DNA levels as a prognostic marker in acute myocardial infarction. *Ann N Y Acad Sci* 1075:278-281.
13. Dwivedi DJ*, et al.* (2012) Prognostic utility and characterization of cell-free DNA in patients with severe sepsis. *Critical care* 16(4):R151.

14. Breitbach S, Tug S, & Simon P (2012) Circulating cell-free DNA: an up-coming molecular marker in exercise physiology. *Sports medicine* 42(7):565-586.
15. Bergman Y & Cedar H (2013) DNA methylation dynamics in health and disease. *Nat Struct Mol Biol* 20(3):274-281.
16. Diehl F*, et al.* (2008) Circulating mutant DNA to assess tumor dynamics. *Nat Med* 14(9):985-990.
17. Lo YM*, et al.* (1999) Rapid clearance of fetal DNA from maternal plasma. *Am J Hum Genet* 64(1):218-224.
18. Akirav EM*, et al.* (2011) Detection of beta cell death in diabetes using differentially methylated circulating DNA. *Proceedings of the National Academy of Sciences of the United States of America* 108(47):19018-19023.
19. Lebastchi J*, et al.* (2013) Immune Therapy and beta-Cell Death in Type 1 Diabetes. *Diabetes* 62(5):1676-1680.
20. Husseiny MI, Kaye A, Zebadua E, Kandeel F, & Ferreri K (2014) Tissue-specific methylation of human insulin gene and PCR assay for monitoring Beta cell death. *PloS one* 9(4):e94591.
21. von Herrath M, Sanda S, & Herold K (2007) Type 1 diabetes as a relapsing-remitting disease? *Nature reviews. Immunology* 7(12):988-994.
22. Shapiro AM*, et al.* (2006) International trial of the Edmonton protocol for islet transplantation. *N Engl J Med* 355(13):1318-1330.
23. Eich T, Eriksson O, Lundgren T, & Nordic Network for Clinical Islet T (2007) Visualization of early engraftment in clinical islet transplantation by positron-emission tomography. *N Engl J Med* 356(26):2754-2755.
24. Huynh JL*, et al.* (2014) Epigenome-wide differences in pathology-free regions of multiple sclerosis-affected brains. *Nat Neurosci* 17(1):121-130.
25. Randall J*, et al.* (2013) Tau proteins in serum predict neurological outcome after hypoxic brain injury from cardiac arrest: results of a pilot study. *Resuscitation* 84(3):351-356.

Legends to figures

Figure 1: beta cell-derived DNA in the circulation of T1D diabetes patients.
A, structure of the insulin gene promoter fragment used as marker. Black dots represent CpG sites, arrows mark positions of PCR primers.
B, methylation status of individual CpG sites in the insulin gene promoter in multiple tissues. Graph shows the percentage of unmethylated molecules in DNA from each tissue. Right columns describe the percentage of molecules in which all 6 CpG sites are unmethylated.
C, beta-cell derived DNA in the plasma of healthy volunteers. The fraction of fully unmethylated insulin promoter DNA molecules (reflective of the fraction of beta-cell derived DNA) was multiplied by the absolute level of cfDNA measured in each individual.
D, beta-cell derived DNA in the plasma of T1D patients, sampled at multiple time points after diagnosis. X axis, months from diagnosis; left Y axis (blue lines), concentration of beta-cell derived DNA in plasma (ng/ml); right Y axis (red), c-peptide levels.
E, beta-cell derived DNA in the circulation of long-time T1D patients sampled at the indicated time points after islet transplantation.

Figure 2: identification of oligodendrocyte-derived cfDNA in multiple sclerosis
A, methylation status of MBP1 and WM1 in multiple tissues. Note lack of methylation in total brain but not in neuron-rich cerebellum, suggesting that unmethylated molecules derived from glia.
B, oligodendrocyte-derived DNA in the plasma of healthy volunteers, remitting and relapsing MS/NMO patients.

Figure 3: identification of brain-derived cfDNA after brain damage.
A, methylation status of CpG sites at the CG0978 locus in multiple tissues, as determined by deep sequencing. Bars represent the percentage of molecules in which all 9 CpGs of the locus are unmethylated.
B, brain-derived DNA in the plasma of healthy volunteers, calculated by multiplying the fraction of fully unmethylated CG0978 by the amount of cfDNA in each individual.
C, brain-derived DNA in the plasma of patients after cardiac arrest. Each patient was sampled immediately after resuscitation ("acute") and at different time points later.
D, brain-derived DNA in the plasma of patients after traumatic brain injury, sampled at different days after admission to a neurotrauma unit.

Supplemental material

Materials and methods

Patients

Recently diagnosed T1D patients:

Islet graft recipients: James

MS/NMO patients: Adi.

Cardiac arrest patients: The cardiac arrest samples were collected at the intensive care unit (ICU) at Uppsala University Hospital, Sweden. Unconscious patients with cardiac arrest were resuscitated with restoration of spontaneous circulation (ROSC). Hypothermia treatment to a body temperature of 32–34 ◦C for 24 h, ventilation, and pharmacologic support were administered immediately after resuscitation as described (Mörtberg E et al., Resuscitation 2011;82:26–31). Patients were defined as comatose if they were (i) not awake, (ii) not following any commands, and (iii) not responding to any stimuli. All patients received an arterial line in the radial or femoral artery for blood sampling. Serial blood samples were collected, starting as soon as possible in the emergency phase and continuing at 24, 48,72, 96 and 108 hours after cardiac arrest. Serum aliquots were frozen at −70◦C until analysis. The study was approved by the Ethics Committee at Uppsala University; informed consent was obtained from the closest relative.

Traumatic brain injury: the 5 patients with traumatic brain injury (TBI) were enrolled from a clinical study at the Neurointensive Care Unit (NICU) at the Sahlgrenska University Hospital, Gothenburg, Sweden. All patients had severe TBI according to the following criteria: 1) Reaction Level Scale (RLS) 4, corresponding to a score of 8 on the Glasgow Coma Scale (1); 2) were in need on ventilator treatment and; 3) monitoring of intracranial pressure (ICP). Venous blood samples were taken on serial days after trauma. Patients were treated following a standardized scheme, ‘‘the Lund concept’’, with the aim to maintain cerebral perfusion pressure above 60 mm Hg and the intracranial pressure of below 20 mm Hg (2). The study was approved by the Ethics Committee at University of Gothenburg; informed consent was obtained from the closest relative.

DNA processing

Cell free DNA was isolated using a kit (Qiaquick, Qiagen), and treated with bisulfite (kit details). Bisulfite treated was PCR amplified, using primers specific for bisulfite-treated DNA but independent of methylation status. Primers were bar-coded, allowing to mix samples from different individuals when sequencing products using MiSeq (Illumina).

Supplemental references

1. Starmark J-E, Stalhammar D, Holmgren E. The Reaction Level Scale (RLS 85). Manual and guidelines. Acta Neurochir (Wien) 1988;91:12– 20.

2. Wahlström MR, Olivecrona M, Koskinen LO, Rydenhag B, Naredi S.Severe traumatic brain injury in pediatric patients: treatment and outcome using an intracranial pressure targeted therapy—the Lund concept. Intensive Care Med 2005;31:832– 9.

Legend to supplemental figures

Figure S1: flow chart of method to detect circulating DNA derived from a specific tissue.
A, procedure to identify tissue-specific methylation markers.
B, procedure to determine levels of tissue-specific DNA in plasma.

Figure S2: methylation of the insulin gene promoter in the plasma of healthy volunteers and recently diagnosed T1D patients.
A, methylation status of individual CpG sites
B, methylation status of an expanded window of 4-6 CpGs

Figure S3: methylation of the MBP3 gene promoter.
A, methylation status of the individual CpG site at the MBP3 gene promoter that is captured in the Illumina 450k array. Data from publicly available 450k arrays.
B, methylation status of individual CpG sites and expanded window of multiple CpGs from the MBP3 gene promoter, in multiple tissues, as determined by deep sequencing.
C, methylation of individual CpG sites from the MBP3 gene promoter in the plasma of healthy controls and relapsing MS/NMO patients.
D, fraction of fully unmethylated MBP3 promoter fragment in the plasma of healthy volunteers and MS/NMO patients.

Figure S4: methylation of CG10809560 and adjacent CpG sites (the WM1 locus).
A, methylation status of CG10809560 in multiple tissues as recorded in publicly available Illumina 450k arrays.
B, methylation status of individual CpG sites and expanded window of multiple CpGs from the WM1 locus, in multiple tissues, as determined by deep sequencing.
C, methylation of individual CpG sites from the WM1 locus in the plasma of healthy controls and relapsing MS/NMO patients.
D, fraction of fully unmethylated WM1 DNA fragments in the plasma of healthy volunteers and MS/NMO patients.

Figure S5: methylation of CG0978 and adjacent CpG sites.
A, methylation status of CG0978 in multiple tissues as recorded in publicly available Illumina 450k arrays.
B, methylation status of individual CpG sites and expanded window of multiple CpGs from the CG0978 locus, in multiple tissues, as determined by deep sequencing.
C, methylation of individual CpG sites from the CG0978 locus in the plasma of healthy controls and patients after cardiac arrest.
D, fraction of fully unmethylated CG0978 DNA fragments in the plasma of healthy volunteers and patients after cardiac arrest.

Figure S6: comparison of total cfDNA and brain-derived cfDNA in the plasma of healthy volunteers, and patients after cardiac arrest or traumatic brain injury.

Additional supplemental figures / tables:

Must have (?):
Clinical data on patients (recently diagnosed T1D patients, islet graft recipients, MS/NMO patients, cardiac arrest, TBI patients.

Hopefully:
Methylation of wrong markers as control.

- unmethylated insulin promoter DNA in plasma of patients with brain pathologies
- unmethylated MBP3 in plasma of patients with recently diagnosed type 1 diabetes
- unmethylated CG0978 in plasma of patients with recently diagnosed type 1 diabetes and MS/NMO patients.