Clinical Epigenetics

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In-utero exposure to maternal diabetes and DNA methylation alterations in the Next Generation birth cohort

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Abstract

Introduction The incidence of type 2 diabetes (T2D) in youth is increasing and *in-utero* exposure to maternal diabetes is a known risk factor, with higher risk associated with pregestational T2D exposure compared to gestational diabetes mellitus (GDM) exposure. We hypothesize this differential risk is reflected in DNA methylation (DNAm) changes induced by differential timing of *in-utero* exposure to maternal diabetes, and that exposure to diabetes throughout pregnancy (T2D) compared to exposure later in development (GDM), induces different DNAm signatures and different T2D risk to offspring. This study presents an epigenome-wide investigation of DNAm alterations associated with *in-utero* exposure to either maternal pregestational T2D or GDM, to determine if the timing of prenatal diabetes exposure differentially alters DNAm.

Methods We performed an epigenome-wide analysis on cord blood from 99 newborns exposed to pregestational T2D, 70 newborns exposed to GDM, and 41 unexposed to diabetes *in-utero* from the Next Generation birth cohort. Associations were tested using multiple linear regression models while adjusting for sex, maternal age, BMI, smoking status, gestational age, cord blood cell type proportions and batch effects.

Results We identified 27 differentially methylated sites associated with exposure to GDM, 27 sites associated with exposure to T2D, and 9 common sites associated with exposure to either GDM or T2D (adjusted p value < 0.05 and effect size estimate > 0.01). One site at *CLDN15* and two unannotated sites were previously reported as associated with obesity. We also identified 87 differentially methylated regions (DMRs) associated with *in-utero* exposure to GDM and 69 DMRs associated with *in-utero* exposure to T2D. We identified 23 DMR sites that were previously associated with obesity, three with T2D and five with *in-utero* exposure to GDM. Furthermore, we identified six CpG sites in the *PTPRN2* gene, a gene previously associated with DNAm differences in blood of youth with T2D from the same population.

Conclusion Our findings support that *in-utero* exposure to maternal diabetes is associated with DNAm alterations in offspring. Moreover, the timing of maternal diabetes *in-utero* exposure (GDM or T2D) produces overlapping but distinct DNAm patterns, suggesting that the window of exposure to maternal diabetes produces different molecular modifications and may reflect, at least in part, the difference in risk for youth-onset T2D in offspring. We also identified

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sites in this study that have been previously associated with T2D or obesity, which may serve as potential early-life biomarkers of exposure and/or risk, warranting further investigation in longitudinal studies.

Keywords Diabetes in youth, DNA methylation, Type 2 diabetes, Epigenetics, In utero programming

Introduction

Youth type 2 diabetes (T2D) is increasing worldwide and is recognized as a public health concern [1]. It is associated with early-onset renal failure and other complications, so identifying risk factors for the development of youth-onset T2D early in life is a priority to inform prevention strategies [2, 3]. *In-utero* exposure to maternal diabetes is one known risk factor for youth-onset T2D, obesity and metabolic diseases in offspring, and aligns with theories of developmental programming, where the early-life period is especially sensitive to long-lasting impacts of exposures [4–9].

Maternal diabetes during pregnancy can be due to pre-existing diabetes (T1D or T2D), where fetal exposure occurs throughout pregnancy, or gestational diabetes mellitus (GDM), a disease of mid to late pregnancy, with fetal exposure in the second or third trimester [10]. *In-utero* exposure to maternal diabetes has previously been associated with offspring's increased risk of obesity [9], cardiovascular diseases [11] and childhood glucose and insulin resistance [12]. A retrospective study found that *in-utero* exposure to T2D conferred a greater risk of developing T2D later in life compared to *in-utero* exposure to GDM [8].

Between 2006 and 2008 in Canada, there were an average of 1.54 cases of youth T2D per 100,000 per year, with the highest incidence of 12.45 cases per 100,000 children per year in the province of Manitoba [13]. Recent reports from Manitoba showed increased incidence and prevalence of T2D in children among First Nation and all other Manitoban children [14]. T2D disproportionally affects First Nation children (1–17 years of age) [1]. In 2017–18, the incidence rate of youth T2D was 10.7 per 100,000 children among non-First Nation Manitoban children, while the incidence was 124.2 per 100,000 among First Nation Manitoban children, making the prevalence of T2D among First Nation in Manitoba among the highest in the world [15]. Given this exceptionally high burden of early-onset T2D among First Nation children in Manitoba, it is critical to explore the underlying biological mechanisms that may contribute to this disparity.

Epigenetic marks are known to reflect prenatal and early-life environmental exposures, and in some cases can persist long enough to be linked to health outcomes [16]. DNA methylation (DNAm) is the best-studied epigenetic modification in an environmental context and is characterized by the addition of a methyl group to the 5' position of a cytosine, mainly located next to a guanine, referred to as a CpG site [17]. DNAm is a mitotically heritable modification that regulates gene expression and accessibility [18], where typically high DNAm at a promoter is associated with transcriptional silencing [18, 19]. Many environmental exposures have been shown to alter DNAm at specific genes and genome-wide, and some studies have proposed that the establishment of differential DNAm also depends on the developmental timing of the exposure. For example, *in-utero* exposure to air pollution was associated with 284 changes in DNAm with 233/284 changes (~82%) associated with exposure during gestational weeks 2–4 while there were no changes in DNAm patterns when the *in-utero* exposure started in the second trimester [20].

Several studies have demonstrated that *in-utero* exposure to maternal diabetes is associated with DNAm alterations in offspring, which is hypothesized to contribute to later-life metabolic risk [21–24]. Some studies have even shown that these DNAm alterations can persist after birth. A recent longitudinal study found that DNAm at a CpG site near *FSD1L* remained associated with maternal gestational diabetes up to 5 years of age [25]. Another study found that DNAm changes associated with *in-utero* exposure to maternal Type 2 diabetes can persist after birth up to the age of 13 years, and are associated with genes in glucose and lipid metabolism [26]. However, the exact molecular mechanisms that link *in-utero* exposure to maternal diabetes and offspring T2D later in life is not clear, nor is whether the timing of exposure in early development due to T1D or T2D results in the same alterations as a later exposure to GDM. Understanding the mechanisms behind youth-onset T2D is crucial to developing successful prevention and management strategies.

This study aims to compare DNAm signatures between *in-utero* exposure to GDM and pre-existing T2D. Here, we identified DNAm changes in the cord blood of the Next Generation (NextGen) birth cohort, composed of offspring born to Indigenous mothers with GDM, T2D or no diabetes, using an epigenome-wide approach. To ensure that we are interrogating environmental and not population-based genetic factors, we tested for effects of the *HNF1a* G319S polymorphism, which has been associated with increased risk of early onset T2D in the Anisininew First Nations populations [27, 28]. By delineating how timing of exposure to maternal diabetes results

in different molecular signatures, our study aimed to contribute to our understanding of how *in-utero* exposure to maternal diabetes alters risk of developing T2D later in life, with the long-term goal of aiding in developing effective early-life screening and prevention strategies.

Materials and methods

NextGen cohort

The Next Generation birth cohort is composed of children born to Indigenous mothers with T2D, GDM, or no diabetes. Pregnant self-declared First Nations and Metis women were recruited either as graduates of the provincial pediatric type 2 diabetes program, through adult diabetes clinics, or through prenatal clinics [29–31]. Women with type 1 diabetes, not residing in Manitoba or not planning to deliver in Manitoba were excluded from the cohort. Ethics approval was provided by the University of Manitoba ethics board (HS16516), and informed consent was obtained prior to enrollment. Cord blood samples were collected in a 4 ml EDTA tube at the time of delivery. Maternal cell contamination was detected by analyzing microsatellite markers unrelated to diabetes, but no samples were removed due to contamination. Genomic DNA was extracted using FlexiGene DNA Extraction Kit (Autogen, Holliston, MA). *HNF1a* was genotyped as previously reported [29].

Means and standard deviation were calculated for all the cohort characteristics. We used Fisher exact tests (categorical variables) or t-test (continuous variables) to compare the general characteristics of the cohort. Due to the small number of missing observations and the relatively low variability in these variables, mean imputation was used to replace nine and five missing values for birthweight and gestational age, respectively. This approach allowed us to retain the full analytical sample without introducing substantial bias.

DNAm analysis

Genome-wide DNAm was measured from 214 cord blood samples using the Infinium MethylationEPIC BeadChip kit (Illumina; San Diego, CA). First, extracted genomic DNA was bisulfite converted using EZ DNA Methylation Gold Kit (Zymo Research; Irvine, CA). The converted DNA was then fragmented and hybridized to the beadchips as per the manufacturer's instructions. After the beadchips were stained and coated, they were scanned using the iScan system (Illumina).

DNAm methylation pre-processing

Data were preprocessed using the R package *minfi* [32]. 150 samples were processed on EPIC v1 chips and 64 samples were processed on EPIC v2 chips. Red and green channel intensity values were extracted from IDAT files for all the samples, comprising 1,051,943 probes from EPIC v1 chips and 1,105,209 probes from EPIC v2 chips. First, a detection *p* value was calculated by comparing the total signal for each probe to the background. No samples were removed by mean probe detection *p* value > 0.05 from the 150 EPIC v1 samples and four samples were removed from the 64 EPIC v2 samples. We modified the *minfi convertArrays()* function—originally developed for converting Illumina 450 k arrays to EPIC v1 format —to convert EPIC v2 RG Channels to EPIC v1 RG Channels. Briefly, we removed probes that do not have the same color in EPIC v1, translated probe addresses, matched probe IDs and removed probes that do not overlap with EPIC v1. The raw combined methylation data of the 150 EPIC v1 samples (866,238 CpGs) were intersected with the 60 converted EPIC v2 samples (703,478 CpGs) using *minfi combineArrays()* function, which combined all of the 210 samples in one RGset (702,950 CpGs overlapped). Probe intensity levels (702,950 CpGs) were normalized for background signal with normal-exponential out-of-band normalization using *preprocessNoob* from *minfi*. Detection *p* values were calculated for each CpG site, and 11,725 CpGs with detection *p* value > 0.01 were removed. Next, 58,229 probes with < 3 bead counts, 13,961 probes localized to sex chromosomes, 1,595 probes with a known single nucleotide polymorphism effect, and 3,043 probes with cross-reactive properties [33–35] were removed, leaving 614,397 probes for downstream analysis. Probe bias correction was accomplished using *BMIQ* from the *watermelon* R package [36, 37]. β values were calculated as described previously [38, 39] and were used to examine DNAm changes.

Cell-type deconvolution

Proportions of 7 cord blood cell types (CD4 T-cells, CD8 T-cells, B-cells, NK cells, monocytes, granulocytes and nucleated red blood cells; Supplementary Fig. 1) from each participant were estimated using *ExperimentHub* [40] and *FlowSorted.CordBlood.EPIC* R packages, and *estimateCellCount2* function using "any" as a probe selection method. Since estimated cell-type proportions are compositional—constrained to the sum of one and inherently correlated—directly including them in regression models can lead to multicollinearity and instability [41]. To address this, we applied an isometric log-ratio transformation to the cell-type proportions using the *robCompositions* R package [42], and then extracted robust principal components (PCs). Standard practice is that PCs accounting for at least 90% of the variance in cell compositions should be included, and in our case the first four PCs accounted for ~ 92% of the variance due to cell type proportions and were included in the linear regression model.

Epigenome-wide analysis

To identify differentially methylated CpG sites that were associated with *in-utero* exposure to GDM or T2D, we performed multivariable linear regressions on DNAm β-values using *limma* [43]. Maternal diabetes status (control, GDM or T2D) was used as the main variable with adjustment for maternal age, BMI before delivery, smoking status, gestational age, birthweight, infant sex, four cell-type PCs, and batch effects (BeadChip number and BeadChip position). One sample with missing smoking status was excluded from the adjusted models. Since we are specifically interested in changes due to *in-utero* exposure to GDM or T2D compared to control, we used *lmFit()* function for model fitting, a contrast matrix in *limma* to specify GDM/control and T2D/control comparisons and *eBayes()* function for empirical Bayes adjustment. Adjusted *p* values were calculated using Benjamini–Hochberg approach [44] with significance set at FDR < 0.05. We used two log fold-change (logFC) cutoffs: a smaller and thus less stringent 0.01, a commonly used minimum effect size [45, 46], and a more stringent 0.05. We also identified differentially methylated regions (DMR). While DMPs assess methylation changes at individual CpG sites, DMR analysis enables the detection of coordinated changes across a genomic region, increasing power to identify biologically meaningful alterations that may be missed at the single-site level. This approach is particularly useful in studies with a small sample size [47, 48]. DMRs were identified using the *combp()* function from the *ENmix* R package [49], and DMR *p* values were calculated using Šidák correction [50]. A DMR was considered significant if it had at least two probes and Šidák < 0.05 [50, 51].

To determine which of our findings had previously been associated with diabetes, in addition to a typical literature search we leveraged the EWAS atlas database [52]. We included the search terms T1D, T2D, obesity or GDM. We identified CpGs with > 1% DNAm difference in the atlas associated with either of those four search terms. For GDM, we excluded studies that examined DNAm only in pregnant women with GDM and focused on studies of in utero GDM exposure. For T1D, T2D, and obesity, we included all studies in the EWAS atlas [53-60].

Since shared genetics could be associated with both the risk of T2D and DNAm patterns, we used the mQTLdb [61] to determine whether any differentially methylated sites are methylation trait quantitative loci (mQTLs), which could indicate a genetic component.

HNF1a is a transcription factor involved in the expression of a number of genes important in insulin secretion, with mutations in *HNF1a* resulting in transcription-related diabetes or maturity-onset diabetes of the young (MODY) [62]. Previously, the *HNF1a* G319S polymorphism was found in 40% of adults living with T2D in the Sandy Lake First Nation in Northwestern Ontario [28]. To control for *HNF1a* G319S genotype, we performed sensitivity analyses. The model was rerun after adding maternal *HNF1a* G319S genotype into the main model, and the effect sizes of the rerun models were compared to the effect sizes of the initial analysis. We also performed the sensitivity analysis for gestational age since it was imbalanced between groups; the main model was rerun after removing gestational age, and the effect sizes of the rerun models were compared to the effect sizes of the initial analysis.

To evaluate whether sites identified in our study exhibit common cross-tissue interindividual variation, we assessed the overlap between DMP or DMR sites and previously reported correlated regions of systemic interindividual variation (CoRSIVs) [63]. We obtained the list of EPIC array probes overlapping CoRSIV regions from Supplementary Table 18 in the original paper [63], which includes probes mapped to hg38 coordinates and identified across multiple tissues as exhibiting coordinated interindividual variability in DNA methylation. Using probe IDs, we checked whether any of the CpGs in our study overlapped with probes in the CoRSIV list.

We also examined whether sites that were unique to either GDM or T2D showed non-significant but trending changes in the other group. For example, if a site was influenced by the length of exposure to diabetes, we might observe a large effect size in children exposed to T2D and a smaller but nonzero effect size in the same direction in GDM. Effect sizes were compared between GDM and T2D by comparing unadjusted Δβ of the differentially methylated sites. Δβ was calculated by subtracting the raw control β from the raw β of GDM or T2D, and the sites were stratified by top hit group (whether the site is a top hit in GDM, T2D or both).

To assess whether the differentially methylated sites identified in the NextGen cohort overlapped with previously reported methylation changes in youth-onset T2D, we compared our results to differentially methylated sites previously identified in the iCARE cohort, a group of First Nations youth (aged 10–18 years) living with youth-onset T2D from Manitoba [64]. For genes found to be common between the two cohorts, we examined chromatin state information from lymphoblastoid cells (GM12878) using the UCSC Genome Browser. Chromatin tracks were visualized using the *UcscTrack()* function from the *Gviz* R package.

Epigenetic gestational age

Epigenetic gestational age (GA) was calculated from unnormalized data using the methylclock R package [65]. We calculated epigenetic GA acceleration (GAA)

as the residuals of a regression between epigenetic and chronological GA ages, and tested for group differences using linear models adjusted for the same covariates as in the EWAS analysis, including infant sex, maternal smoking, maternal age at delivery, pre-pregnancy BMI, birthweight, gestational age, the cell-type PCs, and batch effects (BeadChip number and BeadChip position). We examined three clocks that were trained on cord blood DNAm: the Bohlin [66], Haftorn EPIC [67] and Knight [68] clocks.

Results

The next generation cohort: a cohort with a high risk of developing T2D

The NextGen cohort consists of children born to First Nations and Metis mothers living in Manitoba, Canada who were diagnosed with T2D pre-pregnancy, GDM during pregnancy, or no diabetes (Control) [29]. The subsample examined here consists of cord blood from 210 infants. When stratified by maternal diabetes (Control, GDM or T2D), we did not observe differences in infant sex, maternal smoking status, maternal age at delivery, BMI before delivery or birth weight, but did observe differences between groups in maternal and cord blood *HNF1a* genotype and gestational age (p value < 0.05, Table 1). After removing one sample that was missing

Table 1 NextGen Cohort characteristics

	Total (N=210)	Control (N=41)	GDM (N=70)	T2D (N=99)	*p*-value*	
Infant Sex						Fisher Exact Test
Female	103(49%)	19(46%)	28(40%)	56(57%)	0.55, 0.35	
Male	107(51%)	22(54%)	42(60%)	43(43%)		
Maternal HNF1a						
G/G	129(61%)	41(100%)	48(69%)	40(40%)	1.8e-04, 8.9e-12	
S/G	63(30%)	0(0%)	17(24%)	46(46%)		
S/S	9(4%)	0(0%)	0(0%)	9(9%)		
Missing	9(4.3%)	0(0%)	5(7.1%)	4(4.0%)		
Infant HNF1a						
G/G	148(70%)	39(95%)	53(76%)	56(57%)	0.03, 6.8e-6	
S/G	57(27%)	2(5%)	14(20%)	41(41%)		
S/S	3(1%)	0(0%)	2(3%)	1(1%)		
Missing	2(1.0%)	0(0%)	1(1.4%)	1(1.0%)		
Smoking Status						
No	109(52%)	24(59%)	35(50%)	50(51%)	0.33, 0.35	
Yes	100(48%)	16(39%)	35(50%)	49(49%)		
Missing	1(0.5%)	1(2.4%)	0(0%)	0(0%)		
Chip Version						
EPICv1	150(71%)	28(68%)	50(71%)	72(73%)	0.83, 0.68	
EPICv2	60(29%)	13(32%)	20(29%)	27(27%)		
Delivery Age (yrs)						T-Test
Mean (SD)	28(±6.5)	27(±6.0)	29(±6.5)	27(±6.5)	0.09, 0.93	
Pregnancy BMI Before Delivery						
Mean (SD)	36(±8.0)	34(±7.0)	37(±9.1)	36(±7.4)	0.16, 0.27	
Missing	12(5.7%)	5(12.2%)	1(1.4%)	6(6.1%)		
Gestational Age (wks)						
Mean (SD)	38(±1.5)	39(±0.93)	38(±1.1)	37(±1.4)	2.9e-10, <2e-16	
Missing	5(2.4%)	3(7.3%)	0(0%)	2(2.0%)		
Birth Weight (kg)						
Mean (SD)	3.5(±0.56)	3.5(±0.52)	3.4(±0.44)	3.5(±0.64)	0.30, 0.67	
Missing	9(4.3%)	4(9.8%)	0(0%)	5(5.1%)		

* Two p values are provided: the first one is for GDM vs Control and the second one for T2D vs Control

smoking status, 209 samples were retained for downstream DNAm analysis (Supplementary Fig. 2).

In-utero exposure to GDM or T2D produce different patterns of DNAm in children

To identify differentially methylated sites due to *in-utero* exposure to GDM or T2D, we analyzed DNAm levels at 613,792 sites across the human genome. We used multiple linear regression with adjustment for maternal age, BMI before delivery, smoking status, birthweight, gestational age, infant sex, and cell-type PCs. (Supplementary Fig. 3A & B). We identified a total of 63 differentially methylated sites (also known as differentially methylated positions, DMPs) associated with *in-utero* exposure to GDM or T2D (Supplementary Table 1 & Fig. 1A&B, FDR < 0.05 and effect size ≥ 1%). Of these, 27 sites were unique to GDM exposure (Supplementary Fig. 4), 27 were unique to T2D exposure (Supplementary Fig. 5), and 9 were common between GDM and T2D (Supplementary Fig. 6). The gene with the highest number of CpG hits (3 GDM and 5 T2D) was *PTPRN2*.

In addition to examining each CpG site individually, we also performed differentially methylation region (DMRs) analysis using comb-p to determine whether any regions of DNAm were altered and not identified in the single CpG analysis. We identified 87 DMRs associated with GDM exposure and 69 DMRs associated with T2D exposure (Šidák < 0.05, Fig. 1C & D, Supplementary Table 2). We overlapped the individual DMR CpG sites and identified 245 sites unique to GDM exposure, 180 sites unique to T2D exposure, and 92 sites common between GDM and T2D exposure. As expected, comparison between the sites found in the DMP and DMR analyses found ten T2D sites and seven GDM sites that were common between the two analyses, supporting the robustness and consistency of our findings (Fig. 1E & Supplementary Table 2).

When comparing our results with previous studies, we found two GDM sites (one unannotated and the other in *CLDN15*) and one unannotated T2D site that were previously associated with obesity in our individual CpG analysis [69, 70] and 12 T2D and 11 GDM sites associated with obesity in our DMR analysis [69–72] (Fig. 1E, Supplementary Table 1 & Supplementary Table 2). Interestingly, we also found two T2D DMR sites (one at C20orf3 and the other at RNU5D) and two GDM DMR sites (one at C20orf3 and the other at COMT) that were associated previously with adult T2D [72]. We found five sites (cg21542078 in TRPM4 [57]; cg00484396, cg05754148 and cg22508957 in NAT15 [60]; unannotated cg14906510 [60]) in our GDM DMR analysis and one site in our T2D DMR analysis (unannotated cg14906510 [60]) that were associated previously with *in-utero* exposure to GDM. We identified one mQTL site in our individual CpG analysis and 89 mQTL sites in DMR analysis (Fig. 1F). Interestingly, the two sites (cg06937882 in C20orf3 and cg23425533 in RNU5D) that were associated previously with T2D and the one site (unannotated cg14906510) that were previously associated with *in-utero* exposure to GDM were mQTLs.

To evaluate the potential systemic relevance of our findings, we assessed whether any of the DMP or DMR sites overlapped with previously identified correlated regions of systemic interindividual variation across tissues (CoRSIVs) [63]. In DMP analysis, we found only one T2D site that was known to show cross-tissue correlated inter-individual variability (Supplementary Table 1). In our DMR analysis, we identified 8 such sites unique to GDM exposure, 5 unique to T2D exposure, and 4 sites common between the two exposures (Supplementary Table 2). Interestingly, the 4 common sites were on the same DMR on chromosome 12 and were identified as mQTLs, possibly indicating that sites with cross-tissue common inter-individual variability could be regulated by common genetic variants.

Since cord blood *HNF1a* G319S genotype and gestational age were significantly different between groups in our cohort (Table 1), we performed a sensitivity analysis to ensure that our findings were not influenced by this imbalance (Supplementary Table 1). All of the differentially methylated sites in the sensitivity analyses after adding the *HNF1a* genotype as a covariate (Fig. 2 A & B, Supplementary Fig. 3C & D) or after removing gestational age (Fig. 2C & D, Supplementary Fig. 3E & F) from the main model had a consistent magnitude of change in DNAm in T2D and GDM, indicating that our analysis is robust to these confounders.

Although both GDM and T2D each had 36 differentially methylated sites, the distribution of direction of change compared to control was different. In GDM, 83% (30/36) of sites showed higher levels of DNAm compared to controls, while only 61% (22/36) of sites were higher than control in T2D exposed children (Fig. 1A & B and Supplementary Fig. 4, 5 and 6). We were curious whether sites that were significantly different in T2D but not GDM showed effect sizes in the same directions but of lower magnitude, potentially indicating that the shorter exposure window of GDM resulted in DNAm changes at the same sites but the length of time was insufficient for the change to be significant. As we posited, sites that were significant in only T2D typically had effect sizes significantly smaller but in the same direction in GDM, but sites that were found in GDM had similar effect sizes in T2D (Fig. 3).

Finally, we compared sites identified in this study to previously identified sites in the iCARE cohort, which

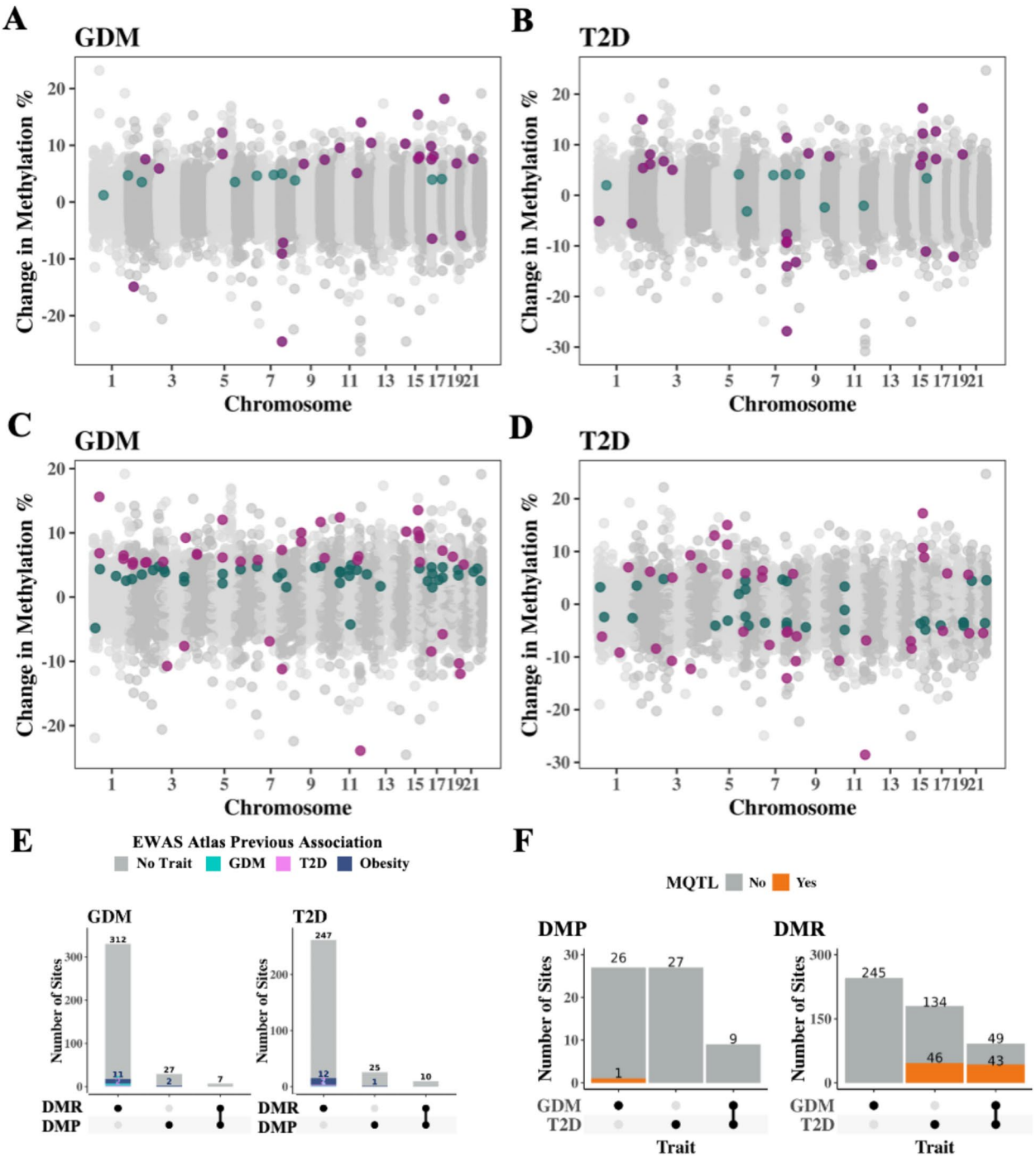

Fig. 1 Differences in timing of in utero exposure to maternal diabetes induces overlapping but independent patterns in cord blood DNAm. 613,792 CpG sites across the genome were analyzed for DNAm changes. Associations were tested using multiple linear regression models from *limma* with adjustments for maternal age, BMI before delivery, smoking status, birthweight, gestational age, infant sex, cell-type PCs and batch effects. **A–D** Purple represents a strong association with *in-utero* exposure (FDR or Šidák < 0.05 and effect size (ES) ≥ 5% change). Green represents a moderate association with *in-utero* exposure (FDR or Šidák < 0.05 and ES ≥ 1% change). The alternating gray of the background points distinguishes the start and end of each chromosome. **A** & **C** 36 differentially methylated sites and 87 differentially methylated regions (DMRs) were identified due to GDM *in-utero* exposure. **B** & **D** 36 differentially methylated sites and 69 DMRs were identified due to T2D *in-utero* exposure. **E** 11 GDM DMR sites and two GDM DMP sites were associated with obesity while 12 T2D DMR sites and one T2D DMP site were associated with obesity. Five GDM and one T2D DMR sites were previously associated with *in-utero* exposure to GDM, while two T2D and two GDM DMR sites were previously associated with T2D **F** 89 mQTLs were identified in the DMR analysis while only one mQTL was identified in the DMP analysis

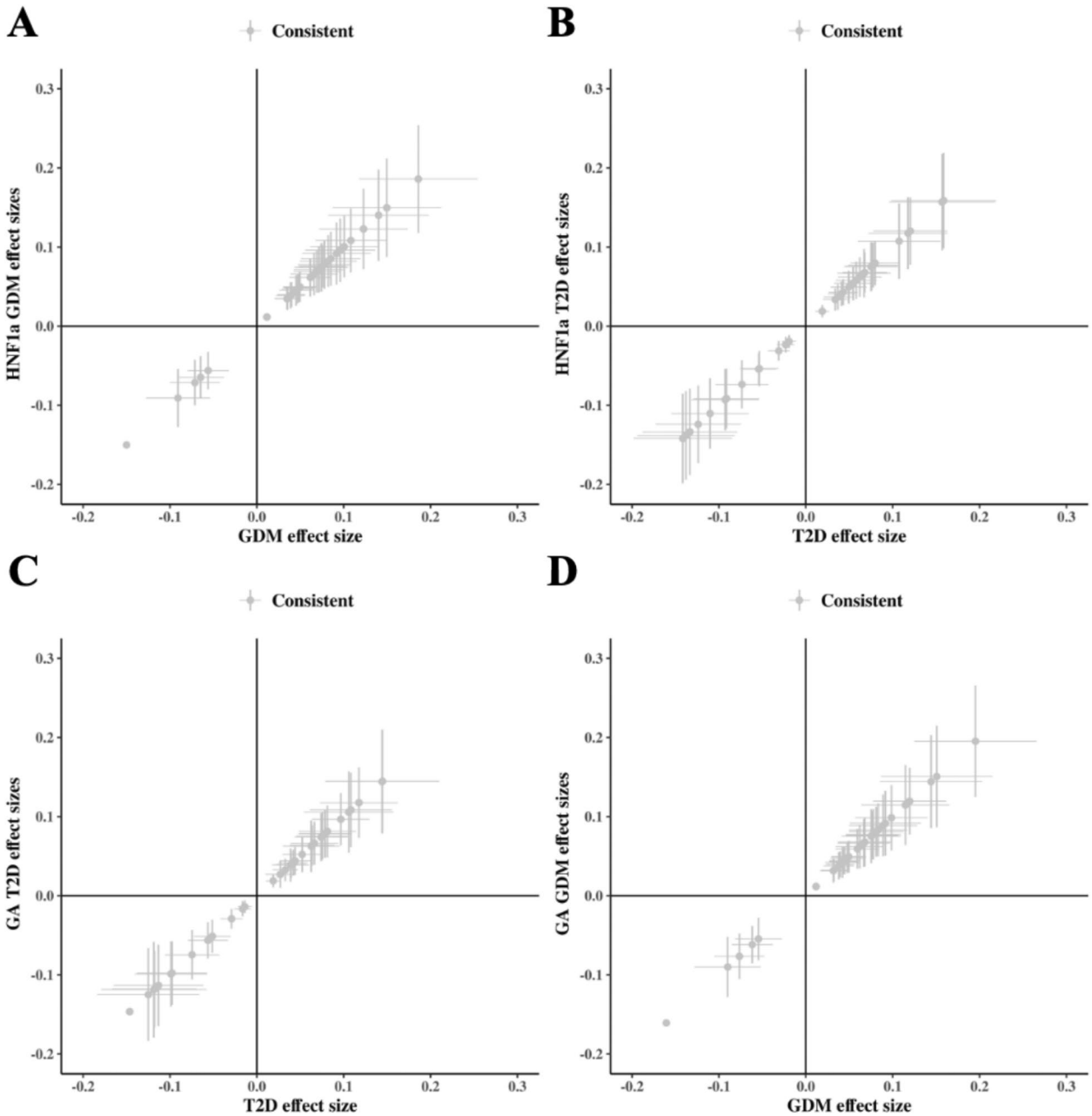

Fig. 2 Epigenome-wide sensitivity analysis shows that the direction of change in DNAm is consistent when adjusting for covariates that are unbalanced between groups. (**A** & **B**) Adding *HNF1α* as a covariate to the linear regression model did not affect the direction or the magnitude of change in DNAm. (**C** & **D**) Removing gestational age (GA) from the linear regression model did not change the direction or the magnitude of DNAm change

compared DNAm patterns of youth with or without T2D and was primarily composed of First Nations youth [64]. We found 11 common genes between NextGen and iCARE cohorts (Table 2, Supplementary Figs. 7, 8, 9, 10, 11 and 12).

Epigenetic gestational age

To test whether in-utero exposure to maternal diabetes would result in epigenetic gestational age acceleration, we used three clocks designed for cord blood: Bohlin, EPIC and Knight GAA clocks. We found that EPIC GA correlated the best with chronological GA (R = 0.71 and

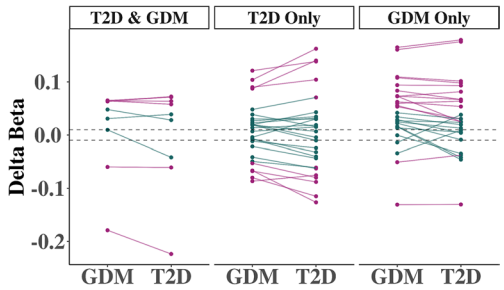

Fig. 3 Comparison of effect sizes between sites associated with GDM or T2D. Effect size (Δβ) was calculated by subtracting the raw control β from the raw β of GDM or T2D, and the sites were stratified by whether the site is significantly different from controls in GDM, T2D or both. Purple represents a strong association with *in-utero* exposure (FDR < 0.05 and effect size (ES) ≥ 5% change). Green represents a moderate association with *in-utero* exposure (FDR < 0.05 and ES ≥ 1% change). The dashed line represents 1% change in ES, the minimum effect size considered

p-value < 2.2e-16), but found no significant difference in epigenetic gestational age acceleration between GDM and Control, or T2D and Control for any of the three clocks used (Supplementary Fig. 13).

Discussion

Early-life exposure to maternal diabetes is a major risk factor for developing youth-onset T2D, with the earlier developmental exposure window of maternal T2D exposure being associated with greater risk than the late second and third trimester exposure of GDM. In this study, we investigated DNAm alterations associated with *in-utero* exposure to either GDM or maternal pregestational T2D, compared to no diabetes, to determine if the timing of prenatal diabetes exposure alters DNAm differently. We identified 27 differentially methylated sites that were associated with *in-utero* exposure to GDM, 27 differentially methylated sites that were associated with *in-utero* exposure to T2D, and nine common differentially methylated sites that were associated with both GDM and T2D *in-utero* exposure. We also identified 245 DMRs sites unique to GDM exposure, 180 DMRs sites unique to T2D exposure, and 92 DMRs sites common between GDM and T2D exposure. A number of our diabetes-associated sites overlapped with prior studies of material diabetes exposure, and interestingly three sites were previously associated with T2D in adults. While additional longitudinal studies are needed to assess the potential

Table 2 Common genes between NextGen and iCARE cohorts

		NextGen				iCARE		
Gene	**Chr**	**CpG**	**Position**	**Effect Size (logFC)**	**Group**	**CpG**	**Position**	**Effect Size (logFC)**
GALNT2	1	cg14280264	230,322,971	−5.55	T2D	cg11527913	230,410,329	3.64
RPIA	2	cg05808646	89,035,504	3.51	GDM	cg10376026	89,035,483	1.72
TFEB	6	cg06911865	41,659,360	−3.18	T2D	cg16111704	41,673,829	1.52
HIVEP2	6	cg21597425	143,195,947	4.63	GDM	cg22858308	143,095,613	−0.43
PTPRN2	7	cg16627193	157,801,404	4.99	GDM	cg00841760	158,362,966	−1.73
		cg15819924	157,886,456	−9.38	T2D			
		cg15819924	157,886,456	−9.09	GDM			
		cg10078132	157,890,798	−7.68	T2D			
		cg20528787	158,032,352	−14.04	T2D			
		cg23066280	158,032,496	−26.86	T2D			
		cg23066280	158,032,496	−24.57	GDM			
		cg23759393	158,110,405	−9.15	T2D			
BCL2L14	12	cg20141578	12,225,262	14.04	GDM	cg27366072	12,222,913	−0.38
IGDCC3	15	cg24741685	65,661,080	6.02	T2D	cg24741685	65,661,080	2.72
MTHFS	15	cg04124281	80,190,137	15.42	GDM	cg17934955	80,142,016	0.83
						cg02196730	80,188,777	0.86
						cg10811429	80,189,771	2.82
						cg20643687	80,189,821	1.04
						cg09284795	80,189,896	1.79
						cg10149159	80,189,934	−0.57
PITPNA	17	cg09822811	1,459,159	7.18	T2D	cg08686850	1,436,624	0.84
DNAH2	17	cg12263130	7,667,626	3.96	GDM	cg22573230	7,649,271	−0.05
PLA2G4C	19	cg17828850	48,597,832	8.10	T2D	cg11854772	48,555,019	−1.58

relationship of these biomarkers to health outcomes later in life, our results show that DNAm profiles in offspring exposed or unexposed to maternal diabetes are detectable at birth and are different between maternal GDM and T2D. Future work may provide an opportunity to develop biomarkers and identify at-risk children who would benefit from preventative strategies and lifestyle modifications starting from early life.

DNAm patterns broadly are established early in development, supporting the importance of the window of *in-utero* exposure [73, 74]. The pathophysiology and window of exposure are different for different types of maternal diabetes, thus we examined maternal GDM and T2D separately. Our hypothesis was that since T2D exposure occurs throughout pregnancy, but GDM occurs exclusively later in gestation, these two conditions might influence DNAm patterns in distinct but overlapping ways. Our results show that *in-utero* exposure to GDM produced a different DNAm profile in offspring than T2D *in-utero* exposure; although both GDM and T2D each had 36 differentially methylated sites, 86% of sites associated with GDM had higher DNAm than controls whereas that proportion was only 61% for T2D exposure. These results suggest that the window of exposure to diabetes produces different molecular modifications and might be linked to the differences in risk of youth-onset T2D after *in-utero* exposure to T2D or GDM. Previous studies showed that offspring who were exposed to T2D *in-utero* were 14 times more likely to develop T2D in adulthood [75], developed T2D at a younger age [76], and had an increased risk of developing cardiovascular diseases compared to offspring who were exposed to GDM *in-utero* [11]. This difference in risk could be attributed partly to genetic predisposition of T2D where the heritability ranges from 40 to 80% [77, 78] and the lifetime risk is 40% if one parent had T2D and 70% if both parents had T2D [79]; but also to exposure to glycemic environment throughout gestation. Taken together, our results underscore how DNAm patterns are shaped by the window of exposure with distinct windows of vulnerability to GDM and T2D resulting in different molecular modifications that could have a lasting impact on health outcomes.

Of the 517 sites we identified in the DMR analysis, 89 of them are mQTLs, or sites of DNAm that are influenced by nearby genetic variation. Interestingly, of the 89 sites associated with *in-utero* exposure to T2D, 46 of them were unique to T2D exposure and the remaining 43 were shared between T2D and GDM exposure. In our DMP analysis, we only identified one mQTL associated with *in-utero* exposure to GDM. This difference in the number of mQTLs identified could be attributed to how those two analyses work. Compared to DMP analysis, DMR analyzes regions rather than individual sites, which increases the statistical power and the ability to detect subtle but biologically meaningful associations [45]. However, genetic effects detected as mQTLs often span multiple CpG sites [80, 81], which in return would make DMR analysis more likely to identify sites of DNAm that are influenced by underlying genetic variation.

Three CpG that were mQTLs were also previously associated with T2D in the EWAS Atlas [72]; one site was unannotated, one was at *C20orf3* (also known as *APMAP*) and the other site was at *RNU5D* gene. APMAP is an adipocyte plasma membrane-associated protein and plays an important role in preadipocyte differentiation into mature adipocytes [82] which is important for reducing insulin resistance. A previous study that examined human pancreatic islets from donors with or without T2D associated the same site we found in *APMAP* with having T2D [83]. In a study that examined DNAm of spermatozoa from men with T2D, they found differential DNAm at 10 paternally imprinted sites, one of which was in *RNU5D* [84]. The fact that we identified differentially methylated sites in those two genes at birth in offspring that have not yet developed T2D hints at the possibility of using DNAm as an early biomarker of increased risk of developing T2D later in life. Moreover, the presence of mQTLs at these sites raises the possibility of gene–environment interactions, where common genetic variants that increase diabetes risk may interact with prenatal diabetes exposure to produce greater or more persistent DNAm changes. This interaction could amplify the impact of in utero exposures on long-term disease risk.

In-utero exposure to maternal diabetes has been theorized to be involved in prenatal programming that involves changes in β cell mass or function and abnormal lipid metabolism [8]. We found 3 GDM and 5 T2D differentially methylated sites in the *PTPRN2* gene. In our previous study in the iCARE cohort of youth living with T2D [64], we also identified differentially methylated sites in the *PTPRN2* gene. Knockdown of *PTPRN2* (protein tyrosine phosphatase receptor type N2), also called phogrin, was previously associated with diabetes [85]. The protein that this gene encodes is enriched in insulin granules and contributes to glucose-stimulated β-cell growth [85], and the deletion of this gene leads to impairment in insulin secretion in mice [86]. Differential DNA methylation in *PTPRN2* has been associated with *in-utero* GDM exposure in cord blood [57, 87], which is consistent with what we found in this study though at a different specific CpG. Differential methylation in *PTPRN2* is also associated with childhood adiposity [53] and childhood obesity [88]. Our findings suggest that DNAm changes could serve as biomarkers that could be detected in cord blood and could be used to identify offspring with an increased risk of developing T2D,

childhood adiposity and/or obesity. This could provide the opportunity of early-life intervention strategies to improve long-term health outcomes.

There are a number of possibilities for why maternal diabetes exposure results in the observed DNAm changes. One is that hyperglycemia could result in alterations to DNA methyltransferase or demethylase activity, though if that were the case we would anticipate broader changes. Another more likely possibility is that the DNAm changes observed are the result of cellular responses to hyperglycemia, in which case the question of why differential timing of exposure results in differential DNAm changes becomes even more interesting. There also remains an interesting question about the direction of DNAm is different depending on whether the exposure was to T2D or GDM. It is not clear why exposure to hyperglycemia in the third trimester only would result in significantly more gains in DNA methylation than losses while exposure throughout development results in more balanced gains and losses. This may be an effect of the gene functions involved rather than a systematic effect, but how the direction of DNAm change interacts with developmental timing is an open question that would warrant further scrutiny.

While our study makes important contributions to our understanding of the molecular outcomes of differential timing of maternal diabetes exposure, it is not without limitations. One primary limitation is the relatively small sample size, which both reduced our power and limited our ability to stratify our analysis. For example, studies have shown that males exposed to GDM *in-utero* have a higher risk for childhood overweight [89, 90] and increased adiposity [91] compared to females exposed to GDM *in-utero*. We were unable to examine this in our study due to sample size. The small sample size of the control group may also have reduced our statistical power to detect demographic differences and could explain why maternal BMI between GDM and control groups was not statistically significant although the difference was clinically relevant (37 vs. 34). Additionally, this study had missing values for birthweight (n = 9) and gestational age (n = 5), and mean imputation was used to retain the full sample for analysis. While this is a simple and commonly used method, it does not account for uncertainty in the imputed values. Although EPIC v1 and v2 arrays were merged and normalization steps were applied, inherent differences in probe design and coverage between the array versions could still introduce technical variability. While we adjusted for batch effects and tested for consistency in effect sizes across EPIC versions, we acknowledge that residual technical differences between array versions may influence DNA methylation measurements and interpretation. The linear models we performed demonstrated some inflation that could be due to population stratification. We were unable to control for genetic ancestry in our study, but endeavored to address genetic contributions to our results by reporting known mQTLs as a proxy for genetic effects. Finally, DNAm was measured in cord blood, and while it is useful for studying prenatal environmental exposures, it is directly influenced by the *in-utero* environment and may reflect short-term, transient changes in DNAm. The extent to which these DNAm signatures persist beyond birth and influence the development of T2D or obesity remains unclear. Future longitudinal studies are needed to assess the temporal stability of these DNAm changes and their relationship to health outcomes later in life, which in return would help determine whether the observed DNAm alterations represent early biomarkers of disease risk.

In conclusion, our study highlights the association of exposure to maternal diabetes and DNAm patterns in cord blood, emphasizing the importance of the timing of *in-utero* exposure to maternal diabetes. Differences in DNAm profiles between GDM and T2D underline the distinct window of vulnerability during gestation, and could help explain why *in-utero* exposure to T2D poses higher risk of developing T2D later in life. We identified DNAm changes in genes such as *APMAP* and *PTPRN2* which have known roles in glucose metabolism, β-cell function, and adiposity, highlighting the potential of using DNAm as a biomarker of increased risk of developing T2D later in life. These early biomarkers of risk could be used to inform screening and early-life intervention platforms and future work in this area may shed light onto the pathophysiology and heterogeneity of youth-onset T2D.

Supplementary Information

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Additional file1.

Additional file2.

Additional file3.

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Author contributions

OES and HK generated the DNA methylation data, and OES conducted all data analysis and drafted the manuscript. YR, PI, CP, EACS recruited the cohort and collected participant data. MJJ and BAW oversaw the funding for the project, conceived the study, supervised the data collection and analysis, and edited the manuscript. All authors approved the final version of the manuscript.

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Data availability
The NextGen cohort is primarily First Nations, and so all data sharing must align with the principles of OCAP (Ownership, Control, Access, and Possession). For information on OCAP, see https://fnigc.ca/. For information on how to apply for access to the data used in this manuscript, please contact the NextGen cohort team at https://www.dreamdiabetesresearch.com/get-involved/#contactus.

Declarations

Ethics approval and consent to participate
The study was approved by the University of Manitoba Research Ethics Board (HS16516). All participants gave full informed consent to participate.

Consent for publication
Not Applicable.

Conmpeting interests
No potential conflicts of interest relevant to this article were reported.

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