Clinical Epigenetics

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Type 2 diabetes impacts DNA methylation in human sperm

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Abstract

Aims/hypothesis Disorders of the reproductive system, including hypogonadism and reduced fertility, are an under-recognized complication of diabetes. Based on experimental data in mice, hyperglycemia and obesity may modify epigenetic marks in sperm and impact health and development of offspring, but data are more limited in humans. Thus, we sought to study the impact of type 2 diabetes and glycemic control on sperm quality and DNA methylation.

Methods In this prospective cohort study, we recruited 40 men with BMI greater than 25 kg/m^2 including 18 with type 2 diabetes, 6 with prediabetes, and 16 normoglycemic controls. Assessments were repeated after 3 months in 9 men with type 2 diabetes and 7 controls. We analyzed reproductive hormones, sperm concentration and motility, and sperm DNA methylation (MethylationEPIC BeadChip).

Results Men with type 2 diabetes had higher levels of follicle-stimulating hormone (FSH), but similar testosterone levels and sperm quality as controls. Sperm DNA methylation was stable with repeat sampling at 3 months in men with and without type 2 diabetes. We identified differential methylation at 655 of 745,804 CpG sites in men with type 2 diabetes versus controls (FDR < 0.05). Of these, 96.5% showed higher methylation in type 2 diabetes, with a mean difference in DNA methylation (beta value, β) of 0.16 ± 0.004 (16 ± 0.4%). Ontology analysis of differentially methylated loci revealed annotation to genes regulating synaptic signaling, actin, cAMP-dependent pathways, and G protein-coupled receptor pathways. 24% of probes differentially regulated in men with type 2 diabetes versus control overlapped with probes associated with HbA1c, suggesting additional factors beyond glycemic control contributed to diabetes-associated differences in DNA methylation.

Conclusions/interpretation Men with type 2 diabetes showed higher DNA methylation levels in sperm relative to normoglycemic controls with similar BMI. Whether these differences are reversible with glucose-lowering treatment or may contribute to post-fertilization transcriptional regulation warrants further investigation.

Trial registration NCT03860558

Keywords DNA methylation, Spermatozoa, Epigenetics, Type 2 diabetes, Semen quality

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Background

The global prevalence of diabetes has risen substantially over the past several decades [1]. One clinically important, but under-recognized, complication in men with type 2 diabetes (T2D) is dysfunction of the reproductive system, including erectile dysfunction, hypogonadism, and infertility [2]. Moreover, emerging data in rodents indicate that paternal diet, hyperglycemia, and obesity may affect sperm epigenetics and impact health and development of offspring [3–5].

Many nutritional or environmental factors can influence transcription and cellular function via epigenetic regulation. Prior studies have demonstrated that type 2 diabetes can affect epigenetic regulation in somatic tissues, including differential DNA methylation at key loci linked to metabolic transcriptional control [6]. Based on experimental data in rodents, a wide range of postnatal exposures, including alterations in dietary macronutrients [5] such as high-fat [7] or low-protein diet [8], cold [9], smoking [10], and endocrine-disrupting chemicals [11], can result in differential DNA methylation of CpG dinucleotides in sperm. Moreover, nutritional exposures during prenatal life can modulate DNA methylation in adulthood [5]; for a subset of loci, methylation changes are associated with altered gene expression in offspring somatic tissues [12].

In humans, aging, lifestyle and environmental exposures, and DNA fragmentation are associated with alterations in the sperm epigenome [13–16]. Likewise, the sperm DNA methylome is altered in men with obesity as compared with lean individuals [15, 17, 18]. Whether alterations in the sperm methylome influence offspring metabolism in humans remains uncertain; however, we recently demonstrated that paternal body mass index (BMI) is associated with umbilical cord blood DNA methylation in offspring, with differential methylation at discrete CpG loci persisting into early childhood [19].

Emerging data suggest that the sperm epigenome is modifiable [20]. Dietary factors including short-term overnutrition, "fast-food" diet, and folate supplementation [5, 21–23] can modify the sperm epigenome. Recent data indicate that sperm DNA methylation can be modified in healthy men after only 1 week of hypercaloric diet achieved by added sugar [24]. Likewise, sperm DNA methylation is altered by exercise [25, 26] and after bariatric surgery [17]. Whether type 2 diabetes, or glycemic control, similarly affects the human sperm methylome has not been fully investigated. A recent study by Chen et al. identified changes in DNA methylation in sperm from men with type 2 diabetes, but did not assess fertility parameters, or effects of glycemic control [27].

We now report results from a human case–control study with repeated measurements after 3 months. We compared DNA methylation patterns in men with type 2 diabetes versus normoglycemic controls with similar BMI, assessed stability of the methylome over time, and probed relationships between glycemic control and the sperm methylome.

Methods

Ethics statement

This study was approved by the Committee on Human Studies of Joslin Diabetes Center, conducted in accordance with the requirements of the Helsinki Declaration, and registered on clinicaltrials.gov (NCT03860558). All participants provided written informed consent.

Study population

Study participants were recruited between 2018 and 2020 (consort diagram, Figure S1) via provider referrals and online ads. Inclusion criteria were age 18 to 65 and BMI $\geq$ 25 kg/m^2. Type 2 diabetes was defined based on prior diagnosis, and normoglycemia (for controls) based on screening fasting glucose < 100 mg/dl and hemoglobin A1c (HbA1c) < 5.7%. Exclusion criteria: (1) chronic systemic disease; (2) severe diabetic retinopathy; (3) congestive heart failure; (4) myocardial infarction, unstable angina or revascularization within the past 6 months; (5) active genitourinary infection; (6) testicular volume < 12 mL; (7) hypogonadism; (8) prolactin > 18 ng/ml; (9) estradiol > 42 pg/ml; (10) cryptorchidism; (11) cigarette smoking; (12) active alcohol or substance abuse; (13) cancer; and (14) use of nitrates, guanylate cyclase stimulators, or steroid hormones (including testosterone). Although recruitment focused on individuals with type 2 diabetes or normoglycemic controls, several participants were identified as having prediabetes during screening according to fasting glucose or HbA1c [28]. Additional information on health and lifestyle was obtained via structured interviews. Weight and height were measured to the nearest 0.1 kg or 0.5 cm, respectively. Body composition was measured by bioimpedance (TANITA). A second visit with identical protocol was conducted 3 months after the first visit in a subset of participants.

Reproductive hormone analysis

Blood was collected after an overnight fast (minimum 8 h) by venipuncture between 9 AM and 12 PM on the same day as semen collection. Plasma glucose, HbA1c, chemistry, and lipids were analyzed using Roche Cobas C501 Chemistry Analyzer; blood count was assessed using Beckman Coulter DxH 600, in the clinical laboratory at Joslin Diabetes Center. Serum reproductive hormones were analyzed by Quest Laboratories; total testosterone was measured by gas chromatography/mass spectrometry, while luteinizing hormone (LH),

follicle-stimulating hormone (FSH), estradiol, prolactin, insulin, leptin, adiponectin, and sex hormone-binding globulin (SHBG) were measured by immunoassay.

Semen analysis and preparation

Semen samples were collected by participants in a polypropylene cup after 2–7 days of abstinence. Semen analysis was performed after a liquefaction period of 30 min at 37 °C. Semen volume was measured by weighing the sample, assuming density to be 1 g/ml [29]. Sperm concentration (million/mL), motility (%), progressive motility (%) and velocity were assessed using computer-assisted semen analysis (CASA; Hamilton Thorne, Version 14 Build 008, MA, USA). Total sperm count was calculated by multiplying sperm concentration by semen volume. Total motile count was calculated by multiplying total count by total motility (sum of progressive and non-progressive).

Isolation of motile sperm

The swim-up method [17] was used to separate spermatozoa from somatic cells and debris, enriching for motile sperm. The semen sample was layered under wash medium (Earle's Balanced Salt Solution, 25 mM HEPES, 3.2% human albumin) and incubated at 37 °C at a 45° angle for 2 h; sperm were harvested from the supernatant. The percentage of residual somatic cells in the final supernatant was assessed by microscopy.

Sperm DNA extraction

Sperm DNA was extracted using the DNeasy Blood/Tissue Kit (Qiagen). Motile sperm previously frozen in Buffer RLT (containing 1% 2-mercaptoethanol) were thawed on ice. 50–100 mg of 0.2 mm steel beads (Next Advance, Inc.) was added prior to homogenization using the TissueLyser II at 30 Hz for 5 min. Samples were incubated at 56 °C overnight with 20 µl proteinase K (Qiagen). Lysates were treated with 4 µl RNAse A (Thermo Scientific) and transferred to spin-filter columns followed by several wash and centrifugation cycles. DNA concentration was determined using Nanodrop ND-1000 spectrophotometer and Qubit® fluorometer.

Illumina methylation EPIC BeadChip

The Infinium Human Methylation EPIC BeadChip was used to assess DNA methylation at the 865,859 CpG sites represented on the array. A total of 32 samples from 16 participants who completed both visits (7 controls, 9 with type 2 diabetes) were randomly chosen for processing (4 arrays) at Partners Personalized Medicine Core, Boston, MA. Bisulfite conversion of 1 µg DNA was performed using EZ-96 DNA Methylation™ Kit (Shallow Well Format) (Zymo, D5003). Bisulfite-converted DNA (200–400 ng) was used as substrate for whole-genome amplification; after fragmentation, the sample was hybridized to 50-mer probes on the array. Single-base extension allows incorporation of a labeled nucleotide, detected via dual color (Cy3/Cy5) staining by the iSCAN reader. Site-specific methylation was quantified using GenomeStudio software.

Bioinformatics analysis of BeadChip data

Quality control steps included principal component analysis by sample plate and SNP matching to confirm paired samples; no samples were excluded.

We preprocessed raw IDAT files using SeSAMe [30]. Standard quality control steps in the SeSAMe analytical pipeline include masking probes determined to be of poor design (e.g., those too close to a SNP), inferring channel for Infinium-I probes, nonlinear correction for dye bias, masking probes not detected in a sample (i.e., assigning a missing value if the detection p value is >0.05), and background correction. A mean of $98.4 \pm 0.6\%$ of probes was successfully detected.

We then transformed methylation beta values, where $\beta = M/[M+U]$, to M values, which are $\log_2(M/U)$. M and U are the proportion of methylated and unmethylated CpGs, respectively. We examined the distribution of M values per probe in the control samples and found that they closely approximated a normal distribution. Beta is not transformable to M values when β is 0 or 1, but all β values were between [0.001, 0.999]. After preprocessing with SeSAMe, some CpGs had missing values for β due to not being detected; we keep those that had at least 25 non-missing values [31].

To assess differential methylation of CpG loci between sperm from men with type 2 diabetes versus controls while adjusting for age, we used Limma, an R package for linear regression modeling that uses empirical Bayes variance estimation, so its tests are termed "moderated." We applied Limma to the M values, which has been recommended for methylation array data analysis [32]. The genomic inflation factor for this analysis was calculated as the ratio of the median squared z-score divided by the 50% percentile of the chi-squared distribution on one degree of freedom. We performed moderated paired t tests to detect probes that are differentially methylated between visits (paired by subject) using false discovery rate (FDR) <0.05 as the significance threshold.

We also performed moderated t tests to detect probes differentially methylated between type 2 diabetes and controls while adjusting for age. Samples from the two visits of the same subject were treated as repeated measurements and used to estimate the correlation between repeated measurements. This correlation was accounted

for in the analysis, which is analogous to a mixed model analysis of variance [33].

We used linear regression to detect probes associated with HbA1c without adjustment, with adjustment for age, and with adjustment for age and BMI, or associated with BMI without adjustment. Likewise, linear regression was used to detect difference of probe *M* value between the two visits that are associated with the difference of HbA1c, with adjustment for the difference of age and BMI.

Relative locations of CpG probes to gene transcripts and genomic feature localization (e.g., 5'UTR, body, 3'UTR, etc.) were annotated based on UCSC_RefGene_Group (hg38 genome). Since some sites have multiple annotations, we tallied all the annotations for sites in each group and determined the percentage of each annotation across all sites in the array and within differentially methylated loci. A proportion test was performed to evaluate enrichment in differentially methylated sites. GO analysis was performed using the missMethyl package [34]. Transcription factor enrichment analysis was performed using ChEA3 [35]. Imprinted genes were defined using the database at http://www.geneimprint.com/.

To assess differentially methylated regions, we used comb-p, which combines *P* values in sliding windows while accounting for spatial correlation across the genome. This technique corrects for multiple testing of probes using the FDR and for multiple testing of regions using the Šidák correction, which behaves similarly to the Bonferroni correction [36, 37].

Bioinformatics analysis of Chen et al.

To complement our analysis, we re-analyzed data from Chen et al. [27] who reported whole-genome bisulfite sequencing (WGBS) of individuals with T2D versus controls without diabetes. We read their supplemental files from GEO GSE138598. We smoothed the total counts using a moving sum method with 500 bp windows from the R package DSS [38] and similarly smoothed the methylated counts, as is recommended for WGBS data, filtered out loci that did not have at least 10 total counts in at least 13 samples, calculated *M* values using an arcsine transformation of one less than twice the beta values, which has been found to be superior in WGBS data due to better variance stabilization [38], and tested differential methylation of sites with DSS, which accounts for unique properties of WGBS data [38]. We tested differential methylation of regions using dmrseq [39]. We tested replication of sites between Chen et al. and our data using ScreenDMT [40], which is based on a directional MaxP test [41] and has good power for detecting sites that are differential in both studies and in the same direction (e.g., increased methylation in T2D).

Statistical analysis

We analyzed demographic, metabolic, and hormonal data for participants with type 2 diabetes, prediabetes, and controls using R/Bioconductor [42] within RStudio. Parameters with skewed distributions were $\log_2$-transformed. Between-group differences were analyzed by one-way analysis of variance followed by Tukey's post-hoc test. Mean and standard deviation are presented. Simple and multiple linear regression and Spearman correlation were performed to assess associations between variables. Significance for non-genome-wide analyses was accepted when $p < 0.05$.

Power considerations

To determine our sample size, we assumed a repeated-measures analysis framework (where subjects' pre-/post-3 month methylation patterns are paired). Based on an alpha of 0.0001 and a standard deviation of the change in proportion of 9%, we expected (using the power.t.test function in R) to detect a change in proportion of 12% (e.g., from 30 to 42%) with power 0.8 with 20 subjects per group.

Results

Baseline characteristics

Forty men, including 16 normoglycemic controls, 6 with prediabetes, and 18 with type 2 diabetes, were included (Table 1, consort diagram in Figure S1). Fasting glucose, HbA1c, and fructosamine were higher in the type 2 diabetes group, while adiposity measures (BMI, waist circumference, and fat mass) did not differ. Controls (42.0 ± 12.9 years) were younger than individuals with prediabetes (51.8 ± 9.5 years) and type 2 diabetes (52.7 ± 7.3 years) ($p = 0.01$). The percentage of participants with offspring was similar across groups. Total and LDL cholesterol was lower in the type 2 diabetes group, consistent with higher statin use in participants with type 2 diabetes. While leptin levels were similar, adiponectin was 36% lower in type 2 diabetes ($p < 0.05$). Participants with type 2 diabetes had higher FSH versus controls (8.9 ± 5.0 versus 4.6 ± 2.4 mIU/mL, $p < 0.01$); LH, prolactin, estradiol, total testosterone, and SHBG were similar across groups (Table 2). There were no differences in semen volume, concentration, total count, motility or progressive motility, or total motile count between groups (Table 3).

Table 1 Baseline characteristics of study population

	Total ($n=40$)	Control ($n=16$)	Prediabetes ($n=6$)	Type 2 diabetes ($n=18$)	ANOVA *P* value
Age (years)	48.3 ± 11.2	42.0 ± 12.9	51.8 ± 9.5	52.7 ± 7.3	0.01[X]
Race	–	–	–	–	0.61
White	28 (71.1)	12 (75.0)	3 (50.0)	13 (75.0)	
Black	9 (21.1)	3 (18.8)	2 (33.3)	4 (18.8)	
Asian	2 (5.3)	0 (0.0)	1 (16.7)	1 (8.3)	
> 1 race	1 (2.6)	1 (8.3)	0 (0.0)	0 (0.0)	
Diabetes duration (years)	–	–	–	12.6 ± 8.2	
Fathered children	20 (50)	6 (30)	2 (10)	12 (60)	1.00
Fasting glucose (mg/dl) [a]	116 ± 44.8	98.1 ± 5.7	97.0 ± 6.4	137.7 ± 56.6	0.01[X]
HbA1c (%)	6.4 ± 1.4	5.2 ± 0.3	5.9 ± 0.1	7.7 ± 1.1	1.84e−10[X,Y]
Fructosamine (umol/L)	274.8 ± 77.9	239.6 ± 21.4	259.8 ± 69.3	311.1 ± 97.4	0.02[X]
BMI (kg/m^2)	32.6 ± 6.2	32.8 ± 6.0	31.0 ± 6.8	33.0 ± 6.5	0.80
Waist circumference (cm)	109 ± 16	110 ± 15	104 ± 19	110 ± 17	0.77
Fat Mass (kg)	34.3 ± 19.9	33.1 ± 13.8	31.4 ± 23.4	36.4 ± 24.0	0.83
Fat %	30.4 ± 7.7	30.71 ± 7.79	30.3 ± 11.1	30.1 ± 6.7	0.98
Fat-free mass (kg)	73.8 ± 18.0	74.1 ± 14.8	76.9 ± 33.0	72.5 ± 14.9	0.88
Insulin (μlU/mL)[a]	13.1 ± 11.7	12.1 ± 8.4	8.8 ± 8.3	16.2 ± 15.9	0.12
Adiponectin (mcg/ml)	6.7 ± 3.2	8.4 ± 3.4	6.2 ± 1.2	5.2 ± 2.7	0.01[X]
Leptin (ng/ml)	7.5 ± 10.0	10.6 ± 14.4	7.3 ± 8.89	4.7 ± 2.6	0.23
Total cholesterol (mg/dl)	173.1 ± 44.5	194.7 ± 39.3	193.0 ± 32.9	147.3 ± 39.7	0.002[X,Y]
LDL-C (mg/dl)	96.1 ± 38.8	110.6 ± 30.7	127.8 ± 29.4	73.4 ± 35.3	0.0008[X,Y]
Triglycerides (mg/dl)	157.0 ± 188.0	171.8 ± 284.2	98.0 ± 22.8	163.5 ± 90.7	0.71
Diabetes medication use, N				Metformin (17) GLP1RA (10) SGLT2i (5) Sulfonylurea (5) Insulin (8)	
Statin use, N	14	2	0	12	

Continuous variables are reported as mean ± standard deviation (SD) and categorical variables reported as count (percentage)

[a] Reduced sample size as data for fasting glucose and insulin were not included for non-fasting participants ($n=5$) and one participant was missing waist circumference measurements

[X] Tukey post-hoc significant difference between men with type 2 diabetes and controls

[Y] Tukey post-hoc significant difference between men with type 2 diabetes and prediabetes

HbA1c hemoglobin A1c, *BMI* body mass index, *LDL-C* low-density lipoprotein cholesterol

Table 2 Reproductive hormone levels of study population at baseline

	Total ($n=40$)	Control ($n=16$)	Prediabetes ($n=6$)	Type 2 diabetes ($n=18$)	ANOVA, *P*
FSH (mIU/mL)	6.6 ± 4.2	4.6 ± 2.4	5.2 ± 1.4	8.9 ± 5.0	0.02[X]
LH (mIU/ml)	4.4 ± 2.0	4.0 ± 2.2	3.6 ± 0.7	4.9 ± 2.0	0.22
Prolactin (ng/mL)	5.8 ± 2.2	6.4 ± 2.6	5.2 ± 1.2	5.4 ± 1.9	0.36
Estradiol (pg/ml)	31 ± 11	28 ± 8	37 ± 11	32 ± 12	0.17
Total testosterone (ng/dL)	399 ± 168	408 ± 175	498 ± 191	359 ± 149	0.21
SHBG (nmol/mL)	27 ± 12	23 ± 9	35 ± 9	28 ± 14	0.13

Continuous variables are reported as mean ± standard deviation (SD)

[X] Tukey post-hoc significant difference between type 2 diabetes and controls

FSH follicle-stimulating hormone, *LH* luteinizing hormone, *SHBG* sex hormone-binding globulin

Table 3 Semen parameters (mean ± SD) at baseline

	Total ($n=38$)	Control ($n=16$)	Prediabetes ($n=6$)	Type 2 diabetes ($n=16$)	ANOVA *P*
Ejaculate volume (mL)	2.7 ± 1.9	3.4 ± 2.2	1.8 ± 0.9	2.3 ± 1.5	0.13
Sperm concentration (mil/mL)	47.7 ± 34.5	41.6 ± 31.4	64.8 ± 52.2	47.4 ± 29.8	0.38
Total sperm count (mil/ejaculate)	120.1 ± 95.9	132.3 ± 94.3	116 ± 86.4	109.5 ± 104.9	0.80
Motility (%)	41 ± 26	42 ± 28	41 ± 21	39 ± 28	0.95
Progressive motility (%)	16 ± 12	18 ± 13	16 ± 11	15 ± 12	0.82
Total motile count (mil/ejaculate)	63.8 ± 66.9	70 ± 67.2	60.7 ± 55.4	58.8 ± 73.8	0.89

Note that two participants with type 2 diabetes were unable to provide semen samples, resulting in $n=16$ for this analysis

Relationship between BMI, glycemic control, reproductive hormones, and sperm parameters

We tested for associations between metabolic variables (including age, HbA1c, fructosamine, BMI, adiponectin, fat percentage, and fat free mass) and reproductive variables using Spearman's rank correlation (heat map, Fig. 1).

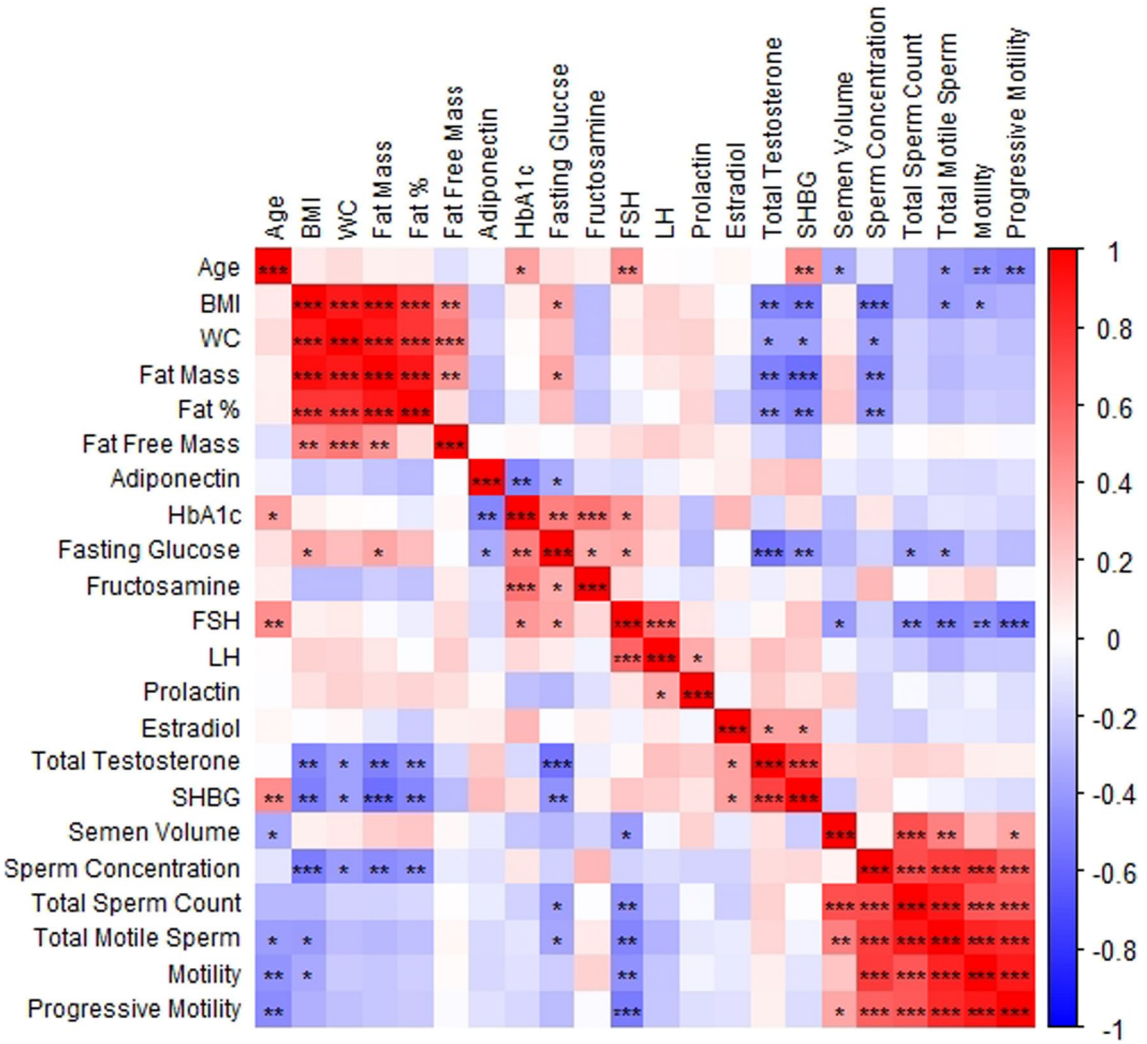

Fig. 1 Associations of metabolic factors, reproductive hormones, and sperm characteristics. Heat map demonstrating results of Spearman's rank correlation analysis of association between metabolic factors and reproductive hormones in study population ($n=16$ control, 6 prediabetes, 18 type 2 diabetes for metabolic and hormonal factors; $n=16$ control, 6 prediabetes, and 16 type 2 diabetes for sperm parameters). *$p<0.05$, **$p<0.01$, *** $p<0.001$

Total testosterone, SHBG, sperm concentration, motility, and total motile sperm were inversely associated with BMI ($p < 0.05$ for all). FSH was positively associated with HbA1c ($\rho = 0.44$, $p = 0.005$) and age ($\rho = 0.49$, $p = 0.002$), while estradiol was also associated with HbA1c ($\rho = 0.36$, $p = 0.027$) (Fig. 1). Linear regression analysis for baseline parameters and change in glycemic control, both raw and age-adjusted, is provided in Table 4.

DNA methylation analysis

Paired semen samples (collected 3 months apart) for DNA methylation analysis were available for nine participants with diabetes and seven BMI-matched controls. Demographic and clinical characteristics for this subset are shown in Table S1 and were similar to the entire cohort. All samples had sperm purity > 98%.

DNA methylation is stable within an individual over 3 months

The DNA methylation pattern in sperm samples demonstrated the expected bimodal distributions, quantified using both the beta and *M* values (Figure S2A/B).

Sample pairs (visit 1 at baseline and visit 2 at 3 months) from an individual participant clustered together in principal components analysis, but T2D did not clearly separate participants (Figure S3). Likewise, DNA methylation showed high stability over time, with no loci reaching array-wide significance for differential methylation in a single individual between baseline and 3 months by

Table 4 Linear regression analysis of metabolic factors and $\log_2$ adjusted reproductive hormones and sperm parameters in whole study population ($n = 38$ for sperm parameters, $n = 40$ for hormones, and $n = 23$ for change in HbA1c)

	Raw				Age-Adjusted			
	HbA1c	**Change in HbA1c**	**Fructosamine**	**BMI**	**HbA1c**	**Change in A1c**	**Fructosamine**	**BMI**
FSH (mIU/mL)	**0.21 (0.03)**	0.48 (0.11)	0.001 (0.41)	0.01 (0.81)	0.11 (0.24)	0.54 (0.06)	0.0004 (0.79)	−0.004 (0.86)
LH (mIU/ml)	0.08 (0.29)	−0.03 (0.91)	−0.0003 (0.86)	0.03 (0.14)	0.08 (0.37)	−0.001 (0.99)	−0.0004 (0.78)	0.03 (0.16)
Estradiol (pg/ml)	0.08 (0.11)	−0.04 (0.76)	0.002 (0.07)	−0.002 (0.89)	0.08 (0.16)	−0.01 (0.91)	0.002 (0.09)	−0.003 (0.80)
Total Testosterone (ng/dL)	−0.10 (0.15)	0.19 (0.17)	0.001 (0.50)	**−0.05 (0.001)**	−0.10 (0.21)	0.18 (0.19)	0.001 (0.42)	**−0.05 (0.002)**
SHBG (nmol/mL) [a]	0.52 (0.70)	−0.29 (0.92)	0.03 (0.30)	**−0.78 (0.01)**	−1.02 (0.44)	0.64 (0.81)	0.01 (0.56)	**−0.92 (< 0.01)**
Ejaculate Volume (mL)	−0.17 (0.21)	−0.07 (0.76)	−0.003 (0.24)	−0.01 (0.74)	−0.10 (0.47)	−0.14 (0.50)	−0.002 (0.38)	−0.001 (0.97)
Sperm Concentration (M/mL)	0.07 (0.67)	−0.14 (0.60)	0.003 (0.22)	**−0.11 (0.001)**	0.14 (0.42)	−0.16 (0.55)	0.004 (0.15)	**−0.105 (0.001)**
Total Sperm Count (mil/ejaculate)	−0.11 (0.63)	−0.21 (0.53)	0.001 (0.90)	**−0.117 (0.01)**	0.03 (0.90)	−0.30 (0.34)	0.002 (0.63)	**−0.105 (0.02)**
Total Motility (%) [a]	−3.3 (0.30)	−2.7 (0.65)	0.01 (0.91)	**−1.7 (0.01)**	−0.38 (0.90)	−4.6 (0.41)	0.04 (0.47)	**−1.5 (0.01)**
Total Progressive Motility (%) [a]	−1.7 (0.25)	−3.7 (0.19)	−0.01 (0.74)	**−0.67 (0.03)**	−0.41 (0.78)	−4.4 (0.11)	0.004 (0.86)	−0.54 (0.06)
Total Motile Sperm Number (M) [a]	−6.85 (0.40)	−15.8 (0.38)	0.09 (0.53)	−2.94 (0.09)	−2.39 (0.78)	−19.3 (0.28)	0.14 (0.32)	−2.50 (0.14)
Adiponectin (ug/mL)	−0.15 (0.07)	−0.18 (0.27)	0.001 (0.37)	−0.002 (0.93)	**−0.18 (0.04)**	−0.18 (0.28)	0.001 (0.40)	−0.002 (0.89)
HDL (mg/dL)	**−0.17 (0.01)**	0.14 (0.16)	−0.0001 (0.88)	−0.01 (0.61)	**−0.14 (0.01)**	0.14 (0.19)	−0.0001 (0.88)	−0.01 (0.61)
HbA1c (%) [a]	–	−0.87 (0.011)	0.009 (0.002)	0.03 (0.39)	–	−0.80 (0.02)	0.01 (0.004)	0.02 (0.58)

Data are presented as beta coefficient (*P* value)

Bolded values indicate statistical significance

[a] Not $\log_2$ transformed

FSH follicle-stimulating hormone, *LH* luteinizing hormone, *SHBG* sex hormone-binding globulin, *HDL* high-density lipoprotein

moderated paired *t*-test analysis in Limma (minimum FDR 0.46). Primary data are provided in Table S2, while Q-Q plots and histograms of significance for the paired comparison are shown in Figure S4A. In parallel, mean glycemic and metabolic parameters (e.g., HbA1c, adiposity, reproductive hormones) remained unchanged over 3 months ($p > 0.05$, paired t test) (Table S1).

DNA methylation patterns are altered in sperm of men with type 2 diabetes

To detect differentially methylated sites between individuals with and without type 2 diabetes, we performed moderated t tests while adjusting for age, given the impact of age on the sperm methylome [13]. We treated samples obtained from the same participant at two visits as repeated measurements. Primary data are provided in Table S3, while Q-Q plots and p value histograms for the comparison between groups are presented in Figure S4B. The genomic inflation factor for this comparison is $\lambda = 0.94$, which is a slight deflation of the median squared z-score. A total of 655 of 745,840 CpG sites were differentially methylated in type 2 diabetes versus control participants (FDR < 0.05). 96.5% of differentially methylated CpG sites had higher methylation in type 2 diabetes (as seen in the probes colored red in Fig. 2A), with a mean + SD per-probe difference in beta values of 0.16 ± 0.10 (16 ± 10%). Probes with higher methylation in type 2 diabetes generally had intermediate-range methylation in controls. A heat map showing *M* values for differentially methylated probes (FDR < 0.05) in individuals without and with type 2 diabetes is shown in Fig. 2B.

We sought to compare these results with the whole-genome bisulfite sequencing data of Chen et al. [27] to test replication of the differentially methylated sites that were in common to the platforms, so we re-analyzed the data of Chen et al. (GEO: GSE138598) to obtain per-site statistics. Consistent with their report, our analysis of their data confirmed more sites with reduced methylation in T2D. Using FDR threshold of 5%, 61% of the significant sites had reduced methylation in T2D. However, of the 25,114 sites that were common to both datasets, 51% had increased methylation

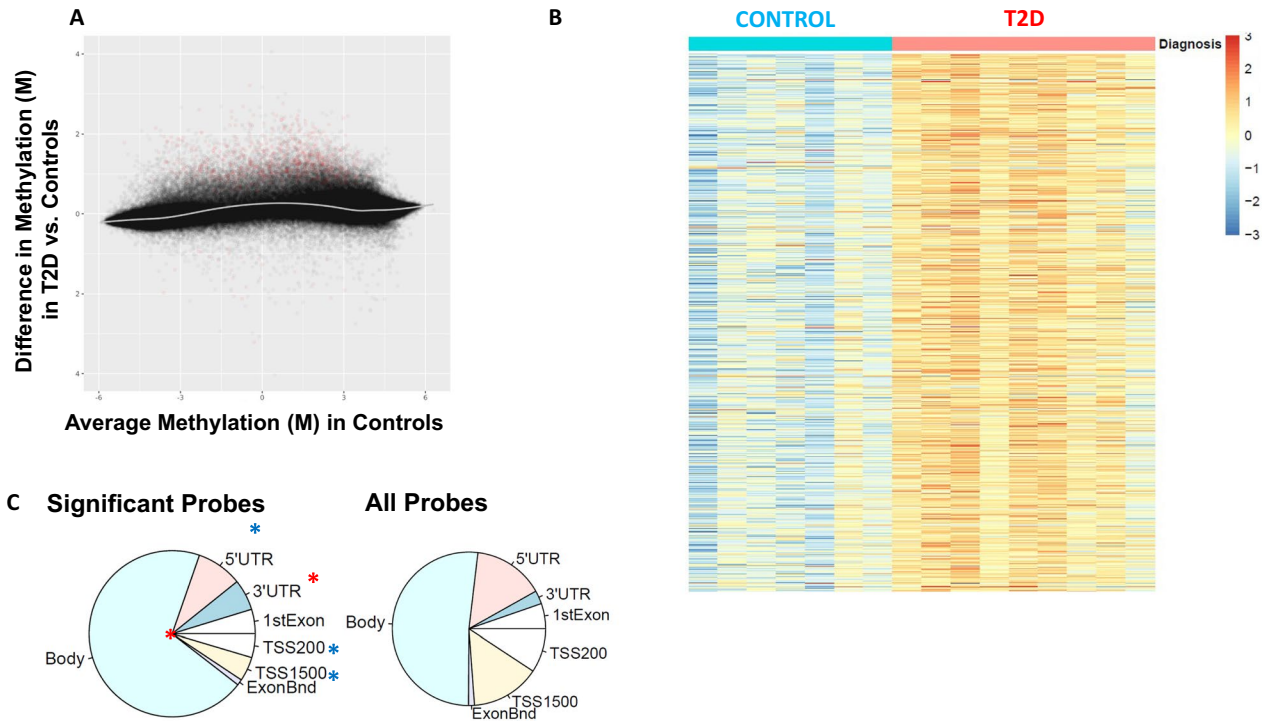

Fig. 2 Epigenome-wide analysis of CpG methylation in individuals with TYPE 2 DIABETES versus BMI-matched controls. **A** Differences in methylation between participants type 2 diabetes versus controls (difference in *M*, *y*-axis) according to levels of methylation in controls at baseline (*x*-axis). Red dots denote loci with FDR < 0.05. **B** Epigenome-wide comparison of CpG methylation (*M* values) in individuals without (blue on top row) and with type 2 diabetes (red on top row). Each column represents a single participant; methylation data from 2 visits per participant were averaged for visualization. Heatmap includes loci with FDR < 0.05 for the comparison between type 2 diabetes versus control groups. **C** Annotation of annotated CpG sites with different methylation in type 2 diabetes (left, $n = 655$ FDR < 0.05), as compared with all sites on the array (right, $n = 739{,}208$). TSS: transcription start site, UTR: untranslated region. * indicates $p < 0.05$ (proportion test) for the comparison of differentially methylated sites in T2D versus all sites on the array. Enrichment is significant within 3'UTR ($p = 6.1\text{E}{-}07$) and gene body ($p = 2.33\text{E}{-}21$) with relative depletion at 5' UTR ($p = 8.95\text{E}{-}6$) and 1500 and 200 bp promoter regions ($p = 2.88\text{E-}5$ and $2.98\ \text{E}{-}13$, respectively)

in T2D in Chen et al.; likewise, in our data 57% of these also had increased methylation in T2D. We next tested if the overlapping sites replicated between datasets using ScreenDMT [40] to find sites that are differential in both studies and in the same direction (e.g., increased methylation in T2D); however, no sites replicated after FDR correction. Lastly, we sought to compare differentially methylated regions between datasets, so we tested for differentially methylated regions in the Chen et al. data using the software dmrseq [39], which accounts for the correlation of methylation between nearby sites and provides stringent FDR control; however, no regions were found to be significant by FDR.

The genomic localization of differentially methylated loci in type 2 diabetes (FDR < 0.05) was not uniformly distributed (Fig. 2C). Differentially methylated loci were enriched at gene bodies ($p = 2.33 \times 10^{-21}$) and 3' UTR ($p = 6.07 \times 10^{-7}$) and depleted at 5' UTR ($p = 8.95 \times 10^{-6}$) and transcription start sites defined by 200 bp and 1500 kb promoters ($p = 2.88 \times 10^{-5}$ and 2.98×10^{-13}, respectively).

We next examined gene ontology for probes significantly different between type 2 diabetes and control (missMethyl analysis tool using probes with FDR < 0.05 as input). While no pathways reached FDR-adjusted significance, top-ranking pathways for each ontology class (nominal $p < 0.05$) included synaptic signaling, actin cytoskeleton, cAMP-dependent pathways, and G protein-coupled receptor pathways (Figure S5).

Since DNA methylation is an important mediator of genomic imprinting, we assessed whether type 2 diabetes was associated with differential methylation at imprinted loci (53 paternally and 71 maternally expressed genes, defined by the Geneimprint database). One paternally expressed gene (*DSCAM*) and two maternally expressed genes (*ADAMTS16, ATP10A*) had significantly higher methylation in type 2 diabetes (FDR < 0.05, change in beta > 0.2). For example, of the 78 sites annotated to the *ADAMTS16* gene, 9 had significantly greater methylation in type 2 diabetes, all located within the gene body (Figure S6).

Differentially methylated probes were located across all chromosomes (Manhattan plot, Figure S7). Regions with differential DNA methylation between control and type 2 diabetes sperm were identified using comb-p [36, 37]. Eighty regions had differential methylation between control and type 2 diabetes (corrected $p < 0.05$). None of the regions with differential DNA methylation overlapped with enhancer regions in human sperm, as defined by Enhancer Atlas; likewise, HOMER tool analysis revealed no significant enrichment of transcription factor binding sites within differentially methylated regions.

Association between hemoglobin A1c, age, and DNA methylation

We hypothesized that differences in sperm DNA methylation between individuals with and without type 2 diabetes may be related to glycemic control. We therefore analyzed associations between DNA methylation at all sites and HbA1c or fructosamine (multivariable linear regression). No probes were significantly associated with HbA1c, HbA1c adjusted for age, or HbA1c adjusted for age and BMI (FDR < 0.05). Since our paired study design included repeat visits for the same individual over 3 months, we also performed linear regression to test whether longitudinal changes in glycemic control were associated with change in DNA methylation between visits 3 months apart (V2–V1). Change in HbA1c or fructosamine was not associated with change in methylation for any probes (none with FDR < 0.05).

Given that glycemic control was not independently associated with DNA methylation in our cohort, we examined associations between DNA methylation and other key variables altered in participants with type 2 diabetes, including age, BMI, lipids, and adiponectin. Only age was significantly associated with DNA methylation in sperm (2382 probes with FDR < 0.05; heat map in Figure S8). Interestingly, probes significantly differentially methylated in type 2 diabetes versus control overlapped minimally with those associated with age. By contrast, 24% of probes differentially methylated in type 2 diabetes overlapped with probes associated with HbA1c. Together, these results suggest that additional, as yet unidentified, factors beyond glycemia or age may contribute to observed differences between groups.

Discussion

We report an analysis of reproductive hormones, sperm quality, and sperm DNA methylation from men with type 2 diabetes, compared with controls of similar BMI (Fig. 3). Type 2 diabetes did not affect sperm concentration or motility, as compared with prediabetes or normoglycemia, at similar BMI. However, we did identify alterations in DNA methylation, with higher levels of methylation in sperm of men with type 2 diabetes compared to controls.

Association between diabetes, sperm, and reproductive hormones

We did not find differences in sperm parameters (total sperm count, concentration, and motility) between controls and individuals with prediabetes or type 2 diabetes. By contrast, a prior meta-analysis including five studies ($n = 719$ with type 2 diabetes; $n = 369$ controls) concluded that type 2 diabetes was associated with reduced semen volume and motility [43]. However, potential

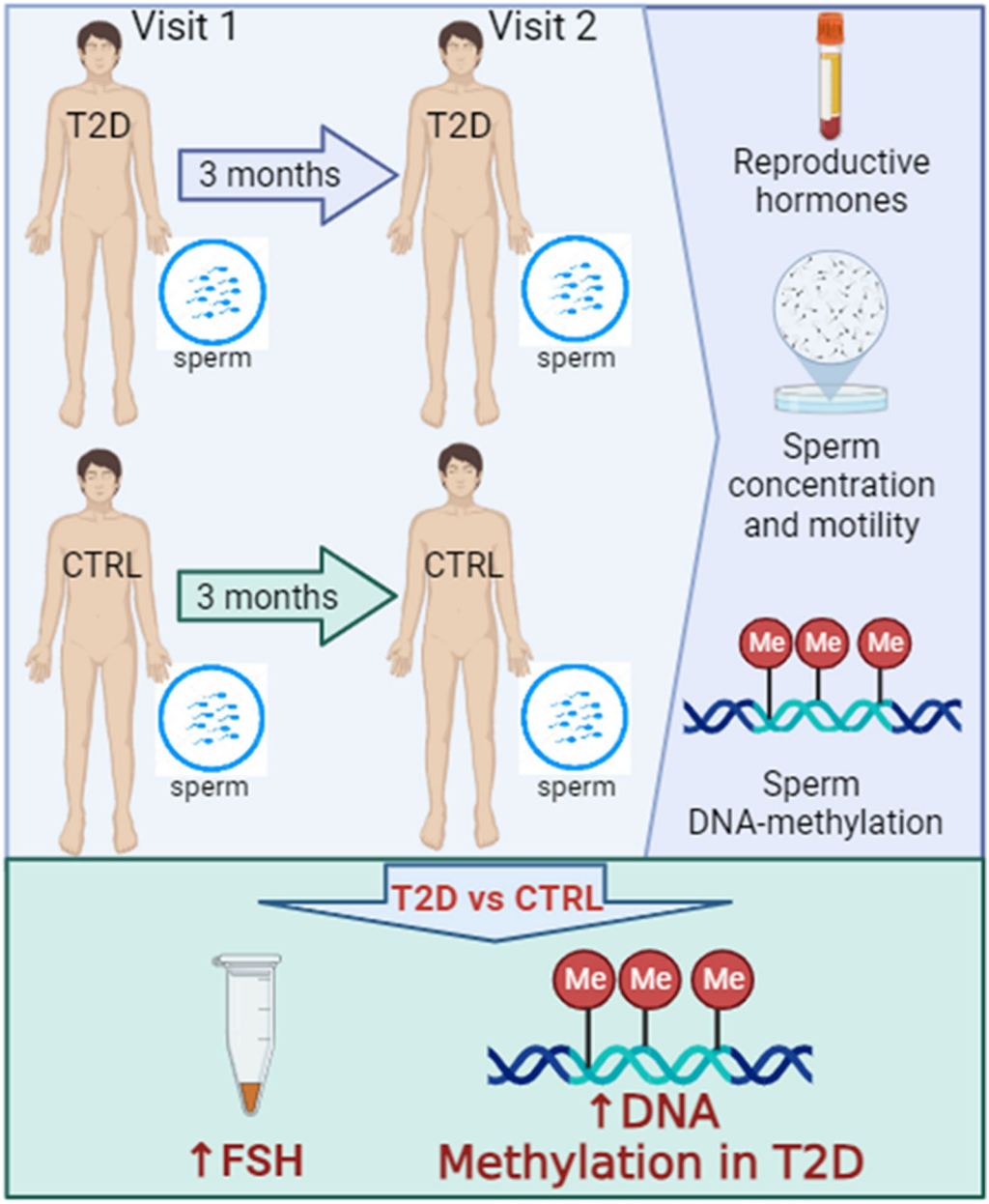

Fig. 3 Graphical abstract

confounders such as HbA1c and BMI were not reported in most studies, and two of the studies were drawn from infertility clinics. While diabetes did not affect sperm parameters in our population, it remains possible that type 2 diabetes may impact spermatogenesis in younger adults without obesity [2].

We did observe a positive association between HbA1c and estradiol, and a modest increase in FSH—within the normal range—in participants with type 2 diabetes, potentially related to the higher age in this group [44]. We also observed an inverse association of BMI with reproductive hormones (testosterone, SHBG), consistent with reported effects of obesity on the reproductive hormone axis. Prior studies have shown that obesity is associated with decreased total sperm number and reduced motility [45, 46]. Thus, our results suggest obesity, but not type 2 diabetes, is associated with reduced sperm quality and reproductive hormones.

DNA methylation is altered in sperm of men with type 2 diabetes

Cytosine DNA methylation (the presence of 5-methylcytosine, 5mC) changes dynamically during development and in response to nutritional and environmental exposures [47]. Our analysis demonstrated stability of sperm DNA methylation in individual men over 3 months. However, we observed differences in DNA methylation in sperm from those with type 2 diabetes versus controls (FDR < 0.05). The vast majority of these differentially methylated CpG had higher methylation levels in type 2 diabetes, which contrasts with Chen et al., who found more hypomethylation in T2D. However, when we analyzed only the sites common to both datasets, we observed that 51% of these sites in Chen et al. had increased methylation in T2D, a value similar to the 57% of the overlapping sites with increased methylation in T2D in our dataset. This analysis suggests that the different patterns are likely to reflect in part the different methodologies (whole-genome bisulfite sequencing versus methylation array) utilized to assess DNA methylation. Additionally, the cohort of Chen et al. had distinct clinical characteristics, including severe degrees of hyperglycemia; resolving this contrast will require future studies.

In our data, differential methylation was more likely to occur at loci for which methylation values in controls were intermediate, suggesting that the observed impact of type 2 diabetes was exerted at sites not fully methylated or demethylated, and thus potentially susceptible to environmental stimuli.

Differential DNA methylation in individuals with type 2 diabetes, and partial overlap of differentially methylated sites with those associated with glycemic control, could implicate a potential role for glucose itself or other components of the metabolic milieu. For example, adiponectin and HDL were both associated with HbA1c in our study; while these were not independently associated with DNA methylation, it is possible that other features of the type 2 diabetes/insulin-resistant state could affect sperm DNA methylation, as suggested by reports that methylation of sperm can be influenced by diet [48], including sugar intake [24], and exercise [49]. Likewise, although we adjusted for age in our primary analysis and found that differentially methylated probes in type 2 diabetes overlapped minimally with age-associated probes, it remains possible that higher age of participants with type 2 diabetes could have contributed to differences in methylation. A recent study employing methyl-capture sequencing to compare DNA methylation in sperm from young versus middle-aged males [13] identified 333 genes adjacent to age-related differentially methylated regions; however, only nine of these were shared with our results.

Due to small sample size, we were unable to assess the potential contribution of differences in medication use. For example, statin use was universal in participants with type 2 diabetes, but not in controls. Statin use has been associated with alterations in DNA methylation in blood [50, 51]. However, we did not observe overlap between DNA methylation sites associated with statin use in those cohorts [50, 51] and differentially methylated sites associated with type 2 diabetes in our study. Similarly, anti-diabetes medications such as metformin [52, 53] could also modulate DNA methylation, but regions (in blood) previously reported to be impacted by metformin were not altered by type 2 diabetes (in sperm) in our study. Thus, whether these medications or others could modulate DNA methylation in sperm remains unknown.

Why more differentially methylated CpG sites had higher average methylation in type 2 diabetes (rather than lower methylation) remains an open question. In the testes, dysregulation of systemic metabolism can induce inflammation, alter metabolism of Sertoli cells, and disrupt spermatogenesis in parallel with changes in the epigenome (reviewed in [54]). Diabetes can impact epigenetic regulation, and specifically DNA methylation, in multiple tissues [6]. We hypothesize that diabetes-associated metabolic disturbances, particularly hyperglycemia, may impact the balance between DNA methylation and demethylation and/or influence methyl donor availability. Experiments using human cell lines demonstrate hyperglycemia can downregulate *TET2* protein, leading to reduced DNA hydroxymethylation (5hmC) [55]. Expression of *TET2* can be detected in human sperm (SpermBase) [56]. Likewise, *TET1/2/3* are expressed in a small percentage of all testes-resident cell types (Human Cell Atlas CellxGene platform). Whether TET-dependent mechanisms could underlie increases in DNA methylation will require additional studies measuring DNA methylation and hydroxymethylation levels simultaneously.

With respect to genomic localization, we found that differential methylation in type 2 diabetes is more likely to occur at 3'UTR and gene bodies. While methylation at promoter CpG islands is typically considered repressive, 3'UTR methylation is associated with increased gene expression [57]. Ontology analysis of genes annotated to differentially methylated CpG loci and genomic regions indicates enrichment for synaptic signaling, actin cytoskeleton, and G protein-coupled receptor pathways. This is particularly interesting as Donkin and colleagues [17] reported enrichment for "nervous system development" genes at obesity-associated differentially methylated CpGs. Moreover, the only other published study of the sperm methylome in men with type 2 diabetes also observed that many of the identified differentially

methylated CpG were annotated to genes involved in neurogenesis [27]. Indeed, comparison of our top-ranking ontology terms with those of Chen et al. [27] revealed overlapping terms including organ development, neuron projection, and neurogenesis. Thus, a growing body of studies of men with metabolic disease identify epigenetic dysregulation in sperm at loci annotated to genes regulating neuronal function or development.

Differences in sperm DNA methylation in type 2 diabetes raise the possibility that sperm methylome changes could modulate transcriptional activity at influential loci during spermatogenesis and fetal development. Some methylation sites escape post-fertilization reprogramming of methylation, as best appreciated at loci regulating genomic imprinting. Notably, we found modest differences in methylation at several predicted imprinted loci, including increased methylation at 9 sites within the gene body of ADAMTS16. Interestingly, ADAMTS16 expression in brain is upregulated in F3 offspring of F1 females exposed to the endocrine disrupter bisphenol A [14], raising the possibility that this site is a hot spot for differential methylation and transcriptional dysregulation in response to environmental exposures. Future studies will be required to assess whether these and other imprinted loci are differentially methylated in parallel in human offspring as a function of paternal type 2 diabetes.

Although we only examined DNA methylation, effects of type 2 diabetes likely also extend to additional epigenetic mediators, such as ncRNA species, which have been reported to be altered by high-fat diet (HFD) in mice, and by bariatric surgery in humans [17, 58]. Future studies are necessary to understand the functional implications of diabetes-associated methylation sites identified in our study, particularly with respect to risk of metabolic disease in offspring. Although human data remain limited, higher paternal BMI is linked to changes in offspring DNA methylation in cord blood [19]. Whether these changes are mediated by alterations in the sperm epigenome will be important for future studies. Moreover, it will be important to determine whether diabetes-related epigenetic changes are modifiable with treatment approaches to improve either glycemic control or obesity. If so, normalization of the sperm epigenome could have implications for interrupting vicious cycles of intergenerational metabolic disease.

Our study has several strengths and limitations. Firstly, our study design with repeated sampling allowed us to demonstrate the stability of DNA methylation over time. Secondly, we enrolled BMI-similar controls, allowing us to isolate the impact of type 2 diabetes and glycemic control from obesity. Thirdly, we included only men with normal reproductive hormones. Fourthly, we analyzed DNA methylation in motile sperm, to assess sperm populations more likely to contribute to fertilization. Moreover, our study population had demographic characteristics representative of persons living with type 2 diabetes (i.e., race/ethnicity, BMI), which may increase the generalizability of our findings. Nevertheless, we acknowledge several limitations, including small sample size and lack of replication cohort. While age of participants was higher for those with type 2 diabetes, we adjusted for age in our analysis. There was an imbalance between statin and anti-diabetes medication use, consistent with established medical practice in T2D. Future studies will be required to robustly test associations with glycemia to analyze the impact of specific medication classes (e.g., GLP1R analogues) and to analyze the functional impact of selected differentially methylated loci on transcriptional regulation, including potential impact on offspring.

Conclusions

In conclusion, we identified differential DNA methylation in motile sperm collected from men with type 2 diabetes, with the majority of significant CpG sites showing higher levels of methylation compared to normoglycemic controls. These differences were more likely to occur at sites of intermediate methylation, demonstrating that differences in type 2 diabetes occur at sites not fully methylated or demethylated. Multigenerational experimental studies are needed to assess whether differential methylation is also observed in early post-fertilization embryonic life, and whether these alterations may contribute to differential transcriptional regulation at developmentally relevant loci. Differential sperm methylation may contribute to the impact of type 2 diabetes and other aspects of paternal metabolism on offspring metabolic phenotypes.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13148-025-01853-9.

Additional file1 (DOCX 705 KB)

Additional file2 (CSV 169985 KB)

Additional file3 (CSV 169882 KB)

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Author contributions

L.S., J.M.D., E.I., R.F. B. and M.E.P. collected the data and wrote the main manuscript text. L.S., V.E. and J.D. collected sperm and extracted DNA. J.P. performed the semen CASA analysis. E.S.G. and C.R. supervised the CASA analysis. G.H., L.R., and C.C. recruited participants and performed study visit procedures. D.W.

performed the association analyses and array data-preprocessing. L.R. and C.C. coordinated the study. R.F. and M.F.H performed the array data-preprocessing. J.M.D and H.P. performed the DNA methylation analysis. A.F.S. and A.G. contributed to study design, manuscript editing, and data analysis. All authors reviewed the manuscript, approved submission, and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Availability of data and materials
Data is provided within the supplementary information files. Primary data for each research participant will be provided via dbGAP (in progress).

Declarations

Competing interests
The authors declare no competing interests.

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