

Epigenetic And Transcriptomic Alterations in Offspring Born To Women With Type 1 Diabetes (The EPICOM Study)

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Abstract

Background: Offspring born to women with pregestational type 1 diabetes (T1DM) are exposed to an intrauterine hyperglycemic milieu and has an increased risk of metabolic disease in later in life. In this present study we hypothesize that in utero exposure to T1DM alters offspring DNA methylation and gene expression, thereby altering their risk of future disease.

Design: Follow-up study using data from the Epigenetic, Genetic and Environmental Effects on Growth, Metabolism and Cognitive Functions in Offspring of Women with Type 1 Diabetes (EPICOM) collected between 2012-2013.

Setting: Exploratory sub study using data from the nationwide EPICOM study.

Participants: Adolescent offspring born to women with T1DM (n=20) and controls (n=20) matched on age, sex and postal code.

Main outcome measures: This study investigates DNA methylation using the 450K-Illumina Infinium assay® and RNA expression (RNA sequencing) of leucocytes from peripheral blood samples.

Results: We identified 9 hypermethylated and 5 hypomethylated positions ($p < 0.005$, $|\text{DM-value}| > 1$). RNA expression profiling identified 38 up- and 1 down-regulated genes ($p < 0.005$, $\log_2\text{FC} \geq 0.3$). Functional enrichment analysis revealed enrichment in ontologies related to diabetes, carbohydrate and glucose metabolism, pathways including MAPK1/MAPK3 and MAPK family signaling and genes related to T1DM, obesity and atherosclerosis. Lastly, by integrating the DNA methylation and RNA expression data, we identified six genes where relevant methylation changes corresponded with RNA expression (*CIITA*, *TPM1*, *PXN*, *ST8SIA1*, *LIPA*, *DAXX*).

Conclusions: Findings suggest the possibility for intrauterine hyperglycemia to impact later life methylation and gene expression, a profile that may be linked to the increased risk of metabolic disease.

Introduction

The “Developmental Origins of Health And Disease” hypothesis describes how an adverse early life environment may lead to increased risk of adult disease¹. Offspring born to women with pregestational type 1 diabetes (T1DM) are exposed to a hyperglycemic intrauterine milieu and recent studies have shown an increased risk of developing type 2 diabetes, cardiovascular disease and obesity²⁻⁴. The pathophysiological mechanisms behind this increased risk in the offspring are unclear, and whether a link to intrauterine T1DM exposure exists remains to be elucidated. Epigenetic modifications have been proposed as a possible mechanism by which maternal diabetes induces long-term effects⁵. However, our knowledge on epigenetic programming of offspring born to women with T1DM is still limited.

Methylation of cytosine is the best-studied epigenetic mark, with the addition of a methyl group to the C5 position of the cytosine ring, and generally occurs on cytosines followed by a guanine (CpG). Genomic regions with an overrepresentation of CpG sites are termed CpG islands and are found at 60-70% of human gene promoters⁶. Hypermethylation of CpG islands is involved in transcriptional repression, thus, changes in DNA methylation adds level of regulation to the DNA molecule without changing the DNA sequence and via this mechanism DNA transcription can be altered⁷. Even though CpG sites are more sparse in gene bodies than in promoters, studies have described CpG hypermethylation in gene bodies to positively correlate with RNA expression⁸. Changes in DNA methylation is well known to be involved in cell differentiation and embryogenesis and is considered to be part of developmental biology⁹. Besides CpG methylation, epigenetic alterations also include histone modifications and non-coding RNA⁵.

Recent studies in cord blood and peripheral blood have linked especially maternal overweight but also, to a lesser degree, maternal gestational diabetes to offspring epigenetic alterations¹⁰⁻¹². Few studies have evaluated the impact of maternal T1DM on offspring epigenetic changes, and included studies exploring an adult cohort including both offspring born to women with gestational diabetes and T1DM, using muscle, adipose tissue or preadipocytes, described increased expression of microRNA-15a and microRNA-15b in skeletal muscle tissue and decreased leptin promoter methylation in subcutaneous adipocytes in offspring of T1DM women¹²⁻¹⁶. Only one study by Gaultier et al. explored the more genome-wide DNA methylation profile using Illumina's Human Methylation 27 Beadchip in describing an association between intrauterine exposure to maternal T1DM and kidney function in the adult offspring¹⁷.

In this study we hypothesized that in utero exposure to maternal T1DM alters global DNA methylation and RNA expression and thereby alters the offspring's risk of later disease development.

Results

Through medical records and the original Diabetes Association registry we retrieved information regarding pregnancy and birth for the majority of both Index and Controls (Table 1). The Index and Controls included in the exploratory cohort were similar on most parameters including BMI, oral glucose tolerance test (OGTT) fasting plasma glucose, OGTT 120 min plasma glucose, fasting insulin, HOMA-IR, HbA_{1c}, systolic and diastolic blood pressure and birth weight, although significant differences were present in some of these parameters in the larger EPICOM group¹⁸. Only gestational age at birth was significantly different for the intrauterine T1DM exposed children compared to the controls (at that time it was common practice to induce labor ~2-3 weeks prior to the expected delivery date in women with diabetes) (Gestational age: 259 (range=217–278) days vs. 278 (258–301) days, $p < 0.001$). Also, maternal age at birth, maternal BMI at birth and parity were similar between the women with T1DM and the mothers of the Controls. For most of the women with T1DM we also had knowledge of their glycaemic regulation pregestationally and during the pregnancy. For the RNAseq data one sample from the control cohort was missing. This however, did not change the overall phenotypic results.

Table 1
Baseline characteristics of diabetes exposed children and controls

	Exposed to T1DM	Control children	P-value
Number of children	20	20	
Male sex	10 (50%)	10 (50%)	
Age (years)	17.8 (0.8)	18.1 (0.8)	0.26
Body mass index (kg/m ²)	23.2 (2.6)	23.4 (2.2)	0.81
Oral glucose tolerance test (mmol/L)			
time = 0 min	5.4 (0.4)	5.3 (0.3)	0.60
time = 120 min	6.0 (0.9)	6.4 (0.8)	0.20
S-Insulin (fasting) (pmol/L)	55.8 (17.3)	57.9 (19.8)	0.72
HOMA-IR	2.2 (0.7)	2.3 (0.8)	0.77
Waist circumference (cm)	75.8 (5.6)	76.4 (6.5)	0.77
Hip circumference (cm)	98.5 (7.4)	98.7 (5.3)	0.93
Systolic blood pressure (mmHg)	120.8 (9.6)	120.8 (11.6)	0.99
Diastolic blood pressure (mmHg)	63.4 (8.2)	63.7 (6.8)	0.89
Total cholesterol (mmol/L)	3.9 (0.7)	4.1 (1.1)	0.55
LDL cholesterol (mmol/L)	2.2 (0.6)	2.3 (1.0)	0.55
HDL cholesterol (mmol/L)	1.4 (0.4)	1.3 (0.4)	0.53
Triglycerides (mmol/L) [‡]	0.8 (0.3-2.5)	0.9 (0.5-1.9)	0.24
HbA _{1c} (%) [mmol/mol]	5.2 (0.2) [33 (2.5)]	5.1 (0.3) [32 (2.8)]	0.15
Gestational age at birth (days)	259 (217–278) (n=18)	278 (258 – 301) (n=17)	< 0.001*
Birth weight (g)	3,482 (711) (n=19)	3,571 (542) (n=18)	0.68
Maternal Characteristics			
Maternal age at birth (years)	28.6 (3.8) (n=20)	29.8 (3.7) (n=20)	0.30
Maternal BMI at birth (kg/m ²)	22.9 (2.3) (n=16)	24.2 (4.4) (n=17)	0.47*
Parity #	1.5 (1–3) (n=20)	1.8 (1–4) (n=18)	0.55*
Pre-eclampsia	5 (26%) (n=19)	2 (12%) (n=17)	0.41 [†]
Maternal HbA _{1c} (%) [mmol/mol]			
Pregestational	7.4 (1.3) [57] (n=18)	(–)	
1st trimester	7.2 (1.2) [55] (n=17)	(–)	
2nd trimester	6.3 (1.0) [45] (n=19)	(–)	
3rd trimester	6.5 (0.9) [48] (n=18)	(–)	

Differentially methylated positions and regions

Performing whole-blood genome-wide DNA methylation analysis using the 450K-Illumina Infinium assay we identified 14 differentially methylated positions (DMPs) with a $p < 0.005$ and $|\Delta M\text{-value}| > 1$ (9 hypomethylated and 5 hypermethylated) between

Index and Controls (Figure 1 and Supplemental Table S1 online) However, after Bonferroni correction, no single DMP reached the threshold of a family wise error rate (FWER) (< 0.05). Differentially methylated regions (DMRs) defined as methylation in groups of nearby positions have been proposed to be involved in transcriptional regulation. Therefore, we extended our analysis to the regional level. However, no DMRs were identified applying a Benjamini-Hochberg false detection rate (FDR) < 0.05 .

RNA expression

Subsequently, we analysed gene expression by RNAseq to test if any differentially expressed genes (DEGs), coding or non-coding RNA, were present when comparing our two cohort groups (Index vs. Controls). We identified 39 coding DEGs (38 up regulated and 1 down regulated) (Table 2, Figure 2a+b and Supplemental Figure S1 online) ($p < 0.005$ and a $\log_2FC \geq 0.3$). In addition, we also found 20 non-coding DEGs ((7 up regulated tags and 13 downregulated tags) ($p < 0.005$ and a $\log_2FC \geq 0.3$) (Supplemental Table S2 online). No DEGs reached an FDR < 0.05 .

Table 2

List of differentially expressed autosomal coding genes between type 1 diabetes exposed offspring and controls.

Gene-ID	Log2Fold Change	Log CPM	P-value	Chromosome Name	Start Position	End Position	External Gene Name
ENSG00000103148.11	1.402	4.682	0.0003637	16	134273	188859	<i>NPRL3</i>
ENSG00000036448.5	1.238	3.216	0.001291	8	1993155	2113475	<i>MYOM2</i>
ENSG00000163735.6	0.778	3.821	0.0003347	4	74861359	74864496	<i>CXCL5</i>
ENSG00000120885.15	0.754	3.931	7.9319E-05	8	27454434	27472548	<i>CLU</i>
ENSG00000259207.3	0.729	5.136	0.001563	17	45331212	45421658	<i>ITGB3</i>
ENSG00000005961.13	0.696	3.951	0.003207	17	42449548	42466873	<i>ITGA2B</i>
ENSG00000184922.9	0.687	5.329	0.004019	17	43298811	43324687	<i>FMNL1</i>
ENSG00000161911.7	0.682	1.937	0.001432	6	41117080	41122075	<i>TREML1</i>
ENSG00000113140.6	0.670	4.702	0.0003625	5	151040657	151066726	<i>SPARC</i>
ENSG00000175746.4	0.670	2.299	0.003372	15	39542885	39547046	<i>C15orf54</i>
ENSG00000138798.7	0.650	3.438	0.0008035	4	110834040	110933422	<i>EGF</i>
ENSG00000122786.15	0.642	2.518	0.004715	7	134429003	134655479	<i>CALD1</i>
ENSG00000100385.9	0.610	3.173	0.001113	22	37521878	37571094	<i>IL2RB</i>
ENSG00000082781.7	0.566	3.489	0.002852	3	124480795	124620265	<i>ITGB5</i>
ENSG00000198478.6	0.565	6.232	0.0013672	6	80341000	80413372	<i>SH3BGRL2</i>
ENSG00000168497.4	0.564	6.971	0.001083	2	192699028	192711981	<i>SDPR</i>
ENSG00000151693.5	0.564	2.812	0.0004620	2	9346894	9545812	<i>ASAP2</i>
ENSG00000185909.10	0.549	2.620	0.001593	3	49209044	49213917	<i>KLHDC8B</i>
ENSG00000140022.5	0.547	4.040	4.5483E-05	14	81727000	81902809	<i>STON2</i>
ENSG00000019582.10	0.545	6.485	0.003180	5	149781200	149792492	<i>CD74</i>
ENSG00000101162.3	0.516	7.425	0.001578	20	57594309	57601709	<i>TUBB1</i>
ENSG00000090975.8	0.506	2.549	0.002654	12	123468027	123634562	<i>PITPNM2</i>
ENSG00000095303.10	0.505	5.532	0.0005005	9	125132824	125157982	<i>PTGS1</i>
ENSG00000111644.3	0.497	2.010	0.001323	12	6747241	6756626	<i>ACRBP</i>
ENSG00000169313.9	0.483	3.076	0.003989	3	151055168	151102600	<i>P2RY12</i>
ENSG00000163737.3	0.471	4.875	0.004818	4	74846794	74847841	<i>PF4</i>
ENSG00000141522.7	0.459	3.114	0.003189	17	79825597	79829282	<i>ARHGDI1A</i>
ENSG00000054793.9	0.441	3.264	0.002419	20	50213053	50385173	<i>ATP9A</i>
ENSG00000107438.4	0.440	3.137	0.004217	10	96997329	97050781	<i>PDLIM1</i>
ENSG00000143368.9	0.429	2.080	0.004332	1	149895209	149900236	<i>SF3B4</i>
ENSG00000153071.10	0.419	4.220	0.0003941	5	39371780	39462402	<i>DAB2</i>
ENSG00000067225.13	0.401	6.571	0.0002313	15	72491370	72524164	<i>PKM</i>
ENSG00000143537.9	0.399	2.580	0.001539	1	155023042	155035252	<i>ADAM15</i>

Gene-ID	Log2Fold Change	Log CPM	P-value	Chromosome Name	Start Position	End Position	External Gene Name
ENSG00000146192.10	0.387	5.061	0.003886	6	36973422	36996846	<i>FGD2</i>
ENSG00000177830.13	0.361	2.254	0.002612	11	867357	915058	<i>CHID1</i>
ENSG00000157823.12	0.339	3.560	0.004107	15	90373831	90437574	<i>AP3S2</i>
ENSG00000075624.9	0.335	9.389	0.003498	7	5566782	5603415	<i>ACTB</i>
ENSG00000172757.8	0.309	6.236	0.000952	11	65590493	65629497	<i>CFL1</i>
ENSG00000185261.9	-0.383	5.944	0.002067	5	93488671	93954309	<i>KIAA0825</i>

Functional enrichment analysis

To gain mechanistic insight into functional importance of the coding DEGs found above, we used enrichment analysis to associate these changes to disease phenotypes (DO), Biological Processes (GOBP) and biological pathways (REACTOME). We observed enrichment in ontologies relating to acquired metabolic disease, glucose metabolism disease, diabetes mellitus, carbohydrate metabolism disease, disease of metabolism and clustering around genes including *CXCL5*, *EGF* and *SPARC* (DO) (Figure 3). For biological processes and pathways, the analysis revealed enrichment relating to striated muscle tissue development, muscle tissue development, growth and developmental growth (GOBP) and biological pathways (REACTOME) including ECM proteoglycans, RAF/MAP kinase cascade, MAPK1/MAPK3 signaling and MAPK family signaling (Figure 3 and Supplemental Figure S2 online (cnet plot of GOBP)).

Functional enrichment analysis was also carried out with identified DMPs as input. Again, enrichment in ontologies and pathways relating to carbohydrate metabolism and glucose transportation and pathways including Glucagon-like Peptide-1 (GLP1) regulated insulin secretion were in the top-enriched terms (Supplemental Table S3 and S4 online).

Correlation between DNA methylation and gene expression

DNA methylation has been shown to play a regulatory role in mediating gene expression, and if located within a gene promotor, higher levels of methylation usually repress transcriptional expression⁸. To further explore the consequences of altered DNA methylation patterns, we therefore compared the DNA methylation data with our gene expression data (Figure 4 and Supplemental Table S5 online). First, we visualized correlations between changes at the group level. That is, the mean change in methylation level plotted against the mean change in gene expression of the gene annotated to the methylation site. DEGs overlapping with DMPs (annotated to closest gene to the methylation site), between T1DM exposed offspring and controls (p-value < 0.05) were plotted in a scatter plot based on log2FC and ΔM -value.

This analysis revealed that 93 DMPs annotated to 76 DEGs showed an inverse correlation where DNA hypomethylation correlated to an increased RNA expression (Figure 4, lower-right (blue)). On the contrary, DNA hypermethylation correlated to a decreased RNA expression, 14 DMPs annotated to 12 DEGs were found to be inversely correlated (Figure 4, upper-left (red)). Also, 201 DMPs annotated to 129 genes showed a positive correlation between DNA hypermethylation and increased RNA expression. As the second step of our correlation analysis, we performed a Spearman rank correlation for each DMP-DEG pair, using each subject's methylation level at the involved DMPs and expression level (normalized counts) for the corresponding RNA expression data as input. Here, our analysis showed that 2 DMPs and their corresponding genes had significant inverse correlation between DNA hypomethylation and increased RNA expression (p < 0.1) (*CIITA* and *TPM1*, Table 3). DNA hypermethylation of CpG sites localized within the gene body has been described as being associated with increased RNA expression and in our analysis, we found 4 annotated DMPs localized within the gene body and their corresponding genes displaying positive correlation between DNA methylation and RNA expression (p < 0.1) (*PXN*, *ST8SIA1*, *LIPA* and *DAXX*, Table 3).

Table 3
Top candidate genes from the two-step correlation analysis.

	Probe name	Gene region	Relation to CPG island	Gene symbol	<i>rho</i>	P-value	FDR-P-value
DNA hypomethylation and positive RNA expression	cg06871213	Body	Nshore	<i>CIITA</i>	-0.3490	0.03002	0.06005
	cg04194852	TSS1500	Island	<i>TPM1</i>	-0.3237	0.04493	0.42438
DNA hypermethylation and positive RNA expression (located in genebodies)	cg04256697	Body;TSS1500;Body	OpenSea	<i>PXN</i>	0.3933	0.01379	0.05514
	cg24112692	Body	OpenSea	<i>ST8SIA1</i>	0.3686	0.02152	0.26539
	cg13931663	Body;Body	OpenSea	<i>LIPA</i>	0.3020	0.06206	0.56834
	cg20618109	Body;Body;TSS1500;Body;Body	N_Shore	<i>DAXX</i>	0.2909	0.07271	0.581641

Discussion

This exploratory study suggests the existence of epigenetic marks that possibly influence the long-term health of offspring born to women with pregestational T1DM and elucidate intuitive pathways to be involved. Combining these epigenetic marks with gene expression data in offspring exposed to T1DM in utero is novel and provides new insight into the mechanistic consequences of epigenetic changes. However, none of the findings were present after correction for multiple testing.

In a cohort of prospectively studied offspring of women with T1DM we identified 14 DMPs ($p < 0.005$ and $|\Delta M\text{-value}| > 1$), 9 being hypomethylated and 5 hypermethylated, between T1DM exposed offspring and their matched unexposed controls. We also sought to identify any overall differences in RNA expression between index and controls, and were able to identify a set of predominantly up regulated genes ($p < 0.005$ and a $\log_2FC \geq 0.3$).

The functional enrichment analysis showed clustering around the genes *CXCL5*, *EGF* and *SPARC* which is associated with diabetes, obesity and insulin secretion and enriched in disease ontology terms of metabolic disease, diabetes and glucose metabolism disease. These findings seem to fit the hypothesis of altered intrauterine programming of offspring born to women with T1DM^{19–21}. The functional enrichment analysis points towards muscle tissue development and developmental growth to be associated with intrauterine exposure to T1DM and that the MAPK signaling pathway could play a role in the pathogenesis. The link between intrauterine exposure to T1DM and later life alterations in skeletal muscle has previously been described by Houshmand-Oeregaard et al. and even though no studies have described a link between intrauterine exposure to diabetes and later life alterations in the MAPK pathway, the central placement of MAPK in insulin signaling makes it a plausible object for pathogenetic changes^{13,22}.

Our access to both DNA methylation and gene expression data made it possible to explore the impact of epigenetic changes induced by intrauterine T1DM exposure on gene expression. We chose to perform a two-step analysis where we first explored the overall correlation between DMPs and DEGs at group level. During step two, we studied the individual correlation between $\Delta M\text{-value}$ at the involved DMP and \log_2FC for the corresponding DEG. No studies within the area of fetal programming as a consequence of maternal T1DM have to our knowledge, performed a similar linkage study between DNA methylation and RNA expression data. However, in cancer studies similar methodology has been used²³. Applying this method, provided us with six genes where changes in DNA methylation between offspring exposed to maternal T1DM and controls correlated with relevant changes in gene expression (Table 3). *PXN*, which is localized at chromosome 12 has been linked to progression of T1DM and *LIPA* at chromosome 10 to the composition of lipoproteins^{24,25}. Mutations in *TPM1* at chromosome 15 is associated with both the development of the metabolic syndrome and cardiac hypertrophy^{26,27}. *CIITA* is associated with MHC class II (MHCII) expression and MHCII antigen presentation in adipocytes and is reported to trigger early adipose inflammation and insulin resistance as well as inducing changes to energy expenditure in skeletal muscle^{28,29}.

In our study we chose to match our participants so that they did not overtly differ in phenotypic appearance. This approach resulted in a very homogenous group where the major difference was in T1DM exposure status. The matching was an attempt to exclude any phenotypic differences to be the cause of differences in methylation status. However, a consequence of the matching could, in

theory, result in the observed changes in methylation and gene expression as being protective towards the development of later life metabolic disease, as none of the participants in the exploratory cohort had overt metabolic disease in contrast to the participants in the full EPICOM study¹⁸. Also, it is well known that gestational age is associated with DNA-methylation and as gestational age differed between our two cohorts, this could have influenced our findings³⁰.

Epigenetic changes in relation to intrauterine exposure to either nutrition or maternal diabetes has previously been described in animal studies but the results have been difficult to apply to human studies^{31,32}. In recent years methylation analyses of cord blood have shown promising results, but the use of cord blood methylation status as a biomarker of long-term offspring risk of disease development has yet to be fully described^{11,33}.

Here we used leucocytes from peripheral blood from both T1DM exposed and non-exposed offspring. The most likely target tissue for the pathogenesis of long-term consequences of being born to a mother with T1DM may, however, be pancreatic β -cells, and muscle- and fat-tissue. These three tissues are not as readily available and it is debated whether peripheral blood leucocytes reflects global epigenetic changes induced by maternal pregestational T1DM. Studies of tissue-specific differentially methylated regions have shown conservation of DNA methylation patterns across different tissues^{34,35}. However, replication of our findings in target tissues would further strengthen our hypothesis. In our study we did not have access to specific white cell counts for each participant. Instead, we used the EstimateCellCounts algorithm from the minfi package, which enables us to minimize confounding related to cell composition, a strength compared to prior studies within the same area.

The samples used in our study were collected at a mean age of ~18 years. This provides time to develop phenotypic characteristics. However, it also provides the possibility that any epigenetic changes have occurred during the time period from birth to follow-up. In the EPICOM study we didn't have access to cord blood samples and it is therefore not possible for us to state, that the epigenetic findings in our study have been present since birth. Lifestyle and diet in families where the mother has T1DM could be different compared to families without diabetes and this could affect the epigenetic findings^{36,37}.

When interpreting our finding one must consider the fact that no single DMP reached the FWER adjusted value of 0.05. This is probably a consequence of our modest sample size in the exploratory cohort and an obvious limitation of our study. Instead we used the same pragmatic threshold for DMPs as West et al. providing an opportunity to compare findings in related studies³⁸.

The strength of this study is that it was performed on a group of prospectively studied offspring born to women with T1DM. For both diabetes exposed offspring and their matched controls, we have extensive knowledge of intrauterine exposure, birth, neonatal period as well as current health status. Our women with T1DM are well characterized, and no women with T2DM or gestational diabetes were included in our cohort. This enables us to explore associations between both fetal and long-term consequences of being born to mother with T1DM and epigenetic and transcriptomic alterations.

In conclusion, our data indicate intrauterine hyperglycemia to impact later life methylation and gene expression, a profile that may be linked to the increased risk of metabolic disease. However comprehensive follow-up studies using a genome-wide approach and including relevant target tissue is needed.

Methods

The EPICOM cohort:

This study is a part of the EPICOM (Environmental Versus Genetic and Epigenetic Influences on Growth, Metabolism and Cognitive Functions in Offspring of Women with Type 1 Diabetes) study (ClinicalTrials.gov registration no. NCT01559181). From 1992-1999, pregnancies in women with pregestational T1DM were prospectively reported to a registry managed by the Danish Diabetes Association. Information regarding diabetes status and pregnancy outcome was reported to the registry by local obstetricians at eight hospitals in Denmark, who were responsible for antenatal care and delivery for pregnant women with T1DM. Coverage of cases from the reporting centers spanned from 75 to 93%, as described by Jensen et al.³⁹. The Danish Diabetes Association Register consists of 1326 records of children born to women with T1DM. Only children born after 24 completed gestational weeks were registered. As part of the EPICOM study we invited one child per mother (the oldest) to participate in a clinical study concerning

metabolic risk. Controls were recruited from the background population and were matched by sex, age and postal number. Details from this study have previously been described by Vlachova et al. ¹⁸.

Sample inclusion

Among the oldest participants of the EPICOM cohort we selected 20 offspring of women with pregestational T1DM (Index children) and their 20 matched controls (Controls) as our exploratory cohort of DNA methylation and RNA expression (Table 1). Besides fulfilling the matching criteria for inclusion in the EPICOM cohort, we made sure that our exploratory cohort did not differ in body mass index (BMI), glucose tolerance, HbA_{1c}, cholesterol or triglyceride levels and that the index child and their control were age matched. DNA methylation and RNA expression profiling were performed on leucocytes isolated from peripheral blood samples.

DNA isolation and the 450K-Illumina Infinium assay®

EDTA treated peripheral blood samples were collected from the participants when they participated in the EPICOM study and were stored as EDTA treated at -80°C until use. Genomic DNA was extracted from peripheral blood using QIAmp® Mini Kit (Qiagen, Germany). For each sample, 1 µg of genomic DNA was bisulfite-converted using Zymo EZ DNA Methylation Kit according to the manufacturer's recommendations. DNA methylation level was measured using the 450K-Illumina Infinium assay (Illumina, Inc.) at Aros Applied Biotechnology A/S.

(Pre-)processing of the 450K-Illumina Infinium assay data

All analyses were performed in R statistics, version 3.6.1 and R package minfi (version 1.32.0) was used for normalization, analysis and visualization ⁴⁰. Detection p-values were calculated to identify failed positions with a p-value cut-off > 0.01. Probes were removed if they failed in more than 20% of the samples (n = 350). No samples were identified as failed, as the proportion of failed probes did not exceed 1% for any single sample. Density-bean-plots were used to identify outliers and minfi's inbuilt function was used to evaluate data with respect to extreme methylation outliers (> 3 SD away from the median). We performed background normalization and control normalization implementing the pre-processing choices of Genome Studio®. Next, we applied subset-quantile-within-array-normalization correcting for technical differences between Infinium type I and II assay design allowing both within-array and between-sample normalization. Cross reactive probes (n = 29,541), probes with SNPs documented in C or G of the target (n = 18,284) probes on sex chromosomes (n = 12,312) were excluded, leaving 415,009 probes. Methylation values were calculated as M-values (logit [beta]) (Equation (1)) ⁴¹.

$$M\text{-value} = \log_2 \left(\frac{\text{Beta}}{1 - \text{Beta}} \right)$$

Multidimensional scaling plots were evaluated to identify clusters of samples behaving differently than expected. Finally, the probes were annotated to the human genome version 19 using the enhanced Illumina annotation method developed by Price et al. ⁴².

Estimated differential cell counts

We adjusted for differences in cell proportions using minfi's estimateCellCounts that implements Houseman et al.'s regression calibration algorithm ⁴³. This algorithm returns relative proportions of CD4+ and CD8+ T-cells, natural killer cells, monocytes, granulocytes and B-cells in each sample.

Identifying differentially methylated positions

To identify positions where methylation is associated with intrauterine exposure to maternal T1DM we fitted a linear model, which utilizes a generalized least squares model (lmFit of R-package *limma*) allowing for missing values ⁴⁴. Significance was evaluated using F-test. The sample variances were estimated using an empirical Bayes approach with shrinkage towards the means. A Bonferroni adjusted FWER below 0.05 was considered significant. We applied the model both without and with adjustment for estimated relative cell proportions (CD4+ and CD8+ T-cells, natural killer cells, monocytes, granulocytes, and B-cells).

Identifying differentially methylated regions (DMRs)

We used DMRcate to identify any autosomal DMRs⁴⁵. DMRcate identifies and ranks the most differentially methylated regions across the genome based on kernel smoothing of the differential methylation signal. The model performs well on small sample sizes and builds on the well-established *Limma* package, allowing us to incorporate estimated cell proportions as covariates. A FDR below 0.05 with a $|\Delta M\text{-value}| < 1$ was considered significant.

RNA sequencing (RNA-seq) sample preparation

Blood samples were drawn using PAXgene Blood RNA Tubes® and placed 2 hours at room temperature, sequentially stored overnight at -20 degrees before being stored at -80 degrees until analysis.

RNA-Seq library construction and sequencing

Whole transcriptome, strand-specific RNA-Seq libraries were prepared from total-RNA using the Ribo-Zero Globin technology (Illumina, Inc.) for depletion of rRNA and globin mRNA followed by library preparation using the ScriptSeq technology (Illumina, Inc.). Depletion and library preparation were automated on a Sciclone NGS (Caliper, Perkin Elmer) liquid handling robot. The total-RNA (1.7 µg per sample) was subjected to Baseline-ZERO DNase prior to depletion. Total-RNA was purified using Agencourt RNAClean XP Beads before and after DNase treatment followed by on-chip electrophoresis on a LabChip GX (Caliper, Perkin Elmer) and by UV measurements on a NanoQuant (Tecan). Cytoplasmic and mitochondrial rRNA as well as globin mRNA were removed from 400 ng DNase treated total RNA using the Ribo-Zero Globin Gold Kit (Human/Mouse/Rat, Illumina, Inc.) following the manufacturer's instructions and quality of the depleted RNA was estimated on a LabChip GX (Caliper, Perkin Elmer). Synthesis of strand-specific RNA-Seq libraries were conducted using the ScriptSeq v2 kit (Illumina, Inc.) following the recommended procedure, and the qualities of the RNA-Seq libraries were estimated by on-chip electrophoresis (HS Chip, LabChip GX, Caliper, Perkin Elmer) of a 1 µL sample. The DNA concentrations of the libraries were estimated using the KAPA Library Quantification Kit (Kapa Biosystems). The RNA-Seq libraries were multiplexed paired-end sequenced on an NextSeq 500 (75 + 6 + 75 bp) using a high output flowcell.

RNA-Seq analysis

Paired de-multiplexed fastq files were generated using bclfastq (v.2.20 Illumina) and initial quality control was performed using FastQC. Adapter trimming was conducted using the GATK ReadAdaptorTrimmer tool followed by mapping to the human genome (hg19) in addition to transcripts from databases on non-coding RNAs (mirbase, mitranscriptome, rfam, snornabase, tjumima and trnascanse) using Bowtie and then further analyzed using Tophat, and Cufflinks and HTSeq-count (union method)^{46,47}. HTSeq-count (union method) was applied to produce raw counts which were then submitted for differential expression analysis in R using edgeR⁴⁸. All non-informative features were filtered out by removing features with less than one counts per million (CPM) in 39 samples removing 98334 features, leaving 13842 for downstream analysis. A generalized linear model was fitted and p-values and log fold changes (Log2FC) were retrieved from the individual comparisons of Index vs. Controls. A Benjamini-Hochberg FDR below 0.05 was considered significant.

Functional enrichment analysis

To investigate possible biological functions of the RNA expression changes in our cohort, we performed gene set enrichment analysis (GSEA) using Clusterprofiler⁴⁹. The input genes were ranked based on the magnitude of changes in the Index vs. Controls comparison (log2FC). Disease associations were identified by Disease Ontology (DO) enrichment analysis, while Gene Ontology Biological Processes (GOBP) and REACTOME were used for functional and pathway enrichment. The top-enriched terms for each enrichment analysis were sorted according to p-value and presented as barplots. Furthermore, gene-concept networks, that depicts linkages of the genes and biological terms (DO, GOBP and REACTOME terms), were made by evoking the cnetplot function of Clusterprofiler.

For methylation data, GOBP and REACTOME enrichment analysis were performed on all differentially methylated positions ($p < 0.005$) using the methylGSA package for R⁵⁰. The probes were annotated to the human genome version 19 as previously described.

Correlation between DNA methylation and gene expression

DNA methylation has been shown to play an important role in modulating gene expression. Therefore, we correlated changes in methylation and gene expression of the gene closest to the methylation site. Shared differentially expressed genes (DEGs) and DMPs (closest gene to methylation site) between Index and Controls ($p < 0.05$) were plotted in a scatter plot based on log2FC and

Δ M-value. In the second part of the analysis, we used Spearman's rank correlation to analyze the correlation between the specific methylation level (M-value) and RNA expression level (normalized counts) for shared genes. We considered the correlations to be interesting (either negative or positive) if $p < 0.1$.

Analysis software

Statistics was done using R 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria) with Bioconductor 3.9.⁵¹ DNA methylation data was analysed using the *minfi*⁴⁰, *DMRcate*⁴⁵ and *Limma*⁵² package 1.32.0, and RNAseq data using edgeR⁴⁸. Functional gene enrichment analysis was carried out using the R packages ClusterProfiler and MethyIGSA^{49,50}. Graphics were made using basic R functions ggbio, Gviz, DEseq, DEXSeq and ggplot2. The package knitr was used for data documentation.

Declarations

Ethics declarations

All participants provided informed consent. The study was approved by The Danish Data Protection Agency and approved by The Danish National Committee on Health Research Ethics (M-20110239). All clinical investigations were conducted according to the Declaration of Helsinki. The research has been registered at ClinicalTrials.gov (no. NCT01559181).

Data availability

All sequence and methylation data from the EPICOM study are deposited at the EGA MOMA repository (<https://ega-archive.org/dacs/EGAC00001000145>) and will be available upon request within the General Data Protection Regulation.

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JJ is acknowledged for generating Fig. 3 and 4 and Supplementary Fig. S1.

Author contributions

SK, ZL and BB contributed with data collection in the EPICOM cohort. SK, CH, ZL, BB, PD, KH, DMJ, KS, and CHG all contributed substantial to conception and design of the study. SK, AS, JJ, CT, SV and CHG analysed the data, and SK drafted the manuscript and designed the tables. All authors were involved in the interpretation of the data and critically revised the article. All authors had full access to the data in the study and take full responsibility for the integrity of the data and the accuracy of the data analysis. SK is the guarantor of the study and, as such, had full access to all data and affirms that the manuscript is honest, accurate, and transparent and no important aspects of the study have been omitted. The data has not been published previously and is not under consideration for publication elsewhere. The data has partially been presented at the 47th Annual Diabetes and Pregnancy Study Group Meeting.

Additional Information

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Competing interests statement: CHG has given talks for Novo Nordisk (NN). PD is participating in multicenter and multinational clinical studies on the use of insulin in pregnant women with pre-existing diabetes in collaboration with NN; no personal honorarium

is involved. None of the study funders had any role in study design, collection, analysis, interpretation, preparation of the manuscript or in the decision to submit the article for publication.

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Figures

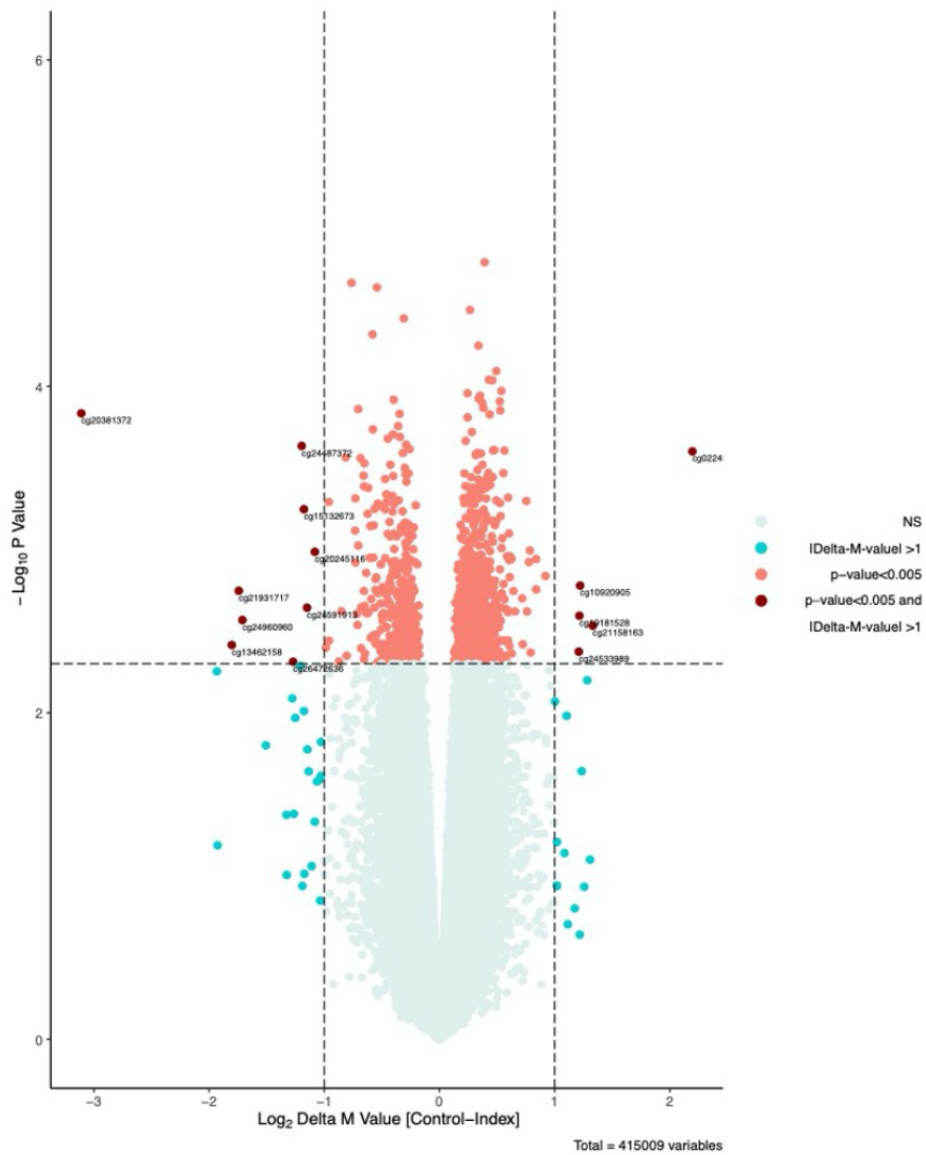


Figure 1

DNA methylation between type 1 diabetes exposed offspring and matched controls. Volcano plot of $-\log_{10}$ p-value against delta-M values of differentially methylated positions. Red dots are DMPs between type 1 diabetes exposed offspring and matched controls with a p-value < 0.005 and an absolute $|\Delta M\text{-value}| > 1$. The horizontal line represents p-value = 0.005.

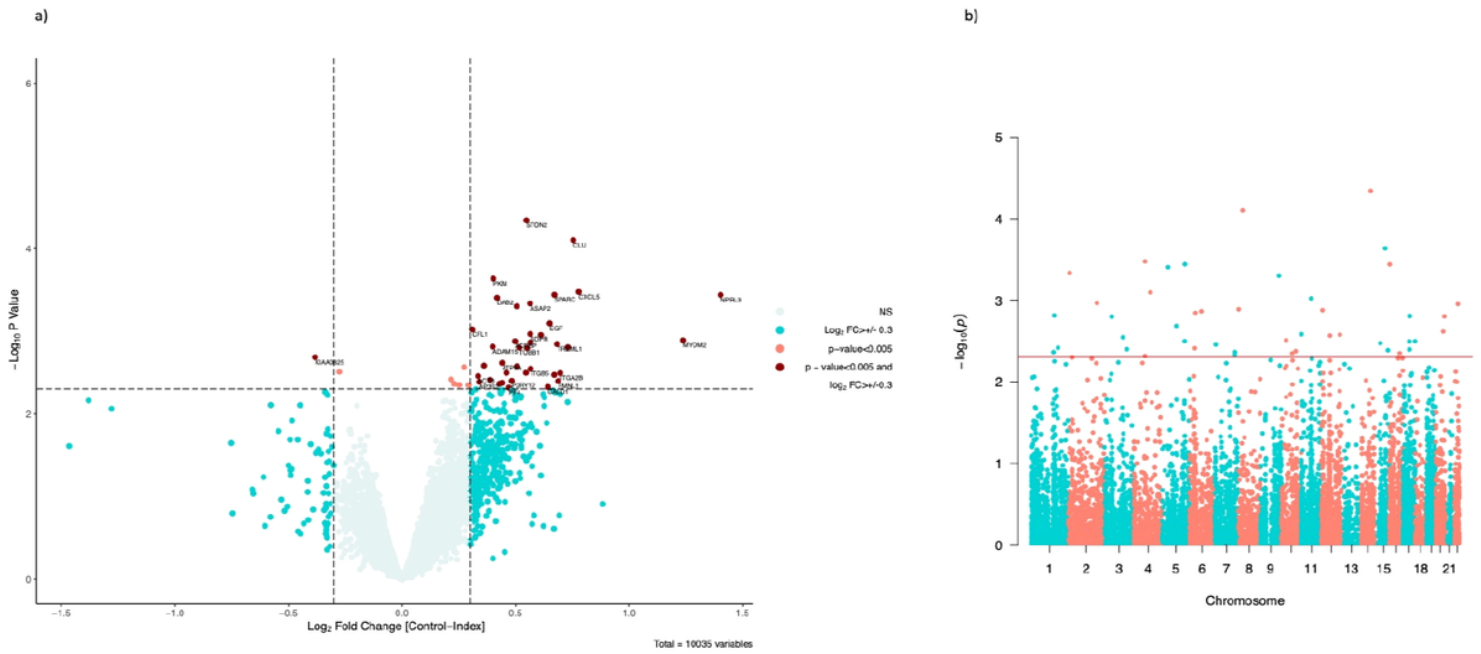


Figure 2

Differentially expressed coding genes between type 1 diabetes exposed offspring and matched controls. (a) Volcano plot of $-\log_{10} p$ -value against \log_2 fold change. Red dots are differentially expressed autosomal coding genes with a $p < 0.005$ and a $\log_2 \text{FC} \geq 0.3$. The horizontal line represents $p = 0.005$. (b) Manhattan plot of chromosomal distribution of differentially expressed genes between type 1 diabetes exposed offspring and matched controls. The red horizontal line illustrates a crude p -value < 0.005 .

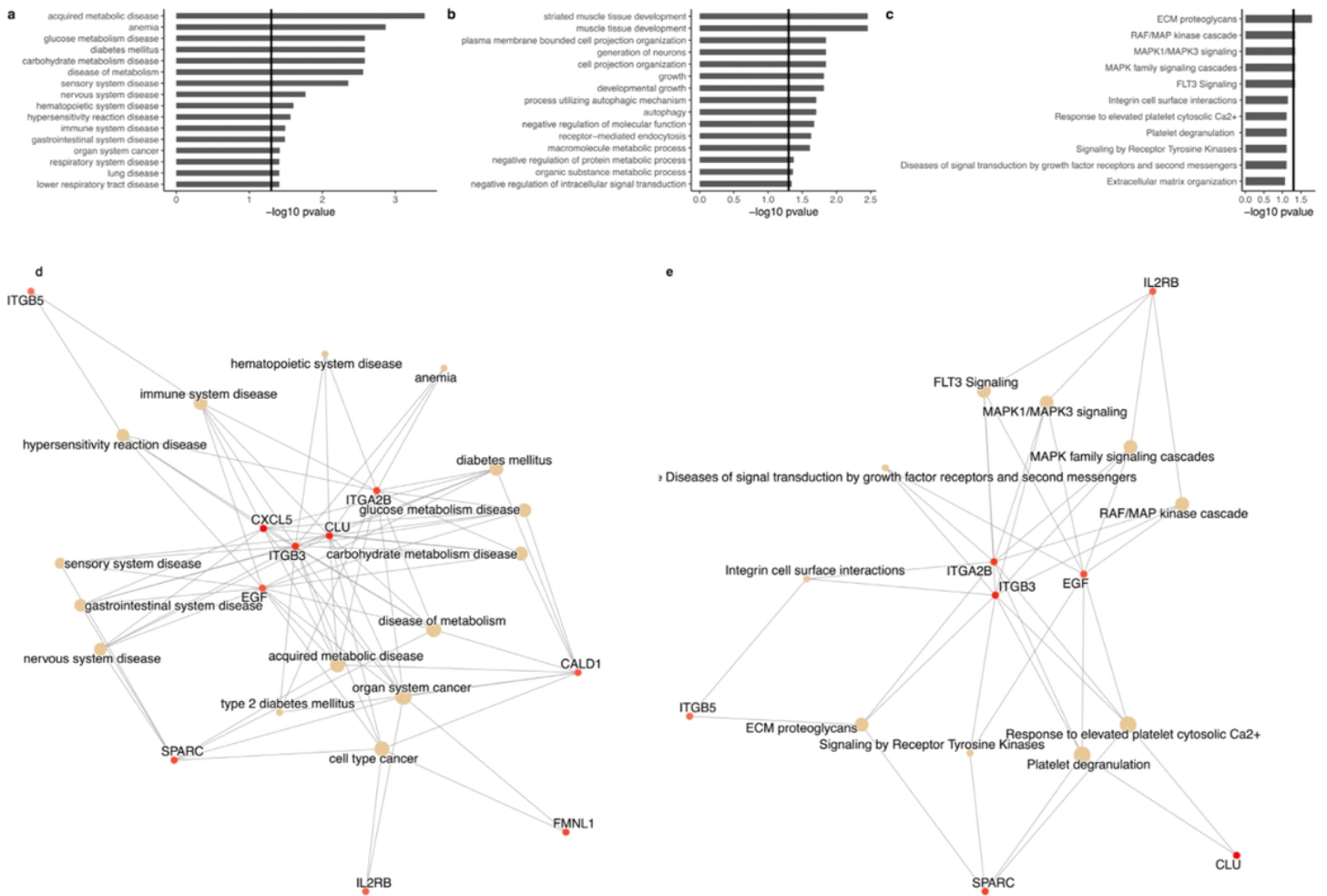


Figure 3

Functional Enrichment Analysis of the gene expression changes observed in type 1 diabetes exposed offspring compared to matched controls. The differentially expressed autosomal coding genes were used as input for gene set enrichment analysis, to identify enriched disease phenotypes (DO) (a), biological processes (GOBP) (b) and biological pathways (REACTOME) (c). The top-enriched terms were sorted according to p-value (vertical black line denotes $p = 0.05$). A network plot (d, e), depicted the genes that were involved in the significant DO terms (e) and REACTOME terms (e).

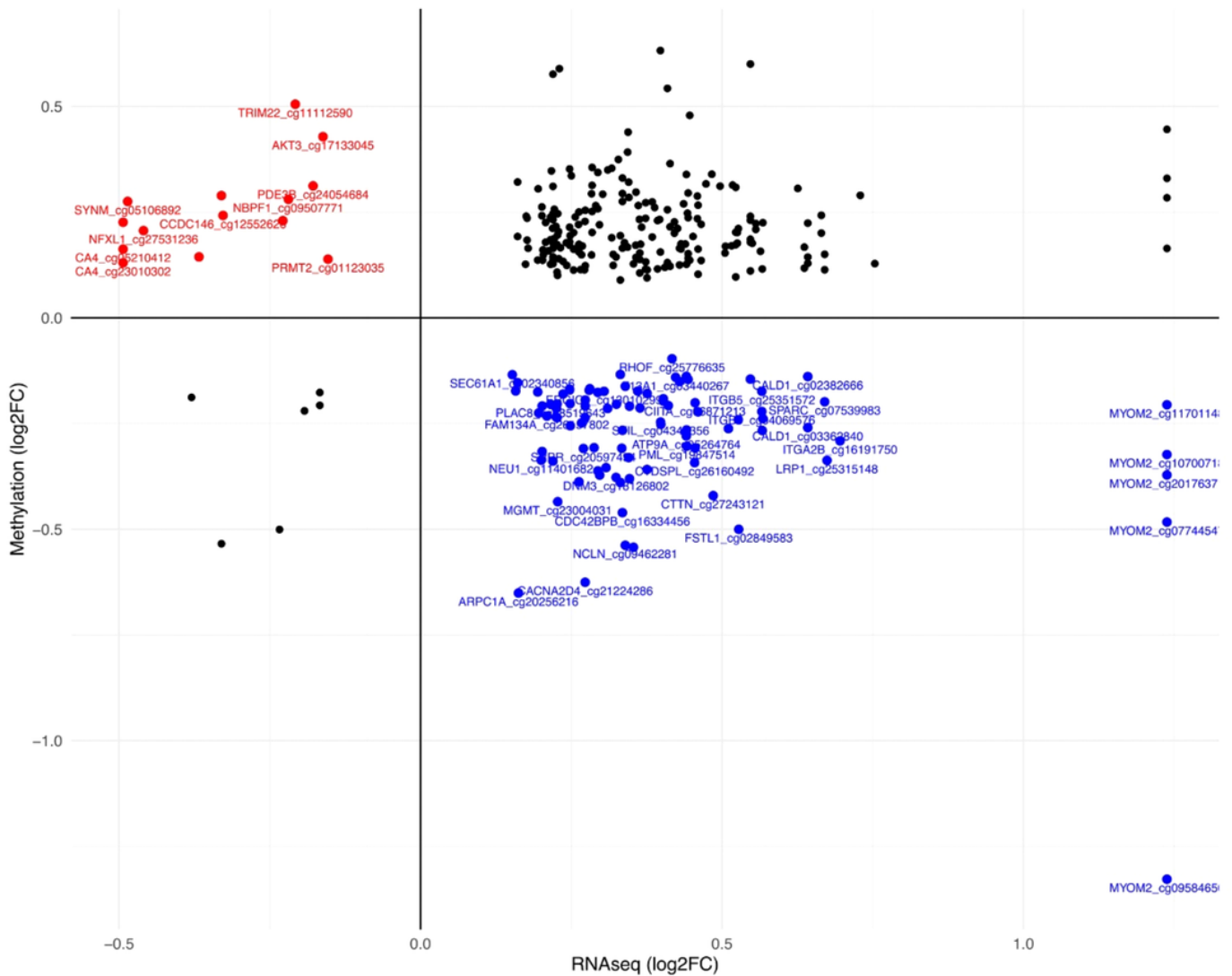


Figure 4

Correlated changes in DNA methylation and gene expression in type 1 diabetes exposed offspring compared to matched controls. Scatter plot depicting mean methylation difference (delta M) versus mean gene expression change (log2FC), of shared DEGs ($p < 0,05$) and DMPs (closest gene to methylation site, $p < 0.05$). Upper-left (red): Increased methylation corresponding to a decreased gene expression. Lower-right (blue): Decreased methylation corresponding to increased gene expression. Upper-right: Increased methylation corresponding to increased gene expression.

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