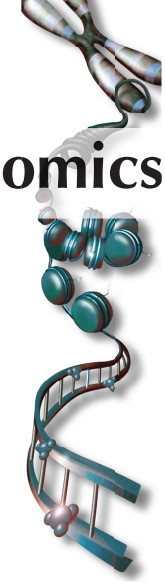
Preliminary Communication

For reprint orders, please contact: reprints@futuremedicine.com

Epigenomics



Epigenetic modification of the pentose phosphate pathway and the IGF-axis in women with gestational diabetes mellitus

Angela Steyn[1], Nigel J Crowther[1,2], Shane A Norris[3], Raquel Rabionet[4], Xavier Estivill[4] & Michèle Ramsay*[,1,5]

[1]Division of Human Genetics, National Health Laboratory Service and the School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, Gauteng, South Africa

[2]The Department of Chemical Pathology, School of Pathology, University of the Witwatersrand, Johannesburg, Gauteng, South Africa

[3]Developmental Pathways for Health Research Unit, University of the Witwatersrand, Johannesburg, Gauteng, South Africa

[4]The Centre for Genomic Regulation, Genes and Diseases Program, Barcelona, Spain

[5]The Sydney Brenner Institute for Molecular Bioscience, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, Gauteng, South Africa

*Author for correspondence: michele.ramsay@wits.ac.za

Aim: Gestational diabetes mellitus (GDM) has been linked with adverse long-term health outcomes for the fetus and mother. These effects may be mediated by epigenetic modifications. **Materials & methods:** Genome-wide RNA sequencing was performed in placental tissue and maternal blood in six GDM and six non-GDM pregnancies. Promoter region DNA methylation was examined for selected genes and correlated with gene expression to examine an epigenetic modulator mechanism. **Results:** Reductions of mRNA expression and increases in promoter methylation were observed for *G6PD* in GDM women, and for genes encoding IGF-binding proteins in GDM-exposed placenta. **Conclusion:** GDM involves epigenetic attenuation of *G6PD*, which may lead to hyperglycemia and oxidative stress, and the IGF-axis, which may modulate fetal macrosomia.

First draft submitted: 20 November 2018; Accepted for publication: 12 June 2019; Published online: 4 October 2019

Keywords: DNA methylation • epigenetics • gestational diabetes mellitus • glucose-6-phosphate dehydrogenase • IGF binding protein • pentose phosphate pathway • RNA sequencing

Changes in adult lifestyle are thought to be the main contributor to the rising prevalence of noncommunicable diseases observed over the past decade [1]. However, events during early development may also play a crucial role in the pathogenesis of chronic conditions [2]. The Developmental Origins of Health and Disease hypothesis suggests that exposure to adverse prenatal and/or neonatal conditions may reprogram the fetus in order to ensure immediate survival [3,4]. However, these adaptions, thought to occur through epigenetic modifications, may enhance disease risk later in life, including Type 2 diabetes (T2D) [5].

Gestational diabetes mellitus (GDM) has been suggested as an appropriate disease model for studying the impact of the fetal environment on gene programming and future disease risk. Gestational diabetes is known to lead to fetal macrosomia and it also increases risk in the offspring of developing obesity, metabolic syndrome, cardiovascular complications and T2D in adult life. Women with GDM also have an increased risk of developing T2D later in life [6–8]. The disease is characterized by high blood glucose levels that first develop during pregnancy and normalize after parturition [9,10]. Although the clinical manifestations and potential implications of GDM for the mother and fetus have been well characterized, the pathogenesis of GDM is still not fully understood [11,12].

The aim of the current study was to investigate the impact of GDM on gene regulation in fetal placental tissue and in blood samples from mothers with GDM. Genes with altered transcription patterns, identified by RNA sequencing, were selected for DNA methylation studies to assess alterations in the epigenome. Using this methodology, we sought to identify complex regulatory changes associated with GDM that may impact fetal (re)programming and give insights into the pathogenic processes involved in the development of GDM.

Future Medicine

Research design & methods

Participant selection

Participant recruitment and sample collection was performed under the umbrella of the Soweto First 1000 Days Cohort [13]. Inclusion criteria were: black South African women over 18 years of age during the first trimester of pregnancy, HIV negative, not taking any medication that may modulate blood glucose levels, not suffering from any other diseases, nonsmokers and singleton pregnancies. Women who met the inclusion criteria were recruited from the Developmental Pathways for Health Research Unit at Chris Hani Baragwanath Academic Hospital and followed from their first trimester of pregnancy (<14 weeks) to delivery. Glucose tolerance was assessed using a 75 g oral glucose tolerance test (OGTT) performed at approximately 24–28 weeks' gestation. The International Association of the Diabetes and Pregnancy Study Groups (IADPSG) criteria were used to diagnose GDM [14]. Only women with female babies were recruited in order to eliminate sex-specific differences in placenta global gene expression as an additional variable in this small study [15–18]. Once the sex of the baby was known, six women with confirmed GDM and six healthy controls with normal glucose tolerance were selected. All study participants provided written informed consent and the study was approved by the University of the Witwatersrand Human Research Ethics Committee (Medical) (Ethics clearance no.: M130420).

Sample collection

Two tubes of whole blood were taken at 29–33 weeks' gestation: one for DNA and one for RNA extraction. At the time of delivery, the placenta of female babies was obtained and two 8-mm vertical placental punch biopsies were taken within 1 h after birth. The biopsies were taken from the placental disc, avoiding the umbilical cord insertion site and approximately 3 cm from the edge of the placenta. The direction of the punch biopsy was from maternal to fetal side. One punch biopsy was used for RNA extraction and one was used for DNA extraction.

DNA & RNA isolation

DNA was extracted from whole blood samples following a salting out method [19] and from placental tissue using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). Total RNA was extracted from whole blood following the Tempus™ Spin RNA Isolation Kit Protocol (Applied Biosystems, Warrington, UK) and from placental tissue using the RNeasy Mini Kit (Qiagen). A DNAse treatment step was included during the RNA extraction protocol (Qiagen) and the RNA integrity number (RIN) for all RNA samples was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies). The RIN values for all samples were above 8.

cDNA library preparation & RNA sequencing

The cDNA libraries were constructed with Illumina's TruSeq stranded mRNA sample preparation kit. The cDNA products were amplified and quality control of the libraries was done with the Agilent 2100 Bioanalyzer (Agilent Technologies). The cDNA samples were indexed and pooled together in equal concentrations into six pools (4 samples per pool as follows: placenta–case, placenta–control, blood–case, blood–control). The blood and placenta transcriptomes were sequenced at the Centre for Genomic Regulation (CRG) in Barcelona, Spain, with the HiSeq 2000 Illumina sequencing platform as paired-end reads of 75 bp.

RNA-sequence data analysis

The quality of the raw sequence reads was determined with the FastQC tool (Babraham Institute). Reads with a low quality score <20 and reads shorter than 25 bp were removed. After trimming, the paired-end reads were aligned to the human genome (version GRCh37/hg19) using TopHat v1.3.1 software [20]. The UCSC hg19 genome sequence indexes and the GTF transcript annotation files provided by Illumina were used in the alignment. A transcriptome index was built with a control pool and the same index was used for the other alignments. The original alignment file was processed to measure transcript abundance using Cufflinks v1.0.3 software. The case and control groups were compared for differential gene expression with Cuffdiff software [21,22]. In the differential expression analysis, a q-value (a false discovery rate [FDR] corrected p-value) of <0.05 was considered statistically significant. Only genes showing a large fold change (of >2 or <-2) between cases and controls were considered for further analyses. The filtered list of genes (Supplementary Tables 1 & 2) were then run through pathway and functional annotation analysis using PANTHER-v8.1 (Protein Analysis through Evolutionary Relationships) Classification System and DAVID (Database for Annotation, Visualisation and Integrated Discovery). Pathway

level analysis of gene expression data was performed by Gene Set Enrichment Analysis (GSEA) in PANTHER. Using all of the above information, potential candidate genes were selected for validation.

Reverse transcription & qPCR

One microgram of total RNA was reverse transcribed into cDNA using SuperScriptIII (Invitrogen, CA, USA) and random hexamers (Sigma-Aldrich, MO, USA). The qPCR reaction was set up using TaqMan chemistry (ThermoFisher Scientific, IL, USA) with primers and a probe designed to specifically target each gene. The qPCR runs were performed on the ABI-7900HT Real-Time PCR machine (Applied Biosystems), in triplicate. Each run included three housekeeping genes (*RPLPO*, *ACTB* and *HPRT1*), and the appropriate controls. The data generated were analyzed using the Biogazelle software (relative quantification method). The normalized C_T values were used to determine the fold change ($\Delta\Delta C_T$) in gene expression between cases and controls. Statistical analysis was performed using GraphPad Prism.

Promoter region methylation analysis of selected genes

The methylation status of selected CpG islands in the promoter region of the *G6PD* (CpG Island 115375), *TKT* (CpG Island 110332), *IGFBP-1* (CpG Island 113146), *IGFBP-2* (CpG Island 108855) and *IGFBP-6* (CpG Island 103158) genes were examined by methylation-specific PCR. This was performed following the EpiTect Methyl II PCR assay (Qiagen) procedure. Real-time PCR was carried out as per the manufacturer's instructions (Qiagen) on an ABI-7900HT Real-Time PCR machine (Applied Biosystems). Samples were analyzed as recommended by the manufacturer (www.sabiosciences.com/dna_methylation_data_analysis.php). Comparisons of gene promoter methylation levels between two groups (cases blood vs controls blood, cases placenta vs controls placenta; cases blood vs cases placenta; controls blood vs controls placenta) were determined using the appropriate Student's t-test.

Correlation analysis

In order to determine whether there is a correlation between the mRNA expression levels of *G6PD*, *TKT*, *IGFBP-1*, *IGFBP-2* and *IGFBP-6* with other measured variables such as maternal glucose levels, maternal body mass index and fetal birth weight, Spearman's correlation analysis was performed using Intellectus Statistics.

Results

Clinical characteristics

The cases and controls differed for pre-pregnancy body mass index and weight gain, however, these values just failed to reach statistical significance. The blood glucose levels at fasting and 1 h post oral glucose load were significantly different between the cases and controls. The blood glucose levels at 2 h post-glucose load were not significantly different between the two groups. All newborns were females and there was no significant difference for gestational age at birth or birth weight between the GDM exposed and unexposed groups (Table 1).

Differential gene expression

The analysis of the blood dataset identified 1088 genes with a significant difference in expression between GDM and normal glucose tolerance women, while in the exposed placentas, 1489 genes passed the significance threshold. Further filtering of genes through gene ontology and enrichment analysis as well as an in-depth literature research identifying genes linked to diabetes and/or glucose metabolism, was performed and reduced the list of potential candidate genes to 60 for blood and 52 for placenta.

Pathway & functional analyses

The list of significantly differentially regulated genes in the blood (Supplementary Table 1) and placenta (Supplementary Table 2) was sorted into gene ontology (GO) term categories for molecular function and biological processes. The GO terms associated with the differentially regulated genes in the blood include a large percentage annotated as 'binding activity' (39.7%) and 'catalytic activity' (34.5%). The most common GO terms associated with the placental genes included 'metabolic process' (26.9%), 'cellular process' (25.8%), 'developmental process' (9.0%) and 'immune system process' (9.0%).

From the gene set enrichment analysis, the carbohydrate and NADP metabolic pathways were among those most significantly enriched (fold enrichment of 48.62 and 21, respectively) in the blood and the five genes clustered within these pathways were *G6PD*, *TKT*, *ALDOA*, *PGLS* and *DCXR*, all of which encode enzymes that function

Table 1. Clinical characteristics of the study participants.

Maternal characteristics	Mothers (whole blood samples)		
	Controls (n = 6) NGT	Cases (n = 6) GDM	p-value
Fasting glucose (>5.1 mmol/l)	4.1	5.2	**0.003**
1 h OGTT† (>10 mmol/l)	5.5	9.5	**0.0001**
2 h OGTT† (>8.5 mmol/l)	5.7	7.5	0.08
Age (years)	26.7	31.3	0.18
BMI (kg/m^2)	30.8	37.9	0.06
Weight before pregnancy (kg)	86.7	87.0	0.21
Weight after pregnancy (kg)	94.8	98.3	0.17
Weight gain (kg)	8.1	11.4	0.052
Perinatal characteristics	**Fetuses (placental samples)**		
	Controls (n = 6)	Cases (n = 6)	p-value
Gestational age at birth (weeks)	38.7	38.5	0.78
Birth weight (kg)	3.36	3.40	0.85
Length (cm)	49.5	45.0	0.41

Data are presented as mean +/- standard deviation. Variables were compared using Student's nonpaired t-test.
†Done at approximately 24 to 28 weeks' gestation.
Bold font: $p < 0.05$; significant difference between cases and controls.
BMI: Body mass index; GDM: Gestational diabetes mellitus; HIV: Human immunodeficiency virus; NGT: Normal glucose tolerance; OGTT: Oral glucose tolerance test.

in the pentose phosphate pathway (PPP; Figure 1). In the placenta, the transmembrane receptor protein kinase signaling pathway was enriched >100-fold ($p = 0.00049$) and the over-represented group of genes are *IGFBP-1*, *IGFBP-2* and *IGFBP-6*. The above-mentioned analyses reduced the list of genes to ten strong functional and biological candidates from both the blood and placenta datasets. Table 2 lists the 20 genes for qPCR validation.

qPCR validation of RNA sequencing results

The ThermoFisher assays for each of the 20 selected genes are listed in Supplementary Table 3. The TaqMan assays were optimized and validation showed complete concordance of expression with the RNA-sequencing results for all 20 selected genes (Figure 2A & B). We assessed the correlation between the FPKM values (obtained with RNA sequencing) with their corresponding ΔΔCT values from the TaqMan assays using Spearman correlation (Rs) analysis. In the blood and placenta respectively, the linear regression analysis of the differentially expressed genes provided Rs values of 0.9377 and 0.923, indicating a strong correlation between the two methods.

Promoter region methylation

From the 20 validated genes, five (*G6PD*, *TKT, IGFBP-1, IGFBP-2* and *IFGBP-6*) were selected for promoter region methylation analysis (Supplementary Figure 1). There was no significant alteration in promoter region methylation for *IGFBP-1*, *IGFBP-2* and *IGFBP-6* in blood samples from GDM patients when compared with the controls ($p = 0.85$; $p = 0.91$ and $p = 0.11$, respectively). However, the promoter regions of *IGFBP-1* and *IGFBP-2* in exposed placenta are significantly hypermethylated compared with the unexposed group ($p < 0.0001$ for both; Figure 3C). For *IGFBP-6*, there appears to be a trend toward increased promoter region methylation in the placenta of the cases when compared with the controls but this was not statistically significant ($p = 0.08$; Figure 3C).

The level of *G6PD* promoter region methylation in both blood and placenta samples from cases was significantly higher ($p < 0.0001$ for both) than in the controls (Figure 3C). The level of *TKT* promoter region methylation was not significant between cases and controls in the blood or placenta (Figure 3C). For the *IGFBPs*, the methylation levels were higher in the placenta than in the blood for both cases and controls. This is not the case for *G6PD*, where the methylation levels are higher in the blood and placenta cases compared with controls (see above and Figure 3C), and for *TKT*, where methylation was not different between groups or tissues (Figure 3C).

Figure 3D plots the relationship between gene expression and promoter region methylation of the differentially expressed genes. We observed a significant negative association between the level of promoter region methylation and the magnitude of gene expression for *IGFBP-1* and *IGFBP-2*, but not for *IGFBP-6*, in the placental samples (Figure 3D). For all three genes, there is no association in the blood samples. There is a significant negative association between *G6PD* mRNA expression and promoter region methylation in both the blood and placenta samples

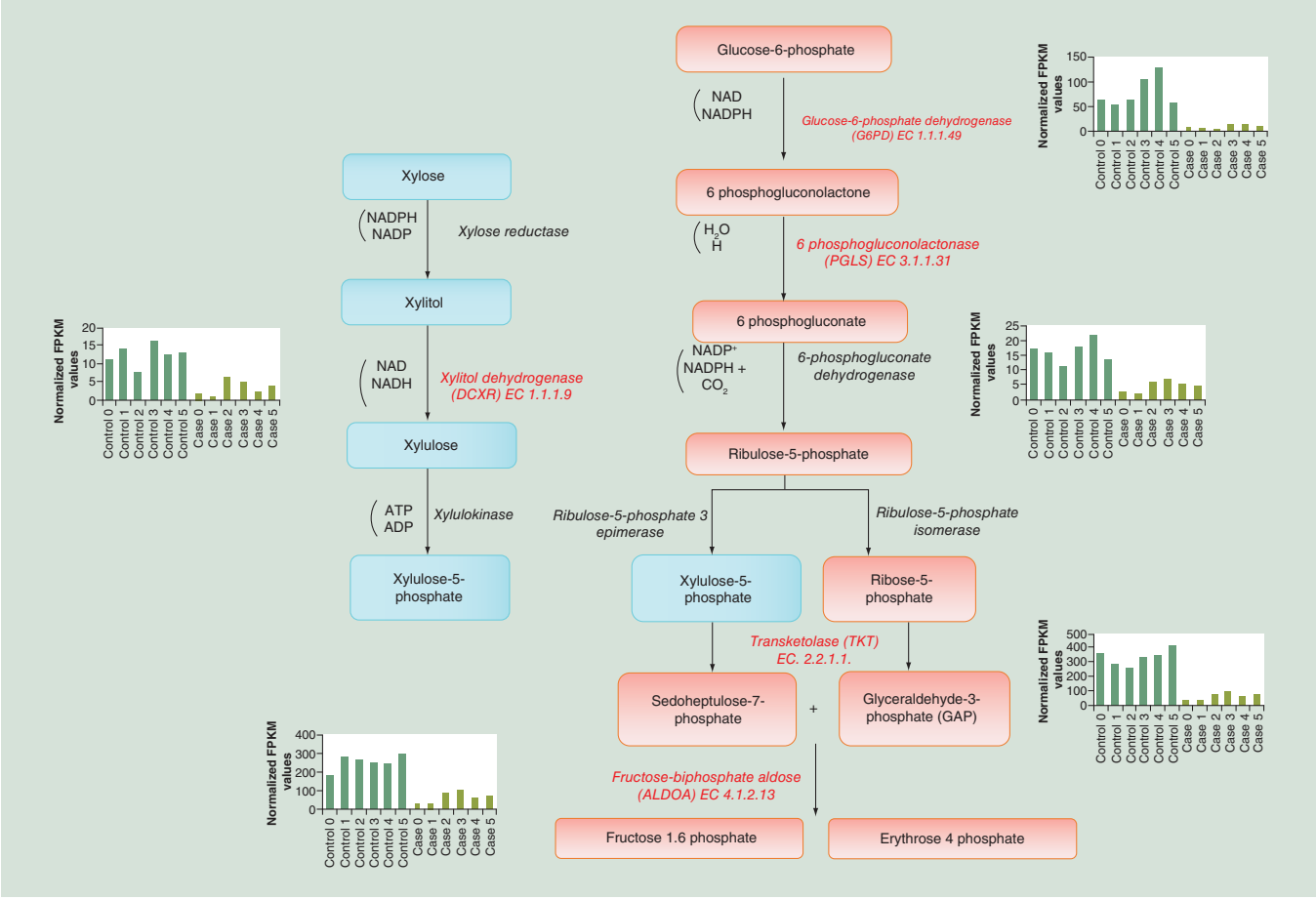


Figure 1. A schematic presentation of the pentose phosphate pathway highlighting the genes found to be differentially expressed between gestational diabetes mellitus cases and control. The genes of interest are marked in red. The full names, gene symbol and their location within the pentose phosphate pathway are shown. The bar charts within the figure represent the normalized gene expression values (FPKM) of each gene in each individual case (in red) and control (in blue) sample.
FPKM: Fragments per Kilobase of transcript per Million mapped reads.

(Figure 3D). There is no significant association between mRNA expression and promoter region methylation for *TKT* in the blood and placenta samples (Figure 3D). The heat map (Figure 4) illustrates the higher promoter region methylation of *IGFBP-1*, *IGFBP-2* and *IGFBP-6* in the placenta exposed to GDM *in utero* in comparison to the control samples. This figure also highlights the higher level of *G6PD* promoter region methylation in the women who develop GDM in comparison to controls.

Relationship of mRNA expression & promoter methylation with other measured variables

There was a significant negative correlation between *G6PD* mRNA expression in the blood and placenta with the level of maternal glucose at fasting, 1 and 2 h post-glucose load (Table 3). We observed a significantly positive correlation between *G6PD* promoter region methylation in both blood and placental tissues with maternal glucose levels at fasting and at 1 h post-glucose load. For *TKT*, significantly negative correlations were observed in the blood between the level of mRNA expression and maternal glucose levels at fasting and at 1 h post-glucose load but no significant correlation was observed between maternal glucose levels and mRNA expression levels for *TKT* in the placenta (Table 3). No correlations were observed for blood or placental *TKT* gene methylation with any of the blood glucose levels.

For the *IGFBPs*, significant negative correlations were observed between the mRNA expression levels of *IGFBP-1* in maternal blood with maternal glucose levels at fasting, 1- and 2 h post-glucose load (Table 3); and for *IGFBP-2* at fasting and 1 h post-glucose load. No significant correlation was observed between mRNA expression levels and maternal glucose levels 2 h post-glucose load for *IGFBP-2*. No significant correlation was observed between

Table 2. Top ranked differentially expressed genes in gestational diabetes mellitus compared to non-gestational diabetes mellitus (RNA-sequencing analysis).

Gene_id ENSG00000	Gene symbol	Gene name	Relative fold change	p-value	Gene enrichment (fold enrichment; significance)
BLOOD					
105221	*AKT2*	RAC-beta serine/threonine-protein kinase	-2.28	0.0005	No result
149925	*ALDOA*	Fructose-bisphosphate aldolase A	-2.26	<0.0001	NADP metabolic process (48.62; 0.014) Carbohydrate metabolic process (21.02; 0.0003)
185347	*C14orf80*	Uncharacterized protein C14orf80	-2.13	<0.0001	No result
169738	*DCXR*	Xylulose reductase	-2.18	0.0003	NADP metabolic process (48.62; 0.014) Carbohydrate metabolic process (21.02; 0.0003)
160211	*G6PD*	Gluscose-6-phosphate dehydrogenase	-3.09	<0.0001	NADP metabolic process (48.62; 0.014) Carbohydrate metabolic process (21.02; 0.0003)
105723	*GSK3A*	Glycogen synthase kinase-3 α	3.20	0.0005	No result
53918	*KCNQ1*	Potassium voltage-gated channel subfam KQT member	-2.02	0.0004	No result
130313	*PGLS*	6-phosphogluconolactonase	-2.52	0.0003	NADP metabolic process (48.62; 0.014) Carbohydrate metabolic process (21.02; 0.0003)
146678	*IGFBP-1*	IGF-binding protein 1	-2.31	0.0003	No result
163931	*TKT*	Transketolase	-2.94	<0.0001	NADP metabolic process (48.62; 0.014) Carbohydrate metabolic process (21.02; 0.0003)
PLACENTA					
163464	*CXCR1*	C-X-C chemokine receptor type 1	-3.37	<0.0001	Cellular response to interleukin-8 (7.67; 0.045)
180871	*CXCR2*	C-X-C chemokine receptor type 2	-2.35	<0.0001	Cellular response to interleukin-8 (7.67; 0.045)
160211	*G6PD*	Glucose-6-phosphate dehydrogenase	-2.68	<0.0001	NADP metabolic process (3.56; 0.01) Carbohydrate metabolic process (5.67; 0.003)
146678	*IGFBP-1*	IGF-binding protein 1	-4.74	<0.0001	Transmembrane receptor protein tyrosine kinase signaling pathway (>100; 0.00049)
115457	*IGFBP-2*	IGF-binding protein 2	-2.65	<0.0001	Transmembrane receptor protein tyrosine kinase signaling pathway (>100; 0.00049)
211896	*IGFBP-6*	IGF-binding protein 6	-2.92	0.0007	Transmembrane receptor protein tyrosine kinase signaling pathway (>100; 0.00049)
110347	*MMP12*	Macrophage metalloelastase	2.03	0.0005	No result
151948	*GLT1D1*	Glycosyltransferase 1 domain-containing protein 1	-2.87	0.0001	No result
197421	*GGT3P*	Putative gamma-glutamyltranspeptidase 3	8.01	0.0002	No result
163931	*TKT*	Transketolase	-2.56	<0.0001	NADP metabolic process (3.56; 0.01) Carbohydrate metabolic process (5.67; 0.003)

Student's t-test used to determine p-value; significance taken as $p < 0.05$. These genes were also selected as potential candidates due to gene ontology and enrichment analysis as well as extensive literature search to identify involvement in metabolic disease.
Relative fold change: GDM relative to controls and normalized to housekeeping genes. No result: the gene is not associated with any annotated pathway.

maternal glucose levels and mRNA expression levels for *IGFBP-6* (Table 3). For the placenta, significant negative correlations were observed between the mRNA expression levels of *IGFBP-1* and maternal glucose levels at fasting, 1- and 2 h post-glucose load; for *IGFBP-2* at fasting and 1 h post-glucose load and for *IGFBP-6* at fasting and at 1 h post-glucose load (Table 3). With regard to methylation, no significant correlation was observed for promoter region methylation of *IGFBP-1*, *IGFBP-2* and *IGFBP-6* in maternal blood with maternal glucose levels. However, in the placenta, there was a significant positive correlation between promoter region methylation and maternal glucose levels for *IGFBP-1*, *IGFBP-2* and *IGFBP-6* at fasting and at 1 h post-glucose load. No significant association was observed between promoter region methylation and glucose levels at 2 h for any of the *IGFBPs* (Table 3).

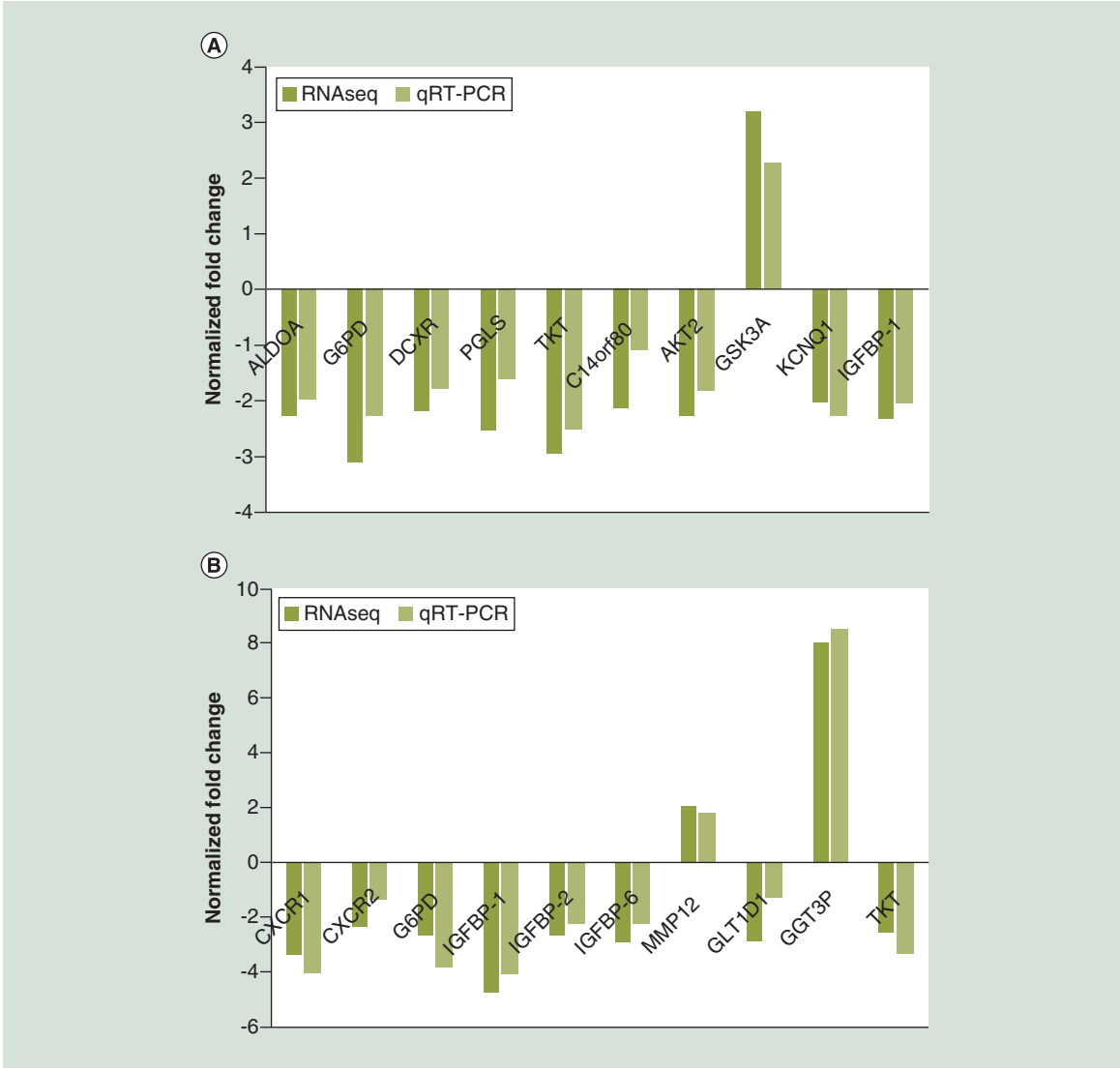


Figure 2. RT-qPCR validation of RNA-seq results using TaqMan probes. Validation of RNA-seq results was performed in the 20 most promising candidate genes (listed in Table 2) **(A)** Fold change comparison between RNA-seq and RT-qPCR fold change results from the same RNA samples in blood samples **(B)** Fold change comparison between RNA-seq and RT-qPCR fold change results from the same RNA samples in placenta samples. Fold changes represented gene expression changes in each group relative to the control group.

There was a tendency for *G6PD* and *TKT* mRNA expression in the mother's blood to fall with birthweight, but these relationships just missed statistical significance. However, in the placenta there was a significant negative correlation observed between *G6PD* mRNA expression and birthweight but a positive correlation with methylation. No significant relationships were observed for placental *TKT* expression or methylation with birthweight.

There was a significant positive correlation of *IGFBP-1* mRNA expression in maternal blood with birthweight. There was no significant correlation observed for *IGFBP-2* and *IGFBP-6* in maternal blood with birthweight. There was a significant positive correlation of birthweight with *IGFBP-1*, *IGFBP-2* and *IGFBP-6* mRNA levels in the placenta (Table 3). With reference to methylation, no significant correlation was observed between methylation of the *IGFBPs* in the blood with birthweight. However, there was a significant positive correlation of the methylation of the *IGFBPs* (*IGFBP-1*, *IGFBP-2* and *IGFBP-6*) in the placenta with birthweight.

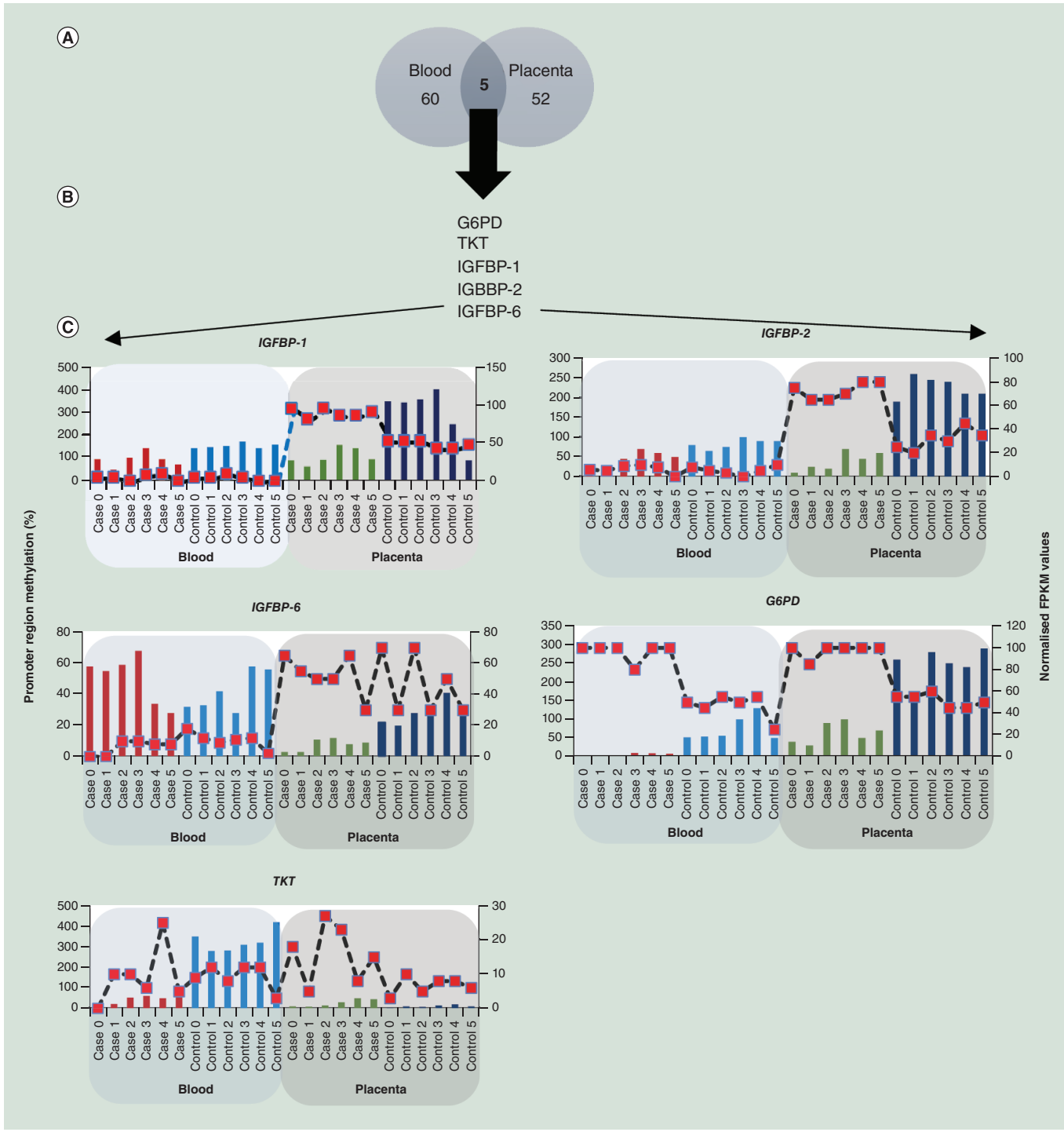


Figure 3. Differentially expressed genes in the RNA-seq blood and placenta dataset. (A) Venn diagram of tissue specific and overlapping differentially expressed genes from the filtered datasets. **(B)** From these datasets, five genes were differentially expressed in both the blood and placenta. They were chosen for promoter region methylation analysis. *G6PD*, *TKT* and *IGFBP-1* were statistically significantly downregulated in blood and placenta while *IGFBP-2* and *IGFBP-6* were downregulated in both datasets but this only reached levels of significance in the placenta. **(C)** Graphic visualization of the relationship between gene expression and promoter region methylation. The columns represent the expression data and black lines represent the methylation data. **(D)** Spearman's correlation rank for each gene was performed using Intellectus Statistics in order to identify a significant correlation between relative mRNA expression of the gene and promoter region methylation between the cases and control samples of each dataset.
FPKM: Fragments per Kilobase of transcript per Million mapped reads.

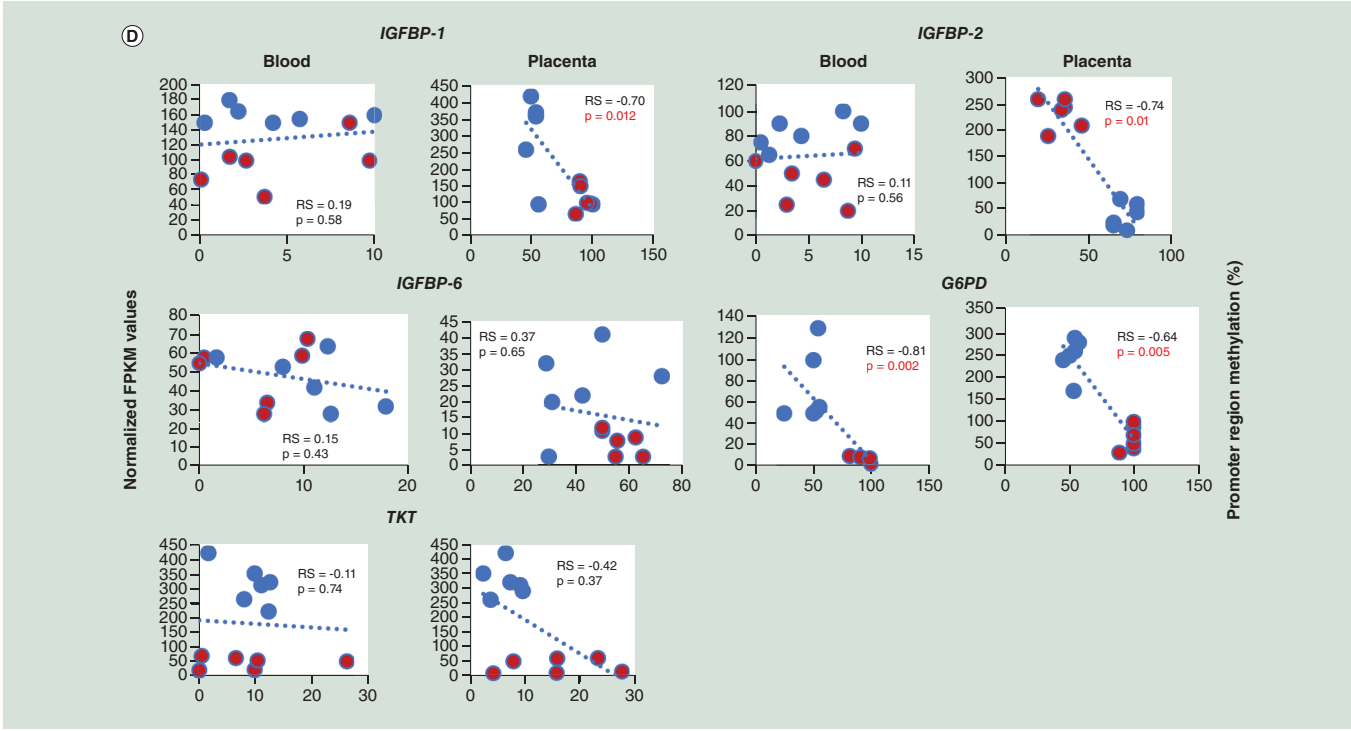


Figure 3. Differentially expressed genes in the RNA-seq blood and placenta dataset (cont.). (A) Venn diagram of tissue specific and overlapping differentially expressed genes from the filtered datasets. **(B)** From these datasets, five genes were differentially expressed in both the blood and placenta. They were chosen for promoter region methylation analysis. *G6PD*, *TKT* and *IGFBP-1* were statistically significantly downregulated in blood and placenta while *IGFBP-2* and *IGFBP-6* were downregulated in both datasets but this only reached levels of significance in the placenta. **(C)** Graphic visualization of the relationship between gene expression and promoter region methylation. The columns represent the expression data and black lines represent the methylation data. **(D)** Spearman's correlation rank for each gene was performed using Intellectus Statistics in order to identify a significant correlation between relative mRNA expression of the gene and promoter region methylation between the cases and control samples of each dataset.
FPKM: Fragments per Kilobase of transcript per Million mapped reads.

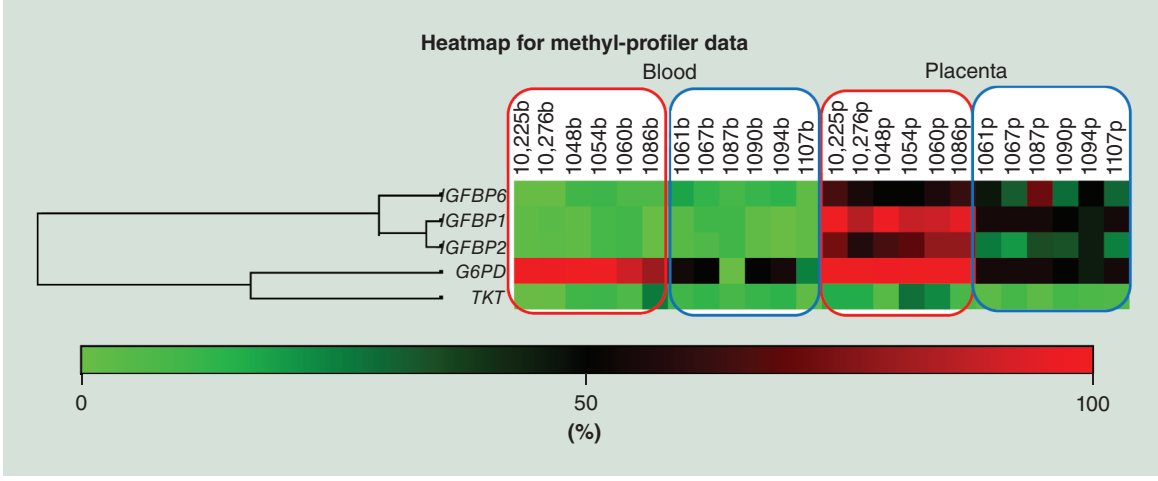


Figure 4. Heat map for blood and placental methylation data for the five most promising candidate genes. The heat map compares the methylation status of five genes in the genomic DNA of blood and placental samples using the EpiTect Methyl II Assays. The results further illustrate the correlation of increased methylation in *G6PD* for both blood and placenta cases and for IGFBPs in the placenta. Red blocks indicate high methylation and green blocks indicate low (or no) methylation.

Table 3. Summary of the correlation analysis of gene expression and methylation with maternal glucose levels, birth weight and maternal BMI in maternal blood and fetal placental tissue.

Maternal blood									
Gene	Expression vs maternal glucose levels			Methylation vs maternal glucose levels			Expression versus birthweight	Methylation versus birthweight	Expression versus BMI†
	Fasting	1 h	2 h	Fasting	1 h	2 h			
G6PD	-0.74 (**0.006**)	-0.67 (**0.016**)	-0.27 (0.84)	0.91 (**0.001**)	0.81 (**0.001**)	0.41 (0.69)	-0.52 (0.08)	0.59 (0.05)	-0.27 (0.39)
TKT	-0.82 (**0.003**)	-0.75 (**0.005**)	-0.41 (0.87)	-0.17 (0.71)	-0.35 (0.56)	-0.22 (0.32)	-0.56 (0.06)	-0.21 (0.50)	-0.41 (0.18)
IGFBP-1	-0.88 (**0.001**)	-0.75 (**0.005**)	-0.73 (0.003)	-0.15 (0.65)	-0.32 (0.71)	-0.10 (0.58)	0.75 (**0.005**)	0.10 (0.75)	0.24 (0.42)
IGFBP-2	-0.85 (**0.001**)	-0.64 (**0.025**)	-0.42 (0.68)	-0.17 (0.39)	0.20 (0.41)	-0.38 (0.71)	0.57 (0.87)	-0.50 (0.87)	0.46 (0.70)
IGFBP-6	0.001 (0.81)	0.004 (0.96)	-0.30 (0.45)	-0.52 (0.74)	-0.39 (0.89)	-0.15 (0.57)	0.13 (0.20)	0.56 (0.30)	0.36 (0.23)
Placental tissue									
Gene	Expression vs maternal glucose levels			Methylation vs maternal glucose levels			Expression versus birthweight	Methylation versus birthweight	Expression versus BMI†
	Fasting	1 h	2 h	Fasting	1 h	2 h			
G6PD	-0.84 (**0.001**)	-0.73 (**0.007**)	-0.59 (**0.045**)	0.65 (**0.023**)	0.76 (**0.004**)	0.23 (0.32)	-0.67 (**0.01**)	0.70 (**0.009**)	-0.46 (0.13)
TKT	-0.26 (0.91)	-0.12 (0.46)	0.02 (0.50)	-0.32 (0.58)	-0.14 (0.89)	-0.09 (0.78)	-0.17 (0.6)	0.39 (0.21)	-0.43 (0.25)
IGFBP-1	-0.96 (**0.001**)	-0.75 (**0.005**)	-0.58 (**0.048**)	0.63 (**0.027**)	0.75 (**0.005**)	0.19 (0.32)	0.67 (**0.017**)	0.77 (**0.003**)	-0.46 (0.13)
IGFBP-2	-0.83 (**0.001**)	-0.89 (**0.001**)	-0.43 (0.84)	0.68 (**0.014**)	0.87 (**0.001**)	0.50 (0.28)	0.66 (**0.021**)	0.73 (**0.007**)	-0.41 (0.19)
IGFBP-6	-0.72 (**0.005**)	-0.70 (**0.012**)	-0.36 (0.62)	0.68 (**0.016**)	0.58 (**0.047**)	0.46 (0.51)	0.59 (**0.043**)	0.61 (**0.034**)	-0.24 (0.45)

†Methylation versus maternal BMI is not shown since there were no significant associations for any of the five candidate genes in blood and placental tissue.
Bold values: $p < 0.05$; significant difference between measured variables
BMI: Body mass index.

Discussion

We have demonstrated significant epigenetic shifts leading to altered gene expression in specific genes in the blood of pregnant women with GDM and in the placental tissues of the fetuses. The level of mRNA expression of genes encoding enzymes within the PPP and for *IGFBPs* was lower in the blood and placenta of women with GDM compared with controls. Quantitative analysis of gene methylation demonstrated that for some of these genes, the mRNA expression levels correlated negatively with gene methylation.

The PPP produces precursors for the synthesis of coenzymes, nucleotides, RNA and DNA and also to generate NADPH. Shunting of accumulated cytosolic glycolytic intermediates into the PPP is proposed to unburden glycolysis and limits processing of glycolytic intermediates into harmful metabolic products. In this way, the PPP represents a 'protective' mechanism against hyperglycemia-induced damage [23]. The *G6PD* gene encodes a cytoplasmic enzyme that catalyzes the rate-limiting step in the oxidative branch of the PPP that generates the first molecule of NADPH, and therefore its expression and activity are tightly regulated [24–26]. One of the major functions of NADPH is to act as a co-factor in cellular pathways that prevent oxidative stress [27].

The expression levels of genes coding for enzymes within the PPP were significantly downregulated in white blood cells and placental tissue taken from women with GDM. Furthermore, in both these tissues expression of *G6PD* correlated negatively with methylation at the promoter region of the gene. It is possible that the attenuation of expression of the other genes in the PPP is a result of *G6PD* downregulation, since this enzyme is the rate-determining step of the pathway [23,24]. It was also found that maternal blood glucose levels correlated negatively with *G6PD* expression and positively with methylation in both the placenta and the white blood cells. Previous studies demonstrated downregulation of *G6PD* activity by glucose [27,28], but this is the first study to show that the effect of hyperglycemia on *G6PD* expression may be mediated via an epigenetic mechanism. These data suggest that elevated maternal glucose levels may reduce activity of the PPP by increasing methylation of the *G6PD* gene hence reducing its expression. However, not all investigations demonstrate a negative effect of glucose on *G6PD* expression or activity, with some studies showing a positive relationship between these factors [29,30].

The consequences of low *G6PD* expression would include a reduction in NADPH production and attenuation of the cellular processes that protect against oxidative stress [31,32]. Oxidative stress is a risk factor for both T2D [33] and GDM [34] and studies have shown that placenta from women with GDM have high levels of oxidative stress [35,36]. Thus, low expression of maternal and placental G6PD may enhance progression of GDM in the mother, while in

the placenta it may be one of the factors that contributes to oxidative stress. It is known that GDM is a risk factor for pre-eclampsia [37,38] and that placental oxidative stress is involved in its etiology [39]. It is therefore possible that low *G6PD* expression in the placenta may mediate the association of GDM with pre-eclampsia, and this warrants further investigation.

In GDM, insulin output is lower than in nondiabetic mothers at each level of insulin resistance [40]. It is possible that the G6PD deficiency we see in GDM women could be involved in this process. Thus, high glucose levels may reduce *G6PD* gene expression in the ß-cells leading to attenuation of the anti-oxidant system in these cells and reduced insulin output [41]. A previous study showed that incubating islets in high glucose medium does reduce *G6PD* expression and increase β-cell apoptosis [42].

The major role of *IGFBP-1* and *IGFBP-2* is the regulation of the bioavailability of IGF (IGF)-1 and IGF-2, although each binding protein also has effects that are IGF independent [43]. Throughout pregnancy, the expression and circulating levels of IGFBPs change in the mother and may influence IGF bioavailability [44]. Reduced levels of these binding proteins will result in an increase in free, unbound IGFs, which are then able to bind to their respective receptors. The maternal IGF system plays a vital role in fetal growth regulation via stimulation of extravillous trophoblast migration/invasion and facilitation of nutrient exchange through the promotion of growth and development of the placenta [45].

Suppression of *IGFBP-1* expression in women with GDM and in exposed placenta was observed. We also observed a negative correlation of maternal and placental *IGFBP-1* and *IGFBP-2* mRNA levels with maternal glucose levels. Furthermore, there was a negative association of birthweight with maternal IGFBP-1 expression and with placental expression of all three binding proteins. These results are supported by a study showing that cord plasma IGFBP-1 levels correlated negatively with birthweight and that IGFBP-1 levels are lower both in women with GDM and in cord blood from such pregnancies [46]. These data suggest that high maternal glucose levels during pregnancy influence the bioavailability of IGFs through attenuation of both maternal and placental *IGFBP* expression which may increase fetal somatic growth.

In the placental but not maternal tissue, the expression of *IGFBP-1* and *IGFBP-2* mRNA is negatively correlated with promoter region methylation. For all three *IGFBP*s, methylation correlates positively with maternal glucose levels and with birthweight. In support of these findings, a recent study showed that gene expression levels of *IGFBPs 1, 2, 3, 4* and *7* were highest in small-for-gestational age neonates and lowest in large-for-gestational age neonates while gene methylation levels followed the opposite trend [47]. Our observations suggest that DNA methylation reduces expression of these binding proteins only in the placenta. It is important for the placenta to be able to respond to prevailing nutrient levels and an epigenetic process may facilitate a more chronic response, while in the adult, there is no gene methylation and this may provide a more acute change in *IGFBP* levels in response to the glucose supply. We conclude that the hypermethylation observed at the promoter region of these binding proteins in the placenta may be a result of the presence of GDM and may be one mechanism through which GDM leads to fetal macrosomia.

A limitation of this study is the small sample size. Although this does limit data interpretation and statistical analysis, it was still sufficient to allow observations of major differences in gene expression and methylation patterns between the cases and controls. Maternal white blood cells were used for gene expression analysis due to ease of access. However, expression patterns in these cells may not reflect those of other more disease-relevant tissues such as pancreatic islets or skeletal muscle. We did not measure the HbA1c levels in the women because, unlike detecting diabetes in a nonpregnant patient, testing HbA1c is ineffective in diagnosing GDM due to red blood cell turnover increasing during pregnancy and naturally reducing HbA1c levels in early and late pregnancy [48].

In conclusion, the present study shows that GDM-associated hyperglycemia is associated with changes in gene methylation levels which cause the attenuation of expression of genes controlling the PPP and IGFBP levels. This may lead to greater maternal and placental oxidative stress and higher levels of free IGF, respectively. Through these mechanisms epigenetic processes may therefore play an important role in GDM disease progression and fetal macrosomia.

Future perspective

Future studies could investigate whether expression levels of *G6PD* in blood correlate with that in more strongly disease-associated tissues such as skeletal muscle and islets of Langerhans. Levels of NADPH and markers of oxidative stress could be measured in the placenta to determine if they correlate with *G6PD* expression and methylation in case-control studies of GDM and pre-eclampsia. The expression level of G6PD could be investigated in Type 2

diabetes and prediabetes and also monitored through pregnancy to find out whether low G6PD levels predate the development of these diseases. The levels of free IGFs in cord blood could be analyzed in relation to expression and methylation levels of the placental *IGFBP* genes. Ultimately these studies will determine whether *G6PD* has a fundamental role in the etiology of GDM, Type 2 diabetes and pre-eclampsia and uncover possible new drug targets for disease intervention.

Summary points

- It has been suggested that gestational diabetes mellitus (GDM) may affect the epigenetic status of key genes of the exposed offspring and the mother, leading to an increased risk of diabetes in both parent and child.
- The purpose of the current study was therefore to compare genome-wide gene expression levels and gene methylation of affected genes in placental tissue and blood samples from mothers with and without GDM.
- We observed a significant decrease in the mRNA expression of genes involved in the pentose phosphate pathway, but most particularly *G6PD*, in blood samples from women who were diagnosed with GDM, as well as in placental tissue taken from these pregnancies.
- The reduced expression of *G6PD* in both the blood of affected women and placental tissue from exposed fetuses was significantly related to an increase in the level of *G6PD* methylation.
- Maternal blood glucose levels correlated negatively with *G6PD* expression and positively with gene methylation in both the placenta and the white blood cells of the GDM cases.
- There was a significant reduction in the expression of the IGF-binding protein genes, *IGFBP1* and *IGFBP2*, and increased gene methylation at these loci in placental tissue from GDM-exposed fetuses.
- Maternal blood glucose levels correlated negatively with *IGFBP1* and *IGFBP2* placental expression but positively with gene methylation.
- In mothers with GDM, *IGFBP* expression in the blood cells was decreased compared with controls and correlated negatively with maternal blood glucose levels. However, gene methylation at the *IGFBPs* did not correlate with gene expression levels in maternal blood cells.
- The fetal macrosomia observed in GDM may partly be explained by reduced placental *IGFBP* expression that is mediated by the effect of maternal glucose levels on gene methylation. Similar mechanisms may drive lower expression of *G6PD* in placental and maternal tissues in GDM possibly leading to lower insulin output and greater oxidative stress.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/epi-2018-0206

Author contributions

A Steyn, M Ramsay and SA Norris conceived and designed the experiments, NJ Crowther contributed to data interpretation and writing of the paper, A Steyn performed the experiments under the supervision of R Rabionet, and X Estivill analyzed the data and wrote the first draft of the paper. All authors edited and approved the final manuscript.

Acknowledgments

The authors thank the CRG-Novartis Africa mobility programme for the opportunity to carry out research at the Centre for Genomic Regulation (CRG) in Barcelona, Spain and to the South African Medical Research Council (MRC) and the National Health Laboratory Service (NHLS) Research Trust for funding. A Steyn was a PhD fellow on an NIH/Fogarty funded training program that is part of the Millennium Promise Awards: noncommunicable Chronic Diseases Research Training (Grant Number: D43TW008330). We would like to thank M Berry for his help in the bioinformatics analysis and S Macaulay for her help with patient recruitment.

Financial & competing interests disclosure

The Soweto First 1000 Days cohort was supported by the UK MRC-DfID Africa Research Leader Scheme and the South African Medical Research Council. M Ramsay is a South African Research Chair in Genomics and Bioinformatics of African populations hosted by the University of the Witwatersrand, funded by the Department of Science and Technology, and administered by the National Research Foundation of South Africa (NRF). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript

Ethical conduct of research

University of the Witwatersrand Human Research Ethics Committee (Medical) (ethics clearance number M130420).

References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1. Rhee CM, Kovesdy CP. Epidemiology: Spotlight on CKD deaths-increasing mortality worldwide. *Nat. Rev. Nephrol.* 11(4), 199–200 (2015).
2. Vaiserman A. Epidemiologic evidence for association between adverse environmental exposures in early life and epigenetic variation: a potential link to disease susceptibility? *Clin. Epigenetics* 7, 96 (2015).
3. Barker DJ, Hales CN, Fall CH, Osmond C, Phipps K, Clark PM. Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. *Diabetologia* 36(1), 62–67 (1993).
4. Gluckman PD, Hanson MA. The developmental origins of the metabolic syndrome. *Trends Endocrinol. Metab.* 15(4), 183–187 (2004).
5. Hanson MA, Gluckman PD. Early developmental conditioning of later health and disease: physiology or pathophysiology? *Physiol. Rev.* 94(4), 1027–1076 (2014).
6. Catalano C, Marshall SM. Epidemiology of end-stage renal disease in patients with diabetes mellitus: from the dark ages to the middle ages. *Nephrol. Dial. Transplant* 7(3), 181–190 (1992).
7. Catalano PM, Kirwan JP, Haugel-de Mouzon S, King J. Gestational diabetes and insulin resistance: role in short- and long-term implications for mother and fetus. *J. Nutr.* 133(5), 1674S–1683S (2003).

•• **Highlights and discusses the possible link between gestational diabetes mellitus and insulin resistance and the short- and long-term implications for mother and fetus.**

8. Friedman JE, Ishizuka T, Shao J, Huston L, Highman T, Catalano P. Impaired glucose transport and insulin receptor tyrosine phosphorylation in skeletal muscle from obese women with gestational diabetes. *Diabetes* 48(9), 1807–1814 (1999).
9. Ben-Haroush A, Yogev Y, Hod M. Epidemiology of gestational diabetes mellitus and its association with Type 2 diabetes. *Diabet. Med.* 21(2), 103–113 (2004).
10. Jawad F, Ejaz K. Gestational diabetes mellitus in South Asia: epidemiology. *J. Pak. Med. Assoc.* 66(9), S5–S7 (2016).
11. Buchanan TA, Xiang AH. Gestational diabetes mellitus. *J. Clin. Invest.* 115(3), 485–491 (2005).
12. Buchanan TA, Xiang AH, Page KA. Gestational diabetes mellitus: risks and management during and after pregnancy. *Nat. Rev. Endocrinol.* 8(11), 639–649 (2012).
13. Macaulay S, Ngobeni M, Dunger DB, Norris SA. The prevalence of gestational diabetes mellitus amongst black South African women is a public health concern. *Diabetes Res. Clin. Pract.* 139, 278–287 (2018).

•• **Focuses on determining the prevalence of gestational diabetes mellitus among black South African women, describing gestational diabetes mellitus-associated risk factors and clinical management, as well as evaluating the efficacy of the fasting plasma glucose reading in diagnosing gestational diabetes mellitus.**

14. Duran A, Saenz S, Torrejon MJ *et al.* Introduction of IADPSG criteria for the screening and diagnosis of gestational diabetes mellitus results in improved pregnancy outcomes at a lower cost in a large cohort of pregnant women: the St. Carlos Gestational Diabetes Study. *Diabetes Care* 37(9), 2442–2450 (2014).
15. Hannum G, Guinney J, Zhao L *et al.* Genome-wide methylation profiles reveal quantitative views of human aging rates. *Mol. Cell* 49(2), 359–367 (2013).
16. Khulan B, Manning JR, Dunbar DR *et al.* Epigenomic profiling of men exposed to early-life stress reveals DNA methylation differences in association with current mental state. *Transl. Psychiatry* 4, e448 (2014).
17. Boks MP, Derks EM, Weisenberger DJ *et al.* The relationship of DNA methylation with age, gender and genotype in twins and healthy controls. *PLoS ONE* 4(8), e6767 (2009).
18. Osei-Kumah A, Smith R, Jurisica I, Caniggia I, Clifton VL. Sex-specific differences in placental global gene expression in pregnancies complicated by asthma. *Placenta* 32(8), 570–578 (2011).
19. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* 16(3), 1215 (1988).
20. Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* 25(9), 1105–1011 (2009).
21. Trapnell C, Hendrickson DG, Sauvageau M, Goff L, Rinn JL, Pachter L. Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nat. Biotechnol.* 31(1), 46–53 (2013).

22. Trapnell C, Roberts A, Goff L *et al.* Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. Protoc.* 7(3), 562–578 (2012).
23. Pacal L, Tomandl J, Svojanovsky J *et al.* Role of thiamine status and genetic variability in transketolase and other pentose phosphate cycle enzymes in the progression of diabetic nephropathy. *Nephrol. Dial. Transplant* 26(4), 1229–1236 (2011).
24. Jiang P, Du W, Wu M. Regulation of the pentose phosphate pathway in cancer. *Protein Cell* 5(8), 592–602 (2014).
25. Jin L, Zhang H, Chen L *et al.* Combined overexpression of genes involved in pentose phosphate pathway enables enhanced D-xylose utilization by *Clostridium acetobutylicum*. *J. Biotechnol.* 173, 7–9 (2014).
26. Wan GH, Tsai SC, Chiu DT. Decreased blood activity of glucose-6-phosphate dehydrogenase associates with increased risk for diabetes mellitus. *Endocrine* 19, 191–195 (2002).

- **Describes how G6PD dehydrogenase deficiency predisposes affected individuals to being highly susceptible to oxidative stress, one of the risk factors for diabetes.**

27. Zhang Z, Apse K, Pang J, Stanton RC. High glucose inhibits glucose-6-phosphate dehydrogenase via cAMP in aortic endothelial cells. *J. Biol. Chem.* 275(51), 40042–40047 (2000).
28. Diaz-Flores M, Ibanez-Hernandez MA, Galvan RE *et al.* Glucose-6-phosphate dehydrogenase activity and NADPH/NADP+ ratio in liver and pancreas are dependent on the severity of hyperglycemia in rat. *Life Sci.* 78(22), 2601–2607 (2006).
29. Kelley DS, Kletzien RF. Ethanol modulation of the hormonal and nutritional regulation of glucose 6-phosphate dehydrogenase activity in primary cultures of rat hepatocytes. *Biochem. J.* 217(2), 543–549 (1984).
30. Tsintzas K, Norton L, Chokkalingam K *et al.* Independent and combined effects of acute physiological hyperglycaemia and hyperinsulinaemia on metabolic gene expression in human skeletal muscle. *Clin. Sci.* 124(11), 675–684 (2013).
31. Pandolfi PP, Sonati F, Rivi R, Mason P, Grosveld F, Luzzatto L. Targeted disruption of the housekeeping gene encoding glucose 6-phosphate dehydrogenase (G6PD): G6PD is dispensable for pentose synthesis but essential for defense against oxidative stress. *EMBO J.* 14(21), 5209–5215 (1995).
32. Tian WN, Braunstein LD, Apse K *et al.* Importance of glucose-6-phosphate dehydrogenase activity in cell death. *Am. J. Physiol.* 276(5), C1121–1131 (1999).
33. Nowotny K, Jung T, Hohn A, Weber D, Grune T. Advanced glycation end products and oxidative stress in Type 2 diabetes mellitus. *Biomolecules* 5(1), 194–222 (2015).
34. Lappas M, Hiden U, Desoye G, Froehlich J, Hauguel-de Mouzon S, Jawerbaum A. The role of oxidative stress in the pathophysiology of gestational diabetes mellitus. *Antioxid. Redox Signal.* 15(12), 3061–3100 (2011).

- **Describes the role that oxidative stress may play in the pathophysiology of gestational diabetes mellitus.**

35. Biri A, Onan A, Devrim E, Babacan F, Kavutcu M, Durak I. Oxidant status in maternal and cord plasma and placental tissue in gestational diabetes. *Placenta* 27(2), 327–332 (2006).
36. Coughlan MT, Vervaart PP, Permezel M, Georgiou HM, Rice GE. Altered placental oxidative stress status in gestational diabetes mellitus. *Placenta* 25(1), 78–84 (2004).
37. Ostlund I, Haglund B, Hanson U. Gestational diabetes and pre-eclampsia. *Eur. J. Obstet. Gynecol. Reprod. Bio.* 113(1), 12–16 (2004).
38. Sandhu MS, Dunger DB, Giovannucci EL. Insulin, IGF-I (IGF-I), IGF binding proteins, their biologic interactions, and colorectal cancer. *J. Natl. Cancer Inst.* 94(13), 972–980 (2002).
39. Matsubara K, Higaki T, Matsubara Y, Nawa A. Nitric oxide and reactive oxygen species in the pathogenesis of pre-eclampsia. *Int. J. Mol. Sci.* 16(3), 4600–4614 (2015).
40. Megia A, Vendrell J, Gutierrez C *et al.* Insulin sensitivity and resistin levels in gestational diabetes mellitus and after parturition. *Eur. J. Endocrinol.* 158(2), 173–178 (2008).
41. Stanton RC. Glucose-6-phosphate dehydrogenase, NADPH, and cell survival. *IUBMB Life* 64(5), 362–369 (2012).

- **A review in which the biochemistry, regulatory signals, physiologic roles and pathophysiologic roles for G6PD that have been elucidated over the past 20 years are discussed. This review also highlights the pathophysiologic roles for G6PD that have been identified in disease processes, one of which is diabetes.**

42. Zhang Z, Liew CW, Handy DE *et al.* High glucose inhibits glucose-6-phosphate dehydrogenase, leading to increased oxidative stress and beta-cell apoptosis. *FASEB J.* 24(5), 1497–1505 (2010).
43. Shen X, Xi G, Wai C, Clemmons DR. The coordinate cellular response to IGF-I (IGF-I) and IGF-binding protein-2 (IGFBP-2) is regulated through vimentin binding to receptor tyrosine phosphatase beta (RPTPbeta). *J. Biol. Chem.* 290(18), 11578–11590 (2015).
44. Sferruzzi-Perri AN, Owens JA, Pringle KG, Roberts CT. The neglected role of IGFs in the maternal circulation regulating fetal growth. *J. Physiol.* 589(1), 7–20 (2011).
45. Qiu HY, Chu YL, Li M, Sun YY, Li HF. Tyrosine phosphorylation and protein expression of insulin receptor substrate-2 in the adipose tissue from patients with polycystic ovary syndrome. *Zhonghua Fu Chan Ke Za Zhi* 40(2), 116–119 (2005).
46. Lappas M. IGF-binding protein 1 and 7 concentrations are lower in obese pregnant women, women with gestational diabetes and their fetuses. *J. Perinatol.* 35(1), 32–38 (2015).

•• **Discusses a potential link between the concentrations of IGFBPs in women with gestational diabetes and their fetuses.**

47. Nawathe AR, Christian M, Kim SH, Johnson M, Savvidou MD, Terzidou V. IGF axis in pregnancies affected by fetal growth disorders. *Clin. Epigenetics* 8, 11 (2016).

48. Renz PB, Cavagnolli G, Weinert LS, Silveiro SP, Camargo JL. HbA1c test as a tool in the diagnosis of gestational diabetes mellitus. *PLoS ONE* 10(8), e0135989 (2015).