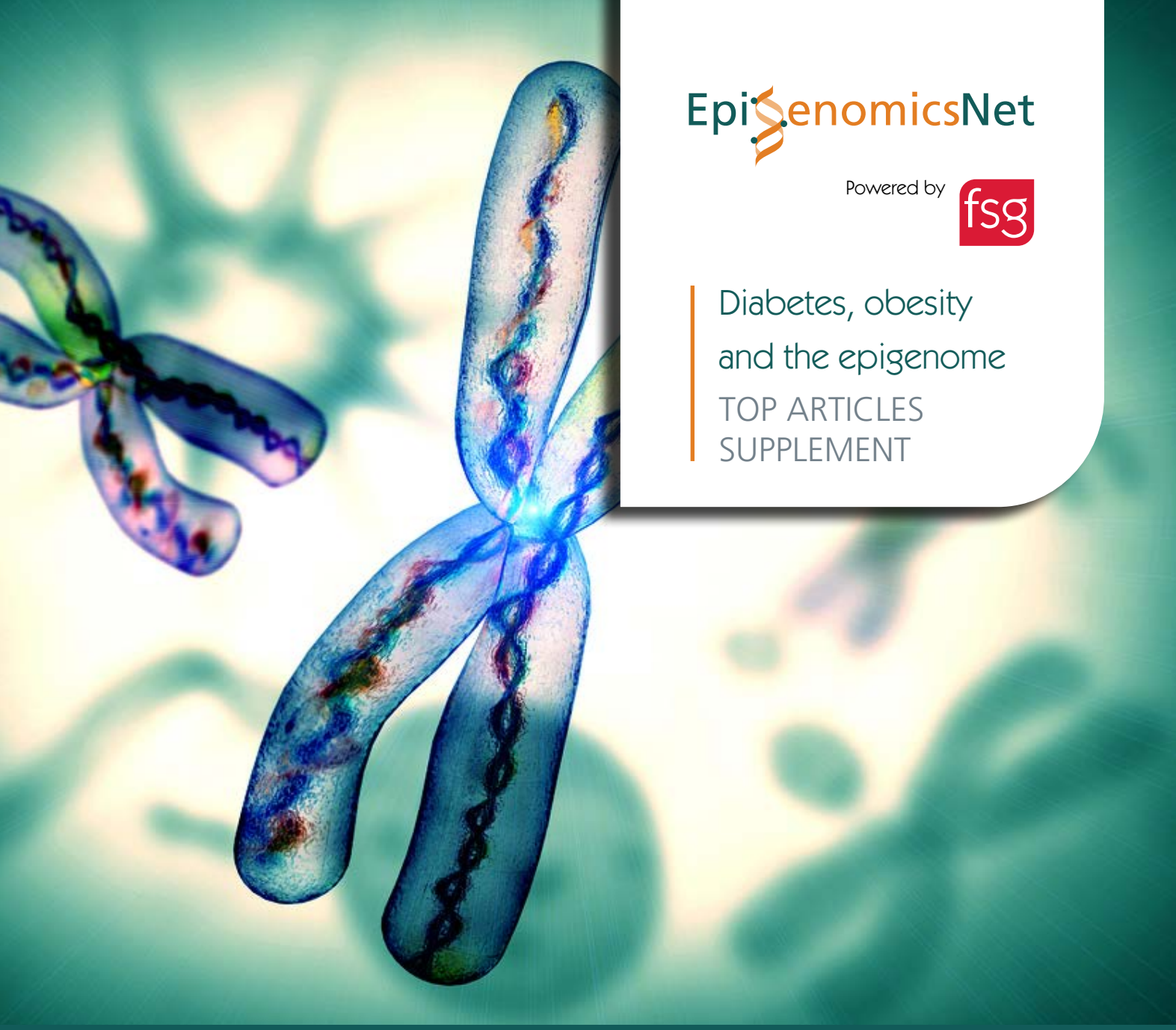

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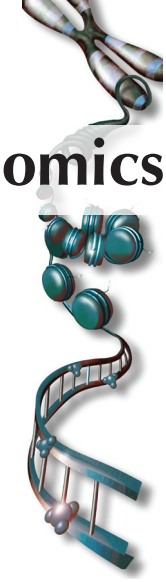

HIF3A association with adiposity: the story begins before birth

Aim: Determine if the association of *HIF3A* DNA methylation with weight and adiposity is detectable early in life. **Material & methods:** We determined *HIF3A* genotype and DNA methylation patterns (on hybridization arrays) in DNA extracted from umbilical cords of 991 infants. Methylation levels at three CpGs in the *HIF3A* first intron were related to neonatal and infant anthropometry and to genotype at nearby polymorphic sites. **Results & conclusion:** Higher methylation levels at three previously described *HIF3A* CpGs were associated with greater infant weight and adiposity. The effect sizes were slightly smaller than those reported for adult BMI. There was also an interaction within *cis*-genotype. The association between higher DNA methylation at *HIF3A* and increased adiposity is present in neonates. In this study, no particular prenatal factor strongly influenced *HIF3A* hypermethylation. Our data nonetheless suggest shared prenatal influences on *HIF3A* methylation and adiposity.

Keywords: birth weight • DNA methylation • embryonic and fetal development • epigenomics • *HIF3A* protein • human • obesity

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DNA methylation states at particular loci have been associated with a range of disease states and environmental exposures. It is desirable to know whether particular DNA methylation changes occur before disease symptoms or after as a consequence of the disease, as epigenetic biomarkers have been suggested as a way of tracking developmental trajectories to disease [1].

A large epigenome-wide association study (EWAS) with replication in two independent cohorts reported that methylation levels at three CpGs in the first intron of the hypoxia inducible factor 3A (*HIF3A*) locus were positively associated with BMI in adult Caucasian whole blood and adipose tissue [2]. The finding has since been independently replicated [3]. The authors postulated that *HIF3A* has a role in acquired obesity, perhaps in regulating adipocyte differentiation.

There is considerable evidence of developmental pathways to obesity [4–7], beginning before birth and at least partly mediated by epigenetics [8–10]. Factors acting prenatally may affect methylation levels at particular loci, which in turn affect transcription and phenotype later in life [1]. Birth weight is often used as a surrogate for the quality of the *in utero* environment and both low and high birth weights are associated with an increased risk of obesity and metabolic syndrome [11,12]. An association of birth weight or neonatal adiposity with *HIF3A* methylation at birth would be compatible with the developmental origins of obesity hypothesis. The effects of the prenatal environment on the epigenome could be genotype dependent [13] as we have previously found that umbilical cord methylation levels can associate with various *in utero* environmental factors in interaction with infant genotype [14]. *HIF3A* methylation was shown to associate with *cis*-genotype but the genotype was not associated with adult BMI [2].

The present study has three objectives. First, we tested if *HIF3A* gene methylation levels in umbilical cords were associated with birth size (weight and length) and adiposity. Second, due to the strong association between

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cis-SNPs and methylation within the *HIF3A* locus, we studied if the association of methylation with birth weight is independent of *cis*-genotype. Third, we sought to identify prenatal environmental variables associated with both birth weight and *HIF3A* methylation.

Materials & methods

Study population

Mother–offspring pairs in our study were drawn from the Growing Up in Singapore towards Healthy Outcomes (GUSTO) birth cohort [15]. Women aged at

Table 1. Characteristics of participants in the GUSTO cohort studied in the analysis.

Characteristic	Time point	n (%)	Mean (standard deviation)
Ethnicity:	Delivery		
Chinese		568 (57)	
Malay		251(25)	
Indian		172 (17)	
Child sex:			
Male		530 (53)	
Female		461 (47)	
Gestational age (weeks)			38.9 (1.0)
Weight (g)	Delivery	961	3150 (376)
	6 months	872	7744 (919)
	12 months	848	9405 (1073)
	18 months	808	10,770 (1304)
	24 months	820	11,996 (1542)
Length (cm)	Delivery	961	49 (2)
	6 months	876	67 (3)
	12 months	850	75 (3)
	18 months	695	82 (3)
	24 months	720	88 (4)
BMI (g/cm^2)	Delivery	961	1.3 (0.1)
	6 months	872	1.7 (0.2)
	12 months	847	1.6 (0.1)
	18 months	693	1.6 (0.1)
	24 months	720	1.6 (0.1)
Subscapular skinfold (mm)	Delivery	962	5.0 (1.2)
	18 months	673	6.4 (1.4)
	24 months	760	6.4 (1.6)
Triceps skinfold (mm)	Delivery	963	5.5 (1.2)
	18 months	714	8.6 (1.7)
	24 months	734	8.8 (1.8)
Subscapular: triceps	Delivery	962	0.93 (0.17)
	18 months	648	0.76 (0.14)
	24 months	723	0.73 (0.14)
Methylation of cg27146050	Delivery	991	15% (4%)
Methylation of cg16672562			19% (5%)
Methylation of cg22891070			31% (6%)
rs8102595 minor allele frequency (G)			0.22
rs3826795 minor allele frequency (T)			0.41

Table 2. Association between umbilical cord methylation at three sites in *HIF3A* and six neonatal anthropometric outcomes (weight, length, BMI, subscapular skinfold, triceps skinfold and ratio of subscapular to triceps skinfold) at birth.

	cg27146050			cg16672562			cg22891070		
	Est.	95% CI	p-value	Est.	95% CI	p-value	Est.	95% CI	p-value
Birth weight (g)	3.61	(0.68–6.63)	**0.015**	3.34	(1.4–5.32)	**0.00068**	2.05	(0.32–3.82)	**0.020**
Birth length (cm)	0.60	(-0.29–1.5)	0.19	0.46	(-0.13–1.05)	0.13	0.35	(-0.18–0.88)	0.20
BMI at birth (g/cm^2)	2.38	(0.23–4.58)	**0.030**	2.4	(0.97–3.84)	**0.00096**	1.35	(0.07–2.64)	**0.039**
Subscapular skinfold at birth (mm)	-0.77	(-5.97–4.72)	0.78	5.44	(1.76–9.25)	**0.0035**	3.27	(0–6.64)	**0.050**
Triceps skinfold at birth (mm)	-1.28	(-6.42–4.14)	0.64	0.75	(-2.75–4.38)	0.68	0.25	(-2.90–3.51)	0.88
Subscapular: triceps at birth	0.50	(-3.85–5.04)	0.83	4.67	(1.66–7.77)	**0.0022**	3.02	(0.34–5.78)	**0.027**

Regression coefficients (Est.) and 95% CI are reported as percentage change in outcome for 10% increase in methylation level. p-values are two-sided and p-values less than 0.05 are shown in bold. Analysis was done by linear regression of log-transformed outcome against methylation at each CpG site, adjusting for child sex, ethnicity, cell type proportions and interactions between ethnicity and cell type proportions.

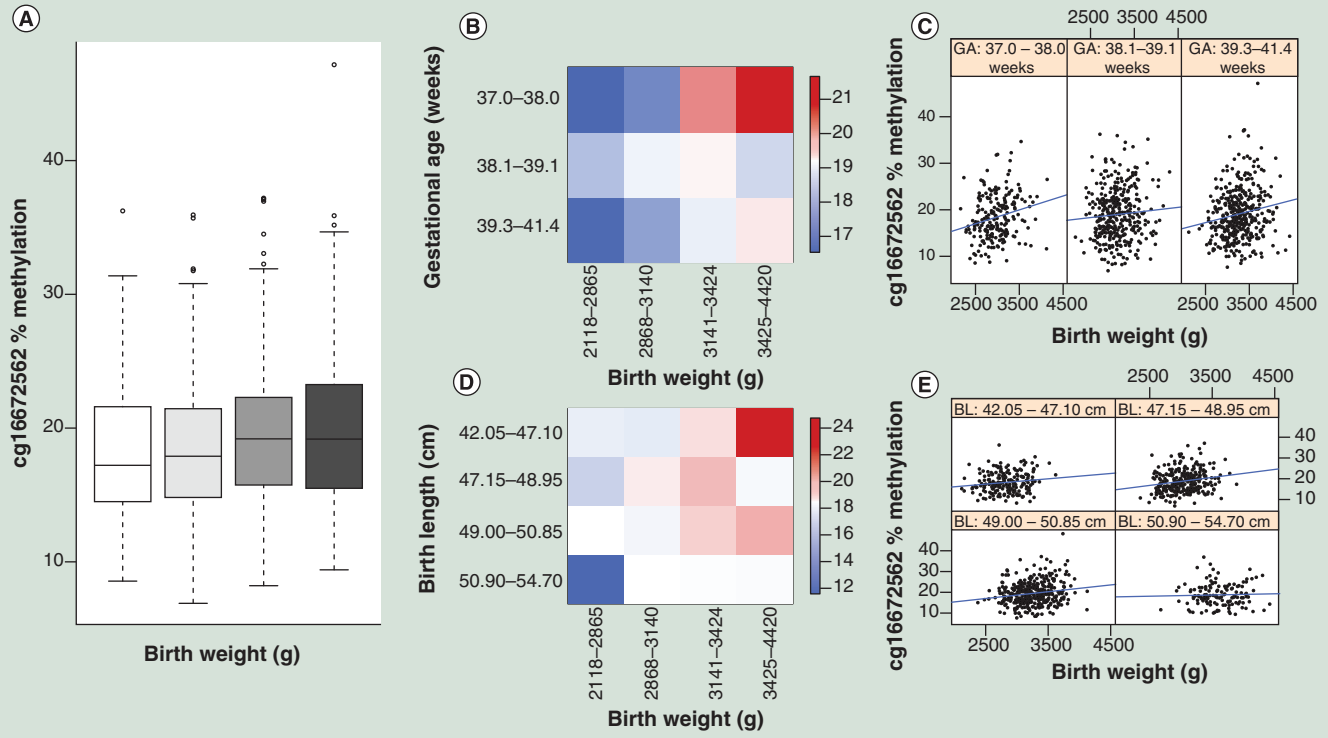
least 18 years were prospectively recruited from the KK Women's and Children's Hospital (KKH) and the National University Hospital (NUH) in Singapore during their first trimester of pregnancy. Written informed consent was obtained. The GUSTO study was approved by the ethics boards of both KKH and NUH. To be eligible, women had to hold Singapore citizenship or permanent residency, intended to reside in Singapore for the next 5 years, were of Chinese, Malay or Indian ethnicity, had homogeneous parental ethnic background and had the intention of delivering at either NUH or KKH. Women who were on chemotherapy, psychotropic drugs or had diabetes mellitus were ineligible. Interviewer-administered questionnaires were used to obtain demographic, medical and obstetric data to assess eligibility. Multiple pregnancies, preterm and intrauterine growth restriction births were excluded from this analysis. There were 1052 live singleton births after excluding 124 preterm and intrauterine growth restriction births. This analysis used 991 offspring with complete information available on *HIF3A* methylation at three sites (cg27146050, cg16672562, cg22891070), genotype at two *cis*-SNPs (rs8102595 and rs3826795) and at least one anthropometric outcome.

Offspring anthropometry

Offspring weight and recumbent length were measured at birth, 6, 12, 18 and 24 months of age. Weight was measured using a calibrated infant scale (SECA 334 Weighing Scale, SECA Corp) and recorded to the nearest gram. Length was measured using a SECA infant mat (SECA 210 Mobile Measuring Mat, SECA Corp) and recorded to the nearest 0.1 cm. Measurements were taken in duplicates for reliability. BMI was derived as weight (g) divided by length^2 (cm^2) at all time points. Subscapular and triceps skinfolds were measured at birth, 18 and 24 months and taken in triplicates using Holtain skinfold calipers (Holtain Ltd, Crymych, UK) on the right side of the body, recorded to the nearest 0.2 mm. Subscapular to triceps skinfold ratio was derived by dividing subscapular skinfold (mm) by triceps skinfold (mm).

Prenatal environmental exposures

An interviewer-administered questionnaire was conducted at 26–28 weeks of gestation to obtain information on occupational activity during pregnancy, exercise during pregnancy, alcohol usage before and during pregnancy and smoking patterns before and during pregnancy (Supplementary File 1; for supplementary information please see online at: www.futuremedicine.com/doi/full/10.2217/EPI.15.45). Maternal height and weight were measured during the same time period. Prepregnancy weight was self-reported during study recruitment in the first trimester of pregnancy. Pregnancy weight gain was calculated as the difference between prepregnancy and 26–28-week weights. Maternal prepregnancy BMI was derived as prepregnancy weight divided by height squared. Maternal glucose levels, indicative of gestational diabetes, (2 h postglucose and fasting) were ascertained at 26–28 weeks using an oral glucose tolerance test (OGTT) of 75 g after overnight fasting (8–10 h). Information on maternal glucose levels (2 h postglucose and fasting) were available for 922 subjects. Using the 1999 WHO standard criteria (≥7.8 mmol/l for 2-h postglucose and/or ≥7.0 mmol/l for fasting glucose), 162 out of 922 subjects would be diagnosed as having ges-



Birth weight (g)	2118–2865	2868–3140	3141–3424	3425–4420
Count	231	253	266	211
Median methylation (%)	17.2	17.9	19.2	19.2
Mean methylation (%)	18.0	18.4	19.4	19.9

Figure 1. Association between percentage methylation at cg16672562 and birth weight. (A) Box plot of birth weight (horizontal axis) binned into four bins of equal numbers of unique values, against % methylation at cg16672562 (vertical axis). Number of individual data points, median and mean % methylation of each bin is displayed in the table below the horizontal axis. **(B)** Heatmap displaying % methylation at cg16672562, maximum, medium and minimum methylation are shown in red, white and blue, respectively. Subjects are split by binned (four bins of equal number of unique values) birth weight (horizontal axis) and binned (four bins of equal number of unique values, last two bins are combined as there are few observations in the last bin) gestational age (vertical axis). The progression from blue to red (low to high methylation) as birth weight increases (left to right) is visible in each gestational age bin (top to bottom). **(C)** Scatter plots of birth weight (horizontal axis) against % methylation at cg16672562 (vertical axis), each panel displays data for binned gestational ages (four bins of equal number of unique values, last two bins are combined as there are few observations in the last bin), ranges for each bin are displayed in panel headers. **(D)** Heatmap displaying % methylation at cg16672562, maximum, medium and minimum methylation are shown in red, white and blue, respectively. Subjects are split by binned (four bins of equal number of unique values) birth weight (horizontal axis) and binned (four bins of equal number of unique values) birth length (vertical axis). The progression from blue to red (low to high methylation) as birth weight increases (left to right) is visible in each birth length bin (top to bottom). **(E)** Scatter plots of birth weight (horizontal axis) against % methylation at cg16672562 (vertical axis), each panel displays data for binned (four bins of equal number of unique values) birth length, ranges for each bin are displayed in panel headers.
For color images please seen online at: www.futuremedicine.com/doi/full/10.2217/EPI.15.45

tational diabetes in this group. Maternal plasma micronutrient levels, including vitamin D, vitamin B12, copper, iron, zinc and folate, were tested using serum drawn at 26–28 weeks of gestation. Birth order and mode of delivery were abstracted from hospitals' medical records.

Methylation & genotyping data

Bisulfite-converted gDNA from umbilical cords was interrogated on Infinium® Human Methylation 450 Bead Chip arrays [16] as per manufacturer's instructions and as described previously [14]. Data were processed as previously described [17]. Briefly, signal extraction was performed in GenomeStudio™ Methylation Module and raw values were extracted. Probes with data from two beads or fewer for any sample, or with signal detection p-values (calculated from signal vs background of the individual bead intensities) greater than 0.01, for any sample, were discarded for all samples. The green

Table 3. Association of two *cis*-single nucleotide polymorphisms (rs8102595, rs3826795) with methylation at three sites in *HIF3A*.

Model	cg27146050			cg16672562			cg22891070		
	Est.	95% CI	p-value	Est.	95% CI	p-value	Est.	95% CI	p-value
CpG ~ rs8102595	1.2	(0.96–1.5)	**5.7E-19**	2.9	(2.5–3.3)	**1.6E-45**	3.2	(2.8–3.7)	**5.7E-47**
CpG ~ rs3826795	0.64	(0.40–0.87)	**1.1E-07**	1.6	(1.2–1.9)	**3.2E-18**	1.5	(1.1–1.9)	**3.2E-14**

Above are results from six different linear regression models. In each linear regression model, methylation at each CpG site is regressed against each SNP. Regression coefficients (Est.) and 95% CI reflect changes in % methylation per copy of the effect allele, adjusting for child sex, ethnicity, cell type proportions and interactions between ethnicity and cell type proportions. p-values are two-sided and p-values less than 0.05 are shown in bold. The effect allele of rs8102595 is G and the effect allele of rs3826795 is C.

and red channel signals were normalized and the background signal from the negative probe control values was removed. Probe β-values or % methylation values were derived from the data. β-values are the ratio of the methylated probe intensity and the overall intensity, β-value for an *i*th interrogated CpG site:

$$\beta_i = \frac{\max(Yi_{i,methy},0)}{\max(Y_{i,unmethy},0) + \max(Y_{i,methy},0) + a}$$

Equation 1

where $y_{i,methy}$ and $y_{i,unmethy}$ are the intensities measured by the *i*th methylated and unmethylated probes, respectively, averaged over the replicate beads; a is a constant offset, which is by default 100. Therefore, β-values range between 0 and 1 with 0 representing no methylation and 1 representing 100% methylation. β-values were further processed to scale the % methylation range of the type 2 probes to the type 1 probes using the procedure suggested by [18]. All data from the sex chromosomes were removed and the remaining data were subjected to quantile normalization. As part of the experimental design, subjects were randomized such that key variables (birth weight, child sex, ethnicity, gestational age) were randomly distributed across batch/chip/position. Batch effects were observed between different runs in the processed data and removed by a commonly used empirical Bayes method known as COMBAT [19]. Even though chip/position were associated with the three *HIF3A* methylation sites in question (Supplementary Table 1), chip/position were not associated with any of the six neonatal outcomes studied in this report (Supplementary Table 2) due to experimental design. As chip/position were associated only with the independent variable (methylation) but not the outcome, this minimizes confounding bias due to chip/position.

The same umbilical cord DNA samples were genotyped using the Illumina Omniexpress + exome arrays. Genotyping was performed by the service provider, Expression Analysis Inc. Data were processed in Genome Studio Genotyping Module™. Genotyping calls were made by the Gen Call software, which incorporates a clustering algorithm (GenTrain) and a calling algorithm (Bayesian model). Gen Call score of each single nucleotide polymorphism (SNP) probe and call rate of each sample are generated. Genotypes with a Gen Call score less than 0.15 were not considered.

Accounting for cellular heterogeneity from methylation data

Umbilical cord tissue consists of a mixture of cell types with differing epigenetic profiles. To control for cellular heterogeneity in our samples, we applied three methods: one requiring a reference panel of methylation levels in discrete cell types [20] and two reference-free methodologies [21,22]. For the former, we estimated the proportion of fibroblasts, B cells and T cells using a reference panel's cell-specific methylation profiles [20]. The methylation dataset obtained from EMBL-EBI European Genome-Phenome Archive [23] under accession number EGAD00010000460 was used as our reference panel [24]. The estimated cell fractions were then adjusted as covariates in the regression models. Interestingly, the associations between the estimated cellular

Table 4. Association between two *cis*-single nucleotide polymorphisms at the *HIF3A* locus (rs8102595, rs3826795) and birth weight.

Single nucleotide polymorphisms	Est.	95% CI	p-value
rs8102595	0.39	(-0.89–1.7)	0.55
rs3826795	0.059	(-1.0–1.2)	0.92

Regression coefficients (Est.) and 95% CI are reported as percentage change in birth weight per copy of the effect allele. p-values are two-sided. Analysis was done by linear regression of log-transformed birth weight against each SNP (additive genetic model), adjusting for ethnicity and child sex. The effect allele of rs8102595 is G and the effect allele of rs3826795 is C.

Table 5. Association between umbilical cord methylation at three sites in *HIF3A* and six neonatal anthropometric outcomes (weight, length, BMI, subscapular skinfold, triceps skinfold and ratio of subscapular to triceps skinfold) at birth, adjusting for rs8102595 and rs3826795.

Outcome	cg27146050			cg16672562			cg22891070		
	Est.	95% CI	p-value	Est.	95% CI	p-value	Est.	95% CI	p-value
Birth weight (g)	3.67	(0.62–6.81)	**0.018**	3.87	(1.7–6.09)	**0.00044**	2.28	(0.34–4.26)	**0.021**
Birth length (cm)	0.63	(-0.3–1.57)	0.18	0.55	(-0.11–1.21)	0.10	0.4	(-0.19–1)	0.18
BMI at birth (g/cm^2)	2.37	(0.13–4.67)	**0.038**	2.74	(1.14–4.36)	**0.00075**	1.46	(0.03–2.91)	**0.046**
Subscapular skinfold at birth (mm)	-0.62	(-6.05–5.11)	0.83	7.08	(2.92–11.41)	**0.00073**	4.31	(0.64–8.11)	**0.021**
Triceps skinfold at birth (mm)	-0.06	(-5.46–5.65)	0.98	2.64	(-1.33–6.77)	0.19	1.7	(-1.85–5.39)	0.35
Subscapular: triceps at birth	-0.6	(-5.06–4.08)	0.8	4.34	(1–7.79)	**0.011**	2.57	(-0.4–5.63)	0.091

Analysis without adjusting for rs8102595 and rs3826795 are shown in Table 2, results of both are similar. Regression coefficients (Est.) and 95% CI are reported as percentage change in outcome for 10% increase in methylation level. p-values are two-sided and p-values less than 0.05 are shown in bold. Analysis was done by linear regression of log-transformed outcome against methylation at each CpG site, adjusting for rs8102595, rs3826795, child sex, ethnicity, cell type proportions and interactions between ethnicity and cell type proportions.

proportions and child anthropometry were generally ethnic-dependent (Supplementary Tables 3 & 4). Therefore, interaction terms between cellular proportions and ethnicity were included in all regression models. As a sensitivity analysis, we also applied two reference-free methods [21,22] to investigate the association between methylation levels at *HIF3A* and log-transformed child anthropometric measures, adjusting for infant sex and ethnicity. Leek *et al.* [22] estimates surrogate variables which capture cellular heterogeneity, the surrogate variables are then adjusted as covariates in regression analysis. We note that this sensitivity analysis using Leek *et al.* [22] would also act to adjust for residual unknown confounding effects, including chip/position effects since the estimated surrogate variables can potentially capture both cellular heterogeneity and residual batch/chip/position effects [21,25–27]. The method proposed by Houseman *et al.* [21] is similar to the surrogate variable analysis proposed by Leek *et al.* [22] with an additional biological mixing assumption. Houseman *et al.* [21] correct the cellular heterogeneity without explicitly estimating the relative cell proportions or returning the surrogate variables.

Statistical analysis

Linear regression models were used to examine the association of child anthropometric measures with *HIF3A* methylation β-values, with adjustment for child sex, ethnicity, cellular composition and interactions between ethnicity and cellular proportions. Child anthropometric measures were log-transformed to satisfy statistical assumptions of normality. Due to the log-transformation, effect sizes are reported as percentage increase in child outcome for 10% increase in methylation β-values. As a sensitivity analysis, we also report results additionally adjusted for gestational age. To examine if the association between child anthropometry with *HIF3A* methylation were independent of *cis*-genotypes, we additionally adjusted for both *cis*-genotypes. To identify prenatal environmental exposures associated with both birth weight and *HIF3A* methylation, we regressed birth weight on various prenatal exposures, and adjusted for child sex and ethnicity. We further regressed *HIF3A* methylation on various prenatal exposures (and their interactions with genotype), adjusting for child sex, ethnicity, cellular composition and interactions between ethnicity and cellular proportions.

Table 6. Two-sided p-values from testing for interaction term between log-transformed birth weight and single nucleotide polymorphism (rs8102595, rs3826795), with methylation as outcome, adjusting for main effect of log-transformed birth weight, main effect of single nucleotide polymorphism, child sex, ethnicity, cell type proportions and interactions between ethnicity and cell type proportions.

Model	cg27146050 p-value	cg16672562 p-value	cg22891070 p-value
log(BW)* rs8102595	0.32	0.077	0.30
log(BW)* rs3826795	0.34	**0.019**	**0.033**

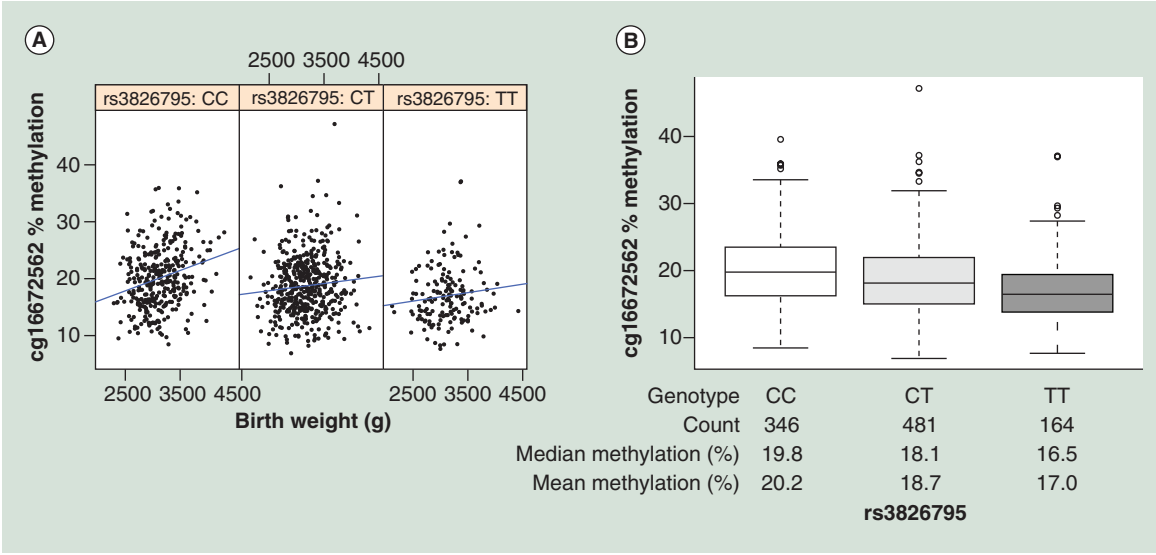


Figure 2. Association between % methylation at cg16672562 and birth weight, stratified by rs3826795 genotype. **(A)** Scatter plots of birth weight (horizontal axis) against % methylation at cg16672562 (vertical axis), each panel displays data for each genotype. **(B)** Box plot of rs3826795 genotype (horizontal axis) against % methylation at cg16672562 (vertical axis). Number of individual data points, median and mean % methylation of each bin is displayed in the table below the horizontal axis.

Results

HIF3A methylation in umbilical cords was positively associated with birth weight & adiposity

Table 1 gives the characteristics of the 991 infants investigated in the current analysis. Higher umbilical cord *HIF3A* methylation level at all three CpGs was associated with greater infant birth weight (Table 2 & Figure 1A), after adjusting for infant sex, ethnicity, estimated cellular proportions and interactions between ethnicity and cellular proportions [20]. There were also significant associations with adiposity at birth measured by BMI and, for two of the three CpGs, by subscapular skinfold and the ratio of subscapular to triceps skinfold (thought to reflect fat deposition on the trunk rather than limbs [28]) (Table 2). Similar results were obtained at two of the CpGs when we used reference-free methodologies [21,22] to account for cellular heterogeneity (Supplementary Tables 5 & 6). Despite the strong association between gestational age and birth weight in our dataset ($r = 0.32$; $p < 0.0005$), the associations of *HIF3A* methylation at all three CpGs with birth weight remained significant, after adjusting for gestational age (Supplementary Table 7). The association between methylation and birth weight was consistent across gestational age categories and was perhaps strongest in neonates with the earliest and latest gestational ages (Figure 1B & C & Supplementary Table 8). Similarly, despite the strong correlation between birth weight and length in the dataset ($r = 0.67$; $p < 0.0005$), *HIF3A* methylation was not significantly associated with birth length and the association between methylation and birth weight was consistent across birth length categories (Figure 1D & E), suggesting a link between *HIF3A* methylation and adiposity at birth rather than birth size in general.

For every 10% increase in methylation of cg27146050, cg16672562 and cg22891070, birth weight was 3.61% (95% CI: 0.68–6.63), 3.34% (1.4–5.32) and 2.05% (0.32–3.82) higher, respectively (Table 2). These effect sizes are slightly smaller than the corresponding effect estimates on adult BMI of 7.8% (5.1–10.4), 3.2% (2.0–4.4) and 3.6% (2.4–4.9), as previously reported [2].

The association of *HIF3A* methylation & birth weight remained significant after adjusting for *cis*-acting SNPs; but birth weight & *HIF3A* methylation was more strongly correlated in one genotypic group

Using the same 991 GUSTO umbilical cord samples, we genotyped the SNPs identified as *cis*-influencing *HIF3A* methylation in the Dick *et al.*'s study [2]. We found that rs8102595 and rs3826795 were strongly associated with methylation levels at all three CpGs sites (Table 3). However, neither SNP was associated with birth weight (Table 4) and the associations of birth weight with methylation at all three sites were similar when adjusted for genotype at both SNPs (Table 5). This mirrors the findings of [2]. We also tested if *HIF3A* methylation levels were explained by an interaction of *HIF3A* genotype and infant birth weight (adjusting for

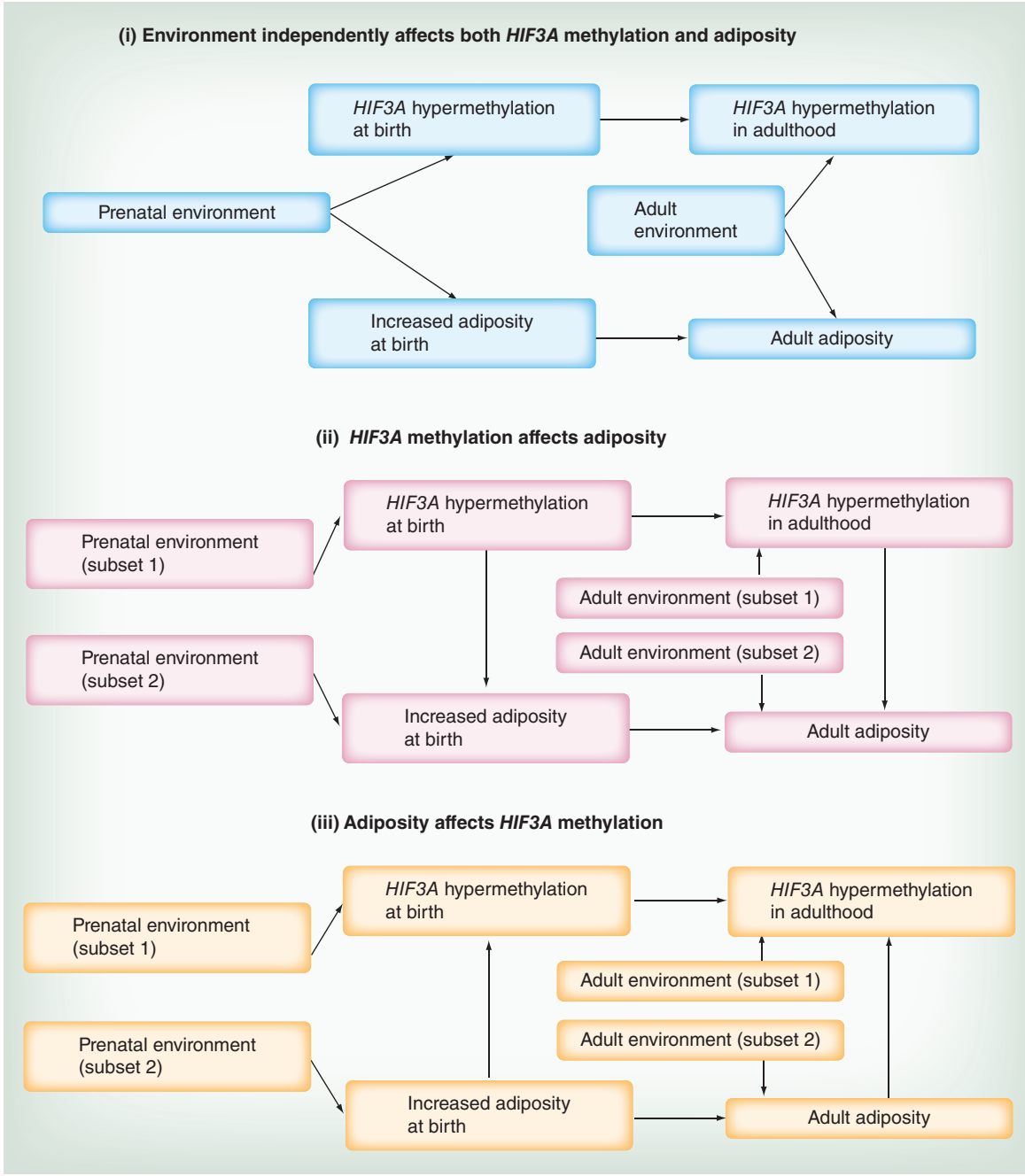


Figure 3. Three of the simplest possible scenarios to explain the relationship between *HIF3A* methylation and adiposity.

main effects of both genotype and infant birth weight), as we have previously found that neonatal methylation patterns are often a product of the interaction of the *in utero* environment and genotype. The interaction term was significant at $p < 0.05$ for two of the CpGs and rs3826795 (Table 6). In the CC genotype group, birth weight was more strongly correlated with *HIF3A* methylation (Figure 2 & Supplementary Table 9). The CC genotype was also associated with higher methylation values at the *HIF3A* locus (Table 3 & Figure 2). This suggests the CC genotypic group could be more plastic to environmental exposures *in utero*.

The prenatal environmental variables examined were not consistently associated with *HIF3A* methylation & birth weight

As we hypothesized that *HIF3A* methylation and birth weight were related to the prenatal environment, we examined for significant associations with both *HIF3A* methylation and birth weight for a range of factors

related to the prenatal environment, both as main effects and in interaction with *HIF3A* genotype. Factors investigated included antenatal lifestyle factors such as activity level and smoking, maternal prepregnancy BMI and pregnancy weight gain, maternal glucose tolerance during pregnancy indicative of gestational diabetes, maternal micronutrient levels, maternal age and parity (Supplementary File 1). There were some suggestive associations; for example, maternal glucose tolerance (indicative of gestational diabetes) was significantly associated with both *HIF3A* methylation at one of the CpGs and birth weight. Furthermore, maternal weight gain during pregnancy was significantly associated both with infant birth weight and *HIF3A* methylation (again at only one CpG) in interaction with genotype. However, we did not find any prenatal environmental variables that showed consistently significant association with all three *HIF3A* sites and birth weight and none yielded substantial effect sizes.

Discussion

We have extended the finding that *HIF3A* DNA methylation in adults associates with BMI [2], to show that the link between *HIF3A* DNA methylation with weight and adiposity can be detected at birth. Interestingly, the association was limited to measures of adiposity (i.e., weight, BMI and skinfolds) and not other determinants of birth size or putative proxies for gestational

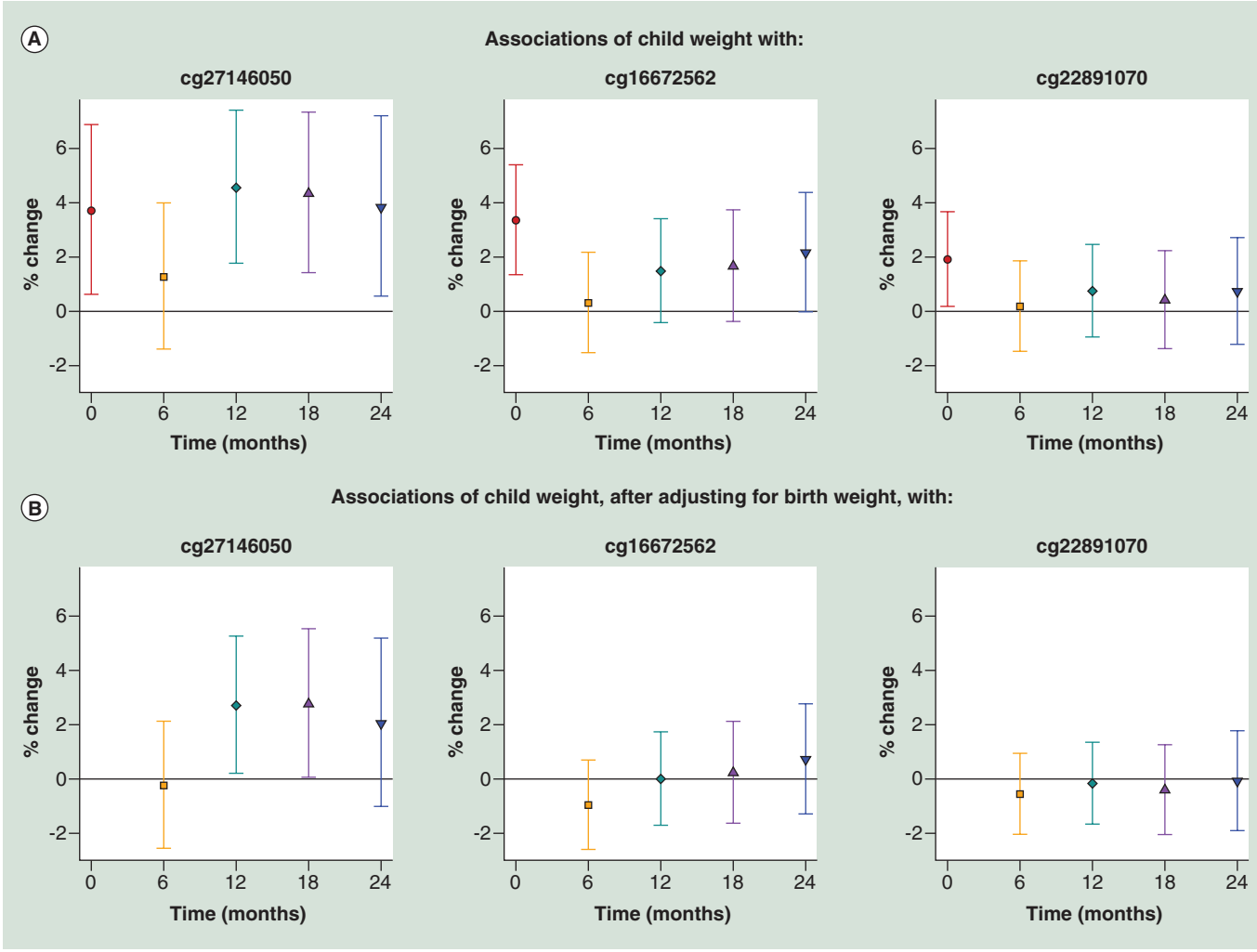


Figure 4. Association between umbilical cord methylation at three sites in *HIF3A* and child weight measured at 0, 6, 12, 18 and 24 months. (A) Without adjusting for birth weight. **(B)** Adjusted for log-transformed birth weight. Regression coefficients (Est.) and 95% CI are reported as percentage change in child weight for 10% increase in methylation level. P-values are two-sided. Using linear regression models estimated using generalized estimating equations to account for repeated measures, we regressed log-transformed weight against methylation at each CpG site, adjusting for fixed effects of time, child sex, ethnicity, cell type proportions and interactions between ethnicity and proportions. Time was coded using a binary variable for each distinct time point and interaction terms of time with all variables (methylation, child sex, ethnicity, cell type proportions and interactions between ethnicity and proportions) were included.

quality such as gestational age and birth length. As such, the relationship of increased methylation at *HIF3A* CpGs with birth outcomes is reminiscent of the effect of maternal diseases, like Type 1 diabetes, on the offspring, whereby weight is increased, but length is not [29].

Although pertaining to the same three CpGs within the *HIF3A* gene that were previously reported, our data were derived from a different tissue (umbilical cord vs blood and adipose), in a different population (Asian vs Caucasian), in a population-based cohort (normative range vs study population for metabolic disorders and controls) and most importantly at a different stage in the lifecourse (neonates vs adults). The effect size reported here are slightly smaller than those reported by Dick *et al.* [2]. Reasons the effect sizes are smaller could be statistical, population dependent (our population are healthy babies, Dick *et al.* studied a disease cohort with a high level of obesity) or developmental (the effect is smaller at birth and increases during the lifecourse as adiposity increases).

Replicated positive associations such as the one described here can guide future hypothesis-free EWAS. Lack of statistical power is a big challenge in EWAS, especially in cohorts of modest size. The integration of effects of cellular heterogeneity, *cis* and *trans* genotype and the potential influence of pregnancy conditions is also challenging. Therefore, a detailed case study of a proposed association (*HIF3A* with adiposity replicated across populations and ages) is important. It will help set the standards for statistical replication and delineate the issues to consider when evaluating hits from more open-ended screening.

Dick *et al.* [2] considered three possibilities that could explain the association between *HIF3A* methylation and adiposity in adults: (i) a confounding factor (e.g., environment) independently affects both *HIF3A* methylation and adiposity, (ii) increased *HIF3A* methylation causes increased adiposity and (iii) increased adiposity causes increased *HIF3A* methylation. To put our findings in neonates in context with the findings in adults, the same three simplified possible scenarios are schematically depicted in Figure 3. We note that more complicated scenarios or combinations of these simplified situations are possible and likely.

Using a Mendelian randomization reasoning [30] from the observation that *HIF3A* genotype was associated with *HIF3A* methylation but not adult BMI, Dick *et al.* [2] suggested that scenario (iii) (adiposity results in *HIF3A* hypermethylation) is more likely than scenario (ii) (*HIF3A* hypermethylation leads to increased adiposity). However, in this context, Mendelian randomization assumes the genotype can affect the phenotype only through DNA methylation and not through other biological pathways. The applicability of this assumption is unknown for *HIF3A* genotype. We cannot confer causality in our dataset but following the same arguments, increased DNA methylation at the *HIF3A* locus could result from increased birth weight/adiposity and not cause it, but the aforementioned limitation also applies in our case.

Dick *et al.* considered scenario (i) less likely as they did not find an association between *HIF3A* methylation and BMI-related characteristics like diabetes. However, the absence of an association between *HIF3A* methylation and diabetes could also be due to the same confounding factor having a direct effect on glucose tolerance that is independent of increased adiposity. We postulate that scenario (i) could involve a prenatal environmental factor that independently affected both birth weight and *HIF3A* methylation. We also find that, as may be expected from other studies [14,31], interindividual variation in *HIF3A* methylation can be explained by an interaction of genotype and birth weight, suggesting there may be a genotype group (CC) particularly sensitive to gestational environment (Table 6 & Figure 2). However, in this study, we were unable to definitively identify a factor acting prenatally that is consistently associated with both *HIF3A* methylation and birth weight, despite the extensive dataset on pregnancy environments collected from the GUSTO cohort and examined in this study. Nevertheless, *HIF3A* methylation at birth after much further study, may prove to be a useful surrogate for likely metabolic trajectory determined by gestational environment [32], and could be more useful than birth weight as it would be independent of the inherited genetic and environmental determinants of birth weight.

Transgenerational transmission of obesity and the importance of the very early environment in determining metabolic trajectories have been much studied [33,34]. If metabolic trajectory is programmed during gestation, a biomarker of likely trajectory would aid intervention [1]. Birth weight has been shown to predict later weight [35] and is strongly associated with later weight in the GUSTO cohort. Interestingly, *HIF3A* methylation levels at birth were also associated with weights at later ages, albeit possibly with slightly lower effect sizes (Figure 4A & Supplementary Table 10). Only a few of the associations survive at borderline significance when adjusted for birth weight (Figure 4B & Supplementary Table 11), thus the association of *HIF3A* methylation with later weights may reflect the growth trajectory set at birth. The 'dip' in the effect size at 6 months could be due to the effects of weaning or the decreased influence of genetic factors [36,37], although it is interesting to note that birth weight is more correlated with weight at 6 months than with weight at any of the other time points (Supplementary Tables 12 & 13).

Future studies should track both *HIF3A* methylation and offspring size and adiposity longitudinally during gestation, at birth, and at subsequent ages to determine if the association of *HIF3A* methylation with adiposity is consistent. As blood and adipose are invasive tissues to sample in infants, *HIF3A* methylation levels should be investigated in other tissue types such as saliva, buccal swabs or circulating fetal cells obtained prenatally. Longitudinal studies can help determine, if *HIF3A* is a biomarker for obesity risk and assess the utility of using *HIF3A* methylation to evaluate the efficacy of interventions such as lifestyle modification. Future studies should also examine the impact of *HIF3A* methylation changes on gene expression in relevant tissues. Dick *et al.* [2] found a negative association between *HIF3A* methylation levels and *HIF3A* gene expression in adipose tissue. It is a limitation of our study that we cannot examine gene expression in a relevant tissue due to the unfeasibility of sampling adipose tissue in neonates or infants.

Conclusion

The association between increased DNA methylation at *HIF3A* and increased adiposity is present in neonates. This suggests *HIF3A* methylation is a potential biomarker for metabolic trajectory and/or implicated in metabolic syndrome and further study is warranted.

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Executive summary

Introduction

- Variation in DNA methylation between individuals is a product of influences from the environment and individual genetic backgrounds.
- DNA methylation levels at particular loci may closely map trajectories to diseases such as obesity, which have environmental and genetic components.
- A recent study linked methylation within the *HIF3A* gene to BMI and obesity in adults.
- Individual susceptibility to obesity is influenced by early environmental exposures.
- This study investigates whether the association of *HIF3A* methylation with adiposity can be detected at birth.

Material & methods

- The study was conducted in the 991 subjects from the GUSTO birth cohort.
- We determined *HIF3A* genotype and DNA methylation patterns in DNA extracted from umbilical cords of the 991 infants using Illumina® Infinium HumanMethylation and Omniexpress arrays.
- Associations of methylation levels and birth outcomes were adjusted for sex, ethnicity, cellular composition of umbilical cords and interactions between ethnicity and cellular composition.

Results

- Higher DNA methylation within *HIF3A* measured in umbilical cord tissue was associated with greater infant birth weight and adiposity.
- The association of *HIF3A* methylation and birth weight remained significant after adjusting for *cis*-acting single nucleotide polymorphisms; but the association was stronger in one genotypic group.
- None of the prenatal environmental variables examined were consistently associated with *HIF3A* methylation and birth weight.

Discussion

- We have extended the previous finding that *HIF3A* DNA methylation in adults associates with BMI to show that the link between *HIF3A* DNA methylation with weight and adiposity can be detected at birth.
- Although pertaining to the same three CpGs within the *HIF3A* gene that were previously reported, our data were derived from a different tissue (umbilical cord vs blood and adipose), in a different population (Asian vs Caucasian), in a population-based cohort (normative range vs study population for metabolic disorders and controls) and most importantly at a different stage in the lifecourse (neonates vs adults).
- This suggests that prenatal factors may influence *HIF3A* methylation but despite the extensive data collected in the GUSTO, we were unable to definitively identify a responsible prenatal factor.
- We were unable to imply causality in our data and do not know whether adiposity causes *HIF3A* hypermethylation, *HIF3A* hypermethylation causes adiposity or if some independent factor influences both.
- In their study of *HIF3A* methylation and adult BMI, Dick *et al.* used Mendelian randomization to suggest that *HIF3A* hypermethylation is a consequence of increased adiposity. We could draw the same conclusion from our data but note that some of the assumptions of Mendelian randomization might not be satisfied.
- We suggest that as the association between *HIF3A* methylation and adiposity is detectable so early in life, *HIF3A* may be a potential biomarker of metabolic trajectory.

M Hanson, CJ Henry, C-Y Hsu, H Inskip, J Kapur, K Kwek, IY-M Lau, BW Lee, N Lek, SB Lim, Y-L Low, I Magiati, LM Daniel, C Ngo, K Naiduvaje, WW Pang, A Qiu, BL Quah, VS Rajadurai, M Rauff, SA Rebello, JL Richmond, A Rifkin-Graboi, LP-C Shek, A Sheppard, B Shuter, L Singh, W Stunkel, LL Su, OH Teoh, HPS van Bever, RM van Dam, IBY Wong, PC Wong, GSH Yeo.

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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Review

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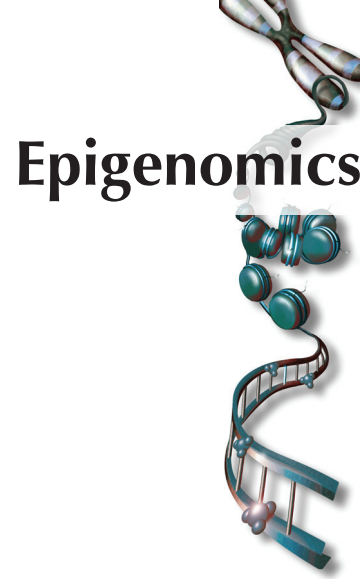

DNA methylation as a diagnostic and therapeutic target in the battle against Type 2 diabetes

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Type 2 diabetes (T2D) develops due to insulin resistance and impaired insulin secretion, predominantly in genetically predisposed subjects exposed to nongenetic risk factors like obesity, physical inactivity and ageing. Emerging data suggest that epigenetics also play a key role in the pathogenesis of T2D. Genome-wide studies have identified altered DNA methylation patterns in pancreatic islets, skeletal muscle and adipose tissue from subjects with T2D compared with nondiabetic controls. Environmental factors known to affect T2D, including obesity, exercise and diet, have also been found to alter the human epigenome. Additionally, ageing and the intrauterine environment are associated with differential DNA methylation. Together, these data highlight a key role for epigenetics and particularly DNA methylation in the growing incidence of T2D.

Keywords: adipose tissue • BMI • CpG-SNPs • epigenetics • exercise • HbA1c • insulin resistance • insulin secretion • pancreatic islets • skeletal muscle

Already in the early 1990s Hales and Barker suggested a role for epigenetics in the development of metabolic diseases, including Type 2 diabetes, when they described how early life events affected health outcome in adulthood [1]. This first hypothesis was confirmed by a number of epidemiological studies, investigating altered fetal, early postnatal and childhood growth on the development of Type 2 diabetes or insulin resistance [2–5]. Clearly this was an important beginning of a field that would start to be experimentally explored in humans many years later, with new laboratory technologies allowing for thorough investigations of epigenetic marks.

Type 2 diabetes is a complex disorder manifested by chronically elevated blood glucose levels, as a result of insufficient insulin secretion from the pancreatic β cells and decreased insulin sensitivity in target tissues, for example, the liver, skeletal muscle and adipose tissue. Genetic as well as environmental factors are involved in the development of Type 2 diabetes, with obesity as one of the main factors for the rapid increase of affected individuals worldwide [6]. Interestingly, the interaction between genetic and environmental factors in disease development most likely involves epigenetic modifications [7].

The most studied epigenetic mark is DNA methylation, the addition of a methyl group to the DNA nucleotide cytosine [8]. A strictly regulated DNA methylation pattern is essential for normal development and has a key role throughout life in tissue specific gene regulation and transcription [9]. Histone modifications are other important epigenetic marks, interacting with DNA methylation and tightly linked to the chromatin structure [10,11]. Also noncoding RNAs, with the ability to post transcriptionally regulate gene expression, is considered an epigenetic modification and implicated in the pathogenesis of Type 2 diabetes [12]. In contrast to the stable DNA sequence, epigenetic modifications are dynamic and reversible and hence promising targets for pharmacological interventions.

This review will summarize published experimentally data supporting a role for epigenetic variation in the development of

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Type 2 diabetes, with a focus on findings from human studies, including both candidate gene approaches and genome-wide studies (Figure 1).

Evidence of epigenetic alterations of genes involved in insulin secretion

The ability of pancreatic β cells to produce and secrete insulin in response to glucose is of utmost importance to maintain normoglycemia. During the development of Type 2 diabetes, the demands of insulin usually increase as a result of increased insulin resistance. Hence, mechanisms that affect β cell function, including cell viability, insulin production and secretion potential, are keys to find new treatment strategies for diabetes.

In a recent study, we investigated the genome-wide DNA methylation pattern in human pancreatic islets obtained from deceased donors [13]. We found altered levels of DNA methylation in islets from Type 2 diabetic versus nondiabetic donors for a total of 1649 CpG sites, corresponding to 853 genes. These genes include known Type 2 diabetes loci such as *TCF7L2*, *KCNQ1*, *THADA*, *FTO*, *IRS1* and *PPARG*, and are enriched in pathways involved in cancer, axon guidance and MAPK (mitogen-activated protein kinase) signaling. Furthermore, 102 of the genes showing differential methylation also displayed altered mRNA expression between islets from Type 2 diabetic versus nondiabetic donors, suggesting epigenetic regulation of transcriptional activity and a possible mechanism for how Type 2 diabetes risk genes contribute to disease development (Figure 1). The majority of these genes showed increased DNA methylation and decreased gene expression in islets from diabetic versus nondiabetic donors. Functional analysis of some of those genes demonstrated, for example, impaired insulin secretion when *Cdkn1a* and *Pde7b*, respectively, were overexpressed in β cells, and also reduced β cell proliferation as a result of overexpression of *Cdkn1a* (p21). Additionally, silencing of *Exoc3l*, a gene encoding one component of the exocyst complex, in the β cells resulted in reduced exocytosis. Importantly, in this study there was no significant difference in the β cell content between diabetic and nondiabetic islets, showing that the identified epigenetic differences are not due to an altered cell type composition. Taken together, this study provides detailed information of the DNA methylome in human pancreatic islets and indeed highlights the importance of epi-

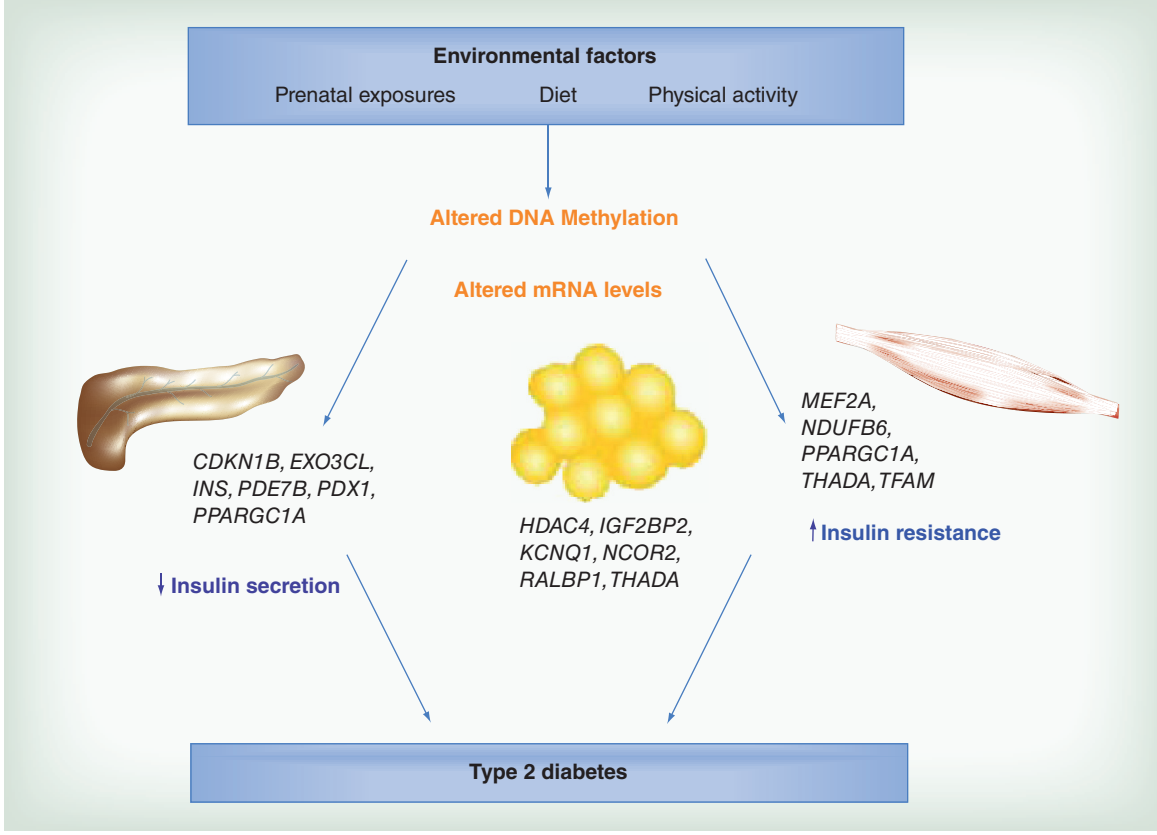

Figure 1. Altered DNA methylation in target tissues for Type 2 diabetes. Altered DNA methylation and transcriptional activity of Type 2 diabetes candidate genes in human pancreatic islets may contribute to reduced insulin secretion. Additionally, environmental factors may induce insulin resistance in adipose tissue and skeletal muscle through epigenetic modifications.

genetic regulation as a mechanism in the pathogenesis of Type 2 diabetes [13].

DNA methylation in human pancreatic islets has also been investigated by Volkmar *et al.* [14]. This study, covering less CpG sites and investigating less human islet donors as compared with the study by Dayeh *et al.*, was still able to detect 276 CpG sites annotated to 254 gene promoters with differential DNA methylation in islets obtained from Type 2 diabetic versus nondiabetic donors. These genes are implicated in β cell function and survival and further support a role for DNA methylation in the pathogenesis of Type 2 diabetes. Interestingly, 27% of the CpG sites identified by Volkmar *et al.* was also differentially methylated in islets from Type 2 diabetics versus nondiabetic donors in our study [13]. Moreover, both studies show that the CpG sites with significantly altered DNA methylation tend to have decreased DNA methylation in islets from Type 2 diabetic compared with nondiabetic donors (96 and 97%, respectively) [13,14]. As aberrant DNA methylation could be either a cause or arise as a consequence of disease, Volkmar *et al.* exposed pancreatic islets from nondiabetic donors to high glucose levels. They found no significant effect on DNA methylation for the 16 CpG sites tested, making the altered methylation pattern in islets from Type 2 diabetic patients unlikely to be a cause of hyperglycemia [14]. However, it remains to be tested if prolonged hyperglycemia, in other words, more than 72 h as tested here, may have a different outcome as diabetes and its complications is known to develop for decades. To approach the same question from a different angle, we tested if the 1649 CpG sites with altered DNA methylation in islets from Type 2 diabetics versus nondiabetic donors were associated with age, BMI or HbA1c, all known risk factors for Type 2 diabetes, in nondiabetics [13]. For HbA1c, 142 CpG sites (8.6%) were found to have significantly associated levels of DNA methylation in islets from nondiabetic donors, and for age and BMI we found 18 and 16 CpG sites, respectively. Importantly, 92% of those sites change in the same direction in islets from Type 2 diabetic donors as with increased age, BMI or HbA1c, hinting that some epigenetic alterations may be seen prior to onset of disease [13].

We have also studied the genome-wide DNA methylation pattern in human pancreatic islets in response to palmitate treatment, to mimic the situation of elevated circulating free fatty acids often seen in Type 2 diabetes patients [15]. Global DNA methylation, in other words, the average of all 483,844 CpG sites analyzed, was significantly higher in islets exposed to palmitate for 48 h compared with control islets. Additionally, exposure to palmitate altered the degree of DNA methylation of 46,977 sites at $p < 0.05$, which is approximately double the expected number and significantly more than expected by chance. However, after correction for multiple testing, no individual CpG site showed a significant difference in DNA methylation in response to palmitate treatment. Nevertheless, of the 1860 genes with significantly altered expression levels due to palmitate treatment, 290 genes (15.6%) had one or more CpG sites with a nominal difference in the level of DNA methylation in palmitate treated compared with control islets [15], including Type 2 diabetes susceptibility genes like *TCF7L2* [16] and *GLIS3* [17]. Although this study suggests global epigenetic alterations in pancreatic islets due to palmitate exposure, further investigations are needed to determine if prolonged elevation of circulating free fatty acids, which is often seen in subjects with Type 2 diabetes, may have stronger effects on the DNA methylation pattern and to what extent the association with Type 2 diabetes is mediated by epigenetic factors [15].

In an early study investigating DNA methylation of *PPARGC1A* as a candidate gene, we detected altered levels of methylation in a region upstream of the gene in human pancreatic islets from Type 2 diabetic versus nondiabetic donors [18]. This increase in DNA methylation observed in islets from Type 2 diabetic donors was further accompanied by reduced *PPARGC1A* mRNA expression and reduced glucose-stimulated insulin secretion (Figure 1). *PPARGC1A* is a master transcriptional regulator of genes participating in oxidative phosphorylation and hence decreased levels may lead to reduced ATP production from the mitochondria, impairing glucose-stimulated insulin secretion [19]. The *KCNQ1* imprinted locus is another candidate gene region associated with Type 2 diabetes, believed to act through impaired islet function [20–22]. Using both adult human islets and fetal pancreas samples, Travers *et al.* were able to show that a Type 2 diabetes genetic risk variant in the *KCNQ1* locus was associated with DNA methylation of a regulatory sequence in fetal samples, and with another region involving a PLAGL1 binding site in adult islets [23]. However, no effect was observed on the level of gene expression. These results highlight the dynamic feature of DNA methylation, with different regulatory roles at different developmental stages.

An obvious target to investigate when studying Type 2 diabetes is the insulin gene (*INS*) and its regulation. As a matter of fact, two studies have shown that *INS* promoter DNA methylation is involved in regulating gene expression of insulin in human pancreatic islets and β cells, respectively [24,25] (Figure 1). Kuroda *et al.* showed that the human *INS* promoter is demethylated specifically in β cells, and that methylation of this region *in vitro* suppresses *INS* gene expression [24]. Further-

more, we have shown increased levels of DNA methylation in 4 CpG sites in the *INS* promoter region in human pancreatic islets obtained from Type 2 diabetic compared with nondiabetic donors [25]. The methylation levels of these CpG sites, together with another 9 sites in the region, were negatively correlated with *INS* gene expression, indeed suggesting the insulin gene to be subject to epigenetic regulation. In further support of this hypothesis, we could show that hyperglycemia directly altered the degree of methylation of the insulin gene in clonal β cells cultured *in vitro*. Also, FACS (fluorescence-activated cell sorting) sorted human alpha cells had much higher degree of methylation of *INS* than β cells, supporting that *INS* methylation regulate cell specific gene expression. Additionally, the islets obtained from Type 2 diabetic donors displayed decreased insulin content and glucose-stimulated insulin secretion [25]. A similar approach was undertaken to investigate *PDX1* [26], a transcription factor important for pancreas development and β cell maturation and function [27]. We found three CpG sites located within the distal promoter and a set of seven CpG sites in the enhancer region of *PDX1* that exhibit significantly increased DNA methylation in islets from Type 2 diabetic compared with nondiabetic donors. Interestingly, all nine analyzed CpG sites in the enhancer region correlated negatively with *PDX1* mRNA expression levels in human islets, whereas this only was seen for one CpG site in the distal promoter and one in the proximal promoter, suggesting that epigenetic regulation of *PDX1* is mediated mainly through the enhancer region. By the use of luciferase constructs, we could functionally demonstrate that methylation of the enhancer region of *PDX1* decreases its transcriptional activity. Moreover, *PDX1* mRNA levels were significantly correlated with *INS* mRNA expression and glucose-stimulated insulin secretion (Figure 1). We also found a significant, positive association between *PDX1* DNA methylation and HbA1c, suggesting that prolonged hyperglycemia may induce epigenetic alterations. This result was further experimentally verified by exposing clonal β cells to high glucose, resulting in increased DNA methylation and decreased mRNA expression of *PDX1* [26].

Incretin hormones are important for stimulating the release of insulin in response to a meal and its mimicking agents are used in Type 2 diabetes treatment [28]. *GLP1R* encodes the receptor for one of the incretin hormones, glucagon-like peptide-1, and is expressed in pancreatic β cells [29]. When investigating the epigenetic component of *GLP1R* gene regulation, we found a modest but significant difference, with increased DNA methylation in human pancreatic islets obtained from Type 2 diabetics compared with nondiabetic donors [30]. Another CpG site in *GLP1R*, within a region containing SP1 and SP3 transcription factor binding sites, was negatively correlated with *GLP1R* expression and positively correlated with HbA1c and BMI [30].

There is also evidence for altered levels of noncoding RNA in pancreatic islets from human Type 2 diabetic organ donors [31]. Kameswaran *et al.* identified an imprinted locus with β cell specific expression of a cluster of miRNAs, which were downregulated in islets from Type 2 diabetic compared with nondiabetic donors. Interestingly, this was strongly correlated with promoter hypermethylation [31]. This region, the *DLK1-MEG3* locus, has previously been associated with different forms of cancer [32]. This study also confirmed differential expression of miRNAs previously implicated in diabetes and β cell function, for example, miR-7 [33,34].

As epigenetics is known to regulate gene expression in a tissue specific manner, it is important to consider which tissue to investigate in relation to disease development. However, to be useful as a clinical biomarker, it is also essential to find markers that are reflected in readily accessible tissues or cells, such as blood samples. A few Type 2 diabetes case-control studies have investigated epigenetic alteration in Type 2 diabetes candidate genes in blood samples [35–39]. Toperoff *et al.* detected differentially methylated regions (DMRs) in blood from patients with Type 2 diabetes compared with control individuals, including 6 DMRs in regions previous genetically associated with Type 2 diabetes (*CENTD2, FTO, KCNJ11, TCF7L2* and *WFS1*) [39]. Additionally, replication of individual CpG sites within the investigated DMRs found 13 CpG sites (intronic sites of *THADA, JAZF1, TCF7L2, KCNQ1* and *FTO*, and 3′-UTR and downstream sites in *SLC30A8*) with a significant difference in DNA methylation between pooled cases and controls. They furthermore investigated DNA methylation in blood samples from a longitudinal study with healthy participants at inclusion, of which some progressed to impaired glucose metabolism. The results showed aberrant DNA methylation prior to disease, suggesting that DNA methylation patterns associated with Type 2 diabetes in tissues not directly involved in insulin secretion or action, like blood, is established early in development, and has a role as a predictive rather than a diagnostic marker [39]. In peripheral blood leukocytes, del Rosario *et al.* demonstrated altered promoter methylation in offspring to mothers with diabetes during pregnancy [36]. No individual genes were significantly different between offspring with (n = 14) and without (n = 14) diabetic mothers. However, differentially methylated genes were enriched for pathways involved in pancreas development and insulin secretion, including the pathways maturity onset diabetes of the young, Type 2 diabetes and Notch signaling. This suggests that the intrauter-

ine environment, acting through epigenetic alterations, may exert an effect on future β cell function and risk of diabetes [36]. DNA methylation of the *TCF7L2* promoter was recently investigated in whole blood from patients newly diagnosed with Type 2 diabetes and controls matched for age and BMI [35]. Here, altered levels of DNA methylation was detected in 13 of the 22 analyzed CpG sites in Type 2 diabetic patients compared with matched controls, after correction for multiple testing and confounding factors. Additionally, specific CpG sites were associated with metabolic traits including fasting glucose (four CpG sites) and total- and LDL-cholesterol (one CpG site). Importantly, the patients were only on diet without any pharmacological treatment affecting the results and this study suggests that DNA methylation alterations of a gene important for β cell function may be reflected in blood DNA [35].

Epigenetic alterations & insulin sensitivity

Insulin resistance is a common feature of Type 2 diabetes, affecting the liver and peripheral tissues like skeletal muscle and adipose tissue. Impaired response to insulin in the peripheral tissues results in reduced capacity to clear the blood stream from glucose, and additionally, makes adipose tissue secrete adipokines which further negatively affect whole body glucose homeostasis. Insulin resistance in the liver results in failure to inhibit glucose output in response to high glucose levels, which adds to an increasing concentration of glucose in the circulation. Consequently, insulin resistance and the tissues involved in this process are, as well as insulin secretion and pancreatic β cells, a key to understand the pathogenesis of Type 2 diabetes.

Skeletal muscle is the main tissue responsible for glucose uptake, which is further stimulated by physical exercise involving muscle contractions. Additionally, individuals with a high genetic predisposition for Type 2 diabetes are characterized by reduced physical fitness [40]. Clearly, exercise is beneficial to improve insulin sensitivity and prevent or delay the onset of Type 2 diabetes. Using an exercise intervention study, we investigated genome-wide DNA methylation in human skeletal muscle from individuals with or without a family history of Type 2 diabetes, in other words, a genetic predisposition for the disease [41]. First, differential methylation at baseline between individuals with or without a family history of Type 2 diabetes was observed for genes involved in, for example, MAPK, insulin, Wnt and calcium signaling, as well as in genes important for muscle function like *MAPK1*, *MYO18B*, *HOXC6* and *PRKAB1*. Of the genes differentially methylated due to a family history of Type 2 diabetes, 40% could be validated in skeletal muscle from monozygotic twin pairs discordant for the disease. Next, 134 genes were found to be differentially methylated in response to the six months exercise intervention, including *MEF2A* (exercise transcription factor), *THADA* (Type 2 diabetes candidate gene), *NDUFC2* (mitochondrial function) and *IL7* (cytokine). Several genes in this study also exhibited changes in both DNA methylation and gene expression levels (Figure 1). Clearly, exercise induces genome-wide changes in DNA methylation in human skeletal muscle; however, the response may differ due to the genetic predisposition for Type 2 diabetes [41]. Barres *et al.* also investigated DNA methylation in human skeletal muscle, and found a global decrease in DNA methylation in response to acute exercise, as well as decreases in promoter methylation of some candidate genes [42]. The latter includes genes with increased expression in skeletal muscle after exercise and which also display altered levels of DNA methylation in Type 2 diabetes, in other words, *PPARGC1A*, *TFAM*, *PPARD*, *PDK4* and *CS* [43] (Figure 1).

Also adipose tissue has a central role in glucose homeostasis, being an endocrine organ affecting many metabolic pathways [44]. We recently investigated genome-wide DNA methylation and mRNA expression in adipose tissue from healthy individuals participating in a six months exercise intervention [45,46]. We found a change in global DNA methylation as well as in 17,975 individual CpG sites in response to exercise [45]. Among the 39 genes with a validated association with Type 2 diabetes at that time [47], 21 had one or more CpG sites with significantly altered levels of DNA methylation in adipose tissue before versus after exercise [45]. *KCNQ1* and *TCF7L2* had most differentially methylated CpG sites, and for *HHEX*, *IGF2BP2*, *JAZF1* and *TCF7L2* increased DNA methylation in response to exercise was accompanied by decreased mRNA expression. To investigate the direct effect of DNA methylation on gene expression, a reporter gene construct of the human *RALBP1* promoter was created where the number of CpG sites methylated can be modified. *RALBP1* is implicated in metabolic disease and GLUT4 trafficking [48,49] and indeed increased DNA methylation of this promoter reduced its transcriptional activity. Furthermore, functional validation of genes (*HDAC4* and *NCOR2*) that exhibit increased DNA methylation and decreased mRNA expression in adipose tissue after six months exercise resulted in increased insulin-stimulated lipogenesis when silenced in an adipocyte cell line [45] (Figure 1). We have also studied the genome-wide DNA methylation pattern in adipose tissue from subjects with Type 2 diabetes compared with nondiabetic controls [50]. DNA methylation was altered in 15,627 individual CpG sites (7046 unique genes) with a false discovery rate of 15%. Replication in adipose tissue from monozygotic twins discordant for Type 2 diabetes

showed a nominal association with disease for 1410 of these CpG sites. These include, for example, the Type 2 diabetes candidate genes *KCNQ1, NOTCH2, TCF7L2* and *THADA* [50] (Figure 1). In adipose tissue from healthy twins, Grundberg *et al.* found 8638 CpG sites in 4133 unique genes where the shared common environment accounted for more than 30% of the methylation variance. A pathway analysis suggested these sites to be associated with metabolic diseases including Type 2 diabetes [51], implying DNA methylation as a link between the environment and disease susceptibility.

Ribel-Madsen *et al.* analyzed genome-wide promoter DNA methylation in both skeletal muscle and adipose tissues from elderly MZ twin pairs discordant for Type 2 diabetes [52]. After correction for multiple testing, only one CpG site in muscle (*IL8*) and seven in adipose tissue were significantly associated with disease, which may be due to the small sample size. However, analyzing Type 2 diabetes candidate genes separately further showed altered levels of DNA methylation in Type 2 diabetic versus control twins for *HNF1A* and *CDKN2A* in adipose tissue. Additionally, global DNA methylation (LINE1) did not associate with Type 2 diabetes status, but with the associated phenotypes BMI and 2 h plasma glucose [52].

Also in DNA from peripheral blood leukocytes there is evidence for a relation between the level of DNA methylation and insulin resistance [53]. Zhao *et al.* examined global DNA methylation (Alu repeats) in monozygotic twins and the relation to insulin resistance as measured by homeostasis model assessment. Each of the four CpG sites investigated was significantly associated with insulin resistance, after correcting for confounding factors including several known risk factors of insulin resistance. However, the mechanism behind this association remains unknown, although the authors speculate that it may be a result of genomic instability due to the altered methylation of the Alu elements [53]. Simar *et al.* went one step further and investigated global DNA methylation in individual cell types in blood [54]. Using this approach, they found increased global DNA methylation in natural killer and B cells from patients with Type 2 diabetes, which correlated positively with insulin resistance. This highlights the need for not only tissue specific but also cell type specific epigenetic analyses and the results potentiate a role for DNA methylation in immune function and metabolic disorders [54].

Low levels of circulating insulin-like growth factor binding protein-1 (IGFBP1) is associated with insulin resistance and Type 2 diabetes [55], and is also a predictor for development of Type 2 diabetes [56]. To investigate how this gene is regulated, Gu *et al.* analyzed *IGFBP1* DNA methylation in blood from patients with Type 2 diabetes and age-matched controls [38]. While the level of DNA methylation was higher, IGFBP1 serum levels were lower in Type 2 diabetic patients compared with controls. Additionally, among individuals newly diagnosed with Type 2 diabetes, a family history of diabetes was associated with higher levels of *IGFBP1* DNA methylation [38], excluding the results to be caused by treatment and suggesting altered methylation of this gene to be a predictive marker of disease. Also IGFBP7 has been linked to insulin resistance and Type 2 diabetes, and is further able to interact both with insulin and insulin-like growth factor-1. In a similar approach as above, Gu *et al.* found increased *IGFBP7* DNA methylation in men newly diagnosed with Type 2 diabetes, however, no correlation with serum IGFBP7 protein levels was observed [37].

Mitochondrial dysfunction likely has a role not only in impairing insulin secretion from the pancreatic β cells, but also in insulin resistance [57]. Interestingly, differential DNA methylation of the promoter of mitochondrial transcription factor A (*TFAM*) in blood samples has been shown to be associated with insulin resistance [58]. Ageing is also associated with insulin resistance and an increased risk for Type 2 diabetes. We have previously found increased DNA methylation in the promoter of two candidate genes for the disease, *NDUFB6* and *COX7A1*, in skeletal muscle from elderly compared with young subjects [59,60]. The increased methylation was further associated with decreased gene expression. While *NDUFB6* encodes a protein which is part of complex one of the mitochondrial respiratory chain, *COX7A1* encodes a member of complex four. Additionally, the expression of both of these genes is decreased in muscle from subjects with Type 2 diabetes compared with nondiabetic controls [61] (Figure 1).

Finally, energy rich diets are associated with obesity, insulin resistance and an increased risk for Type 2 diabetes. Interestingly, a five days high fat diet altered the DNA methylation pattern in skeletal muscle from young healthy men [62]. The 6508 genes with altered levels of DNA methylation were enriched in pathways affecting inflammation, cancer and reproduction. The diet was also associated with metabolic alterations such as hepatic insulin resistance and reduced nonesterified fatty acid levels [62].

Interaction between genetic & epigenetic factors in Type 2 diabetes pathogenesis

There may be an interaction between genetic and epigenetic factors, together affecting physiological processes and disease pathogenesis. Already in 2007, we described the interaction of genetic and epigenetic factors in human skeletal muscle [59]. The investigated gene, *NDUFB6*, is involved in oxidative phosphorylation, and is known to be decreased in insulin resistant and Type 2 diabetic sub-

jects [61]. We found a genetic variant in *NDUFB6* that introduces a CpG site subject to DNA methylation, with the ability to regulate gene expression and glucose uptake in an age dependent matter [59]. Based on this finding, and to reveal a possible disease mechanism, we continued to investigate if genetic variants associated with Type 2 diabetes introduce or remove possible DNA methylation sites [63]. Surpassing our expectations, 19 of the 40 known single nucleotide polymorphisms (SNPs) associated with Type 2 diabetes at that time [47] indeed altered the DNA sequence to introduce or remove a CpG site (also known as CpG-SNPs). We hence analyzed 16 of these CpG-SNPs in human pancreatic islets and found that all were associated with altered levels of DNA methylation in the SNP site. In six of the loci (*CDKN2A*, *TCF7L2*, *HMGA2*, *KCNQ1*, *ADCY5* and *WFS1*), not only the DNA methylation site directly affected by the SNP, but also surrounding CpG sites, showed altered levels of DNA methylation significantly associated with the CpG-SNP. Furthermore, two CpG-SNPs were additionally associated with altered pancreatic islets mRNA expression, four with alternative splicing events (*SLC30A8*, *WFS1*, *CDKAL1* and *TCF7L2*), and four with islet function as measured by insulin secretion and content and glucagon secretion. These findings suggest a mechanism where genetic and epigenetic factors interact to affect gene function and possible risk of disease [63].

As methods for large scale studies improved, we further extended our analyses of genetic and epigenetic interactions by investigating genome-wide SNP (n = 574,533) and DNA methylation (n = 468,787) data in human pancreatic islets [64]. The methylation quantitative trait loci (mQTL) analysis identified 67,438 SNP-CpG pairs in *cis* and 2562 in *trans*, including 11,735 and 383 individual CpG sites, respectively, where genetic variation is significantly associated with altered DNA methylation in human islets. Represented are many previous reported Type 2 diabetes loci, such as *ADCY5*, *KCNJ11*, *INS*, *PDX1* and *GRB10*. A causality inference test further suggested DNA methylation to mediate the effect of genotype on pancreatic islet mRNA expression levels and insulin secretion. Functional validation of candidate genes with genetic variation associated with both DNA methylation and mRNA expression, showed that silencing of *Gpx7* or *Gstt1* results in increased cell death signaling, while silencing of *Snx19* increases β cell proliferation in a β cell line [64]. Indeed, there is evidence for a strong interaction between genetic and epigenetic factors, leading to altered tissue specific gene expression and eventually disease phenotypes.

Using DNA from blood, Bell *et al.* also performed an integrated analysis of interaction between genotypes and epigenotypes in the context of Type 2 diabetes [65]. The method applied in this study investigates the average degree of DNA methylation over larger genomic regions, here exemplified by LD blocks of known Type 2 diabetes associated loci, and not the methylation level of individual CpG sites. Using this approach, they identified haplotype-specific DNA methylation in *FTO*, a locus previously associated with Type 2 diabetes and obesity [66], but not for any of the other investigated Type 2 diabetes regions. Further evaluation of the *FTO* methylation signal pointed to a putative enhancer region, with CpG-creating SNPs affecting the ability for DNA methylation [65], in agreement with enrichment of H3K4me1 in this region as previously described [67,68]. Tobi *et al.* showed that SNPs in *IGF2* and *INS*, respectively, were associated with the level of DNA methylation in blood in gene regulatory regions [69]. There was further an independent and additive effect of prenatal famine on the DNA methylation pattern of these genes.

Conclusion

As methods for analysis of epigenetic modifications have dramatically improved during the last years, maps describing the epigenome in various tissues in health and disease have evolved. In this review, we have specifically described differences in the epigenomes observed in Type 2 diabetic patients compared with nondiabetic control subjects, in tissues relevant for the disease, in other words, pancreatic islets, skeletal muscle, adipose tissue and blood. We have also included associations between DNA methylation and phenotypes or environmental factors relevant for Type 2 diabetes, for example, insulin secretion and action, BMI, HbA1c and exercise.

DNA methylation is highly relevant to serve as a diagnostic tool for Type 2 diabetes, as many epigenetic changes are seen prior to disease development, for example, associated with increased BMI or HbA1c in healthy individuals or detected in prospective studies. The finding of altered DNA methylation in genes relevant for Type 2 diabetes also in blood suggests that at least some of these epigenetic alterations are established in early development. Epigenetics also holds promises for therapeutic advances, with numerous studies of, for instance, HDAC inhibitors clearly demonstrating beneficial effects on diabetic phenotypes. However, there is a significant need to translate the research from cultured cell lines or animals into human clinical investigations.

The results presented in this review show that epigenetics is of major significance for Type 2 diabetes and improves our understanding of the disease, and will guide the development of future drugs.

Future perspective

The arrays investigating genome-wide DNA methylation have greatly contributed to improved knowledge

and understanding of the human epigenome, however, whole genome bisulfite sequencing will take it to the next level by providing details of DNA methylation on single base resolution throughout the complete DNA sequence. As more genome-wide epigenetic data becomes available, there will also be a better understanding of the complex interplay between DNA methylation and the various histone modifications, acting together with genetic variation in gene regulation and establishment of the chromatin structure. Together, these data will help identify and develop novel diagnostic and therapeutic agents that can be used in the battle against Type 2 diabetes.

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Executive summary

- The DNA methylation pattern differs between patients with Type 2 diabetes and matched controls in target tissues for the disease, in other words, human pancreatic islets, skeletal muscle and adipose tissue.
- Both insulin secretion and insulin resistance show associations with altered DNA methylation in multiple human tissues.
- Alterations in the level of DNA methylation are widespread across the entire genome, but also present in numerous candidate genes for Type 2 diabetes.
- Epigenetic variations are present prior to disease development and are found to be associated with risk factors for Type 2 diabetes, including age, BMI and HbA1c.
- Patients with Type 2 diabetes display altered levels of DNA methylation of genes implicated in the disease also in blood samples, providing an opportunity to be used as clinical biomarkers.
- As epigenetic changes are dynamic and potentially reversible, targeting drugs, for example, histone deacetylase (HDAC) inhibitors, are suggested for disease prevention or in the treatment of Type 2 diabetes.

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Review

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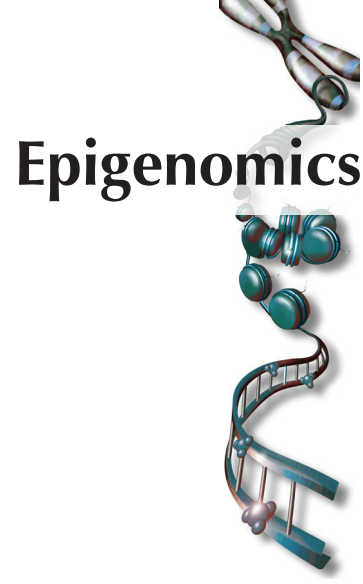

The role of butyrate, a histone deacetylase inhibitor in diabetes mellitus: experimental evidence for therapeutic intervention

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The contribution of epigenetic mechanisms in diabetes mellitus (DM), β-cell reprogramming and its complications is an emerging concept. Recent evidence suggests that there is a link between DM and histone deacetylases (HDACs), because HDAC inhibitors promote β-cell differentiation, proliferation, function and improve insulin resistance. Moreover, gut microbes and diet-derived products can alter the host epigenome. Furthermore, butyrate and butyrate-producing microbes are decreased in DM. Butyrate is a short-chain fatty acid produced from the fermentation of dietary fibers by microbiota and has been proven as an HDAC inhibitor. The present review provides a pragmatic interpretation of chromatin-dependent and independent complex signaling/mechanisms of butyrate for the treatment of Type 1 and Type 2 DM, with an emphasis on the promising strategies for its drugability and therapeutic implication.

Keywords: β-cell • butyrate • diabetes • epigenetics • HDAC inhibitors • histone deacetylase • insulin signaling

Diabetes mellitus (DM) is characterized by chronic hyperglycemia with multiple etiologies and perturbations in carbohydrate, fat and protein metabolism due to absolute or relative deficiency of insulin [1]. DM is a chronic metabolic disorder with complex pathogenesis, and lack of appropriate treatment and management may lead to several micro and macrovascular complications [2,3]. According to the International Diabetes Federation, DM incidence is growing at an alarming rate both in developing and developed countries. Presently, 387 million people worldwide suffer with both Type 1 diabetes mellitus (T1DM) and Type 2 diabetes mellitus (T2DM), which is expected to reach 592 million by 2035. DM is a multifactorial disease and recent reports suggest that the complexity of the disease cannot be entirely accounted for by genetic predisposition [1]. Both genetic and epigenetic factors including lifestyle are implicated in the pathogenesis of DM [4–6]. Environmental factors and life style changes are primarily responsible for the epigenetic modulations like histone modifications, which might be associated with both T1DM and T2DM [5,7]. The epigenetic processes at the chromatin template significantly modulate the transcriptional and phenotypic outcomes to environmental signaling including metabolic state and nutritional requirements [8]. Epigenetic mechanisms may modulate some of the genes associated with DM, which could predispose the individuals for DM during early postnatal development as well as throughout the adult life [9,10]. Furthermore, both genetic and epigenetic changes are responsible for the accelerated rate of chronic micro and macrovascular complications, even in the patients who are on standard antidiabetic therapy [8,11–14].

The histone acetyltransferases and histone deacetylases (HDACs) maintain the acetylation of the histone and nonhistone proteins, thereby modulating the expression of critical subsets of genes associated with the pathogenesis of DM [15,16]. Fundamentally, the activities of these enzymes can affect chro-

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matin at the target genes themselves and/or modulate their activators/suppressors expression, thereby resulting aberrant transcription of a particular gene [9,17,18]. The HDACs are involved in different cellular signaling associated with the pathogenesis of DM and its complications [7,12,19,20]. Recent evidence highlights the link between DM and HDACs, shown by the beneficial effects of HDAC inhibitors (HDACi) in β-cell development, proliferation, differentiation and function, as well as insulin signaling [15,20–22]. Additionally, HDACi prevent cytokine-induced β-cell damage in both *in vitro* and *in vivo* models [21,23,24]. HDACs act through the deacetylation of histones, various transcription factors and other regulatory proteins, which are directly or indirectly involved in glucose metabolism [15,25]. Moreover, glucose-mediated regulation of insulin gene transcription is under the control of histone acetylation, suggesting the role of HDACs in insulin synthesis and function [26,27]. Thus, HDACs play a regulatory role in the development of pancreas, insulin signaling and resistance including translocation of glucose transporter-4 (GLUT-4) [28–31]. The above reports highlighted that HDACs play an important role in the cellular and molecular mechanisms of glucose homeostasis. Based on the recent literature, it can be concluded that butyrate exerts several useful effects in both T1DM and T2DM through its complex chromatin-dependent and independent mechanisms (Table 1). In this review, we discuss the complexity of the disease pathogenesis and the contribution of epigenetic mechanisms, particularly the role of HDACs inhibition (histone modification) in DM. Finally, we highlight the experimental evidences of butyrate intervention for the treatment of both T1DM and T2DM with an emphasis on the possible strategies for its drugability and therapeutic implication.

Butyrate: the rationality for intervention

Butyrate is a short-chain fatty acid (SCFA) naturally produced in the colon of human and rodents from the fermentation of complex dietary fibers by microbiota and also found in butter and cheese [32]. Butyrate has been reported as an HDACi without any toxic effects and its HDAC inhibition potential has been well characterized in various cancerous, noncancerous and metabolic disorders by using *in vitro* and *in vivo* experiments [33,34]. The SCFAs have significant physiological functions, but butyrate plays a pivotal role in the cell proliferation, differentiation, energy metabolism and the maintenance of tight junctions as well as pathogenesis of DM [35,36]. Butyrate activates the genes of early pancreatic development in the embryonic stem cells [37] and increases the β-cell differentiation as well as insulin gene expression in rat islet cell lines [37]. Recently, we have reported that butyrate protects the β-cell death and improves the glucose homeostasis by the modulation of p38/ERK MAPK signaling through HDAC inhibition in juvenile diabetic rat [38]. Moreover, fiber-rich diet has been reported to reduce the daily insulin requirement and thereby decreases the risk of diabetes [39,40]. Further, butyrate reduces the body weight, fasting plasma glucose and insulin sensitivity through increase in energy expenditure in high-fat diet-induced T2DM in mice [33,39]. Notably, it has been reported that butyrate level and butyrate-producing microbes are reduced in subjects with diabetes including children [41–43]. Further, children with β-cell autoimmunity have shown low abundance of butyrate-producing bacteria in the gut [44,45]. Apart from this, butyrate have several other pharmacological activities such as anti-inflammatory, anticancer, antioxidant and immunomodulatory, which in part responsible for its beneficial effects across different experimental and clinical studies [46,47]. From the recent literature, it can be deduced that butyrate can modulate the expression of various regulatory genes and/or proteins, which are directly and/or indirectly involved in the glucose metabolism as well as pathogenesis of DM. Thus, butyrate can exert several beneficial effects by maintaining the fine-tuning of different molecular signaling/pathways such as; increasing the insulin transcription and translation; preventing β-cell apoptosis; increasing β-cell differentiation, proliferation and function and; inhibiting gluconeogenesis and glycogenolysis (indirect glucose production) in the liver (Figure 1). Therefore, it is pertinent that butyrate may be considered one of the promising molecules for the treatment of DM and can be further investigated in the experimental and clinical setups for in-depth mechanistic understanding.

Butyrate & HDAC inhibition: molecular mechanisms for intervention in T1DM

The pancreatic duodenal homeobox 1 (Pdx1) is synthesized in the early pancreatic development, which plays a central role in the differentiation of progenitor cells into endo and exocrine cells [28,48]. In the pancreas, expression of HDACs is tightly controlled at normal physiology for its developmental and functional regulation, but overexpression of HDACs is thought to be involved in the pathogenesis of DM [49]. Moreover, it has been reported that HDAC4, 5 and 9 (class IIa) are key regulators for the pancreatic β/δ-cell lineage control [22]. Another study reports that inhibition of HDAC1 and HDAC3, but not the HDAC2 protect β-cell mass as well as function in clinical islet transplantation and subjects with T1DM [24]. However, the physiological role of the different HDACs isoform as

Table 1. Experimental and clinical evidences of butyrate in various tests systems that highlighted the beneficial effects in both Type 1 diabetes mellitus and Type 2 diabetes mellitus.

Serial number	Mechanisms/inferences	Disease condition	Tests systems	Ref.
1	Reduced β-cell apoptosis and improved the β-cell proliferation, function and glucose homeostasis by the modulation of p38/ERK MAPK and apoptotic pathway	T1DM	Juvenile rats	[38]
2	Insignificant effects on islets protection, but delayed diabetes onset and fixed the diabetes-induced intestinal leakage	T1DM	Diabetes-prone rat pups	[35]
3	Increased the rate of lipolysis by HDAC inhibition in 3T3-L1 adipocytes	*In vitro*	3T3-L1 adipocytes	[70]
4	Attenuated inflammation and lipogenesis generated by the interaction of adipocytes and macrophages	*In vitro*	3T3-L1 adipocytes	[59]
5	Improved the skeletal muscle mitochondrial dysfunction, obesity and insulin resistance through epigenetic mechanisms	HFD-fed obesity	C57BL/6J mice	[72]
6	Alleviated metabolic impairments and protects β-cell function	T2DM	C57BL/6J mice	[76]
7	Improved insulin sensitivity and increased energy expenditure in mice via the promotion of energy expenditure and induction of mitochondrial function	T2DM	Mice	[33]
8	Reduced inflammation parameters, steatosis and glucose tolerance in the liver via the suppression of Toll-like receptors and NF-κB activation	HFD-induced impaired glucose homeostasis	Juvenile male SD rats	[86]
Butyrate mimetic/deficiency approaches/studies				
9	Fermentable dietary fibre decreased weight gain, liver fat, cholesterol and triglyceride content and altered the formation of SCFAs	HFD-fed obesity	Rats	[41]
10	Reduced nonalcoholic fatty liver disease progression by the butyrate-producing probiotics	Diet-induced obesity	Rats	[73]
11	Reduced butyrate-producing microbes species in the diabetic children as compared with age-match healthy children	T1DM	Children (age: 1–5 years)	[43]
12	Children with β-cell autoimmunity have shown low abundance of butyrate-producing bacteria	T1DM	Human and children	[44,45]

HDAC: Histone deacetylase; HFD: High-fat diet; SCFA: Short-chain fatty acid; SD: Sprague–Dawley; T1DM: Type 1 diabetes; T2DM: Type 2 diabetes.

well as their contribution in the β-cell function are not fully explored. Further, HDACi enhance and maintain the expression profile of the proendocrine markers in the pancreas [24,49]. Butyrate activates the genes of early pancreatic development in the embryonic stem cells [37] and increases the β-cell differentiation as well as insulin gene expression in rat islet cell lines [37]. HDACi can lead to an increased pool of endocrine cells through modifying the proliferation/apoptosis balance in the experimental studies [38,49]. Further, butyrate acts as a potent factor for the insulin gene expression in human pancreatic islets [26]. HDACi promote β-cell development, proliferation, differentiation and function in animal models of diabetes [22,23]. Moreover, butyrate increases the ERK phosphorylation and can modulate the MAPK pathway leading to cell proliferation [50]. Recently, we have reported that butyrate protects the β-cell apoptosis and improves the glucose homeosta-

sis by modulating p38/ERK MAPK signaling through HDAC inhibition in juvenile diabetic rat [38]. Butyrate also protects the β-cell damage by reducing diabetes-induced intestinal leakage in the new born rat pups [35]. In isolated β-cells, HDACi increase the acetylation of histone H4 without any toxic response [15,49]. HDACi attenuate the expression of proapoptotic proteins and β-cell dysfunction by preventing the IL-1β-induced activation of NF-κB and apoptosis signaling in experimental studies [23,24].

Glucose activates the transcription and release of insulin through β-cell specific transcription factors

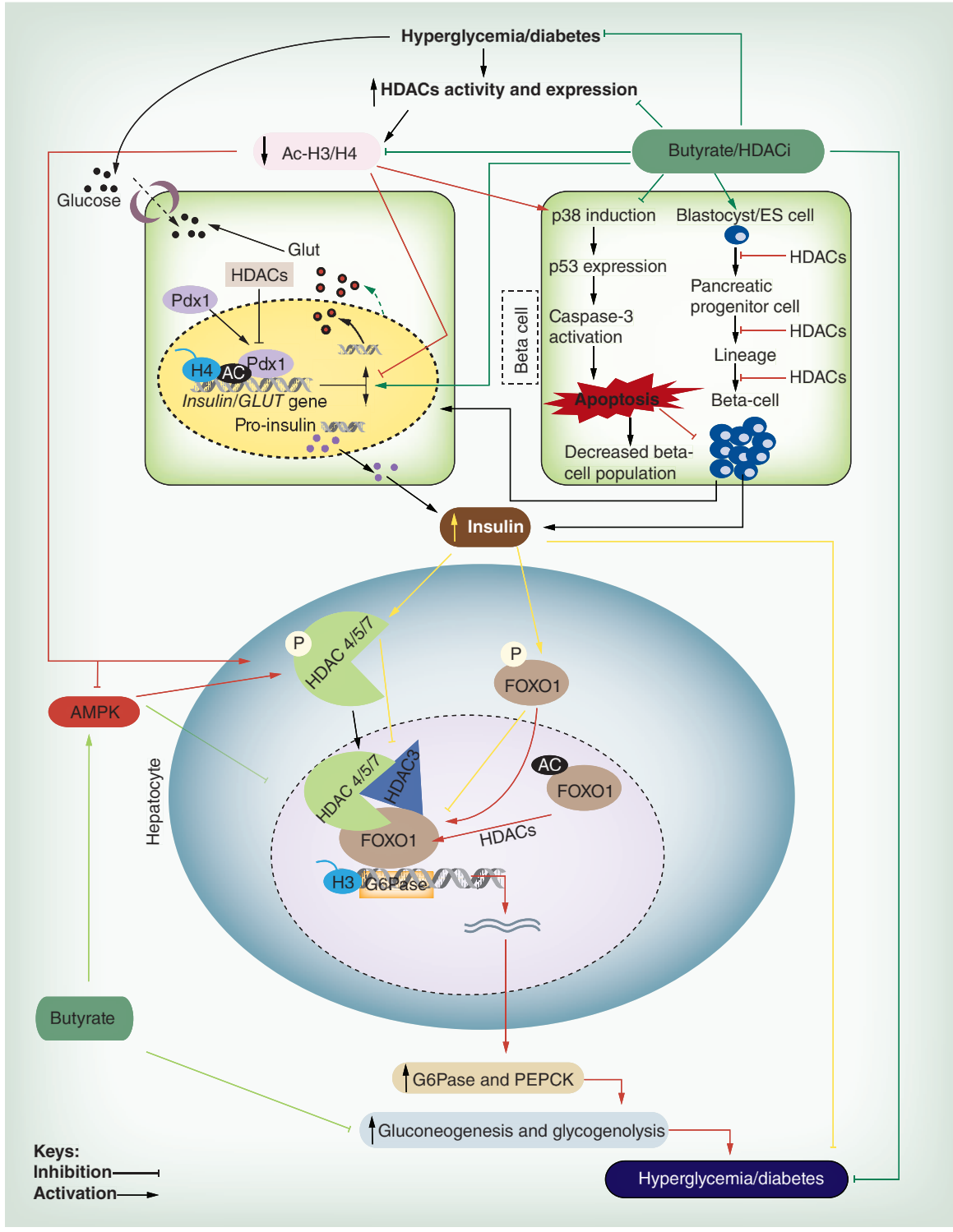

Figure 1. Schematic depiction shows the role of histone modification (epigenetics) and molecular targets in diabetes as well as possible protective effects/mechanisms of butyrate (see facing page). Diabetes increases the expression and activity of histone deacetylases, which perturbs the insulin synthesis, release and hepatic glucose production. Butyrate can modulate the β-cell proliferation, differentiation, function and apoptosis in the pancreas as well as facilitate the glucose homeostasis and insulin signaling in the liver; thereby it can prevent/treat the DM by following ways. Increasing β-cell differentiation, proliferation and function as well as preventing β-cell apoptosis, thereby increasing overall functional β-cell mass and insulin level. Butyrate reduces the gluconeogenesis and glycogenolysis (indirect glucose production) by inhibiting histone deacetylase-mediated over expression of G6pase and PEPCK. Butyrate also facilitates the insulin signaling and GLUT-4 translocation as well as modulates the AMPK activity. Red arrows show the phenomenon under diabetic condition, and green/yellow arrows show protective/preventive effects/mechanisms of butyrate/HDACi, while black arrows indicate the normal physiological process.
DM: Diabetes mellitus; ES: Embryonic stem cell; HDACi: Histone deacetylase inhibitor.
For color images please see online at: www.futuremedicine.com/doi/full/10.2217/EPI.15.20

such as Pdx1, NeuroD1 and musculoaponeurotic fibrosarcoma oncogene homologue A [51]. The above activation of transcription factors (binding) is suppressed by the overexpression of HDACs. Besides direct induction of apoptosis and cytokines, HDACs can perturb the β-cell differentiation by reducing the expression and/or activity of Pdx1, NeuroD1 and musculoaponeurotic fibrosarcoma oncogene homologue A [52,53]. Further, the transcription regulation of insulin gene in response to glucose is mainly regulated by histones H4 acetylation [54]. It has been reported that HDACi treatment increases insulin expression at low glucose level, whereas the release of insulin is less affected [51,55]. Additionally, HDACi prevent the development of virus-induced T1DM by the modulation of immune response during diabetes progression [56]. However, it has also been reported that vorinostat, an HDACi, protects β-cells and prevents diabetes progression by chromatin-independent mechanism [57].

The inflammation and oxidative stress are the major pathological factors in response to chronic hyperglycemia, which finally lead to β-cell apoptosis and dysfunction. Several studies indicate that butyrate may affect the host immune and inflammatory response [57,58]. Suppression of NF-κB activation by HDAC inhibition is the most frequently investigated anti-inflammatory mechanism of butyrate [59,60]. NF-κB modulates the expression of genes encoding the proinflammatory cytokines, chemokines, inducible nitric oxide synthase, COX-2 and adhesion molecules [47]. The anti-inflammatory effect of butyrate is mainly exerted by the inhibition of NF-κB, decreased myeloperoxidase and COX-2 expression [61,62]. Another recent study demonstrates the anti-inflammatory and immunomodulatory effects of butyrate by promoting functional regulatory T cells via epigenetic upregulation of the forkhead box P3 gene in colon of mice, which have a central role in the reduction of inflammatory and allergic responses. [46]. In summary, it can be emphasized that butyrate may exert its beneficial effects in T1DM by promoting β-cell differentiation, proliferation, insulin expression and release as well as preventing β-cell apoptosis through multiple chromatin-dependent and independent mechanisms (Table 1).

Butyrate & HDAC inhibition: molecular mechanisms for intervention in T2DM

T2DM is associated with insulin resistance and β-cell failure with or without obesity. The emerging evidences highlighted that epigenetic mechanisms play critical role in T2DM as well as β-cell reprogramming [63,64]. Interestingly, recent reports have highlighted that butyrate level and butyrate-producing microbes are relatively low in subjects with diabetes [41,45]. Further, HDACs regulate the transcription of GLUT4 enhancer factor and myocyte enhancer factor-2, thereby modulate the transcription of GLUT4 [65,66]. Additionally, activation of HDAC4/5 decreases the GLUT4-mediated glucose metabolism in the skeletal muscle as well as in the liver of mice [31,67]. Gluconeogenesis is another important process in glucose homeostasis that is thought to be regulated by HDACs. HDAC1 induces the expression of hepatocyte nuclear factor 4a and the dephosphorylation of FOXO1 in the hepatocyte leading to the induction of phosphoenolpyruvate carboxykinase expression and gluconeogenesis in the liver [16]. Loss of class IIa HDACs inhibits FOXO targeted gluconeogenetic genes and lowers the blood glucose leading to increase glycogen storage [16]. In mouse models of T2DM, inhibition of class IIa HDACs has been further shown to ameliorate hyperglycemia, indicating that class IIa HDACs regulate glucose-6-phosphate expression and subsequently affect the gluconeogenesis [68].

Insulin signaling plays an important role in the regulation of blood glucose by facilitating glucose uptake in the peripheral tissues and promoting the glycogen synthesis in the liver. Once insulin binds to its receptor, phosphate groups are added to tyrosine on target proteins in the cell including insulin receptor substrate (IRS). Recent report indicates that HDAC2 can bind to IRS-1 in the liver cells of db/db mouse, which leads to decrease acetylation of IRS-1 and the subsequent tyrosine phosphorylation

of the same [27]. Furthermore, HDACi or silencing of HDACs by RNA interference approaches, enhance the acetylation of IRS-1, thereby partially attenuate the insulin resistance [69]. Pharmacological inhibition of class I/II HDACs facilitates Adenosine monophosphate activated protein kinase (AMPK) activity and exerts beneficial effects in DM [66,70]. Similarly, high fiber diet also activates the AMPK in the liver, which may be one of the underline mechanisms for the beneficial effect of butyrate in DM [71]. Additionally, consumption of dietary fibers has shown many positive health effects in the metabolic disorders such as improved satiety as well as decrease in the body weight, blood glucose and cholesterol [36]. The above effects mainly associated with the complex chromatin-dependent and independent pharmacological properties of butyrate. Recently, it has been reported that butyrate improves the skeletal muscle mitochondrial dysfunction and insulin resistance through epigenetic mechanisms in high-fat diet-induced T2DM in mice [72]. Butyrate and other HDACi improve the insulin sensitivity and metabolic abnormities, primarily by chromatin-dependent complex molecular mechanisms [41,42,73].

On the other hand, β-cell dysfunction and failure is a major concern in the progression of T2DM, which involves the epigenetic changes in the various regulatory proteins under chronic diabetic condition. [22,63]. Thus, altered epigenetic landscape ultimately reprogrammed the endocrine pancreas and produces proinsulin (nonfunctional) with augmented glucagon level and/or β-cell death [63,74]. Recent study also reports the inactivation of various β-cell specific transcription factors in T2DM, which might be responsible for β-cell dysfunction and failure [75]. Butyrate can alleviate the metabolic stress and improve the β-cell function as well as protect β-cell from inflammatory response in pregnant obese mouse without fetus toxicity [76]. Further, several reports have analyzed the epigenetic landscapes such as DNA methylation and histone modification in islets from subjects with T2DM and controls, which highlights the aberrant pattern in the subject with diabetes as compared with healthy individuals [6,63,77]. Thus, in-depth understanding of epigenetic landscapes in the islet differentiation and function would be more useful to improve β-cell functionality for the prevention and/or treatment of DM [75,78].

HDACi including butyrate have been shown to reduce the diabetes associated low-grade inflammation [41,79]. Further, tributyrin attenuates the obesity-associated inflammation and insulin resistance in high-fat diet fed mice [80]. Butyrate exerts an anti-inflammatory effect by inhibiting the NF-κB activation and production and/or signaling of IFN-γ as well as the upregulation of PPARγ [58,81]. In the inflammation, NF-kB activation promotes HDAC3 activity leading to suppression of PPARγ function, while HDAC3 inhibition restores the PPARγ function in obese rat [82]. Moreover, butyrate and other SCFAs can act through their free fatty acid receptors 1 and 2 (FFA1 and FFA2) and modulate the inflammatory and immune response [83]. However, the receptor-mediated effects of butyrate and SCFAs may be due to histone acetylation and/or other nonhistone targets of HDACs [84,85]. Recent evidences support that butyrate promotes release of glucagon-like peptide-1 through FFA2 and FFA3 receptor and facilitates the satiety, which can be a potential approach for the treatment of DM [42,86]. Beside the receptors-mediated effects of butyrate, it also increases the satiety and decreases the weight gain by the stimulation of glucagon-like peptide-1 release through entroendocrine L cells, which ultimately facilitate insulin secretion [85]. In summary, butyrate can modulate the fatty acid receptors and chromatin-dependent signaling as well as nonhistone targets in DM.

Advantages & limitations

Butyrate is an attractive candidate for the treatment of both T1DM and T2DM, because of its chromatin-dependent and independent mechanisms [33,38]. Butyrate acts on multiple targets and can modulate both insulin production and glucose utilization in the peripheral tissues. Butyrate concentration can be achieved by the supplementation of fiber-rich diet and butyrate-producing probiotics, which is easy for intervention and economically viable (Figure 2). The above strategy might be more applicable, which has been successfully proven to reduce the insulin resistance and metabolic impairments in different *in vivo* experimental setups [41,45,87]. Additionally, butyrate has several other pharmacological properties such as anti-infllamatory, antioxidative, immunomodulatory and chemoprotective, which can add further value to subjects with diabetes. However, butyrate has short half-life of 10–15 min due to its rapid uptake and metabolism by normal cells, which dampens its therapeutic utilization [88]. This problem can be overcome with more stable butyrate derivatives like N-(1-carbamoyl-2-phenyl-ethyl) butyramide (FBA or phenylalanine-butyramide), which can provide a consistent effective concentration in systemic circulation [89]. FBA is capable to release butyric acid in small and large intestine at a constant rate, and its toxicological profile is comparable to butyrate. Physicochemical characteristics of FBA are more suitable than butyrate for dosing and clinical development [89]. Further, microencapsulated sodium butyrate, a hydroxy propyl

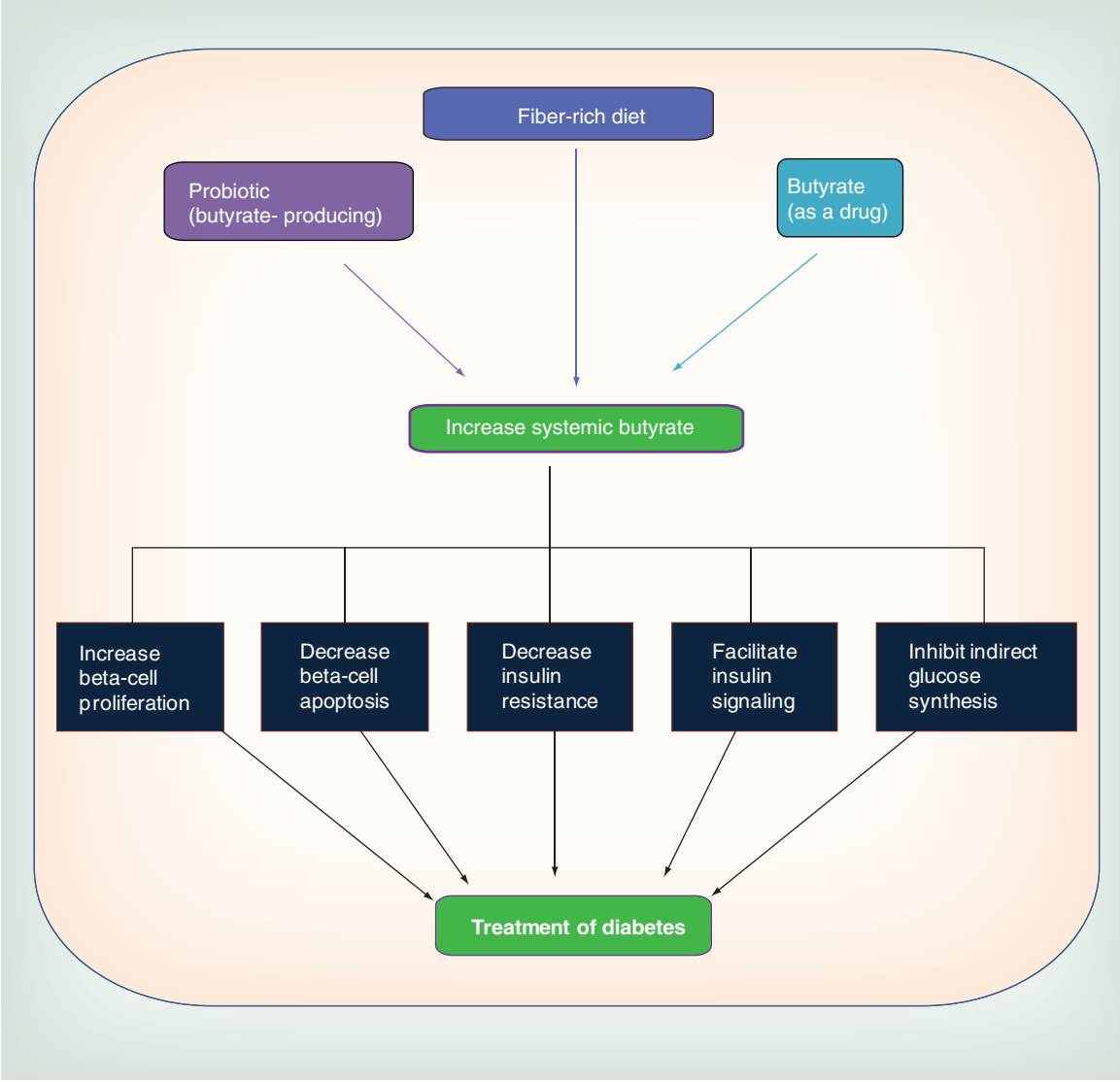

Figure 2. The strategies for the therapeutic implication of butyrate and its beneficial effects in both Type 1 diabetes mellitus and Type 2 diabetes mellitus. Schematic presentation of the possible approaches for the butyrate supplementation/administration, which can provide desired effective concentration for the pharmacological effects and subsequent therapeutic benefits in the pancreas and peripheral tissues for the prevention/treatment of diabetes mellitus.

methyl cellulose and shellac coating of butyrate has been developed for extended and selective delivery of butyrate in colon [90,91]. Although, butyrate-based products are available in the market, but their use is very limited in chronic diseases due to lack of orally administrable formulations as well as poor palatability [32]. Apart from this, another major problem for butyrate drugability is its unpleasant taste and odor, which make oral administration very difficult, especially for the children [32,89]. Thus, new formulations of butyrate with a better palatability as well as sustain/control drug delivery dosage form are needed, which can be orally administered and ultimately improve the patient compliance. Although, butyrate is a non-selective HDACi and preferably inhibited class I and II HDACs at millimolar concentrations, thereby modulates the wide range of cellular and molecullar signaling/pathways [38,42]. Further, it is postulated that two molecule of butyrate can inhibit HDACs by occupying its hydrophobic pocket as similar with the trichostatin A [62,92]. However, the exact mechanism of HDAC inhibition by butyrate is yet to be explored. Additionally, the global alterations in histone acetylation may or may not be significant, but it may significantly modulates the expression of target gene [93]. Thus, gene specific histone acetylation is more appropriate to explore the exact epigenetic targets in DM, which can improve the selectivity for therapeutic

Executive summary

Histone deacetylases & histone deacetylases inhibitors in diabetes mellitus

- The emerging evidences highlighted that histone deacetylases (HDACs) and histone acetylation (epigenetic mechanisms) play a critical role in the pathogenesis of diabetes mellitus (DM), which is still unexplored.
- The HDACs are involved in the different cellular signaling associated with the pathogenesis of DM and its complications.
- Butyrate can modulate the expression of various regulatory genes and/or proteins, which directly and indirectly involved in the glucose metabolism as well as pathogenesis of DM.

Butyrate, HDAC & histone modification in Type 1 DM

- Recent evidences suggested that HDACs play a critical role in the modulation of β-cell fate, because HDAC inhibitor (HDACi) promote β-cell differentiation, proliferation, function and insulin synthesis as well as its release.
- HDACi can prevent the β-cell apoptosis through various chromatin-dependent and independent mechanisms.

Butyrate, HDACi & histone modification in Type 2 DM

- Butyrate level and butyrate-producing microbes are decreased in subjects with diabetes including children.
- HDACs are associated with an inactivation of various β-cell-specific transcription factors in T2DM, which are responsible for reprogramming of β-cell function.
- HDACi prevent β-cell dysfunction, improve the insulin resistance and satiety as well as inhibit the indirect glucose production in the liver.

Conclusion & future perspective

- Butyrate exerts several pharmacological effects through its chromatin-dependent and independent complex signaling/mechanisms, which are viable for the treatment of both absolute and relative deficiency of insulin in DM.
- Butyrate can be an ideal agent to treat DM, because it can act on the pancreas (β-cell modulation) and peripheral tissues (insulin sensitivity) and CNS (satiety).
- The future studies will elucidate that how histone acetylation/modification (epigenetic mechanisms) involved in DM and its complications.

intervention [78,93]. Therefore, newer, stable and orally applicable derivatives of butyrate and/or probiotic strategies alone or in combination can be employed for improving the pharmacokinetic and pharmacodynamic profile of butyrate, which directly or indirectly improves its effective concentration.

Conclusion & future perspective

The emerging evidences highlighted that epigenetic mechanisms play a critical role in the pathogenesis of DM, which are still unexplored [6,74,75]. The future studies will elucidate how epigenetic mechanisms involved in DM and its complications. It is plausible that a number of epigenetic processes like histone acetylation, methylation and RNA interferences might associate with DM [64]. The detail understanding of the epigenetic processes responsible for reprogramming of the endocrine pancreas and the contribution of the same in DM can provide novel treatment strategy. Moreover, both animal and human studies highlight that alter gut microbiota is associated with DM. Thus, exploring the contribution of gut microbiota can provide better understanding to the complex pathogenesis of DM. Use of advance technologies that allow the study of complete epigenome during development and disease progression as well as interpretation of complex interactions of environment with gut microbiota can offer a new strategy for exploring the epigenetic mechanisms in the pathogenesis of DM and β-cell reprogramming. Moreover, advancement in the pharmaceutical technologies and drug delivery methods can generate newer and more stable derivatives. Thus, drugability of butyrate should improve to deliver the desired concentration for the therapeutic benefits.

In conclusion, here we present the potential benefits of butyrate and its therapeutic implications for clinical application, which can be further investigated using different experimental and clinical setups for the treatment of T1DM and T2DM. Further, exploring and understanding the tissue/cell specific physiological roles of different HDACs in DM as well as designing and synthesizing the selective HDACi would contribute in the management of DM. Furthermore, an emphasis should be given on the *in vivo* animal and human studies in DM to elucidate the exact molecular mechanisms of butyrate. Finally, we suggest that future studies should be designed to investigate the chromatin-dependent and independent mechanisms of butyrate/fiber and the association of gut microbiota for the successful intervention in DM. Thus, butyrate can reduce the disease burden and its complications as well as heath expenses particularly in the developing countries.

Authors contributions

Both authors equally contributed to the content, review/edited and approved the final manuscript.

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• of interest; •• of considerable interest

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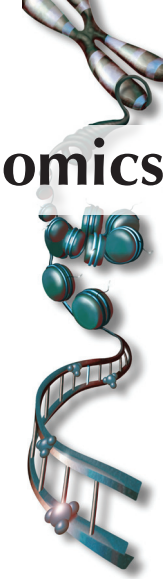
Review

Epigenomics



Circulating miRNAs as intercellular messengers, potential biomarkers and therapeutic targets for Type 2 diabetes

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miRNAs have emerged as key epigenetic regulators of metabolism. Their deregulation contributes to metabolic abnormalities, proposing their potential role as therapeutic targets for Type 2 diabetes. The exciting finding that miRNAs exist in the bloodstream suggests that circulating miRNAs may act in a hormone-like fashion. Despite the fact that significant progress has been made in understanding circulating miRNAs, this topic is full of complexities and many questions remain unanswered. The goal of this review is to bring together up-to-date knowledge about circulating miRNAs and their role as intercellular communicators as well as potential biomarkers and therapeutic targets in metabolic diseases, providing examples of possible clinical applications for circulating miRNAs in diabetes and cardiovascular complications.

Keywords: biomarkers • cell-to-cell communication • circulating miRNAs • therapeutic targets • Type 2 diabetes

Introduction to miRNAs

In the postgenomic era, epigenetics has emerged as one of the most exciting scientific breakthroughs with the challenge of understanding the intriguing mechanisms involved in the origin, progression and drug resistance of a wide range of diseases. Epigenetics is generally defined as the study of inheritable changes in gene expression that are not established in the DNA sequence, explaining much of the environmental contribution to the biology of an individual organism. Disruption or alteration of the epigenetic machinery may induce developmental defects and cancer, as well as metabolic diseases [1,2]. Epigenetic regulation is complemented by different mechanisms and increasing emphasis is being placed on the ability of noncoding RNA (ncRNA) transcripts to modulate gene expression. ncRNAs include multiple classes of RNA transcripts that do not encode proteins but instead regulate gene expression. To date, the most commonly studied ncRNAs are miRNAs (usually 19–24 nucleotides in length). The biogenesis of miRNAs has been described in great detail in [3]. Mature miRNAs regulate gene expression through sequence-specific binding to the 3′ untranslated region (3′ UTR) of mRNA targets. This binding activates the RNA-induced silencing complex (RISC) through which miRNAs act as post-transcriptional repressors, affecting translation and/or transcript decay [4].

Some of the transcripts regulated by miRNAs are epigenetic enzymes, which are necessary for the proper targeting of histone modifying complexes or play a role in DNA methylation. Conversely, the expression of many miRNAs is regulated by epigenetic processes. Indeed, genomic loci producing noncoding transcripts, such as miRNAs, are subject to histone modification and DNA methylation similar to protein-coding genes [5,6]. Therefore, as in the case of coding genes, environmental factors may lead to changes in miRNA levels and, subsequently, in their function. Considering the reciprocal regulation of miRNAs and epigenetic key enzymes, altered miRNAs may be referred as both cause and effect of changes in gene expression. For this reason, they may contribute to explain environmental influences

Future Medicine part of fsg

in diseases that are passed on through cell divisions and across generations. The miRNA repression or overexpression, additionally, may be an effective way of amplifying changes in gene expression through the downstream epigenetic enzymes.

It is now regarded as common knowledge that cellular miRNAs are powerful regulators of metabolism. Furthermore, circulating miRNAs are involved in cell-to-cell communication and show promise as a new class of biomarkers for metabolic diseases, due to their stability in biological fluids. In addition, miRNA-based therapies may have a good chance of success shortly. For these reasons, with the present review, we will focus our attention on circulating miRNAs and, in particular, on their possible role in controlling recipient cell phenotype and their great potential in prevention, diagnosis and treatment of metabolic diseases. We will also highlight that the research in circulating miRNA biomarkers as well as the manipulation of potential therapeutic miRNAs are still in their infancy and currently represent bottlenecks for molecular diagnostics.

miRNAs in metabolism & metabolic diseases

miRNAs are crucial regulators of different aspects in development and homeostasis. In addition, their importance in the control of metabolism has recently been revealed. Not surprisingly, therefore, an aberrant miRNA expression and function induce metabolic homeostasis loss and disease [7,8]. However, miRNAs typically have rather modest effects on target protein levels. For this reason, for a single miRNA, several actions on functionally related targets in one pathway are required in order to significantly influence complex processes. On the other hand, as one target contains multiple predicted miRNA binding sites, it is possible that groups of miRNAs act in concert to exert more potent effects on a specific target. Altered miRNAs, targeting the components of the insulin signaling pathway or the GLUT4-mediated glucose uptake and metabolism, have been demonstrated to play a key role in diminishing insulin sensitivity of peripheral tissues and thus cause insulin resistance and the onset of Type 2 diabetes (Table 1) [9–27]. In an attempt to compensate for insulin resistance, the failure of pancreatic β cells occurs (Table 1) [28–43], leading to chronic hyperglycemia and the appearance and progression of diabetic vascular complications. Numerous studies have addressed the role of miRNAs in the glucose-induced vascular dysfunction (Table 1) [44–52]. For example, Togliatto *et al.* demonstrated the contribution of miR-221/222 in vascular damage mediated by a high-glucose environment, evaluating cell-cycle progression and chemotaxis in human mature endothelial cells and endothelial progenitor cells treated with high glucose and advanced glycation end-products (AGEs). They also showed that high glucose and AGEs-mediated inhibition of vessel formation in mice by miR-221/222 downregulation [51]. Furthermore, the persistence of these miRNA alterations may contribute to the 'metabolic memory', which results in chronic inflammation and vascular dysfunction in diabetes even after achieving glycemic control. For example, miR-125b, upregulated in diabetic *db*/*db* mice, was demonstrated to be responsible for the reduced expression of the histone methyltransferase Suv39h1 and the subsequent decrease in the repressive histone H3 lysine-9 trimethylation (H3K9m3) at the promoter of inflammatory genes in vascular smooth muscle cells (MVSMCs) from *db*/*db* mice. The persistent increase in miR-125b levels was observed even after culturing cells for several passages *in vitro*, suggesting a potential memory of the deregulated miR-125b expression [44]. In another recent article, Togliatto *et al.* demonstrated that unacylated ghrelin (UnAG) was able to protect endothelial cells from glucose-mediated oxidative stress, one of the most relevant mechanism in the 'metabolic memory', when administered in diabetic *ob*/*ob* mice subjected to hind-limb ischemia. In diabetic conditions, UnAG restored miR-126 levels. The resulting increase in the histone de-acetylase sirtuin 1 (Sirt1) levels was responsible for the de-acetylation of p53 and histone 3 (H3K56) and the upregulation of superoxide-dismutase-2, which represents an important defense against reactive oxygen species production in hyperglycemia [48].

This emerging role of miRNAs in metabolism has gathered much interest not only from a scientific point of view but also from a clinical perspective, attracting interest on their potential applications in the treatment of metabolic diseases. However, a number of open questions remain on the impact of miRNAs in metabolic homeostasis and they will undoubtedly be topics for intense and exciting research for years to come.

Circulating miRNAs

Distinctive features of circulating miRNAs

The first evidence that miRNAs exist outside the cell was reported by Valadi *et al.* in 2007, who showed that exosomes contained both mRNAs and miRNAs, and proposed the name 'exosomal shuttle RNA' (esRNA) [53]. Since 2008, a growing number of reports have consistently revealed the presence of miRNAs in blood and in other body fluids [54–60]. The existence of circulating miRNAs is due to their astonishing stability to RNase activity, long-term storage at room temperature, multiple cycles of freeze-thawing as well as extreme pH conditions [54,57]. Evidence is now accumulating that circulating miRNAs are pro-

Table 1. Roles of miRNAs in diabetes and in its complications.

miRNA	Target tissue(s)	Function(s)	Ref.
let-7	Adipose tissue, muscle	Insulin resistance	[9,10]
miR-1	Endothelium	Endothelial dysfunction	[45]
miR-9	Pancreas	Deficit of insulin release	[28,29]
miR-21	Adipose tissue	Insulin resistance	[11,46]
miR-29a/b	Liver, adipose tissue, muscle, pancreas	Insulin resistance, deficit of insulin release	[12–14,30,31]
miR-30d	Pancreas	Deficit of insulin release	[32,33]
mir-33a/b	Liver	Insulin resistance	[15–17]
miR-34a	Liver, pancreas	Insulin resistance, deficit of insulin release	[18,34–37]
miR-93	Adipose tissue	Insulin resistance	[19]
miR-103/107	Liver, adipose tissue	Insulin resistance	[13,20]
miR-122	Liver	Insulin resistance	[92–94]
miR-124a	Pancreas	Deficit of insulin release	[30]
miR-125b	Vascular smooth muscle	Endothelial dysfunction	[44]
miR-126	Liver, endothelium	Insulin resistance, endothelial dysfunction	[21,47,48,82–84]
miR-146a	Endothelium	Endothelial dysfunction	[49]
miR-221/222	Endothelium	Endothelial dysfunction	[50,51]
miR-223	Muscle	Insulin resistance	[24]
miR-320	Adipose tissue	Insulin resistance	[25]
miR-335	Liver, adipose tissue, pancreas	Insulin resistance	[38]
miR-375	Pancreas	Deficit of insulin release	[39–43]
miR-494	Muscle	Insulin resistance	[26]
miR-503	Endothelium	Endothelial dysfunction	[52]
miR-802	Liver	Insulin resistance	[27]

tected by packaging in membrane-derived vesicles or by association with proteins or lipoproteins, which are defined as carriers for miRNAs (Figure 1). miRNA expression profiles are distinctive for each class of carriers, although some miRNAs have been found in all carriers and in equal amounts. In more detail, it has been demonstrated that the majority of plasma miRNAs (about 90%) are not encapsulated within vesicles, but instead are stably associated with the protein argonaute 2 (Ago2), which is the key intracellular effector component of the RISC [61,62]. However, it is still unclear how Ago2-miRNA complexes are exported from cells. Based on current assumptions, they are passively released as nonspecific 'by-products' of cellular activity or in the process of cell death, even though the experimental methods used to isolate vesicles may cause their damage with the release of enclosed functional Ago2-miRNA complexes. It is also unknown whether Ago2-miRNA complexes not contained within vesicles can be uptaken by recipient cells to regulate their gene expression, as is the case of vesicles, whose role as 'on-purpose' released cell-to-cell signaling messengers is already well established. Only a very small proportion of miRNAs seem to be stored in membrane-derived vesicles, which are secreted by many (if not all) cell types under either physiological or pathological conditions [53,55,63]. Depending on their size and mode of release from the cell, these particles are classified as [64]:

- Exosomes, the smallest vesicles (40–100 nm), of endocytic origin, derived from multivesicular bodies that fuse with the plasma membrane;
- Microparticles (MPs), membranous vesicles that are larger (0.1–1 μm) than exosomes and are released from the cell through membrane blebbing;
- Apoptotic bodies, the largest vesicles (0.5–5 μm), which are shed from the plasma membrane of dying/apoptotic cells via blebbing.

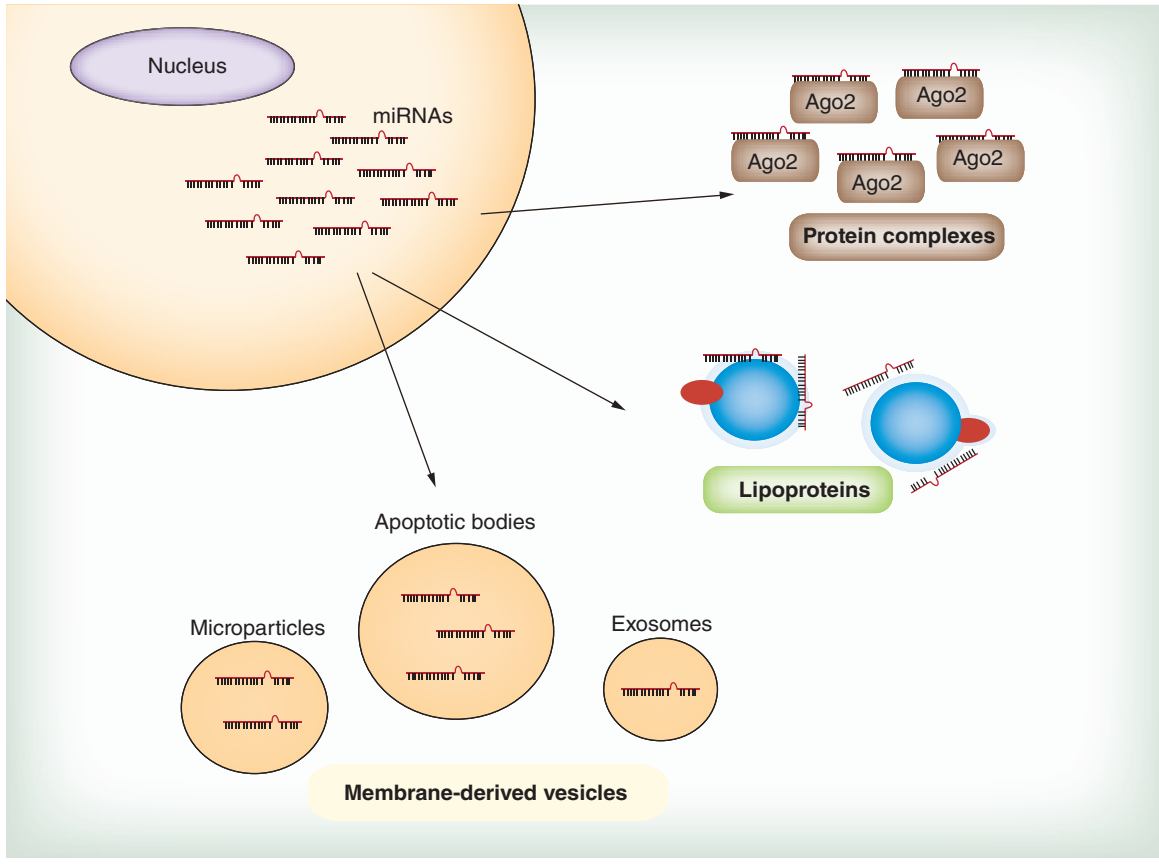

Figure 1. Carriers for circulating miRNAs. Mature miRNAs are released into the circulation associated with carriers. The majority of them bind RNA-binding proteins such as Ago2 (up to 90%) or lipoproteins such as the high-density lipoproteins (approximately 8%). In addition, a very small amount of miRNAs is released in membrane-derived vesicles, classified for their size as exosomes (40–100 nm), microparticles (0.1–1 μm) and apoptotic bodies (0.5–5 μm).

It is also known that nucleic acids, including miRNAs, can bind lipids within lipoproteins via divalent cations with the formation of stable ternary complexes. Recent studies have revealed that miRNAs can also be found in high-density lipoproteins (HDLs), suggesting new potential roles for HDLs in transporting not only cholesterol and other lipids and proteins, but also regulatory miRNAs [65,66]. The HDL-miRNA profile in plasma was different from the profile of miRNAs associated with other carriers and HDLs isolated from familial hypercholesterolemia subjects had a higher concentration of miRNAs and contained more individual miRNAs than HDLs from healthy subjects, such as miR-223, miR-105 and miR-206a. In addition, this study provided evidence for the transport of miRNAs by HDL generated from J774 mouse macrophages to cultured human hepatocytes (Huh7) with consequently alteration in gene expression, although these HDL-bound miRNAs were not found to be associated with Ago2 [65]. Follow-up analyses are required to determine whether the circulating miRNAs associated with HDLs may contribute to molecular etiology of diseases. Moreover, plasma HDL–miRNA profiling may serve as a novel biomarker tool for detecting or monitoring the progression of cardiovascular diseases.

Numerous recent investigations have demonstrated that miRNA signatures in blood may reflect the expression profile observed in disease tissues. To date, altered profiles of circulating miRNAs have been reported for cardiovascular disease [67,68], diabetes [47], cancer [57,69,70] and other pathologies. For this reason, miRNAs may be used as novel biomarkers for diagnosis, prediction and treatment of diseases. In addition, circulating miRNAs may serve as mediators in cell-to-cell communication [53,62,71]. Since miRNAs derived from parental cells are carried by membrane-derived vesicles to act as intercellular mediators, the awareness of vesicle-mediated epigenetic effects by their miRNA content has revolutionized the general thinking about possible mechanisms based on vesicle signaling and has also suggested a potential use of circulating miRNAs as therapeutic targets in several diseases.

Intercellular communication by circulating miRNAs

Increasing evidence suggests that circulating miRNAs may not merely be vesicular passengers but rather active players, which deliver regulatory messages from donor to recipient cells [58,71–73], thereby resembling secreted soluble factors for their paracrine and/or endocrine function. More precisely, specific miRNAs, which are highly abundant in vesicles and are directly delivered, may exert mass action effects on protein output in specific organs or cell types similar to classic hormones. Despite the lack of detailed information, carriers are in charge of circulating miRNA transport. With regard to packaging and export, some miRNAs are selected for cellular release while others are retained, implying that active, but still unknown mechanisms exist to promote selected miRNAs toward carriers. It has been demonstrated that, although 66% of the miRNAs are released in quantities that reflect their intracellular levels, 13% are selectively retained by cells and thus come out of cells at very low levels, whereas 21% seem to be actively released and appear at disproportionately higher levels in vesicles. Concerning the mechanisms of secretion, it has been shown that exosomal miRNAs are released by a ceramide-dependent secretory machinery, as the inhibition of neutral sphyngomyelinase 2 (nSMase2), which regulates the biogenesis of ceramide, impaired the cellular export of exosomal miRNAs [73–75]. However, further studies are needed for a systematic dissection of the mechanisms underlying the selective release of miRNAs in vesicles. Furthermore, the role of circulating miRNAs as messengers is attributed to their uptake by recipient cells, which probably depends on cell-specific membrane proteins and lipids, even if the participating receptors and transmembrane proteins remain to be identified. To date, *in vitro* studies have used conditioned media exchange and coculture experiments to investigate the possible miRNA uptake by cells [59,76,77], and gene reporter assays to show that uptaken miRNAs are able to silence transcripts in recipient cells [53,63,71,73,75,78,79]. For the first time in 2007, a study demonstrated that exosomes from both mouse and human mast cell lines (MC/9 and HMC-1, respectively), as well as primary bone marrow-derived mouse mast cells, contained both mRNAs and miRNAs. Exosome-derived RNAs from MC/9 were transferred not only to the same cell type but also to human mast cells and the presence of mouse proteins was examined in the human recipient cells [78]. Several further reports, mainly in cancer research, have revealed that vesicular miRNAs are mediators in cell-to-cell communication, showing that delivered miRNAs alter gene expression and phenotype in recipient cells. Indeed, tumor cells are capable of constitutively secreting vesicles, known as tumor-released vesicles, which potentially exert a paracrine influence on the surrounding cells to promote proliferation, stromal remodeling, angiogenesis and impairment of the immune system response. Conversely, activated human monocyte-derived macrophages were demonstrated to promote invasion and metastasis in cocultivated human breast cancer cell lines, SKBR3 and MDA-MB-231, by exosome-mediated delivery of oncogenic miRNAs, such as miR-223 [77]. In addition, tumor-released vesicles are also able to interact to and release their cargo into cells located at distant organs, affecting the metastatic process. For example, miR-150 was selectively packaged into MPs and actively secreted by a human monocytic cell line (THP-1). Uptaken by human microvascular endothelial cells (HMEC-1), miR-150 was responsible for the enhanced migration of the recipient cells. Moreover, after intravenous injection of fluorescently labeled MPs produced by THP-1 cells in C57BL/6 mice, the isolated endothelium of mouse blood vessels showed fluorescence and miR-150 levels were increased. In addition, MPs isolated from plasma of human patients with atherosclerosis contained higher levels of miR-150 and promoted HMEC-1 migration more effectively than MPs from healthy donors [80]. In another study, miR-150 secreted from monocytes enhances angiogenesis both *in vitro* and *in vivo*. HMEC-1 cells were used again as recipient cells for MPs released from THP-1 cells and the miR-150 levels were dramatically increased in these endothelial cells, facilitating migration and capillary tube formation. C57BL/6J mice, tumor-implanted mice or *ob*/*ob* mice, were injected with MPs both released from THP-1 cells and collected from plasma of normal donors as well as atherosclerosis patients. As a consequence, angiogenesis enhanced in presence of high miR-150 levels [81]. Because angiogenesis contributes to a number of diseases, including cancers, atherosclerosis and diabetes, the search for novel angiogenic targets, like secreted miR-150, may have significant clinical applications in the future. Several other circulating miRNAs have been identified as critical regulators of vascular development and angiogenesis. For example, endothelial cell-derived apoptotic bodies were generated during atherosclerosis and conveyed alarm signals to human umbilical vein endothelial cells (HUVECs), where higher levels of miR-126 were observed after incubation with these apoptotic bodies. In addition, the injection of endothelial cell-derived apoptotic bodies enriched with miR-126 in different mouse models of atherosclerosis increased the number of endothelial progenitor cells and stabilized plaques, limiting atherosclerosis [82]. Moreover,

Jansen *et al.* showed that endothelial MPs generated from human coronary artery endothelial cells (HCAECs) contained and transferred functional miR-126 to the same endothelial cells, inducing migration and proliferation and thus regulating vascular endothelial cell repair. These endothelial MPs were also injected intravenously in C57BL/6 mice after carotid artery injury, promoting re-endothelialization *in vivo*. Interestingly, MPs derived from high glucose-damaged HCAECs, simulating the diabetic condition, enclosed less miR-126 and lost their regenerative capacity. Furthermore, the expression analysis of miR-126 in plasma of human patients revealed that diabetes is associated with a significantly reduced miR-126 expression in circulating vesicles [83]. Peripheral blood mononuclear cells (PBMCs) were isolated from human patients with Type 2 diabetes and age-matched healthy subjects and subpopulations were separated for their $CD34^+$ and $CD14^+$ surface expression. MiR-126 levels were substantially increased in both $CD34^+$ PBMCs and their supernatant, and these results were consistent with a more potent pro-angiogenic effect tested *in vitro* and *in vivo*. Indeed, angiogenesis assays were performed in human aortic endothelial cells (HAECs) and male *NRMI nu/v* mice; as a diabetic mouse model, C57BL/6 mice treated with streptozotocin were also used. In addition, the reduced miR-126 expression was proposed as a novel mechanism leading to an impaired pro-angiogenic capacity in high glucose-treated or diabetic patient-derived $CD34^+$ PBMCs [84]. Therefore, all these studies suggested that secreted vesicles may act as key information vectors between hyperglycemia and the development of diabetic vasculopathy, and that the manipulation of their miRNA content may contribute to maintain cell homeostasis or favor cell repair and angiogenesis. According to another study, as the initiation of lesion formation occurs only in areas of disturbed flow-like bends or bifurcations of vessels, where endothelial cells are exposed to low shear stress, both the shear-responsive transcription factor Krüppel-like factor 2 (KLF2) overexpression and shear stress stimulus were used in HUVECs. Both KLF2 and shear stress regulated a number of miRNAs, mainly the miR-143/145 cluster, which is atheroprotective and plays a critical role in modulating vascular smooth muscle cell phenotype. Interestingly, the vesicles secreted by KLF2 overexpressed or flow-stimulated HUVECs were demonstrated to be enriched in miR-143/145 and delivered to human aortic smooth muscle cells (HASMCs), where these miRNAs changed the gene expression pattern toward a contractile phenotype. Coculture of KLF2 overexpressed endothelial cells isolated from miR-143/145 knockout mice with HASMCs failed, showing the important role of miR-143/145 transfer. In addition, fed a high-fat diet over a period of 6 weeks, apolipoprotein E ($Apoe^{-/-}$) null mice were injected intravenously with extracellular vesicles derived from mock or KLF2 overexpressed mouse endothelial cells or vesicles from KLF2 overexpressed mouse endothelial cells after treatment with anti-miR-143/145. Mice were then killed and fatty lesions in the aorta showed a significant reduction of the atherosclerotic lesion formation in the case of vesicles from KLF2 overexpressed cells. These results provided evidence for an atheroprotective communication between endothelial cells and vascular smooth muscle cells via a miRNA- and vesicle-mediated mechanism, which may turn out to obtain a promising strategy and combat atherosclerosis [85]. Adipocytes may also use circulating miRNAs for both local and systemic communication. Indeed, 3T3-L1 mouse adipocytes are able to secrete adipocyte-derived microvesicles, containing also adipocyte-related miRNAs, whose abundance is mostly correlated with that in the donor cells. As adipocyte-derived microvesicles mediated transport into RAW264.7 mouse macrophages, their role as RNA transporter in paracrine and possibly endocrine manners was demonstrated, establishing a network between systemic fat storage and inflammation by miRNA transfer [86]. Furthermore, MPs and exosomes derived from large rat adipocytes were demonstrated to contain several miRNAs (miR-16, miR-27a, miR-146b and miR-222), which were transferred into other adipocytes of the same species with upregulation of lipogenesis and cell size. The miRNA transfer and lipogenic activity were more efficient for small rather than large acceptor adipocytes and significantly upregulated by physiological (palmitate, H2O2) and pharmacological (antidiabetic sulfonylurea drug glimepiride) stimuli. Therefore, vesicles released from large adipocytes stimulated lipid storage in small ones by a horizontal gene transfer of lipogenic information, which was due to relevant miRNAs. Therefore, this paracrine and endocrine regulation of lipid storage and cell size of white adipocytes by specific circulating miRNAs may represent a novel target to interfere with metabolic diseases, such as obesity and metabolic syndrome [87]. Although all these *in vitro* studies and many others have provided fundamental insights into the intercellular communication, showing that lipid-based carriers can transfer sufficient amounts of miRNAs to repress gene expression and alter phenotype in recipient cells, little is known about the exact nature of circulating miRNAs in healthy and diseased states *in vivo*. For this reason, the main challenges will be to reveal the physiological and pathological importance of the miRNA-mediated intercellular communication and, most significantly,

to consider the possibility of modifying the miRNA signals for therapeutic benefits. The major issue associated with the switch from the *in vitro* to *in vivo* studies consists on the small amounts of miRNAs in serum and plasma, as carriers transport only a small number of specific miRNAs, each of which in relatively low quantities. Some researchers argue that there are not sufficient amounts of circulating miRNAs in the body fluids to achieve the same regulatory effects in recipient cells as demonstrated *in vitro*, believing that the impact of miRNAs depends specifically on their concentration. However, recipient cells may have an increased sensitivity for circulating miRNAs or the impact of miRNA low quantities in gene regulation may have been underestimated. Another issue in studying the transferred miRNAs is that each class of carriers also contains a diversity of bioactive lipids, proteins and mRNAs [78]. For this reason, assuming that gene expression changes in recipient cells are exclusively due to the delivered miRNAs remains a big challenge. However, the most important facet of intercellular communication is the selective export of specific miRNAs from cells. Current evidence, indeed, suggests that the circulating miRNA profile may not be exactly representative of the parent cells and is distinct in abundance and content for each carrier, because of the differences in their biogenesis, delivery mechanisms and sources. On the other hand, rapid analysis of miRNAs in circulation is neither feasible nor available at this time, and purification of the lipid-based carriers is time-consuming. Therefore, future studies will need to identify altered miRNAs within specific carrier classes and to demonstrate that these alterations are representative of diseases, such as diabetes and its cardiovascular complications.

Circulating miRNAs as a novel class of biomarkers

One of the major challenges in research is the identification of valid and reliable biomarkers for diseases, which can be measured in routine clinical laboratories. The importance of biomarkers is highlighted by their application for detecting early onset and risk stratification with impact on treatment decision, for monitoring disease progression and response to treatment, and for identifying early relapse, eventually. An ideal biomarker fulfills the following criteria:

- Specificity – the molecule should be specific to the organ or tissue affected by the disease and able to distinguish between different types and states. Therefore, its levels should be proportional to the degree of severity of the pathology in order to allow a better classification into pathological subtypes. Numerous studies have shown unique patterns of aberrant miRNAs in each type of cancer; on the contrary, there is a lack of consistent and strong evidence that serum or plasma miRNAs are fingerprints in diabetes;

- Sensitivity – the molecule levels should reflect the dynamic pathological changes within a very short time period to provide information regarding disease evolution or therapeutic effects;

- Noninvasiveness – the molecule should be present in easily accessible samples, like blood and urine. Profiles in such body fluids are useful for the analysis of disease states especially when the disease does not originate from one distinct cell type and when the tissue is not readily accessible. To date, miRNAs have been detected in a number of body fluids, including serum and plasma, whole blood, urine, feces, saliva and breast milk;

- Stability – the molecule should be detected in samples which may have not been stored in ideal conditions. Serum miRNAs have been shown to be resistant to RNase digestion and stable to a wide range of harsh conditions, including high temperatures, extreme pH values and repeated freeze–thaw cycles;

- Robustness – detection and quantification techniques should be simple, rapid, accurate, reproducible and inexpensive to enable the clinical use of the molecule as a biomarker. Quantitative real-time PCR (qPCR) is the method of choice for circulating miRNAs because of the limited amounts of starting material usually available;

- Reproducibility – concerning the discovery of a new molecular biomarker, a confirmation of the results by several independent replications should be made to minimize pre-analytical and biological variation.

In 2008, the presence of miRNAs was first described in serum from diffuse large B-cell lymphoma (DLBCL) patients (n = 60) and healthy controls (n = 43), demonstrating higher levels of miR-21, miR-155 and miR-210 in patients and suggesting miRNAs as potential diagnostic markers [56]. Since then, hundreds of papers have been published on the potential use of circulating miRNAs as biomarkers, tied to their ability to comply with the required features of 'the good biomarker', at least in part. Therefore, miRNAs have been profiled in serum and plasma for several pathological conditions,

although most of these studies have focused on circulating miRNAs in various cancer types, as reviewed in [88]. In these observational studies, a miRNA profiling has been first performed and compared between a small group of patients with the disease of interest and a control group. Then, those miRNAs with significant variation between groups have been selected and validated on larger populations by individual qPCRs. However, in order to identify miRNAs as potential biomarkers for diseases, a large number of individuals must be included in longitudinal or cohort study settings. Another issue that needs to be addressed is the number of miRNAs to consider as a set in order to make an accurate risk assessment, because only a combination of multiple miRNAs may improve the diagnostic accuracy. In the case of diabetes, it is still unclear whether the miRNAs, which have been identified so far, can be considered reliable as a test, because the clinical trials used for the identification were not sufficiently powered to detect statistically significant differences between groups. For example, to explore the clinical significance of seven diabetes-related serum miRNAs (miR-9, miR-29a, miR-30d, miR-34a, miR-124a, miR-146a and miR-375) during the pathogenesis of diabetes, only 56 subjects were recruited, divided into three groups (newly diagnosed diabetic patients, prediabetes individuals and predisposed individuals with normal glucose tolerance). The results from this study indicated that the expression levels of these miRNAs were elevated in diabetic patients, while there was no significant difference during the pathogenesis of diabetes [89]. In another study, significant deregulation of seven candidate miRNAs was found to be associated with the risk of metabolic syndrome. In addition, a plausible association of miR-27a and miR-320a with metabolic syndrome and diabetes patients was also suggested, as these miRNAs remained deregulated in both the pathological conditions. In this case, a total of 265 participants were recruited and characterized into distinct groups, with only 50 individuals included in the diabetic group [90]. In the prospective study on the Bruneck population (Bolzano Province, Italy) were considered over 800 individuals, even if data were only available on a small number who developed diabetes during the course of the analysis. In diabetic patients, qPCR assessment revealed lower plasma levels of miR-15a, miR-20b, miR-21, miR-24, miR-126, miR-191, miR-197, miR-223, miR-320 and miR-486, but a modest increase of miR-28-3p. The authors also proposed a set of five most significantly altered miRNAs (miR-15a, miR-126, miR-223, miR-320, miR-486 and miR-28-3p) as minimal requirement for the classification of diabetes cases (70%). Furthermore, in subjects with normal glycemic levels (52%), the development of diabetes was predicted by this miRNA set and confirmed in the 10-year follow-up. In conclusion, a plasma miRNA signature for diabetes was revealed by this study, including loss of endothelial miR-126 [47].

The clinical application of circulating miRNAs as biomarkers is still a work in progress with several critical issues that need to be solved. Indeed, to ensure accurate measurements of circulating miRNAs, rigorous determinations of quantity and quality for miRNAs isolated from biological samples are demanded, facing interferences of degraded mRNA in RNA preparations and difficulties due to the low concentration of RNA typically obtained from this type of samples. Moreover, problems involved in the quantification of circulating miRNAs make the sample-to-sample and cross-comparisons challenging. Another relevant issue consists on how normalize miRNA quantitative data, since currently no references for small RNAs are available in the case of circulating miRNAs. In many cases, normalization by input volume remains the best option when measuring circulating miRNAs, using equal amounts of all samples and adding a spike-in control. On the other hand, equal volumes of serum may generate different amounts of total RNA; in addition, spike-in normalization does not account for internal variations in circulating miRNA levels between distinct individuals. Combined methods should always be applied to assure robustness of findings, regardless of the normalization approach used. To this end, an interesting possibility may be a correlation between circulating miRNA levels and previously established markers for the same disease, preferentially found in the same biological fluid. Moreover, as no standardized methods for measuring circulating miRNAs in serum and plasma have been established until now, it is difficult to compare miRNA expression profile data obtained in different laboratories worldwide. Nevertheless, circulating miRNAs hold great promise for diagnostics, early prediction strategies and pharmacologic assessments. Furthermore, although these preliminary results require confirmation and cross-validation in follow-up studies as well as in independent cohorts, the picture which is emerging is that some miRNAs in plasma and serum are quite specific for several diseases, such as diabetes and its cardiovascular complications. Therefore, they may not only be useful for diagnostic and monitoring purposes but also provide much needed intermediate end points for clinical trials.

Circulating miRNAs as novel therapeutics

In May 2013, Mirna Therapeutics (Austin, TX, USA) announced the advance into the clinic for MRX34, a liposome-formulated miR-34 mimic-based drug to treat patients with primary liver cancer or meta-

static cancer with liver involvement [91]. According to Mirna's strategy, MRX34 is used to induce apoptosis in tumor cells by restoring the lost function of the endogenous, tumor suppressor miR-34a. A synthetic double-stranded RNA oligonucleotide, which is then processed into a single-strand form to act in a miR-34a-like manner, is delivered by a liposome formulation, which naturally accumulates in the liver. The liposomal encapsulation has been shown to facilitate cellular uptake by endocytosis and protect the miRNA mimic from degradation. The neutral lipid emulsion is anionic at normal body pH (7–7.5), thus preventing unwanted interactions with negative charges of cellular membranes; on the other hand, the lipids become cationic in a tumor area, where the pH tends to be lower, and so the construct uptake is enhanced only in tumor cells. As another example, Miravirsen (SPC3649), an anti-miR drug candidate developed by Santaris Pharma (Hørsholm, Denmark), is currently in the most advanced miRNA clinical trial for the treatment of hepatitis C virus (HCV) infection. Data from the Phase IIa trial showed that Miravirsen given as a 4-week monotherapy treatment provided robust dose-dependent antiviral activity and HCV RNA became undetectable in four of nine treated patients. Therefore, Miravirsen may offer high barrier to viral resistance and the potential for cure with monotherapy. It was also well tolerated in patients with HCV, signaling a possible advantage over the standard treatment. Miravirsen has been designed to specifically recognize and sequester miR-122, a liver-specific miRNA with an important role in the life cycle of HCV. As a result, miR-122 becomes unavailable to the virus and its replication is effectively inhibited [92–94]. Its chemistry is based on locked nucleic acids (LNAs) with a methylene bridge connecting the 2′-oxigen and the 4′-carbon atoms in the ribose ring, which confers a locked conformation with resistance to degradation and increased hybridization affinity toward complementary single-stranded RNA. The unique combination of small size and very high affinity allows this drug candidate to potently and specifically inhibit the complementary miRNA in the liver without an additional delivery formulation. Apart from treating HCV infection, miR-122 has been shown to function as an important factor in modulating cholesterol metabolism. Indeed, treatment of high-fat diet-fed mice with Miravirsen resulted in long-lasting decrease in serum cholesterol [95]. Other miRNA-based treatments currently in preclinical development are also in the area of cardiovascular diseases. Therapeutic inhibition of miR-208a by the LNA-modified anti-miR during hypertension-induced heart failure in *Dahl* hypertensive rats dose-dependently prevented pathological myosin switching and cardiac remodeling while improving cardiac function, overall health and survival [96]. Follow-up studies showed that long-term treatment with anti-miR-208 prevented age-induced weight gain normally observed in mice [96]. To further investigate this phenotype, the effect of anti-miR-208 was also tested in a mouse model of Type 2 diabetes. Mice fed a high-fat diet and treated with anti-miR-208 showed remarkable resistance to high-fat diet-induced obesity by reducing the increase in body weight and improvements in systemic insulin sensitivity and glucose tolerance. These results suggested that miR-208a inhibitors, in addition to providing benefit in the setting of heart disease, may also have therapeutic utility in metabolic disorders, such as obesity, hypercholesterolemia, Type 2 diabetes, hepatic steatosis and hyperlipidemia [97]. Therefore, these exciting results are paving the way for the development of miRNA-based therapeutics against several diseases, including diabetes and its complications.

Advances in the understanding of the miRNA role in pathological processes have significantly contributed to the identification of alternative molecular pathways that will definitely influence the selection of new therapeutic approaches. The appealing strength of the miRNA therapeutic option dwells in their ability to concurrently regulate multiple targets, frequently in the context of a specific pathway, making miRNA-based therapy extremely efficient in the control of distinct biological processes relevant to the normal and pathological cell homeostasis. There are two main therapeutic strategies based on the 'corrective use' in the case of pathological miRNA level profiles: miRNA replacement and miRNA reduction. MiRNA replacement treatment (e.g., MRX34) involves the reintroduction of the miRNA which is downregulated in disease by either synthetic double-stranded miRNAs or viral expression vector in order to reactivate cellular pathways and drive a therapeutic response. miRNA reduction treatment consists in inactivating the miRNA which is upregulated in disease by either antisense oligonucleotides, known as anti-miRs (e.g., miravirsen), or competitive miRNA inhibitors, known as miRNA sponges. In more detail, miRNA sponges are multiple, tandemly repeated binding sites for the miRNA of interest and are overexpressed into the cells by high-copy number expression vectors or vectors equipped with a strong promoter to compete with *bona fide* miRNA targets. However, it is difficult to determine the degree of miRNA inhibition due to sponge vectors, this is why alternative strategies are preferred.

To date, some fundamental issues have impeded the development of miRNA-based treatments. The most challenging problem to overcome is the biological instability after intravenous injection of unmodi-

fied, saline-formulated oligonucleotides due to RNAse-mediated degradation and rapid renal excretion. In this case, frequent administrations and huge doses of miRNA drugs would be requested. In order to increase the miRNA half-life and make the miRNA-based treatment effective, various chemical modifications in synthetic oligonucleotides have been investigated. Among others, the LNA constructs provide the most promising results, conferring the following advantages: high hybridization affinity toward complementary sequences; excellent mismatch discrimination; high aqueous solubility. Another hurdle toward clinical application of miRNAs is the targeted, systemic delivery of these agents *in vivo*. The available delivery agents have a variety of targeting difficulties and unwanted side effects, which seriously limit their long-term use. Indeed, because many ubiquitous expressed mRNAs are targeted by one miRNA, the high risk for off-target effects in the miRNA modulation exists, especially during chronic treatments. Apart from the rapid localization to the kidney and liver for molecules delivered systemically and addresses to the kidney and liver, many peripheral tissues have been successfully targeted by a conjugation strategy with the nucleic acid linked to compounds with specific tissue affinity, such as antibodies or other bioactive molecules. Alternatively, the nucleic acid is encapsulated into a lipid-based formulation that enhances cell-specific uptake. However, in order to obtain appreciable patient benefits and reduced drug toxicity, there is a strong need for an immunological inert, nano-sized delivery tool, which can be loaded with RNA and targeted *in vivo* upon intravenous applications, delivering its cargo to the cytosol of recipient cells. Exosomes have the potential to address these needs and to revolutionize targeted delivery of nucleic acids. As physiologic liposomes with nano-scaled size, exosomes have reduced immunogenity and increased half-life in the circulation, in addition to an intrinsic ability to cross biological barriers and specific antigens on their surface to target the delivery. Thereby, they are able to assure protection for their therapeutic cargos, particularly in the case of miRNAs. The chance of manipulating *ex vivo* the exosome miRNA content as well as exosome membrane proteins enables the delivery of specific miRNAs to specific organs and cell types, suggesting a new and alternative therapeutic intervention for a variety of human diseases. However, their use still have several difficulties to face. First, exosomes must be abundant or highly enriched in order to utilize them in therapy: when exosome production mechanisms are clarified, 'exosome-producing cells' will be developed by cell engineering. Also more effective isolation exosome methods are required. Second, methods to introduce desired miRNAs into exosomes need improvements. Currently, the miRNA is overexpressed in exosome donor cells, resulting in an increased amount of the same miRNA inside the exosomes, or it is exogenously introduced into the exosomes by electroporation. At the present time, there are only a few studies that have employed exosomes to deliver therapeutic agents. Recently, exosome targeting was achieved by engineering the exosome donor cells to express the GE11 peptide, which binds specifically to EGFR. Intravenously injected in a xenograft breast cancer mouse model, exosomes delivered let-7a miRNA to EGFR-expressing cells of the breast cancer tissue, resulting in a significant antitumoral activity caused by the targeting EGFR receptors [98].

The research on new miRNA-based treatments is still in its infancy, but is running fast. Before providing approved and widespread therapeutic tools, new technologies and new strategies need to be developed and side effects need to be evaluated. However, there is rising optimism in the scientific community that these objectives will be achieved in the next few years.

Conclusion & future perspective

Over the last decade, innovative methods have opened up new possibilities for harnessing the potential of post-genomic research to study disease processes in clinical samples and to treat patients. From this perspective, circulating miRNAs currently provide an attractive choice as noninvasive biomarkers for a more accurate diagnosis of several diseases. In the emerging era of personalized medicine, miRNA biomarkers may also contribute to understand the individual response to a given treatment plan and to make decisions about the management of patients. Additionally, in the case of metabolic diseases, a number of candidate miRNA signatures are emerging and clinical trials are underway in order to validate their utility and to obtain the approval as diagnostic panels by regulatory authorities. With regards to diabetes, it will be crucial to carry out prospective trials employing well-defined, large-scale groups in multicenters across different countries worldwide in the forthcoming years. Moreover, it is reasonable that results based on the integration of clinicopathological clues with mRNA and miRNA expression data are highly informative, relevant and useful compared with the single miRNA signature. However, only a few studies nowadays incorporate more than one of these levels and, thus, the low power of available data is denounced.

Furthermore, miRNAs have many advantages as therapeutic tools. The mature miRNA sequences are short and often completely conserved across multiple vertebrate species. These characteristics make miRNAs relatively easy to target therapeutically and allow to use the same compound for miRNA modulation in preclin-

ical efficacy and safety studies as well as in clinical trials. Moreover, miRNAs have typically many targets within cellular networks, which, in turn, enable modulation of entire pathways in a disease state via therapeutic targeting of disease-associated miRNAs. Therefore, because of the great number of *in vitro* studies, knowledge about the potential use of miRNAs for therapeutic purposes is growing rapidly and technological advancements in miRNA-based therapies have accelerated this progress. As a consequence, miRNAs are being currently evaluated as potential drug candidates in several pathologies. Indeed, a number of animal studies have already produced impressive results; hence, clinical trials have been started or are ready to get started by pioneering pharmaceutical organizations. However, many challenges remain to be overcome before miRNA-based therapeutics become a reality. As the specific cellular context affects miRNA-target interactions, unexpected off-targets may make it difficult to predict the spectrum of side effects and toxicity profiles associated with a miRNA-based therapy. In addition, the ubiquitous expression patterns reported for many miRNAs increase the risk of off-target effects by a miRNA modulator, especially in long-term treatments. Thus, delivery of miRNA modulators to the cell type or tissue of interest is a key factor for the successful development of miRNA-based therapeutics, even though safe and reliable cell- and organ-specific delivery systems to use *in vivo* are still missing. Furthermore, due to the variation in miRNA levels across different cell and tissue types under physiological conditions as well as in disease, extensive preclinical studies are required to determine the optimal level of inhibition in miRNA reduction treatments. Similarly, miRNA replacement therapies will require optimization for restoring the activity of a downregulated or lost miRNA in order to prevent toxic effects due to the overloading of RISC with exogenous miRNA mimics. Issues related to the pharmacokinetic profile, including tissue distribution and cell penetration, also represent potential obstacles to the therapeutic use of nucleic acids that need to be resolved. Therefore, to advance a miRNA toward the clinic, a well-defined preclinical process is required in order to optimize suitable drug candidates and safety tests in animals must be performed to receive the approval from Research Ethics Committees and other regulatory authorities. Despite promising results

Executive summary

Introduction to miRNAs

- miRNAs are regulators of and are regulated by the epigenetic machinery. As epigenetic machinery disruption or alteration induces diseases, also miRNAs may be involved.

miRNAs in metabolism & metabolic diseases

- The importance of miRNAs in the control of metabolism has recently been revealed. An aberrant miRNA expression and function may induce metabolic homeostasis loss and disease.

Circulating miRNAs

- A growing number of reports have consistently revealed the presence of miRNAs in the blood and in other body fluids.

Distinctive features of circulating miRNAs

- The existence of circulating miRNAs is due to their resistance to RNase digestion and stability in a wide range of harsh conditions. A reasonable explanation for this characteristic may be that circulating miRNAs are packaged in membrane-derived vesicles or are associated with proteins or lipoproteins, which are defined as carriers.

Intercellular communication by circulating miRNAs

- Circulating miRNAs deliver regulatory messages from donor to recipient cells. Lipid-based carriers seem to be crucial for the miRNA role in cell-to-cell communication. These findings have been revealed by both *in vitro* studies and *in vivo* research on animal models.

Circulating miRNAs as a novel class of biomarkers

- Hundreds of papers have been published about the role of circulating miRNAs as potential biomarkers in humans, complying with the required features of 'the good biomarker'. However, several critical issues need to be resolved and, thus, their clinical application is still a work in progress.

Circulating miRNAs as novel therapeutics

- The research on miRNA-based therapies is still in its infancy. Several preclinical trials in animal models are currently being performed. New technologies and new strategies need to be developed and the side effects need to be evaluated, even if the exciting results so far obtained have given rise to optimism in the scientific community.

Conclusion & future perspective

- Based on the studies conducted up to now with cultured cells and animal models, there is the expectation that miRNAs may enter the clinic as diagnostic and therapeutic tools for several diseases, included diabetes and cardiovascular complications.

have been reported in both *in vitro* and *in vivo* studies and have provided the rationale for conducting human clinical trials, prudence and caution are recommended when interpreting and extrapolating experimental findings related to miRNAs to human disease, especially in the context of multifactorial, age-related diseases such as diabetes, which may arise from an intricate interplay of genetic and environmental factors.

In conclusion, the initial data required for the transfer of miRNA-based products from the laboratory to the pharmaceutical industry are encouraging and there is the expectation that miRNAs may enter the clinic as diagnostic and therapeutic tools for several diseases, included diabetes and cardiovascular complications, in the near future.

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