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DNA methylation partially mediates antidiabetic effects of metformin on HbA1c levels in individuals with type 2 diabetes

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ABSTRACT

Aims: Despite metformin being used as first-line pharmacological therapy for type 2 diabetes, its underlying mechanisms remain unclear. We aimed to determine whether metformin altered DNA methylation in newly-diagnosed individuals with type 2 diabetes.

Methods and Results: We found that metformin therapy is associated with altered methylation of 26 sites in blood from Scandinavian discovery and replication cohorts (FDR < 0.05), using MethylationEPIC arrays. The majority (88%) of these 26 sites were hypermethylated in patients taking metformin for ~ 3 months compared to controls, who had diabetes but had not taken any diabetes medication. Two of these blood-based methylation markers mirrored the epigenetic pattern in muscle and adipose tissue (FDR < 0.05). Four type 2 diabetes-associated SNPs were annotated to genes with differential methylation between metformin cases and controls, e.g., *GRB10*, *RPTOR*, *SLC22A18AS* and *TH2LCRR*. Methylation correlated with expression in human islets for two of these genes. Three metformin-associated methylation sites (*PKNOX2*, *WDTC1* and *MICB*) partially mediate effects of metformin on follow-up HbA1c levels. When combining methylation of these three sites into a score, which was used in a causal mediation analysis, methylation was suggested to mediate up to 32% of metformin's effects on HbA1c.

Conclusion: Metformin-associated alterations in DNA methylation partially mediates metformin's antidiabetic effects on HbA1c in newly-diagnosed individuals with type 2 diabetes.

1. Introduction

Metformin is globally used as first-line pharmacotherapy for type 2 diabetes. The main effects of metformin are considered to be decreased hepatic gluconeogenesis and increased glucose uptake in peripheral tissues through activation of AMP-activated protein kinase (AMPK) [1]. However, its mechanisms are more complex and certain effects of metformin may be independent of AMPK. To ensure an optimal use of metformin, it is essential to ascertain its underlying mechanisms. Interestingly, metformin may act through epigenetic mechanisms [2]. For example, we previously found decreased methylation of genes encoding the metformin transporters *OCT1*, *OCT3*, and *MATE1* in liver from 20 individuals with type 2 diabetes taking metformin [3]. Additionally, short-term metformin administration in 12 healthy participants altered methylation in blood [4], and differentially methylated regions were found in blood of 12 long-term metformin-treated individuals with type 2 diabetes compared to 12 drug-naïve type 2 diabetes individuals [5]. Moreover, metformin increased the level of the methyl donor SAM [6] and altered the activity of several epigenetic enzymes, including DNA methyltransferases (DNMT), which may thereby affect DNA methylation levels [7,8]. Although these data suggest that metformin could act through epigenetic mechanisms in humans, previous studies were of limited sample size, lack replication and did not test if metformin's function on clinical outcomes could be mediated through DNA methylation.

Our aim was therefore to first explore whether metformin therapy

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affects the genome-wide methylation pattern in blood from a discovery and a replication cohort including a total of 322 newly-diagnosed individuals with type 2 diabetes, of whom 92 were cases who had been taking metformin for ~ 3 months and 230 were controls who had not been taking any diabetes medication. We further performed a causal mediation analysis to test if methylation could mediate the effect of metformin on glycated haemoglobin (HbA1c) levels.

2. Material and Methods

2.1. Study populations

Newly-diagnosed individuals with type 2 diabetes from two prospective cohorts, ANDIS discovery cohort (n = 171) and ANDiU replication cohort (n = 151), and with DNA methylation data available, were included to investigate the effects of metformin on genome-wide methylation in blood (Fig. 1). Individuals with type 2 diabetes were diagnosed by clinical examination including blood glucose levels, HbA1c measurements, c-peptide values, and GADA antibodies to exclude subjects with type 1 diabetes and latent autoimmune diabetes in adults (LADA).

Participants in the discovery cohort were selected from the ANDIS (All New Diabetics in Scania) project (http://andis.ludc.med.lu.se), a prospective cohort aiming at registering all new diabetics within the Scania region [9]. This register intends to provide a better understanding of the heterogeneity of diabetes to ameliorate its diagnosis and treatment. At registration, patients filled out a questionnaire and blood samples were taken. Information on patients' medication is gathered from the drug registry recording when patients collect their medication from the pharmacy. Written consent was obtained from all participants within the ANDIS study and it adheres to the Declaration of Helsinki. The research protocol was approved by the Regional Ethical Review Board in Lund (numbers 584/2006, 2011/354, 2014/198).

To replicate our findings, an independent cohort was obtained from the ANDiU (All New Diabetics in Uppsala) study (https://www.andiu.se/) [9]. Anyone who was diagnosed with diabetes according to the WHO classification and resides in the County of Uppsala, Sweden, was eligible to participate. Within twelve months after diagnosed with diabetes each patient was provided with written information and asked for a written consent to participate in the study. Then blood tests (fasting plasma glucose, HbA1c, GADA antibodies, c-peptide, sample for genotyping and for biobanking) were given at the local primary health care center. The diabetes nurse completed the online questionnaire together with the patient. Follow-up was by automatic synchronization to the National Diabetes Registry. The design of ANDiU has been granted acceptance by the Swedish Data Inspection Board and by the Regional Ethical Review Board in Uppsala (2011/155). Written consent was obtained from all participants within the ANDiU study and it adheres to the Declaration of Helsinki.

A statistical power of 88% and 89% ($\alpha = 0.05$) was achieved to find 1% differences in methylation (SD = 2) between cases and controls in the ANDIS and ANDiU cohort, respectively, using the pwr package in R (Fig. A.1).

2.2. Study design

Subjects were considered metformin positive cases if they were on metformin for > 7 days before their DNA samples were taken. Controls were randomly selected and matched to cases for HbA1c levels, age and BMI at inclusion to a ratio of 1:3 for the discovery and 1:2 for the replication cohort. Patients with HbA1c levels > 13.1% (120 mmol/mol) at inclusion, patients with missing phenotype information, patients on other diabetes medication or on metformin for 1–6 days were excluded from this study (Fig. 1). Patient characteristics are presented in Table 1 and no differences in any of the studied variables were observed at inclusion between cases and controls. Written consent was obtained from all participants following ethical guidelines.

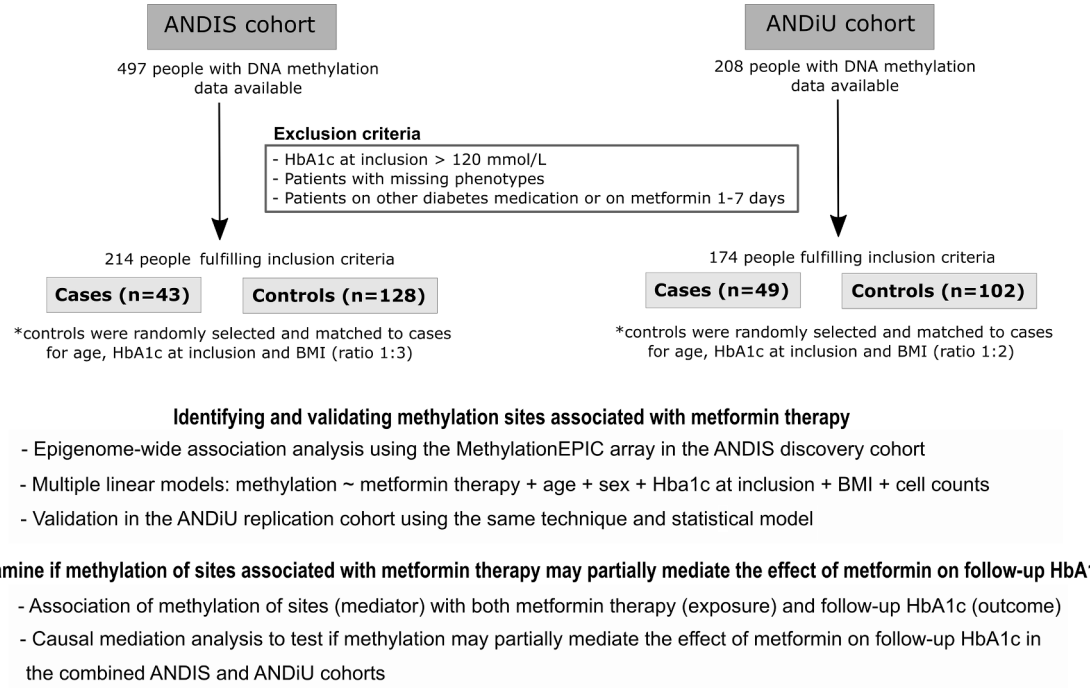

Fig. 1. Diagram of the study design including selection of participants and analyses. We included 171 and 151 individuals from the ANDIS discovery and ANDiU replication cohorts respectively, with available DNA methylation data, fulfilling the inclusion criteria, and controls were randomly selected and matched to cases for age, HbA1c at inclusion and BMI. The analyses in this study can be divided into two steps: 1) Identifying and validating methylation markers associated with metformin therapy by epigenome-wide analyses in discovery and replication cohorts; 2) Examine if methylation markers identified and validated in step 1 may partially mediate the effect of metformin on follow-up HbA1c levels by performing causal mediation statistical analyses in the combined ANDIS and ANDiU cohorts.

Table 1
Baseline clinical characteristics of metformin positive cases and diabetes drug naïve controls.

		ANDIS discovery cohort (n = 171)			ANDiU replication cohort (n = 151)		
		Controls (n = 128)	**Cases (n = 43)**	**p**	**Controls (n = 102)**	**Cases (n = 49)**	**p**
Time on metformin therapy (days)	*mean (SD)*	–	71.51 (93.4)		–	96.0 (85.4)	
	min–max		8–517			7–376	
Sex	*male (%)*	42.2	46.5	0.751	49	55	0.484
	male (n)	54	20		50	27	
Age (years)	*mean (SD)*	60.14 (10.94)	61.93 (10.05)	0.334	63.02 (9.48)	59.82 (12.22)	0.147
	min–max	38–85	43–81		40–83	33–85	
BMI (kg/m2)	*mean (SD)*	31.25 (5.26)	31.64 (6.33)	0.936	31.65 (5.66)	32.14 (4.84)	0.415
	min–max	20.38 – 48.43	18.56 – 49.45		20 – 48	21 – 41	
HbA1c (% and mmol/mol) at inclusion	*mean (SD)*	8.1 % (64.86 mmol/mol, SD 17.10)	8.2 % (65.63 mmol/mol, SD 18.88)	0.563	7.3 % (56.09 mmol/mol, SD 17.02)	7.3 % (56.37 mmol/mol, SD 16.79)	0.417
	min–max	41–116	34–114		30–118	30–115	
Antihypertensives	*Yes (%)*	54.7	48.8	0.625	45.1	55.1	0.249
	Yes (n)	70	21		46	27	
Lipid lowering drugs	*Yes (%)*	23.4	37.2	0.118	23.5	36.7	0.090
	Yes (n)	30	16		24	18	

Phenotypes were measured in individuals with newly-diagnosed diabetes from the ANDIS (All New Diabetics in Scania) study and the ANDiU (All New Diabetics in Uppsala) study. P-values were calculated with Mann-Whitney *U* test for continuous variables and Pearson's chi-square test for binary variables. $p < 0.05$ was considered significant. Cases and controls were matched for the relevant phenotypes (age, bmi and hba1c) to minimise confounding in the analysis.

2.3. Phenotypes measurements

Information regarding the patients' age, sex and BMI, were registered at the same time point as blood samples for DNA methylation analysis were taken. Standard protocols were used to measure height and weight and calculate BMI (kg/m^2). Information regarding diabetes medication, lipid lowering drugs and antihypertensive drugs were obtained from the drug registry linked to ANDIS and ANDiU. Patients on another diabetes medication were excluded from the study, as well as patients who had picked up metformin ≤ 7 days before blood samples for DNA methylation analysis were taken. HbA1c was measured using Variant II Turbo HbA1c Kit 2.0 (Bio-Rad Laboratories, Copenhagen, Denmark). Basal HbA1c levels included in Table 1 were analysed before or at the same time point as blood samples for DNA methylation analysis, while follow-up HbA1c levels, used for the causal mediation analysis, were measured at the same time as methylation data was generated or afterwards. For cases taking metformin, the follow-up HbA1c was always measured after at least 3 months on metformin monotherapy to give metformin enough time to reduce HbA1c. While, for controls who were not on any diabetes medication, the follow-up HbA1c could be analysed at the same time point as blood samples for DNA methylation analysis or shortly later.

Regarding the analyses of response to metformin treatment in the ANDIS discovery cohort, the glycaemic response of the participants was based on the change in HbA1c levels after ~ 1–1.5 years of metformin monotherapy. It should be noted that most of the 128 individuals in the metformin control group of the ANDIS discovery cohort (Table 1) started metformin therapy after blood samples for analysis of DNA methylation were taken, and they were therefore also included in this analysis. We used EASD and ADA guidelines for definition of glycaemic response, along with the criteria presented in our previous study on metformin response [9]. Hence, glycaemic responders were those who had a HbA1c < 6.5–7% (<48–53 mmol/mol) and reduction in HbA1c ≥ 1% after ~ 1–1.5 years therapy, while non-responders were those who had a HbA1c ≥ 6.5–7 % (≥48-53 mmol/mol) and reduction in HbA1c < 1% after ~ 1–1.5 years therapy. Based on these criteria, among the 43 metformin cases in the ANDIS discovery cohort, 10 were glycaemic responders and 5 non-responders, while among the 128 metformin controls, 31 were glycaemic responders and 13 non-responders. These responders and non-responders were on metformin monotherapy during ~ 1–1.5 years without starting any other diabetes medication.

2.4. Genome-wide DNA methylation analysis in whole blood

Blood samples for the analysis of DNA methylation were taken at registration. The Gentra Puregene Blood kit (Qiagen, Hilden, Germany) was used to extract the DNA according to the manufacturer's instructions. Nucleic acid concentration and purity were determined using the NanoDrop 1000 spectrophotometer (NanoDrop Technologies). The genome-wide DNA methylation analysis was carried out at the SCIBLU genomics centre and at Lund University Diabetes Centre. DNA methylation in whole blood samples was analysed in type 2 diabetes metformin-positive cases and metformin-negative controls with the Illumina MethylationEPIC BeadChip microarray (Illumina, Inc., San Diego, CA, USA) covering a total of 853,307 CpG sites. 500–1000 ng of genomic DNA was bisulfite treated using the EZ DNA methylation kit (Zymo Research, Orange, CA, USA). DNA samples were then randomised across chips and DNA was amplified, fragmented, and hybridised to the BeadChips following the Infinium HD assay methylation protocol (Illumina). The BeadChips' images were acquired using the Illumina iScan after single base extension and staining. Raw methylation score for each DNA methylation site was obtained from GenomeStudio methylation module software (Illumina). ß-values for these methylation scores were calculated with the following equation: β-value = intensity of the Methylated allele (M) / (intensity of the Unmethylated allele (U) + intensity of the Methylated allele (M) + 100), ranging from

0 (completely unmethylated) to 1 (completely methylated). Samples with a high bisulfite conversion efficiency (intensity signal > 4,000) were subsequently included in further analyses after passing the GenomeStudio (Illumina) quality control steps using built-in control probes for staining, hybridisation, extension and specificity. Raw methylation data was exported from GenomeStudio and analysed with Bioconductor. We filtered away rs-probes, cross-reactive probes and polymorphic probes based on annotation from McCartney et al. [10], Y chromosome and non-CpG probes, as well as individual probes based on mean detection p-values > 0.01. In order to remove heteroscedasticity in further analyses, ß-values were converted into M−values [11]. ComBat was used to correct for batch effect [12]. Since whole blood contains multiple cell types, we also used the reference-free Houseman method [13] in some models to correct for potential effects of cellular heterogeneity. In order to easier interpret the data, M−values were reconverted into ß-values using the equation: $M = \log_2$ ß-value/(1 – ß-value), which were then used for the data description and creating tables and figures.

DNA methylation data are deposited at the LUDC repository (https://www.ludc.lu.se/resources/repository) under the following accession numbers: LUDC2023.02.1 (ANDIS discovery cohort), and LUDC2023.02.2 (ANDiU replication cohort), and are available upon request.

2.5. DNA methylation in other tissues

The Monozygotic Twin cohort [14] was used to assess if metformin-associated methylation in blood mirror methylation in key tissues for type 2 diabetes, i.e. adipose tissue (n = 28) and skeletal muscle (n = 36), and to correlate methylation with expression for these two tissues. In this cohort including monozygotic twin pairs discordant for type 2 diabetes, methylation from the Illumina 450 K is available for the three cell-types from the same individual (blood, adipose tissue, muscle), while expression data is only available from adipose tissue and muscle. Methylation data were extracted if the methylation sites significantly associated with metformin therapy were also covered by the 450 K array. In total 12 out of 26 methylation markers were available in the 450 K array. Moreover, we correlated DNA methylation of these 12 sites with expression data of annotated genes available in the GeneChip Human Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA), using Pearson correlations and the data from skeletal muscle and adipose tissue of the twins. Additional information of this cohort is published elsewhere [9,14,15].

We also assessed whether DNA methylation of the metformin-associated sites correlated with gene expression in human pancreatic islets [16]. Here, we included 102 donors not diagnosed with diabetes (34% females, age = 59 ± 10.8, BMI = 26.2 ± 3.9) with available DNA methylation (MethylationEPIC array) and gene expression data (RNA-Seq on a HiSeq 2000 or NextSeq 500) (Illumina).

2.6. Causal mediation analysis

A causal mediation analysis was performed to study if metformin-associated alterations in methylation could be a potential mechanism through which metformin may partially act to achieve its antidiabetic effect. This analysis requires that the exposure (in our case metformin treatment including cases and controls) has a significant effect on the outcome (in our case follow-up HbA1c measured at the same as methylation or afterwards) and then it tests if methylation of sites significantly associated with metformin treatment may partially mediate the effect of metformin on HbA1c. Subsequently, both metformin naïve controls and cases need to be included in the causal mediation analysis. Overall, mediation helps to find out how metformin treatment might influence HbA1c levels. Follow-up HbA1c levels were measured at the same time as methylation data or afterwards. Controls have a mean follow-up HbA1c of 7.7% (61 mmol/mol, SD 17.6). For cases, the mean follow-up HbA1c was 6.8% (51 mmol/mol, SD 9.8) and it was always measured after at least 3 months on metformin monotherapy to give metformin enough time to reduce HbA1c. For this analysis, the ANDIS and ANDiU cohorts were combined (n = 310), and some cases were excluded (n = 12) because they did not have available follow-up HbA1c levels that fulfil the requirements. Age, sex, BMI and cohort were included as confounders. The required sample size to detect the mediated effect with 0.8 statistical power was calculated using information from Fritz and MacKinnon's tables [17]. Using the standardized coefficients obtained from the linear models in the causal mediation analysis and looking at the percentile bootstrap approach, 126 participants are needed to reach 80% power. Since the number of participants is higher for this analysis (n = 310), the statistical power needed to reach this aim is guaranteed.

This analysis was performed in R using the R mediation package [18] which runs a nonparametric bootstrap using the default settings. For a variable to be considered a mediator, it needs to be associated with both the exposure (in our case, metformin therapy) and the outcome (in our case, follow-up HbA1c levels). Therefore, this analysis was done only for the four methylation sites found to be associated with both metformin and follow-up HbA1c levels (q < 0.05) in a previous step. We also tested whether a methylation score, including the three methylation sites found to be significant partial mediators, could better mediate the association between metformin and follow-up HbA1c levels. The score was calculated as the sum of the standardized methylation values for each of the included methylation sites, and this was multiplied by the B-coefficient for the respective site in the discovery cohort [9,15]. This methylation score was calculated to test if when combining the three methylation sites, independently of genomic location, results in a stronger mediator effect than assessing one by one, which is a similar concept to the widely known genetic risk scores.

2.7. Statistical analyses

Multiple linear regression models were applied to assess associations between DNA methylation and metformin therapy. The models were adjusted for sex, age, BMI and HbA1c levels at inclusion to minimise confounding effects. We further adjusted the analysis for cell composition using the reference-free Houseman method in both cohorts. In all regression analyses, methylation levels were the dependent outcome and metformin therapy the independent variable. False Discovery Rate (FDR) below 5% (q < 0.05) based on Benjamini-Hochberg was applied. Moreover, Pearson analyses were performed to assess the correlations between methylation in blood and methylation in other tissues, and between methylation and expression of the respective annotated gene in these other tissues (q < 0.05).

The ANDIS discovery and ANDiU replication cohorts were combined for following downstream analyses. Linear regression models to assess the association between methylation sites and follow-up HbA1c were adjusted for age, sex, BMI and cohort, and q < 0.05 was applied. Causal mediation analyses were run using the R mediation package including age, sex, BMI and cohort as confounders.

3. Results

To assess the effects of metformin therapy on DNA methylation in blood, we conducted two separate analyses in the ANDIS discovery cohort (n = 171, Table 1). The first linear model, adjusted for sex, age, BMI and HbA1c at inclusion, resulted in 7,957 differentially methylated sites (q < 0.05) between metformin positive cases and controls (Fig. 2**A**). The second linear model, also including the reference-free Houseman method to adjust for cell composition, resulted in 24,857 differentially methylated sites (q < 0.05) between cases and controls. 5,371 methylation sites were found in both of these analyses. To capture robust differences in methylation between cases and controls, we considered methylation of these overlapping 5,371 sites to be affected by metformin

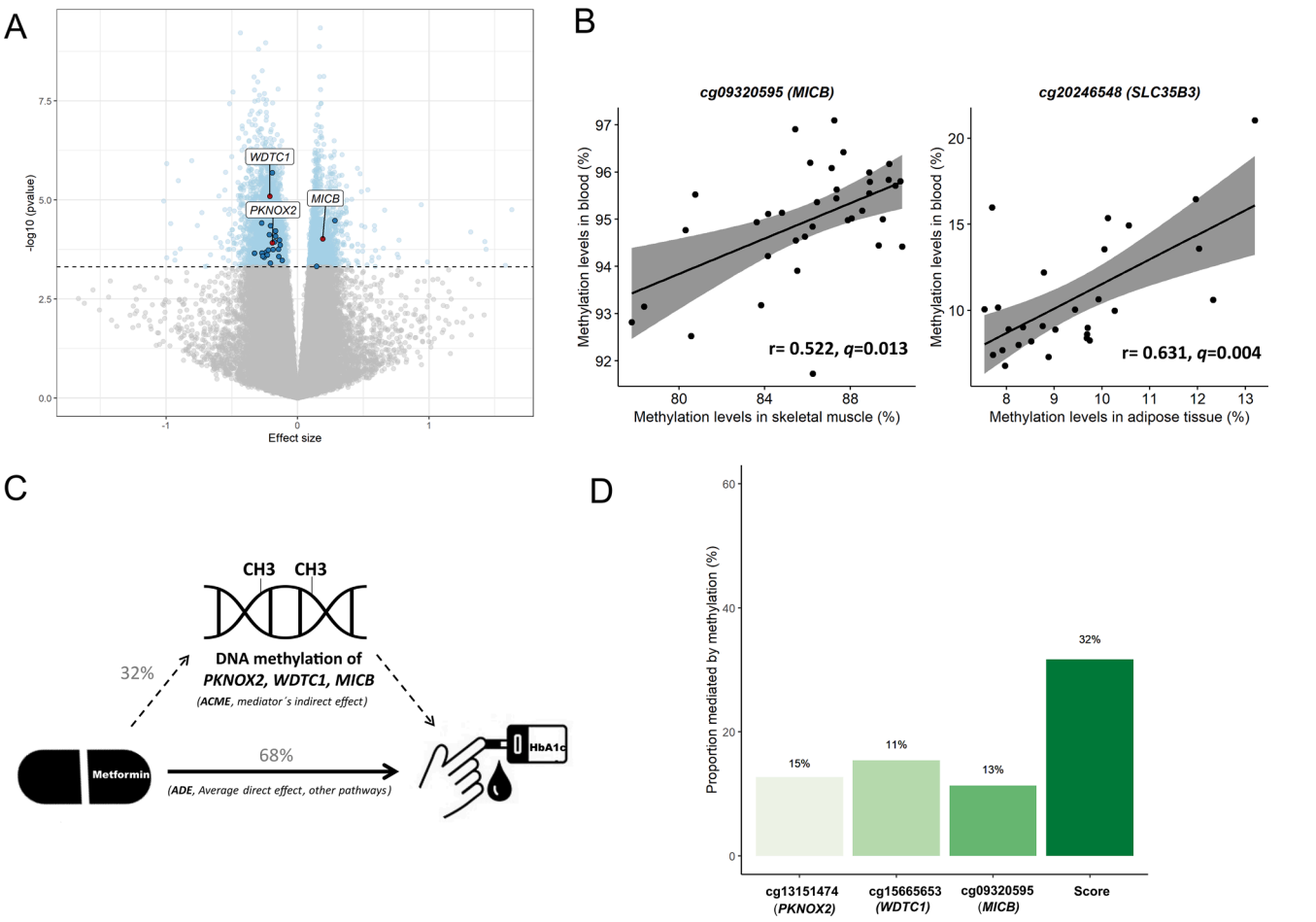

Fig. 2. Methylation markers associated with metformin therapy in newly-diagnosed individuals with type 2 diabetes. (A) Volcano plot showing the effect size (comparing controls versus metformin positive cases) and significance level of all methylation sites analyzed with the Illumina MethylationEPIC BeadChip microarray (~850,000 sites) in the ANDIS discovery cohort (n = 171). The significance cut off indicated with a dotted line is FDR < 5%. In the plot, 7,957 sites were found to be significant between metformin cases and controls in the linear model adjusted for sex, age, BMI and HbA1c. Highlighted in dark blue are the 26 methylation sites replicated in the ANDiU replication cohort, and among these ones three sites are highlighted in red which are found to mediate the effect of metformin on follow-up HbA1c levels. **(B)** Methylation in blood of 2 out of 26 sites associated with metformin therapy (12 sites were available in the Illumina 450 K array used for this data) mirror methylation in target tissues of type 2 diabetes, i.e. adipose tissue and skeletal muscle, after correcting for multiple testing (q < 0.05). **(C)** Causal mediation analysis was performed in the combined ANDIS and ANDiU to assess the role of methylation as a mediator of the effect of metformin on follow-up HbA1c levels. In the scheme, the solid black arrow shows the effect of metformin therapy on follow-up HbA1c levels that operate directly or through a pathway different from methylation (ADE) which is 68%. The dotted black arrows show a suggested alternative pathway, where an indirect effect (ACME) of metformin therapy on HbA1c levels is mediated by DNA methylation representing 32% of the total effect. (**D**) Three methylation sites partially mediated the association of metformin and HbA1c, and when combining these sites into a score, 32% of the effect of metformin on HbA1c could be explained by DNA methylation. Causal mediation analyses were adjusted for age, sex, BMI and cohort.

therapy independently of cell composition in the ANDIS discovery cohort (Table A.1). We then validated these sites in an independent cohort, showing that 26 methylation markers were associated with metformin therapy (q < 0.05) also in the ANDiU replication cohort (n = 151) after adjusting for the same confounders and cell composition (Table 2). We next performed additional models in which we further adjusted for other potential confounders, i.e., lipid-lowering medication, antihypertensives or time from diabetes diagnosis, and then methylation of all 26 sites was associated with metformin therapy in the ANDIS and ANDiU cohorts, with $P = 3.35 \times 10^{-6} - 5.85 \times 10^{-2}$ (Table A.2). Moreover, the methylation levels of the 26 sites were not associated with time on metformin treatment among the cases (q > 0.05, Table A.3).

The majority (88%) of the 26 sites were hypermethylated in cases taking metformin compared to the controls, and 20 of them were annotated to gene regions. Notably, SNPs annotated to four of these 20 genes with differential methylation after metformin therapy, *GRB10*, *RPTOR*, *SLC22A18AS* and *TH2LCRR*, have been associated with type 2 diabetes in previous genome-wide association studies (GWAS catalogue, accessed 13/09/2022, Table A.4). We further tested if methylation of the metformin-associated sites correlates with gene expression in human pancreatic islets, skeletal muscle, and adipose tissue [14,16]. Interestingly, expression of *SLC22A18, SLC22A18AS, OLFM1* and *GRB10* correlated with methylation of respective metformin-associated site in human pancreatic islets, while expression of *TPCN1* correlated nominally with methylation in muscle (Table A.5), implying a biological function of these methylation sites also in other tissues.

Moreover, blood-based methylation for two of these 26 sites, cg09320595 annotated to *MICB* (r = 0.522, $q = 0.013$) and cg20246548 annotated to *SLC35B3* (r = 0.631, $q = 0.004$), correlated positively with methylation in skeletal muscle and adipose tissue (Fig. 2**B**), respectively, suggesting that these sites may play a role also in muscle and adipose tissue.

We then examined whether methylation of any of the validated 26 metformin-associated sites also correlated with follow-up HbA1c levels, which were analysed at the same time as DNA methylation analyses or afterwards, i.e., after ≥ 3 months of metformin therapy for the cases, in

Table 2
Validated DNA methylation sites associated with metformin therapy in newly-diagnosed individuals with type 2 diabetes from both the ANDIS discovery (n = 171) and ANDiU replication (n = 151) cohorts, including cases on metformin monotherapy for > 7 days and controls who had not taken any diabetes medication.

				ANDIS (n = 171)											ANDiU (n = 151)							
Target ID	Gene	Gene region	CpG island group	B-coeff	SEM	p- value from linear model adjusted for sex, age, BMI and HbA1c	q-value from linear model adjusted for sex, age, BMI and HbA1c	Cases		Controls		Difference case -control	p-value *	q-value†	Cases		Controls		Difference case -control	p-value *	q-value†	p- value from linear model adjusted for sex, age, BMI and HbA1c
								Mean	SD	Mean	SD				Mean	SD	Mean	SD				
cg00292857	*RNF139*	TSS200	Island	–0.118	0.032	3.37E-04	4.34E-02	4.66	0.6	4.31	0.5	0.35	3.23E-04	2.29E-02	4.65	0.85	4.20	0.73	0.45	1.31E-05	1.16E-02	7.26E-04
cg04176235	*PTK2B*	5′UTR	open_sea	–0.239	0.063	2.08E-04	3.66E-02	92.84	1.34	91.6	1.95	1.24	4.55E-07	8.57E-04	91.06	1.82	90.05	2.28	1.01	1.76E-04	3.29E-02	5.87E-03
cg04223026	*GRB10*	Body;5′UTR	open_sea	–0.138	0.035	1.03E-04	2.82E-02	54.16	3.42	52.03	3.59	2.13	3.04E-04	2.23E-02	58.01	4.35	56.04	3.62	1.96	1.31E-04	2.93E-02	6.65E-03
cg04656692	*MRGPRG; C11orf36*	1stExon;Body	Island	–0.144	0.039	2.66E-04	4.00E-02	64.63	3.19	62.53	3.92	2.1	9.23E-04	3.88E-02	68.90	3.77	66.24	4.19	2.66	3.01E-04	4.14E-02	7.95E-05
cg04986290	*MMP2*	5′UTR; TSS1500;Body	S_Shore	–0.208	0.058	3.95E-04	4.62E-02	89.54	2.64	88.3	2.37	1.24	7.66E-04	3.55E-02	88.64	1.99	86.91	2.82	1.72	4.44E-04	4.88E-02	1.11E-04
cg05929100		intergenic	open_sea	0.284	0.067	3.32E-05	1.96E-02	12.12	3.64	14.65	4.46	–2.53	3.32E-04	2.33E-02	11.15	2.70	12.10	3.51	–0.95	2.17E-04	3.59E-02	9.75E-03
cg07018107	*MIR1208*	Body	open_sea	–0.215	0.053	7.45E-05	2.52E-02	71.13	4.49	67.98	5.06	3.15	2.27E-05	6.09E-03	74.98	4.16	71.55	6.25	3.43	4.23E-05	1.85E-02	5.98E-04
cg08915125		intergenic	open_sea	–0.188	0.049	1.78E-04	3.44E-02	87.42	2.6	86.09	2.24	1.33	2.64E-05	6.60E-03	84.10	2.10	82.44	2.68	1.66	1.34E-05	1.17E-02	3.29E-04
cg09198782	*SLC22A18AS; SLC22A18*	5′UTR; TSS1500	N_Shore	–0.168	0.041	6.04E-05	2.35E-02	83.99	2.06	82.39	2.41	1.6	6.35E-04	3.22E-02	85.13	2.15	83.76	2.34	1.37	1.92E-05	1.36E-02	3.81E-04
cg09320595	*MICB*	3′UTR	open_sea	0.191	0.048	9.65E-05	2.78E-02	90.54	1.68	91.61	1.41	–1.07	2.62E-05	6.57E-03	87.09	3.00	88.33	2.53	–1.24	8.86E-05	2.50E-02	1.97E-03
cg10495402	*CHD6*	Body	open_sea	–0.274	0.065	3.81E-05	2.04E-02	89.39	3.06	87.61	2.55	1.78	6.80E-07	1.08E-03	84.44	3.64	82.50	2.90	1.93	5.06E-05	1.98E-02	2.39E-04
cg12174736	*TH2LCRR*	TSS200;Body	Island	–0.271	0.072	2.19E-04	3.72E-02	53.32	8.44	48.72	6.67	4.6	5.39E-05	9.47E-03	57.74	6.21	55.16	6.40	2.58	2.68E-05	1.54E-02	3.28E-03
cg12914100	*TPCN1*	3′UTR	open_sea	–0.193	0.039	2.06E-06	7.26E-03	81.23	2.46	79.23	2.63	2	1.45E-06	1.62E-03	81.95	2.49	80.35	2.46	1.60	5.28E-05	2.02E-02	3.20E-04
cg13151474	*PKNOX2*	Body	open_sea	–0.192	0.049	1.21E-04	2.98E-02	67.33	4.36	64.34	4.25	2.99	1.40E-04	1.52E-02	70.01	4.32	68.29	4.50	1.72	3.14E-04	4.23E-02	4.80E-03
cg13572380	*KCNN3*	Body	open_sea	–0.175	0.044	1.14E-04	2.93E-02	76.7	3.19	74.72	3.57	1.98	2.04E-04	1.84E-02	79.09	2.96	77.80	3.33	1.29	6.16E-05	2.16E-02	4.80E-02
cg15665653	*WDTC1*	5′UTR;1stExon	Island	–0.212	0.046	8.02E-06	1.15E-02	17.54	2.39	15.65	2.52	1.89	1.32E-05	4.65E-03	13.91	2.30	13.01	2.60	0.91	1.14E-04	2.77E-02	8.05E-03
cg15904623	*FLNC*	Body	open_sea	–0.222	0.058	1.83E-04	3.49E-02	94.79	1	93.95	1.35	0.84	1.29E-03	4.60E-02	93.78	0.95	93.00	1.46	0.78	2.50E-05	1.50E-02	1.73E-03
cg16027745	*OLFM1*	Body	N_Shelf	–0.328	0.087	2.19E-04	3.73E-02	83.05	4.99	79.69	5.67	3.36	2.46E-04	2.01E-02	84.22	4.04	80.80	4.88	3.42	7.60E-08	1.41E-03	2.61E-05
cg19537308	*EPS8L3*	Body	open_sea	–0.205	0.049	4.46E-05	2.12E-02	91.15	1.47	89.94	1.85	1.21	2.73E-04	2.12E-02	90.48	1.63	89.59	1.47	0.89	6.16E-05	2.16E-02	2.12E-04
cg20023120		intergenic	N_Shore	0.144	0.04	4.72E-04	4.93E-02	7.77	1.36	8.41	1.35	-0.64	1.32E-03	4.65E-02	8.43	1.47	9.26	1.79	–0.83	3.13E-04	4.23E-02	1.03E-02
cg20246548	*SLC35B3*	TSS200	Island	–0.255	0.069	2.77E-04	4.06E-02	6.75	1.95	5.7	1.51	1.05	3.30E-05	7.37E-03	10.40	3.16	9.05	2.47	1.35	1.47E-04	3.07E-02	2.58E-03
cg20337105		intergenic	open_sea	–0.131	0.034	1.38E-04	3.13E-02	69.2	2.99	67.38	3.01	1.82	2.11E-06	1.93E-03	66.51	2.76	65.17	3.24	1.34	4.09E-04	4.72E-02	2.92E-02

(*continued on next page*)

Table 2 (*continued*)

Target ID	Gene	Gene region	CpG island group	ANDIS (n = 171)								ANDiU (n = 151)							
				B-coeff	SEM	p- value from linear model adjusted for sex, age, BMI and HbA1c	q-value from linear model adjusted for sex, age, BMI and HbA1c	Cases		Controls		Difference case -control	p- value *	q- value†	Cases		Controls		Difference case -control	p- value *	q- value†	p- value from linear model adjusted for sex, age, BMI and HbA1c
								Mean	SD	Mean	SD				Mean	SD	Mean	SD				
cg22035374	*RPTOR*	Body	open_sea	–0.146	0.038	1.76E-04	3.44E-02	91.97	1.29	91.21	1.15	0.76	5.67E-04	3.04E-02	89.40	1.35	88.88	1.51	0.52	1.07E-04	2.69E-02	1.25E-02
cg23483081	*ZNF516*	5'UTR	S_Shelf	–0.168	0.041	8.16E-05	2.61E-02	84.19	2.3	82.55	2.41	1.64	6.50E-06	3.26E-03	85.35	2.46	84.31	2.37	1.04	1.01E-04	2.62E-02	7.46E-03
cg23513222		intergenic	open_sea	–0.266	0.071	2.61E-04	3.97E-02	85.12	3.58	82.83	4.16	2.29	1.89E-04	1.76E-02	86.26	2.86	84.24	3.53	2.02	1.10E-04	2.73E-02	9.77E-04
cg25069770		intergenic	open_sea	–0.231	0.062	2.44E-04	3.87E-02	85.88	2.91	83.89	3.28	1.99	6.04E-04	3.14E-02	84.21	3.92	81.16	5.18	3.05	2.63E-04	3.89E-02	3.43E-04

Models are adjusted for age, sex, BMI and HbA1c at inclusion.
B-coefficients are shown comparing controls vs. metformin-positive cases.
* p-values from Houseman reference-free method.
† q-values from Houseman reference-free method.

the combined ANDIS + ANDiU cohorts. DNA methylation of four sites correlated with follow-up HbA1c levels after adjusting for age, sex, BMI and cohort ($q < 0.05$) (Table A.6). The four methylation sites associated with both metformin therapy and follow-up HbA1c levels, were then included in a causal mediation analysis to test if methylation could mediate the effect of metformin on HbA1c levels. This analysis showed that; **a**) metformin therapy has a significant overall effect of B = -9.48 (95% CI: -12.48 to -6.37) on follow-up HbA1c levels (Total effect, Table 3), **b**) part of that effect goes directly or via mediator(s) other than methylation, called average direct effect (ADE), **c**) this effect may operate via an indirect path (indirect effect), possibly through methylation, with a significant average causal mediator effect for 3 methylation sites annotated to *PKNOX2*, *WDTC1* and *MICB* (ACME, Table 3), and **d**) consequently, the total effect of metformin therapy on follow-up HbA1c levels, 11 to 15%, is suggested to act via these 3 methylation sites (Table 3, Fig. 2**C-D**). When combining methylation of these three sites into a score, which was then used in the causal mediation analysis, methylation could explain up to 32% (95% CI: 14 to 56, $p < 0.001$) of the effect of metformin on follow-up HbA1c, indicating methylation as a partial mediator of this association (Table 3, Fig. 2**C-D**).

We next examined whether methylation levels of these 26 sites differed between glycaemic responders and non-responders to metformin based on reduction in HbA1c after 1–1.5 years treatment. Here, we studied both the metformin cases and controls (Table 1), since also the controls eventually were treated with metformin, but they started their treatment after blood samples for DNA methylation analysis were taken. However, methylation of the 26 sites did not differ between glycaemic responders and non-responders to metformin in a subset of the ANDIS cohort, neither in the metformin cases nor in controls ($q > 0.05$, Table A.7).

We finally examined if genes annotated to metformin-associated differentially methylated regions (DMRs) previously identified in 24 women [5], were also annotated to our methylation sites presented in Table 2 and Table A.1. Eight genes had both metformin-associated DMRs [5] and methylation sites in ANDIS (Table A.1), but these were not among the 26 sites replicated in ANDiU.

4. Discussion

This study shows differences in DNA methylation in newly-diagnosed individuals with type 2 diabetes taking metformin compared to controls. We identified and validated 26 methylation markers to be associated with metformin therapy. Notably, hypermethylation was detected on most sites (88%) affected by metformin treatment in this study, which concurs with the assumption that metformin increases the SAM:SAH ratio, with SAM being the primary methyl donor for DNA methylation [6]. Similarly, metformin may also affect the activity of epigenetic enzymes, and notably it could decrease the influence of DNMT inhibitors inducing hypermethylation [7,8]. Interestingly, metformin-associated methylation markers are annotated to genes previously associated with glucose homeostasis and type 2 diabetes in GWAS and other studies. For example, *GRB10* is a type 2 diabetes susceptibility gene encoding an inhibitor of insulin receptor signaling, and the liver of individuals with type 2 diabetes showed decreased methylation of *GRB10* [19]. Other genes with altered methylation by metformin treatment in our study, *PTK2B, KCNN3* and *RPTOR*, have been previously activated or downregulated by metformin treatment in cell lines and animal models [20–22]. While metformin activated *PTK2B* expression related to drug resistance in breast cancer cell lines [20], it downregulated KCNN3 protein expression in the rat heart [21]. Metformin has an antitumor potential through activation of AMPK, which is a main negative regulator of the mTOR pathway where RPTOR is involved [22]. Hence, the epigenetic regulation of these genes might contribute to metformin's antidiabetic and potential anticancer effects.

Other studies have assessed the effect of metformin on blood DNA

Table 3
Causal mediation analysis on the significant associations between metformin therapy and Hba1c-related methylation sites as mediators and HbA1c levels (mmol/mol) as the outcome.

CpG site	Gene	ACME estimate of methylation as mediator (95% CI)	ACME p-value	ADE estimate (95% CI)	Total effect (95% CI)	Total effect p-value	Proportion mediated by methylation (95%CI)
cg13151474	*PKNOX2*	–1.46 (–2.91 to –0.36)	0.004	–8.01 (–11.06 to –4.96)	–9.48 (–12.48 to –6.37)	<0.001	0.15 (0.04 to 0.31)
cg15665653	*WDTC1*	–1.07 (–2.21 to –0.20)	0.014	–8.40 (–11.66 to –5.24)	–9.48 (–12.48 to –6.37)	<0.001	0.11 (0.02 to 0.25)
cg09320595	*MICB*	–1.20 (–2.49 to –0.17)	0.022	–8.27 (–11.36 to –5.25)	–9.48 (–12.48 to –6.37)	<0.001	0.13 (0.02 to 0.27)
cg04656692	*MRGPRG; C11orf36*	–1.09 (–2.71 to 0.13)	0.096	–8.38 (–11.84 to –5.24)	–9.48 (–12.48 to –6.37)	<0.001	0.11 (–0.01 to 0.29)
Score with the 3 significant methylation sites		–3.00 (–4.92 to –1.35)	<0.001	–6.47 (–9.61 to –3.33)	–9.48 (–12.48 to –6.37)	<0.001	0.32 (0.14 to 0.56)

Models are adjusted for age, sex, BMI and cohort (80 cases and 230 controls from ANDIS and ANDiU).
ACME, average causal mediator effect; ADE, Average direct effect.

methylation in healthy individuals (n = 12) [4] or long-term metformin-treated women with type 2 diabetes (n = 24) [5]. There was some overlap with our metformin-associated methylation sites in ANDIS (Table A.1) and the results in the two smaller published studies, but none of them were among our 26 replicated sites. However, differences in experimental design (population, technique to measure DNA methylation, smaller sample size, no replication cohorts included) could explain the heterogenicity of the results between studies. On the other hand, we previously showed that blood-based DNA methylation of 11 sites in drug-naïve newly diagnosed individuals with type 2 diabetes could predict the glycemic response to metformin, suggesting that these may be used for precision medicine [9]. Importantly, and as one may expect, these 11 sites were not influenced by metformin therapy and did not overlap with the 26 methylation sites identified in the present study. Also, since the study design between the studies is different, one would not expect an overlap. Moreover, based on published data, type 2 diabetes was not found to impact methylation of the 26 metformin-associated sites in liver [19], pancreatic islets [23] or blood [24], and methylation of 25 of the 26 sites in human adipose tissue since methylation of *WDCT1* was higher in adipose tissue from overweight individuals without diabetes vs. individuals with diabetes [14].

Metformin has direct effects on glycemia and on reducing HbA1c levels. Whether DNA methylation mediates this effect is unknown. Methylation has been shown to mediate effects of other drugs, e.g., statins on LDL-cholesterol [25]. Here, we demonstrate for the first time that methylation of *PKNOX2*, *WDTC1* and *MICB* acts as a partial mediator of metformin's impact on HbA1c. Combining methylation of these three sites into a score suggests that methylation explains 32% of the effect of metformin on lowering Hba1c. Interestingly, overexpression of *WDTC1* in adipose tissue is associated with lower adiposity and enhanced glucose utilization [26], and here we show that *WDTC1* methylation could mediate the effect of metformin on HbA1c. Moreover, methylation of *WDCT1* in adipose tissue was higher in overweight individuals without diabetes (52.21 ± 4.32) vs. individuals with diabetes (48.70 ± 4.81) [14] which is in accordance with our study (metformin cases showed higher *WDTC1* methylation), suggesting that metformin might normalize DNA methylation status of this gene. *PKNOX2* encodes a tumor suppressor [27], and *MICB* is expressed in human cancer due to cellular stress [28], and the methylation changes of these genes may thus contribute to metformin's impact on cancer. Indeed, increased insulin levels are associated with cancer [29], and thus by reducing insulin and glucose levels, metformin-induced AMPK activation reduces cancer-associated cell proliferation [30]. Moreover, we showed that *MICB* methylation in blood mirrored methylation levels in skeletal muscle in monozygotic twin pairs discordant for type 2 diabetes, suggesting potential biological roles of this methylation marker in a target tissue for type 2 diabetes. This is in line with studies showing correlation between methylation in blood and target tissues, which support the usefulness of analyzing DNA methylation in minimally invasive tissues such as blood [31,32].

This study has strengths, including identification and validation in two different cohorts, use of a homogenous population of newly-diagnosed individuals with type 2 diabetes, and the matching case–control design may decrease possible bias regarding clinical phenotypes such as age, sex, HbA1 or BMI. Moreover, the causal mediation analysis allowed us to better understand the biological role of epigenetic mechanisms. Limitations include no access to the dosage or compliance of metformin or smoking data from our participants. DNA methylation was measured at one time-point in individuals either taking metformin or not, and evidence for metformin-mechanistic conclusions can therefore not be provided. Modest differences in methylation between groups were found but they can still be biologically relevant since epigenetic changes due to environmental factors are commonly small but can have additive and strong effects on transcriptional activity, can persist across time and are based on the number of cells present in a sample which can considerably affect that cell's function depending on the nature and characteristics of that cell [33]. Our findings have been replicated in an independent cohort, suggesting the robustness of these relatively small effects. Moreover, it is possible that the identified changes in methylation increase with longer exposure to metformin. Regarding the mediation analysis, for some individuals the mediator was measured at the same time as the outcome, thus reverse causation cannot be completely ruled out. Northern Europeans were included in this study and further research is needed in other ethnicities to validate our findings.

In conclusion, metformin-associated alterations in DNA methylation in blood from newly-diagnosed individuals with type 2 diabetes partially mediate the effect of the drug on HbA1c levels.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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SG, SS and CL contributed to the conception of the work. SG, SS, MM, EA, and CL contributed to the data collection. SG, SS, and AP contributed to the data analysis. SG, SS and CL drafted the article. All authors contributed to the interpretation of data and critical revision of the article. All authors gave final approval of the version to be published. S. G. and C.L. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. We declare no competing interests.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diabres.2023.110807.

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