Human Molecular Genetics, 2015, 1–22

doi: 10.1093/hmg/ddv124
Original Article

ORIGINAL ARTICLE

Impact of age, BMI and HbA1c levels on the genome-wide DNA methylation and mRNA expression patterns in human adipose tissue and identification of epigenetic biomarkers in blood

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Abstract

Increased age, BMI and HbA1c levels are risk factors for several non-communicable diseases. However, the impact of these factors on the genome-wide DNA methylation pattern in human adipose tissue remains unknown. We analyzed the DNA methylation of ~480 000 sites in human adipose tissue from 96 males and 94 females and related methylation to age, BMI and HbA1c. We also compared epigenetic signatures in adipose tissue and blood. Age was significantly associated with both altered DNA methylation and expression of 1050 genes (e.g. *FHL2*, *NOX4* and *PLG*). Interestingly, many reported epigenetic biomarkers of

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Received: January 29, 2015. **Revised and Accepted:** April 7, 2015

aging in blood, including *ELOVL2*, *FHL2*, *KLF14* and *GLRA1*, also showed significant correlations between adipose tissue DNA methylation and age in our study. The most significant association between age and adipose tissue DNA methylation was found upstream of *ELOVL2*. We identified 2825 genes (e.g. *FTO*, *ITIH5*, *CCL18*, *MTCH2*, *IRS1* and *SPP1*) where both DNA methylation and expression correlated with BMI. Methylation at previously reported *HIF3A* sites correlated significantly with BMI in females only. HbA1c (range 28–46 mmol/mol) correlated significantly with the methylation of 711 sites, annotated to, for example, *RAB37*, *TICAM1* and *HLA-DPB1*. Pathway analyses demonstrated that methylation levels associated with age and BMI are overrepresented among genes involved in cancer, type 2 diabetes and cardiovascular disease. Our results highlight the impact of age, BMI and HbA1c on epigenetic variation of candidate genes for metabolic diseases and cancer in human adipose tissue. Importantly, we demonstrate that epigenetic biomarkers in blood can mirror age-related epigenetic signatures in target tissues for metabolic diseases such as adipose tissue.

Introduction

Epigenetic factors, including DNA methylation, histone modifications and various RNA-mediated processes, are involved in tissue-specific gene regulation and have been suggested as mechanisms for interaction between environmental factors and the genome (1). As epigenetic variation affects genome function, it may also contribute to common human diseases (2). Indeed, a number of factors support the involvement of epigenetic components in common complex diseases, e.g. monozygotic twins do not show 100% concordance for common diseases and indeed display epigenetic differences (3–6), the incidence of several complex diseases is rising in the general population (7), and there is an association between *in utero* environment or early development and diseases in adult life (8–10). DNA methylation is an easily accessible epigenetic mark for laboratory investigations and thereby suited for epigenome-wide association studies (EWAS) and may be used as an epigenetic biomarker (2). However, the fact that epigenetic alterations may be either causal or arise as a consequence of disease needs to be accounted for. It is hence important to study the impact of non-genetic risk factors for disease, e.g. age, BMI and HbA1c (a measure of long-term glycemia) (11–15), on epigenetic modifications prior to disease development. These three non-genetic risk factors are known to increase the risk for several non-communicable diseases such as type 2 diabetes (T2D), cardiovascular disease and cancer (11–14,16). It is also critical to consider tissue specificity of the epigenome and to test if epigenetic modifications in blood may be used as biomarkers to mimic epigenetic signatures in target tissues for disease.

Adipose tissue is the main energy store in the human body, but also a metabolically active tissue which acts both as an endocrine and an immune organ, and contributes to whole body energy homeostasis (17). Dysfunction of the adipose tissue, e.g. promoted by excessive energy intake, is commonly seen in genetically and environmentally predisposed individuals (18). Adipose tissue gene expression and hormone secretion influence various metabolic phenotypes which, in turn, are associated with human complex traits involved in obesity, T2D and cardiovascular diseases. Epigenetic modifications in adipose tissue may contribute to these phenotypes. Indeed, we recently identified altered gene expression and differential DNA methylation in adipose tissue from subjects with T2D compared with non-diabetic controls (5). We have also shown that regular exercise contributes to extensive transcriptional and DNA methylation changes in human adipose tissue (19,20). Additionally, increased BMI has been associated with increased DNA methylation of *HIF3A* in both human adipose tissue and blood cells (21). However, the potential associations between estimates of obesity or glycemia and the genome-wide DNA methylation pattern in human adipose tissue from non-diabetic subjects have not yet been investigated.

Several studies further point to the importance of epigenetic modifications in the process of aging (3,15,22). We have previously identified age-associated changes in DNA methylation in human skeletal muscle, pancreatic islets and blood cells (3,23–25). More recently, genome-wide, well-powered cross-sectional DNA methylation studies have been performed in leukocytes and whole blood, showing that almost 30 and 15%, respectively, of the analyzed DNA methylation sites were associated with age (26,27). This finding has also been verified in a longitudinal study (28). The age-associated changes in DNA methylation may be influenced by the underlying genetic architecture (24), resulting in both common and tissue-specific alterations (29). However, whether age affect the genome-wide DNA methylation pattern in human adipose tissue and if any of these age-associated epigenetic changes can also be found in blood cells is not known.

The aim of this study was to perform EWAS in human subcutaneous adipose tissue obtained from a discovery cohort of 96 males (male discovery cohort) and in a validation cohort of 94 females (female validation cohort) and relate the genome-wide DNA methylation pattern to three selected known risk factors for common complex diseases (age, BMI and HbA1c). This study design gives us the opportunity to test for both common and gender-specific effects on epigenetic variation. We also investigated the association between the same phenotypes (age, BMI and HbA1c) and genome-wide mRNA expression in adipose tissue from the 96 males. We finally tested if epigenetic variation in blood cells can mirror epigenetic signatures in adipose tissue and potentially be used as epigenetic biomarkers, using adipose tissue and blood cells from a mixed validation cohort (37 males and 67 females) and published data obtained from blood cells.

Results

Analysis of DNA methylation and gene expression in human adipose tissue

To study if known risk factors for common complex diseases, i.e. age, BMI and HbA1c levels (11–14,16), may mediate their effects via epigenetic modifications, we analyzed DNA methylation genome-wide in adipose tissue from 96 males without known disease and with a broad range in age, BMI and HbA1c (male discovery cohort; Table 1). We proceeded to study the impact of age, BMI and HbA1c on DNA methylation levels in adipose tissue from the male discovery cohort using a random effect mixed model, including cohort as the random effect variable and age, BMI and HbA1c as fixed factors. However, we first calculated variance inflation factors (VIFs), which provides information about potential multicollinearity of the studied phenotypes (i.e. age, BMI and HbA1c) (30). Importantly, in the male discovery cohort, all calculated VIFs were close to 1 (1.04–1.18), demonstrating

Table 1. Clinical characteristics of study participants

Characteristic	Male discovery cohort ($n = 96$ males)	Female validation cohort ($n = 94$ females)	Mixed validation cohort ($n = 67$ females, 37 males)
Age (years)	32.4 ± 12.8 (23–80)	29.2 ± 4.2 (21–37)	52 ± 11 (32–83)
BMI (kg/m^2)	25.6 ± 3.7 (17.5–39.0)	27.2 ± 6.7 (18.2–44.9)	27.6 ± 5.2 (18–47)
IFCC HbA1c (mmol/mol)	34 ± 4 (28–46)	31 ± 3 (25–39)	34 ± 3 (22–44)
NGSP HbA1c (%)	5.3 ± 0.3 (4.7–6.4)	5.0 ± 0.3 (4.4–5.7)	5.3 ± 0.3 (4.2–6.2)

Data are expressed as the mean ± SD (range). IFCC HbA1c (mmol/mol), reference value < 50 years: 27–42 and >50 years: 31–46; NGSP HbA1c (%), reference value: 4.0–6.0.

that there are no problems with multicollinearity among the studied phenotypes (age, BMI and HbA1c). Genomic DNA from adipose tissue of these 96 males successfully generated DNA methylation data for 456 800 CpG sites throughout the genome. After correction for multiple testing, we found 62 496 CpG sites significantly associated with one or more of the three phenotypes studied (age, BMI and HbA1c; $q < 0.05$), representing all chromosomes (Supplementary Material, Tables S1–S3). Due to the possible interaction between epigenetic modifications and gene expression (31), we also generated mRNA microarray data from adipose tissue of 94 of the males with available DNA methylation data. Here, mRNA expression data were obtained from a total of 28 499 probe sets representing 22 115 annotated transcripts and 20 246 unique genes. Moreover, after quality control and filtering of probes, genomic DNA from adipose tissue of 94 females included in the female validation cohort (Table 1) (32,33) successfully generated DNA methylation data for 460 973 CpG sites throughout the genome.

Adipose tissue DNA methylation and age

Aging is associated with numerous diseases and it has also been suggested to increase epigenetic variability, including altered levels of DNA methylation (3). This phenomenon includes both common and tissue-specific events (29); however, the specific effect of age on the genome-wide DNA methylation pattern in human adipose tissue is not known. In the male discovery cohort, including 96 males with a range in age between 23 and 80 years, we found that the average DNA methylation level for all 456 800 CpG sites throughout the genome correlated positively with age ($P = 1.1 \times 10^{-5}$, Supplementary Material, Table S4). When dividing the sites based on their relation to the nearest gene (TSS1500, TSS200, 5′UTR, 1st exon, gene body, 3′UTR, intergenic) or in relation to CpG islands (northern shelf, northern shore, CpG island, southern shore, southern shelf, open sea), the average methylation levels were positively and significantly associated with age for all tested regions (Supplementary Material, Table S4). After correction for multiple testing, we found that DNA methylation of 31 567 individual CpG sites in adipose tissue from the male discovery cohort was significantly associated with age ($q < 0.05$), indeed suggesting that the human DNA methylome in adipose tissue changes with age (Supplementary Material, Table S1). Among these sites, 24 514 are annotated to 11 036 unique genes, whereas 7053 of the CpG sites are intergenic. Most of the CpG sites significantly associated with age showed a positive relation between age and methylation level ($n = 28\,605$; 90.6%), whereas only 2962 (9.4%) of the CpG sites showed a negative relation (Fig. 1A). The most significant association between age and DNA methylation was seen for a CpG site upstream of *ELOVL2* (cg21572722, TSS1500, $q = 8.5 \times 10^{-24}$; Fig. 2A, Supplementary Material, Table S1). Altogether, methylation of eight CpG sites annotated to *ELOVL2* correlated significantly with age ($q < 0.05$, Fig. 2A, Supplementary Material, Table S1).

We proceeded to study the genomic distribution of all individual CpG sites significantly associated with age in adipose tissue from the male discovery cohort, either based on their relation to the nearest gene or in relation to CpG islands (Fig. 1C and D and Supplementary Material, Table S5). When comparing the distribution of the significant age-associated CpG sites with the distribution of all analyzed CpG sites across the different genomic regions using χ^2-tests, we found an under-representation of significant CpG sites within TSS1500, TSS200, 5′UTR and intergenic regions, and an over-representation within the 1st exon, gene body and 3′UTR (Supplementary Material, Table S5 and Fig. 1C). We also observed a pronounced effect of age on DNA methylation for CpG sites in regions in relation to CpG islands, with a strong over-representation of significant CpG sites within CpG islands and under-representations in the open sea and shelf regions (Supplementary Material, Table S5 and Fig. 1D).

To test for general and gender-specific effects on epigenetic variation, we next studied the impact of age on DNA methylation in adipose tissue from 94 females (the female validation cohort, Table 1). It should be noted that the span in age was smaller in the female validation cohort (21–37 years) compared with the male discovery cohort (Table 1). Nevertheless, we found DNA methylation of 62 CpG sites to be significantly associated with age in the female validation cohort ($q < 0.05$), where 60 (96.8%) show positive and 2 (3.2%) negative correlations (Supplementary Material, Table S6). In agreement with our result in the male discovery cohort, the most significant association in the female validation cohort was seen for a CpG site upstream of *ELOVL2* (cg16867657, $q = 4.5 \times 10^{-7}$), and methylation in three CpG sites annotated to *ELOVL2* correlated significantly with age in both cohorts, suggesting some common effects of age on methylation in adipose tissue from both males and females ($q < 0.05$, Fig. 2A and B and Supplementary Material, Table S6). Additionally, the majority (42 CpG sites, 70%) of the CpG sites showing a positive correlation with age in the female validation cohort ($q < 0.05$) were also positively correlated with age in the male discovery cohort ($q < 0.05$, Supplementary Material, Table S6). These include CpG sites annotated to *SPATA18*, *PATZ1*, *ANK1*, *NPAS4* and *CADPS2*, genes previously associated with aging (34–38).

To test if epigenetic modifications in blood cells may mirror epigenetic signatures in target tissues for metabolic diseases such as adipose tissue and potentially be used as epigenetic biomarkers, we further compared our results identified in adipose tissue with data from a recent study examining the impact of age on the genome-wide DNA methylation pattern in white blood cells from 421 individuals ranging in age from 14 to 94 years (27). In white blood cells, Johansson *et al.* found that age affected DNA methylation at 137 993 sites, which corresponds to almost one-third of the investigated sites. Interestingly, the

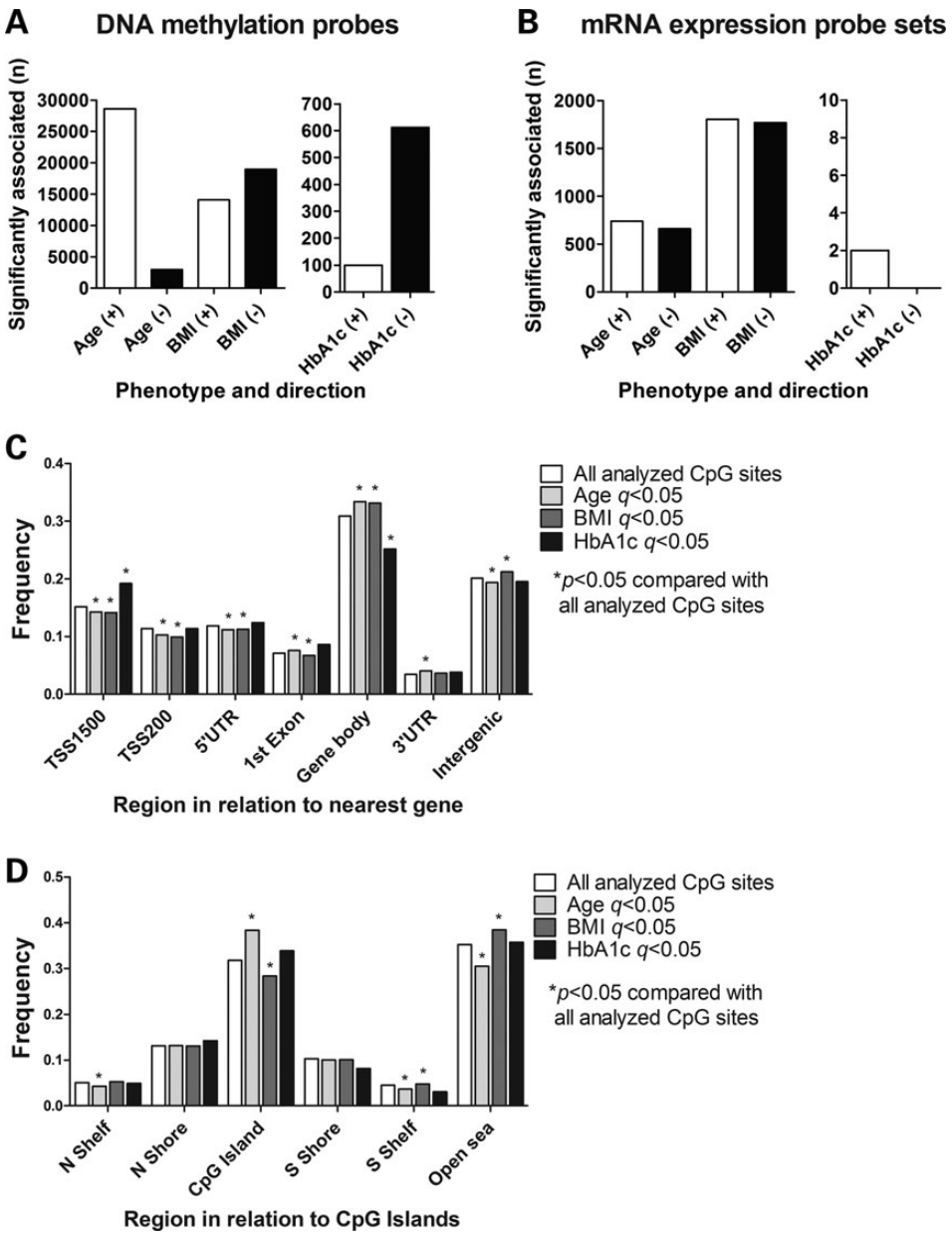

Figure 1. Distribution of significant CpG sites and mRNA expression probe sets in the male discovery cohort. Number and distribution of positive versus negative significant associations between DNA methylation of individual CpG sites (**A**) or mRNA expression probe sets (**B**) and the phenotypes age, BMI and HbA1c. +, positive association; –, negative association. Distribution of CpG sites significantly associated with age, BMI or HbA1c compared with all analyzed CpG sites in relation to gene region (**C**) and CpG island region (**D**).

DNA methylation level of 12 708 of these sites was also significantly associated with age in adipose tissue in the male discovery cohort in the present study, 9897 (78%) in the same direction as in blood (Supplementary Material, Table S7). Moreover, 51 (82%) of the 62 CpG sites significantly associated with age in the female validation cohort were also significantly associated with age in the study of white blood cells (27), all in the same direction in the two studies. All together, the overlap between our two adipose tissue cohorts and blood data by Johansson *et al.* was 37 CpG sites including sites annotated to *ELOVL2*, *SPATA18*, *ANK1*, *NPAS4* and *CADPS2* showing associations between age and DNA methylation (Table 2). Moreover, Steegenga *et al.* (39) have recently summarized data from several studies where the impact of age on DNA methylation in whole blood or purified blood cells was investigated. They presented a list of 14 genes, *ELOVL2*, *FHL2*, *PENK*, *KLF14*, *SST*, *GLRA1*, *TP73*, *GATA4*, *THRB*, *DLX5*, *NEFM*, *TMEM179*, *ATP8A2* and *FOXE3*, displaying age-related changes in DNA methylation based on previous published studies (26,28,40–45). Importantly, all of these previously reported epigenetic biomarkers of aging in blood did also show significant associations with age in adipose tissue from our male discovery cohort (Supplementary Material, Table S1, Table 3 and Fig. 2A, C and D). Also, despite the limited age span in our female validation cohort, four of these genes (*ELOVL2*, *FHL2*, *KLF14* and *GLRA1*) were among the genes significantly associated with age (Supplementary Material, Table S6 and Fig. 2B–D).

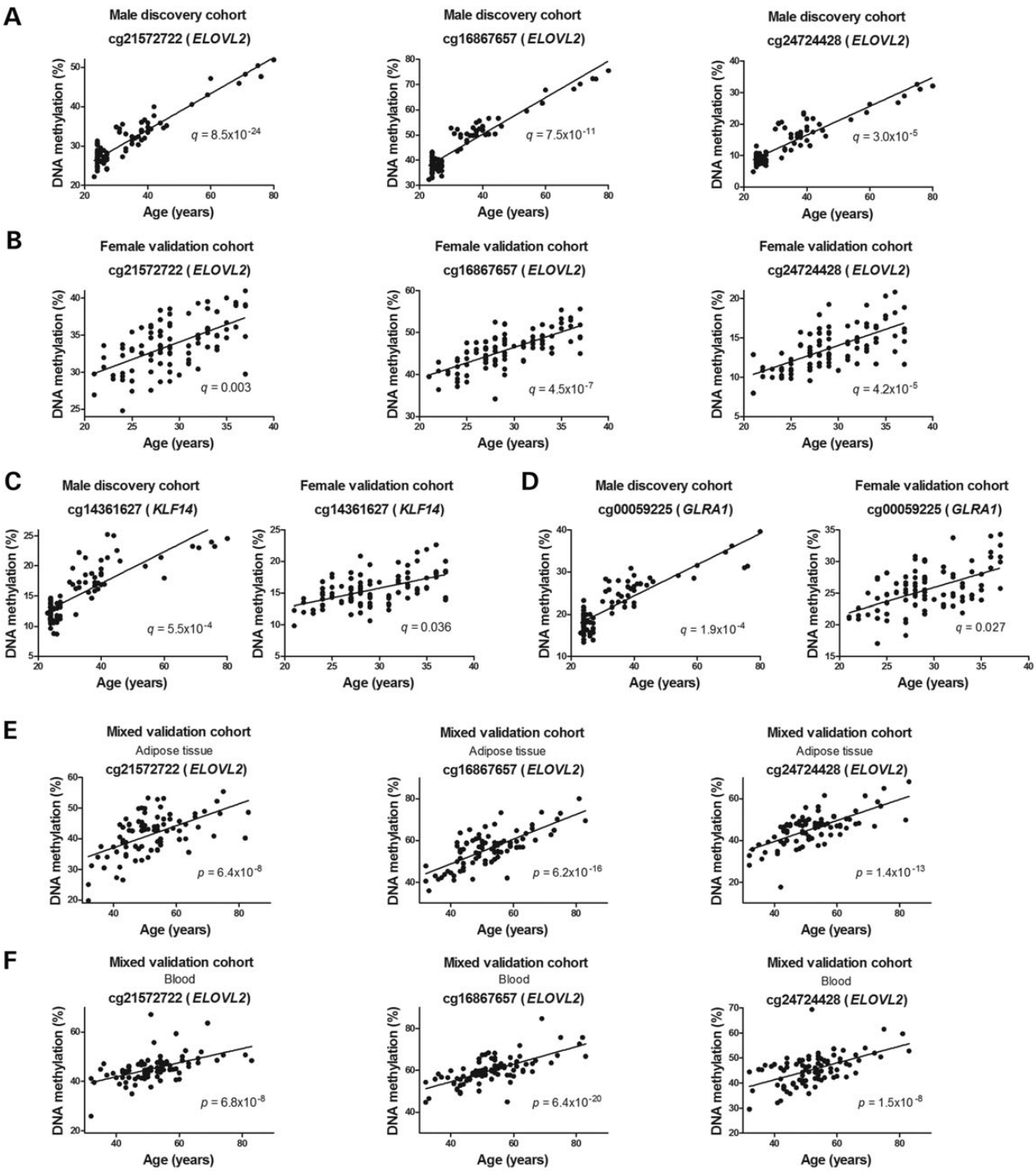

Figure 2. Age correlates with DNA methylation and mRNA expression of specific genes in adipose tissue and blood. DNA methylation at cg21572722, cg16867657 and cg24724428 in *ELOVL2* correlated significantly with age in both the male discovery cohort (**A**) and the female validation cohort (**B**). DNA methylation at CpG sites in *KLF14* (**C**) and *GLRA1* (**D**) correlated significantly with age in both the male discovery cohort and the female validation cohort. DNA methylation at cg21572722 (adipose tissue $n = 81$, blood $n = 83$), cg16867657 ($n = 90$) and cg24724428 (adipose tissue $n = 87$, blood $n = 89$) in *ELOVL2* correlated with age in both adipose tissue (**E**) and blood (**F**) in the mixed validation cohort. DNA methylation in adipose tissue correlated significantly with DNA methylation in blood at cg21572722 ($n = 62$), cg16867657 ($n = 77$) and cg24724428 ($n = 74$) in *ELOVL2* in the mixed validation cohort (**G**). DNA methylation at cg14361627 (adipose tissue $n = 94$, blood $n = 108$) in *KLF14* correlated with age in both adipose tissue (**H**) and blood (**I**) in the mixed validation cohort, and DNA methylation in adipose tissue correlated significantly with DNA methylation in blood ($n = 90$, **J**). For *NOX4* (**K**) both mRNA expression and DNA methylation correlated significantly with age in the male discovery cohort (the most significant CpG site is shown). (**L**) Selected significantly enriched KEGG pathways (FDR adjusted *P*-values < 0.05) of genes that exhibit associations between DNA methylation and age in the male discovery cohort. A complete list of significantly enriched KEGG pathways is presented in Supplementary Material, Table S20.

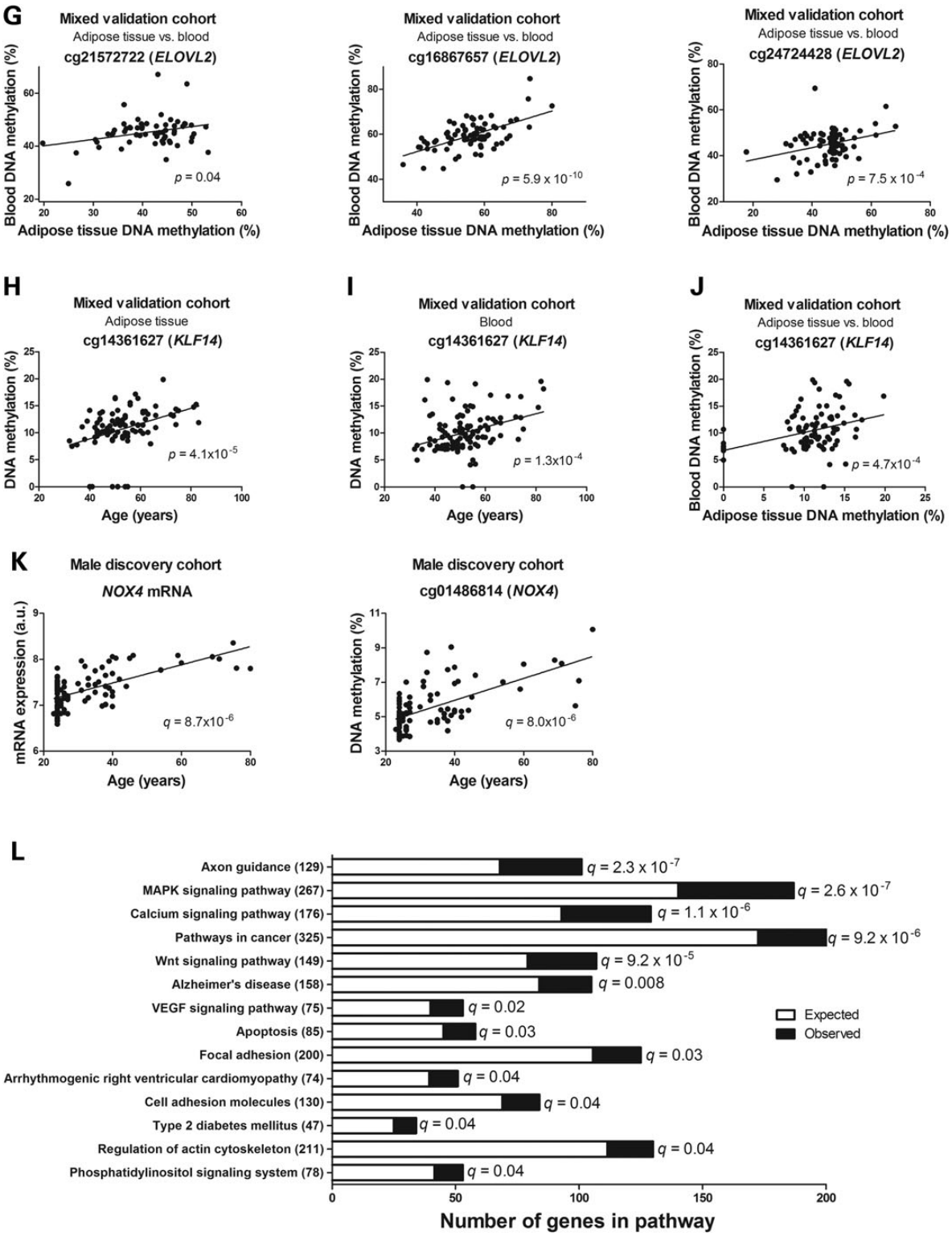

Figure 2. Continued.

We proceeded to test if we could identify age-related epigenetic changes in both adipose tissue and blood cells taken from the same subjects. Here, we used pyrosequencing to analyze DNA methylation of three CpG sites in *ELOVL2* and one CpG site in *KLF14* in both adipose tissue and whole blood cells taken from a mixed validation cohort including 37 males and 67 females with an age span of 32–83 years (Table 1). These CpG sites were selected based on the age-associated changes in DNA methylation in our male discovery cohort (Fig. 2A and C), female validation cohort (Fig. 2B and C) and blood cells in previous published studies

Table 2. Overlap of CpG sites with DNA methylation levels significantly altering with age in adipose tissue from our male discovery cohort and female validation cohort (present study, $q < 0.05$) as well as in white blood cells (27)

Target ID	Chromosome and position	Gene	Relation to gene region	Relation to CpG island	Adipose tissue (96 men)		Adipose tissue (94 women)		Blood (Johansson *et al.*)	
					FDR *q*-value	Regression coefficient	FDR *q*-value	Regression coefficient	*P*-value	Regression coefficient
cg16867657	6:11 044 877	*ELOVL2*	TSS1500	Island	7.5E−11	0.034	4.5E−07	0.038	1.8E−231	0.0051
cg24724428	6:11 044 888	*ELOVL2*	TSS1500	Island	3.0E−05	0.036	4.2E−05	0.043	1.3E−161	0.0043
cg03036557	13:92 050 720	*GPC5*	TSS1500	N_Shore	2.7E−07	0.045	1.7E−04	0.037	7.7E−13	0.0004
cg13516820	9:37 036 494		Intergenic	Island	0.009	0.010	0.001	0.027	2.3E−28	0.0002
cg04880546	12:1 20 868 468		Intergenic	Open Sea	5.6E−04	0.025	0.001	0.039	2.1E−18	0.0005
cg01429039	4:52 918 065	*SPATA18*	Body	Island	0.002	0.019	0.002	0.035	1.1E−23	0.0008
cg23193759	10:71 389 896	*C10orf35*	TSS200	Island	2.1E−06	0.025	0.003	0.037	2.6E−23	0.0008
cg12589298	19:50 828 905	*KCNC3*	Body	Island	0.008	0.022	0.003	0.029	9.5E−13	0.0003
cg23606718	2:1 31 513 927	*FAM123C*	5′UTR;1st Exon	Island	1.8E−06	0.033	0.003	0.030	2.1E−113	0.0026
cg07547549	20:44 658 225	*SLC12A5*	Body	Island	1.0E−13	0.026	0.003	0.033	9.7E−112	0.0032
cg21572722	6:11 044 894	*ELOVL2*	TSS1500	Island	8.5E−24	0.028	0.003	0.026	2.6E−164	0.0025
cg12934382	3:51 741 135	*GRM2*	1st Exon;5′UTR	Island	0.042	0.032	0.004	0.076	1.6E−107	0.0053
cg25090514	5:20 38 743		Intergenic	Island	1.2E−03	0.033	0.008	0.037	2.8E−52	0.0011
cg01908954	15:51 973 764	*SCG3*	5′UTR;1st Exon	Island	9.0E−10	0.026	0.010	0.031	2.0E−57	0.0021
cg00303541	3:51 741 280	*GRM2*	5′UTR	Island	0.042	0.017	0.012	0.045	2.8E−69	0.0037
cg02650266	4:1 47 558 239		Intergenic	Island	5.8E−04	0.030	0.012	0.027	1.6E−93	0.0021
cg25287474	9:14 693 538	*ZDHHC21*	TSS200	Island	0.004	0.024	0.013	0.028	1.1E−29	0.0006
cg27320127	2:47 798 396	*KCNK12*	TSS1500	Island	5.5E−10	0.023	0.019	0.029	1.9E−80	0.0018
cg00590036	6:1 58 957 433	*TMEM181*	TSS200	Island	2.9E−04	0.027	0.022	0.029	9.3E−52	0.0010
cg16547027	18:24 127 588	*KCTD1*	5′UTR;1st Exon	Island	1.8E−04	0.015	0.024	0.026	2.5E−81	0.0016
cg23483765	5:1 56 886 996	*NIPAL4*	TSS200	Island	9.0E−04	0.032	0.026	0.040	7.2E−17	0.0007
cg00059225	5:1 51 304 357	*GLRA1*	1st Exon;5′UTR	Island	1.9E−04	0.022	0.027	0.028	8.1E−99	0.0021
cg11896587	19:39 440 734	*FBXO17*	Body	Island	1.5E−08	0.020	0.027	0.028	6.5E−47	0.0013
cg10947146	8:11 058 710	*XKR6*	1st Exon	Island	0.009	0.022	0.031	0.035	5.6E−14	0.0004
cg19891728	8:41 754 871	*ANK1*	TSS1500	Island	5.0E−06	0.023	0.031	0.033	2.0E−61	0.0012
cg02383785	7:1 27 808 848		Intergenic	Island	3.9E−10	0.023	0.035	0.023	8.3E−98	0.0019
cg14361627	7:1 30 419 116	*KLF14*	TSS1500	Island	5.5E−04	0.022	0.036	0.030	1.5E−142	0.0025
cg01685883	4:1 55 254 497	*DCHS2*	Body	Island	0.026	0.009	0.036	0.035	4.9E−10	0.0004
cg07665387	4:1 34 069 388	*PCDH10*	TSS1500	Island	4.5E−05	0.020	0.036	0.026	1.8E−54	0.0016
cg20899581	6:27 841 230	*HIST1H4L*	1st Exon;TSS1500	Island	1.5E−05	0.026	0.036	0.025	2.3E−16	0.0004
cg26158959	1:2 10 111 162	*SYT14*	TSS1500	N_Shore	9.4E−04	0.020	0.037	0.025	2.5E−52	0.0006
cg14704921	4:53 728 654	*RASL11B*	1st Exon;5′UTR	Island	2.3E−07	0.032	0.039	0.027	2.8E−53	0.0009
cg04768203	11:66 189 188	*NPAS4*	Body	Island	4.4E−04	0.011	0.041	0.025	2.0E−16	0.0003
cg22736354	6:18 122 719	*NHLRC1*	1st Exon	Island	0.041	0.016	0.041	0.029	1.8E−116	0.0021
cg20665157	7:1 21 969 421	*CADPS2*	Body	Open Sea	0.007	0.011	0.048	0.030	1.2E−91	0.0033
cg00664406	3:51 740 875	*GRM2*	TSS1500	Island	0.012	0.021	0.0495	0.035	5.8E−104	0.0037
cg11991058	2:1 68 150 602		Intergenic	Island	0.035	0.013	0.0496	0.028	4.0E−40	0.0012

FDR *q*-value, false discovery rate adjusted *P*-value.

Table 3. DNA methylation and association with age ($q < 0.05$) in adipose tissue from the male discovery cohort among genes displaying multiple probes associated with age in blood or blood cells in previously reported studies

Gene	Identified in	Target ID	*P*-value	FDR *q*-value	Regression coefficient	SEM	Chromosome and position	Relation to gene region	Relation to CpG island
ELOVL2	4, 5, 6, 7, 9, 10	cg21572722	1.9E−29	8.5E−24	0.0278	0.0016	6:11 044 894	TSS1500	CpG island
		cg16867657	1.3E−15	7.5E−11	0.0343	0.0035	6:11 044 877	TSS1500	CpG island
		cg24724428	4.5E−08	3.0E−05	0.0357	0.0060	6:11 044 888	TSS1500	CpG island
		cg16323298	4.2E−04	1.4E−02	0.0134	0.0037	6:11 044 974	TSS1500	CpG island
		cg23642061	1.1E−03	2.5E−02	0.0069	0.0020	6:11 043 631	Gene body	N_Shore
		cg25151806	1.4E−03	2.9E−02	0.0160	0.0048	6:11 045 370	TSS1500	S_Shore
		cg21649660	2.7E−03	4.3E−02	0.0058	0.0019	6:10 995 125	Gene body	Open sea
		cg22143569	7.4E−04	2.0E−02	0.0079	0.0023	6:11 044 541	5′UTR;1st Exon	CpG island
FHL2	4, 5, 6, 7, 9, 10	cg19850931	7.7E−05	5.0E−03	0.0120	0.0029	2:1 05 993 347	Gene body	Open sea
		cg06907053	2.8E−04	1.1E−02	0.0110	0.0029	2:1 06 015 869	TSS200;5′UTR;TSS1500	CpG island
		cg26344233	2.4E−03	4.0E−02	0.0061	0.0019	2:1 06 015 817	TSS200;5′UTR;TSS1500	CpG island
PENK	1, 5, 6, 7, 8, 9, 10	cg16219603	9.4E−07	2.5E−04	0.0179	0.0034	8:57 360 586	TSS1500	CpG island
		cg16419235	4.5E−05	3.5E−03	0.0215	0.0050	8:57 360 613	TSS1500	CpG island
		cg16072688	8.2E−05	5.2E−03	0.0101	0.0024	8:57 360 711	TSS1500	CpG island
		cg19414741	1.5E−06	3.5E−04	0.0123	0.0024	8:57 358 240	Gene body	CpG island
		cg12877723	1.5E−05	1.7E−03	0.0274	0.0060	8:57 358 312	Gene body	CpG island
		cg18742346	6.1E−05	4.3E−03	0.0106	0.0025	8:57 358 625	5′UTR;TSS200	CpG island
		cg04127342	5.0E−04	1.6E−02	0.0160	0.0044	8:57 358 130	Gene body	CpG island
		cg21694941	1.4E−04	7.4E−03	0.0147	0.0037	8:57 358 590	5′UTR;1st Exon	CpG island
KLF14	1, 3, 5, 7, 8, 9, 10	cg08097417	4.4E−15	1.5E−10	0.0177	0.0019	7:1 30 419 133	TSS1500	CpG island
		cg07955995	1.7E−08	1.5E−05	0.0140	0.0023	7:1 30 419 159	TSS1500	CpG island
		cg04528819	2.0E−07	8.5E−05	0.0178	0.0032	7:1 30 418 315	1st Exon	CpG island
		cg14361627	2.8E−06	5.5E−04	0.0221	0.0044	7:1 30 419 116	TSS1500	CpG island
		cg22285878	3.1E−06	5.8E−04	0.0165	0.0033	7:1 30 419 173	TSS1500	CpG island
		cg18751682	9.7E−06	1.3E−03	0.0119	0.0025	7:1 30 419 066	TSS200	CpG island
		cg20426994	5.6E−05	4.0E−03	0.0156	0.0037	7:1 30 418 324	1st Exon	CpG island
		cg21449170	4.8E−04	1.5E−02	0.0134	0.0037	7:1 30 419 062	TSS200	CpG island
		cg00094518	1.3E−03	2.8E−02	0.0201	0.0060	7:1 30 418 549	1st Exon	CpG island
		cg25109431	1.3E−03	2.8E−02	0.0076	0.0023	7:1 30 419 057	TSS200	CpG island
		cg06533629	1.4E−03	2.9E−02	0.0128	0.0039	7:1 30 419 370	TSS1500	CpG island
SST	1, 3, 5, 7, 8, 9, 10	cg25478614	6.1E−14	8.4E−10	0.0281	0.0032	3:1 87 387 866	Gene body	N_Shore
		cg00481951	1.8E−13	2.1E−09	0.0318	0.0037	3:1 87 387 650	Gene body	N_Shore
		cg14703224	7.9E−05	5.0E−03	0.0106	0.0026	3:1 87 389 415	TSS1500	S_Shore
		cg16927040	2.7E−04	1.1E−02	0.0127	0.0034	3:1 87 388 128	1st Exon;5′UTR	CpG island
		cg02164046	2.4E−03	4.0E−02	0.0098	0.0031	3:1 87 388 148	1st Exon;5′UTR	CpG island
GLRA1	1, 2, 3, 4, 8, 9, 10	cg02071447	9.0E−09	9.3E−06	0.0175	0.0028	5:1 51 304 542	TSS200	CpG island
		cg08316825	2.7E−05	2.5E−03	0.0177	0.0040	5:1 51 304 547	TSS200	CpG island
		cg26419265	1.2E−03	2.8E−02	0.0124	0.0037	5:1 51 303 982	Gene body	N_Shore
		cg00059225	6.1E−07	1.9E−04	0.0224	0.0042	5:1 51 304 357	1st Exon;5′UTR	CpG island
TP73	1, 2, 3, 4, 8, 9, 10	cg20677901	9.1E−04	2.3E−02	0.0183	0.0053	1:3 568 210	TSS1500	CpG island
		cg01915516	2.8E−03	4.4E−02	−0.0309	0.0100	1:3 568 243	TSS1500	CpG island
		cg07178825	1.2E−05	1.5E−03	0.0214	0.0046	1:3 649 574	Gene body;3′UTR	CpG island
		cg19692322	1.9E−03	3.6E−02	0.0057	0.0018	1:3 621 048	Gene body	N_Shelf
		cg03664527	2.4E−03	4.1E−02	0.0058	0.0019	1:3 648 749	Gene body;3′UTR	N_Shore

Table 3. Continued

Gene	Identified in	Target ID	P-value	FDR *q*-value	Regression coefficient	SEM	Chromosome and position	Relation to gene region	Relation to CpG island
		cg16554148	3.3E−03	4.8E−02	0.0061	0.0020	1:3 630 882	Gene body	N_Shelf
		cg07382920	1.3E−05	1.5E−03	0.0173	0.0037	1:3 567 646	TSS1500	CpG island
		cg24073122	1.4E−05	1.6E−03	0.0121	0.0026	1:3 567 986	TSS1500	CpG island
		cg01434649	2.3E−03	3.9E−02	0.0193	0.0061	1:3 567 870	TSS1500	CpG island
GATA4	1, 2, 3, 8, 9, 10	cg05930881	2.5E−05	2.4E−03	0.0296	0.0067	8:11 560 540	TSS1500	CpG island
		cg13434842	1.3E−04	7.0E−03	0.0246	0.0062	8:11 567 896	Gene body	S_Shore
		cg24646414	6.3E−04	1.8E−02	0.0152	0.0043	8:11 565 725	5′UTR	CpG island
		cg16803596	2.7E−03	4.3E−02	0.0084	0.0027	8:11 561 498	TSS1500	CpG island
THRB	1, 2, 3, 8, 9, 10	cg04928005	2.8E−06	5.5E−04	0.0128	0.0026	3:24 536 812	TSS1500	CpG island
		cg13790603	5.0E−06	8.1E−04	0.0167	0.0034	3:24 536 478	TSS200;TSS1500	CpG island
		cg24120841	2.6E−05	2.5E−03	0.0096	0.0022	3:24 536 562	TSS1500	CpG island
		cg27622506	4.2E−04	1.4E−02	0.0321	0.0087	3:24 537 160	TSS1500	CpG island
		cg09805010	1.0E−03	2.5E−02	0.0070	0.0021	3:24 536 077	5′UTR	CpG island
		cg27526665	1.1E−03	2.5E−02	0.0194	0.0057	3:24 537 050	TSS1500	CpG island
		cg20985755	1.5E−03	3.1E−02	0.0108	0.0033	3:24 536 474	TSS200;TSS1500	CpG island
		cg07647822	2.5E−03	4.1E−02	0.0240	0.0077	3:24 537 349	TSS1500	CpG island
		cg26139133	3.3E−03	4.8E−02	0.0059	0.0020	3:24 536 765	TSS1500	CpG island
		cg02333852	1.5E−04	7.4E−03	0.0088	0.0022	3:24 536 252	1st Exon;5′UTR	CpG island
DLX5	1, 2, 3, 8, 9, 10	cg08101303	1.5E−03	3.1E−02	0.0114	0.0035	7:96 652 222	Gene body	CpG island
NEFM	1, 3, 7, 8, 9, 10	cg18898125	9.7E−08	5.1E−05	0.0119	0.0021	8:24 770 381	TSS1500	N_Shore
		cg26330518	1.1E−05	1.4E−03	0.0082	0.0017	8:24 770 342	TSS1500	N_Shore
		cg07502389	2.5E−07	9.8E−05	0.0220	0.0039	8:24 771 259	TSS200;TSS1500	CpG island
		cg17078116	1.3E−06	3.1E−04	0.0150	0.0029	8:24 772 344	TSS200;1st Exon	CpG island
		cg02106941	1.5E−05	1.7E−03	0.0150	0.0033	8:24 772 137	TSS1500;1st Exon	CpG island
		cg23290344	5.3E−05	3.9E−03	0.0150	0.0035	8:24 771 466	TSS1500;1st Exon	CpG island
		cg20585869	1.2E−03	2.7E−02	0.0115	0.0034	8:24 772 333	TSS200;1st Exon	CpG island
		cg22562942	2.1E−03	3.8E−02	0.0116	0.0037	8:24 772 309	TSS200;1st Exon	CpG island
		cg18267374	1.2E−03	2.7E−02	0.0156	0.0047	8:24 771 273	TSS1500;5′UTR;1st Exon	CpG island
		cg26980244	2.2E−03	3.9E−02	0.0105	0.0033	8:24 772 513	5′UTR;1st Exon;Gene body	CpG island
TMEM179	1, 3, 7, 8, 9, 10	cg00107187	9.8E−10	1.7E−06	0.0151	0.0022	14:1 05 070 998	1st Exon	CpG island
		cg10281977	2.2E−07	9.1E−05	0.0111	0.0020	14:1 05 070 864	1st Exon	CpG island
ATP8A2	1, 2, 3, 4, 8, 9, 10	cg13096859	1.6E−08	1.4E−05	0.0150	0.0024	13:26 310 861	Gene body	Open sea
		cg00255889	1.3E−05	1.5E−03	0.0168	0.0036	13:26 586 288	Gene body	CpG island
		cg18836626	4.2E−05	3.3E−03	0.0104	0.0024	13:26 042 746	Gene body	CpG island
		cg07973479	1.6E−04	7.7E−03	0.0108	0.0027	13:25 945 255	TSS1500	N_Shore
		cg13537353	2.6E−04	1.1E−02	0.0196	0.0051	13:26 586 651	Gene body	CpG island
		cg02057782	4.1E−04	1.4E−02	0.0118	0.0032	13:26 042 598	Gene body	N_Shore
		cg25516742	2.1E−03	3.7E−02	0.0081	0.0025	13:26 503 010	Gene body	Open sea
FOXE3	1, 3, 4, 7, 8, 9, 10	cg22796507	1.5E−11	6.6E−08	0.0144	0.0019	1:47 882 739	1st Exon	CpG island
		cg18815943	2.5E−09	3.5E−06	0.0177	0.0027	1:47 882 314	1st Exon	CpG island
		cg01281911	1.1E−07	5.5E−05	0.0148	0.0026	1:47 882 686	1st Exon	CpG island
		cg19809499	4.0E−04	1.4E−02	0.0130	0.0035	1:47 882 265	1st Exon	CpG island

1, Teschendorff *et al.* (27 K) (44); 2, Raykan *et al.* (27 K) (43); 3, Bell *et al.* (27 K) (40); 4, Heyn *et al.* (450 K) (42), 5, Hannum *et al.* (450 K) (26); 6, Garagnani *et al.* (450 K) (41); 7, Florath *et al.* (450 K) (28); 8, Xu *et al.* (27 K) (45); 9, Steegenga *et al.* (450 K) (39); 10, Johansson *et al.* (450 K) (27); FDR *q*-value, false discovery rate adjusted *P*-value; SEM, standard error of the mean.

(Table 3). Importantly, increased age was significantly associated with increased DNA methylation in these three *ELOVL2* sites and the *KLF14* site in both adipose tissue and blood cells taken from the mixed validation cohort, and the DNA methylation in adipose tissue correlated significantly with the methylation in blood (Fig. 2E–J). Together these data show that age-associated methylation changes found in blood cells can mirror epigenetic signatures in target tissues such as adipose tissue and potentially be used as epigenetic biomarkers to predict susceptibility and progression of disease.

We therefore proceeded to test if age-associated methylation differences also can be identified in diseased subjects, i.e. in patients with T2D. We recently published a case–control study identifying 15 627 CpG sites with differential DNA methylation in adipose tissue from subjects with T2D compared with non-diabetic age-matched controls (5). As aging is a known risk factor for T2D, we further investigated the overlap between the 31 567 CpG sites showing significant association between DNA methylation and age in adipose tissue from our discovery cohort of 96 non-diabetic males in the present study (Supplementary Material, Table S1) and the 15 627 sites showing differential DNA methylation in adipose tissue from subjects with T2D compared with controls in our previous study (5). Notably, DNA methylation of 1278 CpG sites was both significantly associated with age in the 96 non-diabetic males and displayed differential DNA methylation between T2D subjects and age-matched controls. Importantly, DNA methylation of as many as 90% of these CpG sites changed in the same direction due to increasing age or T2D (Supplementary Material, Table S8). These include CpG sites annotated to *IRS1* and *KCNQ1*, genes previously implicated in the pathogenesis of T2D (46,47), as well as to *TMEM17*, an age-associated blood-based epigenetic biomarker (Table 3). In total, DNA methylation of 188 CpG sites was significantly associated with age in both adipose tissue (male discovery cohort) and blood (in the study by Johansson *et al.*) and displayed differential DNA methylation between T2D subjects and controls in the same direction (Supplementary Material, Table S7). These data support the use of blood-based epigenetic biomarkers to foresee epigenetic changes that take place in target tissues of diseased subjects, i.e. with T2D.

Adipose tissue mRNA expression and age

We proceeded to test if age was associated with altered gene expression in human adipose tissue. Interestingly, we also found a striking effect of age on adipose tissue mRNA expression levels in the male discovery cohort, with expression of 1400 probe sets of which 1130 are annotated to 1084 unique genes significantly associated with age ($q < 0.05$, Supplementary Material, Table S9). The number of positive and negative correlations between mRNA expression and age is shown in Figure 1B. The most significant correlations between age and mRNA expression were observed for *NOX4* (positive correlation, $q = 8.7 \times 10^{-6}$, Fig. 2K) and *PLG* (negative correlation, $q = 8.7 \times 10^{-6}$, Supplementary Material, Table S9). As epigenetic modifications are known to regulate tissue-specific gene expression (31), we further tested if genes with significant correlations between mRNA expression and age also showed age-related changes in DNA methylation. Indeed, in 1050 of the 1084 genes with age-associated changes in mRNA expression, DNA methylation was also associated with age in the male discovery cohort (Supplementary Material, Table S10) when using CpG sites within the *cis* distance 500 kb upstream and 100 kb downstream of each gene. These include *NOX4* (Fig. 2K), *PLG*, *ETS2*, *CCR2* and *CXCR2* (Supplementary Material, Table S10).

Adipose tissue DNA methylation and BMI

BMI is a simple measurement of body size and widely used for population based studies, as well as for diagnosis of overweight (BMI > 25 kg/m^2) and obesity (BMI > 30 kg/m^2). Increased BMI is associated with the development of several common diseases (12,13). There are metabolic differences observed in adipose tissue from obese compared with lean subjects (48) and these changes may partly be due to epigenetic modifications. In the adipose tissue from the male discovery cohort with a range in BMI between 17.5 and 39.0 kg/m^2, the average DNA methylation level for all 456 800 CpG sites throughout the genome did not correlate significantly with BMI ($P = 0.3$, Supplementary Material, Table S4). However, we found that DNA methylation of 33 058 individual CpG sites was significantly associated with BMI in the male discovery cohort ($q < 0.05$), of which 24 939 sites are annotated to 12 325 unique genes and 8119 sites are intergenic (Supplementary Material, Table S2). In contrast to our identified associations between adipose tissue DNA methylation and age, there were slightly more negative ($n = 18\,972$, 57.4%) than positive ($n = 14\,086$, 42.6%) significant associations between DNA methylation and BMI (Fig. 1A). Among annotated genes, the most significant correlation between BMI and DNA methylation was observed for a CpG site in the promoter of *CCRL2* (cg18599081, negative correlation, $q = 9.0 \times 10^{-6}$; Fig. 3A). Among all CpG sites significantly associated with BMI, we observed an over-representation within the gene body and intergenic regions, and less significant CpG sites compared with the distribution on the array in the region surrounding transcription start, i.e. TSS1500, TSS200, 5′UTR and 1st exon (Supplementary Material, Table S5 and Fig. 1C). In contrast to what was seen for associations between age and DNA methylation, CpG sites with DNA methylation associated with BMI were under-represented within CpG islands and over-represented within the open sea and southern shelf (Supplementary Material, Table S5 and Fig. 1D).

We next investigated the impact of BMI on DNA methylation in adipose tissue from the female validation cohort including 94 females with a range in BMI between 18.2 and 44.9 kg/m^2. Here, we found that DNA methylation of 39 533 CpG sites was significantly associated with BMI ($q < 0.05$), of which 30 507 sites are annotated to 11 766 unique genes and 9026 sites are intergenic (Supplementary Material, Table S11). These include 27 809 (70%) positive and 11 724 (30%) negative correlations between DNA methylation and BMI. The most significant correlation was observed for a CpG site in the body of *PLEC1* (negative correlation; $q = 1.8 \times 10^{-12}$, Fig. 3B). This site was also negatively correlated with BMI in the male discovery cohort (Fig. 3B). The overlap between the male discovery cohort and the female validation cohort was 4979 CpG sites, 2756 sites with positive and 2223 sites with negative correlations between BMI and DNA methylation (Supplementary Material, Table S12). These include sites annotated to genes previously linked to obesity, T2D and/or fat metabolism, e.g. *FTO*, *TCF7L2*, *FASN*, *IRS1*, *IRS2*, *MTCH2* and *PPARGC1B*. Many of the most significant sites in the male discovery cohort could be validated in the 94 females ($q < 0.05$), including cg18599081 in *CCRL2* (Fig. 3A), cg12492380 in *MYH16*, cg27115863 (intergenic) and cg11151251 in *DCAF5* among the 10 most significant sites. Importantly, since the discovery cohort only includes males and the validation cohort only females, the overlapping sites are most likely gender-unspecific, while the sites correlating only in males or females might represent gender-specific associations between BMI and adipose tissue DNA methylation. Impressively, we were able to replicate the strong, gender-unspecific association between BMI and DNA

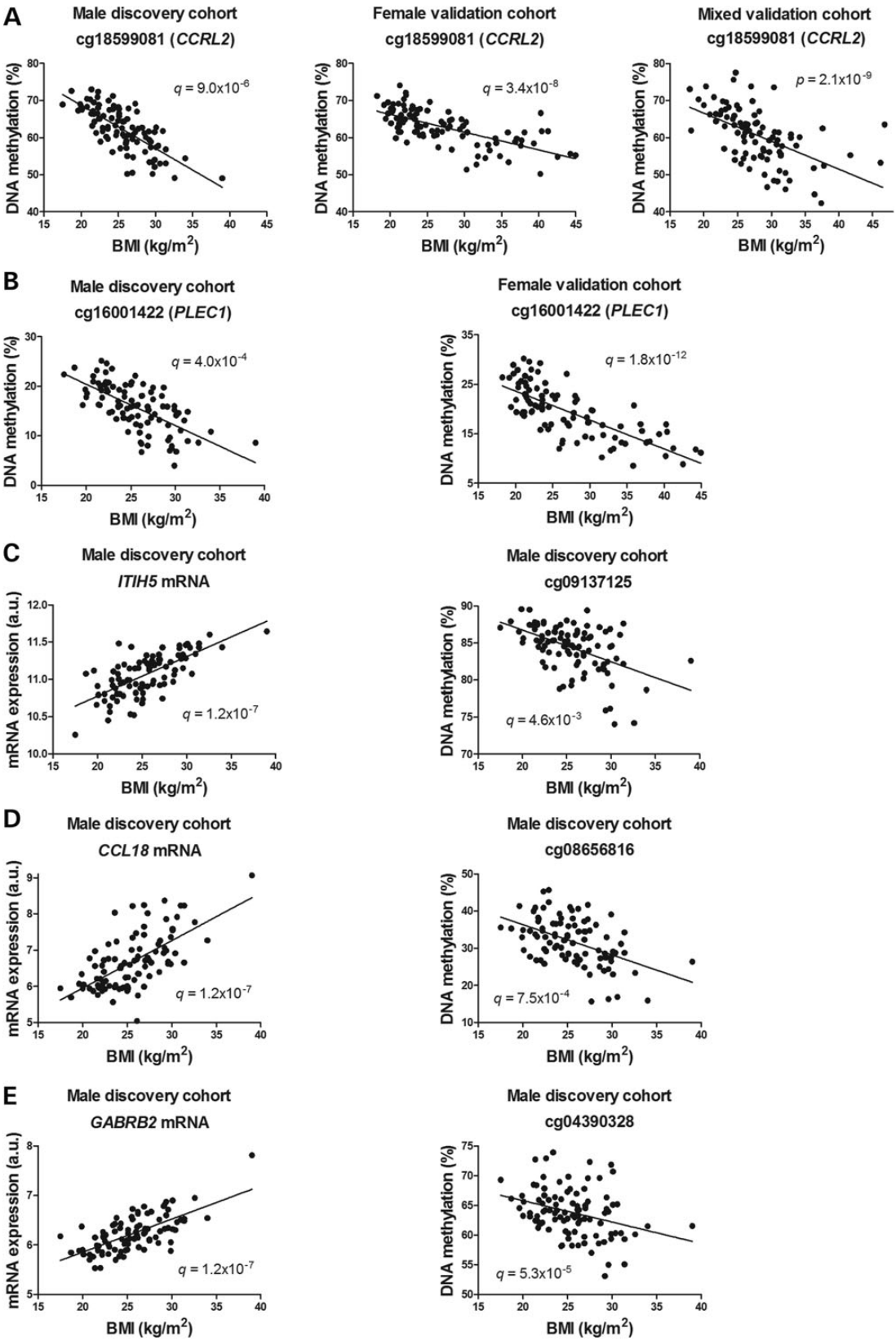

Figure 3. Correlations between BMI and DNA methylation and BMI and mRNA expression in human adipose tissue. DNA methylation at cg18599081 in *CCRL2* (**A**) correlated significantly with BMI in the male discovery cohort, the female validation cohort as well as the mixed validation cohort ($n = 91$). DNA methylation at cg16001422 in *PLEC1* (**B**) correlated significantly with BMI in both the male discovery cohort and the female validation cohort. For *ITIH5* (**C**), *CCL18* (**D**) and *GABRB2* (**E**), both DNA methylation and mRNA expression correlated with BMI in the male discovery cohort (the most significant CpG site within the *cis* distance 500 kb upstream and 100 kb downstream of each gene is shown). (**F**) Selected significantly enriched KEGG pathways (FDR adjusted *P*-values < 0.05) of genes that exhibit correlations between DNA methylation and BMI in the male discovery cohort. A complete list of significantly enriched KEGG pathways is presented in Supplementary Material, Table S21.

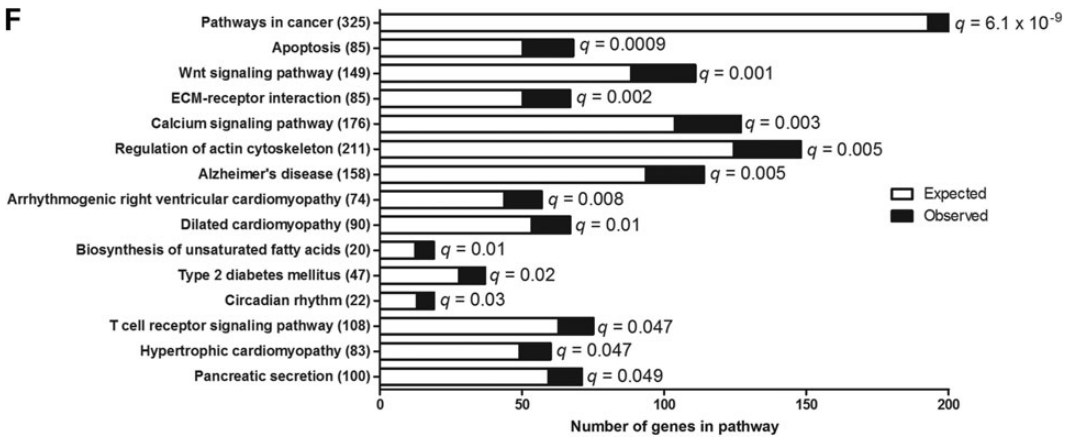

Figure 3. Continued.

Table 4. DNA methylation in *HIF3A* in relation to BMI, in adipose tissue from the male discovery cohort and the female validation cohort

Target ID	Chromosome and position	Gene	Relation to gene region	Relation to CpG island	Adipose tissue Male discovery cohort		Adipose tissue Female validation cohort	
					FDR *q*-value	Regression coefficient	FDR *q*-value	Regression coefficient
cg01552731	19:46 806 907	*HIF3A*	1st Exon;5′UTR;Body	N_Shore	0.039	0.045	0.0007	0.033
cg23548163	19:46 807 119	*HIF3A*	5′UTR;Body	Island	ns (0.59)		0.015	0.019
cg16672562[a]	19:46 801 672	*HIF3A*	5′UTR;Body;1st Exon	S_Shore	ns (0.77)		0.019	0.029
cg12068280	19:46 804 528	*HIF3A*	5′UTR;Body	N_Shelf	ns (0.30)		0.028	0.014
cg22891070[a]	19:46 801 642	*HIF3A*	Body;TSS200	S_Shore	ns (0.83)		0.031	0.027
cg07684068	19:46 807 660	*HIF3A*	Body	S_Shore	0.026	0.047	0.032	0.019
cg27146050[a]	19:46 801 557	*HIF3A*	Body;TSS200	S_Shore	ns (0.90)		0.042	0.016

The seven CpG sites where DNA methylation correlated significantly with BMI ($q < 0.05$) in the female validation cohort are presented.
[a]CpG sites reported to be significantly associated with BMI in the study by Dick *et al.* (21). FDR *q*-value, false discovery rate adjusted *P*-value; ns, non-significant *q*-values (>0.05).

methylation of cg18599081 in *CCRL2* in adipose tissue from the mixed validation cohort using pyrosequencing ($P = 2.1 \times 10^{-9}$, Fig. 3A).

We proceeded to compare our results with data from a recently published study, which identified positive correlations between BMI and DNA methylation at three CpG sites in *HIF3A* in adipose tissue (only females) and blood (21). In adipose tissue from our female validation cohort, we observed that DNA methylation of seven CpG sites annotated to *HIF3A*, including the same three CpG sites as reported by Dick *et al.*, were significantly associated with BMI ($q < 0.05$, Supplementary Material, Table S11, Table 4). In contrast, the 96 males of our discovery cohort did not show any significant association between DNA methylation of these *HIF3A* CpG sites and BMI ($q > 0.05$, Table 4). However, DNA methylation of two other sites within the *HIF3A* gene was significantly associated with BMI in our male discovery cohort ($q < 0.05$, Supplementary Material, Table S2, Table 4). These data support both gender-specific and general effects of BMI on the DNA methylation pattern in human adipose tissue.

As obesity, as well as age, is a known risk factor for T2D, we investigated how many of the CpG sites significantly associated with BMI in adipose tissue from non-diabetic subjects in the present study that also show differential DNA methylation in adipose tissue from subjects with T2D compared with non-diabetic controls in our recently published case–control study (5). We found that BMI was significantly associated with DNA methylation of 988 and 3425 CpG sites in the male discovery and female validation cohort, respectively, that also displayed differential DNA methylation between subjects with T2D and controls (Supplementary Material, Tables S13 and S14). The majority (60% in the male discovery cohort and 99% in the female validation cohort) of CpG sites that exhibit BMI associated changes in DNA methylation in non-diabetic subjects changed in the same direction in subjects with T2D compared with controls (Supplementary Material, Tables S13 and S14). These include CpG sites annotated to genes previously linked to T2D, obesity and/or energy metabolism such as *PC*, *FOXO1* and *HSF1* in both cohorts and *IGF2*, *VEGFA*, *IRS1*, *IL1RN*, *IGF1R*, *ELOVL6* and *KCNQ1* in the female validation cohort.

Adipose tissue mRNA expression and BMI

We further studied the association between BMI and gene expression in adipose tissue from the male discovery cohort. Also BMI was associated with transcriptional changes in adipose tissue, with significant associations to 3575 probe sets, of which 3104 are annotated to 2936 unique genes (Supplementary Material, Table S15). The most significant correlations between BMI and mRNA expression were observed for *ITIH5*, *CCL18* and *GABRB2* (positive correlations, $q = 1.2 \times 10^{-7}$, Fig. 3C–E), and *SNORD115-1* (negative correlation, $q = 1.2 \times 10^{-7}$, Supplementary Material, Table S15). Furthermore, 2825 of the 2936 genes with significant associations between BMI and mRNA expression ($q < 0.05$) had one or more CpG sites within the *cis* distance 500 kb upstream

and 100 kb downstream of each gene significantly associated with BMI (Supplementary Material, Table S16). These include *ITIH5*, *CCL18*, *GABRB2, FTO*, *MTCH2*, *IRS1* and *SPP1* (*OPN*) (Fig. 3C–E).

Adipose tissue DNA methylation and HbA1c

Glycated hemoglobin (HbA1c) is a long-term measure of average blood glucose levels and elevated HbA1c levels are associated with an increased risk of T2D, cardiovascular disease and cancer (11,14,16). Additionally, glucose-induced epigenetic changes have been suggested to explain the so called metabolic memory, which may increase the risk for cardiovascular disease and T2D (49–52). As adipose tissue has a role in whole body glucose homeostasis, we investigated the association between adipose tissue DNA methylation and HbA1c as a continuous variable. In the male discovery cohort, including 96 non-diabetic males with a range in HbA1c between 28 and 46 mmol/mol (representing 4.7–6.4%), we found that the average DNA methylation level for all 456 800 CpG sites throughout the genome correlated negatively with HbA1c ($P = 0.025$, Supplementary Material, Table S4). Furthermore, we found that DNA methylation of 711 individual CpG sites was significantly associated with HbA1c ($q < 0.05$), of which 541 are annotated to 583 unique genes and 170 CpG sites are intergenic (Supplementary Material, Table S3). Among these CpG sites, 99 (14%) showed positive and 612 (86%) showed negative correlations between adipose tissue DNA methylation and HbA1c (Fig. 1A). The most significant correlation between HbA1c and adipose tissue DNA methylation was seen for a CpG site upstream (TSS1500) of *ANKRD11* (negative correlation; Fig. 4A and Supplementary Material, Table S3), located in a CpG

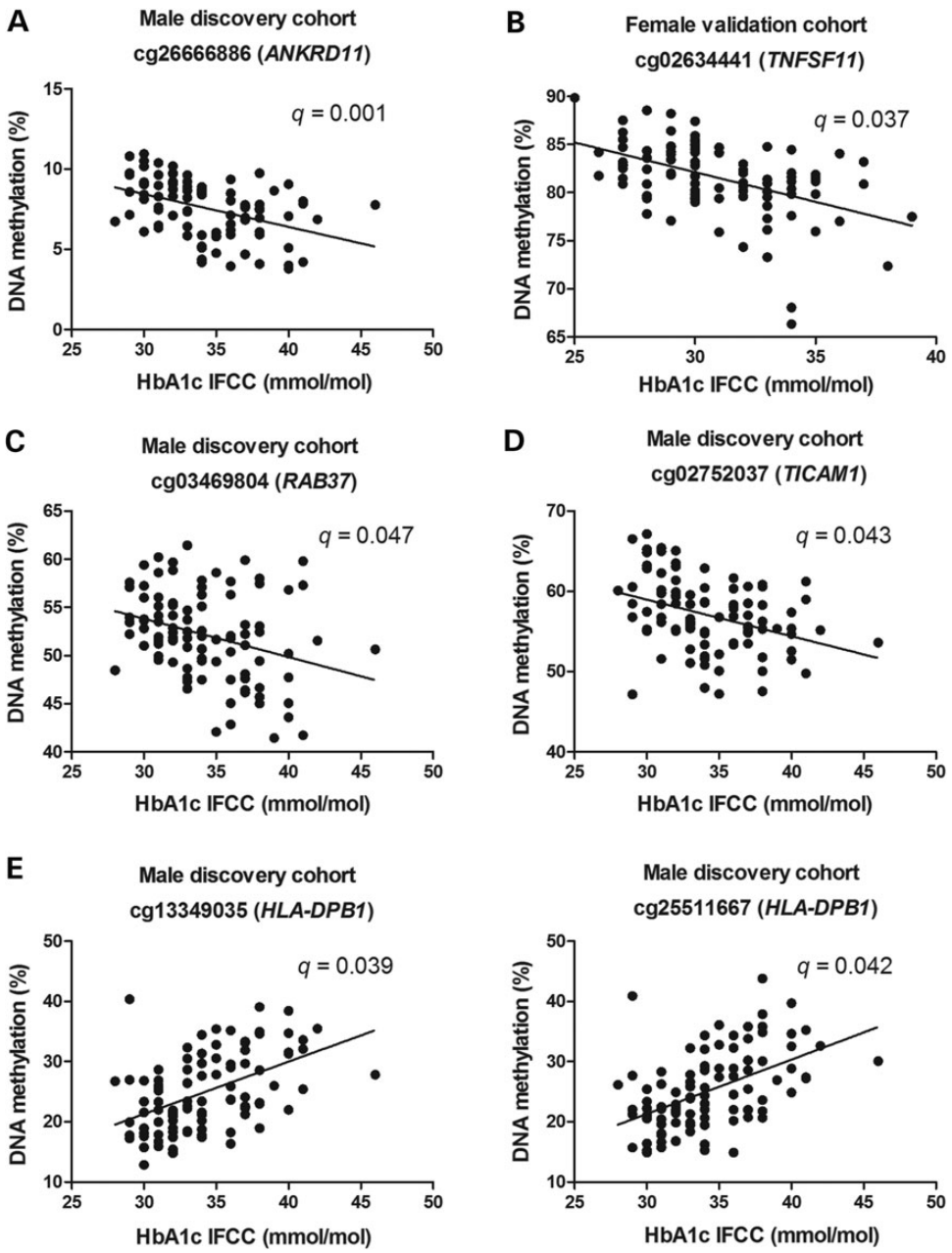

Figure 4. Correlations between HbA1c and DNA methylation in human adipose tissue. DNA methylation at CpG sites in *ANKRD11* (**A**), *TNFSF11* (**B**), *RAB37* (**C**), *TICAM1* (**D**) and *HLA-DPB1* (**E**) correlated significantly with HbA1c.

island shore. Moreover, CpG sites with DNA methylation significantly associated with HbA1c were over-represented within the TSS1500 and under-represented within the gene body regions, whereas no significant difference was observed for the distribution in regions in relation to CpG islands (Supplementary Material, Table S5, Fig. 1C and D).

In our female validation cohort, consisting of 94 non-diabetic females with a span in HbA1c between 25 and 39 mmol/mol (representing 4.4–5.7%), we identified seven CpG sites with DNA methylation significantly associated with HbA1c ($q < 0.05$), two with a positive and five with a negative coefficient (Supplementary Material, Table S17). The strongest correlation was observed for a CpG site upstream *TNFSF11* ($q = 0.04$, Fig. 4B). None of these seven sites were significantly associated with HbA1c in the male discovery cohort.

Elevated HbA1c levels within the non-diabetic interval predict future risk of T2D (16). We therefore tested if CpG sites significantly associated with HbA1c levels in the present study were also found to be differentially methylated in adipose tissue from subjects with T2D compared with controls in our previous case–control study (5). Indeed, we found 30 among the 711 CpG sites significantly associated with HbA1c in the male discovery cohort also to have differential DNA methylation levels in subjects with T2D compared with controls. The majority (28 sites; 93%) of the CpG sites that exhibit differential DNA methylation due to increased HbA1c in non-diabetic subjects changed in the same direction in subjects with T2D compared with non-diabetic controls (Supplementary Material, Table S18). These include CpG sites annotated to genes previously linked to T1D or T2D such as *RAB37*, *TICAM1* and *HLA-DPB1* (53–55) (Fig. 4C–E).

Adipose tissue mRNA expression and HbA1c

We found significant associations between mRNA expression of two transcripts, annotated to LOC100288814 and *CLLU1*, respectively, and HbA1c levels in adipose tissue from the discovery cohort, both with positive correlations (Fig. 1B). However, neither LOC100288814 nor *CLLU1* had CpG sites with DNA methylation significantly associated with HbA1c levels.

Overlap between associations of DNA methylation and age, BMI and HbA1c

It has been suggested that certain regions or positions in the genome are more prone to epigenetic variation (56). Based on this, we investigated the overlap of CpG sites with DNA methylation significantly associated in the same direction with more than one of the phenotypes examined in the male discovery cohort. Most overlap was found between age and BMI, namely 1334 CpG sites in the same direction (Supplementary Material, Table S19). The overlap between age and HbA1c included 2 CpG sites, and between BMI and HbA1c, we found 12 overlapping CpG sites in the same direction. The overlap between associations of DNA methylation and the different phenotypes is hence modest, but likely a result of the statistical model where all three phenotypes are included and thereby adjusted for. However, the overlaps we do detect thereby represent independent effects of the different phenotypes on the DNA methylome.

Pathway analysis

To gain further biological relevance of the significant associations between DNA methylation and the studied phenotypes, we performed KEGG pathway analyses with WebGestalt (http://www.webgestalt.org). We included genes with one or more CpG site(s) annotated to the gene significantly associated with respective studied phenotypic trait ($q < 0.05$). For associations between DNA methylation and age, 31 KEGG pathways involved in, for example, cancer, signal transduction, cardiovascular disease and T2D were significantly enriched in the male discovery cohort (Supplementary Material, Table S20 and Fig. 2L). Among these 31 pathways, only one was also enriched in the female validation cohort (Neuroactive ligand-receptor interaction pathway). For associations between DNA methylation and BMI, 47 KEGG pathways involved in, for example, cancer, signal transduction, cardiovascular disease, T2D and inflammation were significantly enriched in the male discovery cohort (Supplementary Material, Table S21 and Fig. 3F). Among these 47 pathways, 41 (87%) were also enriched in the female validation cohort (Supplementary Material, Table S21). For associations between DNA methylation and HbA1c, no KEGG pathways were significantly enriched.

For transcripts with positive associations between mRNA expression and age, three KEGG pathways involved in cardiovascular disease were significantly enriched (Supplementary Material, Table S22). Pathway analysis of transcripts with mRNA expression negatively associated with age revealed three significant pathways, of which two are involved in chemokine signaling and cytokine–cytokine receptor interaction (Supplementary Material, Table S22).

For positive associations between mRNA expression and BMI, 29 KEGG pathways involved in, for example, the immune system and glycan biosynthesis were significantly enriched (Supplementary Material, Table S23). Pathway analysis of transcripts with mRNA expression negatively associated with BMI revealed six significant pathways involved in, for example, fatty acid metabolism, glucose metabolism, amino acid metabolism and translation (Supplementary Material, Table S23).

Due to uneven genomic distribution of CpG sites and CpG islands and the design of the Illumina 450k array, conclusions based only on pathway analysis could be severely biased (57). However, the pathway results in our study in many cases mirror the results obtained from the analysis of individual genes; hence, we use this additional method in support of our findings and to share lights on metabolic pathways and biological function.

Correlations between DNA methylation of CpG sites significantly associated with age, BMI or HbA1c and mRNA expression in human adipose tissue

DNA methylation is known to regulate gene expression and depending on the genomic location of a CpG site, methylation may be either negatively or positively associated with transcriptional activity (31). To study the direct correlations between DNA methylation and mRNA expression, we performed Spearman's correlations between DNA methylation of individual CpG sites significantly associated with age, BMI or HbA1c (Supplementary Material, Tables S1–S3) and expression of nearby mRNA probe sets in adipose tissue from 94 individuals in the male discovery cohort with genome-wide data available for both DNA methylation and mRNA expression. In this analysis, we included mRNA probe sets within a *cis* distance from the significant CpG sites, i.e. CpG sites within a distance 500 kb upstream and 100 kb downstream of mRNA probe sets. Based on these inclusion criteria, 794 515 CpG–mRNA combinations, including 61 932 unique CpG sites and 28 041 mRNA probe sets, were included in the correlation analysis. After correction for multiple testing, 199 450 of these CpG–mRNA combinations showed a significant correlation ($q < 0.05$) between DNA methylation and mRNA expression.

We further separated the significant correlations between DNA methylation and mRNA expression based on what phenotype the included CpG sites were associated with. For CpG sites associated with age, we found 33 000 positive and 43 825 negative CpG–mRNA correlations including 17 710 unique CpG sites and 17 056 unique mRNA probes. Thus, 56% of all CpG sites significantly associated with age also show a correlation with one or more mRNA expression probe sets (Supplementary Material, Table S24). For example, the CpG site most significantly associated with age, cg21572722 in *ELOVL2* and several other CpG sites in *ELOVL2* showed significant correlations to mRNA expression (Supplementary Material, Table S24). Some of these correlations are presented in Figure 5A. Additionally, among the 33 058 CpG sites significantly associated with BMI, 23 361 unique sites (71%) were also significantly associated with expression of one or more mRNA probe sets, giving rise to 62 134 positive and 66 679 negative CpG–mRNA correlation combinations (Supplementary Material, Table S25). Included are, for example, cg18599081 in *CCRL2* (Fig. 5B) and cg01552731 (four CpG–mRNA combinations) and cg07684068 (nine CpG–mRNA combinations) in *HIF3A*. For HbA1c, we found 891 positive and 1095 negative correlations between DNA methylation and mRNA expression, including 397 unique CpG sites (Supplementary Material, Table S26), i.e. 56% of all the CpG sites significantly associated with HbA1c.

Enzymes regulating DNA methylation

DNA methylation in mammalian cells is carried out by DNA methyltransferases (DNMTs) (1), making this family of enzymes inevitable for a functional epigenome. We therefore investigated if age, BMI or HbA1c were associated with altered DNA methylation and/or mRNA expression of the genes encoding these enzymes in human adipose tissue. The Infinium HumanMethylation450 BeadChip array analyzes DNA methylation of 134 CpG sites annotated to *DNMT1*, *DNMT3A*, *DNMT3B* or *DNMT3L*. In the male discovery cohort, we found DNA methylation of 11 CpG sites annotated to these genes significantly associated with age (all with a positive coefficient) and another 10 sites associated with BMI (6 negatively and 4 positively associated; $q < 0.05$), (Supplementary Material, Tables S1 and S2). We also found that age has a negative impact on *DNMT3A* mRNA levels in the male discovery cohort ($q = 0.04$, Supplementary Material, Table S9). In the female validation cohort, we found DNA methylation of 21 CpG sites annotated to genes encoding DNMTs significantly associated with BMI (6 negatively and 15 positively associated; $q < 0.05$; Supplementary Material, Table S11).

We finally investigated the TET enzymes, which have a role in DNA demethylation by creating hydroxymethylation (58). Of the 63 CpG sites analyzed in *TET1*, *TET2* and *TET3* in the male discovery cohort, we found four sites with DNA methylation positively associated with age as well as six sites positively and one negatively associated with BMI ($q < 0.05$; Supplementary Material, Tables S1 and S2). Furthermore, *TET3* mRNA expression showed a positive association with BMI ($q = 0.02$, Supplementary Material, Table S15). In the female validation cohort, we found four CpG sites annotated to genes encoding TET enzymes with DNA methylation positively associated with BMI ($q < 0.05$; Supplementary Material, Table S11).

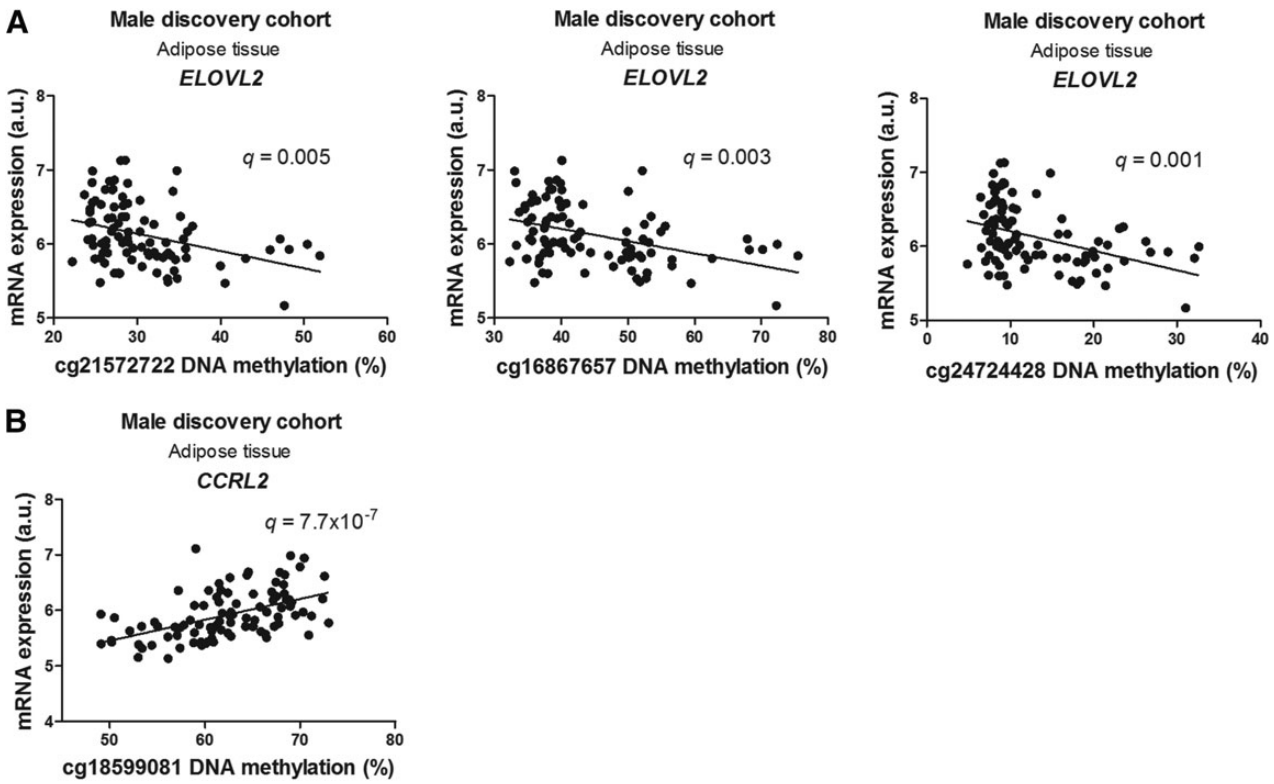

Figure 5. Correlations between DNA methylation of CpG sites significantly associated with age or BMI and mRNA expression in human adipose tissue. DNA methylation at cg21572722, cg16867657 and cg24724428 in *ELOVL2* correlated significantly with mRNA expression of *ELOVL2* (probe set 8123920) in adipose tissue from the male discovery cohort (**A**). DNA methylation at cg18599081 in *CCRL2* correlated significantly with mRNA expression of *CCRL2* (probe set 8079407) in adipose tissue from the male discovery cohort (**B**).

Discussion

In this study, we have shown that three known risk factors for common diseases, i.e. age, BMI and HbA1c play important roles in determining the pattern of DNA methylation and mRNA expression in human adipose tissue. These changes take place in genes known to contribute to development of age- and obesity-related diseases such as T2D, cardiovascular disease and cancer. We also demonstrate for the first time that age-associated epigenetic variation in blood can mirror epigenetic signatures in adipose tissue and potentially be used as epigenetic biomarkers for metabolic diseases.

Identification of subjects with a high risk of developing metabolic disease is a criterion for disease prevention. Genetic and environmental factors have been shown to predict T2D and obesity (13). However, their capacity to predict disease is still suboptimal and there is a need for new biomarkers with a high capacity of predicting metabolic disease. These may potentially include epigenetic biomarkers. Importantly, the epigenome is dynamic and changes due to environmental exposures (3,19,24,25,59–62) but once epigenetic modifications are introduced they may be stable and inherited through cell divisions (63,64), making epigenetics a potentially important pathogenic mechanism in complex diseases. However, it is important to be aware of the tissue-specific nature of the epigenome. Although the nucleotide sequence is identical in most human cells, the epigenetic pattern is highly cell specific as it contributes to the diverse phenotype and the expression pattern seen in different cell types (6,51,65). In most large well-phenotyped cohorts, blood is often the only source of biological material available, and so far little is known about the correlation between DNA methylation in target tissues such as adipose tissue and more accessible cell types such as blood cells. Additionally, blood-based biomarkers have an important clinical relevance since they are easy to analyze in patients and high-risk populations. In this study, many epigenetic biomarkers of aging in blood, i.e. *ELOVL2*, *FHL2*, *KLF14* and *GLRA1*, also showed significant correlations between adipose tissue DNA methylation and age. Notably, our data demonstrate that epigenetic biomarkers in blood can mirror age-related epigenetic signatures in biologically relevant target tissues such as adipose tissue.

Many of the genes associated with age in our study have functions related to the aging process and/or are previously reported biomarkers of aging in blood. For example, DNA methylation of *ELOVL2*, encoding fatty acid elongase 2, has previously been shown to increase with age in blood cells (41) and in our study the most significant association between age and DNA methylation was found upstream of *ELOVL2*. Importantly, we found significant correlations between methylation in several CpG sites annotated to *ELOVL2* and age in adipose tissue from three different cohorts as well as in whole blood cells, supporting the use of blood-based epigenetic biomarkers to mirror some epigenetic signatures in target tissues. Also, *KFL14* belongs to the genes showing increased DNA methylation in both human adipose tissue and blood cells with increased age. *KFL14* encodes Kruppel-like factor 14 which has been suggested to be a master regulator of gene transcription in human adipose tissue (66) and GWAS have identified SNPs near/in *KFL14* associated with T2D and HDL cholesterol levels (47,67). Additionally, DNA methylation of a CpG site in the gene body of *SPATA18* correlated positively with age in both adipose tissue from our two cohorts and white blood cells in a previous study by Johansson *et al.* (27). *SPATA18* (*MIEAP*) encodes a p53-inducible protein that controls mitochondrial quality by repairing or eliminating unhealthy mitochondria (36). Maintenance of healthy mitochondria prevents aging, cancer and a variety of degenerative diseases that are due to the result of defective mitochondrial quality control. For *NOX4*, both DNA methylation and mRNA expression were associated with age. Nox4 (NAPDH oxidase 4) has previously been shown to regulate mitochondrial function in an aging-induced senescence model of cultured endothelial cells (68). Interestingly, cellular aging seems to promote Nox4 interaction with mitochondria. This disrupts complex I in the electron transport chain and increases mitochondrial reactive oxygen species (ROS). This could be a contributing factor in the loss of replicative lifespan seen in senescence. A CpG site in the first exon of *PATZ1* correlated with age in adipose tissue of both our cohorts. The POZ/BTB and AT-hook-containing zinc finger protein 1 (PATZ1) seems to have an important role in the regulation of endothelial cell senescence through an ROS-mediated p53-dependent pathway and contribute to vascular diseases associated with aging (35).

It should be noted that our male discovery cohort includes adipose tissue from 96 non-diabetic males with a wide range in all studied phenotypes (age, BMI and HbA1c), whereas our female validation cohort includes 94 non-diabetic females with a smaller age span, but a wide range in BMI and HbA1c. This is a likely explanation for why we identify a smaller number of CpG sites significantly associated with age in the female validation cohort compared with the male discovery cohort. Nevertheless, the majority of CpG sites associated with age in the female validation cohort were also significant in the male discovery cohort, suggesting a general effect of aging on DNA methylation in both genders. Additionally, our male discovery cohort consists of combined data from four different subcohorts (5,9,10,69). Here, we included males without known disease and with DNA available from subcutaneous adipose tissue. It is known that artifacts such as batch effects may reduce the statistical accuracy in genomic data. To adjust for this potential problem, the association between DNA methylation data or mRNA expression data and studied phenotypes was analyzed using a random effect mixed model, including cohort as the random effect variable and age, BMI and HbA1c as fixed factors. We can still not exclude that our data include some false-positive results due to batch effects rather than biological variation. Nevertheless, the fact that we are able to validate many of our results in adipose tissue from a validation cohort as well as in blood from previously published studies strengthens our data. Of note, 9897 methylation sites were significantly associated with age in the same direction both in adipose tissue in our male discovery cohort and in blood in the study by Johansson *et al.* (27).

We also found a strong effect of increased BMI on the degree of DNA methylation in human adipose tissue, indeed proposing that obesity can mediate some of its effects via altering the epigenome. Importantly, DNA methylation of ~5000 CpG sites was associated with BMI in adipose tissue from both our male discovery cohort and female validation cohort. Interestingly, a large number of these CpG sites did also show differential DNA methylation in adipose tissue from subjects with T2D compared with non-diabetic controls (5), suggesting that BMI associated changes in DNA methylation may predispose to T2D. We could also link BMI associated DNA methylation to differential expression of 2825 genes. The strongest correlation between mRNA expression and BMI was seen for *ITIH5*. This gene encodes inter-alpha-trypsin inhibitor heavy chain family member 5 and it is highly expressed in subcutaneous adipose tissue, increased in obesity, down-regulated after weight loss and associated with measures of body size and metabolism (70). Also, eight CpG sites annotated to *ITIH5* correlated with BMI, suggesting a key epigenetic

mechanism for regulation of this gene. Other genes showing both altered DNA methylation and expression in human adipose tissue based on increased BMI include *FTO*, *CCL18*, *MTCH2*, *IRS1* and *SPP1 (OPN)*. Interestingly, we recently found that *CCL18*, encoding CC chemokine ligand 18, and *SPP1*, encoding osteopontin, were the most up-regulated genes in adipose tissue from subjects with T2D compared with non-diabetic controls and both have previously been linked to inflammation (71,72). Additionally, genetic variation in *FTO* has previously been linked to both obesity and T2D, although recent data suggest that *IRX3* rather than *FTO* is mediating the effects of this SNP (73,74). We also identified methylation sites only associated with BMI in the male discovery cohort or the female validation cohort. These differences may be due to gender-specific effects on DNA methylation. Indeed, gender differences in the DNA methylation pattern have previously been reported in human pancreatic islets, the liver, heart muscle, blood and saliva (75–81).

Moreover, a recent investigation related BMI to DNA methylation in whole blood cells from 479 individuals and identified three CpG sites annotated to *HIF3A* with increased DNA methylation associated with increased BMI. The association between BMI and methylation of *HIF3A* was further validated in adipose tissue from 635 females (21). Interestingly, we found that DNA methylation of the same three CpG sites was positively associated with BMI in our female validation cohort, consisting of 94 females, but not in our male discovery cohort, consisting of 96 males. Nevertheless, DNA methylation in two other sites in *HIF3A* was significantly associated with BMI in the 96 males. The protein encoded by *HIF3A*, hypoxia inducible factor 3 alpha subunit, has been shown to play a role in the cellular response to glucose and insulin and to function as an accelerator of adipocyte differentiation (82,83). However, the possible gender-specific relation between *HIF3A* methylation and BMI is novel and has to be validated further.

We also studied the association between HbA1c and DNA methylation in human adipose tissue from non-diabetic subjects. However, the effect of glucose on both DNA methylation and gene expression in human adipose tissue seems less strong than those of BMI and age. Whether this is also the case in other tissues with a key role in whole body glucose homeostasis, i.e. the liver, skeletal muscle and pancreatic islets, remains to be tested. This result may be due to a smaller span in HbA1c compared with age and BMI, since only subjects without known diabetes were included in this study. Importantly, the strong effects of age and BMI on DNA methylation presented in this study can introduce a bias in studies of the DNA methylation pattern in disease, when cases are not carefully matched for age and BMI or when the statistical analyses are not adjusted for these phenotypes, a factor that needs to be considered in the design of future epigenetic studies.

It should also be noted that the impact of age, BMI and HbA1c on the degree of DNA methylation in human adipose tissue in the present study is quite large compared with some previous studies in human adipose tissue where the greatest absolute differences in methylation were ~20% (5,19). Here, we observed larger absolute differences in methylation, e.g. ~40% absolute difference in the methylation of *ELOVL2* between young and elderly subjects and ~25% absolute difference in the methylation of *CCRL2* between lean and obese subjects.

Interestingly, mRNA expression correlated significantly with the degree of DNA methylation for a large proportion of the CpG sites identified in this study. Since all included CpG sites already were shown to be associated with the phenotypes investigated, these results truly supports an interaction between adipose tissue DNA methylation and mRNA expression in establishing metabolic phenotypes.

In addition to adipocytes, adipose tissue comprises a mixture of different cell types, and changes in cell type composition could potentially be responsible for some of the observed changes in DNA methylation. However, as exemplified by the overlap between adipose tissue and blood, some DNA methylation patterns may also be tissue and cell type unspecific. Additionally, when we investigated mRNA expression for cell type-specific markers, no significant associations were found between BMI or HbA1c and *PNPLA2*, *FAS*, *LIPE* and *RETN* as markers of adipocytes, *DLK1* as a marker of preadipocytes, *PRDM16* and *UCP1* as markers of brown adipocytes, *EMR1* as a marker of macrophages, *TNF* and *IL6* representing cytokines and finally *CASP3*, *CASP7* and *LGALS3* as markers for inflammation. We further compared our findings of altered DNA methylation in adipose tissue with cell type-specific methylation sites of candidate genes in inflammatory complex diseases observed in white blood cells (84). Among 8252 analyzed CpG sites in 343 genes, they found 1865 CpG sites differentially methylated between the different cell types in blood. Among our 33 058 CpG sites significantly associated with BMI in adipose tissue, we found 173 CpG sites overlapping with the 1865 cell type-specific methylation sites observed in white blood cells by Reinius *et al*. For HbA1c, only 9 cell type-specific methylation sites were found among our 711 CpG sites associated with HbA1c in adipose tissue. Taken together, these results suggest that there is no major impact of cellular composition or inflammatory response on the observed associations in adipose tissue DNA methylation and BMI or HbA1c. Anyway, although future studies should aim to investigate the DNA methylome in adipocytes isolated from both subcutaneous and intra-abdominal fat tissue, it should be noted that cell isolation processes may alter both gene expression and DNA methylation.

In conclusion, we demonstrate for the first time an impact of age, BMI and HbA1c on the genome-wide DNA methylation pattern in human adipose tissue. Our data support an important function of altered DNA methylation in the development of several non-communicable diseases such as T2D, obesity, cardiovascular disease and cancer. Finally, we demonstrate that epigenetic variation in blood cells can mirror age-related epigenetic signatures in target tissues of important biological function, i.e. adipose tissue. This opens up for the future development and use of blood-based epigenetic biomarkers to predict disease and altered metabolic function.

Materials and Methods

Study participants

The male discovery cohort consists of 96 males from Sweden and Denmark without known disease and with a broad range in age (23–80 years), BMI (17.5–39.0 kg/m^2) and HbA1c levels (28–46 mmol/mol). It should be noted that the range in HbA1c is represented by variation in non-diabetic subjects. This cohort includes males without known disease from four subcohorts, all previously described (5,9,10,69), with DNA available from subcutaneous adipose tissue biopsies taken in the fasted state (19,60,85). Their clinical characteristics are presented in Table 1, and the characteristics of the subjects included in the present study from the four subcohorts are presented separately in Supplementary Material, Table S27. The female validation cohort consists of 94 Swedish females with a broad range in BMI (18.2–44.9 kg/m^2) and HbA1c levels (25–39 mmol/mol), but a more even distribution in age (21–37 years) and with DNA available

from subcutaneous adipose tissue biopsies taken at the fasted state. This cohort is part of a clinical study examining the impact of polycystic ovary syndrome on female metabolism as described previously (32,33). The characteristics of the female validation cohort are shown in Table 1. The mixed validation cohort consists of 37 males and 67 females from Denmark without known disease and with DNA available from subcutaneous adipose tissue biopsies and blood samples taken at the fasted state. This cohort is part of a Danish family study previously described (86,87) and includes 42 families with genetic risk for T2D and with a broad range in age (32–83 years), BMI (18–47 kg/m^2) and HbA1c levels (22–44 mmol/mol). Their characteristics are shown in Table 1.

In all study cohorts, height and weight were measured wearing light clothing and no shoes, and BMI was calculated as weight divided by the square of the height (kg/m^2). Written informed consent was obtained from all participants and the research protocol was approved by the local human research ethics committees.

DNA methylation analysis

DNA was extracted from subcutaneous adipose tissue biopsies and blood using Qiagen DNA extraction kits (Qiagen, Hilden, Germany). DNA methylation was analyzed genome-wide using the Infinium HumanMethylation450 BeadChip assay (Illumina, San Diego, CA, USA), covering a total of 485 577 probes corresponding to 21 231 (99%) RefSeq genes [88,89]. Five hundred nanograms of genomic DNA from adipose tissue was bisulfite-converted using the EZ DNA methylation kit (Zymo Research, Orange, CA, USA) and used with the Infinium® assay, with all other procedures following the standard Infinium HD Assay Methylation Protocol Guide (Part # 15 019 519, Illumina). The Illumina iScan system was used for imaging data on the BeadChips.

GenomeStudio® Methylation module software was used to calculate the raw methylation score for each probe, represented as methylation β-values [β = intensity of the Methylated allele (M)/intensity of the Unmethylated allele (U) + intensity of the Methylated allele (M) + 100]. All samples passed the GenomeStudio quality control steps based on built in control probes for staining, hybridization, extension and specificity, and the bisulfite conversion efficiency was high (intensity signal >4000) (90). The DNA methylation data were exported from GenomeStudio and subsequently analyzed using Bioconductor (91). β-Values were converted to M-values [$M = \log_2(\beta/(1-\beta))$] using the lumi package (92), to make data more homoscedastic and appropriate for further bioinformatical and statistical analyses (93). Next, the probes on the array targeting SNPs (rs; $n = 65$) and non-CpG sites (ch; $n = 2757$) were removed. Additionally, 5448 probes with SNPs in the target CpG (MAF > 0.1 based on dbSNP) (94) and 14 316 probes reported to be cross-reactive with 50 bp (95) were removed. Finally, 6191 probes were filtered away based on Illumina detection P-value (mean $P \geq 0.01$), resulting in a total of 456 800 individual CpG sites from adipose tissue of 96 men for subsequent analyses. After the same quality control and filtering of probes, 460 973 individual CpG sites generated successful DNA methylation data in adipose tissue of 94 women. Of note, probes targeting the Y-chromosome were also filtered away in these women. The DNA methylation data were background corrected by subtracting the median M-value of the 600 built in negative controls and was further normalized using quantile normalization. BMIQ was used to correct for the bias of the two different probe types on the array (96). As 12 samples are analyzed on each Infinium HumanMethylation450 BeadChip, the generated DNA methylation data needed to be batch-corrected. Correction for batch effects within the methylation array data of each cohort was performed using COMBAT (97).

DNA methylation of specific CpG sites was analyzed in bisulfite-treated genomic DNA from adipose tissue and blood cells using pyrosequencing together with the PyroMark PCR kit, PyroMark Gold Q96 reagents and the PyroMark ID 96 (Qiagen) according to the manufacturer's instructions. Primers were designed using the PyroMark Assay design Software 2.0 and data were analyzed with the PyroMark Q96 2.5.7 software program. The following primer sequences were used for pyosequencing of *ELOVL2*: F-primer 5′-GAGGGGAGTAGGGTAAGTGAG-3′, R-primer 5′Biotin-CATTTCCCCCTAATATATACTTCAA-3′, sequencing primer 5′-GGGAGGAGATTTGTAGGTTT-3′, *KLF14*: F-primer 5′-GGTTTTTAGGTTAAGTTATGTTTAATAGT-3′, R-primer 5′Biotin-AAACTACTACAACCCAAAAATTCC-3′, sequencing primer 5′-ATAGTTTTAGAAATTATTTTGTTT-3′, *CCRL2*: F-primer 5′-AGTTTTAGTTTGGGGTTAAATTTGT-3′, R-primer 5′Biotin-ACAACCAAAAATAATTAATACTATAACTCA-3′, sequencing primer 5′-ATATTTTTTTTTATTTAATTTGATG-3′.

mRNA expression analysis

RNA was extracted from subcutaneous adipose tissue biopsies using miRNeasy kit followed by RNeasy MinElute Cleanup kit (Qiagen; subcohort 1, 2 and 3) or using the RNeasy Lipid Tissue Mini Kit (Qiagen; subcohort 4). The BioAnalyzer (Agilent, Santa Clara, CA, USA) was used to measure RNA quality, requiring a RNA integrity number >7 for each sample to be included. Total RNA (200 ng) from subcutaneous adipose tissue biopsies was used for analysis using the GeneChip Human Gene 1.0 ST whole transcript based array (Affymetrix, Santa Clara, CA, USA), following the Affymetrix standard protocol. The Expression Console Software was used for basic Affymetrix chip and experimental quality, and for background correction, data normalization and probe summarization the robust multi-array average method was used (98). Also here, we applied COMBAT to correct for batch effects within each cohort (97).

Statistical analysis

The association between DNA methylation data from the Infinium HumanMethylation450 BeadChip and studied phenotypes was in the male discovery cohort analyzed using a random effect mixed model, including cohort as the random effect variable and age, BMI and HbA1c as fixed factors. Also the association between mRNA expression and age, BMI and HbA1c was analyzed using the same model. In the female validation cohort, the association between DNA methylation data and studied phenotypes was analyzed using the R package 'limma' and linear model including age, BMI, HbA1c and polycystic ovary syndrome status as variables. To account for multiple testing, we applied false discovery rate (FDR) analysis and $q < 0.05$ (FDR < 5%) was considered significant (99). For the overlap between mRNA expression and DNA methylation as well as for pathway analyses, a CpG site was annotated to a gene if it was located between a distance of 500 kb upstream and 100 kb downstream of the gene. Associations between age or BMI and DNA methylation in the mixed validation cohort was analyzed using random effect mixed linear models in R i386 3.1.0 (http://www.r-project.org) and sex, age, BMI and HbA1c were included as fixed factors in all models, whereas family number/pedigree was included as a random factor. Also, associations between DNA methylation levels in adipose tissue and blood in the mixed validation cohort were analyzed with random

effect mixed linear models adjusted for sex (fixed factor) and family number/pedigree (random factor).

Supplementary Material

Supplementary Material is available at *HMG* online.

Acknowledgements

This study would not be possible without the help from participating clinicians and laboratory technicians, who performed clinical studies and provided the clinical materials, including Targ Elgzyri and Ylva Wessman at Scania University Hospital, Malmö, Sweden, and Marianne Modest and Lars Sander Koch at Steno Diabetes Center, Denmark. We thank SCIBLU (Swegene Center for Integrative Biology at Lund University) Genomics Facility for help with DNA methylation and mRNA expression analyses.

Conflict of Interest statement. None declared.

Funding

This work was supported by grants from the Swedish Research Council, Region Skåne (ALF), Knut and Alice Wallenberg Foundation, Novo Nordisk Foundation, EFSD/Lilly Fellowship, Söderberg Foundation, The Swedish Diabetes foundation, Påhlsson Foundation, EXODIAB, Linné grant (B31 5631/2006), The Danish Strategic Research Council, The Danish Council for Independent Research, Rigshospitalet, University of Copenhagen, Steno Diabetes Center, Danish Diabetes Academy, Jane and Dan Olsson Foundation, Swedish federal government under the LUA/ALF agreement ALFGBG-136481 and the Regional Research and Development agreement (VGFOUREG-5171, –11296, and –7861). The study behind the mixed validation cohort was supported by the Lundbeck Foundation (The Lundbeck Foundation Centre for Applied Medical Genomics in Personalised Disease Prediction, Prevention and Care [LuCamp], www.lucamp.org) and The Danish Council for Independent Research. The Novo Nordisk Foundation Center for Basic Metabolic Research is an independent Research Center at the University of Copenhagen partially funded by an unrestricted donation from the Novo Nordisk Foundation (www.metabol.ku.dk).

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