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RESEARCH PAPER

DNA methylation and obesity traits: An epigenome-wide association study. The REGICOR study

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ABSTRACT

Obesity is associated with increased risk of several diseases and has become epidemic. Obesity is highly heritable but the genetic variants identified by genome-wide association studies explain only limited variability. Epigenetics could contribute to explain the missing variability. The study aim was to discover differential methylation patterns related to obesity. We designed an epigenome-wide association study with a discovery phase in a subsample of 641 REGICOR study participants, validated by analysis of 2,515 participants in the Framingham Offspring Study. Blood DNA methylation was assessed using Illumina HumanMethylation450 BeadChip. Next, we meta-analyzed the data using the fixed effects method and performed a functional and pathway analysis using the Ingenuity Pathway Analysis software. We were able to validate 94 CpGs associated with body mass index (BMI) and 49 CpGs associated with waist circumference, located in 95 loci. In addition, we newly discovered 70 CpGs associated with BMI and 33 CpGs related to waist circumference. These CpGs explained 25.94% and 29.22% of the variability of BMI and waist circumference, respectively, in the REGICOR sample. We also evaluated 65 of the 95 validated loci in the GIANT genome-wide association data; 10 of them had Tag SNPs associated with BMI. The top-ranked diseases and functions identified in the functional and pathway analysis were neurologic, psychological, endocrine, and metabolic.

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Introduction

Obesity is associated with increased risk of several diseases, including diabetes, cardiovascular disease, stroke, and cancer.[1] With estimates that 58% of the world's adult population will be overweight or obese in 2030,[2] obesity has become epidemic and is the fourth leading risk factor attributable to disability-adjusted life years.[3]

Obesity is highly heritable[4] and genome-wide association studies have identified several genetic variants linked with obesity-related traits, but these variants explain only limited variability.[5] Epigenetics could contribute to explanation of this missing heritability and provide some insights into the mechanisms related to obesity. DNA methylation is the most-studied epigenetic marker that regulates gene expression without altering the primary DNA sequence. It is heritable, but it is also modified by lifestyle and environmental factors. Thus, DNA methylation could encompass the interaction between genetics and environment. Numerous studies have associated DNA methylation patterns with obesity, based on body mass index (BMI) or waist circumference (WC).[6-16] A recent meta-analysis identified 187 methylation markers associated with BMI.[17]

Our aim in this project was to identify genetic loci showing differential methylation in relation to BMI and WC. We used an epigenome-wide association approach with a discovery and a validation sample.

Results

Discovery phase

After excluding participants with BMI ≤18.5 (4 individuals) and applying Illumina HumanMethylation450 BeadChip (450K) array quality controls (after which 3 additional participants were excluded), 641 individuals and 427,948 probes (88.7% of the total probes) were included in the analysis. Sociodemographic, clinical, and anthropometric characteristics of participants are shown in Table 1. Participant characteristics across BMI groups are shown in Supplementary Table 1 and 2.

In the discovery phase, 40 CpGs showed differential methylation associated with BMI and 7 with WC using model 1. Using model 2, we identified differential methylation in 214 CpGs associated with BMI and 36 with WC. In total, 237 CpGs related to BMI or WC were identified, located in 182 genes or coding regions (13 of them in non-protein coding regions: 7 CpGs in long noncoding RNA, 4 in microRNA, and 2 in small nucleolar RNA genes) and in 58 intergenic regions. All the Manhattan and q-q plots are available in Supplementary Figure 1. The lambda values of the q-q plots ranged between 1.075 and 1.474.

Validation phase

We included 2,515 individuals (53 individuals were excluded: 2 with a BMI ≤18.5 and 51 after applying the 450K array quality

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Table 1. Characteristics of the participants in the discovery (REGICOR study) and replication (Framingham Offspring Study) cohorts.

	REGICOR n = 641	FOS n = 2515	*P* value
Age	63.2 (11.7)	66.2 (8.91)	<0.001
Sex, female, n (%)	325.0 (50.7)	1354.0 (53.8)	0.169
BMI, Kg/m^2*‡	27.0 (4.0)	28.4 (5.3)	<0.001
Waist, cm*	94.3 (11.4)	102.0 (14.3)	<0.001
BMI categories, n (%)‡			<0.001
Normal (BMI: ≥ 18.50 and <25)	210.0 (32.9)	686.0 (27.4)	
Overweight (BMI: ≥ 25 and <30)	301.0 (47.1)	1029.0 (41.0)	
Obese (BMI: ≥ 30)	128.0 (20.0)	792.0 (31.6)	
Total cholesterol, mg/dL*	208.0 (36.5)	186.0 (37.3)	<0.001
LDL cholesterol, mg/dL*‡	135.0 (32.3)	105.0 (31.4)	<0.001
HDL cholesterol, mg/dL*‡	52.9 (12.3)	57.1 (18.0)	<0.001
Triglycerides, mg/dL†	89.0 [67.0;121.0]	102.0 [74.0;142.0]	<0.001
SBP, mmHg*‡	131.0 (18.5)	126.0 (17.1)	<0.001
DBP, mmHg*‡	76.1 (9.91)	71.6 (10.1)	<0.001
Hypertension, n (%)‡	301.0 (47.1)	1432.0 (57.2)	<0.001
Diabetes, n (%)‡	63.0 (9.9)	—	—
Glucose, mg/dL*	97.8 (20.4)	107.0 (23.8)	<0.001
Smoking status, n (%):			<0.001
Current smokers	106.0 (16.5)	247.0 (9.9)	
Former 1 to 5 years	31.0 (4.8)	55.0 (2.2)	
Former >5 years	165.0 (25.7)	10.0 (0.4)	
Never smokers	339.0 (52.9)	2191.0 (87.5)	
Cholesterol treatment, n (%)	153.0 (23.9)	1090.0 (43.4)	<0.001
Diabetes treatment, n (%)	44.0 (6.9)	—	—
Blood pressure treatment, n (%)	199.0 (31.2)	1228.0 (49.0)	<0.001

*Mean (Standard deviation)
†Median [Interquartile range]
‡BMI, Body mass index; LDL, Low density lipoprotein; HDL, High density lipoprotein; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; Hypertension, defined as previous treatment or SBP ≥ 140 mmHg or DBP ≥ 90 mmHg; Diabetes, defined as previous treatment or glycemia ≥ 126 mg/dL.

control) and all the CpGs selected in the discovery phase, after applying again the quality controls used in the discovery phase. The main characteristics of this population are shown in Table 1.

Meta-analysis

After meta-analysis of the results observed in the Framingham Offspring and REGICOR studies, we validated 51 CpGs with differential methylation associated with BMI in model 1; 26 of them were also associated with WC (Supplementary Table 3 and 4). In model 2, 94 CpGs located in 95 loci were significantly associated with BMI; 49 of them were also associated with WC (Supplementary Tables 3 and 4). These CpGs were located in 72 genes or known coding regions (2 non-coding RNA) and 23 intergenic regions. Among these CpGs, 70 related to BMI and 33 related to waist were new discoveries (Table 2 and 3).

The validated CpGs explained 25.94% and 29.23% of the variability of BMI and WC, respectively, in the REGICOR Study. The same set of CpGs explained 14.18% and 16.73% of the total variability of BMI and WC, respectively, in the Framingham Offspring Study (Supplementary Table 5).

Association between genetic variants in the validated loci and obesity traits

We were able to evaluate 65 of the 95 validated loci in the GIANT genome-wide association data. Ten out of 65 loci presented SNPs associated with BMI, with a *P* value $<7.7 \times 10^{-4}$ (0.05/65) (Table 4, Supplementary Table 6 and Supplementary Figure 2).

Functional and enriched pathway analysis

We used the IPA software to further explore our findings and describe the relationship between the genes associated with BMI or WC. The top 10 canonical pathways selected according to Fisher exact test are shown in Supplementary Table 7. The most remarkable pathways in the top 10 ranking for BMI were "LXR/RXR Activation" (rank 3), "Gustation Pathway" (rank 4), and "PPARα/RXRα Activation" (rank 6). The top 10 ranking for WC included "LXR/RXR activation" (rank 1), "Nitric Oxide Signaling" (rank 4), "Gustation Pathway" (rank 6), and "Cardiac β-adrenergic Signaling" (rank 8). However, none of them were significant after Benjamini-Hochberg correction.

We found 72 and 63 diseases and functions enriched for BMI and WC genetic loci, respectively, when we applied the Fisher exact test; after adjusting for Benjamini-Hochberg, 55 and 42, respectively, remained statistically significant. The 10 top-ranked diseases and functions according to Benjamini-Hochberg test are shown in Supplementary Table 8. The top 4 diseases and functions (neurologic, psychological, endocrine, and metabolic) were consistent for both traits; the gene overlap is shown in Supplementary Figure 3. Six genes were found in all the pathways enriched for BMI: *ITGB5, SREBF1, SLC7A11, GRIK1, CACNA1C*, and *NOTCH4*. The results were the same for WC, with the exception of *NOTCH4*.

We drew the top 4 networks (neurologic, psychological, endocrine, and metabolic) for BMI and for WC using the IPA information (Supplementary Figure 4). These networks showed that the genes we identified are involved in diabetes mellitus, metabolic syndrome, hypercholesterolemia, nonalcoholic liver disease, polycystic ovary syndrome, depression, dementia,

Table 2. Novel CpG sites with differential methylation significantly associated with BMI in the meta-analysis phase.

Features			Meta-analysis		
CpG	CHR*	Gene	Coef.*	SE*	*P* value
cg15459104	15	*MAP1A*	1.18	0.16	3.50E-13
cg00134210	10	*FAM107B*	−0.81	0.11	8.60E-13
cg25404397	10	*C10orf26*	1.21	0.17	1.60E-12
cg02008402	11	*FLJ32810*	−0.89	0.13	4.80E-12
cg03717755	6	*MYLIP*	0.9	0.13	6.40E-12
cg07217499	12	*CACNA1C*	−0.78	0.11	7.10E-12
cg06734985	11	NA	−0.75	0.11	8.60E-12
cg09572125	6	*SYNGAP1*	1.11	0.16	9.70E-12
cg00171092	3	*ITGB5*	−0.94	0.14	2.60E-11
cg13840239	9	NA	−0.85	0.13	6.00E-11
cg15857470	7	*SRPK2*	−0.92	0.14	7.00E-11
cg01172150	16	NA	0.98	0.15	7.80E-11
cg01581222	19	*C19orf38*	1.07	0.17	1.80E-10
cg00585790	2	*LIMS1*	1.01	0.16	2.60E-10
cg05628049	3	NA	1.01	0.16	3.20E-10
cg08215255	5	NA	−0.82	0.13	3.60E-10
cg15674825	5	*MYOZ3*	1.04	0.17	3.70E-10
cg09047573	5	*NME5*	0.95	0.15	3.80E-10
cg03310939	7	*CUX1*	0.94	0.15	4.00E-10
cg12153755	5	*ARAP3*	0.85	0.14	4.80E-10
cg17526229	11	NA	0.83	0.13	4.90E-10
cg15835542	6	NA	0.89	0.15	8.10E-10
cg10094443	4	*UGDH*	0.94	0.15	1.00E-09
cg26766064	17	*MIR657;AATK;MIR338*	−0.99	0.16	1.00E-09
cg20118717	6	*SYNGAP1*	1.17	0.19	1.20E-09
cg16599983	6	*NOTCH4*	−0.9	0.15	1.40E-09
cg18500988	4	NA	0.9	0.15	1.50E-09
cg08877257	16	*MAZ*	−0.89	0.15	1.60E-09
cg09956615	7	*TTYH3*	0.85	0.14	2.60E-09
cg24340572	15	NA	0.97	0.16	2.90E-09
cg15997548	1	NA	−0.7	0.12	3.00E-09
cg01300684	4	*SCOC*	−0.93	0.16	3.30E-09
cg05918312	2	*PDE1A*	−0.8	0.13	3.60E-09
cg03508235	14	*JUB*	−0.7	0.12	3.70E-09
cg04264638	4	*CLOCK*	1.05	0.18	4.20E-09
cg11986385	11	*NAV2*	−0.82	0.14	4.40E-09
cg07950000	21	*GRIK1*	0.84	0.14	5.70E-09
cg11714752	2	*TMEM127*	0.97	0.17	6.10E-09
cg02426464	17	*SLC43A2*	−0.82	0.14	6.30E-09
cg00234616	2	*TLX2*	0.79	0.14	7.00E-09
cg04797846	6	*BTNL2*	−0.76	0.13	7.50E-09
cg16003913	16	*MPG*	1.11	0.19	7.60E-09
cg21390682	13	*MCF2L*	−0.66	0.11	8.10E-09
cg27577928	4	NA	0.79	0.14	8.20E-09
cg17822325	1	*SERINC2*	0.53	0.09	8.50E-09
cg13597054	6	*DDAH2*	1	0.17	8.80E-09
cg12976145	7	*CTTNBP2*	−0.76	0.13	1.20E-08
cg09689944	12	*SUOX*	0.8	0.14	1.20E-08
cg18862566	6	*RUNX2*	0.77	0.13	1.30E-08
cg20981127	19	*NR2F6*	0.82	0.14	1.30E-08
cg15442888	4	NA	−0.66	0.12	1.40E-08
cg14286682	9	*TPD52L3*	−0.8	0.14	1.40E-08
cg23893346	6	*NOTCH4*	−0.81	0.14	1.70E-08
cg03370106	3	*VGLL4*	0.81	0.14	1.80E-08
cg17478979	6	*ZC3H12D*	0.82	0.15	1.80E-08
cg15548101	3	*DGKG*	0.65	0.12	2.10E-08
cg19936757	11	NA	−0.8	0.14	2.30E-08
cg04726013	10	*LHPP*	0.79	0.14	2.50E-08
cg16611352	17	*P2RX1*	0.82	0.15	2.50E-08
cg13084458	4	*INTU*	0.79	0.14	3.30E-08
cg07800670	6	*DST*	−0.86	0.16	3.50E-08
cg08540100	1	NA	0.74	0.13	3.90E-08
cg08120831	12	*LRRC43*	1	0.18	4.10E-08
cg23417875	2	*MAP4K4*	0.83	0.15	4.50E-08
cg19574327	11	NA	−0.75	0.14	4.60E-08
cg23884217	4	*APBB2*	−0.85	0.16	5.70E-08
cg12917475	14	*BCL2L2*	0.81	0.15	6.50E-08
cg16721489	2	NA	−0.63	0.12	6.70E-08
cg12978214	11	*NCAM1*	−0.67	0.12	6.80E-08
cg24824917	19	NA	0.67	0.12	9.00E-08

*CHR = Chromosome; Coef. = Linear regression coefficient; SE = Standard Error

Table 3. Novel CpG sites with differential methylation significantly associated with waist circumference in the meta-analysis phase.

Features			Meta-analysis		
CpG	CHR*	Gene	Coef.*	SE*	*P* value†
cg03717755	6	*MYLIP*	2.55	0.36	2.93E-12
cg06734985	11	NA	−1.97	0.3	2.80E-11
cg07217499	12	*CACNA1C*	−2.12	0.33	8.16E-11
cg15459104	15	*MAP1A*	3.05	0.47	8.76E-11
cg25404397	10	*C10orf26*	3.09	0.49	2.22E-10
cg00134210	10	*FAM107B*	−2.06	0.33	2.81E-10
cg00171092	3	*ITGB5*	−2.44	0.39	5.01E-10
cg13840239	9	NA	−2.27	0.37	1.04E-09
cg09572125	6	*SYNGAP1*	2.74	0.46	2.28E-09
cg02008402	11	*FLJ32810*	−2.12	0.36	3.39E-09
cg17526229	11	NA	2.26	0.38	3.40E-09
cg01300684	4	*SCOC*	−2.62	0.45	5.70E-09
cg00585790	2	*LIMS1*	2.64	0.45	6.14E-09
cg20118717	6	*SYNGAP1*	3.07	0.53	9.59E-09
cg19936757	11	NA	−2.3	0.4	1.12E-08
cg16003913	16	*MPG*	2.94	0.52	1.29E-08
cg01581222	19	*C19orf38*	2.62	0.46	1.43E-08
cg05918312	2	*PDE1A*	−2.11	0.38	2.32E-08
cg26766064	17	*MIR657;AATK;MIR338*	−2.44	0.44	2.42E-08
cg15857470	7	*SRPK2*	−2.27	0.41	2.57E-08
cg15674825	5	*MYOZ3*	2.62	0.47	3.12E-08
cg04797846	6	*BTNL2*	−2.02	0.37	3.30E-08
cg03508235	14	*JUB*	−1.84	0.34	4.87E-08
cg15997548	1	NA	−1.73	0.32	6.50E-08
cg07950000	21	*GRIK1*	2.22	0.41	7.64E-08
cg08215255	5	NA	−1.98	0.37	8.08E-08
cg01172150	16	NA	2.3	0.43	1.04E-07
cg10094443	4	*UGDH*	2.33	0.44	1.05E-07
cg20981127	19	*NR2F6*	2.13	0.4	1.10E-07
cg24340572	15	NA	2.41	0.45	1.16E-07
cg09956615	7	*TTYH3*	2.14	0.41	1.32E-07
cg17822325	1	*SERINC2*	1.39	0.26	1.34E-07
cg15835542	6	NA	2.1	0.41	3.48E-07

*CHR = Chromosome; Coef. = Linear regression coefficient; SE = Standard Error

Alzheimer, and schizophrenia. Moreover, in the psychological network for WC, *GRIK1* was related to compulsive gambling and bulimia nervosa.

Discussion

In the present study, we investigated the association between DNA methylation and obesity traits (BMI and WC) using an epigenome-wide approach. We replicated 24 CpGs associated with BMI and 16 with WC, previously reported by others,[6-16] and newly identified and validated 70 CpGs associated with BMI and 33 with WC. Collectively, these CpGs explained around 26% and 29% of the variability of the analyzed obesity

Table 4. Significant association between the top leading SNPs in those genes validated in the meta-analysis and BMI in the GIANT study.

CHR	Gene	rsID	*P* value
17	*KRT16*	rs11079001	0.000044
12	*CACNA1C*	rs215992	0.00011
4	*SFRP2*	rs10007443	0.00018
16	*MPG*	rs6600233	0.00036
6	*RUNX2*	rs16873740	0.0005
4	*INTU*	rs1033175	0.00063
7	*CTTNBP2*	rs11975899	0.00063
14	*BCL2L2*	rs7157207	0.00067
13	*MCF2L*	rs4907596	0.00068
11	*CPT1A*	rs4930248	0.00075

traits (BMI and WC) in the discovery cohort and 14% and 17% in the validation cohorts, respectively. Ten of the 93 identified loci (*INTU, SFRP2, RUNX2, CTTNBP2, CPT1A, CACNA1C, MCF2L, BCL2L2, MPG*, and *KRT16)* have tag SNPs associated with obesity, strengthening the association between those genes and obesity. The top 4 networks suggest a common link between obesity and both endocrine and metabolic diseases (diabetes mellitus, metabolic syndrome, hypercholesterolemia, non-alcoholic liver disease) and psychological and neurologic processes (depression, dementia, Alzheimer, schizophrenia, compulsive gambling, bulimia nervosa).

Recently, the CHARGE consortium published the first meta-analysis assessing the relation between DNA methylation and BMI, validating 187 methylation markers.[17] We replicated 6 CpGs described in this meta-analysis (cg12593793, cg06690548, cg00574958, cg11024682, cg24679890, cg06500161) and 7 loci (*SLC7A11, CUX1, CPT1A, SREBF1, MYO9B, SLC1AS, ABCG1*). Several reasons could explain the limited overlap between our results and those recently presented by the CHARGE Consortium. First, the statistical power of our study is lower than that of the CHARGE Consortium; therefore, we cannot expect to replicate all the loci reported by this larger study. Second, we replicated 20 CpGs previously reported in other studies. The proportion of replicated CpGs from other studies vs. CHARGE is 2.3 times higher (14 vs. 6 CpGs). This could be related to the heterogeneity introduced in this type of meta-analysis when the included studies used different methods to measure body mass index or waist circumference. This is a well-known limitation of the GWAS meta-analysis that could also affect the EWAS meta-analysis.[18] Finally, our results show a high inflation rate, with a lambda value higher than 1.2 in most of the discovery analyses. This suggests a higher proportion of false positive results than would be expected by chance. However, the results were replicated in an independent cohort and some of the known and newly identified loci point to pathways with a potentially relevant pathological role in obesity.

The first locus reported to have differential methylation associated with BMI was *HIF3A*.[8] This locus has been replicated in a cohort of women[19] but not in other studies[9,14] or the recent meta-analysis.[17] We also did not replicate this result in our study. In addition, the association between methylation levels in *HIF3A* and BMI has been questioned and a transgenerational effect of maternal BMI affecting the association between *HIF3A* methylation and the descendent BMI has been proposed.[19]

In total, we found 55 CpGs associated with BMI and 30 with WC that have been previously associated with age in individuals at high risk for metabolic syndrome.[20] It is known that age affects DNA methylation, as well as WC. Therefore, obesity traits could act as confounders of the association between age and DNA methylation. In our work, we included age as a covariate in the multivariate models to remove its potential confounding effect on the association between DNA methylation and obesity traits.

Regarding our novel results, 19 (27%) of the 70 CpGs were located in intergenic regions. In the same way, methylation changes in adipocyte differentiation mostly occur in intergenic regions, suggesting that those regions could have an important function in obesity.[21]

Among the new CpGs associated with BMI or WC and located in gene regions, we will focus our discussion on those with greater potential for clinical or functional impact. We report that *CUX1* methylation is associated with obesity-related traits. This gene was proposed as a regulator of *FTO* and *RPGRIP1L* expression,[22] and experimental studies downregulating *CUX1* expression also reduced *FTO/RPGRIP1L* expression.[23] Both *FTO* and *RPGRIP1L* are located in one of the most important loci related to obesity.[5] Although *FTO* and *RPGRIP1L* functions in specific tissues are controversial,[24] some studies suggest that the expression of these genes regulates appetite by modulating leptin sensitivity at the hypothalamus: a decrease in their expression reduces the sensitivity to leptin, increasing appetite and food intake and contributing to an increase in body weight.[25] Our study found that hypermethylation in a CpG on *CUX1* is associated with higher BMI, while the recent meta-analysis reported another CpG on *CUX1* with an inverse effect on BMI.[17] Both CpGs are located in the gene body of *CUX1*. Although we do not have a clear explanation for these contradictory results, it could be related to differential transcripts or isoforms. Two isoforms of *CUX1* (P110 and P200) with opposite effects have been described: P110 activates and P200 represses *FTO/RPGRIP1L* expression.[26] In summary, all these data suggest that *CUX1* hypermethylation reduces *CUX1* expression, which in turn decreases *FTO* and *RGRIP1L* expression, reducing satiation in response to leptin and causing an increase in food consumption that leads to obesity. Therefore, methylation in *CUX1* could be a key element to understand the relationship between *FTO* and *RGRIP1L* and obesity.

DDAH2 encodes an enzyme that regulates concentrations of methyl arginine, which in turn inhibits nitric oxide synthase, altering processes such as vasodilation, respiration, cell migration, immune response and apoptosis.[27,28] High expression of *DDAH2* has been associated with reduced WC and higher values of HDL-cholesterol.[29] Our study found a direct association between *DDAH2* methylation and obesity traits. All these results suggest that low levels of DNA methylation could be associated with higher gene expression, and as a result lower BMI and higher values of HDL-cholesterol. In the REGICOR data, this CpG site was associated in the opposite direction with HDL-cholesterol levels (coefficient $= -1.970$; P value $=$ 0.007). In addition, some *DDAH2* genetic variants have been associated with a lower risk of obesity and myocardial infarction, suggesting a possible causal effect between *DDAH2* and obesity—and highlighting this gene as a possible target against obesity and cardiovascular risk.[30]

Obesity is a risk factor for cardiovascular diseases, an association that could be mediated by several mechanisms, including lipid metabolism and regulation of ionic balance, among others. Our study identified some genes showing differential methylation associated with BMI and WC that also have been associated with lipid traits[31-34]: *ABCG1, SREBF1* and *SYNGAP1* methylation have been associated with HDL cholesterol, and methylation in *ABCG1, SREBF1, SLC7A11, MYLIP*, and *CPT1A* with triglycerides.[31-34] In addition, *CPT1A* methylation has been associated with BMI and WC in previous studies.[7,9,15]

We identified 2 genes (*CACNA1C* and *PDE1A*) related to intracellular calcium balance that are associated with obesity.

CACNA1C encodes an α subunit of a voltage-dependent calcium channel. In animal models, obese and diabetic rats show an increase in expression of *CACNA1C* in myocardial tissue.[35-38] *PDE1A* is a Ca(2+)/calmodulin-dependent phosphodiesterase that is activated by calmodulin in the presence of Ca(2+),[39] and its regulation has an important role in neointima formation in atherosclerosis and restenosis.[40] Moreover, lifestyles have an impact on the circadian rhythm which could in turn modify behavior, metabolism, and weight control.[41] In our analyses, we identified a direct association between obesity and differential methylation in the gene *CLOCK* that regulates circadian rhythms. This result is consistent with a candidate gene study showing lower methylation levels in lean individuals compared with obese individuals.[42,43]

Finally, in the IPA network analyses we identified *GRIK1* as one of the genes having a role in the top 4 networks. *GRIK1* has been associated with compulsive gambling, bulimia nervosa and hyperactive-impulsive symptoms.[44] This result highlights the relevance of impulse control as a mechanism related to obesity.

The major strengths of the study were the use of standardized methodology implemented to remove the non-biologic source of variation and the use of a large external population, which allowed us to replicate and meta-analyze the data, thereby strengthening the evidence of our study. In addition, we used robust multivariate regression to reduce outlier effects and adjusted for residual confounding factors to improve accuracy. However, some limitations must be considered. We used REGICOR and Framingham Offspring data to perform the discovery, the replication, and the meta-analysis; the results of these studies present some heterogeneity. We controlled this limitation using meta-analyses with random effects in CpGs with coefficients that were statistically different between studies, and the results did not change. Another limitation is inherent to the cross-sectional design of the study, which hampers our capacity to infer causality of the reported associations.

In summary, the present study identified 95 CpGs associated with obesity traits, of which 70 are new. This study contributes to increased knowledge about the epigenetic basis of obesity and identified several genes and pathways related to obesity and its associated traits. The new findings should be replicated in independent studies, and further studies to assess the direction of the association (causal, reversal, or both) and the functional mechanisms are warranted.

Methods

Study design and participants

A cross-sectional epigenome-wide association study was designed. We randomly selected 648 individuals from the REgistre GIroni del COR (REGICOR) population-based cohort study for the discovery stage and 2,568 participants from the Framingham Offspring Study's population-based cohort for the validation stage.

The REGICOR participants were selected from those individuals enrolled in an initial survey performed during 2003–2005 (n = 6,352; response rate >70%) who attended a second visit in 2008–2013 (n = 4,980; response rate >75%). The first REGICOR survey included participants aged 35–79 years, not institutionalized, and residing in Girona Province in Catalonia (Spain). Participants with a BMI <18.5 kg/m^2 were excluded.

The Framingham Offspring Study sample was obtained through the Database of Genotypes and Phenotypes (dbGAP, available at http://dbgap.ncbi.nlm.nih.gov; project number #9,047). The sample included offspring of the original Framingham Heart Study who attended examination 8 and had available DNA methylation data (n = 2,568). Participants with a BMI <18.5 kg/m^2 were excluded.

The study was developed according to the Declaration of Helsinki and was approved by the local ethics committee. All the participants gave written informed consent before their inclusion.

Obesity-related traits

The methods have been described previously in detail.[45] In summary, the REGICOR-trained team of nurses collected clinical and sociodemographic information following standardized protocols and questionnaires. Weight and height were measured using a precision scale of easy calibration, with participants in underwear and barefoot, and WC at umbilicus with a standard measuring tape.

In the Framingham Offspring Study, the relevant information from examination 8 was obtained through dbGAP. Weight and height were measured barefoot and rounded to the nearest pound and the next lower $^1/_4$ inch, respectively; WC at umbilicus was also recorded to the next lower $^1/_4$ inch. All measurements were converted to international system units (Kg and cm) for analysis.

In both studies, BMI was estimated as weight divided by squared height (Kg/m^2). Underweight participants (BMI <18.50 Kg/m^2) were removed for this analysis.

DNA methylation array

Both the REGICOR Study and Framingham Offspring Study extracted DNA from whole peripheral blood and buffy coat, respectively, using standardized methods (Puregen TM; Gentra Systems). DNA methylation was assessed using the Infinium HumanMethylation450 BeadChip Kit (Illumina) according to Illumina Infinium HD Methylation protocol in both studies. This array allowed assessment of DNA methylation in 485,577 CpGs.[46,47]

REGICOR samples were processed in 2 different laboratories of the Spanish National Genotyping Center, 188 in the Center for Genomic Regulation in Barcelona and 460 in the Centro Nacional de Investigaciones Oncológicas in Madrid. As an internal quality control, each batch contained 2 duplicate samples. The Framingham Offspring Study analyzed 2,568 samples, also in 2 different laboratories (n = 509 and n = 2,059) and also including duplicated samples in each batch.[48]

We used the M-value as an estimation of DNA methylation (Equation 1). An M-value close to 0 means that the CpG is half-methylated. A positive M-value means that the CpG has more methylated than unmethylated cytosines and a negative M-value indicates the opposite ratio.[49,50] To avoid batch effect and other potential technical sources of variation, and as the

participants were randomly assigned to batches, we standardized M-values for batches (Equation 2).

Equation 1 :

$$M_{value} = \log_2\left(\frac{M_i + \alpha}{U_i + \alpha}\right) \quad \begin{matrix} Mi = \text{ intensity of methylated probes.} \\ Ui = \text{ intensity of unmethylated probes.} \\ \alpha = 1. \end{matrix}$$

Equation 2 :

$$Z = \frac{(X - \overline{X})}{\sqrt{\frac{\sum (X - \overline{X})^2}{(n-1)}}} \quad \begin{matrix} X = M - value \text{ for a specific individual.} \\ \overline{X} = mean \text{ of } M - value \text{ for a specific batch.} \\ n = \text{ sample size.} \end{matrix}$$

Finally, we assessed the quality control of the methylation data using a well-defined pipeline (see Supplementary material).

Other covariates

Relevant sociodemographic, lifestyle, and cardiovascular risk factor data were collected using standardized and validated questionnaires. Smoking exposure was categorized into 4 groups: current smokers (smoked ≥1 cigarette/day at the time of the visit, on average, or gave up smoking within the year of the visit); former smokers, between 1 and 5 years (gave up smoking up to 5 years before the visit); former smokers, more than 5 years; and never smokers.

Statistical analysis

To assess the association of methylation with BMI and WC, we used robust linear regression. To identify and remove potential sources of variation (technical and biologic confounders), we estimated surrogate variables using the *R::sva* package.[51-53] The surrogate variable analysis (SVA) method estimates variables, directly constructed from high-throughput data, which provide information related to potential unmeasured confounder variables. These surrogate variables can be included in multivariate models as covariates to control for unmeasured confounding.

We considered methylation as an independent variable and BMI or WC as a dependent variable, according to each analysis. We created 2 models for BMI and 4 models for WC. Model 1 was adjusted for sex, age, smoking exposure, and estimated cell count and model 2 was further adjusted for surrogate variables. These models were fitted for BMI and for WC. Two additional models were defined for WC: model 3 was adjusted as in model 1 (sex, age, smoking exposure, estimated cell count) with the addition of BMI, and model 4 was further adjusted for surrogate variables. We estimated cell count proportion using *R::minfi* package.

From the discovery phase in the REGICOR study, we selected those CpG sites associated with BMI or WC exceeding a *P* value threshold of 1×10^{-05}. We established an arbitrary threshold on the discovery to be less restrictive in this phase. The association between this group of CpG sites and BMI / WC was also assessed in the Framingham Offspring Study.

Finally, we performed fixed-effects meta-analyses using data from the REGICOR and Framingham Offspring studies. In the meta-analyses, we were very strict about declaring statistical significance and only those CpG sites that fulfilled the Bonferroni criteria applied for all the CpG sites assessed in the discovery phase (0.05/427,948; *P* value $<1.17 \times 10^{-07}$) were considered significant. In addition, we estimated the variability of the obesity-related traits explained by each validated CpG, and the variability of BMI and WC explained jointly by the validated CpGs. The regression coefficient value (effect size) that could be detected as statistically significant in the meta-analyses accepting an α risk of 1.17×10^{-07}, in a 2-sided test and with an 80% power, for each of the CpGs selected in the discovery phase is shown in Supplementary Table 10.

Association between genetic variants and obesity traits

We accessed the GIANT public database to identify single nucleotide polymorphisms (SNPs) in the validated loci associated with BMI, using the LocusZoom tool available in http://locuszoom.sph.umich.edu/locuszoom/. We considered as statistically significant those SNPs associated with BMI with a *P* value inferior to the threshold defined by the Bonferroni criteria (*P* value <0.05/number of validated loci).

Functional and pathway analysis

We performed a functional analysis of the genes showing differential methylation related to obesity traits. We uploaded the gene symbol identifiers of the validated genes to the Ingenuity Pathway Analysis (IPA) software (http://www.ingenuity.com/; QIAGEN, Redwood City, CA, USA). The aim was to identify the canonical pathways, and diseases and functions terms showing overrepresentation of the genes of interest.

In the "Canonical pathway" analysis, we selected all metabolic and those signaling pathways related to cellular growth, proliferation and development; cellular immune response; cellular stress and injury; cell cycle regulation; atherosclerosis, cardiovascular, cardiac hypertrophy, nuclear receptor, intracellular and second messenger signaling; and transcription regulation. In the "Diseases and functions" analysis, we selected CHD- and atherosclerosis-relevant terms to create functional networks linking the input genes to functions or diseases. We also created networks for the top 4 enriched "diseases and functions" terms for both traits. All terms with a *P* value <0.05 in the Fisher exact test or after Benjamini-Hochberg multiple testing correction were considered as a significant overrepresentation of input genes in a given process.

Disclosure of interest

The authors report no conflict of interest.

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References

1. Must A, Spadano J, Coakley EH, Field A E, Colditz G, Dietz WH. The disease burden associated with overweight and obesity. JAMA : the journal of the American Medical Association. 1999;282:1523-29. doi:10.1001/jama.282.16.1523
2. Smith K, Smith M. Obesity Statistics. Prim Care. 2016;43:121-35. doi:10.1016/j.pop.2015.10.001. PMID:26896205
3. GBD2015. Global, regional, and national comparative risk assessment of 79 behavioural, environmental and occupational, and metabolic risks or clusters of risks, 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015. Lancet. 2016;388:1659-724. doi:10.1016/S0140-6736(16)31679-8. PMID:27733284
4. Min J, Chiu DT, Wang Y. Variation in the heritability of body mass index based on diverse twin studies: a systematic review. Obes rev. 2013;14:871-82. doi:10.1111/obr.12065. PMID:23980914
5. Locke AE, Kahali B, Berndt SI, Justice AE, Pers TH, Day FR, Powell C, Vedantam S, Buchkovich ML, Yang J, et al. Genetic studies of body mass index yield new insights for obesity biology. Nature. 2015;518:197-206. doi:10.1038/nature14177. PMID:25673413
6. Arner P, Sinha I, Thorell A, Rydén M, Dahlman-Wright K DI. The epigenetic signature of subcutaneous fat cells is linked to altered expression of genes implicated in lipid metabolism in obese women. Clin Epigenetics. 2015;7:1-13. doi:10.1186/s13148-015-0126-9. PMID:25628764
7. Demerath EW, Guan W, Grove ML, Aslibekyan S, Mendelson M, Zhou Y-H, Hedman ÅK, Sandling JK, Li L-A, Irvin MR, et al. Epigenome-wide Association Study (EWAS) of BMI, BMI Change, and Waist Circumference in African American Adults Identifies Multiple Replicated Loci. Hum Mol Genet. 2015;24:4464-79. doi:10.1093/hmg/ddv161. PMID:25935004
8. Dick KJ, Nelson CP, Tsaprouni L, Sandling JK, Aïssi D, Wahl S, Meduri E, Morange P-E, Gagnon F, Grallert H, et al. DNA methylation and body-mass index: a genome-wide analysis. Lancet. 2014;383:1990-98. doi:10.1016/S0140-6736(13)62674-4. PMID:24630777
9. Aslibekyan S, Demerath EW, Mendelson M, Zhi D, Guan W, Liang L, Sha J, Pankow JS, Liu C, Irvin MR, et al. Epigenome-wide study identifies novel methylation loci associated with body mass index and waist circumference. Obesity (Silver Spring, Md). 2015;23:1493-501. doi:10.1002/oby.21111. PMID:26110892
10. Almen MS, Nilsson EK, Jacobsson JA, Kalnina I, Klovins J, Fredriksson R, Schiöth HB. Genome-wide analysis reveals DNA methylation markers that vary with both age and obesity. Gene. 2014;548:61-7. doi:10.1016/j.gene.2014.07.009. PMID:25010727
11. Ollikainen M, Ismail K, Gervin K, Kyllönen A, Hakkarainen A, Lundbom J, Järvinen E a, Harris JR, Lundbom N, Rissanen A, et al. Genome-wide blood DNA methylation alterations at regulatory elements and heterochromatic regions in monozygotic twins discordant for obesity and liver fat. Clin Epigenetics. 2015;7:39. doi:10.1186/s13148-015-0073-5. PMID:25866590
12. Voisin S, Almén MS, Zheleznyakova GY, Lundberg L, Zarei S, Castillo S, Eriksson FE, Nilsson EK, Blüher M, Böttcher Y, et al. Many obesity-associated SNPs strongly associate with DNA methylation changes at proximal promoters and enhancers. Genome med. 2015;7:103. doi:10.1186/s13073-015-0225-4. PMID:26449484
13. Mansego ML, Milagro FI, Zulet MA, Moreno-Aliaga MJ, Martinez JA. Differential DNA Methylation in Relation to Age and Health Risks of Obesity. Int J Mol Sci. 2015;16:16816-32. doi:10.3390/ijms160816816. PMID:26213922
14. Rönn T, Volkov P, Gillberg L, Kokosar M, Perfilyev A, Jacobsen A, Jorgensen S, Brons C, Jansson P, Eriksson K, et al. 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. Hum Mol Genet. 2015;24:3792-813. PMID:25861810
15. Al Muftah W, Al-Shafai M, Zaghlool S, Visconti A, Tsai P, Kumar P, Spector T, Bell J, Falchi M, Suhre K. Epigenetic associations of type 2 diabetes and BMI in an Arab population. Clin Epigenetics. 2016;8:1-10. doi:10.1186/s13148-016-0177-6. PMID:26753011
16. Huang RC, Garratt ES, Pan H, Wu Y, Davis EA, Barton SJ, Burdge GC, Godfrey KM, Holbrook JD, Lillycrop KA. Genome-wide methylation analysis identifies differentially methylated CpG loci associated with severe obesity in childhood. Epigenetics 2015;10:995-1005. doi:10.1080/15592294.2015.1080411. PMID:26646899
17. Wahl S, Drong A, Lehne B, Loh M, Scott WR, Kunze S, Tsai PC, Ried JS, Zhang W, Yang Y, Tan S, Fiorito G, Franke L, Guarrera S, Kasela S, Kriebel J, Richmond RC, Adamo M, Afzal U, Ala-Korpela M, Albetti B, Ammerpohl O, Apperley JF, Beekman M, Bertazzi PA, CJ. Epigenome-wide association study of body mass index, and the adverse outcomes of adiposity. Nature. 2017;541:81-86. doi:10.1038/nature20784. PMID:28002404
18. Burmeister M, McInnis MG, Zöllner S. Psychiatric genetics: progress amid controversy. Nat Rev Genet 2008;9:527-40. doi:10.1038/nrg2381. PMID:18560438
19. Richmond R, Sharp G, Ward M, Fraser A, Lyttleton O, McArdle W, Ring S, Gaunt T, Lawlor D, Smith G, et al. DNA methylation and body mass index: investigating identified methylation sites at HIF3A in a causal framework. Diabetes. 2016;65:1231-44. doi:10.2337/db15-0996. PMID:26861784
20. Ali O, Cerjak D, Kent JWJ, James R, Blangero J, Carless MA, Zhang Y. An epigenetic map of age-associated autosomal loci in northern European families at high risk for the metabolic syndrome. Clin Epigenetics. 2015;7:12. doi:10.1186/s13148-015-0048-6. PMID:25806089
21. van den Dungen M, Murk A, Kok D, Steegenga W. Comprehensive DNA Methylation and Gene Expression Profiling in Differentiating Human Adipocytes. J Cell Biochem. 2016;117:2707-18. doi:10.1002/jcb.25568. PMID:27061314
22. Stratigopoulos G, Leduc C a, Cremona ML, Chung WK, Leibel RL. Cut-like homeobox 1 (CUX1) regulates expression of the fat mass and obesity-associated (FTO) and retinitis pigmentosa gtpase regulator interacting protein-1 like (RPGRIPIL) genes, and co-ordinates leptin receptor signaling. J Biol Chem. 2010;1:1-26
23. Stratigopoulos G, Padilla SL, LeDuc C a, Watson E, Hattersley AT, McCarthy MI, Zeltser LM, Chung WK, Leibel RL. Regulation of Fto/Ftm gene expression in mice and humans. Am J Physiol Integr Comp Physiol. 2008;294:R1185—96. doi:10.1152/ajpregu.00839.2007
24. Gan XT, Zhao G, Huang CX, Rowe AC, Purdham DM, Karmazyn M. Identification of Fat Mass and Obesity Associated (FTO) Protein Expression in Cardiomyocytes: Regulation by Leptin and Its Contribution to Leptin-Induced Hypertrophy. PLoS One. 2013;8:e74235. doi:10.1371/journal.pone.0074235. PMID:24019958
25. Hulea L, Nepveu A. CUX1 transcription factors: From biochemical activities and cell-based assays to mouse models and human diseases. Gene. 2012;497:18-26. doi:10.1016/j.gene.2012.01.039. PMID:22306263
26. Stratigopoulos G, Burnett L, Rausch R, Gill R, Penn D, Skowronski A, LeDuc C, Lanzano A, Zhang P, Storm D, et al. Hypomorphism of Fto and Rpgrip1l causes obesity in mice. J Clin Invest. 2016;126:1897-910. doi:10.1172/JCI85526. PMID:27064284
27. Khor GH, Froemming GRA, Zain RB, Thomas Abraham M, Omar E, Tan SK, Tan AC, Vincent-Chong VK, Thong KL. DNA methylation profiling revealed promoter hypermethylation-induced silencing of p16, DDAH2 and DUSP1 in primary oral squamous cell carcinoma. Int J Med Sci. 2013;10:1727-39. doi:10.7150/ijms.6884. PMID:24155659
28. Leiper J, Nandi M. The therapeutic potential of targeting endogenous inhibitors of nitric oxide synthesis. Nat Rev Discov. 2011;10:277-91. doi:10.1038/nrd3358
29. Puchau B, Hermsdorff HHM, Zulet MÁ, Martínez JA. DDAH2 mRNA expression is inversely associated with some cardiovascular risk-related features in healthy young adults. Dis Markers. 2009;27:37-44. doi:10.1155/2009/978157. PMID:19822957
30. Perez-Hernandez N, Vargas-Alarcon G, Arellano-Zapoteco R, Martinez-Rodriguez N, Fragoso JM, Aptilon-Duque G, Posadas-Sanchez R,

Posadas-Romero C, Juarez-Cedillo T, Dominguez-Lopez ML, et al. Protective role of DDAH2 (rs805304) gene polymorphism in patients with myocardial infarction. Exp Mol Pathol. 2014;97:393-8. doi:10.1016/j.yexmp.2014.09.015. PMID:25236572

31. Irvin MR, Zhi D, Joehanes R, Mendelson M, Aslibekyan S, Claas SA, Thibeault KS, Patel N, Day K, Jones LW, et al. Epigenome-wide association study of fasting blood lipids in the genetics of lipid-lowering drugs and diet network study. Circulation. 2014;130:565-72. doi:10.1161/CIRCULATIONAHA.114.009158. PMID:24920721
32. Pfeiffer L, Wahl S, Pilling LC, Reischl E, Sandling JK, Kunze S, Holdt LM, Kretschmer A, Schramm K, Adamski J, et al. DNA methylation of lipid-related genes affects blood lipid levels. Circ Cardiovasc Genet. 2015;8:334-42. doi:10.1161/CIRCGENETICS.114.000804. PMID:25583993
33. Dekkers KF, van Iterson M, Slieker RC, Moed MH, Bonder MJ, van Galen M, Mei H, Zhernakova D V, van den Berg LH, Deelen J, et al. Blood lipids influence DNA methylation in circulating cells. Genome biol. 2016;17:138. doi:10.1186/s13059-016-1000-6. PMID:27350042
34. Sayols-Baixeras S, Subirana I, Lluis-Ganella C, Civeira F, Roquer J, Do A, Absher D, Cenarro A, Muñoz D, Soriano-Tárraga C, et al. Identification and validation of seven new loci showing differential DNA methylation related to serum lipid profile: an epigenome-wide approach. The REGICOR study. Hum Mol Genet. 2016;25:4556-65
35. Gaber EM, Jayaprakash P, Qureshi MA, Parekh K, Oz M, Adrian TE, Howarth FC. Effects of a sucrose-enriched diet on the pattern of gene expression, contraction and Ca2+ transport in Goto-Kakizaki type 2 diabetic rat heart. Exp Physiol. 2014;99:881-93. doi:10.1113/expphysiol.2013.077594. PMID:24681897
36. Howarth FC, Qureshi M a, Hassan Z, Al Kury LT, Isaev D, Parekh K, Yammahi SRKD, Oz M, Adrian TE, Adeghate E. Changing pattern of gene expression is associated with ventricular myocyte dysfunction and altered mechanisms of Ca2+ signalling in young type 2 Zucker diabetic fatty rat heart. Exp Physiol. 2011;96:325-37. doi:10.1113/expphysiol.2010.055574. PMID:21216827
37. Ojo O, Srinivasan D, Owolabi B, McGahon M, Moffett R, Curtis T, Conlon J, Flatt P, Abdel-Wahab Y. Molecular mechanisms mediating the beneficial metabolic effects of [Arg4]tigerinin-1R in mice with diet-induced obesity and insulin resistance. Biol Chem. 2016;397:753-64. doi:10.1515/hsz-2016-0120. PMID:26966929
38. Sahraoui A, Dewachter C, de Medina G, Naeije R, Aouichat Bouquerra S, Dewachter L. Myocardial Structural and Biological Anomalies Induced by High Fat Diet in Psammomys obesus Gerbils. PLoS One. 2016;11:e0148117. doi:10.1371/journal.pone.0148117. PMID:26840416
39. Michibata H, Yanaka N, Kanoh Y, Okumura K, Omori K. Human Ca2+/calmodulin-dependent phosphodiesterase PDE1A: Novel splice variants, their specific expression, genomic organization, and chromosomal localization. Biochim Biophys Acta. 2001;1517:278-87. doi:10.1016/S0167-4781(00)00293-1. PMID:11342109
40. Nagel DJ, Aizawa T, Jeon K-I, Liu W, Mohan A, Wei H, Miano JM, Florio VA, Gao P, Korshunov VA, et al. Role of nuclear Ca2+/calmodulin-stimulated phosphodiesterase 1A in vascular smooth muscle cell growth and survival. Circ Res. 2006;98:777-84. doi:10.1161/01.RES.0000215576.27615.fd. PMID:16514069
41. Albrecht U. The circadian clock, metabolism and obesity. Obes Rev. 2017;18:25-33. doi:10.1111/obr.12502. PMID:28164453
42. Milagro FI, Gómez-Abellán P, Campión J, Martínez JA, Ordovás JM, Garaulet M. CLOCK, PER2 and BMAL1 DNA Methylation: Association with Obesity and Metabolic Syndrome Characteristics and Monounsaturated Fat Intake. Chronob Int. 2012;29:1180-94. doi:10.3109/07420528.2012.719967
43. van Dijk SJ, Molloy PL, Varinli H, Morrison JL, Muhlhausler BS. Epigenetics and human obesity. Int J Obes. 2015;39:85-97. doi:10.1038/ijo.2014.34
44. Lasky-Su J, Neale BM, Franke B, Anney RJL, Zhou K, Maller JB, Vasquez AA, Chen W, Asherson P, Buitelaar J, et al. Genome-wide association scan of quantitative traits for attention deficit hyperactivity disorder identifies novel associations and confirms candidate gene associations. Am J Med Genet B Neuropsychiatric Genet. 2008;147:1345-54. doi:10.1002/ajmg.b.30867
45. Grau M, Subirana I, Elosua R, Solanas P, Ramos R, Masiá R, Cordón F, Sala J, Juvinyà D, Cerezo C, et al. Trends in cardiovascular risk factor prevalence (1995-2000-2005) in northeastern Spain. Eur J Cardiovasc Prev Rehabil. 2007;14:653-9. doi:10.1097/HJR.0b013e3281764429. PMID:17925624
46. Bibikova M, Barnes B, Tsan C, Ho V, Klotzle B, Le JM, Delano D, Zhang L, Schroth GP, Gunderson KL, et al. High density DNA methylation array with single CpG site resolution. Genomics. 2011;98:288-95. doi:10.1016/j.ygeno.2011.07.007. PMID:21839163
47. Sandoval J, Heyn HA, Moran S, Serra-Musach J, Pujana MA, Bibikova M, Esteller M. Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. Epigenetics. 2011;6:692-702. doi:10.4161/epi.6.6.16196. PMID:21593595
48. Kannel WB, Feinleib M, McNamara PM, Garrison RJ, Castelli WP. An investigation of coronary heart disease in families. The Framingham offspring study. Am J Epidemiology. 1979;110:281-90. doi:10.1093/oxfordjournals.aje.a112813
49. Du P, Zhang X, Huang C-C, Jafari N, Kibbe W a, Hou L, Lin SM. Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis. BMC bioinformatics. 2010;11:587. doi:10.1186/1471-2105-11-587. PMID:21118553
50. Dedeurwaerder S, Defrance M, Bizet M, Calonne E, Bontempi G, Fuks F. A comprehensive overview of Infinium HumanMethylation450 data processing. Brief in bioinform. 2014;15:929-41. doi:10.1093/bib/bbt054
51. Houseman EA, Accomando WP, Koestler DC, Christensen BC, Marsit CJ, Nelson HH, Wiencke JK, Kelsey KT. DNA methylation arrays as surrogate measures of cell mixture distribution. BMC Bioinformatics. 2012;13:86. doi:10.1186/1471-2105-13-86. PMID:22568884
52. Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, Feinberg AP, Hansen KD, Irizarry RA. Minfi: A flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. Bioinformatics. 2014;30:1363-69. doi:10.1093/bioinformatics/btu049. PMID:24478339
53. Leek JT, Johnson WE, Parker HS, Jaffe AE, Storey JD. The sva package for removing batch effects and other unwanted variation in high-throughput experiments. Bioinformatics. 2012;28:882–3. doi:10.1093/bioinformatics/bts034. PMID: 22257669