

Sex differences in DNA methylation in autism spectrum disorder: an epigenome-wide association study

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Abstract

Background

Autism Spectrum Disorders (ASD) represent a heterogeneous group of neurodevelopment conditions with a significant sex disparity—males are affected 3–4 times more frequently than females. The current study aims to explore the role of DNA methylation, an epigenetic modification, in the underlying mechanisms of ASD and its sex differences.

Methods

Blood DNA from 95 ASD probands (48 males, 47 females) and 49 age-matched controls was analysed. Using the Infinium MethylationEPIC BeadChip, we assessed over 845,000 CpG-sites to conduct an Epigenome-Wide Association Study (EWAS). The EWAS results were further evaluated through Gene Set Enrichment Analysis to identify significantly enriched pathways. DNA methylation clocks were calculated, and differences between ASD boys and girls, as well as between ASD probands and controls, were assessed.

Results

Seven significant CpG-sites associated with ASD were identified, with a lower mean methylation compared with controls. The gene set enrichment analysis revealed ‘multicellular organismal signalling’ as the most significant pathway. While no sex-specific significant associations emerged, we identified global hypomethylation in boys compared with girls. The sex-specific pathway analyses revealed enriched pathways linked to cellular signalling and metabolic functions. The analysis of DNA methylation clocks (Levine’s clock and telomere length’s (TL) clock) showed sex differences in the TL clock, with ASD boys exhibiting shorter telomeres compared with ASD girls.

Conclusions

The findings of the study indicate that DNA methylation could be implicated in the sex-differences in ASD.

BACKGROUND

Autism spectrum disorders (ASD) encompass a complex and diverse range of neurodevelopment conditions characterized by language and communication alterations (1), impaired social interactions, and repetitive patterns of behaviour and restricted interests (2). The prevalence of ASD has risen to about 1–2% in world populations, with a distinct sex disparity with males being affected 3–4 times more than females (3, 4). Notably, Maenner and collaborators were the first to report a prevalence of ASD

exceeding 1% in girls. The presentation of ASD is highly heterogeneous, ranging from high functioning subjects with poor social and communication skills to severely cognitively impaired subjects (5), which hampers the diagnosis, treatment and investigation of risk factors. Furthermore, there are clear clinical, cognitive, and biological differences between sexes. ASD females show better social skills, higher social motivation, higher ability to accommodate symptoms, higher associated emotional disorders, and higher levels of self-injury and epilepsy than males. ASD males have higher levels of social communication functional difficulties, higher prevalence of repetitive behaviours and hyperactivity in childhood (6). Also, males display heightened aggressiveness, while females experience greater anxiety and depression (7). Biologically, there are differences in brain morphology and genetic load, with ASD females presenting higher levels of risk variants than males (6, 8, 9, 10). However, the precise mechanisms underlying these differences remain to be clearly discerned (11).

Twin and family studies have provided evidence of a strong genetic component in the development of ASD, with an estimated heritability of 40–90% (9). Common, rare and de novo structural variants have been associated with risk of ASD (12). More than 1,000 genes with ASD risk variants have been implicated in ASD, as reported in the SFARI (www.sfari.org) and

AutismKB2.0 databases (<http://db.cbi.pku.edu.cn/autismkb> v2CNTNAP2). *CNTNAP2*, *MTHFR*,

OXTR, *SLC25A12* and *VDR* were highlighted as the top genes containing common risk single nucleotide polymorphisms (SNP) for ASD in recent meta-analyses (13, 14). A review of published studies revealed that genes involved in synaptic integrity, neurotransmitter metabolism and cell adhesion were frequently associated with Autism development (15). However, the ASD variants discovered to date account for approximately 30–40% of heritability (16–18). Deep learning strategies combining information from big data sets have obtained moderate levels of prediction (19).

There is evidence of the contribution of other non-genetic factors playing an important role in ASD aetiology. Non-genetic perinatal factors such as paternal age, mother's BMI, diabetes and hypertension have been associated with ASD (20, 21). There is also strong evidence linking environmental factors such as air pollution, pesticides, valproic and folic acids with ASD development (20, 21). Further information, including underlying mechanisms of sex differences, may be obtained from epigenomic studies. Epigenomic events may be influenced by environmental factors and better reflect the gene x environment interaction that results in neurodevelopment disorders. Neuronal differentiation processes are modulated by epigenetic mechanisms, and their dysregulation may cause abnormal neurodevelopment which can result in disorders such as ASD and other psychiatric disorders (22). Among various epigenetic modifications, DNA methylation involving the addition of a methyl group to the 5' carbon of cytosine stands out as the most stable and easily detectable. Methylation alterations in sperm increase with age, which may partially account for the relation between paternal age and ASD risk (23, 24). DNA methylation is a key mechanism in the regulation of tissue-specific cell differentiation and may play a pivotal role in sexual differentiation (25). Furthermore, epigenetics emerges as one of the

mechanisms underlying sex biases in psychiatric conditions and may contribute to the differences in ASD prevalence between males and females (26).

DNA methylation patterns have been observed to differ between ASD patients and controls. Initial studies centred on post-mortem brain samples from various brain regions, albeit with limited sample sizes. DNA methylation has been correlated with increased polygenic burden (27), though pinpointing specific differentially methylated CpG-sites (DMP) has proven elusive (27, 28). Previous studies highlighted differential methylation in genes such as *PRRT1*, *ZNF57*, and *C11orf21* in the temporal cortex, as well as *SDHAP3* in the cerebellum (29). Differential methylation in the genes *DDL1*, *AUTS2*, *CYP2E1* and *IRS2* in ASD probands when compared to controls has been reported in several studies (30, 31). Several differentially methylated genes in ASD coincide with those identified in genetic studies, such as *OTX1*, *GRIK4*, *ADCY5*, *PAK2*, *GTF2I* and *FOXP1* highlighting the interplay between genetic and epigenetic factors in the regulation of complex diseases (32, 33). Sex specific epigenetic differences have been scarcely investigated, despite some interesting results. Utilizing artificial neural networks, methylation in genes like *MECP2*, *HTR1A*, and *OXTR* was linked to ASD in females, while methylation in genes like *EN2*, *BCL2*, and *RELN* was linked in males (34). A region of the gene *ZFP57* and one its targets, *RASGRF2*, were differentially methylated between sexes in ASD subjects (35). A previous study observed differently methylated genes related to mitochondrial function in ASD females that may protect against ASD (36). Several differentially methylated regions between male and female ASD subjects were reported in a recent study (Mouat 2025).

Despite these encouraging results, no accurate epigenetic patterns that could facilitate ASD early diagnosis or explain sex differences in presentation have been detected (37). Most epigenomic studies on ASD have been conducted in relatively small or moderate size cohorts, requiring further investigation to confirm findings. A meta-analysis of epigenomic studies in ASD individuals reveal no overall significant findings, but suggestive evidence for enrichment of CpG-sites controlled by SNPs (meQTL targets) (28). The contribution of methylation alterations to ASD and sex differences is yet to be discerned. The aim of our study is to identify epigenetic differences that may explain the discordant sex prevalence and symptomatology in ASD probands using a clinically well-defined ASD cohort and population controls matched for sex, age and ethnic origin to increase the probability of significant findings.

METHODS

Clinical sample

A total of 95 probands (48 males, 47 females, average age: 9.40 ± 3.56 S.D.) with ASD diagnosis according to DSM-5-TR and attending the Child and Adolescent Mental Health Unit of the Hospital Universitari Mutua de Terrassa (HUMT) were included in the study. A control group consisting of $N = 49$ control individuals without mental disorders (27 males, 22 females, average age: 9.89 ± 2.96 S.D.) was recruited amongst outpatients of the paediatric ward of our hospital. All the participants or their carers

gave informed consent to participate in this study, which was approved by the HUMT Ethics Committee. A whole blood sample was obtained from all participants and DNA extracted using a commercial kit (SQ Blood DNA kit, Omega Bio-Tek, USA) and following the manufacturers indications. DNA extractions were conducted simultaneously for probands and controls, using the same methods and reagents to minimize experimental background.

Methylation analysis

The methylation pattern of 144 samples was assessed using the Infinium MethylationEPIC BeadChip (Illumina) that interrogates $N > 850\,000$ CpG-sites. First, we transformed the raw methylation data into β values. CpG-sites were filtered out using ChAMP (version 2.32.0) in R (version 4.3.3) following the following conditions: probes with a detection p-value $> 5\%$, probes with < 3 beads in at least 5% of samples per probe, non-CG probes and multi-hit probes were removed. CpG-sites predicted to be located in a SNP by methyltnsnp package (version 0.99) (38) were also filtered out. The missing β values after applying these filters were imputed using ChAMP package (39) in R using K nearest neighbours (KNN) method. Samples with more than 5% missing probes and samples with discordances between the genetic and the phenotypic sex were also removed. Data was analysed including the X and Y chromosomes. The detection p-value in men and women was calculated separately as previously recommended by Inkster et al. (40). The β values were normalized using Noob and BMIQ and the batch effect was corrected using Combat from ChAMP package. The cell-type proportion was estimated using the RefbaseEWAS method with the whole blood reference-database DNA methylation profile of the major cell types from blood. Then, β values were adjusted by cell-type estimates. The statistical association analysis was performed using general linear regression (glm) with sex, age and sample origin (batch in which the blood was extracted) as covariates in the ASD vs controls analysis (Analysis 1). We also stratified this analysis by sex and is analysis adjusted by age and sample origin (Analysis 2). P-values $< 9.4e-8$ were considered epigenome-wide significant based on the recommendations from Mansell et al. (41), while p-values $< 10e-5$ were considered nominal associations. The bias and the inflation of the results were evaluated using Bacon in R (version 1.30.0) (42).

Additionally, the genes previously associated with ASD from SFARI (www.sfari.org) ($n = 1,191$)

were downloaded and the association with ASD in the analyses 1 and 2 for the CpG-sites located in these genes was evaluated.

Metabolic pathway analysis

The function `champ.GSEA` from the ChAMP package in R was implemented to analyse metabolic pathway enrichment from the EWAS results. Using Gene Set Enrichment Analysis (GSEA), all the genes after ranking them by z-scores were considered in the analyses. This function corrects the results by the number of CpG-sites in each gene.

Biological DNA methylation (DNAm) clocks analysis

We selected the two biological DNAm clocks implemented in Methylclock package (version 1.8.0) in R, as they correlate with biological mechanisms and healthspan (43). Levine’s clock (44) and Telomere length’s (TL) clock (45) were estimated using the DNA methylation data from the 91 ASD patients and 49 controls that passed quality controls. Both methods are valid in children (44, 45). Specifically, Levine’s clock uses 513 CpG-sites while TL clock uses 140 CpG-sites, and both clocks were trained in blood samples. Clock values were adjusted by age and cell type. We also assessed the difference between DNAm age and chronological age (DNAm acceleration 1), the residuals after regressing chronological age and DNAm age (DNAm acceleration 2) and the residuals after regressing DNAm age and chronological age but adjusting for cell count (DNA acceleration 3). The association of these measures with ASD was investigated via Student’s t- tests. We also evaluated whether DNAm clocks differed between girls and boys with ASD and in controls with Student’s t-tests. Finally, the possible association of DNAm clocks with IQ levels was assessed using Pearson’s correlation.

RESULTS

ASD vs CONTROLS (Analysis 1)

A total of 95 ASD patients (n = 48 males and n = 47 females) and 55 controls (n = 30 males and n = 25 females) were analyzed (Table 1). The mean intelligence quotient (IQ) was lower in ASD males than ASD females (Table 1). After QCs, a total of 845,444 CpG-sites were analysed in 91 ASD patients (3 samples were removed for discordant sex and 1 for missingness > 1%) and 49 controls. Seven CpG-sites were significantly associated with ASD (p-value < 9.4e-8). The results did not show inflation based on the calculations from Bacon (λ = 0.81). The top CpG site was located in the gene body of the zinc finger FYVE-type containing 21 (*ZFYVE21*) in the chromosome 14 (Table 2 and Fig.S1). The mean methylation for the seven significant CpG-sites was lower in ASD patients compared with controls (0.47 vs 0.72 in ASD vs controls, respectively).

Table 1
Sample description

	CONTROLS		ASD	
	MALES	FEMALES	MALES	FEMALES
N	30	25	48	47
Age (median)	9.63 ± 0.53	10.1 ± 0.61	9.58 ± 0.47	9.19 ± 0.62
IQ (mean)			86.9 ± 5.3	90.6 ± 3.8
IQ: intelligence <i>quotient</i>				

Table 2
Top EWAS results from analysis 1:

CpG	CHR	BP	Std. Error	t_value	P_value	Gene	Feature
<i>cg06946814</i>	<i>14</i>	<i>1040000</i>	<i>0.0067</i>	<i>-6.85</i>	<i>2.73E-10</i>	<i>ZFYVE21</i>	<i>Body</i>
<i>cg06658698</i>	<i>12</i>	<i>15816305</i>	<i>0.0140</i>	<i>-6.55</i>	<i>1.26E-09</i>	<i>EPS8</i>	<i>Body</i>
<i>cg21375582</i>	<i>10</i>	<i>77796358</i>	<i>0.0046</i>	<i>6.48</i>	<i>1.78E-09</i>	<i>C10orf11</i>	<i>Body</i>
<i>cg21507153</i>	<i>7</i>	<i>189357</i>	<i>0.0087</i>	<i>-6.46</i>	<i>2.00E-09</i>		<i>IGR</i>
<i>cg11330075</i>	<i>1</i>	<i>567438</i>	<i>0.0040</i>	<i>-6.45</i>	<i>2.08E-09</i>		<i>IGR</i>
<i>cg00394062</i>	<i>19</i>	<i>18601728</i>	<i>0.0033</i>	<i>5.89</i>	<i>3.20E-08</i>	<i>ELL</i>	<i>Body</i>
<i>cg16267121</i>	<i>20</i>	<i>55935398</i>	<i>0.0048</i>	<i>-5.69</i>	<i>8.15E-08</i>	<i>MTRNR2L3</i>	<i>TSS1500</i>
cg05812299	10	57358588	0.0051	-5.66	9.65E-08	<i>MTRNR2L5</i>	TSS200
cg23492258	10	57357709	0.0074	-5.62	1.14E-07	<i>MTRNR2L5</i>	TSS1500
cg12124386	1	19000000	0.0023	-5.61	1.22E-07	<i>FAM5C</i>	5'UTR
cg16581032	8	47742015	0.0042	-5.58	1.38E-07		IGR
cg15890734	5	79947234	0.0037	-5.47	2.28E-07	<i>DHFR</i>	Body
cg16008511	20	1299450	0.0005	5.36	3.73E-07	<i>SDCBP2</i>	Body
cg25310459	6	119000000	0.0027	5.36	3.75E-07		IGR
cg25340674	1	145000000	0.0024	5.32	4.59E-07	<i>PDE4DIP</i>	Body
cg15057325	2	198000000	0.0055	5.31	4.62E-07		IGR
cg02947129	6	145000000	0.0067	5.27	5.67E-07		IGR
cg12143918	13	37011756	0.0064	5.23	6.64E-07	<i>CCNA1</i>	ExonBnd
cg23856707	9	129000000	0.0015	5.20	7.67E-07		IGR
cg11212237	6	116000000	0.0014	5.19	8.13E-07	<i>FRK</i>	Body
cg07399224	1	245000000	0.0014	5.15	9.52E-07		IGR

The table shows the top CpG-sites associated with ASD with a p-value < 10e-8. The significant CpG-sites (p-value < 9.4e-8) are highlighted in italics. Negative t-values indicate hypomethylation.

The evaluation of genes previously associated with ASD (as reported by Sfari and AutismKB 2.0) in the analysis 1 revealed a total of 4,861 CpGs in 954 different genes (193 with Score 1, 571 with Score 2 and 125 with Score 3, considering the evidence supporting a gene's relevance to ASD risk) significantly associated with ASD (p-value < 0.05). However, only 1 CpG site (in *FRK*) was significantly associated with ASD after Bonferroni correction (p-value = 8.13e-7) (Table S1).

The pathway enrichment analysis showed a significant enrichment of some pathways, being the multicellular organismal signalling the most significant. Other pathways such as action potential and insulin secretion were also enriched (Fig.S2).

ASD vs CTLS by sex (Analysis 2)

The sex-specific analysis (analysis 2) did not reveal any significant association with ASD at the EWAS significant level (Table 3–4 and Fig. S3 and S4). A total of 213 and 63 CpG-sites in boys and girls, respectively, were nominally associated with ASD. From them, 142 and 47, respectively, were also nominally associated with ASD in the analysis 1.

Table 3
EWAS results in boys with ASD vs controls (Analysis 2):

CpG	CHR	BP	Std. Error	t_value	P_value	Gene	Feature
cg26120787	2	178516770	0.0081	5.96	1.06E-07	<i>PDE11A</i>	Body
cg12508639	3	58157070	0.0021	5.50	6.44E-07	<i>FLNB</i>	3'UTR
cg05094970	16	26799507	0.0073	5.44	8.20E-07		IGR
cg18135853	16	1402335	0.0009	5.43	8.27E-07	<i>GNPTG</i>	Body
cg16531305	5	180146243	0.0077	5.31	1.32E-06		IGR
cg00293718	11	10662026	0.0021	5.25	1.71E-06	<i>MRVI1</i>	5'UTR
cg00100948	3	186382460	0.0124	5.21	1.94E-06	<i>HRG</i>	TSS1500
cg11330075	1	567438	0.0054	-5.10	2.96E-06		IGR
cg27289295	8	49013813	0.0100	5.08	3.26E-06		IGR
cg00394062	19	18601728	0.0038	5.05	3.58E-06	<i>ELL</i>	Body

The table shows the top CpG-sites associated with ASD in boys. Negative t-values indicate hypomethylation.

Table 4
EWAS results in girls with ASD vs controls (Analysis 2):

CpG	CHR	BP	Std. Error	t_value	P_value	Gene	Feature
cg00729928	17	25790061	0.0021	5.41	1.32E-06		IGR
cg10182839	14	73254773	0.0033	5.20	2.79E-06	DPF3	Body
cg06946814	14	1,04E + 08	0.0096	-5.10	4.04E-06	ZFYVE21	Body
cg00577284	22	26235575	0.0046	5.06	4.68E-06	MYO18B	Body
cg06658698	12	15816305	0.0207	-5.02	5.41E-06	EPS8	Body
cg20212082	17	5800808	0.0012	-5.02	5.49E-06	LOC339166	Body
cg18914226	9	1,3E + 08	0.0054	5.01	5.69E-06	RALGPS1	Body
cg25911881	10	88509742	0.0062	4.95	6.98E-06		IGR
cg14552376	2	56412597	0.0012	-4.93	7.42E-06	CCDC85A	Body
cg22370005	7	89749098	0.0050	-4.89	8.65E-06	DPY19L2P4	Body

The table shows the top CpG-sites associated with ASD in boys. Negative t-values indicate hypomethylation.

The global mean methylation in ASD girls was slightly higher than in ASD boys (0.6235 vs 0.6230, respectively) and the same was identified in controls (0.624 in girls vs 0.621 in boys). Similarly, when only the nominal CpG-sites were considered, the mean methylation in girls vs boys showed the same pattern, with higher methylation in girls than in boys in both, in ASD (0.736 in girls vs 0.735 in boys) and in controls (0.721 in girls vs 0.719 in boys).

From the seven significant CpG-sites in the analysis 1, all were nominally associated with ASD in boys and three in girls. All but one of the seven significant CpG-sites associated with ASD were hypomethylated in boys compared with girls (Fig. 1). When the CpG-sites nominally associated with ASD in analysis 1 were studied in analysis 2, a higher number of CpG-sites were nominally significant in the boy's analysis compared with the girl's analysis (71 vs 16) (Fig S3A). Additionally, a higher percentage of the sex-specific CpG-sites with at least a nominal association in the analysis 1 was observed in boys (n = 210, 33.8%) compared with girls (n = 60, 21.6%) (Fig S4B).

Girls and boys mean methylation in each of the seven significant CpG-sites with differential DNA methylation associated with ASD from analysis 2.

The analysis of genes previously associated with ASD revealed a total of 3,176 CpGs in 890 different genes (184 with Score 1, 522 with Score 2, 120 with Score 3 and 64 without assigned score) significantly associated with ASD in girls (p-value < 0.05) (Table S2) and a total of 3,411 CpGs in 882 different genes (186 with Score 1, 517 with Score 2, 117 with Score 3 and 62 without assigned score) significantly

associated with ASD in boys (p -value < 0.05) (Table S3). From the 890 and 882 significant genes in the girls' and boys' analyses, 807 and 804 were also significant in the analysis 1, respectively.

The pathway enrichment analysis for the genes whose methylation was nominally associated with ASD in boys showed an enrichment of the insulin pathway (Fig. S5A). The apical junction pathway was the most enriched pathway from the significant ASD genes in the girls (Fig. S5B).

Biological DNAm clocks analysis

The percentage of missing CpG-sites from the considered clocks were 2.9% for the TL clock and 0.4% for Levine's clock. None of the clocks or the acceleration calculated from these clocks were associated with ASD (Table S1). A significant association of the telomere clock with sex in ASD patients was observed but not in controls. Specifically, ASD boys exhibited shorter telomeres compared with ASD girls (t -value = -2.59, p -value = 1.11×10^{-2}) (Table S4). Similarly, a deacceleration (using the age acceleration 2 and 3) in the telomere biological clock in ASD boys compared with ASD girls was observed (Table S4 and Fig. 2). However, no differences in acceleration were found in controls. This result suggests that boys with ASD are aged compared with affected girls.

Telomere acceleration from boys and girls are presented in green and red, respectively.

DISCUSSION

Alterations in DNAm have been previously associated with neurodevelopmental disorders such as ASD in several studies (20, 46, 47). Dysregulation in parental methylation have also been associated with an increased risk of ASD in their offspring (37, 48, 49). However, there is great variability in the results, probably due to the heterogeneity of autistic disorders. Additionally, information on the contribution of methylation dysregulation to the differences in ASD presentation and prevalence between sexes is limited.

In our study, we identified 7 CpGs differentially methylated in ASD subjects in comparison with controls at EWAS level (p -value $< 10^{-8}$) and 14 additional CpGs associated with ASD at nominal level (p -value $> 10^{-6}$). Mean methylation levels were lower in ASD than in controls, in agreement with previous studies (50, 51). Five of the significantly associated CpGs were located within genes previously associated with ASD (*ZFYVE21*, *EPS8*, *C10orf11*, *ELL*, and *MTRNR2L3*). Genetic variants and methylation levels of *ZFYVE21*, a zinc finger protein involved in cell adhesion, had previously been associated with ASD risk (52). The second most significant site is located in the EGFR pathway substrate 8 (*EPS8*) gene, a signalling protein involved in synaptic function (53). Reduced levels of *EPS8* have been observed in the brain of ASD subjects (53) and splicing variants of this gene have been associated with hearing loss (54). The leucine rich melanocyte differentiation associated gene (*C10orf11* or *LRMDA*) codes for a protein that plays a role in cell differentiation, and mutations in this gene have been associated with ASD (AutismKB2.0) and with intellectual disability (55). The elongation factor for RNA polymerase II (*ELL*) is

involved in positive regulation of transcription, and *de novo* and micro-satellite variants of this gene have been related to autism (56, 57). Finally, mutations in the MT-RNR2 like 3 (*MTRNR2L3*) gene have been included in an algorithm to distinguish between ASD and non-ASD development delay (58). Of note, most of the nominally associated CpG-sites are also located in genes previously associated with ASD. The concurrence of genetic mutations and methylation alterations in the genes associated with ASD may reflect the interplay gene x methylation in the aetiology of the disease.

Our study also revealed nominal associations ($p\text{-value} < 0.05$) with several genes previously associated with ASD in different EWAS: *PRRT1*, *ZNF57*, *C11orf21*, *SDHAP3*, *AUTS2* and *CYP2E1* (29). Our results also corroborated the sex-specific differential methylation of *MECP2*, *HTR1A* in ASD females and of *EN2*, *BCL2*, and *RELN* in ASD males (32).

Enrichment analyses showed an overrepresentation of differentially methylated sites in inner ear development and insulin pathways, amongst others. Disruption of auditory brain structure and peripheral auditory nerves resulting in abnormal auditory processing and hypersensitivity has been observed in ASD animal models (59, 60). Sensorial hyper and hyposensitivity is a core diagnostic feature in ASD (60). Genetic variants influencing insulin secretion have been observed in ASD (61), whereas Song and collaborators (2022) detected dysregulation of insulin signalling in fragile X syndrome (48). Finally, enrichment of methylation alterations in genes involved in lipid synthesis and immune response, two common observations in ASD (62, 63), was also detected. Interestingly, obesity during pregnancy is a causal factor associated to ASD (64). These findings contribute to the growing evidence supporting the contribution of methylation alterations to the development of ASD.

ASD vs CTLS by sex

No statistically significant associations ($p\text{-value} < 10e-8$) were observed when conducting separated analyses by sex, probably due to the reduced sample size. A higher number of nominally differentiated positions was observed in ASD males than in ASD females when comparing with controls. Alteration patterns in ASD males were a better representation of the differences with controls than the patterns observed in ASD females. This finding disagrees with the results of a recent study by Mouet and collaborators (51) that reported more methylation alterations in ASD females than in ASD males. Differences in the methodology and the reduced female sample size used in their study may explain the discrepancies. Nevertheless, the mean methylation levels were lower in ASD males than in ASD females, a finding also observed by Mouet and collaborators (51). As females require a higher genetic load than males to be diagnosed, it may be that differential methylation plays a role on the expression of ASD risk genes contributing to a higher expression of risk variants in males than in females.

Enrichment analyses showed overrepresentation of insulin pathway genes when comparing ASD and controls, and when considering ASD and controls males only. However, no significant differences were observed between ASD and control females. Insulin-like growth factors (IGF) promote the development and maintenance of neural circuits, and alterations in IGFs have been observed in ASD. IGF-1 genetic

variants have been associated with IGF-1 serum levels and may constitute a risk factor for ASD (65). Furthermore, maternal alterations in metabolism may induce downregulation of the insulin pathway causing aberrant methylation that can be transferred to the offspring (66). In recent years it has been postulated that IGF1 alterations can be used as a diagnostic tool for ASD (67). Finally, differences in regulation of cell-adhesion, transit of ions, and apical junction pathways were observed between ASD and control females.

We evaluated participants' biological age rather than their chronological DNAm age (as in Horvath's (68) or Hannon's (69) clocks) because it correlates better with biological processes such as inflammation and DNA damage response, making it a valuable measure of healthspan (44, 45). Both Levine's and TL's clocks are valid biological age measures in children and adolescents (44, 45). Additionally, Levine's clock has been shown to outperform chronological DNAm age measures in predicting health outcomes (44). Our results of biological DNAm clock analyses revealed no association with ASD. These findings agreed with previous studies where no epigenetic age acceleration was observed in the blood of individuals with ASD (70, 71). However, decreased RTL and LINE-1 methylation in ASD was reported by Salem et al. (72). Although a shorter TL has been associated with ASD (73, 74, 75), we did not observe significant differences between ASD subjects and controls. However, ASD males presented shorter TL than ASD females, in agreement with the results of a previous study by Panahi et al. (76). Furthermore, according to their findings, shorter TL than normal were observed in ASD boys, but not in ASD females. An interplay of TL and oxidative stress may contribute to the differences in ASD prevalence between males and females: male hormones may exacerbate oxidative stress and TL erosion, whereas female hormones have a protective effect against Telomere shortening (76). However, further studies to confirm this hypothesis are required.

Key strengths of this study include the use of a balanced sex cohort, genome-wide DNA methylation profiling, and an integrative analytical framework combining EWAS, pathway analysis, and epigenetic clocks. Taken together, our results suggest that methylation dysregulation contributes to the risk of ASD and to the differences in ASD presentation and prevalence between males and females.

The main limitations in the study are that epigenomic studies were conducted on peripheral blood, which may not accurately reflect methylation status in the brain. However, DNA methylation studies including samples from newborns and young infants with ASD, have revealed correlations between differential methylation in blood and brain (32). Although the sample size was small for an EWAS study, it is one of the largest individual epigenomic studies conducted in ASD subjects. Furthermore, our study included a balanced representation of males and females and was also matched for age and ethnic and geographical origin, whereas most previous studies included an overrepresentation of males and/or demographically heterogeneous cohorts. Another limitation of this study is the lack of replication in an independent cohort. However, the availability of comparable datasets with similar clinical characterization, balanced sex distribution, and matched demographic features remains limited. Despite this limitation, we applied stringent quality control procedures, adjusted for relevant covariates, and observed no inflation in the association results, supporting the robustness of our findings

CONCLUSIONS

In summary, our results contribute further evidence of the aberrant methylation observed in ASD subjects (20, 32) and provide new evidence on sex methylation differences that may contribute to the different presentation and prevalence of ASD in males and females.

Abbreviations

Autism Spectrum Disorders (ASD)

Epigenome-Wide Association Study (EWAS)

Telomere length's (TL)

Single nucleotide polymorphisms (SNP)

Differentially methylated CpG-sites (DMP)

Hospital Universitari Mutua de Terrassa (HUMT)

K nearest neighbours (KNN)

General linear regression (glm)

Gene Set Enrichment Analysis (GSEA)

DNA methylation (DNAm)

Elongation factor for RNA polymerase II (ELL)

Insulin-like growth factors (IGF)

Declarations

Ethics approval and consent to participate

This study was conducted in accordance with the principles of the Declaration of Helsinki. All the participants or their carers gave informed consent to participate in this study, which was approved by the HUMT Ethics Committee

Consent for publication

Not applicable

Competing interests

The authors declare that they have no conflicts of interest related to this work.

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Author Contribution

AH, NC, and MJA conceptualized the project; NC, ASL, and MJA developed the methodology; NC, AH, and MJA performed the investigation; NC prepared the figures; AH and MJA acquired funding and administered the project; AH and MJA supervised the work; NC, AH, and MJA wrote the original draft; NC, AH, MJA, AA, CGF, EDT, and VB reviewed and edited the manuscript.

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Data Availability

The data that support the findings of this study are available from the corresponding author (MJA or NC) upon reasonable request. The data are not publicly available due to confidentiality restrictions.

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Figures

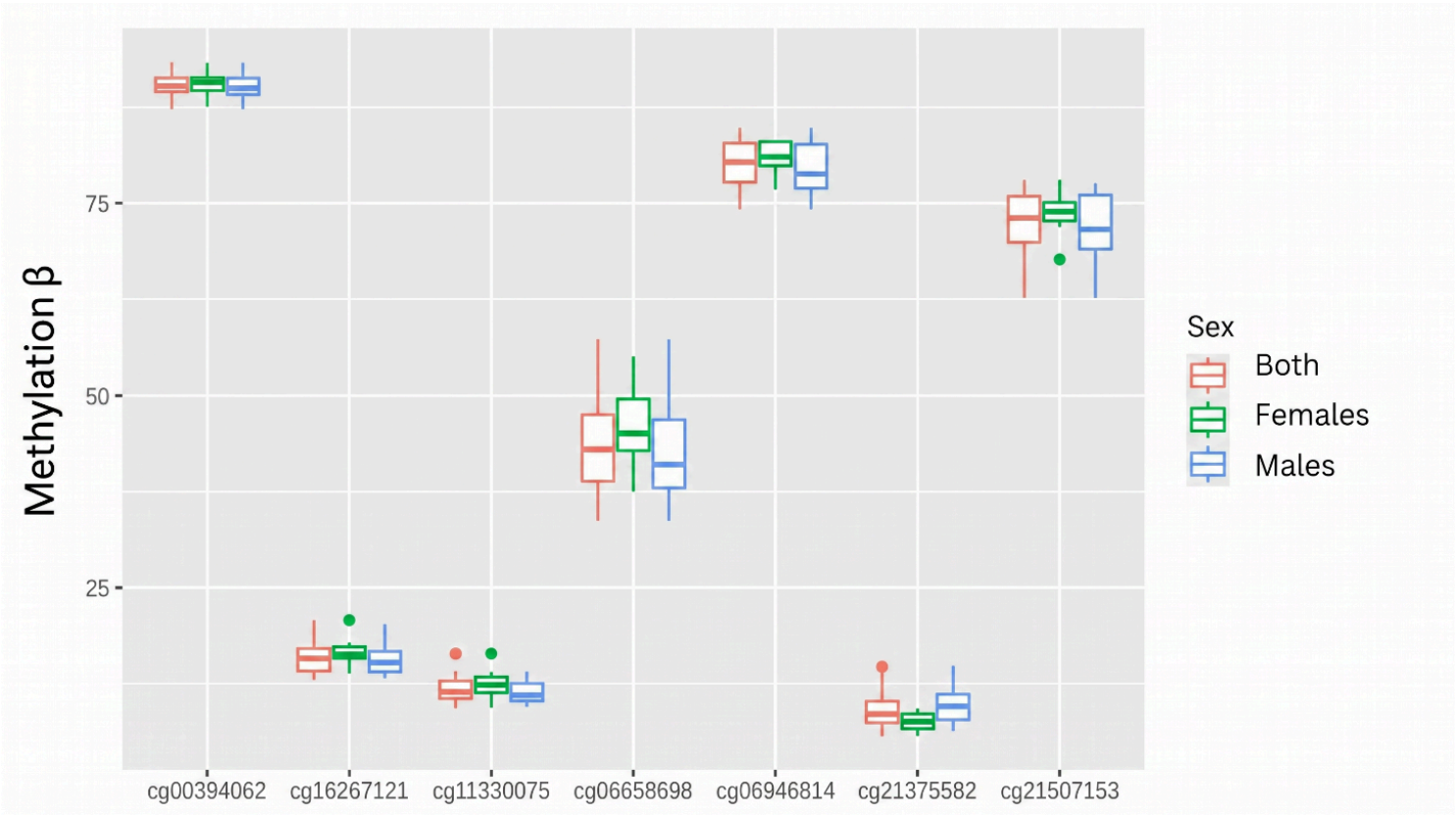


Figure 1

Sex differences in the top CpG-sites from Analysis 2.

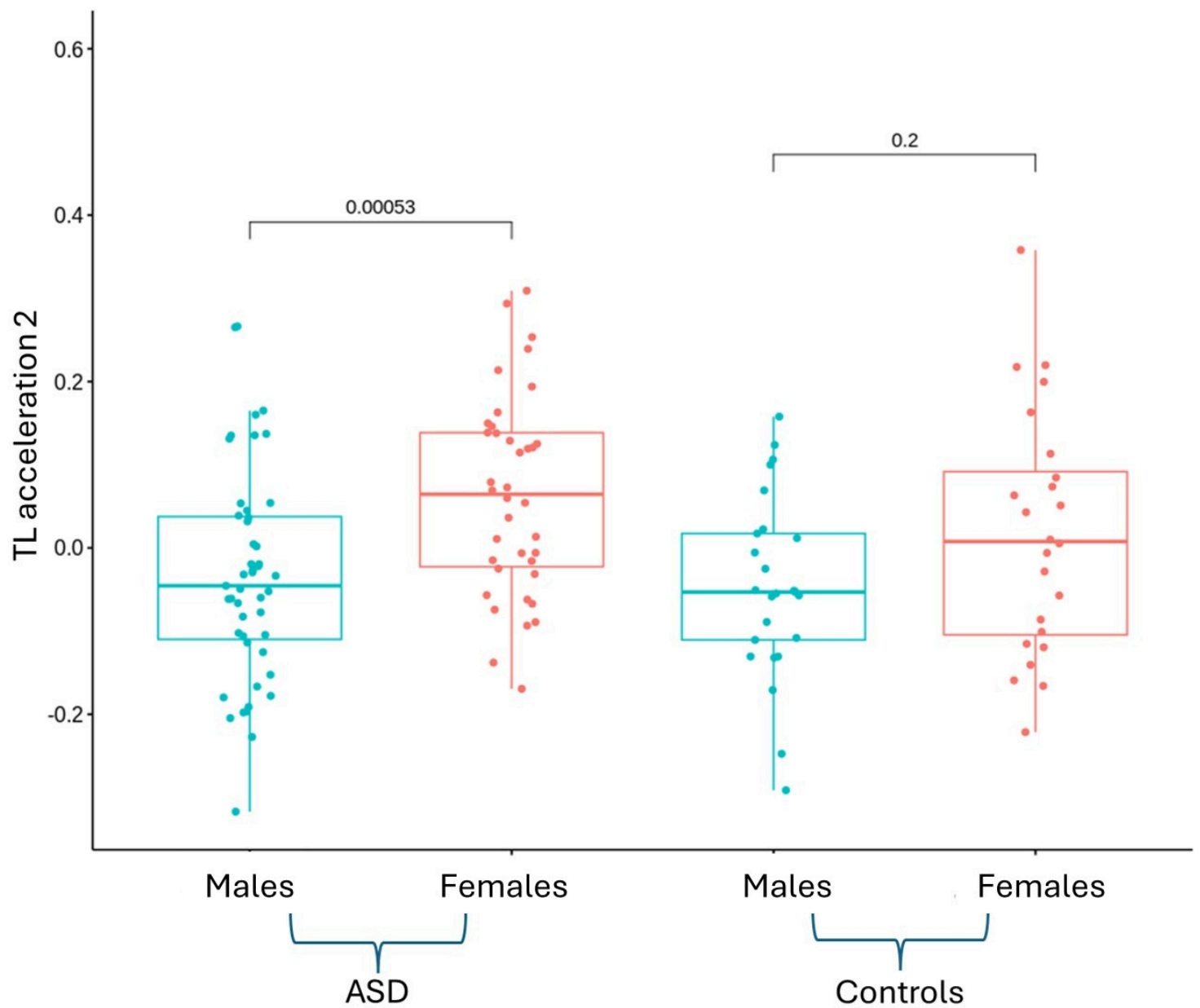


Figure 2

Telomere age acceleration in ASD patient's vs controls stratified by sex.

Supplementary Files

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