

1 Childhood DNA methylation as a marker of early life rapid weight 2 gain and subsequent overweight

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13

14 [Abstract](#)

15 **Background**

16 High early postnatal weight gain has been associated with childhood adiposity, however the
17 mechanism remains unknown. DNA methylation is a hypothesised mechanism linking early life
18 exposures and subsequent disease. However, epigenetic changes associated with high early weight
19 gain have not previously been investigated. Our aim was to investigate the associations between
20 early weight gain, peripheral blood DNA methylation, and subsequent overweight/obese.

21

22 Data from the UK Avon Longitudinal study of Parents and Children (ALSPAC) cohort were used to
23 estimate associations between early postnatal weight gain and epigenome-wide DNA CpG site
24 methylation (Illumina 450K Methylation Beadchip) in blood in childhood (n= 125) and late

25 adolescence (n= 96). High weight gain in the first year (a change in weight z-scores >0.67), both
26 unconditional (rapid weight gain) and conditional on birthweight (rapid thrive), were related to
27 individual CpG site methylation and across regions using the meffil pipeline, with and without
28 adjustment for cell type proportions, and with 5% false discovery rate correction. Variation in
29 methylation at high weight gain associated CpG sites were then examined with regards to body
30 composition measures in childhood and adolescence. Replication of the differentially methylated
31 CpG sites was sought using whole-blood DNA samples from 104 children from the UK Southampton
32 Women's Survey.

33 **Results**

34 Rapid infant weight gain was associated with small (+1% change) increases in childhood methylation
35 (age 7) for two distinct CpG sites (cg01379158 (*NT5M*) and cg11531579 (*CHFR*)). Childhood
36 methylation at one of these CpGs (cg11531579) was also higher in those who experienced rapid
37 weight gain and were subsequently overweight/obese in adolescence (age 17). Rapid weight gain
38 was not associated with differential DNA methylation in adolescence. Childhood methylation at the
39 cg11531579 site was also suggestively associated with rapid weight gain in the replication cohort.

40

41 **Conclusions**

42 This study identified associations between rapid weight gain in infancy and small increases in
43 childhood methylation at two CpG sites, one of which was replicated and was also associated with
44 subsequent overweight/obese. It will be important to determine whether loci are markers of early
45 rapid weight gain across different, larger populations. The mechanistic relevance of these
46 differentially methylated sites requires further investigation.

47

48 Keywords

49 Rapid weight gain, conditional weight gain, epigenetics, EWAS, DNA methylation, DOHAD, ALSPAC,

50 SWS

51 Background

52

53 Children are becoming obese at younger ages (1), suggesting that factors in early life may play a role
54 in obesity development. The developmental origins of health and disease (DOHaD) hypothesis
55 proposes that early life environmental exposures have the potential to modify the risk of later-life
56 diseases, such as obesity (2). Rapid weight gain (RWG) is an early life factor that has been
57 consistently associated with childhood adiposity both dependently and independently of birthweight
58 (3-5). Weight gain in the first year specifically (opposed to change in weight over periods greater or
59 less than 1 year), has been found to be most predictive of childhood obesity (6), suggesting this is a
60 critical period.

61 Given the responsiveness to environmental stimuli, the capacity to alter gene expression and their
62 stability over time, epigenetic changes are a proposed mechanism underlying the DOHaD
63 hypothesis. Through programming effects, epigenetic marks laid down at an early developmental
64 stage could elicit effects at a later stage (7). DNA methylation (DNAm), is the most stable and widely
65 studied epigenetic modification and is a key mechanism regulating gene expression. DNAm involves
66 the covalent addition of a methyl group to cytosine residues adjacent to guanine in DNA (CpG sites)
67 and is associated with changes in gene transcription (8). If early life factors lead to stable changes in
68 DNAm, these changes could be used as biomarkers and to identify individuals who may benefit from
69 intervention prior to disease onset.

70 BMI has been associated with variation in DNAm from birth to adulthood (9-12). Epigenome-Wide
71 Association Studies (EWAS) are a comprehensive approach to identify epigenetic variation
72 associated with a biological trait or exposure (13, 14). In EWAS, other early life risk factors for
73 childhood obesity such as birthweight and maternal BMI have been associated with variation in

74 DNAm (15, 16). To our knowledge, there have been no EWAS to date on early life rapid growth and
75 DNAm.

76 Our first aim was to identify DNAm changes associated with early life growth. In this study we
77 hypothesised that early life rapid growth is associated with DNAm changes. Using epigenome-wide
78 DNAm array data from the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort, we
79 investigated if early life rapid growth is associated with variation in childhood methylation, and if
80 methylation changes persist into adolescence. As catch up growth is more likely in low birthweight
81 infants, we investigated rapid growth both adjusted (rapid thrive, RT) (17) and unadjusted for
82 birthweight (rapid weight gain, RWG)(4). We also examined differential methylation in a subset of
83 known BMI-associated CpG loci (12) with the aim of identifying differentially methylated loci more
84 likely to be related to body composition, and by analysing fewer loci, to offset the multiple
85 comparison problem often associated with null findings in EWAS.

86 An important consideration is that not all children with rapid infancy weight gain will have increased
87 adiposity in childhood (18), and previous studies have highlighted the necessity to distinguish infants
88 at greatest risk of overweight/obesity. Therefore, we also aimed to explore if differential
89 methylation was associated with later life BMI and overweight/obesity in those who experienced
90 early rapid weight gain to determine potential risk markers. Finally, we sought replication in an
91 independent cohort, the UK Southampton Women's survey.

92 [Methods](#)

93 Cohort

94 We performed our initial analysis in the Avon Longitudinal Study of Parents and Children (ALSPAC)
95 cohort, which has detailed early life, anthropometric and epigenome-wide DNAm data at multiple
96 time points. This ongoing longitudinal birth cohort, based in Bristol, England (UK), initially invited
97 pregnant women resident in Avon, UK with expected dates of delivery 1st April 1991 to 31st
98 December 1992 (19, 20). There were 14,541 initial pregnancies enrolled (for these at least one
99 questionnaire has been returned or a "Children in Focus" clinic had been attended by 19/07/99),

100 with a total of 14,676 foetuses, resulting in 14,062 live births and 13,988 children who were alive at
101 1 year of age.

102 The cohort have had extensive questionnaires as well as clinical assessments (including measures of
103 height & weight) throughout childhood. The Accessible Resource for Integrated Epigenomic Studies
104 (ARIES) is a subset of the ALSPAC cohort (21), for which epigenome-wide DNAm analysis was carried
105 out for 1,018 mother and child pairs. Ethical approval for the study was obtained from the ALSPAC
106 Ethics and Law Committee and local research ethics committees. The study website contains details
107 of all the data that are available through a fully searchable data dictionary accessible
108 at www.bris.ac.uk/alspac/researchers/data-access/data-dictionary/.

109

110 Early life data

111 Birthweight and gestational age were taken from medical records. At 12 months, infants were
112 weighed using the Seca 724 (or Seca 835 for children who could only be weighed with a parent).
113 Birthweight and weight-for-age (12 months) z-scores were calculated using the British 1990 growth
114 reference (22), and were used to determine the rapid growth variables: RWG and RT. Whilst
115 birthweight is an important factor in childhood obesity (23), it has previously been examined in the
116 ALSPAC cohort (15, 24). However, in order to differentiate the effects of birthweight on early life
117 rapid growth, both RWG (not conditional on birthweight) and RT (conditional on birthweight) in the
118 first year were examined (Figure 1). Conditional weight gain (also known as thrive index), accounts
119 for normal catch-up growth from low birthweight as a linear measure of weight gain adjusted for
120 regression to the mean (17). Rapid thrive was determined as $z\text{-score}_{12m} - r \times z\text{-score}_{\text{birth}}$ (17), where r
121 is the cohort regression coefficient ($r=0.35$) of birthweight-z on weight-z (12 months). RWG is most
122 often defined as a $>+0.67$ standard deviation change in weight-for-age z-score, equivalent to
123 crossing a growth centile band on a standard child growth chart (4). Both RWG and RT were analysed
124 as a dichotomised variables of a $>+0.67$ standard deviation change (Figure 1).

125

126 Anthropometric data
127 Anthropometric measures at (approximately) age 7 and 17 were also analysed as outcomes in
128 analyses. At age 7, height was measured to the nearest millimetre without shoes or socks using a
129 Holtain stadiometer (Holtain Ltd, Crymych, Pembrokeshire, UK), whilst weight was measured using Tanita
130 THF 300GS body fat analyser and weighing scales (Tanita UK Ltd, Yewsey, Middlesex, UK). At age 17,
131 height was measured with a Harpenden stadiometer to the nearest mm, and weight using the Tanita
132 Body Fat Analyser (Model TBF 401A) to the nearest 50g.

133 BMI and overweight/obese were examined as outcomes at both time points. Body mass index
134 (BMI) was calculated as weight (kg) divided by height (m) squared and was transformed to age and
135 sex standardised BMI z-scores using the British 1990 growth reference with the zanthro program in
136 Stata (25). Clinical cut-offs were used to determine weight categories, whereby healthy weight was
137 between the 2nd and 91st centiles, and overweight/obese as greater than the 91st centile (26) .

138

139 Epigenetic data
140 ALSPAC collected (peripheral) blood at ages 7 and 17 and DNA was extracted. Epigenome-wide
141 DNAm at specific CpG sites was measured for ~1000 individuals using the
142 Infinium® HumanMethylation450K BeadChip assay (Illumina, Inc., CA, USA). DNAm data were pre-
143 processed, including background correction and subset quantile normalization using the pipeline
144 described by Touleimat and Tost (27) (further details in the ARIES cohort profile (21)). Estimation of
145 white blood cell counts (CD8T cells, CD4T cells, Natural Killer, B cells, Monocytes and
146 Granulocytes) was done using the Houseman algorithm (28). Cross-reactive and polymorphic probes
147 identified by Chen *et al.*, (29) and probes on sex chromosomes were removed prior to downstream
148 analysis ($n=453,723$ probes). Due to few participants with non-Caucasian or missing ethnicity, and
149 few non-singleton births, these were removed.

150 Statistical analysis
151 To examine if childhood or adolescent DNAm in peripheral blood (around ages 7 and 17) was
152 associated with early life rapid growth, three different analyses were undertaken, including: analysis
153 of differentially methylation positions, differentially methylated regions, and differentially
154 methylated positions in a subset of candidate loci.

155 For each analysis, DNAm was the outcome and the independent variable was either RWG or RT, with
156 models adjusted for sex and age at blood collection. Cell type composition is a significant source of
157 variation in DNAm analysis (30), however chronic, low level inflammation is a component of the
158 obesity phenotype, therefore, to find novel biomarkers associated with this phenotype, DNAm was
159 investigated in models with and without adjustment for cell composition (all 6 cell types). Correction
160 for multiple testing was applied using a false discovery rate (FDR) threshold of $p < 0.05$ (31).

161 First, epigenome-wide association studies (EWAS) were conducted for rapid growth (first year) and
162 DNAm outcomes in childhood (age 7.4) and adolescence (age 17.6) in the Meffil R package (32).
163 Estimation of differentially methylated sites was carried out using the beta values as the outcome
164 and rapid growth (RWG/RT) as the exposure adjusted for age and sex. Surrogate variable analysis
165 (SVA) and independent surrogate variable analysis (ISVA) methods were utilised to control for
166 unmodelled or unknown confounding factors (such as batch) (33, 34). Meffil simultaneously
167 computes unadjusted, adjusted, SVA and ISVA models, thereby allowing results to be compared (32).
168 In order to minimise the influence of outliers in methylation data, beta values were winsorised at the
169 level of 5% (95th percentile cut-off).

170 Second, in order to detect a DNAm signature of rapid growth, differentially methylated regions
171 (DMRs) were analysed. DMRs, which are stretches or clusters of neighbouring CpG probes, may have
172 more of a functional effect on gene expression than individual CpG loci (35). Additionally, if changes
173 in DNAm are small but persistent across a region, there may be more statistical power to detect
174 them collectively as DMRs (36). The R package DMRcate was used for the estimation of DMRs (37),

175 using the M-values and default settings. The surrogate variables (that were calculated using Meffil in
176 the EWAS models) were included as covariates in the DMR models.

177 Finally, in order to focus on loci with anticipated associations with adiposity, a candidate gene
178 approach was taken using a subset of CpG sites robustly associated with BMI. The candidate sites
179 were selected from a large-scale meta-analysis EWAS which utilised data from multiple cohorts of
180 European and Indian-Asian descent (12). After validation, 187 CpG sites were associated with BMI.
181 We conducted EWAS for both RWG and RT using the 187 identified CpG sites as candidates, at both
182 time points using the Meffil R package (32).

183 Any significantly differentially methylated sites were analysed further to determine if DNAm was
184 also associated with body composition (at age 7 and 17). Using linear regression, significant CpG
185 sites were examined with respect to childhood and adolescent BMI (dependent variable). Similarly,
186 as differentially methylated loci were identified in the RWG EWAS, the CpG sites were also examined
187 with regards to RT in adjusted linear models. All models were adjusted for age and sex. Differences
188 in DNAm by phenotype (RWG, overweight/obese) were assessed using ANOVA tests between groups
189 (with Bonferroni correction for multiple testing).

190 All EWAS and bioinformatic analyses were done in Rstudio version 3.3.2. The human reference
191 genome (GRCh37/hg19 assembly) was used to determine the location and features of the gene
192 region using the UCSC Genome Browser (38). Recoding of the variables and statistical analysis of
193 differentially methylated sites was done in Stata version 15.1 (StataCorp, College Station, Texas,
194 USA).

195 Replication analysis

196
197 CpG sites with FDR P -values <0.05 were carried forward for replication using DNA methylation data
198 from the children from the Southampton Women's Survey (SWS), a similar UK-based cohort (39).

199 Blood methylation measures (EPIC array) and early life weight data for 104 of the SWS children (age
200 = 11-13 years, all Caucasian) were available. Further details of the replication cohort and

201 methylation data processing are provided in the Supplementary text. The exact same models were
 202 estimated for the differentially methylated CpG sites in childhood (age 11-13) and early life rapid
 203 growth (RWG and RT, 0-12 months) using the meffil R package with both SVA and ISVA. Models were
 204 all adjusted for child's sex and age at DNAm measurement and were run with and without
 205 adjustment for cell type composition.

206

207 Results

208 Descriptive characteristics

209 Early life growth and 450K array data were available for 125 ALSPAC children at age 7 and 96 at
 210 age 17 (Table 1). Weight at 12 months was measured in a fraction (n=1,432, 9.3%) of ALSPAC
 211 children, thereby limiting the sample size for the methylation analysis. The ARIES sub-sample was
 212 mostly representative of the main study population; however, ARIES mothers were slightly older,
 213 less likely to have a manual occupation and were less likely to smoke during pregnancy (21). At age
 214 7, 13% of study members were overweight/obese, and 19% were at age 17 (Supplementary Table 1).

215

216 **Table 1 The proportion of individuals in the study sample with RWG or RT at age 7 and 17**

Age	Variable	Models adjusted for cell counts					Models not adjusted for cell counts				
		Total	No	%	Yes	%	Total	No	%	Yes	%
7	RWG	116	75	64.7	41	35.3	125	84	67.2	41	32.8
	RT	116	65	56.0	51	44.0	125	73	58.4	52	41.6
17	RWG	89	54	60.7	35	39.3	96	61	63.5	35	36.5
	RT	89	50	56.2	39	43.8	96	56	58.3	40	41.7

217 Presented for models adjusted or unadjusted for cell counts. RWG, rapid weight gain; RT, rapid thrive.
 218 **94/125 (75%) with measures at age 7 had DNAm measures at age 17.**

219

220 EWAS results

221 We observed associations ($P_{FDR}<0.05$) between RWG and individual CpG loci at age 7. Across the
 222 adjusted models there were 4 associations identified for RWG ($P_{FDR}<0.05$) corresponding to 2 unique
 223 CpG sites (Table 2). These loci were cg01379158 (*NT5M*) and cg11531579 (*CHFR*), and both were
 224 associated with a 1% increase in methylation ($p=0.02$) in those who had RWG. In the models without

225 cell counts, two of the model p values were also below the Bonferroni p value threshold (1.04×10^{-7}).
 226 There were no associations ($P_{FDR} < 0.05$) between RT and individual CpG loci at age 7 in the EWAS. We
 227 examined whether methylation at the RWG-associated CpG sites was also associated with RT using
 228 regression analysis, however the magnitude of the coefficients was lower and less statistically robust
 229 than for RWG (Supplementary Table 2). There was no evidence that RWG or RT was associated (P_{FDR}
 230 < 0.1) with differential DNAm in adolescence for the EWAS, or for the 2 CpG sites identified as
 231 differentially methylated at age 7 using linear regression. In the DMR analysis, there were no overall
 232 DMRs identified; all Stouffer corrected p values were non-significant, suggesting a lack of
 233 consistency in the direction of the methylation changes.

234

235 **Table 2 Associations (FDR p<0.05) between individual CpG sites (age 7, n=453,723) and the early life growth**
 236 **in models**

Exposure	n	CpG name	Nearest gene	Gene region	CpG island name	Model	Coef	SE	P	P_{FDR}
With cell counts										
RWG	116	cg01379158	<i>NT5M</i>	TSS200	chr17:17206527-17207306	ISVA	0.011	0.0018	2.91×10^{-7}	0.02
Without cell counts										
RWG	125	cg01379158	<i>NT5M</i>	TSS200	chr17:17206527-17207306	ISVA	0.011	0.0017	1.41×10^{-8}	0.01
RWG	125	cg11531579	<i>CHFR</i>	Island	chr12:133484658-133485739	SVA	0.011	0.0019	4.16×10^{-8}	0.02
RWG	125	cg11531579	<i>CHFR</i>	Island	chr12:133484658-133485739	ISVA	0.011	0.0019	1.26×10^{-7}	0.03

237 All associations FDR p<0.05 are presented from the ISVA and SVA models, and the models with and without
 238 adjustment for cell types. Chr, chromosome; P_{FDR} , FDR p value; P, unadjusted p value, Coef, coefficient;
 239 TSS200, transcription start site; RWG, rapid weight gain; SVA, Surrogate variable analysis, and ISVA,
 240 independent surrogate variable analysis. Bonferroni p value threshold = 1.04×10^{-7} .

241

242 The candidate gene analysis
 243 The aim of the candidate gene analyses was to select CpG loci already known to be associated with
 244 the outcome phenotype of interest (body composition). Using a smaller subset of loci as candidates
 245 has the advantage of reducing the stringent p value threshold when correcting for multiple tests.
 246 The candidate gene analysis utilised findings from a consortium, which integrated data from 4

247 discovery cohorts and replicated findings in 9 cohorts, and found 187 validated methylation markers
248 associated with BMI (12).

249 The associations between the candidate CpG loci ($n=187$) and early life rapid growth were examined
250 using the ALSPAC methylation childhood and adolescent data, however there were no associations
251 identified (Bonferroni p value $>3 \times 10^{-4}$).

252

253 Investigating phenotypic differences in DNAm associated with RWG
254

255 Methylation at either site was not directly associated with BMI_z at age 7 or 17 in regression analyses.
256 For both CpG sites, highest methylation was in those who had RWG and were subsequently
257 overweight/obese (compared to healthy weight), both in childhood and adolescence
258 (Supplementary Table 3). At the cg11531579 site, childhood methylation was higher in those who
259 were subsequently overweight/obese (age 17) and had RWG compared to those who did not have
260 RWG (Figure 2, ANOVA $p < 0.05$). However, the sample sizes for these groups were small and
261 therefore results are inconclusive.

262

263 Furthermore, those who were healthy weight at age 7 but were overweight/obese at age 17 had
264 higher methylation at age 7 (Figure 3). Whereas, those who had RWG but were a healthy weight (at
265 either time point) had consistently lower levels of methylation. On average, methylation was lower
266 in those who did not have RWG regardless of weight status. Although group sizes were small ($n=6$),
267 methylation at age 7 could have indicated future risk of overweight/obesity in the 'high-risk' group.

268

269 Methylation change over time within individuals
270

271 Overall, the two CpG sites which were positively associated with RWG tended to increase in
 272 methylation over time from childhood to adolescence within individuals (Figure 4). However, when
 273 stratifying by RWG, in those who had RWG there was a decrease in methylation over time compared
 274 to those who did not experience RWG, particularly for cg11531579 ($p < 0.001$, Figure 4).

275
 276 Childhood methylation in the replication cohort
 277
 278 Replication of the significant CpG sites was carried out using a similar UK-based cohort with data on
 279 growth in early life and epigenetic data in childhood ($n=104$ at age 12). Compared to ALSPAC
 280 children, fewer experienced rapid growth in the first year in the SWS cohort; 29.7% had RWG
 281 (30/101) and 22.8% (23/101) had RT. Similar to the findings in ALSPAC, there was evidence of an
 282 association between RWG and DNA methylation at cg11531579 in the ISVA ($p=0.02$) and SVA
 283 ($p=0.04$) models, although the coefficients were smaller (0.005 and 0.004 in the ISVA and SVA
 284 models respectively) (Table 3). There was no association for the either the cg01379158 site or the RT
 285 models.

286

287 **Table 3 Associations between early life rapid growth and childhood methylation in the replication cohort**
 288 **(SWS children age 11-13, $n=104$)**

Models with cell counts			ISVA			SVA		
Exposure	CpG site	Nearest gene	Coef	SE	P	Coef	SE	P
RWG	cg11531579	CHFR	0.0045	0.0020	0.02	0.0045	0.0021	0.04
RWG	cg01379158	NT5M	0.0004	0.0029	0.90	-0.0022	0.0026	0.40
RT	cg11531579	CHFR	0.0022	0.0026	0.39	0.0029	0.0024	0.23
RT	cg01379158	NT5M	-0.0002	0.0036	0.96	-0.0030	0.0029	0.31
Models without cell counts								
RWG	cg11531579	CHFR	0.0005	0.0020	0.82	-0.0002	0.0020	0.92
RWG	cg01379158	NT5M	0.0019	0.0023	0.40	0.0020	0.0023	0.40
RT	cg11531579	CHFR	-0.0013	0.0020	0.52	-0.0020	0.0022	0.37
RT	cg01379158	NT5M	0.0043	0.0027	0.12	0.0018	0.0026	0.48

289 P, unadjusted p value, Coef, coefficient; RWG, rapid weight gain; SVA, Surrogate variable analysis, and ISVA,
290 independent surrogate variable analysis.

291

292 Genomic location of the differentially methylated CpG sites

293 The CpG site; cg01379158 was located upstream of the transcriptional start site in a CpG island

294 (chr17:17206527-17207306). The nearest gene to cg01379158 is *NT5M*, also known as 5',3'-

295 Nucleotidase, Mitochondrial. The second CpG site (cg11531579), was positively associated with

296 RWG ($p_{FDR} < 0.05$, Table 2). This CpG (cg11531579) is located within a CpG island on chromosome 12:

297 upstream 30+ kilobases is the protein coding gene Checkpoint With Forkhead And Ring Finger

298 Domains (*CHFR*) (Table 2), whilst 558 base pairs downstream, is a small (2 exons) non-coding region

299 (AK055957), for which there is limited information.

300

301 Discussion

302

303 Summary

304

305 In this study, we identified that RWG in the first year of life was associated with small but significant

306 increases in childhood DNAm (age 7) at two CpG sites (cg01379158 and cg11531579). The highest

307 levels of methylation at the cg11531579 locus (age 7) were in those who had RWG and were either

308 currently (age 7) or subsequently overweight/obese (age 17). Furthermore, there was suggestive

309 evidence that this site was differentially methylated in the replication cohort. We did not find

310 evidence of differentially methylated regions, of differential methylation associated with RT, or of

311 differentially methylated BMI-associated candidate sites.

312 Interpretation and comparison with previous findings

313

314 To our knowledge this is the first EWAS to identify differential DNAm associated with early life rapid

315 weight gain. DNAm at the locus near *CHFR* (cg11531579) was higher in those who had RWG and who

316 were overweight/obese in childhood or adolescence. Early life RWG was associated with small
317 changes in childhood methylation, but not with methylation in adolescence, which could have been
318 partly due to a smaller sample size or a lack of persistence in the differential DNAm seen in early life.
319 Indeed, in those who had RWG there was a decrease in methylation over time (age 7 to 17), which
320 may reflect the 'recovery' of hypermethylation in childhood. This phenomenon of attenuation of
321 DNAm over time from signals detected in early life has been reported previously (24). There is the
322 possibility that methylation changes may be greater earlier in childhood closer to the timing of the
323 exposure.

324 There are inherent links between birthweight and postnatal growth, and birthweight associated
325 DNAm changes are also often related to growth control (40). Rapid thrive accounts for catch-up
326 growth from low birthweight, whereas RWG includes some of the effects of low birthweight.
327 Although birthweight influences RWG, associations between RWG and adiposity remain after
328 adjustment for birthweight (41). As associations were stronger between DNAm (at the identified
329 CpG sites) and RWG (rather than RT), it is plausible that methylation at these sites also encompasses
330 some of the effects of catch-up growth from low birthweight.

331 Neither early life growth nor birthweight have been previously associated with DNAm at either of
332 the identified sites. We searched the EWAS Catalogue (<http://ewascatalog.org/>) to assess whether
333 any of the CpG sites had been previously identified in other EWAS, with results suggesting that both
334 of these loci may be linked to bone composition and cholesterol metabolism, factors which could
335 plausibly be linked to growth. As the DNAm changes identified were small, it is perhaps speculative
336 to discuss the impact on gene expression.

337 The cg11531579 site, which was positively associated with RWG in ALSPAC and the SWS children,
338 has nearby transcripts with cancer-associated roles (42-48). The *CHFR* (cg11531579) gene encodes a
339 E3 ubiquitin-protein ligase which regulates the cell cycle at the antephasis checkpoint (prior to cell
340 division) (42). Differential epigenetic regulation of *CHFR* has been identified in cancer as a result of

341 promoter hypermethylation (43, 44), or deacetylation of histones in the promoter region (45),
342 however it is unclear whether changes in expression are a cause or consequence of cancer. The CpG
343 site is located within a DNase I hypersensitivity cluster, which may suggest a transcription factor
344 binding region, and a H3K27Ac histone mark, which is often found near regulatory elements and is
345 thought to be a transcription enhancer, suggesting regulatory functions. Downstream of cg11531579
346 is AK055957, a small non-coding RNA regulatory sequence, with an uncharacterised biological role.
347 Recently, this CpG (in combination with others) has been identified as a potential DNAm biomarker
348 for use in detection panels for hepatocellular carcinoma (46) and pancreatic ductal adenocarcinoma
349 (47), and is differentially methylated in children with acute myeloid leukaemia post-chemotherapy (-
350 0.24 change in beta value, $p=0.004$) (48). These findings suggest this locus may have a role in
351 carcinogenesis, which may suggest a weak link with rapid growth.

352 The cg01379158 site is located in the transcriptional start site of the *NT5M* gene, a gene involved in
353 nucleotide metabolism. The gene is located on chromosome 17 in the Smith-Magenis syndrome-
354 critical region, which is a rare condition characterised by inverse circadian rhythm and disturbed
355 sleep, factors which have also been linked to child obesity (49, 50). At this CpG site there was no
356 evidence of differential methylation in the SWS cohort, and methylation at the cg11531579 site was
357 around half that observed in ALSPAC. There are several possible reasons for this, such as the
358 moderate sample sizes, or smaller proportion of those who had rapid growth in the SWS. From the
359 ALSPAC data it was evident that there is loss of methylation over time (age 7-17) at these loci in
360 those with RWG, therefore as the SWS were also slightly older (+5 years) this may also explain less
361 differential methylation. This may suggest that a biomarker for early rapid growth could have
362 greater utility in early childhood and warrants further investigation in younger cohorts.

363 There were no associations between previously reported BMI-related CpG sites and RWG or RT
364 arising from our candidate analysis, which could be for various reasons. First, the candidate loci
365 mapped to genes with specific roles, which could be different to the mechanisms and pathways of
366 RWG. Secondly, although some of the associations have been replicated in pre-school children (9),

367 primarily the candidate loci were relevant to an adult population, whereas this cohort were sampled
368 and analysed at a much younger age. Finally, RWG has been associated with subsequent changes in
369 BMI, whereas, Wahl and colleagues conclude that the majority of the identified BMI-related CpGs
370 were a consequence (rather than a cause) of changes in BMI (12). Thus, if rapid growth was
371 associated with DNA changes in this subset of CpGs, this perhaps would have been more likely to
372 have been as a consequence of changes in BMI. Indeed, current evidence suggests that the direction
373 of the effect is from BMI to DNAm (12, 51), therefore RWG (i.e. early life increases in BMI)
374 associated DNAm changes may also be consequential of the phenotype rather than causal.
375 Similar to Reed *et al.*, (51), we did not identify strong direct associations between childhood
376 methylation at these CpGs and later BMI. They did however identify associations between a DNAm
377 score for BMI and health outcomes where BMI is a risk factor, suggesting methylation may have
378 more utility as a biomarker of BMI-related morbidity than as a predictor of BMI itself (51).

379

380 Strengths and limitations

381 A limitation of our study is the small sample size, however despite this, results were independently
382 replicated at 1 of the CpGs. It will be important to replicate these findings in other cohorts with
383 much larger sample sizes and a range of ages. This study has a number of strengths, principally the
384 rare combination of detailed early life phenotypic and anthropometric data, as well as epigenetic
385 data, as cohorts with longitudinal and epigenetic data of this nature are scarce. Other studies will
386 undoubtedly also be limited by the lack of available early life weight measures, and future birth
387 cohort studies should strive to collect these vital data. Whilst our analysis may have been
388 underpowered, robust associations were still identified, although it is possible that other
389 differentially methylated sites may have been missed.

390 Here we investigated RWG in the first year, however the entire childhood period could also be a
391 critical period for growth and development of obesity (52). There is the possibility that associations

392 may be stronger in early life (before age 7), closer to the timing of the exposure, although this is not
393 possible to test without multiple measures of DNAm throughout childhood.

394 There were differences in the associations identified in the models with and without adjustment for
395 cell types. Whole blood represents a mixed cell population with varying proportions of white blood
396 cells. Phenotypic variation in cell-type composition could confound analyses, or it could also
397 represent an important physiological change in response to an exposure or disease, which may be
398 related to the phenotype of interest. Obesity is an acknowledged chronic, inflammatory condition,
399 and has been associated with inflammatory indicators including C-reactive protein (53) and white
400 blood cell counts (54, 55). When investigating biomarkers (related to an exposure) that are
401 associated with an inflammatory disease outcome, to remove variation from cell counts could
402 potentially disregard important loci.

403 We utilised SVA and ISVA to remove unwanted variation from confounders (which cannot always be
404 adequately corrected for), whilst retaining differences due to the exposure of interest (56). SVA finds
405 sources of variation from the methylation data itself, and models these as linearly uncorrelated
406 singular vectors (surrogate variables) which are then included as covariates in the regression model
407 (33). ISVA is a modified version of SVA where surrogate variables are deemed independent. In
408 support of ISVA, known confounding factors such as age and batch are linearly uncorrelated
409 statistically independent variables, therefore it would be appropriate to model these as independent
410 variables. ISVA was shown to perform best at capturing a known specific biological signature when
411 compared to other adjustment methods (34), although this may not hold true for all datasets (34,
412 57). A thorough simulation study comparing each of the common adjustment methods (Houseman's
413 reference-based method, RefFreeEWAS, SVA, ISVA, EWASher and RUV) and found no method
414 performed perfectly for all parameters, but concluded SVA was most robust (and 'safest') (58). In
415 summary, as high-performing adjustment methods both were utilised in these analyses.

416 Conclusion

417 Our findings suggest that differential DNAm at 2 loci could be markers of early weight gain. At one
418 CpG site, the highest levels of childhood methylation were in those who had RWG in early life and
419 were subsequently overweight/obese in childhood or adolescence, therefore this site may have use
420 as a biomarker of subsequent overweight/obesity in those who experience RWG. The EWAS
421 identified 2 potentially important candidate sites, which could be the focus of further investigation.
422 Further work is required to determine if these CpG sites are consistently, differentially methylated in
423 different populations, time points, and ages.

424

425

426 [Declaration](#)

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481

482 Contributions

483 NR was responsible for the data analysis, prepared the tables/figures, drafted the manuscript, and
484 reviewed and edited the manuscript. JAM, HB, VA and MSP were responsible for the concept and
485 design, and critical revision of the manuscript. CLR contributed to the early appraisal of the methods.
486 KMG, KAL, MAH, RM are responsible for the SWS study design, concept and/or data collection, EA
487 performed the statistical replication analysis in the SWS. All authors participated in manuscript
488 editing and read/approved the final version.

489

490 Ethics approval and consent to participate

491 Informed consent for the use of ALSPAC data collected via questionnaires and clinics was obtained
492 from participants following the recommendations of the ALSPAC Ethics and Law Committee at the
493 time. Follow up of the children and sample collection/analysis was carried out under Institutional
494 Review Board approval (Southampton and SW Hampshire Local Research Ethics Committee) with
495 written informed consent obtained from parents or guardians.

496 Consent for publication

497 Manuscript was approved by the ALSPAC Executive prior to journal submission.

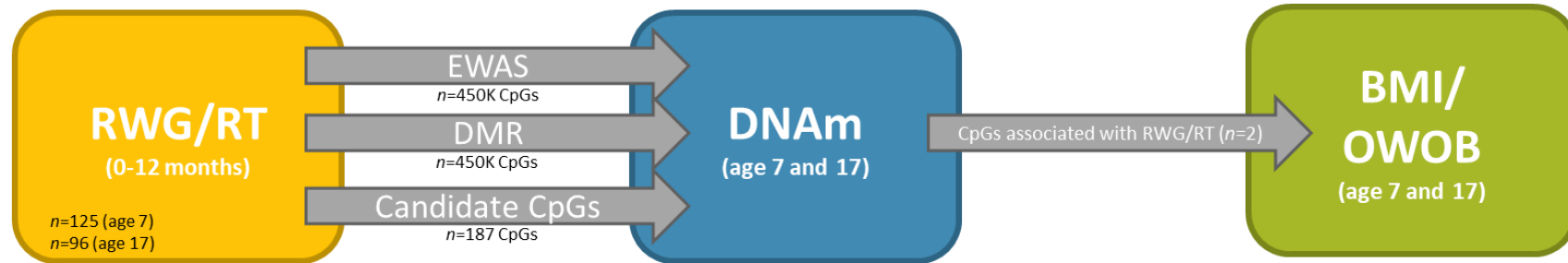
498 Competing interests

499 The authors declare that they have no competing interests.

500

501 Figures

502



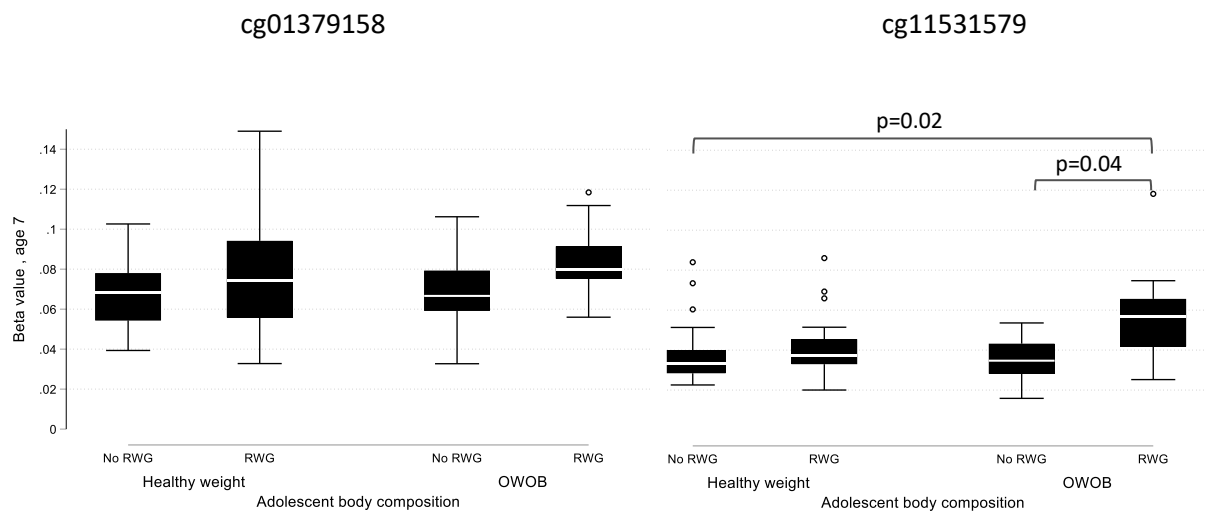
Early life rapid growth	Description	DNAm data	Description	Anthropometric outcomes	Description
Rapid weight gain (RWG)	If experienced a +0.67SD change in weight for age z-score ($z\text{-score}_{12m} - z\text{-score}_{\text{birth}}$)	Epigenome-wide methylation data (450K)	Investigated individual CpG sites, differentially methylated regions, and a subset of BMI-associated methylation markers (12)	BMIz	BMI (kg)/height (m) ² transformed to age and sex standardised z-scores using the UK90 growth reference (22)
Rapid thrive (RT)	If experienced a +0.67 SD change in conditional weight gain ($r = \text{birthweight correlation coefficient}$) ($z\text{-score}_{12m} - r \times z\text{-score}_{\text{birth}}$) (16)			Overweight/obese (OWOB)	Overweight/obese defined as BMIz > 91 st centile

503

504 **Figure 1 Description and measurement of rapid infant weight gain exposures, and epigenome-wide DNA methylation and anthropometric outcomes, and an**
 505 **overview of the analysis. Participants were measured at around age 7 (mean 7.4 (SD 0.10) years) and 17 (mean 17.7 (SD 0.31) years).**

506 **RWG, rapid weight gain; RT, rapid thrive; BMIz, BMI z-scores; OWOB, overweight/obese; DNAm, DNA methylation; EWAS, Epigenome-wide association study;**
 507 **UK90, the British 1990 growth reference.**

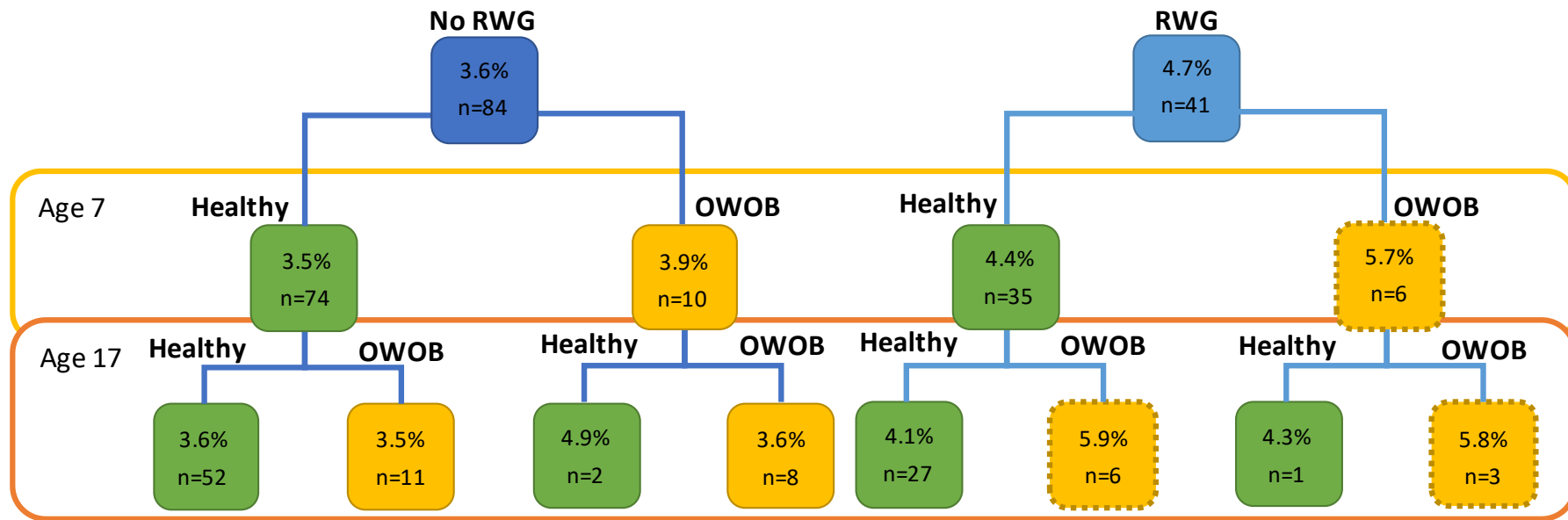
508



509 **Figure 2 Childhood methylation and adolescent body composition.**
 510 **Methylation at the cg01379158 locus (left plot), and cg11531579 (right plot). There were no differences between groups for cg01379158 ANOVA ($p > 0.05$).**
 511 **The cg11531579 plot presents tests for significance (p values) from the Kruskal Wallis test with Bonferroni correction for multiple testing.**
 512 **RWG, rapid weight gain; OWOB, overweight/obese.**

513

514

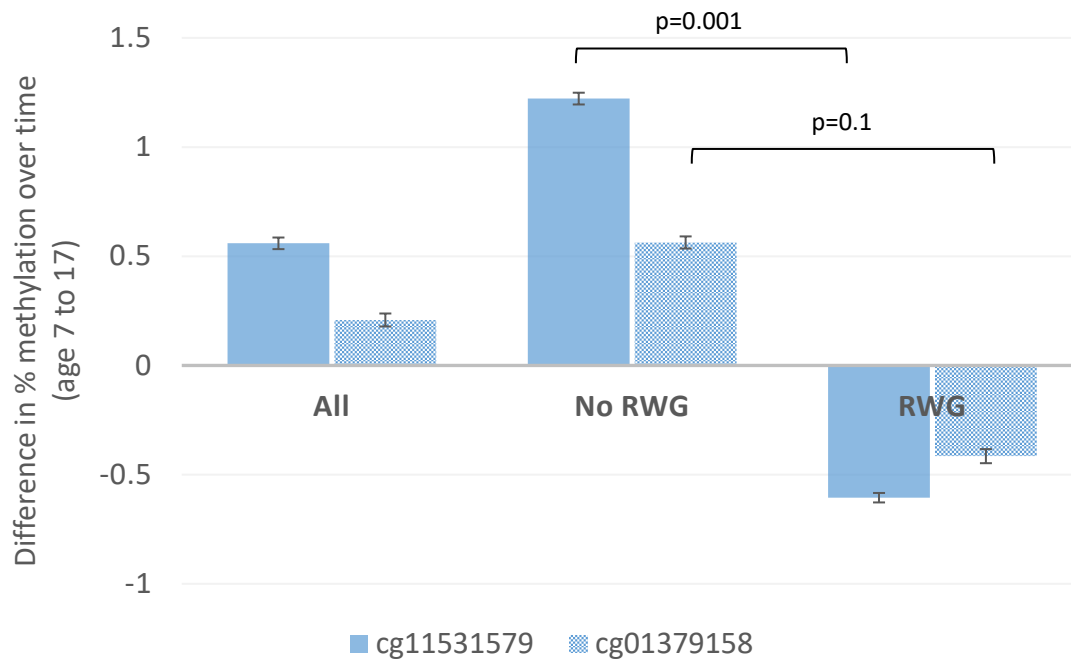


515

516 Figure 3 Pathways of mean methylation (cg11531579) at age 7 (%) and body composition (at ages 7 and 17).
 517 Dotted outline indicate the 'high-risk' individuals (i.e. those who had RWG and were subsequently overweight/obese (OWOB)).
 518 Sample sizes are for those with complete data. Group sizes were small for some phenotypes.

519

520



521

522 **Figure 4** Change in methylation at the loci within individuals from age 7 to 17 by RWG.

523 Test for differences using the Student's t-test. Those who did not have RWG ($n=60$) demonstrated small mean increases (cg01379158, +0.6%; cg11531579, +1.2%) in
 524 methylation, whereas those who had RWG ($n=34$) demonstrated small (cg01379158, -0.4%; cg11531579, -0.6%) decreases in methylation between ages 7 and 17. Error
 525 bars represent standard deviation.

526 [Supplementary text](#)

527

528 **SWS Cohort**

529 The Southampton Women's Survey (SWS) is an ongoing, prospective cohort study of 12,583, initially
530 non-pregnant, women aged 20–34 years, living in the city of Southampton, UK (39). Assessments of
531 lifestyle, diet and anthropometry were performed at study entry (April 1998–December 2002).

532 Women who subsequently became pregnant were followed through pregnancy and their offspring
533 through infancy and childhood. Follow-up of the children and sample collection/analysis was carried
534 out under Institutional Review Board approval (Southampton and South West Hampshire Research
535 Ethics Committee, references 276/97, 307/97, 153/99w, and 10/H0504/30) with written informed
536 consent. Here we focus on the follow-up of the children aged 11-13 years which included epigenetic
537 data.

538 **Early life measures**

539 Birth weights were recorded by midwives attending the birth and weight at 12 months using hospital
540 digital scales (Seca Ltd, London) that were regularly calibrated. In order to adjust for sex and
541 gestational age and also to compare with reference values for the population, birth weight
542 measurements were expressed as z-scores compared with the 1990 British Growth Foundation (CGF)
543 data, and were used to determine the rapid weight gain and rapid thrive variables. Rapid thrive was
544 determined as $z\text{-score}_{12m} - r \times z\text{-score}_{\text{birth}}$, where r was the cohort regression coefficient ($r=0.276$) of
545 the linear model with z-score at 12month as the outcome with birthweight z-score as the exposure.
546 RWG and RT were analysed as categorical variables of a $>+0.67$ standard deviation change.

547 **DNA extraction**

548 Genomic DNA (gDNA) was extracted from whole blood samples using the QIAamp Blood DNA mini
549 kit (Qiagen). Quality of the genomic DNA was assessed by agarose gel electrophoresis and quantity
550 of gDNA was checked on the NanoDrop ND-1000 (NanoDrop Technologies).

551 **Infinium Human MethylationEPIC BeadChip array**

552 DNA methylation using the Infinium Human MethylationEPIC BeadChip array was used to
553 interrogate DNA methylation in 107 whole blood samples. 1µg of the genomic DNA was treated with
554 sodium bisulfite using Zymo EZ DNA Methylation-Gold kit (ZymoResearch, Irvine, California, USA,
555 D5007) and processing of the Human MethylationEPIC (Infinium Methylation EPIC; Illumina, Inc. CA,
556 USA) platform was carried out by the Centre for Molecular Medicine and Therapeutics (CMMT)
557 (<http://www.cmmt.ubc.ca>). The idat files were processed in R v3.5.2. Estimation of white blood cell
558 counts was done using the Houseman algorithm. CpGs with a high detection p-value ($p > 0.01$),
559 beadcount < 3, cross-reactive and polymorphic probes identified by Pidsley et al., (59) and probes on
560 sex chromosomes were removed prior to downstream analysis (final number of CpGs=792,718).
561 However, only CpG sites that were differentially methylated ($p_{FDR} < 0.05$) in the ALSPAC analysis were
562 examined in the SWS samples.

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569 Supplementary tables and figures

570

571 **Table 1 Body composition of ARIES participants age 7 and 17**

	Age 7		Age 17	
	n	Mean (SD)	n	Mean (SD)
BMIz	870	0.15(1.03)	749	0.36(1.12)
	n	%	n	%
Healthy weight	753	87.25	597	80.68
Overweight/obese	110	12.75	143	19.32
Total	863		740	

572 Proportion (%) of study members in healthy weight or overweight/obese and mean BMIz and
 573 standard deviation (SD). n, sample size; %, column percentage.

574

575

576 **Table 2 Linear associations between CpG sites (age 7) and RT.**

Exposure	n	CpG name	Chr	Nearest gene	Model	Coefficient	SE	p value
With cell counts								
RT	116	cg01379158	17	<i>NT5M</i>	SVA	0.0078	0.0025	0.0022
Without cell counts								
RT	125	cg01379158	12	<i>NT5M</i>	SVA	0.0065	0.0025	0.0107
RT	125	cg11531579	12	<i>CHFR</i>	SVA	0.0056	0.0028	0.0493

577 Models are adjusted for age, sex, and surrogate variables, both with or without adjustment for cell
 578 counts. Estimates represent beta coefficients. n, sample size; SE, standard error; SVA, surrogate
 579 variable analysis.

580

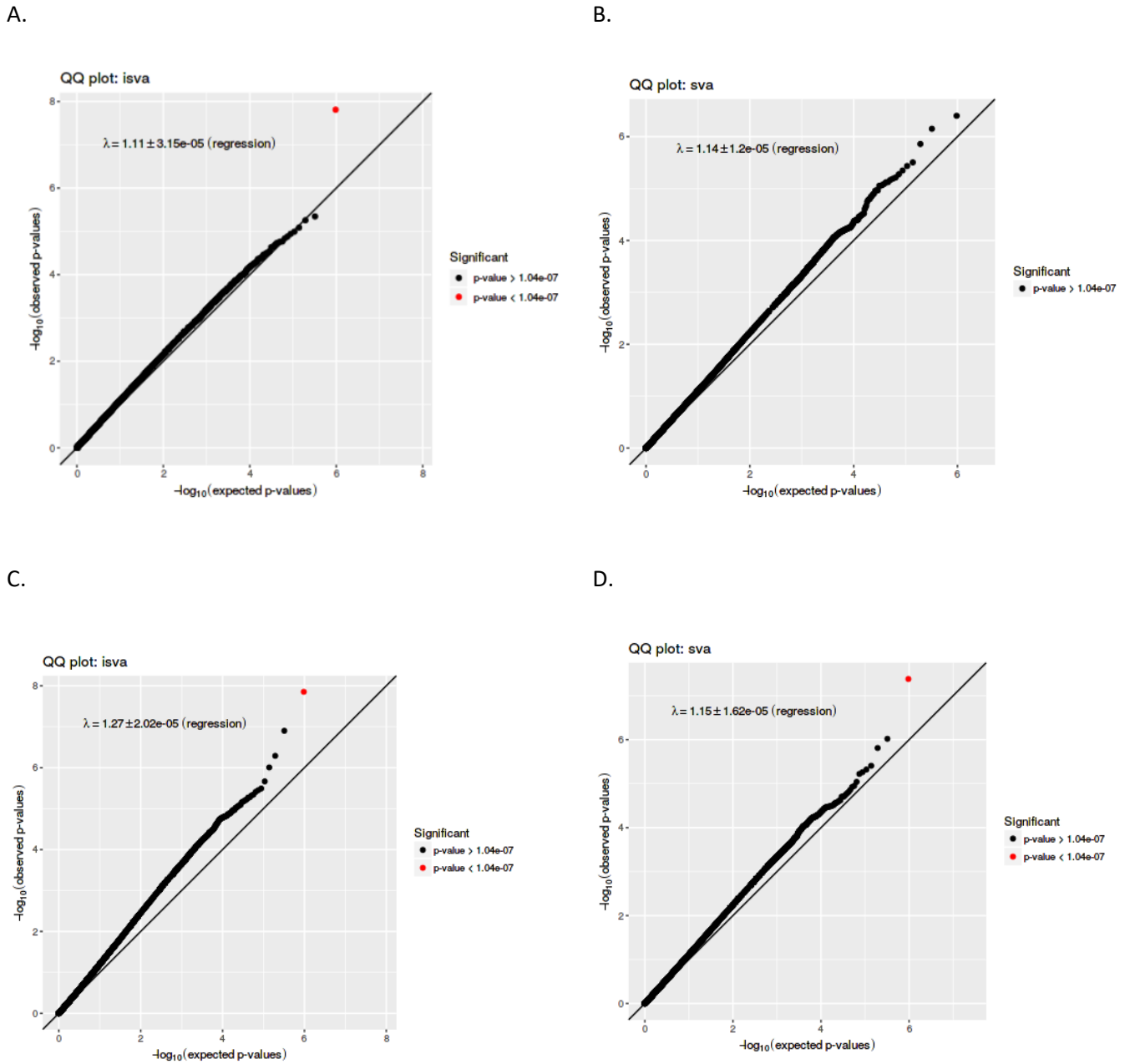
581

582 Table 3 Summary statistics of CpG methylation (age 7) at the identified differentially methylated loci (age 7)
 583 by phenotype (healthy weight not healthy weight) at ages 7 and 17

		N	Mean	SD	Median	Min	Max	N	Mean	SD	Median	Min	Max						
Age 7		cg01379158						Age 7						cg11531579					
No RWG	Healthy weight	74	0.069	0.017	0.068	0.033	0.125	74	0.035	0.011	0.033	0.016	0.084						
	OWOB	10	0.075	0.021	0.071	0.042	0.106	10	0.039	0.011	0.041	0.024	0.054						
RWG	Healthy weight	35	0.078	0.025	0.076	0.033	0.149	35	0.045	0.02	0.039	0.02	0.118						
	OWOB	6	0.091	0.019	0.09	0.064	0.114	6	0.057	0.013	0.059	0.042	0.075						
	Total	125	0.073	0.021	0.073	0.033	0.149	125	0.04	0.015	0.035	0.016	0.118						
p value		0.017						< 0.001											
Age 17		cg01379158						Age 17						cg11531579					
No RWG	Healthy weight	54	0.069	0.017	0.069	0.039	0.103	54	0.036	0.012	0.033	0.023	0.084						
	OWOB	19	0.068	0.018	0.067	0.033	0.106	19	0.036	0.01	0.035	0.016	0.054						
RWG	Healthy weight	28	0.076	0.026	0.074	0.033	0.149	28	0.041	0.014	0.037	0.02	0.086						
	OWOB	9	0.085	0.021	0.08	0.056	0.118	9	0.059	0.027	0.057	0.025	0.118						
	Total	110	0.072	0.02	0.071	0.033	0.149	110	0.039	0.015	0.035	0.016	0.118						
p value		0.0801						< 0.001											

584 Values represent beta values. RWG, rapid weight gain; n, total in each group; SD, standard deviation; min,
 585 minimum; max, maximum. P value from ANOVA. Bold represents the high-risk phenotype of RWG in infancy
 586 and subsequent OWOB, which has higher DNAm levels (relative to the other categories).

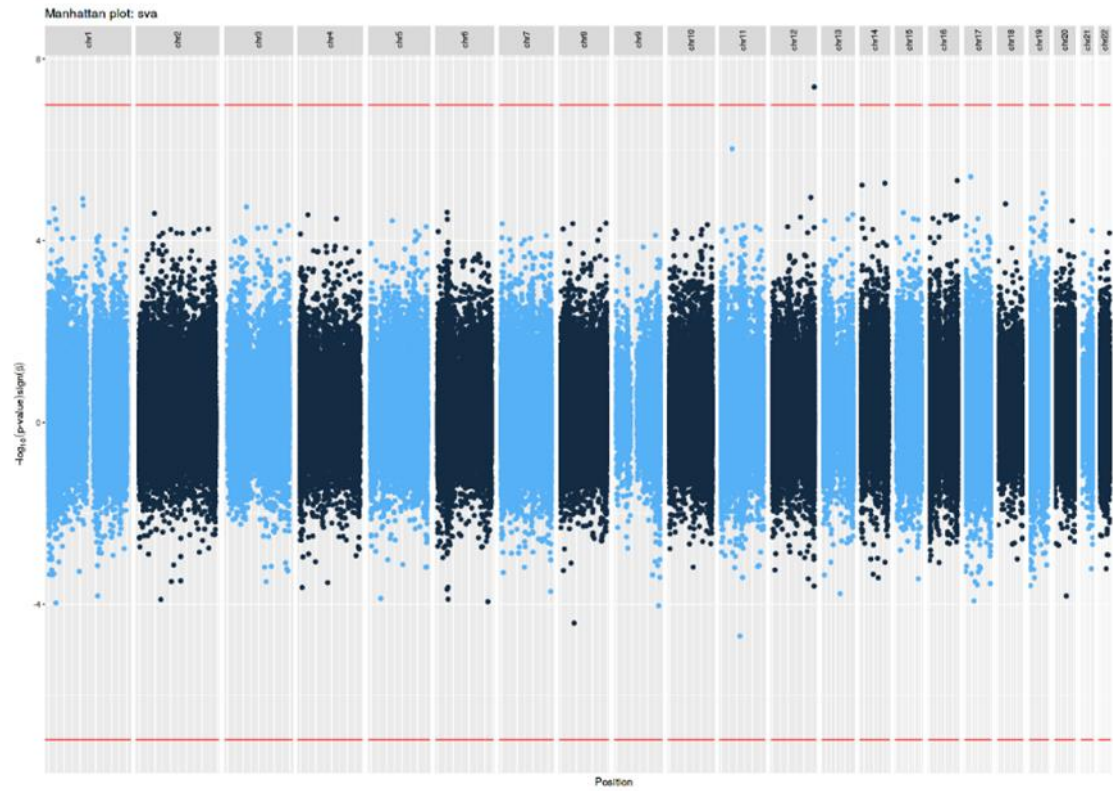
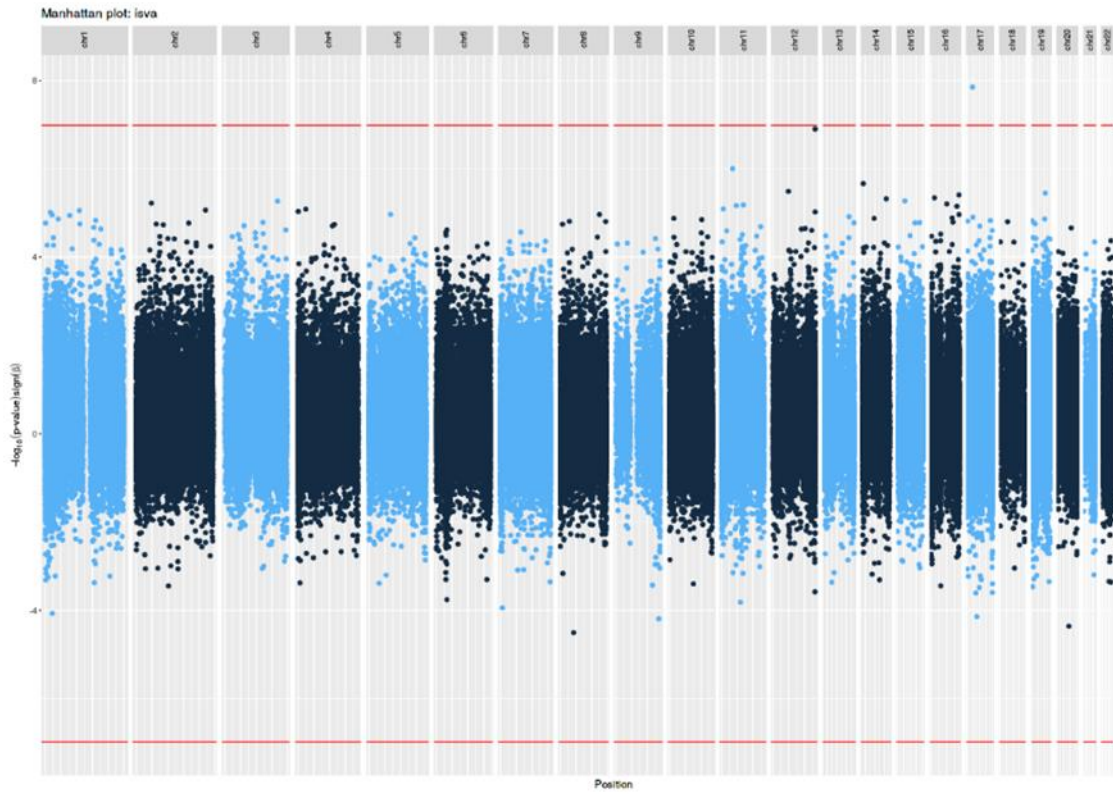
587



588 *Figure 1* Q-Q plots for RWG models (SVA, ISVA) both with (A, B) and without (C,D) adjustment for cell counts.
 589 *The Q-Q plots present the distribution of the p value for the association between CpG site methylation and RWG. The*
 590 *straight line is the expected distribution under the null hypothesis.*

591

Age 7, RWG



Age 7, RT

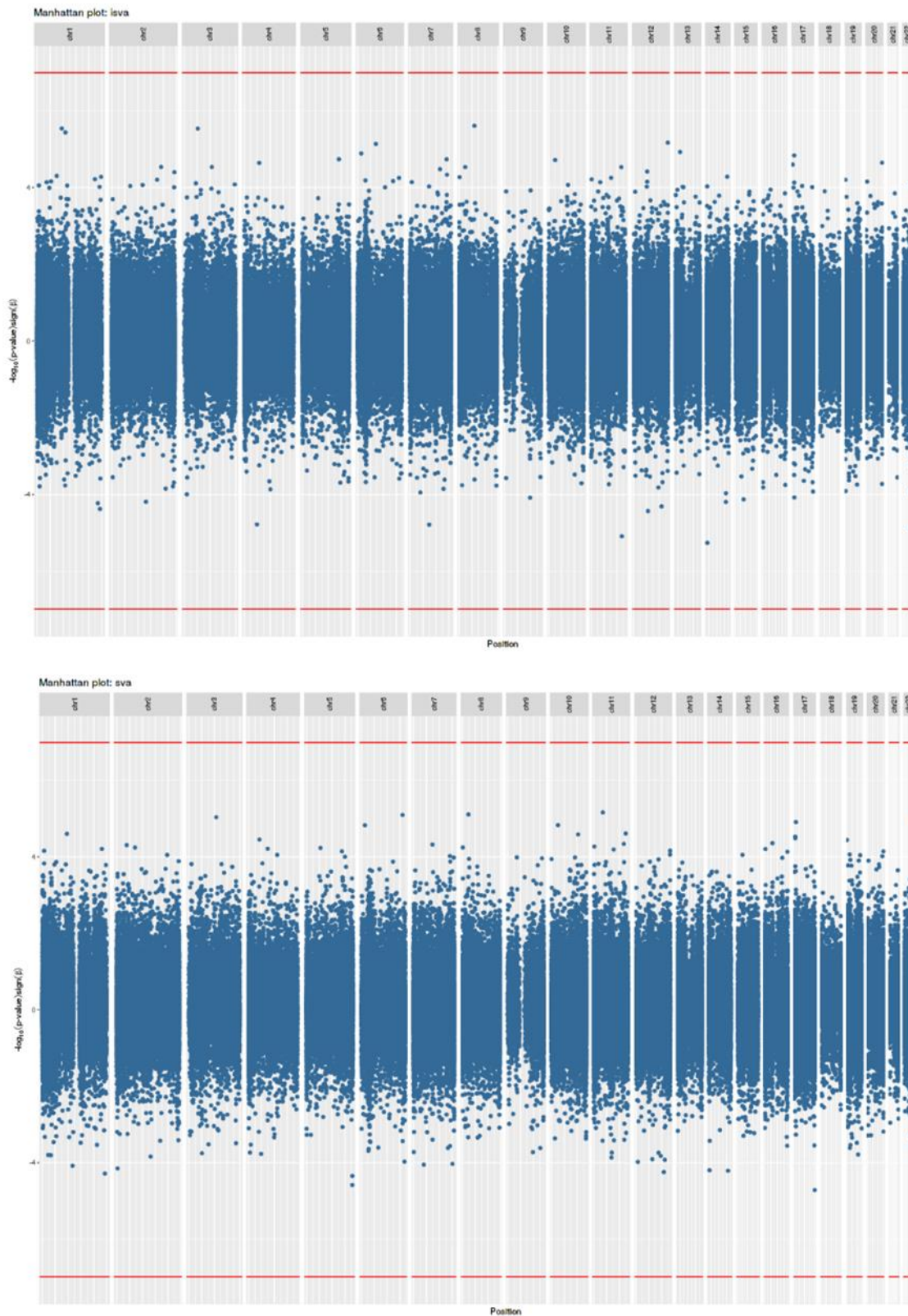


Figure 2 Bi-directional Manhattan plots of the EWAS analysis of RWG and RT for the ISVA and SVA models without cell counts.

The x-axis represents the chromosomes and the y-axis shows the $-\log_{10}(P)$. The red line indicates the Bonferroni-corrected epigenome-wide threshold ($p < 1.04 \times 10^{-7}$). Positively associated loci are displayed in the positive y-axis and negatively associated loci are displayed in the negative y-axis.

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