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Report on the persistence of DNA methylation differences related to early-life stressors

Work package 8 – Task 8.3 – Deliverable 8.3

Authors:
Rae-Chi Huang (UWA), in collaboration with the WP8 partners

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LIFECYCLE Project
Coordination and leadership:

Erasmus MC
Generation R (Na-2918)
PO box 2040
3000 CA Rotterdam
The Netherlands

Phone: +31 (0)10 704 3405
Email: lifecycle@erasmusmc.nl
Web: lifecycle-project.eu

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LIFECYCLE Project
Coordination and leadership:
Erasmus MC
Generation R (Na-2918)
PO box 2040
3000 CA Rotterdam
The Netherlands
Phone: +31 (0)10 704 3405
Email: lifecycle@erasmusmc.nl
Web: lifecycle-project.eu

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1. Summary

Background: Differential DNA methylation at birth, which may underlie associations of early-life stressors and later health variably persists through to childhood and early adulthood.

Aim: Task 8.3 aims to assess the persistence of differences in DNA methylation related to various early-life stressors into childhood and young adulthood. It follows on directly from Task 8.2 which studied the associations of early-life stressors with DNA methylation at birth.

Method:
Across the LifeCycle project, DNA methylation array data are available at various ages, including childhood (5-10 years), adolescence (15-17 years) and adulthood (31- 46 years).

We examined
a. whether DNA methylation differences related to early-life stressors detected at birth, were also present in childhood to early adulthood.
b. whether early-life stressors were associated with DNA methylation changes in childhood and early adulthood.

In the first instance, specific DNA methylation sites associated with early-life factors at birth in cord blood are tested as a look up at later ages. In the second, early-life factors were related to DNA methylation at later ages by epigenome-wide association studies (EWAS).

For the EWAS analyses, each individual cohort runs the analyses on their own data, using a structured analysis plan. After the cohort-specific analyses, results (summary statistics) are shared with the leading center and combined in a meta-analysis. Some projects have been performed in single studies or represent collaborations of a LifeCycle partner with one or more external cohorts.

As DNA methylation is known to be age-related, the meta-analyses are performed within age brackets.

Results: Multiple studies have been done or are ongoing. The cumulative results to date, which are part of this report, are summarised by **Table 1** below.

LIFECYCLE Project
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Erasmus MC
Generation R (Na-2918)
PO box 2040
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Early life factor	Persistence			Number of CPGs persisting from birth	Note
	Childhood	**Adolescence**	**Late adult life**		
Robust Persistence					
Maternal Smoking in Pregnancy (8)	-	**Y**	**Y**	3/8	
Maternal Smoking in Pregnancy (9)	-	**Y**	-	23/23	
No Persistence					
Gestational Age (4)	N	N	-	0/1276	
GWG (unpublished)	N	N	-	0/310	
Some Persistence, possible waning over time or insufficient powered studies					
Maternal BMI (1)	-	**Y**	-	72/86	
Prenatal air pollution (2)	**Y**	N	-	2/6	
Residential proximity to roadway (3)	**Y**	-	-	4/4	Not replicated in 2nd Cohort
Hypertensive disorders of pregnancy (5)	**Y**	**Y**	-	4/43	Single cohort only
Maternal antidepressant use (6)	**Y** (early childhood only)	-	-	1/1	Single Cohort only
IVF versus ICSI (unpublished)	**-**	**Y**	-	5 CpGs differentially methylated	Single Cohort only
Null finding at birth					
Maternal Haemoglobin (7)	N	N	-		No associations detected in childhood and adolescence separate from persistence
Paternal BMI (10)	N	-	-		No associations detected in childhood separate from persistence

Table 1: Cumulative results to date of analyses of persistence of differential DNA methylation in relation to early-life exposures into childhood, adolescence and adulthood.
BMI: body mass index, GWG: gestational weight gain, ICSI: intra-cytoplasmic sperm injection, IVF: in vitro fertilization

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Conclusion: Some DNA methylation changes detected at birth associated with early-life stressors persist into older age.
Three major patterns of DNA methylation persistence are observed.

1. robust persistence, such as that observed with maternal smoking.
2. no persistence whatsoever. The most extreme example of this is that observed with gestational age.
3. some persistence into childhood that wanes with increasing age. Examples of this include particulate air pollution and prepregnancy maternal BMI.

It may become apparent with increasingly larger studies that some of the waning persistence is due to underdetection with insufficient power of studies.

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2. Introduction

Work package 8 of the LifeCycle Project focuses on using the DNA methylation and RNA expression data to assess biological pathways underlying associations of early-life stressors and later life health outcomes. The **specific objective of Task 8.3** is to assess the persistence of any differences in DNA methylation related to early-life factors.

To date, evidence suggests that some DNA methylation differences related to early-life experience persist into adolescence and are stable throughout life, for example those related to maternal smoking. Others appear to have diminishing effects as the offspring become older.

In this report, we first report follow-up look ups of those changes related to early-life exposures. We also report EWAS between early-life factors and DNA methylation in childhood and adolescence.

Where appropriate, we have expanded the collaboration to other studies beyond the LifeCycle partners, mostly through links with the Pregnancy And Childhood Epigenetics (PACE) Consortium, in which multiple LifeCycle partners have leading roles. This increases sample size and thus power in the analyses, while at the same time allowing for a more detailed exploration of heterogeneity and consistency of effects between different populations.

3. Description of progress and results

Below, we present the progress and results for this deliverable. There are multiple completed and ongoing projects under this task. For each completed project, we present a short description of the work performed. Following that, we briefly describe the ongoing projects.

Where the initial data for early-life factor association with DNA methylation at birth (cord blood) has previously been reported for task 8.2, this is indicated and we will only report the relevant task 8.3 associations with DNA methylation in childhood and adult life. The original associations between early-life factors and cord blood DNA methylation will not be repeated other than to give context.

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Erasmus MC
Generation R (Na-2918)
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3000 CA Rotterdam
The Netherlands
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Scientific output

Maternal BMI at the start of pregnancy and offspring epigenome-wide DNA methylation (1)

Partner(s) involved: ERASMUS, ISGLOBAL, UNIVBRIS, UMCG, NIPH, UWA, INSERM;
Background results reported in task 8.2: The meta-analysis of 19 cohorts (9,340 mother-newborn pairs) revealed small methylation variation at 86 CpG sites throughout the epigenome before and after adjustment for cell proportions.
Summary for task 8.3: At 72 of the 86 CpG sites, the direction of the association was the same in newborns and adolescents. These suggest persistence of signals at these 72 sites. (**Figure 1**)
The effect estimates were remarkably consistent between the two age groups at some sites. In particular, six of the top 10 sites with the largest effect size in the cell-adjusted newborn analysis also had the largest effect size amongst adolescents.
Conclusion: There is consistency from birth to adolescence of 72 sites, and similar effect estimates in particular for 6 of 10 top sites. This could be due to either i) an intrauterine influence of maternal pre-pregnancy BMI on variation in offspring DNA methylation that persists to adolescence, ii) confounding by shared familial genetic and/or environmental influences on maternal BMI and offspring methylation that remain stable over time, or iii) the possibility that both maternal pre-/early-pregnancy and the adolescent's own BMI have independent effects on the child's methylation. This study cannot comment on which of the possibilities is most likely.

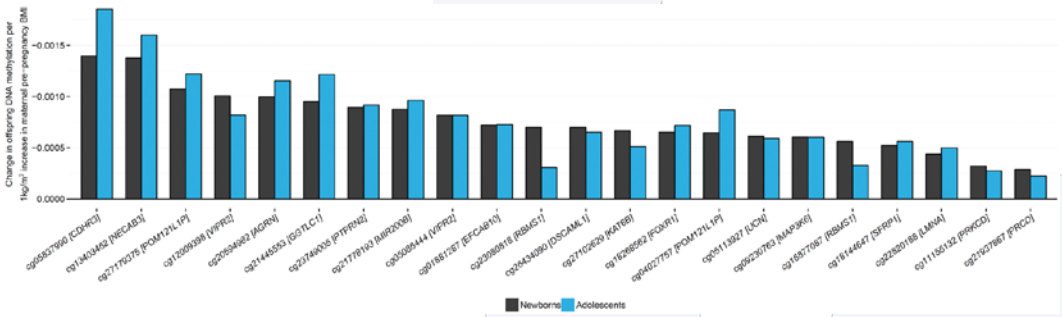

Figure 1: Comparison of estimates of the effect of maternal BMI on offspring DNA methylation at birth and at adolescence. Of the 86 sites where maternal BMI at the start of pregnancy was associated with newborn blood methylation, 72 had the same direction of association in the analysis of adolescents. Plotted here are the 22/86 methylation sites with a *P*-value < 0.05 in the analysis of adolescents, ordered by effect size in newborns.

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Prenatal particulate air pollution and DNA methylation in newborns: an epigenome-wide meta-analysis (2)

Partner(s) involved: ERASMUS, ISGLOBAL, UNIVBRIS, INSERM, UNITO, UOC;

Background: Prenatal exposure to air pollution has been associated with childhood respiratory disease and other adverse outcomes. Epigenetics is a suggested link between exposures and health outcomes.

Background results reported in task 8.2: We investigated associations between prenatal exposure to particulate matter (PM) with diameter <10 (PM_{10}) or < 2.5 µm ($PM_{2.5}$) and DNA methylation in newborns and children.

We meta-analyzed associations between exposure to particulate matter (PM) with diameter <10 (PM_{10}) (n = 1,949) and < 2.5 µm ($PM_{2.5}$) (n = 1,551) at maternal home addresses during pregnancy and newborn DNA methylation assessed by Illumina Infinium HumanMethylation450K BeadChip. Six CpGs were significantly associated (false discovery rate (FDR) < 0.05) with prenatal PM_{10} and 14 with $PM_{2.5}$ exposure.

Summary for task 8.3: Four of the six FDR-significant CpGs identified as differentially methylated in relation to prenatal PM_{10} in our discovery meta-analysis sample of 1,949 newborns showed significance later in childhood in associations with prenatal PM_{10} exposure; cg00905156 (*FAM13A*) and cg06849931 (*NOTCH4*) showed increased methylation in relation to PM_{10} exposure during pregnancy in the combined BAMSE Epigene and MeDALL samples (n=692) of 7- to 9-y-olds (P=0.03), although the direction of association for cg06849931 was opposite to the one in the discovery analysis. Furthermore, cg06849931 was also differentially methylated in the HELIX study (P=0.002), along with cg18640183 (*P4HA2*) (P=0.03), both demonstrating the same direction of association as those in the discovery meta-analysis. In addition, cg15082635 (*GNB2L1*; *SNORD96A*) was also nominally significant in 7-to 9-y-olds from the ALSPAC study with the same direction of association (P=0.02). None of these six associations was present in adolescents from the BAMSE (n=198) and ALSPAC (n=903) studies (P>0.05). (**Table 2**) Interestingly, the children's concurrent PM_{10} exposure at the time of biosampling was not significantly associated with any of these six CpGs (p>0.05).

Conclusion: Two of the functionally plausible PM_{10}-related CpGs mapped to *FAM13A* (cg00905156) and *NOTCH4* (cg06849931) identified in newborns appeared to persist into middle childhood, but were not detected in adolescence. The direction of the association with the *NOTCH4* site was inconsistent, which needs further investigation. The persistence of these CpG marks may be attenuated with increasing age and time distance from prenatal exposure.

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3000 CA Rotterdam
The Netherlands
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				Discovery: newborns[a]		Replication: newborns	Replication: age 7–9 years				Replication: age 15–16 years	
				(n = 1,949)		ALSPAC (n = 688)	BAMSE EpiGene + MeDALL (n = 692)	HELIX (n = 525)	ALSPAC (n = 901)	BAMSE 16 years (n = 198)	ALSPAC 15 years (n = 903)	
Chr	Position[b]	CpG	Gene[c]	β (p-value)	Direction[d]	β (p-value)	β (p-value)	β (p-value)	β (p-value)	β (p-value)	β (P-value)	
5	180670110	cg15082635	*GNB2L1; SNORD96A*	0.001 (8.29E-08)	↓↑↓↑↑↑↑	−0.0004 (0.17)	<0.0001 (1.00)	0.0001 (0.75)	0.0006 (0.02)	−0.0001 (0.63)	0.00006 (0.05)	
17	9559558	cg20340716	*USP43*	−0.002 (1.50E-07)	↓↑↓↓↓↓↓	0.0011 (0.50)	<0.0001 (0.73)	−0.0013 (0.39)	0.0002 (0.89)	0.0003 (0.19)	0.0004 (0.15)	
4	89744363	cg00905156	*FAM13A*	0.001 (3.55E-07)	↓↑X↑↑↑	−0.0003 (0.33)	0.0017 (0.03)	−0.0001 (0.84)	0.0004 (0.15)	0.0001 (0.72)	0.00001 (0.90)	
3	133524572	cg24127244	*SRPRB*	0.001 (7.33E-07)	↓↑↑↑↑↑↑	−0.00001 (0.97)	<0.0001 (0.77)	0.0002 (0.61)	−0.0003 (0.28)	−0.0002 (0.12)	0.00004 (0.42)	
6	32165893	cg06849931	*NOTCH4*	−0.001 (1.72E-06)	↓↓↓↑↑↓↓	0.0003 (0.81)	0.0022 (0.03)	−0.0023 (0.002)	0.0010 (0.33)	0.00002 (0.95)	−0.0002 (0.30)	
5	131563610	cg18640183	*PAIP2*	0.001 (1.80E-06)	↑↑↑↑↑↑↑	0.0003 (0.44)	0.0006 (0.61)	0.0009 (0.03)	−0.0001 (0.82)	0.0001 (0.53)	0.00001 (0.86)	

Note: β, coefficient for methylation with an IQR increase in prenatal PM_{10} exposure; CHR, chromosome.
[a]Discovery meta-analysis does not include the PRISM or Project Viva cohorts due to missing prenatal PM_{10} data.
[b]Chromosomal position based on NCBI human reference genome assembly Build 37.
[c]UCSC annotated gene.
[d]Direction of methylation for each cohort included in the analysis (INMA, Generation R, CHS, ENVIRONAGE, Rhea, Piccolipiù, EARLI): ↑ = increased methylation, ↓ = decreased methylation, X = not available.

Table 2: Statistically significant CpGs (FDR P < 0.05) associated with IQR increases in prenatal PM_{10} (5.6 µg/m^3) exposure and DNA methylation in newborns (discovery meta-analysis) and replication analyses in newborns, children (age 7-9 years) and adolescents (age 15-16 years).

Residential Proximity to Major Roadways at Birth, DNA Methylation at Birth and Midchildhood, and Childhood Cognitive Test Scores: Project Viva (Massachusetts, USA) (3)

Partner(s) involved: ERASMUS;

Background results reported in task 8.2: As reported in task 8.2, 4 individual CpGs within the *LAMB2* gene were associated with residential-proximity-to-roadways at birth (measured by Geographic Information System) using robust linear regression (FDR < 0.05) within Project Viva. These were not replicated in 641 mother-child pairs in the Generation R Study.

Summary for task 8.3: To evaluate whether the association of those differentially methylated CpG sites at *LAMB2* persisted in midchildhood, we evaluated the association between residential proximity at birth and peripheral blood DNA methylation in blood samples collected when participants were 7–11 y old in Project Viva (n = 415). Associations between roadway proximity at birth and methylation in midchildhood samples differed from corresponding associations with methylation at birth, but remained positive for each CpG site, with each halving of prenatal residential proximity to major roadways associated with increased DNA methylation:

1. 0.37% (95% CI: 0.01,0.76%) at cg05654765 (vs. 0.82% in cord blood);
2. 0.53% (95% CI: 0.07, 1.00%) at cg14099457 (vs. 0.88% in cord blood);
3. 0.22% (95% CI: 0.01, 0.45%) at cg03732535 (vs. 0.19% in cord blood);
4. and 0.56% (95% CI: 0.02, 1.10%) at cg02954987 (vs. 1.08% in cord blood).

Cord blood and midchildhood methylation levels were positively correlated for each of the FDR-significant CpG sites, with Spearman correlation coefficients ranging from 0.53 (for cg03732535) to 0.83 (for cg05654765) (**Figure 2**).

Conclusion: Living close to major roadways at birth was associated with the persistence of cord blood methylation of sites in *LAMB2* to middle childhood in Project Viva. These results need replication in further cohorts.

LIFECYCLE Project
Coordination and leadership:
Erasmus MC
Generation R (Na-2918)
PO box 2040
3000 CA Rotterdam
The Netherlands
Phone: +31 (0)10 704 3405
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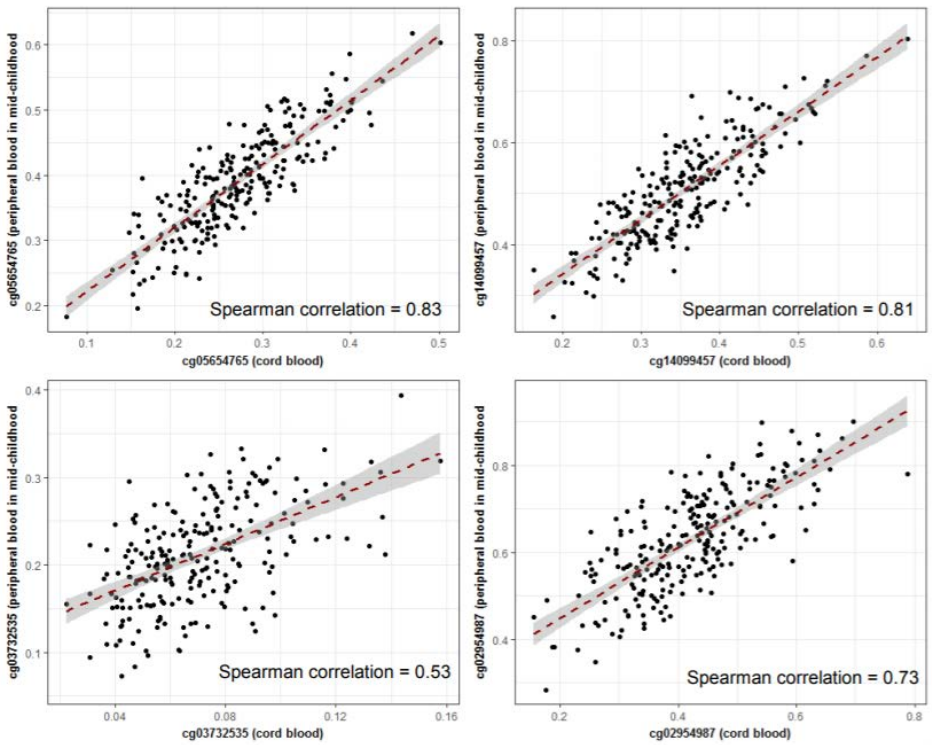

Figure 2: Cord blood and midchildhood methylation levels are positively correlated for each of the FDR-significant CpG sites within *LAMB2*.

Epigenome-wide meta-analysis of blood DNA methylation in newborns and children identifies numerous loci related to gestational age (4)

Partner(s) involved: ERASMUS, ISGLOBAL, UNIVBRIS, NIPH, UOULU, UWA, INSERM;

Summary: Preterm birth and shorter duration of pregnancy are associated with increased morbidity in neonatal and later life. As the epigenome is known to have an important role during fetal development, we investigated associations between gestational age and blood DNA methylation in children.

Background results reported in task 8.2: We performed meta-analysis of Illumina's HumanMethylation450-array associations between gestational age and cord blood DNA methylation in 3,648 newborns from 17 cohorts without common pregnancy complications, induced delivery or caesarean section.

We identified 8,899 CpGs in cord blood that were associated with gestational age (range 27-42 weeks), at Bonferroni corrected significance, $P < 1.06 \times 10^{-7}$, of which 3,343 were novel which were annotated to 4,966 genes. After restricting findings to at least three significant adjacent CpGs, we identified 1,276 CpGs annotated to 325 genes.

LIFECYCLE Project
Coordination and leadership:
Erasmus MC
Generation R (Na-2918)
PO box 2040
3000 CA Rotterdam
The Netherlands
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Summary for task 8.3: We also explored associations of gestational age with DNA methylation measured at 4-18 years in additional pediatric cohorts in a total of 2,481 children. This included 4 cohorts in early childhood (n= 453), 5 cohorts in middle childhood (n=899) and 5 cohorts in adolescence (n=1,129).
Of the 1,276 three or more adjacent genome-wide significant CpGs from our analyses in cord blood, 1,258 CpGs were available for analyses in all older age groups. Out of these CpGs, we observed 40 sites in early childhood, 60 sites in school age, and 60 sites in adolescence to be associated with gestational age at the nominal significance level, $P < 0.05$ with the same direction of effect. However, no CpG survived Bonferroni look-up level correction ($0.05/1258$; $P < 3.97 \times 10^{-5}$). One CpG (cg26385222 annotated to *TMEM176B*) previously associated with gestational age at birth was nominally significant in all age groups with same direction of effect.
Conclusion: The numerous CpGs differentially methylated in relation to gestational age at birth tended not to persist into childhood and adolescence.

Hypertensive disorders of pregnancy and DNA methylation in newborns (5)

Partner(s) involved: ERASMUS, UNIVBRIS, NIPH;
Summary: Hypertensive disorders of pregnancy (HDP) are associated with low birth weight, shorter gestational age, and increased risk of maternal and offspring cardiovascular diseases later in life. The mechanisms involved are poorly understood, but epigenetic regulation of gene expression may play a part.
Background results reported in task 8.2:
We performed meta-analyses to test the associations between either maternal HDP (10 cohorts; n=5,242 [cases=476]) or preeclampsia (PE) (3 cohorts; n=2,219 [cases=135]) and epigenome-wide DNA methylation in cord blood using the IlluminaHumanMethylation450 BeadChip.
In models adjusted for confounders, and with Bonferroni correction, HDP and PE were associated with DNA methylation at 43 and 26 CpG sites, respectively. HDP was associated with higher methylation at 27 (63%) of the 43 sites.
Summary for task 8.3:
Longitudinal analyses (n=108 HDP cases and 550 controls) were performed to examine whether the associations of HDP on offspring differential DNA methylation at CpGs at birth were observed at the same CpGs in blood cells at age ≈7 years (mean age, 7.5 years; SD, 0.1) and at age ≈17 years (mean age, 17.1 years; SD, 1.0). These analyses were conducted in 1 cohort only (ALSPAC) and were restricted to the Bonferroni significant CpGs from the main HDP EWAS. Multilevel models were used, level 1 being the repeat assessments (at birth, 7 years, or 17 years) and within participants (level 2).
Figure 3 shows longitudinal changes in methylation for the top 4 CpGs that reached Bonferroni-corrected P threshold in the main adjusted HDP cord blood EWAS meta-analysis. There were similar increases in methylation levels between birth and adolescence for most of the 43 CpGs in offspring of mothers who experienced HDP and those who did not. For a small number of CpGs, this age-related change was weaker and

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Generation R (Na-2918)
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The Netherlands
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less consistent between 7 and 17 than between birth and 7 years. For all but 1 of the 43 CpGs, there was no strong statistical evidence that age-related change differed between offspring of cases and controls, suggesting that differences persisted but that this was because of general age-related change rather than any further long-term effect of exposure to HDP *in utero*. For the CpG cg08274637 (near *DLEU7* gene), there was evidence that offspring of HDP mothers (compared with those whose mothers who did not experience HDP) had a slightly faster increase in methylation between birth and age 7 (0.27% increased methylation change per year; 95% CI, 0.13%–0.41% methylation change per year; P=0.0002).

Conclusion: In longitudinal analyses conducted in 1 study (n=108 HDP cases; 550 controls), there were similar changes in DNA methylation in offspring of those with and without HDP up to adolescence.

The findings suggest that the association of HDP with differential methylation in cord blood remains, but there is no further effect (beyond what is seen at birth) of HDP on differential DNA methylation at these CpGs. These findings do need replication in larger numbers and other cohorts.

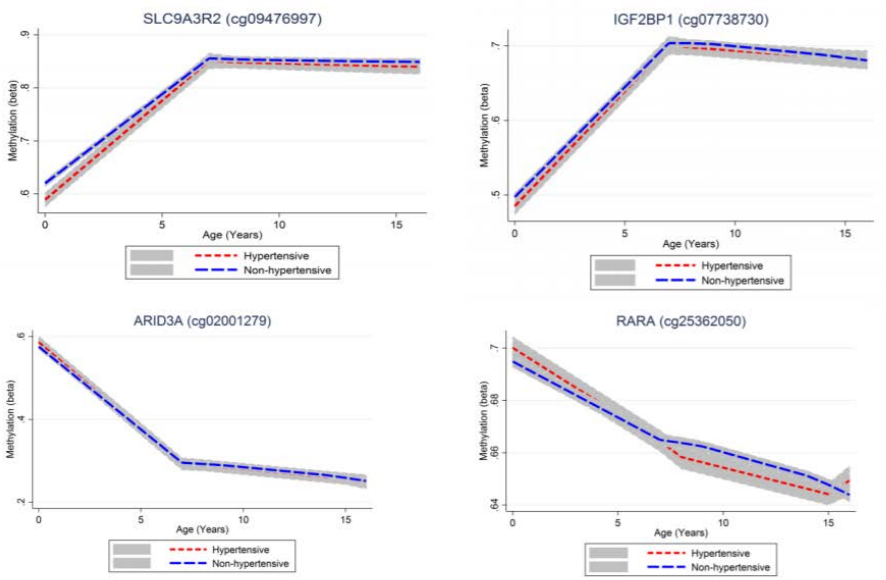

Figure 3: Methylation (proportion of methylated cells) over time for offspring of HDP mothers (dashed (red) line) compared with offspring of non-HDP mothers (dashed (blue) line). Ribbons indicate 95% confidence intervals.

Prenatal maternal antidepressants, anxiety, and depression and offspring DNA methylation: epigenome-wide associations at birth and persistence into early childhood (6)

Partner(s) involved: ERASMUS;

LIFECYCLE Project
Coordination and leadership:
Erasmus MC
Generation R (Na-2918)
PO box 2040
3000 CA Rotterdam
The Netherlands
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Summary: Maternal mood disorders and their treatment during pregnancy may have effects on the offspring epigenome. We aimed to evaluate associations of maternal prenatal antidepressant use, anxiety, and depression with cord blood DNA methylation across the genome at birth and test for persistence of associations in early and mid-childhood blood DNA.
Background results reported in task 8.2: Newborns from Project Viva (n=479) exposed to antidepressants in pregnancy had 7.2% lower DNA methylation (95% CI, - 10.4, - 4.1; P = 1.03×10^{-8}) at cg22159528 located in the gene body of *ZNF575*, and this association replicated in the Generation R Study (β = - 2.5%; 95% CI - 4.2, - 0.7; P = 0.006).
Summary for task 8.3: We evaluated the persistence of the observed association at cg22159528 in the *ZNF575* gene for antidepressants and DNA methylation in Project Viva, in blood collected in early and mid-childhood. In adjusted models, prenatally exposed children (n = 4 out of 120) had 6.2% lower DNA methylation (95% CI − 10.7 to − 1.6; P = 6.70×10^{-3}) compared to non-exposed children in early childhood. This association was in the same direction but attenuated and non-significant in mid-childhood (β = − 3.7, 95% CI − 8.8 to 1.4; P = 0.16) (n = 12 exposed out of 460). Unadjusted differences in DNA methylation were similar to adjusted differences for exposed and unexposed infants at birth, early, and mid-childhood (**Figure 4**).
Conclusion: In Project Viva, the association of maternal antidepressant use with DNA methylation in the *ZNF575* gene persisted in early (β = - 6.2%; 95% CI - 10.7, - 1.6) but not mid-childhood.

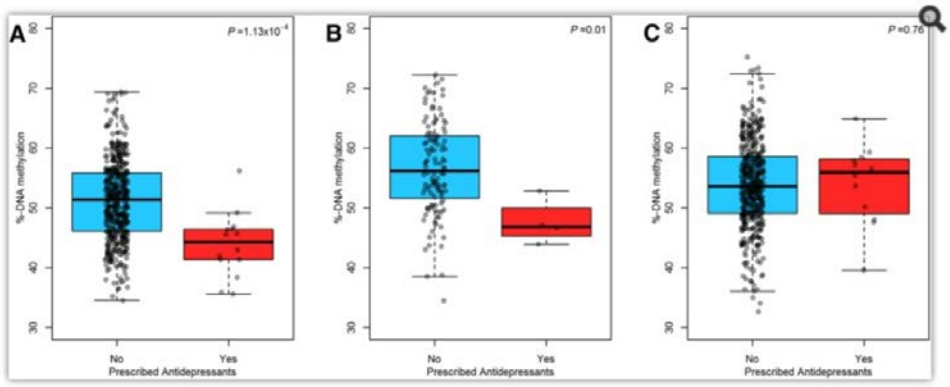

Figure 4: Unadjusted %-DNA methylation distribution for antidepressants exposed and unexposed infants at the replicated CpG site (cg22159528) in the *ZNF575* gene and unadjusted Wilcoxon-rank sum test P value in the discovery cohort, Project Viva, measured at three time points: a. umbilical cord blood (n = 479), b. early childhood (n = 120), and c. mid-childhood peripheral blood (n = 460). One hundred twelve participants in early childhood also had cord blood measurements, and 235 participants from mid-childhood also had cord blood measurement

LIFECYCLE Project
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Maternal haemoglobin levels in pregnancy and offspring DNA methylation in the offspring (7)

Partner(s) involved: UOULU (lead), ERASMUS, ISGLOBAL, UNIVBRIS, UWA, INSERM;
Summary: Altered maternal haemoglobin levels during pregnancy are associated with pre-clinical and clinical conditions affecting the fetus. Evidence from animal models suggests that these associations may be partially explained by differential DNA methylation in the newborn with possible long-term consequences. To test this in humans, we meta-analysed the epigenome-wide associations of maternal haemoglobin levels during pregnancy with offspring DNA methylation in 3,967 newborn cord blood and 1,534 child and 1,962 adolescent whole-blood samples derived from ten cohorts. DNA methylation was measured using Illumina Infinium Methylation450K or MethylationEPIC arrays covering 450,000 and 850,000 methylation sites, respectively. There was no statistical support for association of maternal haemoglobin levels with offspring DNA methylation either at individual methylation sites or clustered in regions.
Conclusion: No association between maternal haemoglobin in childhood or adolescent was detected. However, since no associations were detected in cord blood either, the conclusion maybe that maternal haemoglobin does not appear to induce DNA methylation changes and conclusions on persistence of changes cannot be made. For most participants, maternal haemoglobin levels were within the normal range in the current study, whereas adverse perinatal outcomes often arise at the extremes. Thus, this study does not rule out the possibility that associations with offspring DNA methylation might be seen in studies with more extreme maternal haemoglobin levels.

Association of maternal prenatal smoking GFI1-locus and cardio-metabolic phenotypes in 18,212 adults (8)

Partner(s) involved: ISGLOBAL, UNIVBRIS, NIPH, UOULU (lead), UWA, INSERM;
Summary: DNA methylation at the *GFI1*-locus has been repeatedly associated with exposure to smoking from the fetal period onwards.
We meta-analysed the association between DNA methylation at the *GFI1*-locus (8 CpGs) with maternal prenatal smoking, adult own smoking in five studies (ALSPAC, NFBC1966–31 yr, NFBC1966–46 yr, NFBC1986, and RAINE) participated in the meta-analysis of associations between the eight *GFI1*-CpGs and maternal prenatal smoking (n = 4,230). DNA methylation at the *GFI1*-locus was measured in whole-blood. Multivariable regression models were fitted to examine its association with exposure to prenatal and own adult smoking.
Findings for Task 8.3: Following meta-analysis from five studies, the prenatal maternal smoking exposure status was associated with lower DNA methylation at cg14179389 ($P = 6 \times 10^{-30}$), cg09935388 ($P = 9 \times 10^{-11}$), and cg12876356 ($P = 0.008$). (**Figure 5**) Cg14179389 was found to be the strongest maternal smoking locus and the association was not attenuated when adjusted for age, sex, and adult own smoking ($\beta = -0.03$, $P = 2.0 \times 10^{-27}$, $I^2 = 19.3$). Cg14179389 also did not show association with adult smoking

LIFECYCLE Project
Coordination and leadership:
Erasmus MC
Generation R (Na-2918)
PO box 2040
3000 CA Rotterdam
The Netherlands
Phone: +31 (0)10 704 3405
Email: lifecycle@erasmusmc.nl
Web: lifecycle-project.eu

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status when conditioned by the DNA methylation at the other seven *GFI1*-CpGs. In fact, of the eight CpGs studied, only three of them remained associated with adult smoking following conditional analysis including cg09935388, cg18316974, and cg18146737 ($P < 0.001$).

Conclusion: The association between maternal smoking and three CpGs in the *GFI1*-locus persist into adolesence and into late adult life.

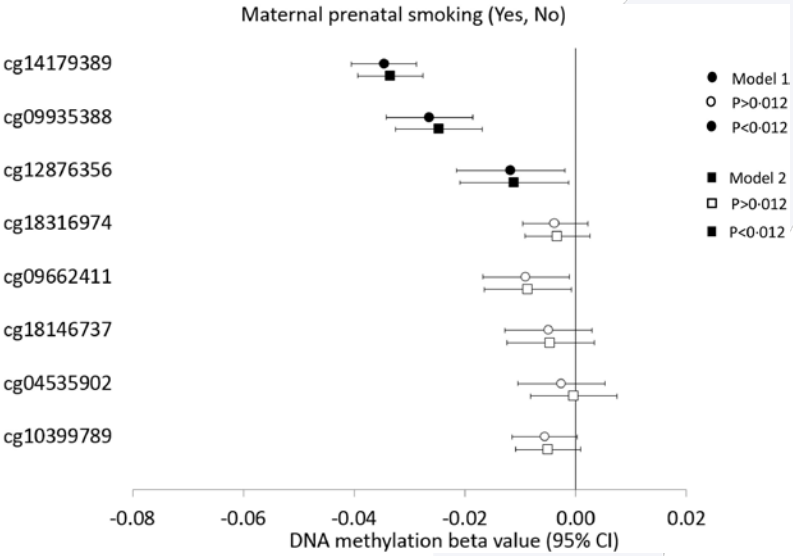

Figure 5: Forest plot showing meta-analysis effect sizes of DNA methylation at eight *GFI1* CpGs by maternal prenatal smoking across five studies (n = 4,230).
Model 1: CpG = maternal prenatal smoking + technical covariates; Model 2: CpG = maternal prenatal smoking + technical covariates + sex + age + adult own smoking.

LIFECYCLE Project
Coordination and leadership:
Erasmus MC
Generation R (Na-2918)
PO box 2040
3000 CA Rotterdam
The Netherlands
Phone: +31 (0)10 704 3405
Email: lifecycle@erasmusmc.nl
Web: lifecycle-project.eu

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Maternal Smoking During Pregnancy Induces Persistent Epigenetic Changes Into Adolescence, Independent of Postnatal Smoke Exposure and Is Associated With Cardiometabolic Risk (9)

Partner(s) involved: UWA;

Summary: Several studies have shown effects of current and maternal smoking during pregnancy on DNA methylation of CpG sites in newborns and later in life. In this study we tested that there are long-term and persistent epigenetic effects following maternal smoking during pregnancy on adolescent offspring DNA methylation, independent of paternal and postnatal smoke exposure.

DNA methylation was measured using the Illumina HumanMethylation450K BeadChip in whole blood from 995 participants attending the 17-year follow-up of the Raine Study. Linear mixed effects models were used to identify differential methylated CpGs, adjusting for parental smoking during pregnancy, and paternal, passive, and adolescent smoke exposure. Additional models examined the association between DNA methylation and paternal, adolescent, and passive smoking over the life course.

We identified 23 CpGs (genome-wide P level: 1.06×10^{-7}) that were associated with maternal smoking during pregnancy, including associated genes *AHRR* (cancer development), *FTO* (obesity), *CNTNAP2* (developmental processes), *CYP1A1* (detoxification), *MYO1G* (cell signalling), and *FRMD4A* (nicotine dependence). A sensitivity analysis showed a dose-dependent relationship between maternal smoking and offspring methylation. (**Figure 6**) These results changed little following adjustment for paternal, passive, or offspring smoking, and there were no CpGs identified that associated with these variables.

Conclusion: This study demonstrates that cigarette smoke exposure establishes persistent changes in DNA methylation into adolescence in a dose-dependent manner. Future studies on current smoking habits and DNA methylation should consider the importance of maternal smoking during pregnancy and explore how the persistent DNA methylation effects of *in utero* smoke exposure increase cardiometabolic risk.

LIFECYCLE Project
Coordination and leadership:
Erasmus MC
Generation R (Na-2918)
PO box 2040
3000 CA Rotterdam
The Netherlands
Phone: +31 (0)10 704 3405
Email: lifecycle@erasmusmc.nl
Web: lifecycle-project.eu

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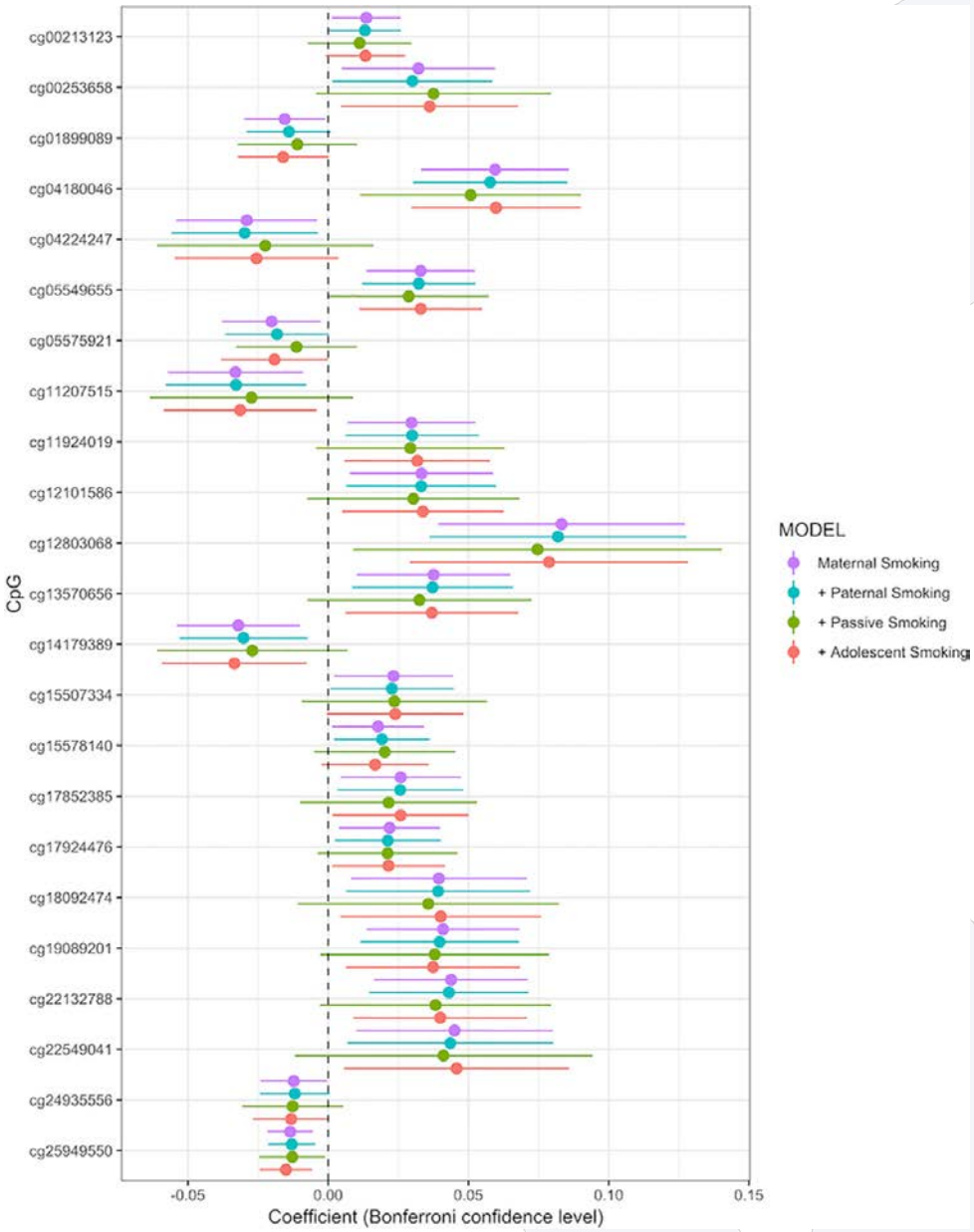

Figure 6: Forrest plot for the epigenome-wide association study with maternal smoking as predictor and individual CpG sites as outcome, adjusted for sex, offspring age, age of the mother, birthweight, gestational weight gain, maternal alcohol consumption during pregnancy, maternal school level, maternal prepregnancy BMI, family income during pregnancy, cell count, and batch effects. Stratified by models without further adjustment (n = 790), adjustment for paternal smoking (n = 692), passive smoking (n = 530), and adolescent smoking (n = 663). X-axis: effect size from the linear mixed effects model and confidence interval; Y-axis: individual CpGs.

LIFECYCLE Project
Coordination and leadership:
Erasmus MC
Generation R (Na-2918)
PO box 2040
3000 CA Rotterdam
The Netherlands
Phone: +31 (0)10 704 3405
Email: lifecycle@erasmusmc.nl
Web: lifecycle-project.eu

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Paternal body mass index before birth and offspring DNA methylation (10)

Partner(s) involved: UNIVBRIS (lead), ERASMUS, ISGLOBAL, UNITO, BTHFT, UOC, NIPH;
Summary: Accumulating evidence links paternal adiposity in the peri-conceptional period to offspring health outcomes. DNA methylation has been proposed as a mediating mechanism, but very few studies have explored this possibility in humans. In this project, we conducted a meta-analysis of epigenome-wide association studies (EWAS) of paternal prenatal Body Mass Index (BMI) (with and without adjustment for maternal BMI) in relation to DNA methylation in offspring blood at birth (13 datasets; total n= 4,894) and in childhood (six datasets; total n = 1,982).
Findings for Task 8.3: We found little evidence of association at either time point: for all CpGs, the False Discovery Rate-adjusted P-values were >0.05. In secondary sex-stratified analyses, we found just four CpGs where there was robust evidence of association in female offspring. To compare our findings to those of other studies, we conducted a systematic review, which identified seven studies, including five candidate gene studies showing associations between paternal BMI/obesity and offspring or sperm DNA methylation at imprinted regions. However, in our own study, we found very little evidence of enrichment for imprinted genes.
Conclusion: Our findings do not support the hypothesis that paternal BMI around the time of pregnancy is associated with offspring blood DNA methylation, even at imprinted regions.

Ongoing projects

Attainment of maternal education associated epigenome-wide DNA methylation changes in the offspring

Partner(s) involved: UOULU (lead), ERASMUS, ISGLOBAL, UNIVBRIS, UMCG, NIPH, INSERM, UWA;
Summary: Maternal education is an important indicator of socio-economic status. It has also been observed to be an important predictor of offspring health outcomes across the life course, such as obesity and type 2 diabetes, and cognitive function. However, the underlying biological mechanisms remain poorly understood. In this study, we aim to explore associations of maternal educational attainment with offspring methylation markers in cord blood and childhood whole blood. In total 25 population-based studies are participating in this project from the PACE Consortium. All cohort data has been received and passed the QC. UOULU and ERASMUS are shadowing each other to work on the data. This includes some analysis to include attrition analysis to account for over representation of some high SES strata.

Exposure to green spaces and genome-wide DNA methylation in placenta and blood

Partner(s) involved: ISGLOBAL (lead), all partners with relevant data have been invited to participate;

LIFECYCLE Project
Coordination and leadership:
Erasmus MC
Generation R (Na-2918)
PO box 2040
3000 CA Rotterdam
The Netherlands
Phone: +31 (0)10 704 3405
Email: lifecycle@erasmusmc.nl
Web: lifecycle-project.eu

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Summary: Urban exposome (built environment, air pollution, road traffic noise, meteorological, natural space and road traffic) affects health outcomes. For instance, we have reported an association between increasing green space exposure and increased birth weight and decreased term low birth weight in 32,000 mother-child pairs. Here we aim to investigate the epigenetic mechanisms that might mediate this association. To this end, we will analyse the association between exposure to green spaces during pregnancy and genome-wide methylation levels, both in the placenta and in cord blood. We will compare significantly associated CpGs between the two tissues and explore persistent effects over time in child blood, also considering postnatal exposures.

This will be the first EWAS in LifeCycle to be done through DataSHIELD. DataSHIELD is an infrastructure and series of R packages that enables the remote and non-disclosive analysis of sensitive research data (https://www.datashield.ac.uk/). To be able to run the EWAS through DataSHIELD we will need (**Figure 7**):

1) A specific R package for omics data in the client site, which is named dsOmicsClient (https://github.com/isglobal-brge/dsOmicsClient) and which has been developed by ISGlobal(https://rpubs.com/jrgonzalezISGlobal/omicsDataSHIELD).
2) A specific R package for omics data in the server site, which is named dsOmics (https://github.com/isglobal-brge/dsOmics) and which has been developed by ISGlobal.
3) A specific R package for dealing with ExpressionSets the server site, which is named resourcer (https://isglobal-brge.github.io/resource_bookdown/resources.html) and which has been developed by EPIGENY, DataSHIELD and ISGlobal

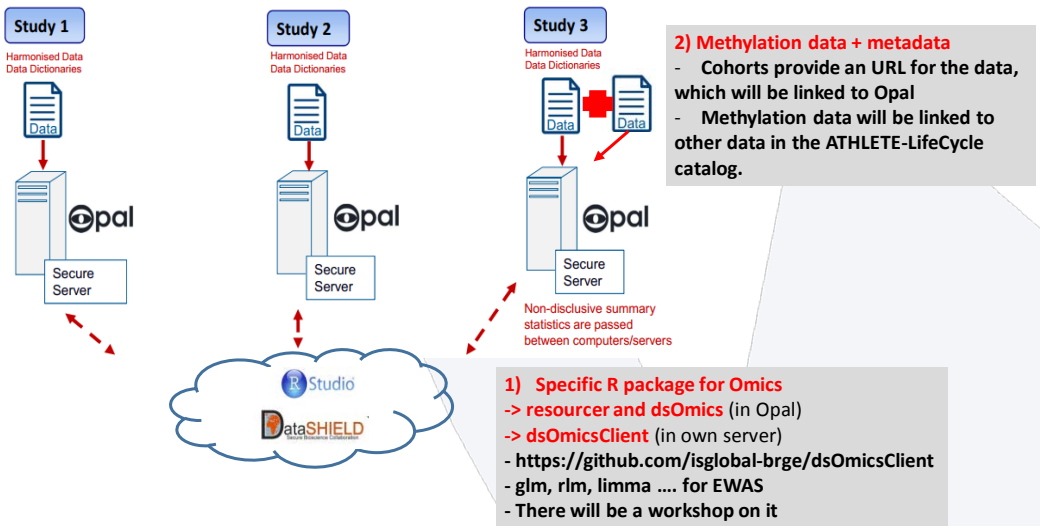

Figure 7: DataSHIELD infrastructure for EWAS, but it can be extended to any other omics

Also, the cohorts will have to save the DNA methylation and some covariates as an ExpressionSet and give the URL link to the leading team to access it through Opal. An

LIFECYCLE Project
Coordination and leadership:
Erasmus MC
Generation R (Na-2918)
PO box 2040
3000 CA Rotterdam
The Netherlands
Phone: +31 (0)10 704 3405
Email: lifecycle@erasmusmc.nl
Web: lifecycle-project.eu

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ExpressionSet is an R object designed to combine several different sources of information into a single convenient structure (**Figure 8**). It contains:

1) A matrix with the DNA methylation data (CpGs in rows and samples in columns)
2) A dataframe, specifically an AnnotadedDataFrame, with metadata (samples in rows and variables in columns). Column names of the methylation matrix have to be the same row names of the metadata.
3) An annotation dataframe describing the probes/CpGs included in the ExpressionSet.

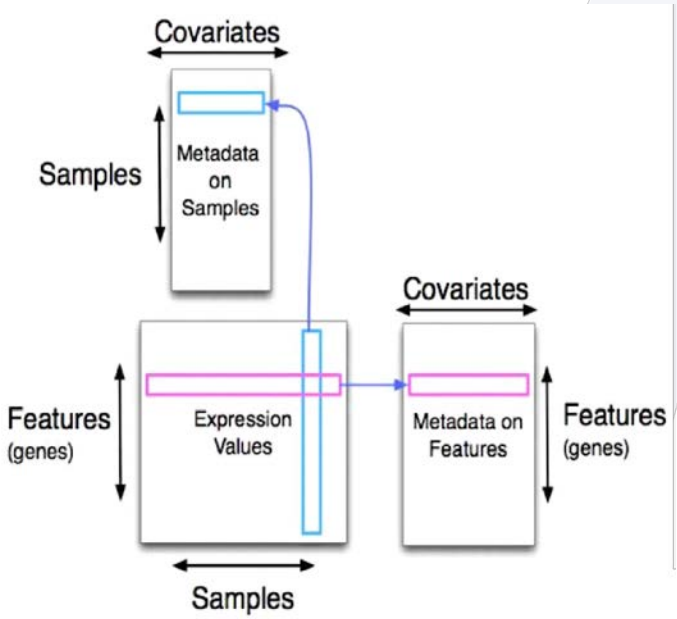

Figure 8: ExpressionSet representation

Exposure to mercury (Hg) during pregnancy and offspring DNA methylation

Partner(s) involved: ISGLOBAL (lead), UNIVBRIS, INSERM;

Summary: Mercury (Hg) is considered by the World Health Organization as one of the chemicals of major public health concern. Exposure to Hg, especially during prenatal life, may cause serious health problems, including impairment of the immune and nervous systems. DNA methylation may mediate the effects of the exposure to Hg during pregnancy. Here, we conducted meta-analysis of EWAS of prenatal Hg (with and without adjustment for maternal fish consumption as its main source) in relation to DNA methylation in offspring blood at birth (5 datasets; total n= 1,500) and in childhood (4 datasets; total n = 1,000).

Only one CpG at *GRK1* gene passed Bonferroni multiple-testing correction, however several top CpGs (P-value < 0.0001) were close to genes previously reported to be affected by mercury in in vitro models. Moreover, some of these CpGs correlated with the expression of the Hg-related genes in blood from HELIX children (HELIX eQTM

LIFECYCLE Project
Coordination and leadership:
Erasmus MC
Generation R (Na-2918)
PO box 2040
3000 CA Rotterdam
The Netherlands
Phone: +31 (0)10 704 3405
Email: lifecycle@erasmusmc.nl
Web: lifecycle-project.eu

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catalog, available at https://helixomics.isglobal.org/). In the next step, we will validate these 5 regions in mouse and human cell lines in collaboration with Joelle Ruegg (Uppsala University) and Karin Broberg (Karolinska Institute).

Gestational weight gain in pregnancy and offspring DNA methylation

Partner(s) involved: UWA (lead), ERASMUS, UOULU, ISGLOBAL (lead), UNIVBRIS;
Summary: Gestational weight gain (GWG) in pregnancy is associated with fetal programming, potentially mediated by DNA methylation.

Eleven Cohorts (n = 4,771) participated in an EWAS meta-analysis to test the effect of GWG on DNA methylation in cord blood.
In the meta-analysis of GWG and DNA methylation measured from cord blood, a total of 102 CpGs were differentially methylated accounting for FDR in cell-adjusted models. With addition adjustment for maternal prepregancy BMI a total of 310 CpGs were significantly differentially methylated with GWG after FDR. Further stratification by investigating maternal prepregnancy overweight/obese (n=1,366) versus normal weight (n=2,225) demonstrated significant associations were mostly observed amongst those with mothers who were overweight/obese (n=11,569 CpGs FDR-corrected).
In the follow-up meta-analysis EWAS of whole-blood in of 7 Cohorts (Raine Study, ALSPAC, Eden, GenR, INMA, VIVA, NFBC) (n=3,395) of children and adolescents, no CpGs were identified associated with GWG that passed FDR.
Conclusion: GWG is associated with DNA methylation at 310 CpGs at birth (cord blood) following FDR adjustment. Differential DNA methylation with GWG was present when prepregnancy overweight/obesity was stratified. None of these CpGs were found to be significantly differential methylated in whole-blood within childhood and adolescence.

DNA methylation patterns within whole blood of adolescents born from IVF are not different from adolescents born from natural conception [Penova-Veselinovic et al. submitted 2020]

Partner(s) involved: UWA;
Summary: Short-term and longer-term studies have investigated the general health outcomes of children born from assisted reproductive technology (ART) treatment, albeit without common agreement as to the cause and underlying mechanisms of these adverse health findings. Growing evidence suggests that the reported adverse health outcomes in in vitro fertilization (IVF)-born offspring might have underlying epigenetic mechanisms.
A total of 288 adolescents and young adults, conceived through IVF treatment, were compared with age-matched counterparts from Generation 2 from the Raine Study.

LIFECYCLE Project
Coordination and leadership:
Erasmus MC
Generation R (Na-2918)
PO box 2040
3000 CA Rotterdam
The Netherlands
Phone: +31 (0)10 704 3405
Email: lifecycle@erasmusmc.nl
Web: lifecycle-project.eu

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The effect of IVF on DNA methylation levels of 238 adolescents mean age 16.06 ± 1.67 years (52.94% male) was compared to 1,188 naturally conceived, age-matched controls, 17.25 ± 0.58 years (50.93% male) from the Raine Study.
Between the two cohorts, no CpGs reached a Bonferroni correction of 1.24×10^{-7} (0.05/402,022), required for statistical significance. When comparing IVF vs. intra-cytoplasmic sperm injection (ICSI) conceived adolescents, after adjustment for participant age, sex, maternal smoking, multiple births and batch effect, five CpGs (cg15016734, cg26744878, cg0331628, cg20235051 and cg20233073) reached a Bonferroni correction of 6.31×10^{-8}. Gene-set enrichment analysis identified one significant gene-ontology pathway neuroactive ligand–receptor interaction (P= 0.00048) after adjusting for age and sex. This pathway has been identified as being linked to addictive disorder.
Conclusion: We observed no significant differences in the DNA methylation profiles of adolescents born from IVF when compared to their naturally conceived, age-matched counterparts, although some differences in the methylation profiles between IVF and ICSI conceived adolescents were detected.
Our findings concur with previous studies that techniques in ART, namely ICSI, may affect DNA methylation levels in neonates that persist to adolescence and diminish with adulthood.

An epigenome-wide meta-analysis of the associations of vitamin B12 concentrations in pregnancy and in newborns with newborn DNA methylation

Partner(s) involved: ERASMUS (lead), ISGLOBAL, UNIVBRIS;
Summary: Suboptimal vitamin B12 concentrations in pregnancy have been associated with lower birth weight, higher body mass index and lower kidney function in the children. Vitamin B12 is a crucial co-factor in the one-carbon metabolism, which comprises several interlinking cyclic metabolic pathways essential for cellular growth and differentiation, nucleic acid synthesis and DNA methylation, among others. As such, concentrations of vitamin B12 *in utero* may affect newborn DNA methylation. We have performed a meta-analysis of epigenome-wide association studies (EWAS) of concentrations of vitamin B12 in pregnancy and in cord blood with offspring DNA methylation. So far, we have identified 109 CpG sites in cord blood at which DNA methylation is associated with maternal vitamin B12 levels in pregnancy. We will perform a look-up of these sites in childhood and adolescence.

LIFECYCLE Project
Coordination and leadership:
Erasmus MC
Generation R (Na-2918)
PO box 2040
3000 CA Rotterdam
The Netherlands
Phone: +31 (0)10 704 3405
Email: lifecycle@erasmusmc.nl
Web: lifecycle-project.eu
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3. Unexpected issues and adaptive action

Analyses have overall been performed according to plan. In general, running these analyses in larger collaborations with external partners may lead to longer timelines than those for studies in single cohorts. However, we do believe that the statistical power that is gained by the (much) larger sample sizes leads to more solid scientific conclusion of this work.
In the last year of the work, COVID-19 related issues have affected the ability of LIFECYCLE members to meet face to face, and in some cases to dedicate as much time to usual research activities. Nevertheless, much of the work has continued by telecommunication and is still largely on track.

4. Conclusion

These analyses as part of Task 8.3, show that there are three major patterns of DNA methylation persistence.

The first pattern is that of robust persistence, such as that observed with maternal smoking. Here the DNA methylation changes appear to persist from birth, throughout adolescence and into middle age and beyond. The work on prenatal smoking within this Task 8.3, show that the DNA methylation changes are stable, persist into later life, are independent of passive smoke and own smoking. As a consequence of this stability, we have created a smoking methylation score through machine learning which predicts maternal smoking from offspring DNA methylation.

The second pattern is that of no persistence whatsoever. The most extreme example of this that observed with gestational age, where thousands of CpGs are differentially methylated according to gestational age at birth, but this is completely attenuated past the neonatal period.

The third pattern is that of some persistence into childhood, but progressive disappearance with increasing age. Examples of this include particulate air pollution, and gestational weight gain and ART (ICSI) where some persistence is noted in early childhood, but is no longer detected by adolescence.

Larger studies with greater power and mechanistic studies to explain the phenomenon on persistence will be required in the future.

LIFECYCLE Project
Coordination and leadership:
Erasmus MC
Generation R (Na-2918)
PO box 2040
3000 CA Rotterdam
The Netherlands
Phone: +31 (0)10 704 3405
Email: lifecycle@erasmusmc.nl
Web: lifecycle-project.eu

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5. List of abbreviations

ART – Assisted Reproductive Technologies
BMI – Body mass index
CI – Confidence interval
CpG site – Cytosine-phosphate-guanine site
DNA – Deoxyribonucleic acid
EWAs – Epigenome-wide association study
FDR – False Discovery Rate
GWG – Gestational weight gain
HDP – Hypertensive Disorders of Pregnancy
ICSI – Intra-cytoplasmic sperm injection
IVF – In vitro fertilization
PACE – Pregnancy And Childhood Epigenetics
PE - Preeclampsia
PM – Particulate Matter
QC – Quality Control
RNA – Ribonucleic acid
SD – Standard deviation

LIFECYCLE Project
Coordination and leadership:
Erasmus MC
Generation R (Na-2918)
PO box 2040
3000 CA Rotterdam
The Netherlands
Phone: +31 (0)10 704 3405
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LIFECYCLE Project
Coordination and leadership:
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Generation R (Na-2918)
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The Netherlands
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