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# EN BIOLOGIE

by

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Born on 20 March 1995 in Thionville, FRANCE

# THE ROLE OF DNA METHYLATION IN THE LIFE COURSE AND AGE ACCELERATION

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# Affidavit

I hereby confirm that the PhD thesis entitled "*The role of DNA methylation in the life course and age acceleration*" has been written independently and without any other sources than cited. All necessary ethical approvals in regards to The ALSPAC human cohort were obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics Committees. Consent for biological samples has been collected in accordance with the Human Tissue Act (2004).

Luxembourg, 19.03.2024 Cyrielle Holuka

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A mes anges gardiens qui m'ont encouragé depuis ma plus tendre enfance, m'ont appris l'importance de prendre le temps et qui désormais veillent chaque jour sur moi

## *Prendre le temps*

*Prendre le temps pour travailler*
*c'est le prix du succès*

*Prendre le temps pour penser*
*c'est la source de la puissance*

*Prendre le temps de la détente*
*c'est le secret de la jeunesse*

*Prendre le temps pour vivre*
*c'est le fondement de la sagesse*

*Prendre le temps pour rire*
*c'est la musique de l'âme*

*Prendre le temps d'être aimable*
*c'est le chemin du bonheur*

*Prendre le temps pour regarder*
*c'est le remède de l'égoïsme*

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# Abstract

Worldwide, ageing represents a major concern. Indeed, ageing is not only characterized by the accumulation of cellular or molecular damage over time and the decrease of physiological capacities, but it induces a higher health care burden, putting considerable pressure on public finances. Early life adversity (ELA) regroups different types of exposure all influencing individual ageing. The ELA label refers to socio-economic status, emotional and environmental exposures. As demonstrated in the Developmental Origins of Health and Diseases (DOHaD) concept, the most sensitive period of life covers the time between conception and age 2 where ELA is mainly occurring. Nonetheless, from conception to adulthood remains a period when ELA can occur. As life circumstances directly interact with biological processes, in this thesis I considered how socio-economic stress as well as early maternal experience can be perceived by their offspring and how this may have epigenomic effects. Following the DOHaD concept, we used the Avon Longitudinal Parents and Children (ALSPAC) cohort to investigate how maternal trauma can affect their child's epigenomes as well as the epigenetic ageing process. Here, we used different approaches to investigate this mother-child association. Firstly, the use of epigenetic clocks (EC) using DNA methylation pattern and considered as an accurate age biomarker, allowed us to demonstrate that child ageing is not associated with maternal trauma exposure. Secondly, the epigenome wide association study (EWAS) approach investigated whether the ageing process is associated to underlying biological mechanisms. Our EWAS models highlighted that maternal experience induces epigenetic imprints, for example in DNA methylation (DNAm), in their children remaining over years. Additionally, we demonstrated that those imprinted marks are associated with biological pathways such as Parkinson disease or oxidative phosphorylation pathways. Within those pathways of interest, we also observed that associated genes such as COX7C known to be associated with ageing were up regulated. On the contrary other genes were down regulated. Finally, we shed into light that mothers possess "directing CpGs" mainly associated with imprinted child's DNAm, suggesting that an epigenetic transmission mechanism took place. Here, in the ALAC project, we answered to three key points of maternal-child association: 1) the in-utero transmission from the individual's mother, 2) the effects of individual life events and statuses prior to the measurement of the epigenetic clock, and 3) changes evaluation in the EC within the same individual as well as the association between levels and changes in the clock between mother and child.

Keywords: DNAm, biological pathways, EWAS, maternal transmission, ALSPAC, socio-economic status

# Aims & Objectives

Early life adversity has been described as a series of environmental, socioeconomic, psychological events representing one of the major type of exposition affecting health trajectories over time. Ageing represents personal experiences that have or might not have affected the immune system leading to an advanced/accelerated senescence process. As personal experiences are unique, it is evident that people will age differently over time as those experiences will be interpreted differentially by their body. Indeed, the ageing process reflects the accumulation of molecular and cellular damages over time and will differ between individuals. The ALAC project is an interdisciplinary approach analysing the effects of socioeconomic factors on the ageing process. Here, we focused on epigenetic modifications such as DNAm, an accurate age biomarker, captured by epigenetic clocks. DNAm changes with age and reflect the life event consequences by each individual. The first part of the project was to investigate how DNA methylation at CpG dinucleotides evolves through adulthood and ageing. By using the large Avon Longitudinal Parents and Children (ALSPAC) cohort and the sub cohort *Accessible Resource of Integrated Epigenomic Studies* (ARIES), and their extraordinary set of lifestyle questionnaires and epigenetic variables, we first calculated the epigenetic age of the participants. The second part of the project assesses the biological consequences due to DNA methylation modifications. To investigate those consequences, we set up case control epigenome-wide association studies to identify biological associations/links between methylation and cellular/molecular processes. Finally, the third part of the project highlights the maternal implication and how maternal experiences define children growth trajectories later in life. The idea behind this final part is to draw specific mechanism of maternal inheritance. Indeed, all along the project we found key elements confirming the epigenetic imprint on the child's genomes. This imprinting mechanism remains over time and continues to evolve over time due to children's experiences i.e. demethylation or re methylation of different CpG sites.

# List of Abbreviations

| | |
|---|---|
| 5mC | 5 methyl Cytosine |
| 6mA | Adenine methylation |
| ACE | Adverse Childhood Experiences |
| ALAC | Age Acceleration and Life Course |
| ALSPAC | Avon Longitudinal Study of Parents and Children |
| ARIES | Accessible Resource of Integrated Epigenomic Studies |
| BER | Base Excision Repair |
| BP | Biological Pathways |
| CMV | Cytomegalovirus |
| COVID-19 | Coronavirus Disease 19 |
| CS | C Section |
| CSE | Childhood Sexual Experiences |
| CTL | Cytotoxic T-Lymphocytes |
| DNAm | DNA methylation |
| DOHaD | Developmental Origins of Health and Diseases |
| EAA | Epigenetic Age Acceleration |
| EC | Epigenetic Clocks |
| ELA | Early Life Adversity |
| EWAS | Epigenome Wide Association Studies |
| GenR | Generation R |
| HIMA | High Dimensional Mediation Analysis |
| HIV | Human Immunodeficiency Virus |
| LGCM | Latent Growth Curve Modelling |
| MBD | Methyl-CpG-Binding Domain proteins |
| MBE | Maternal Birth Experience |
| MD | Maternal Deprivation |
| MeCP2 | Methyl-CpG Binding Protein 2 |
| MFP | Major Financial Problem |

| | |
|---|---|
| OP | Oxidative Phosphorylation |
| PD | Parkinson Disease |
| PoAm | Pace of Ageing |
| PTSD | Post-Traumatic Stress Disorder |
| RNAi | RNA interference |
| sCTL | Cytotoxic Lymphocyte |
| SES | Socio-Economic Status |
| UHFR | Ubiquitin Like With PHD And Ring Finger Domains |
| WHO | World Health of Organization |
| XCI | X Chromosome Inactivation |
| α-HBCDD | α-hexabromocyclododecane |

# Synopsis

Early life adversity (ELA), described as traumatic events encountered during childhood are known to be associated with chronic disease development during adulthood. ELA regroups many different categories such as economic, social or environmental stressors. Adversity occurs throughout the human life but appears to be particularly triggering during sensitive period such as the two first years of life (Gershon et al., 2013). It is well established in the literature that an exposure to such trauma during the very early period of life is critical for the development of the human body and leads to a more severe effect on the adult immune function (Wadhwa et al., 2009).

# I. Early life adversity: The Developmental Origins of Health and Diseases

In Chapter 1, we defined four key components of external stressors all playing a major role in ELA exposure. The four groups are 1. Psychological stress, 2. Infection, 3. Nutrition/Microbiome and 4. Pollution. As described by David Barker in the Developmental Origins of Health and Diseases (DOHaD) theory, the first one thousand days of life are determinant for the optimal development of various systems such as the immune system or the neurological system that are not yet fully developed at birth (Wadhwa et al., 2009). All four can directly alter the immune-senescence mechanism by increasing the number of senescent cytotoxic lymphocyte (sCTL) that can express a large quantity of pro-inflammatory cytokines. Early exposure to ELA can result in a later-life phenotype (modification) with a higher susceptibility to chronic diseases (Dock & Effros, 2011).

As the body is exposed to the external environment exposures throughout life, it remains challenging to fully understand what the consequences of such exposure will be. Indeed, those experiences can differently modulate the genetic programming of individual leading to potentially severe consequences. Although, the individual genetic predisposition playing a major role in the process of “exposure-consequences”, has been already highlighted in the three-hit concept (Daskalakis et al., 2013).

This has been developed into the current three-hit models of susceptibility to diseases development. Those hits are 1. The genetic predisposition, 2. The early life environment and 3. The later life environment (Figure 1).

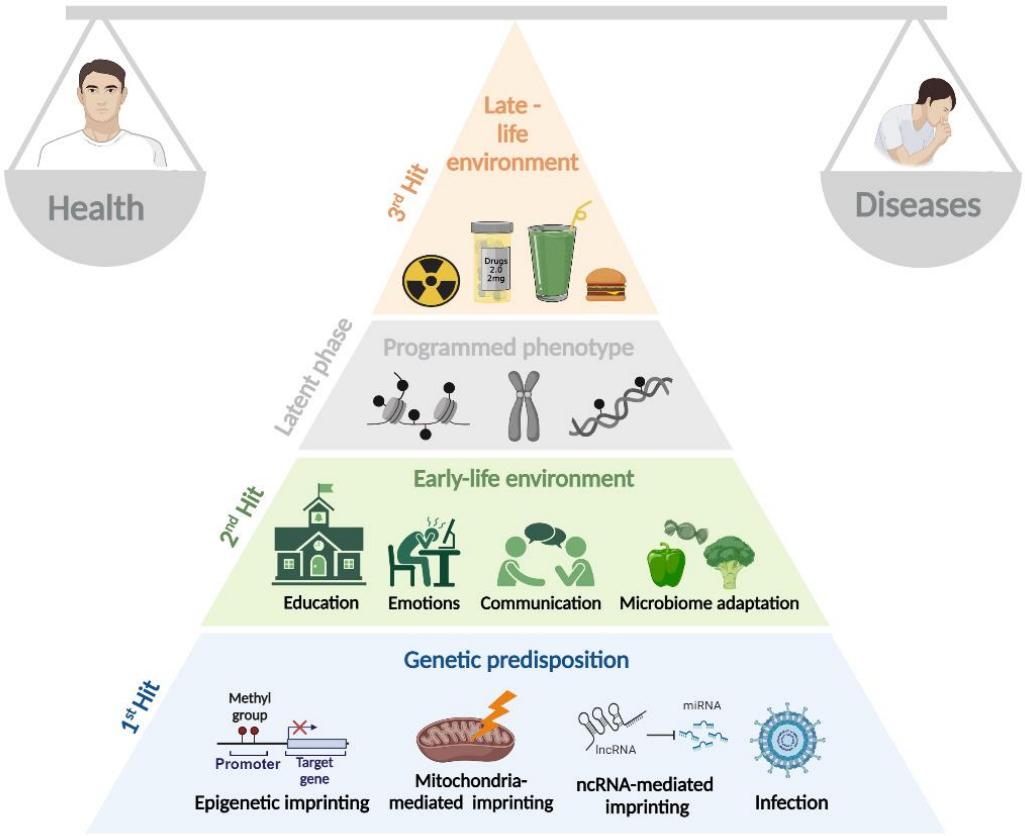


**Figure 1. The three-hit model.**

The bottom of the pyramid in blue represents the 1st and the 2nd hit corresponding to genetic predisposition and early-life environment. The green part represents the latent phase of life while the top of the pyramid in orange represents the 3rd hit describing the late-life environment. The grey bar represents the balance between healthy and sick individuals depending on the three-hit model. (Created with BioRender.com).

## 1. The genetic predisposition

This first hit represents the genetic predisposition. At birth, the infant already possesses genetic material inherited/transmitted by the parents during conception. This genetic material comes from various mechanisms such as epigenetic imprinting, mitochondria mediating imprinting as well as ncRNA-mediated imprinting that might lead to genetic variations development.

Indeed, during the epigenetic imprinting/programming and reprogramming process, specific epigenetic modifications such as DNA demethylation and re methylation occur to allow the establishment of lineage-specific gene expression patterns (Potabattula et al., 2018). Nevertheless, it has been already demonstrated that the DNA demethylation step can fail. This failure can lead to the conservation of parental epigenetic marks inducing a specific genetic predisposition on the

offspring genome (Heard & Martienssen, 2014). However, a genetic predisposition itself unlike an inherited genetic disease i.e. fragile X syndrome, do not assure the development of a specific disease even under external influences also occurring during the first hit. In Chapter 2, we reviewed how epigenetic mechanisms and environmental, social, as well as socioeconomic stressors promote the ELA phenotype. This Chapter focuses on the role played by parental genetic material in the transgenerational inheritance mechanism fully influenced by environmental stressors.

Indeed, in addition to those mechanisms, the first days of life are very sensitive to external stressors such as bacterial or viral infections or lifestyle such as smoking, alcohol, hormonal changes, diet, as well as environmental pressures i.e. pollutants. As we explained in Chapter 1, an early exposure to such determinants will consequently amplify the susceptibility to diseases development as they can alter systems (bio) integrity i.e. biological, physiological and immune systems.

The first hit is considered as the starting point of life as it represents individual's genetic predisposition and might explain individual's susceptibility later in life. As the first hit, both second and third hit respectively representing the overall early life environment and the latest life exposure, are major health determinants later in life.

## 2. The early life environment & programmed phenotype

David Barker reported in the DOHaD theory that the early life period, and more precisely, the first thousand days of life represent one of the most critical period of life. During this period, the human body "encounters" environmental, social, psychological and physical pressures/stressors that will shape each individual. To face those pressures, biological systems constituting the human body will maintain an "alert mode" and continue to evolve. This adaptation process will allow a faster immune response when a similar exposure will occur.

After the second hit, there is "latent phase" where a programmed phenotype lies dormant, although with an increased disease risk. This hit mostly depends on the first hit where the latent epigenetic phenotype set up takes place. Here, the infant possesses (epi) genetic material necessary to maintain an optimal development while being exposed to external stressors. However, many studies stated that ELA induces immune phenotypes differences that will explain disease susceptibility (Hong & Medzhitov, 2023). When exposed to ELA, physiological responses representing the first "adaptive" process take place to promote immediate biological responses. The second mode of adaptation is the "acclimatization" inducing changes in physiology such as

high production of red blood cells. Those changes can be the direct consequence of epigenetic modifications such as DNA methylation.

### 3. The later life environment

As much as the early exposures are crucial for the health "determinant" later in life, every exposure after the first 2 years of life are important as they act as additional pressures on the body. Indeed, all over the life, the human body will face external stressors influencing differently the body and more precisely the immune response. Those late exposures complement the existing phenotype shaped by early life adversity occurring during the childhood and may crystallise the disease risk and "tip the balance" towards disease.

More recently the COVID-19 pandemic encouraged us to take another look at the associations between chronic diseases mainly affecting the immune system associated to a higher susceptibility to develop severe form or not of COVID-19. As well as for chronic diseases, ELA plays a major role in non-communicable diseases such as the COVID-19 infection due to its direct link on the immune system, leading to important "immune" deficiencies i.e. chronic low grade inflammation as well as accelerated immune-senescence. More precisely, we mainly focused on the consequences of socio-economic status (SES) so far underestimated and poorly considered as an official ELA form in the literature. As explained in Chapter 3, socio-economic factors should be considered as a new category of ELA as they induce metabolic changes, especially in the stress response by increasing the cortisol level. Beyond, the stress response, every actual form of SES triggers the biological response and increase the susceptibility to develop infections and/or diseases. As already demonstrated in Chapter 1, early exposure to every type of adversity and throughout life can shape the human responses making each individual unique. Indeed, the same exposure will not affect equally everyone and the consequences can either appear instantly or in a latent form, justifying the complexity of fully understanding the underlying mechanism of ELA.

## II. DNA methylation: structures and functions

Epigenomic including DNA methylation (DNAm), histone modification and microRNA modulation, represents modifications that influence gene expression but do not change the genetic code itself (L. Zhang et al., 2020). DNAm represents one of the most common adaptation mechanism due to external environment. DNAm is an epigenetic mechanism referring to the

transfer of a methyl group ($CH_3$) on the C5 position of the cytosine via DNA methyltransferase enzymes called DNMT (Figure 2, Panel A). DNAm depends on enzymes that can establish, recognize and remove DNAm, respectively called writers, erasers and readers.

The writer enzymes (i.e. methyltransferase) catalysing the addition of the methyl on the cytosine are divided in three components of the DNMT family. DNMT3a or DNMT3b consider as *de novo* DNMT, inducing a new methylation pattern to unmodified DNA strand. DNMT3a and DNMT3b share very similar structural composition, however their gene expression patterns differ. DNMT3a is mostly ubiquitously expressed while DNMT3b remains poorly expressed by most of the differentiated tissues, except for testes, bone marrow and thyroid (Xie et al., 1999). The last writer methyltransferase is DNMT3L that will associate to DNMT3a and DNMT3b to stimulate their methyltransferase activity (Jia et al., 2007). Additionally, studies demonstrated that DNMT3L is indispensable for establishing parental genomic imprinting, compaction of the X chromosome and finally for the methylation of retrotransposons (Kaneda et al., 2004; H. Wu & Zhang, 2011). On the opposite and during the replication process, DNMT1 will copy the existing parental DNAm pattern onto the newly synthesized strand (Figure 2, Panel B and C). Additionally, to mimic the existing DNAm pattern while conserving the original DNAm pattern during cell lineage, DNMT1 has also the capacity to repair DNA methylation. Studies have demonstrated that DNMT1 plays an important role in the dividing cell process as well as in cellular differentiation (Jackson-Grusby et al., 2001).

There are both active and passive DNA erasing processes. For example, the passive DNA demethylation takes place during the cell division. As DNMT1 can maintain DNAm during the replication phase, its inhibition will allow the synthesis of unmethylated cytosine, reducing the methylation level during each division phase leading to the total depletion of the 5-methylcytosine (5mC). On the contrary, active DNA demethylation can take place in dividing and non-dividing cells, but requires additional enzymatic reaction (Drohat & Coey, 2016). One of the most described erasing mechanism is base excision repair (BER) that converts the 5-methyl cytosine to the original cytosine (C) (Bellacosa & Drohat, 2015).

While DNAm can directly reduce gene expression by blocking the binding of transcriptional activators, it can be recognized by different class of proteins themselves inhibiting the transcription factor binding. Those classes are the methyl-CpG-binding domain proteins (MBD) the UHRF and the zinc-finger proteins, all targeting DNAm pattern and sharing a methyl binding activity. The MBD include methyl-CpG binding protein 2 (MeCP2) targeting DNMT1 in order to maintain the DNAm. Similarly to MeCP2, the ubiquitin like with PHD and ring finger domains (UHRF) protein can also bind to DNMT1 to maintain DNAm during the replication phase. Finally,

zinc-finger domain proteins share similarities with the MBD family as they can also repress the transcription via the conservation of DNAm patterns (Moore et al., 2013).

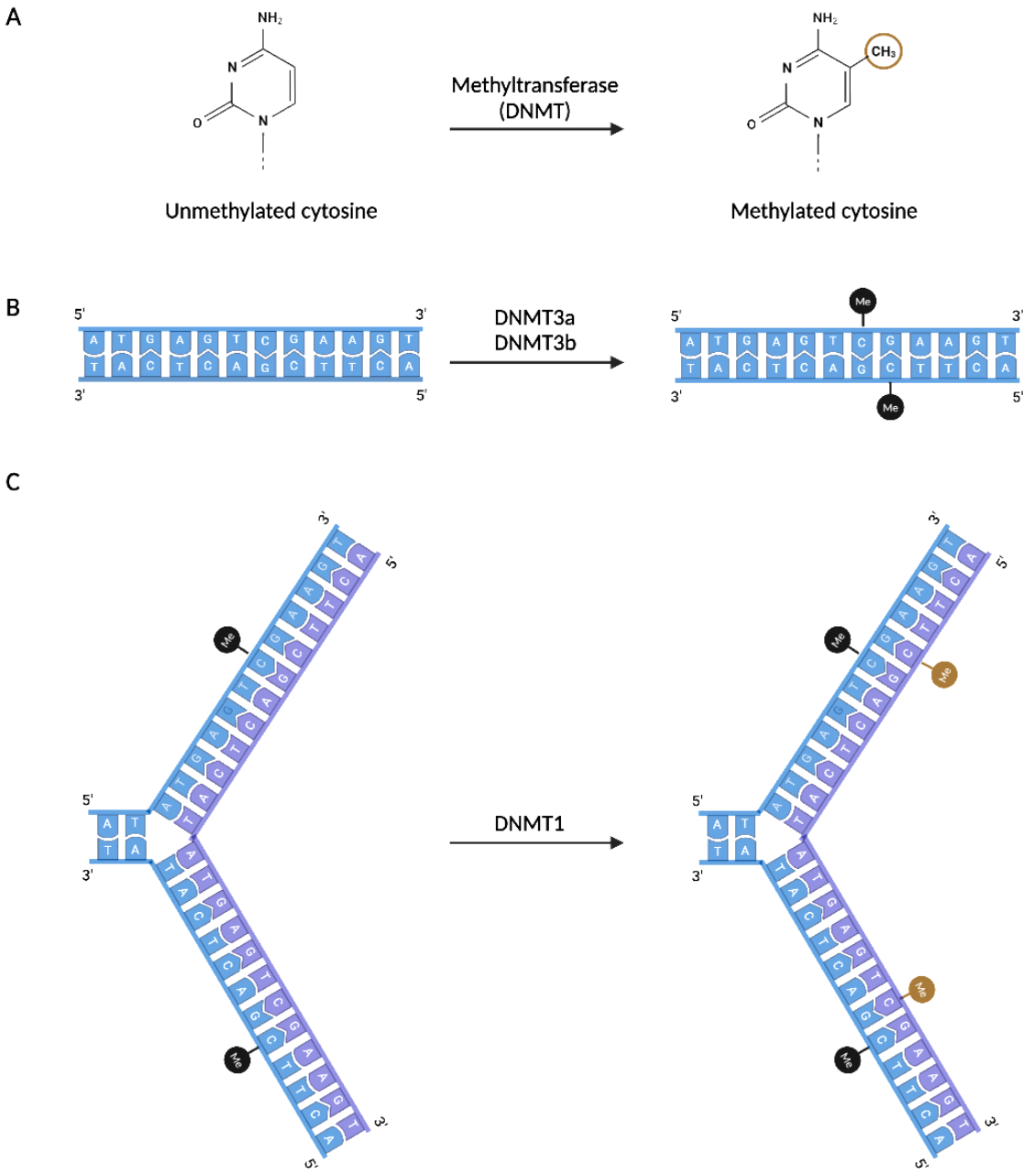


**Figure 2. The DNA methylation pathways.**

Panel A represents the methyl group addition on the cytosine via the methyltransferase. Panel B represents DNMT3a and DNMT3b transferring the methyl group on the DNA strand. Panel C represents DNMT1 which allows the conservation of existing methyl group (in black circle) on parental strand during replication while adding new methyl group (in brown circle) on the newly synthetized strand. The blue strand represents parental strand while the purple strand corresponds to the daughter strand. (Created with BioRender.com).

Those epigenetic modifications are involved in gene regulation as well as in cell differentiation (Holliday & Pugh, 1975; Moore et al., 2013). Each cell possesses the same genetic sequence, however the DNAm pattern itself will differ from a tissue to another (Razin & Riggs, 1980) (Figure 3). As already described in the literature, social and physical environments can influence the human development right after birth. For example, it has been demonstrated that social adversity leads to life-long physical health and behaviour outcomes (Hertzman et al., 2001). However, the phenotype differentiation will depend on external signals/stressors from the social environment (Szyf, 2011).

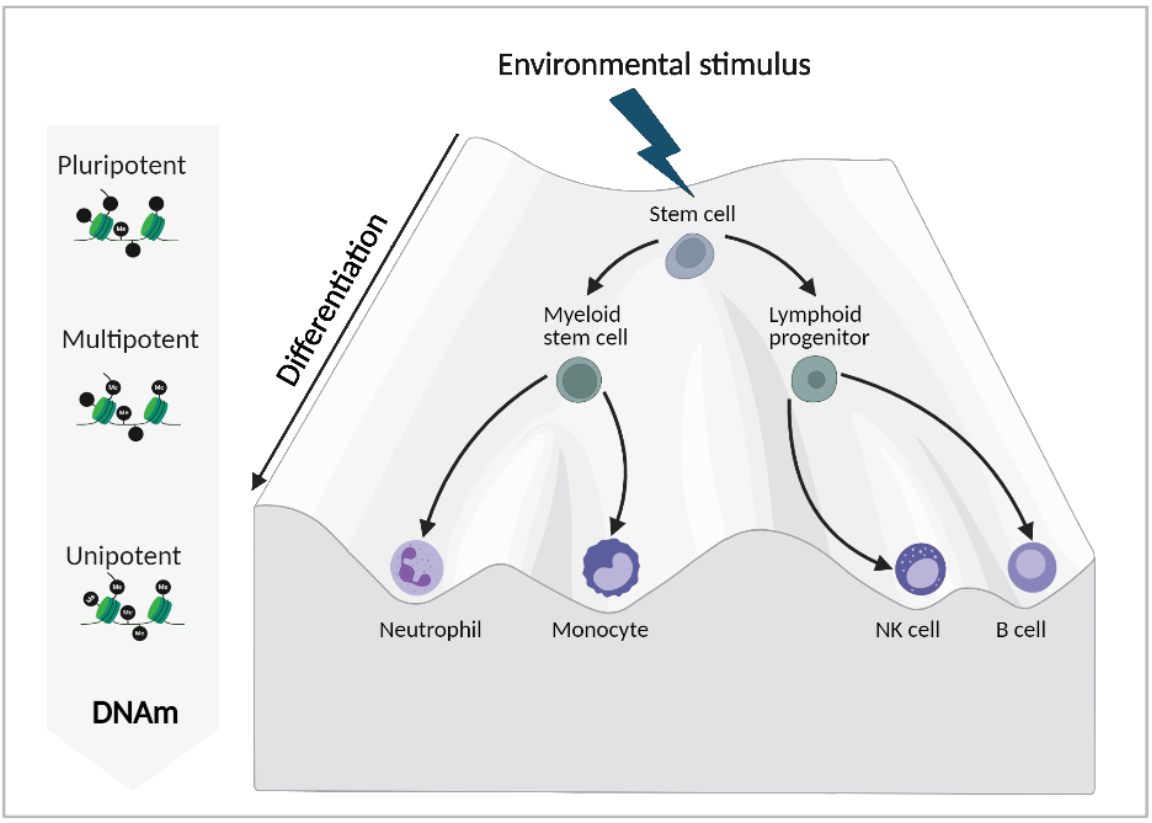


**Figure 3. The Waddington landscape.**

The Figure represents the different differentiation steps of a cell. The top of the Figure shows the differentiation steps from the pluripotent cell (i.e. stem cell) to multipotent cell (i.e. lymphoid or myeloid stem cell), to finally differentiate into unipotent cell (i.e. monocyte cell or B cell). The grey arrow on the left side represents the level of DNA methylation all along the different steps of cell differentiation. (Adapted from Sanchez-Romero, 2021). (Created with BioRender.com).

DNA methylation of the 5 methylcytosine is involved in many cellular processes such as embryonic development, transcription, gene expression, genomic imprinting and chromatin structure but remains poorly understood (Meng et al., 2015). More recently, epigenetic modifications i.e. DNAm have been recognized as major actor of the ageing process due to the genomic changes, such as change in nucleotide sequence or histone modification, that it can induce (Unnikrishnan et al., 2019).

The methylation of adenine (6mA) is an established epigenetic modification. This was long thought to occur exclusively in prokaryotes, and was associated with transcriptional processes in bacteria i.e. DNA replication or gene expression. More recently, it has been admitted that 6mA, is also present in eukaryotes, and that it plays a determinant role in the gene repression and silencing (i.e. X chromosome) in mice (T. P. Wu et al., 2016). In addition, it has been demonstrated that 6mA levels increase during embryogenesis, inducing changes in the cerebellum of female rate after early exposure to brominated flame retardants, clearly linking exposure and developmental processes. This confirmed the role of 6mA in the DOHaD theory (Fernandes et al., 2021). As we suggested in our recent study, early life exposure to α-HBCDD induces the over methylation of genes on 6mA position leading to behavioural and mental problems. As for 5mC, 6mA should be considered as a reliable epigenetic marker in eukaryotic organisms (Holuka et al., 2023).

## III. DNAm & the Ageing process

Ageing is characterized as decline of organ's physiological functions and by consequence increase of the ageing-related chronic diseases such as cancer, metabolic and neurodegenerative diseases (Kennedy et al., 2014). Many studies investigated how DNA methylation can change in specific regions associated to ageing of the genome. Those studies demonstrated that specific methylated loci were associated with age (Bjornsson et al., 2008). Additionally, Bocklandt demonstrated that approximatively 90 CpG sites were age related and could represent accurate biomarker for ageing (Bocklandt et al., 2011). Indeed, epigenetic modifications such as DNAm could change the chromatin organization leading to acceleration of ageing (Wagner, 2019). In 2013, DNAm has been defined as one of the "hallmark of ageing" among DNA instability, telomere attrition, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and finally altered intercellular communication. Each hallmark possesses the three following criteria 1. time dependant manifestation of alteration, 2. possibility

to accelerate ageing when accentuating the hallmark (in vitro), 3. ability to decelerate/halt or reverse the ageing process via therapeutic actions (López-Otín et al., 2023).

In order to track individual chronological age changes, machine learning methods such as elastic net regression based on DNAm and chronological age have been used to develop the epigenetic clocks (EC). Those clocks allow the estimation of epigenetic age based on the instantaneous rate of DNAm change in 3 to 513 CpG sites from tissue or blood. Since 2011, after Bocklandt created the first EC, improved versions of this clock including more tissues, CpG sites and bigger participants' age range have been developed to better track epigenetic age. Those clocks represent the first generation of EC where we can find the famous Horvath and Hannum clocks (Hannum et al., 2013; Horvath, 2013). More recently, a new generation of epigenetic clocks such as the Levine and Dunedin clocks capturing the rate of acceleration/deceleration of ageing have been developed and are now considered more accurate tool than the first generation of EC (Belsky et al., 2020; Fiorito et al., 2019). Since 2011, at least 12 different clocks have been built in order to better capture the change in epigenetic ageing and to better characterize individual ageing pattern rather than only using the chronological age (Figure 4).

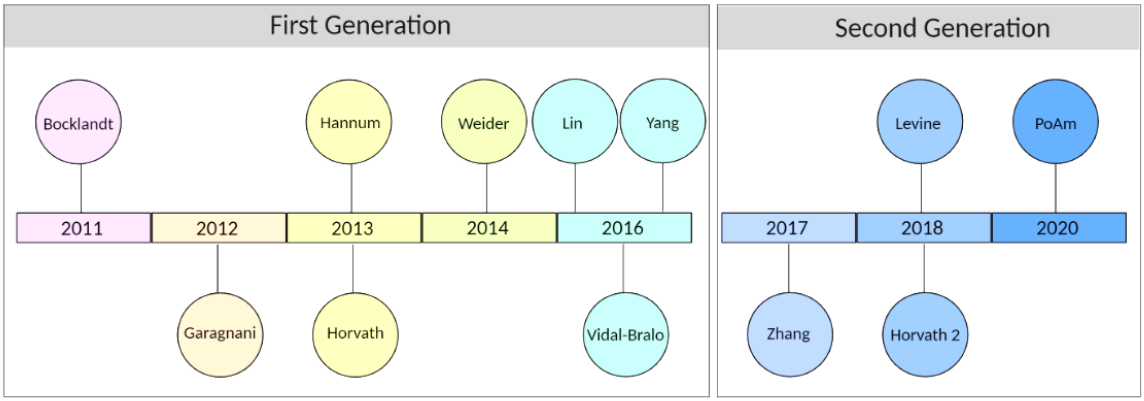


**Figure 4. The evolution of epigenetic clocks.**

The Figure represents the timeline of development and improvements of each epigenetic clocks from 2011 to 2020. (Adapted from Liu et al. 2020). (Created with BioRender.com).

Ageing is associated with immune dysregulation i.e. high pro-inflammatory level leading to cellular senescence also known as an irreversible state of cell cycle as well as cell division alteration. In one hand, studies demonstrated that inflammation becomes detrimental when ageing even though its beneficial effects during early life (Franceschi et al., 2017). The "inflammageing" term, referring to low-grade inflammation characterising ageing. As it represents a risk factor for cardiovascular diseases, cancer as well as mental health problems, it

is now considered as a marker of accelerated ageing (Ferrucci & Fabbri, 2018). In another hand, cellular senescence associated to the prevention of the damaged cells propagation, is involved in many biological pathways (BP) regulation such as embryogenesis, tissue repair or even tumour suppression (Tan et al., 2022). The senescence mechanism remains poorly understood however it has been demonstrated that extracellular stressors such as DNA damages or oxidative stress both acting via specific mechanisms can induce it. Additionally, senescent cells induce the secretion of soluble molecules usually including interleukins (IL-1α, IL-1β, and IL-6), chemokines (IL-8) or growth factors, and can either facilitate the development of cellular senescence or contribute to inflammageing (Borodkina et al., 2018). Human studies demonstrated that senescent cells will more likely accumulate in the skin, T lymphocytes, liver, cardiac muscle, etc. (Rossman et al., 2017). Such accumulation in T cells has been highlighted in patients with chronic infections i.e. cytomegalovirus (CMV) or human immunodeficiency virus (HIV) infection. This could explain why those patients present high level of pro-inflammatory markers as well as weaker vaccine efficacy (Fülöp et al., 2013).

To conclude, ageing involving both inflammageing and cellular senescence mechanisms, is a major risk factor for the development of neurodegenerative diseases (Kritsilis et al., 2018). The overall picture of ageing and immune mechanisms dysregulation remains poorly described, however studies over the past decades highlighted the implication of some external stressors (i.e. lifestyle, personal experiences), immune components i.e. interleukins, cytokines, etc. that all play a considerable role in the ageing process. In order to define a shaper and clearer picture, it is indispensable to consider other aspects of early life adversity that can directly induce individual's epigenome modifications resulting in increase of pace of ageing.

## IV. ALAC. The role of DNAm in the Ageing process

The overall age acceleration and life course (ALAC) project refers to the developmental origins of heath and diseases concept known as Barker's theory. This theory considers that early life conditioning including epigenetic determinants (i.e. DNA methylation or histone modification, etc.) can influence participant's life-long health trajectory. On one hand, we considered the healthy life trajectory and on the other, the unhealthy life trajectory leading to chronic diseases development such as cardiovascular diseases or diabetes. In addition, we also consider the role of environmental exposure (stress, nutrition, pollution, etc.) in the chronic diseases development (Figure 5).

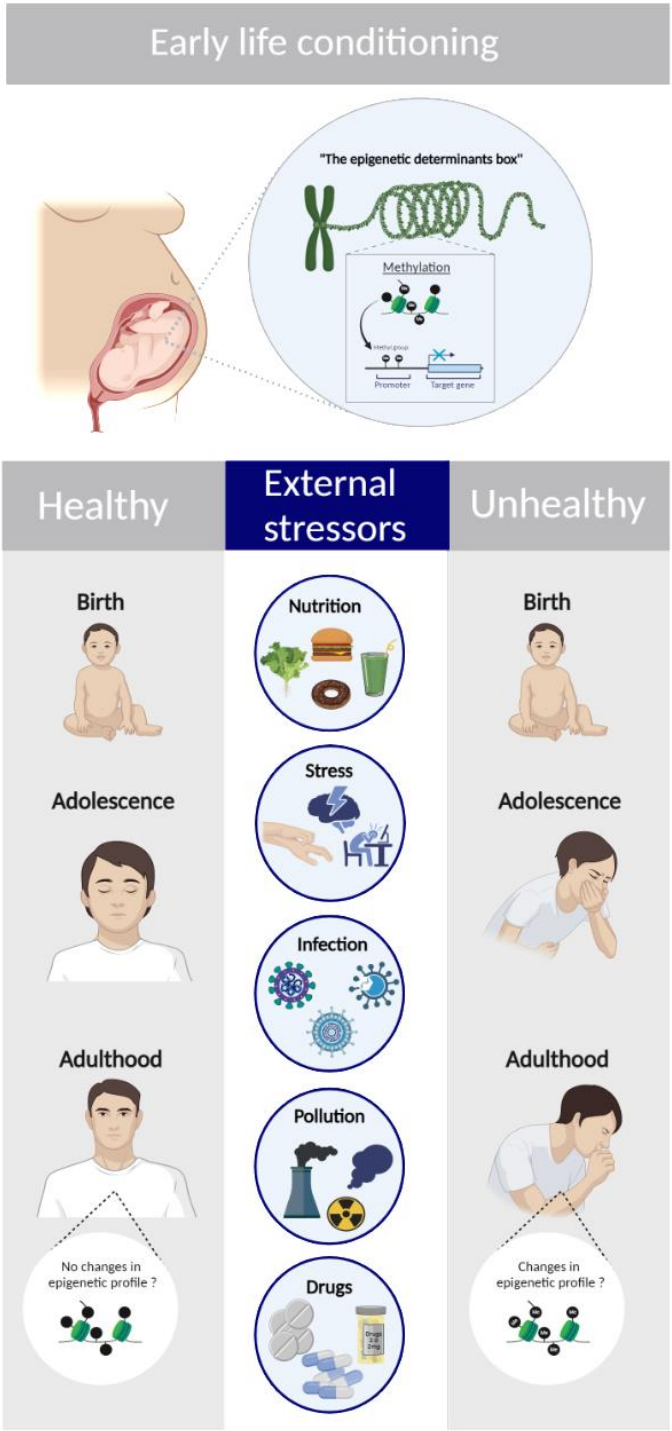


**Figure 5. Maternal early life conditioning impacts infant health trajectories.**

The top box represents the early life conditioning where epigenetic mechanisms occur during pregnancy and can shape infant epigenome. The bottom boxes represent the health (left) and unhealthy (right) health trajectory of the infant. The middle blue box represents external stressors influencing infant's epigenome throughout life. (Created with BioRender.com).

The first aim of the project was to determine whether a traumatic maternal experience can influence their offspring epigenetic age later in life. Indeed, socio-economic environments play a major role in the ageing process and thus at early stage of life such as conception. The first hypothesis we raised was: *"how CpG methylation evolves through adulthood and ageing?"*. We used an accurate biomarker of age called epigenetic clocks based on epigenetic alterations (DNA methylation: covalent attachment of a methyl group on CpG island). Until now, two generations of clocks have been developed to measure epigenetic age. Briefly, the first generation links DNAm to epigenetic age while the second generation links biomarkers of ageing such as leucocyte telomere length. The second generation clock allows the study of the pace of ageing and decline in system integrity. We selected two first generation clocks (Hannum and Horvath) and two second generation (Levine and PoAm) as described in Chapter 4.

Here, we used the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort which follows over 14 000 participants throughout life. ALSPAC regroups over 90 000 variables regarding lifestyle questionnaires and medical records. From this cohort, we are using the sub cohort called Accessible Resource of Integrated Epigenomic Studies (ARIES) regrouping 1022 dyads of mother-children and including epigenetic data taken on cord blood at birth and on whole blood age 7 and 15-17 of children.

In order to investigate how maternal traumata influences children epigenetic profile, we first selected a financial stressor, Major Financial Problem (MFP). MFP total score represents the number of financial events the mother experienced during a specific period of life (i.e. from conception to age 7, conception to birth, and birth to age 7). As described in Chapter 4, none of the correlation analysis between children epigenetic ageing and maternal MFP exposure provided any significant results. These preliminary results suggested that somehow the underlying mechanism might correspond to a biological process rather than an increase of the epigenetic age. The second hypothesis we raised was: *"Is the underlying mechanism a biological process or an increase of the epigenetic age?"*

We used an epigenome wide associations study (EWAS) that allowed us to identify specific phenotype to better characterize individuals by highlighting differentially methylated CpG sites. Additionally, we investigated if those DNAm on specific CpG sites could act as a mediator between maternal exposure such as an economic hardship i.e. MFP and an outcome such as a specific phenotype i.e. decrease of head circumference at birth. As mentioned in the Chapter 4, the first EWAS returned more than 3000 differentially methylated CpGs on children's genome at age 7, however we did not find significant results at birth and age 15. We then questioned the role of

those methylated sites on biological consequences. The third hypothesis we raised was: "*What are the biological consequences of those CpGs methylations?"*

The biological pathways analysis demonstrated that the highlighted CpGs found in the EWAS model were associated with immune and physiological pathways i.e. Parkinson disease (PD), Oxidative phosphorylation (OP), etc. We also demonstrated that the pathway's associated genes were mostly located in the promoter region and were differently regulated (either up or down regulated). This difference in regulation could induce a change of gene expression in the future.

Finally, we hypothesised if the maternal epigenome could act as a "directing" mediator in the offspring genome imprinting. As we already mentioned previously, maternal implication will mostly determine the offspring phenotype later in life. As depicted in Chapter 5, there is a plethora of criteria that need to be to consider to better visualize what can influence the vertical transmission between a mother and her child. As explained in the Chapter 5, the time of exposure as well as the severity of the exposure will determine the importance of maternal imprinting and by consequent its transmission to the next generation. Here, we used a mediation model also called High Dimensional Mediation Analysis (HIMA) (Figure 6) to investigate whether after maternal exposure to trauma, the maternal epigenome induces an imprint on children's genome. In Chapter 4, the first HIMA analysis returned a high number of associations (up to 30 times) between maternal CpGs and children CpGs.

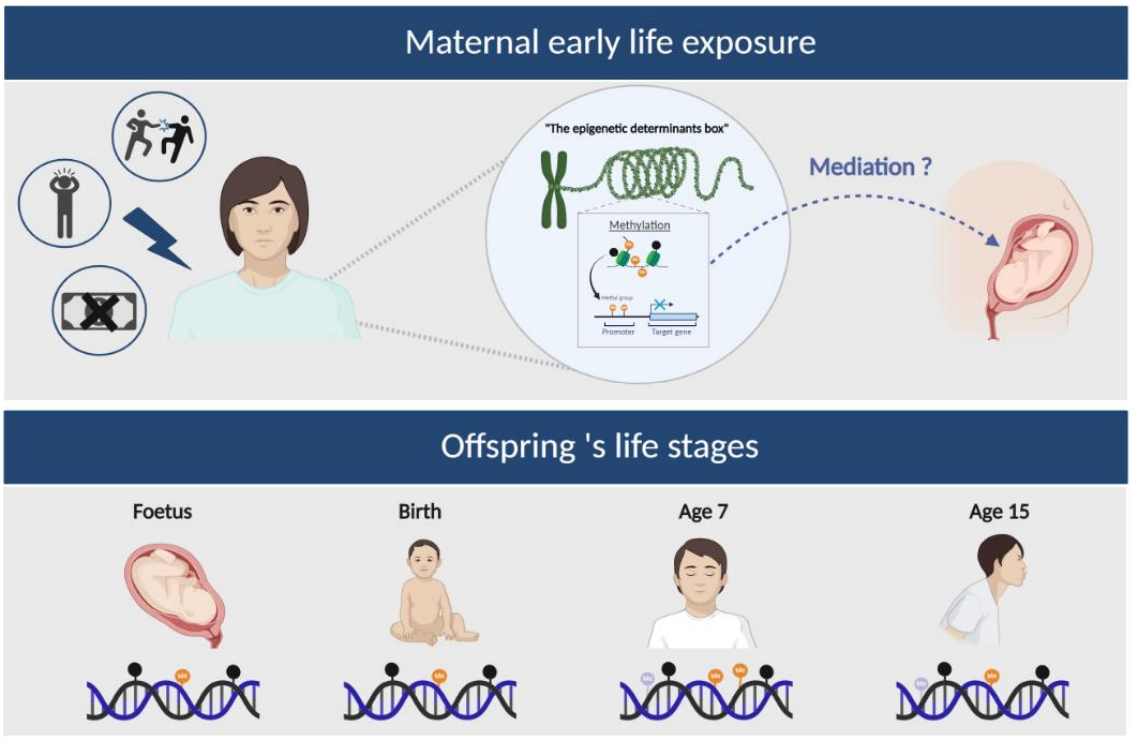


**Figure 6. Mediation model between maternal epigenome and offspring's genome.**

The top box represents the maternal early life exposure when the mother experienced different traumatic events leading to maternal epigenetic imprinting. The bottom box represents the offspring's life stages where all over the years the infant conserves the imprinted maternal epigenetic marks. (Created with BioRender.com).

The results of the EWAS model using an economic hardship as maternal trauma exposure confirmed that such traumatic events are directly imprinted on maternal genome and can be transmitted to the next generation. In Chapter 6, we tested whether a more "stronger/sensitive" variable such as a physical and/or mental exposure can also alter the maternal genome and by consequences affect the child genome. We selected childhood sexual experiences (CSE) variable that regroups five sexual experienced encountered by the mum when she was under 16 years old. As previously observed with MFP, the EWAS returned approximatively 400 differently methylated CpGs on the genome of children at birth and age 7 within 11 remain identical over time. As for MFP, those CpGs are associated with different BP including Parkinson disease and Oxidative phosphorylation directly associated to a gene regulation modification. Finally, the mediation model highlighted 3 directing maternal CpGs highly associated with offspring's CpGs.

Altogether, every tested models demonstrated that maternal pre or post gestational life exposure leads to the specific shaping of their offspring's genome later in life. As mainly mentioned

in the literature, the underlying mechanism of vertical transmission remains poorly understood even if many leads have been considered. In Chapter 2, we highlighted how important maternal environment and experiences were crucial for children development, nevertheless paternal influences need to be also considered. Indeed, the inheritance process requires half genetic material from both parents suggesting that they equally influence the offspring (epi) genetic profile. Here, we demonstrated that maternal exposure seems to be more determinant for the evolution of the child as they share a very strong bond during pregnancy. Additionally, as depicted by David Barker, the early period of life remains the most "sensitive" period for parental exposure and is decisive for all futures steps in life.

Furthermore, there is specific maternal bonds such a breastfeeding described as decisive drivers in the epigenetic shaping of offspring's genome. Breastfeeding is a major factor of early childhood development as it provides a full nutritional support assuring the complete neuronal and biological development of the infant. In addition, it represents one of the strongest bond in the world as it provides an intimate and unique connection with the new born that will last for life (Westerfield et al., 2018).

Following the literature's evidences of maternal benefits through breastfeeding as well as the first interactions with their infant at birth, we hypothesised that the overall birth experience, and more precisely how the mother felt about it might trigger their offspring development. Our fourth hypothesis was: "*Are very early stage of life and maternal behaviour a proxy for children epigenetic imprint?"*. Breastfeeding and birth mode including elective C-section (CS), emergency CS or natural birth variable, both represent the very first step of life and contribute to the offspring health. As mentioned in Chapter 7, our EWAS model returned two differently methylated CpGs at birth (cg05230316 and cg13230077) both associated with ageing and ATP binding activity. However, our model did not allow us to show any correlations between breastfeeding and differently methylated sites on children's genome.

In addition to birth mode and breastfeeding, we considered the maternal birth experience (MBE) mostly representing maternal mental state rather than physical aspect as birth mode as a determinant of children epigenetic profiles modification. However the EWAS models returned no differentially methylated CpG sites (data not shown, manuscript in preparation).

As previously explained, the maternal early life experiences (from conception to age 2) as well as the first external stressors will epigenetically, physically and mentally shape the infant's phenotype. Nevertheless, it is possible to identify from birth which developmental trajectory the offspring might take based on the type and intensity of stressors. The World Health of Organization

(WHO) stated that each individuals develop their own specific speed of development depending on biological factors and environmental exposures (Irwin et al., 2007). As suggested in the literature, studies mostly focused on academic and cognitive scores but, so far, did not consider epigenetic marks as a good candidate to determine individual health developmental trajectory. The use of DNA methylation in addition to cognitive and non-cognitive scales allowed us the build a new measure on a single time point of the pace of development in children capturing the speed of child development. We demonstrated that epigenetic data such as DNA methylation predicts child development and can now be considered as an accurate child development predictor (data not shown, manuscript in preparation). More specifically, DNAm predictor appeared to better predict economic and mental health outcome later in life. This single time point approach demonstrated the power of epigenetic marks when considered as developmental biomarker rather than a DNAm alteration. However, this first statistical approach did not show consistency over time as the CpGs of interest changed over time, especially between cord blood taken at birth and peripheral blood taken at age 7 or 15-17. As DNA methylations are very dynamic over time, it is not surprising to find such differences, nevertheless it remains triggering to be able to predict with certitude how will behave the child 's development trajectory in the next 15 years of life.

To encounter the issue, we then adapted our previous model to catch a latent growth curve pattern. We used a Latent Growth Curve Modelling (LGCM) that estimates the in between-person differences of individuals based on a series of repeated measures (Howard & Curran, 2014). The within-persons patterns captured by the LGCM are called growth curves or latent trajectories. Unlike similar statistical approaches, the latent growth model allowed us to be more flexible in terms of inclusion of missing data, time-lapse between time point and data distribution. Due to the originality as well as specific characteristics of the ALSPAC cohort regarding the distribution of the social variables measures used, the LGCM was a better fit for our analysis. At this time of the analysis, we demonstrated that epigenetic surrogates can predict up to 50% of variation in the child's pace of development. Additionally, we confirmed that these surrogates based on cognitive and non-cognitive measures can predict economic and mental health outcomes in adulthood.

## V. Discussion

Worldwide, people are currently ageing rapidly but more importantly differently. Ageing is a global phenomenon tracking and including individual's age. The European Commission (2018) estimated that the expected number of older people (over 60 years old) will double by 2050 and

triple by 2100. Due to this high increase, additional pressure on public spending such as health care system are now observed. To reduce this pressure, promoting a good health to every individual became a priority over the world as it is essential.

On the biological lead, the ageing process do not only depend on molecular and cellular alterations, but will also be affected by external stressors such as environmental factors as well as SES. More precisely, SES are one of the most important component of the ageing process as they can appear from conception to death. Nevertheless, the interaction between SES and bio-physiological processes remains unknown. A multidisciplinary approach combining socio-economic status, biological and epigenetic measures can allow a better comprehension of the ageing process. As already mentioned by Crimmins in 2018, the ageing process can be studied by using a new multidisciplinary model where "*numerous social and behavioural sciences were natural allies in building this model, but they have been increasingly joined by the biological sciences, so that the view of health and age is at once more similar across disciplines yet more complex than it was a decade ago*" (Crimmins, 2018) (Figure 7). The Crimmins model shows how biological and physiological factors considered as mediators can influence the ageing process and thus health changes. This model represents the complexity of understanding the overall trajectories from early life experience (social or physical) and health outcomes later in life and thus provide information on individual's pace of ageing.

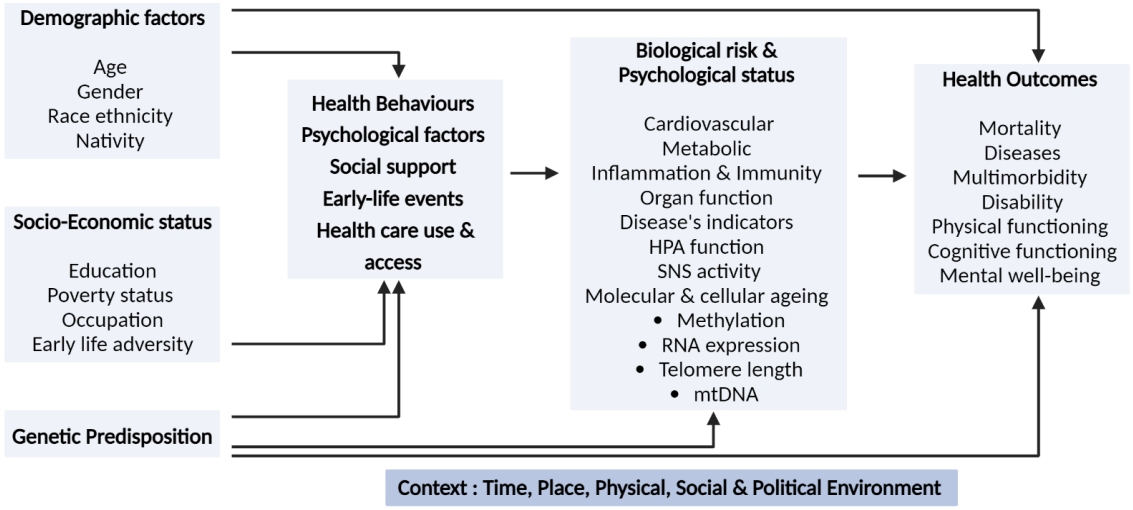


**Figure 7. The multidisciplinary model for examining individual trajectories of health with ageing.**

The figure represents the role of biological mechanisms (i.e. biological risk and physiological status) considered as mediators between SES and health outcomes. (Adapted from Crimmins, 2018). (Created with BioRender.com).

As the ALAC project, using a multidisciplinary approach, allowed us to provide a thorough work on how ELA leads to epigenetic modifications later in life. In the project, we firstly hypothesised that change of epigenetic age can be associated to SES such as economic hardship. The use of the epigenetic clocks mostly used in the literature to capture specific ageing pattern did not show strong and significant associations between maternal exposure and children's DNAm. Even though we used two strong financial stressors i.e. MFP and maternal deprivation (MD), we here demonstrated that these factors are not traumatic enough to alter the pace of ageing. Nevertheless, those results indicated that an underlying mechanism can exist and needed to be investigated.

When investigating epigenetic modifications using EWAS model, we confirmed that maternal socioeconomic and psychological adversity alter offspring's epigenetic profiles later in life. The EWAS model did not capture DNAm modification at birth, however it highlighted a specific epigenomic imprint at age 7 and thus later in life. The non-significant result at birth do not indicate that nothing happened but rather it is too early in the process of imprinting to observe DNAm directly after birth. We hypothesised that those marks can appear later in life, especially during the early development and then remain for years before disappearing during adolescence. During the analysis, we demonstrated that those imprints are reversible as some remained during 8 years (until adolescence at least) while some disappeared after a certain amount of time. The investigation of these epigenetic marks suggested that in addition to a main exposure (i.e. MFP, MD or CSE), environmental stressors as well as lifestyle play a role in the conservation or not of DNAm marks. The exact reversibility mechanism of the epigenetic marks remains unclear however we can affirm that before disappearing over the years, they can alter major biological pathways as well as their associated genes regulation. The conducted analysis showed that OP and PD genes were up or downregulated leading to a partial or complete repression of the gene expression as some of the methylations were found in the promoter region. Indeed, epigenetic modifications such as DNAm are associated with genes silencing or gene downregulation and gene transcription alterations when occurring in promoter regions (Yang & Park, 2012) (Figure 8). The exact silencing mechanism remains poorly described, however it is well known that methylation encourages chromatin to remain condensed (euchromatin form) and thus less accessible for transcription factors. Additionally, DNAm can recruit proteins participating in gene repression or is able to repress the binding of transcription factors usually binding to specific DNA sequences such as enhancer and promoter sequences (Chmielowiec et al., 2022).

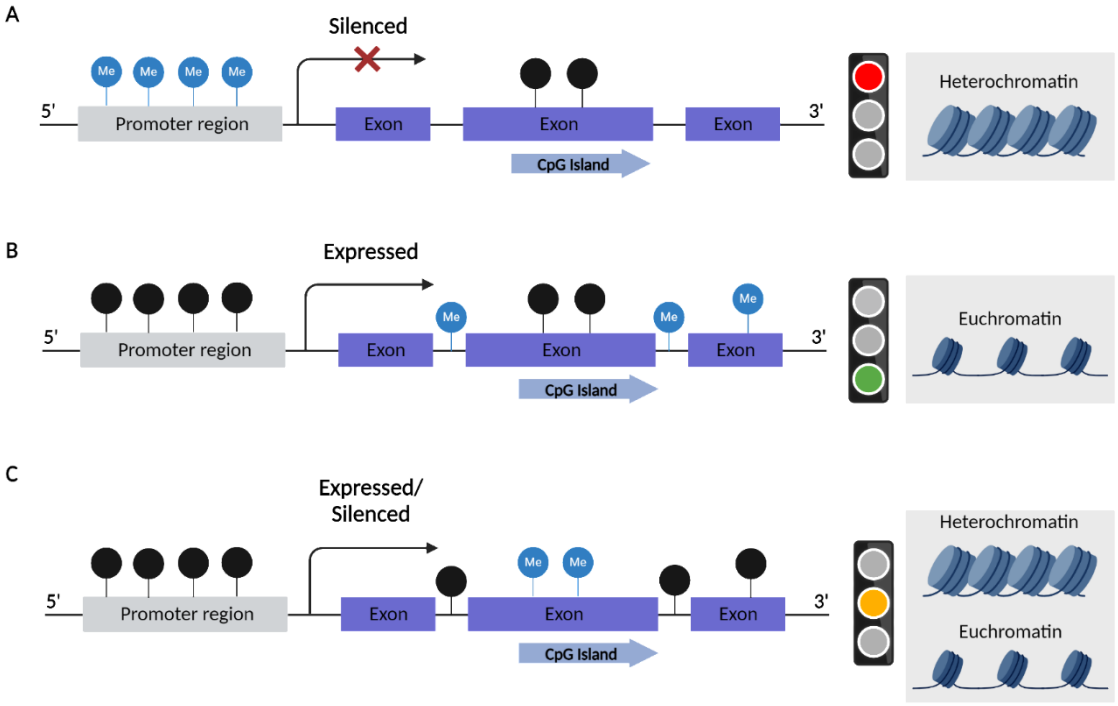


**Figure 8. Correlations between DNAm and gene expression.**

Panel A: DNAm silences the genes expression. Panel B: No DNAm in CpG Island or gene body, the gene expression is active. Panel C: DNAm in the CpG Island and gene body, gene's expression is repressed. (Adapted from Yang, 2016). (Created with BioRender.com).

The ALAC project shed into light the epigenetic mechanism behind the maternal exposure and the child's health life trajectory. As suggested in the DOHaD concept, it is now evident to affirm that each form of ELA either economic, physical or emotional can be encoded in the genome acting as a memory/defence mechanism. Here, we provided a clearer image on how pre and postnatal experience can influence either the foetus during the pregnancy but also after birth and thus remain for years. We also demonstrated that those epigenetic marks are reversible but more importantly, there are not all "coming" from (epi) genetic profiles. Indeed, as mainly suspected in the literature the overall lifestyle and early environmental exposure can somehow reverse the existing DNAm or on the contrary induce new DNAm. Nevertheless, we considered that even if these imprinted marks are personal and reflect a consequence of trauma, only one traumatic experience is enough to "activate" the imprinting mechanism. As we highlighted in the previous chapter, the overall DNAm modification mechanism depends an on combination of different parameters as well as an accumulation of external stressors.

At this stage of the project there is now several key messages that we highlighted in our investigations even though the project presented important limitations. The key messages are presented below and limitations will be discussed in the "Conclusions & Perspectives" part.

Key messages:

- Economic hardship exposure from conception to age 7 does not play a role in the acceleration of the ageing process
- At an epigenome-wide level, SES exposure *in utero* induce DNA methylation on the child's genome
- Methylation occurs at different CpGs sites and affected biological pathways as well as gene regulation
- These DNAm are reversible as they change over time (i.e. adolescence)
- Maternal epigenetic marks induced by early trauma are highly associated with child's CpGs
- Maternal pre-natal exposure does not induce additional epigenetic marks at birth
- Child's health trajectory can be predicted from birth following their induced epigenetic profile

# Materials and methods

**ALSPAC cohort**: We used the Avon Longitudinal Study of Parents and Children that examines how both environmental and genetic factors can influence health development over time (Fraser et al., 2013). ALSPAC regroups 14, 541 women in the South West of England who were pregnant between April 1991 and December 1992. Over the past 30 years, mothers and children were interviewed about their lifestyle, socioeconomic positions and health conditions (Relton et al., 2015). Within ALSPAC, the ARIES subset regroups 1022 mother-child dyads that provided blood sample for DNA extraction from children at birth, age 7 and 15-17. DNA was extracted from either cord blood (at birth) or whole blood (age 7 or age 15) according to a standard protocol. Maternal blood was collected from 4484 mothers between 9-13 weeks of gestation (Golding et al., 2013).

*The "ALSPAC cohort" section refers to Chapters 4, 6, 7 and 8.*

**Bisulphite conversion and DNA methylation:** Extracted genomic DNA underwent bisulfite conversion using the Zymo EZ DNA MethylationTM kit (Zymo, Irvine, CA) (de Vocht et al., 2018). Samples underwent whole genome amplification and hybridised onto HumanMethylation450 BeadChips using the Illumina HT-12 V3 BeadChip preparation Kit (Illumina Inc, Ca). Beadarrays were scanned using an iScan (Illumina). Initial quality control was performed using GenomeStudio. ALSPAC staff distributed samples from different time points and generations (mother/child) across slides in a "semi-random approach to minimise the potential relationship between batch effects and other variables" (Relton et al., 2015). As previously reported, a wide range of technical covariates were collected and integrated into the quality control from the standard control probes on the 450k BeadChip by ALSPAC. As previously reported samples failing quality control (average probe detection p-value ≥0.01) were excluded from further analyses. Methylation levels available from ALSPAC were Beta values, calculated using the standard ratio of methylated probes intensities to total probes intensities. Total probe intensities were calculated as methylated probe fluorescent levels plus unmethylated probe fluorescent levels plus alpha (a technical constant equal to 100). ALSPAC staff provided Beta values pre-processed in R (version 3.0.1) that had undergone a subset quantile normalization (Touleimat & Tost, 2012). Additionally, probes with p-values ≥0.01 for more than 5% subjects were excluded as were CpG loci containing or adjacent to single nucleotide polymorphisms, small insertions and deletions, repetitive DNA, and regions with reduced genomic complexity (Naeem et al., 2014). Cell counts and batch effects have been controlled during the normalization process by regression and the Houseman method respectively.

*The "Bisulphite conversion and DNA methylation" section refers to Chapters 4, 6, 7 and 8.*

**Main variables:** Major financial problems (MFP), Maternal deprivation (MD), Childhood sexual experience (CSE), Birth mode, Maternal birth experience (MBE).

*The "Main variables" section refers to Chapters 4, 6, and 7.*

**Co variables:** The listed variables have been used in the EWAS model to adjust models when necessary. Smoking, alcohol consumption, ethnicity, mother age, BMI, maternal bonding.

*The "Co variables" section refers to Chapters 4, 6, and 7.*

**Epigenetic clocks:** Briefly the Horvath and Hannum first generation clocks were trained on chronological age changes in DNA methylation in order to estimate "epigenetic age". They respectively used DNAm at 353 CpGs and 71 CpGs dinucleotides (Hannum et al., 2013; Horvath, 2013). The age acceleration for those clocks was then calculated as the difference between individual DNAm age and chronological age (Simpson & Chandra, 2021). The second generation clocks such as Pace of Ageing (PoAm) clock developed by Belsky or Levine's clock were trained on a longitudinal change in age-related biomarkers such as leucocyte telomere length (Belsky et al., 2020; Fiorito et al., 2019). PoAm clock is considered as a speedometer and quantifies the rate of decline in biology systems rather than epigenetic age. This specific clock uses 18 biomarkers of system integrity following the Dunedin Longitudinal Study Birth cohort. In R, the R script from supplementary material of Belsky et al. was used to calculate the second generation PoAm clock as described by the authors. As for the previous first generation clocks, the Levine clock using 513 CpGs was calculated with the Watermelon package.

*The "Epigenetic clocks" section refers to Chapters 4, 6, 7 and 8.*

**Statistical analysis**: All analysis were performed in R studio (version 4.1.1 and 4.3.0). Dplyr and smjics packages were used for data cleaning and dichotomisation of the data when necessary (Lüdecke, 2018; Wickham et al., 2020). R Watermelon package was used to calculate the Horvath and Hannum first generation clocks (version 4.1.1) (Noroozi et al., 2021). EpiTools and epiDisplay packages were used to generate uncorrected relative risks and Odds ratio (Aragon, 2020; Chongsuvivatwong, 2018). PathfindR package (version 1.6.3) was used to generate biological pathways plots. The R meffil package was used to remove unwanted technical variation by regressing the variability.

*The "Statistical analysis" section refers to Chapters 4, 6, and 7.*

**Case control EWAS:** The EWAS analysis were performed on the ARIES normalised beta values with a technical detection p value < 0.01. Minfi and DMRcate packages were used to extract differentially

methylated CpGs sites and regions (Peters et al., 2015). Probes statistics were extracted with missMethyl package. GOfuncR and pathfindR packages were used to extract biological pathways associated to significant CpGs (Cabrera et al., 2019; Ulgen et al., 2019).

*The "Case control EWAS" section refers to Chapters 4, 6, and 7.*

**Data transformation:** When necessary a base 10-exponentional function in R was applied to our data to ensure that data meet the statistical assumptions. After transformation and when possible, the R/Bioconductor package was used to adjust the p value inflation (van Iterson et al., 2017).

*The "Data transformation" section refers to Chapters 4, 6, and 7.*

**High-dimensional Mediation Analysis (HIMA):** The R HIMA package investigates the high-dimensional mediation effects between Mother's CpGs and child's epigenome (H. Zhang et al., 2016). HIMA package uses a three steps approach to identify significant CpGs and will provide a list of association and p-value between two lists of the tested CpGs. The 3 steps are: 1. Identify CpGs with the largest effect sizes for the response variable based on a "sure independence screening", 2. Estimate the mediation effect following a minimax concave penalty and 3. Joint significance-testing step (Kilanowski et al., 2022; H. H. Zhang, 2008). HIMA provides a list of association and p-value between maternal CpGs and offspring's CpGs.

*The "High-dimensional Mediation Analysis (HIMA)" section refers to Chapters 4 and 6.*

**Data visualisation:** SigmaPlot (version 14)/Adobe Illustrator CS6 (version 16.0.0) were used to produce graphical figures. VennDiagram and ggplot2 packages were used to respectively generate Venn diagram and Karyogram/Manhattan plots.

*The "Data visualisation" section refers to Chapters 3, 4, 6 and 7.*

# Results

# *Chapter 1*

# *The COVID-19 Pandemic: Does Our Early Life Environment, Life Trajectory and Socioeconomic Status Determine Disease Susceptibility and Severity?*

**My contribution to this Chapter:**

Conceptualisation, Literature review, Making of all figures, and Writing of the article.

**This Chapter has been published as:**

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The coronavirus disease (COVID-19) initially reported in December 2019 in China and declared a pandemic on 11 March 2020 by The World Health of Organization, lead to multiple research projects over the past 4 years (Shah & Farrow, 2020). Indeed, laboratories and institutes all over the world started new investigations to better understand the (underlying) causes and their consequences. The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) caused almost 7 million of deaths the past 4 years. The first biological cause investigated in most of the studies was the individual genetic predisposition that could explain the "immune responses" alterations. However, there is a long-established epidemiological observation that social experiences (adversity) is associated with weaker resistance to disease. Indeed, literature have demonstrated that ELA exposure influences immune function and disease risks (Merz & Turner, 2021; Seeman, 1996). At birth, the human body is not fully developed, i.e. nervous and immune systems continue to evolve until age 2. This concept is now described as the Barker Theory (DOHaD) (Gluckman et al., 2010; Wadhwa et al., 2009).

Nowadays the Baker Theory became the "three-hit model" (Grova et al., 2019). It depicts 3 hits: Genetic predisposition, early life environment and later life environment, all linking the early life period and adult diseases. This model demonstrates the clear implication of the immune system such as inflammation and senescence processes playing a key role in the disease development (Merz & Turner, 2021). As the Barker theory has been refined and clearly established a link between early life exposure and later adult diseases, we here payed a particular attention to whether or not socio-economic status could act as a risk factor in the susceptibility to COVID-19.

Early life adversity have always been described as a major risk for chronic disease development later in life. ELA-associated diseases such as diabetes, obesity or even cancer all share a common aspect which is a bias of the immune system towards a pro-inflammatory and senescent phenotype. More precisely, early life environment can trigger the immune cells such as cytotoxic T-lymphocytes (CTL) (Hertzman & Boyce, 2010). During the COVID-19 pandemic, we discovered that in the immune response to SARS-CoV-2, a functional CTL response (CD8+) is necessary to tackle the infection. Additionally, the severity of COVID-19 increases when the CD8+ response diminishes (Xu et al., 2020). Regarding those elements, we investigated whether ELA can trigger the course of COVID-19 by inducing a pro-inflammatory and a senescent phenotype.

The following Chapter sheds into light the role play by ELA in the COVID-19 pandemic by demonstrating the underlying mechanisms influencing the immune system. For a better understanding of ELA implication we considered four principal components: 1. Psychological stress, 2. Infection, 3. Nutrition/Microbiome and 4. Pollutant exposure. For each sub categories we

reviewed their potential influence on the immune system and their consequences on the susceptibility and severity to diseases such as COVID-19.

Here, we demonstrated how “underestimated” external factors such as socioeconomic factors as well as ELA can influence the later health trajectories. As mentioned in this Chapter, the need of the correct data remains the main problem to efficiently link ELA to viral infections over the next generations. As we demonstrated, there is a considerable part of the population that tend to be more vulnerable to COVID-19 despite showing any physical/biological weaknesses. To shed into light mechanisms linking ELA and specific later phenotype, the collection of more socioeconomic factors, now recognized as strong ELA determinants, became indispensable.

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*Review*

# The COVID-19 Pandemic: Does Our Early Life Environment, Life Trajectory and Socioeconomic Status Determine Disease Susceptibility and Severity?

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**Abstract:** A poor socioeconomic environment and social adversity are fundamental determinants of human life span, well-being and health. Previous influenza pandemics showed that socioeconomic factors may determine both disease detection rates and overall outcomes, and preliminary data from the ongoing coronavirus disease (COVID-19) pandemic suggests that this is still true. Over the past years it has become clear that early-life adversity (ELA) plays a critical role biasing the immune system towards a pro-inflammatory and senescent phenotype many years later. Cytotoxic T-lymphocytes (CTL) appear to be particularly sensitive to the early life social environment. As we understand more about the immune response to SARS-CoV-2 it appears that a functional CTL (CD8+) response is required to clear the infection and COVID-19 severity is increased as the CD8+ response becomes somehow diminished or exhausted. This raises the hypothesis that the ELA-induced pro-inflammatory and senescent phenotype may play a role in determining the clinical course of COVID-19, and the convergence of ELA-induced senescence and COVID-19 induced exhaustion represents the worst-case scenario with the least effective T-cell response. If the correct data is collected, it may be possible to separate the early life elements that have made people particularly vulnerable to COVID-19 many years later. This will, naturally, then help us identify those that are most at risk from developing the severest forms of COVID-19. In order to do this, we need to recognize socioeconomic and early-life factors as genuine medically and clinically relevant data that urgently need to be collected. Finally, many biological samples have been collected in the ongoing studies. The mechanisms linking the early life environment with a defined later-life phenotype are starting to be elucidated, and perhaps hold the key to understanding inequalities and differences in the severity of COVID-19.

**Keywords:** COVID-19; SARS-CoV-2; socioeconomic status; early life adversity; psychosocial stress; immunosenescence; immune exhaustion; health inequalities

## 1. Introduction

The ongoing outbreak of coronavirus disease (COVID-19) was first reported in December 2019 in Wuhan, China. COVID-19 is caused by a betacoronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), that affects the respiratory system [1]. Despite draconian sanitary measures being applied worldwide, COVID-19 was declared a pandemic on 11 March 2020 by The World Health

of Organization (WHO) [2]. By May 13th the outbreak had infected over 4 million people and caused almost 300,000 deaths worldwide (World Health of Organization, 2020).

There is a long-established epidemiological observation that social adversity associates with reduced host resistance to infection and disease [3] which goes back as far as 1976 [4]. More recently, it was recognized that the effect on adult immune function and disease risk was much stronger when the exposure to adversity occurred during early life [5,6]. Humans are not fully developed at birth. Nervous and immune systems are gradually developed and educated up to the age of two. In fact, human life commences and develops for the first 1000 days starting from fetal conception. Any pre-natal complications and post-natal adversity faced defines the lifelong health trajectory [7]. As the COVID-19 pandemic has progressed, it has become clear there are many inequalities in susceptibility and severity of the disease. The recent flurry of pre-print clinical data from many countries worldwide including China, UK, US, are strongly concordant; the lower the current socioeconomic status (SES), the greater the risk [8], however, the role of the early life period and the resultant life-course has so far not been investigated. To understand the mechanisms underlying these differences, we need to dissect the exposome and environmental factors (i.e., pollutants, stress situation, etc.) that patients may be, or have previously been exposed to.

There is a well-established literature on the role of the overall trajectory from early life through to adulthood and the risk of non-communicable diseases such as cardiovascular disease, diabetes, obesity and depression [9], however there is no data on how it affects COVID-19. Although current SES has been associated with the risk, progression and even survival of non-communicable diseases [10], it is now becoming clear that during an individual's life there are periods of increased susceptibility, and the overall trajectory of SES may be more important. This has led to the "Barker theory", or the Developmental Origins of Health and Disease (DOHaD) [11]. In addition, environmental influences which act during early development/life may determine our susceptibility to the disease many years later [11–13].

Over time, the Barker theory has been refined. Currently, this is thought of as a "three hit model". The three "hits" are generally accepted as: (1) genetic predisposition, (2) early life environment and, (3) later life environment [14,15]. As high-quality mechanistic studies have addressed the link between the early-life period and adult disease, it is becoming clear that the immune system, particularly through chronic low-grade inflammation and accelerated immuno-senescence is, mechanistically, in the heat of the action. In addition, we know that stressful experiences during early life induce adaptive responses that are often mediated by the immune system [16].

In this manuscript, we examine the data linking early life adversity to life-long disturbances in the immune system that may play a role in determining its ability to fight SARS-CoV-2 infection, potentially determining the severity of COVID-19 disease and expanding DOHaD to cover infectious diseases later in life.

Furthermore, we review known factors of ELA and their potential influence on the adult immune system and contemplate what kind of data should be collected to understand how SES and ELA influence disease susceptibility and severity of COVID-19 and other diseases. We hope this work will contribute in protecting and treating people at risk of developing severe COVID-19 symptom.

## 2. The Role of Current SES in COVID-19 Morbidity and Mortality

Socioeconomic status (SES) or gradient is a combination of education, incomes, occupation and reveal inequities to privileges or resources between individuals [10]. Indeed, socioeconomic factors (i.e., race/ethnicity) are considered as fundamental determinants in human life span, well-being and health [10]. Data from influenza pandemics of 1918 and 2009 showed that socioeconomic factors may determine both disease detection rates and overall outcomes [17–19]. In the early phase of the COVID-19 pandemic many studies focused on basic criteria (i.e., age, sex, and gender) to investigate coronavirus spread, transmission routes and potential high-risk populations. Socioeconomic data were, unfortunately, missing as they are not considered as data of clinical interest [16]. However, socioeconomic data regroup many relevant factors as daily situations (i.e., stressful job, pollution, etc.)

that directly interact with human health [16]. Evidence is now starting to emerge that COVID-19 mortality is increased in ethnic minority populations. US data indicates that, for example, in Chicago approximately 70% of the deaths were from ethnic minorities [20]. Detailed data from New York showed that the number of COVID-19 cases associated with the percentage of dependents in the local population, the male:female ratio, and low-income neighborhoods [21]. United States-wide data gave a similar result, with proportion of residents >65 years old, ethnic minorities, male:females ratio, and the overall population density associating with increased frequency of COVID-19 [22]. The United Kingdom followed a similar profile. Although the recent UK data only looked at mortality, there was a stronger link between COVID-19 mortality and SES than ethnic background. A 1% increase in the lower socioeconomic class increased COVID-19 mortality by 2% (95% Confidence interval of 1% to 4%) while a 1% increase in ethnic minority increase mortality by only 1% (95% confidence interval 1% to 2%) [8]. Although these are preliminary (pre-print) data, they agree with Shi et al., who reported that the most severe cases were mostly agricultural laborers [23]. The link between the incidence of COVID-19 and lower income neighborhoods and lower SES is most likely due to the overall economic conditions such as poverty, performing essential public tasks, poor quality and over-populated housing as well as an obligation to use public transport [8] as well as higher rates of known comorbidities including type 1 and 2 diabetes, as well as cardiovascular disease and hypertension [24]. Overall, despite the scarcity of the data, we interpret what is available as a suggestion that current SES and neighborhood influence the morbidity of SARS-CoV-2 infection and COVID disease rather than the mortality rate.

## 3. The Role of Early Life in Determining Lifelong Health Trajectories

When considering the early-life environment, many measures such as SES are broad and encompass many concurrent elements. We have previously found it useful to separate these into four principal sub-categories [15] (Figure 1). Although determining the contribution of each of the four elements (psychosocial stress, infection, nutrition and microbiome, and pollutant exposure) is difficult, there are data on well-defined exposure conditions that fit into these sub-categories as well as insidious, general measures like SES.

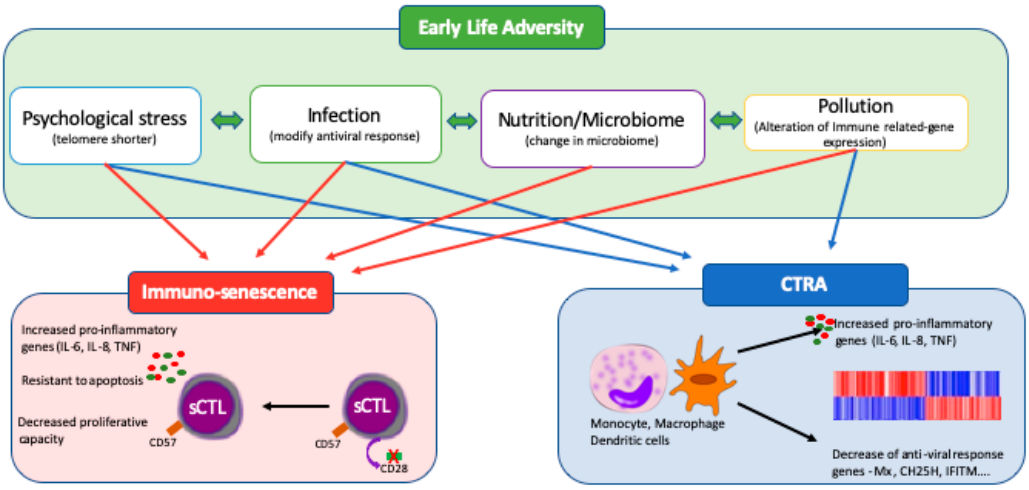


**Figure 1.** Immune adaptation mediated by early life adversity. Early-life adversity (ELA) is broken down into its four key components: psychosocial stress, infectious stress, nutrition and the microbiome; and pollutant exposure. They are linked to increases in the numbers of senescent cytotoxic lymphocyte (sCTL) which, upon stimulation are resistant to apoptosis and release large quantities of expression of pro-inflammatory. Certain elements have also been shown to alter the underlying transcriptional identity of leucocytes such as macrophages, dendritic cells or T lymphocytes. This phenomenon is called "the conserved transcriptional response to adversity" (CTRA).

### 3.1. Early Life Psychosocial Stress

There is now a growing literature on the effects of early-life psychosocial adversity on the immune system. We have previously reported the immunophenotype of young adults that had experienced ELA as institutionalization after separation from their parents and subsequently adopted in early childhood compared to those reared by their biological parents (EpiPath cohort) [25]. In this cohort, we surveyed the innate, humoral, and adaptive immune system. We observed an increase in activated and senescent pro-inflammatory T cells, particularly those, expressing HLA-DR/CD25 and CD5. Senescence is a natural aging process affecting all cells including immune cells. These begin to deteriorate and this leads to weakened immune responses [26]. Furthermore, there was a trend toward an increase in the number of circulating Th17 cells [27,28]. ELA clearly accelerated T-lymphocyte maturation and senescence, although did not affect B cells. T- lymphocytes were accelerated through their maturation cycle from naïve to effector memory and aggregating in the terminally differentiated effector memory cells re-expressing CD45RA (TEMRA) cell phase [27,28]. This skewing of the immune system, in particular the cytotoxic CD8+ T-cells was confirmed in an independent cohort, of teenagers approximately 15 years after a similar form of ELA [29].

Telomere length decreases with chronological and biological age, after cell division, and is a hallmark of cellular senescence. Exposure to stressful events during childhood showed that the telomere length is shorter in these individuals when compared to the control group [30–33], confirming that ELA negatively contributes to an imbalanced immune system [34]. Furthermore, Cohen et al. showed that low childhood SES significantly decreased the telomere length later in life of a CD8+CD28- T cell population, which play major role in the response to viral infections [35].

Studies with rodents produced the predominant hypothesis that the mechanism by which ELA impacts the function of CD8+ cells and, consequently, viral responses, may be through the HPA axis. ELA negatively impacts the HPA axis, which programs its effects and responses later in life. This normally results in a decreased release of corticosterone or cortisol after exposure to stress which consequently has a great impact on the peripheral immune system, leading to compromised viral responses [36–39]. However, results from mechanistic studies in our EpiPath cohort have excluded this. We were able to show that despite an altered HPA axis [25], glucocorticoid signaling and the peripheral HPA-axis stress system were not epigenetically programed [40], implying that the immune system was directly impacted.

### 3.2. Early Life-Infections

It is well known that an early life exposure to infection and inflammation can have devastating effects. One example would be that neonates suffering from bacterial or viral sepsis are about threefold more likely to die within the first 120 days [41]. There is also evidence showing that sepsis in new-borns was associated with poor long-term neurodevelopment [42]. The immediate risk of infection to the organism, especially for those more vulnerable, seems obvious. The long-term consequences of an infection prove far more difficult to grasp.

Bilbo and Schwarz reviewed available data on the connection between perinatal infection and long-term effects on stress reactivity and cytokine production [43] showing that early life infection leads to a cytokine storm (the most prominent being interleukin 1β [IL-1β], IL-6 and tumor necrosis factor α [TNFα] which can pass the blood-brain-barrier and cause long term memory impairment in the hippocampus. Similarly, we found a blunted response to stress and a higher number of exhausted T-lymphocytes in our EpiPath adoptee cohort, which had a higher incidence of cytomegalovirus (CMV) infection and an overall higher risk of childhood infections due to the institutionalization [25,27]. A very recent study in zebrafish shows that expression of several pro-inflammatory genes is increased in adult fish after early life bacterial infection [44]. This study also showed that the age of the first infection is a crucial factor for the adult immune response. Other studies have specifically linked early-life respiratory viral infection with a higher likelihood to develop diseases like childhood asthma

or allergies [45–47] or the chance to develop type 1 diabetes [48]. These chronic conditions are known risk factors for a more severe outcome of COVID-19 disease.

Currently, the molecular mechanisms in which an early life infection distorts the immune system are only partially understood. In in-vitro experiments, Fonseca et al. demonstrated that early-life exposure to bacteria in combination with respiratory syncytial virus (RSV) later in life can lead to epigenetic modifications impacting bone marrow progenitor cells and therefore causing long-term re-shaping of inflammatory mediators and metabolic profiles [49]. Subsequently, all daughter cells of these progenitors would be ill-equipped to handle subsequent infections [47].

Certainly, early life infections present a specific type of early life adversity. It is indubitably linked to the overall health of the individual (immune system) and the social environment, given that host-to-host transmission of pathogens are by far the most prevalent form of infection. In the previous section, we showed the impact of psychosocial stress on the immune system. However, the overlap does not end there: sickness, in humans and animals, also changes their social behavior. Well known behavioral changes include a decrease in activity and expanded sleeping periods [50]. Therefore, social behavior and infection should not be treated as two distinct adversities, but as two sides of the same coin.

*Early life nutrition and the microbiome*: Over the last decade it has become clear that once the microbiome is established it is shaped by the exposome and the ~9 million microbial genes it encodes and play a crucial role in determining host development and health [14,51–53]. Modulating the host most probably protects the natural enteric symbiotic microbial community, and disturbing the established microbiome, producing a dysbiosis, results in disease and may even be fatal [54,55]. The microbiome established is dependent on the route of birth, and is then modulated by nutritional intake, living conditions, the polluted environment and the presence of pets [56,57]. As SARS-CoV-2 appears to persist in the GI tracts and can be detected in human feces [58,59], it will interact, affect, and be affected by the microbiome. Indeed, diarrhea is now recognized by the Centers for Disease Control and Prevention (CDC) as a COVID-19 symptom and it is a clear sign of microbial dysbiosis [60]. The interaction and effects of SARS-CoV-2 will almost certainly depend on both the microbiome that has been established and how the host has adapted to its microbiome.

The LPS content and immunostimulatory potential of the initial early-life microbiome depends on the birth route [51]. The microbiome is established during a sensitive period in which the new-born immune system is primed [61], and may explain why babies born by caesarean section have a significantly increased risk of allergy or asthma later in life [62]. Exposure of new-borns to a more diverse microbiota soon after birth altered both the disease susceptibility and maturation of specific immune cell subsets, whereas if the first encounter occurred later, immune dysfunction was not corrected [63,64]. Regulatory T cells ($T_{reg}$) play a significant role in the host adaptation to the microbiome, recognize host-specific commensal bacteria derived antigens [65], and result in long-term tolerance to the enteric microbiome [66]. It would appear that adverse microbiota is essential for the immune system to fully mature [67].

Peri-natal viral infections, such as CMV have been extensively studied and linked to lifelong changes in the microbiome [68] and common viruses such as influenza are known to affect the development of the immune system when acquired at birth and during infancy [69]. The angiotensin-converting enzyme 2 (ACE2) receptor may play a role in determining microbiome-immune-interactions. In the GI tract ACE2 is expressed in enterocytes and is important for maintaining both antimicrobial peptide expression, and the overall health of the microbiome [70,71]. Mice lacking Ace2 develop gut absorption related diseases [70,72]. As Sars-Cov-2 uses ACE2 receptor to enter cells [73,74] it would be logical to assume that there is a link between the virus and the microbiome that was established in early life, immune cells resident in the GI tract and the overall outcome of COVID-19.

*Early life-pollution exposure*: There is emerging evidence that environmental exposure to pollutants during sensitive developmental periods like early life could be a strong factor of susceptibility,

predisposing the individual to birth outcomes and disease onset in later life [15]. Prenatal exposure to airborne pollutants could affect fetal reprograming by epigenetic modifications (e.g., DNA methylation) and may therefore explain the potential link between air pollutant exposure and adverse pregnancy outcomes. Epidemiological studies have pointed out causal association between fine particulate matter (2.5 μm; PM2.5) and neurodevelopmental (ADHD, autism)/neurodegenerative (Parkinsons, Alzheimers) [15], metabolic, cardiovascular [75] and lung pathologies [76]. Air pollutants were therefore proved to affect key cellular/molecular targets during the perinatal period, which are susceptible to alter immune responses link to abnormal respiratory functions and lung diseases later in life [77]. For instance the EDEN birth cohort study, focusing on determining peri-natal factors that influence childhood health and social development, pointed out that a pre-natal exposure to PM10 (particles with diameter less than 10 μm) was linked to an increased in CD8+ T cell and a decreased in regulatory T cells in infants at birth, leading to a potential increase in the susceptibility of viral infection responses as well as atopy development in children [78]. The impact of traffic pollutants and tobacco smoke on regulation of numerous Immune related-genes, such as cytokines (e.g., IL-4, IL-6, and IFNg), TLR2, nitric oxide synthases (NOSs), and several factors of transcription (e.g., Runx3 and Foxp3), has also been demonstrated [77]. It is now well established that modifications in DNA methylation patterns due to PM 2.5 exposure are frequently associated with the development of lung pathologies [79]. However, it remains difficult to assess whether exposure during early life has a stronger impact on development of diseases than that of the adulthood, or whether substantial morbidity is the result of accumulated exposure [76].

In the context of COVID-19, Zhu et al. demonstrated significant associations between air pollution and COVID-19 infection. High concentration levels of $PM_{2.5}$, $PM_{10}$, CO, $NO_2$ and $O_3$ were therefore positively linked to a risk of COVID-19 infection, whereas high concentration levels of $SO_2$ were negatively linked to the number of daily COVID-19 confirmed cases [80]. These results are supported by those obtained in February 2020 by Martelletti et al., who showed that in the industrialized regions of Northern Italy, those most affected by COVID-19, the concentration levels of PM10 and PM2.5 were above the legislative standard limit of 50 μg per day [81]. The adsorption of SARS-CoV-2 RNA on airborne PM (PM2.5 and PM10) was established in these regions by Setti et al. who suggested that, "in conditions of atmospheric stability and high concentration of PM, SARS-CoV-2 could create clusters with outdoor PM, and, by reducing their diffusion coefficient, enhance the persistence of the virus in the atmosphere." [82]. In a cross-sectional observational study conducted in the United States, Wu et al. showed, by taking into account 20 potential confounding factors in their main analysis, that a slight increase in PM2.5 (+1 $\mu g/m^3$) was linked to an 8% increase in the rate of COVID-19 death [83]. Although all this data results from preliminary investigations, it tends to suggest a positive relationship between ambient air pollution exposure and COVID-19 mortality rate. Confirming the direct impact of airborne pollutants on the COVID-19 severity could prove an asset in terms of public health and prevention strategy in places with poor air quality.

We have previously highlighted the role of early-life pollution exposure and a potential "second hit" in the "three-hit" model producing a quiescent phenotype, likely encoded in the epigenome, which might become vulnerable in later life to a "third environmental hit" such as COVID-19 [15]. Given the long-term effects on health of early-life pollutant exposure and the linkage with the development and progression of pulmonary pathologies in later-life, it is reasonable to assume that early-life pollutant exposure will affects the course of COVID-19.

## 4. Early Life Origins of COVID Co-Morbidities

If the early life environment plays a role in determining the outcome of COVID-19, examining its role in the key comorbidities is essential. The three key comorbidities determining COVID-19 severity are cardiovascular disease, hypertension and diabetes. The seminal work of David Barker clearly identified the role of the in-utero environment, another source of early life adversity, in determining the risk of both cardiovascular disease and hypertension. While this has been extensively reviewed elsewhere [84–86] it is worth noting that the relative risk associated with birthweight and ponderal index is by far larger than

any other risk factor identified for either disease to date. There is now a large body of evidence showing diabetes to be a major risk of complications and death after SARS-CoV-2 infection [87], as in previous coronavirus outbreaks [88], while the risk of SARS-CoV-2 infection appears to be similar [89]. Like the other elements discussed here, type 2 diabetes (T2D) may have its origins in early life. There are well-established, classical risk factors that contribute to T2D including obesity, age, stress, inflammation, diet, lifestyle and environment (both early and late life), however there is growing recognition for non-classical factors such as pollution, exposure to ionizing radiations and low socio-economic status (SES). The classical and non-classical factors are intimately intertwined. SES is a broad measure encompassing prior life history, and low SES also increases the risk for obesity, stress, environmental and lifestyle factors (BMI, smoking, alcohol . . . ) as well as a pro-inflammatory phenotype [90].

The importance of T2D in determining COVID-19 severity may in part be due to treatment strategies currently used in T2D together with another severe co-morbidity, hypertension. Both are often treated with ACE (angiotensin converting enzyme) inhibitors and ARBs (angiotensin II receptor blockers). These increases ACE2 (angiotensin converting enzyme 2) expression in pancreatic islets, lungs, intestines, etc. [91]. SARS-CoV-2 exploits these ACE2 receptors to enter host cells, thus potentially increasing the risk of infection in T2D patients [92]. Increased pancreatic ACE2 activation has been reported to inflict beta cell damage complicating the prognosis [93] and further contributing to the characteristic "cytokine storm" observed in COVID-19 cases. Other T2D drugs that induce ACE2 expression include pioglitazone, liraglutide, gliflozins, and DPP4 (dipeptidyl peptidase 4) inhibitors and have also been implied to promote coronavirus predisposition [94]. This may be further accentuated by hyperglycemia-induced ACE2 glycosylation. ACE2 glycosylation is also a prerequisite for the virus to latch onto the ACE2 receptors [95]. This enhancement is reversible by strict glycemic control [95]. As such, glycemic and overall diabetic status have been proposed as predictors of COVID-19 severity and mortality [96].

Although current T2D status may play an important role in SARS-CoV-2 susceptibility and COVID-19 severity, it is part of a larger etiopathological risk complex. T2D may have its origins in the early life social environment. Low early-life SES showed a clear, strong, association with individual metabolic profiles that was not true for current SES [97]. This result has been replicated by another study that highlighted the effect of SES during adolescence on the development of T2D up to fifty years later [98]. More recently, Chandan et al. (2020) reported a retrospective population-based cohort of 80,657 adults that had been exposed to ELA and 161,314 unexposed controls. This seminal study clearly demonstrated the link between childhood maltreatment and cardiovascular disease, hypertension, and T2D. In a population where ELA rates may reach 25%, their data clearly shows that "a significant proportion of the cardiometabolic and diabetic disease burden may be attributable to maltreatment" [99].

There is now some mechanistic evidence to back up the link between ELA and T2D. Needham et al. investigated the transcriptional effects of low SES [100]. They reported that low (current) adult SES altered the expression of several genes intimately linked to inflammation that are all linked to T2D: *F8* [101], *CD1D* [102], *KLRG1* [103], *NLRP12* [104], and *TLR3* [105] and stress related gene *AVP* [106]. Furthermore, low early-life SES was also shown to affect the expression of stress related genes: *FKBP5* [107] *OXTR* [108] and *AVP* and inflammation associated genes: *CD1D* and *CCL1.* As such, SES would appear to act on inflammatory pathways that are common to low SES environments and eventually T2D, and may worsen the T2D etiopathology by targeting prominent pathophysiological factors like stress and inflammation. The mechanistic link between ELA and T2D is re-enforced by the immune disturbances reported. Patients with T2D have a larger number senescent CD8+ cytotoxic T cells and higher levels of systemic inflammation [109,110] that may explain the higher incidence of viral and bacterial infections in diabetic patients [111].

Although there is no data currently available, it is logical to assume that although T2D may predict COVID-19 severity, the origins of this link may lie in the lifelong pro-inflammatory environment

induced by ELA. T2D may be the adult manifestation of the poor early life social environment which then mediates the effect between ELA and COVID-19.

## 5. The COVID-19 Immune Response, SES and Early Life Adversity

*The immune response to COVID-19:* The SARS-CoV-2, like other viruses, is considered immunologically as an intracellular parasite. In general, the viral infectious-cycle starts with a short-lived extracellular period, followed by cell entry, with a final, longer, intracellular replicative period. In the classical anti-viral immune response, the immune system attacks all phases of the viral cycle using both antigen specific and non-specific mechanisms. The non-specific immune response, particularly effective in the early phase of infection, is mainly mediated through natural killer cells and interferons. Production and/or secretion of type-1 interferons (i.e., all the interferons proteins except IFN-γ) enhances NK cell ability to lyse infected cells as well as inhibits viral reproduction and cellular proliferation. When an adaptive immune response has been mounted, the most effective antibodies are the so-called neutralizing antibodies which block viral entry into the host cell by binding to viral surface proteins such as the envelope or capsid protein (Figure 2). When the subsequent cell-mediated immunity enters into force, it is principally CD8+ cytotoxic T lymphocytes (CTLs) that are the effector cells. CTLs recognize MHC class-I presented antigens, to lyse the presenting cell, a response that is not always beneficial as the damage done by the cytotoxic cells is occasionally greater than that of the virus itself.

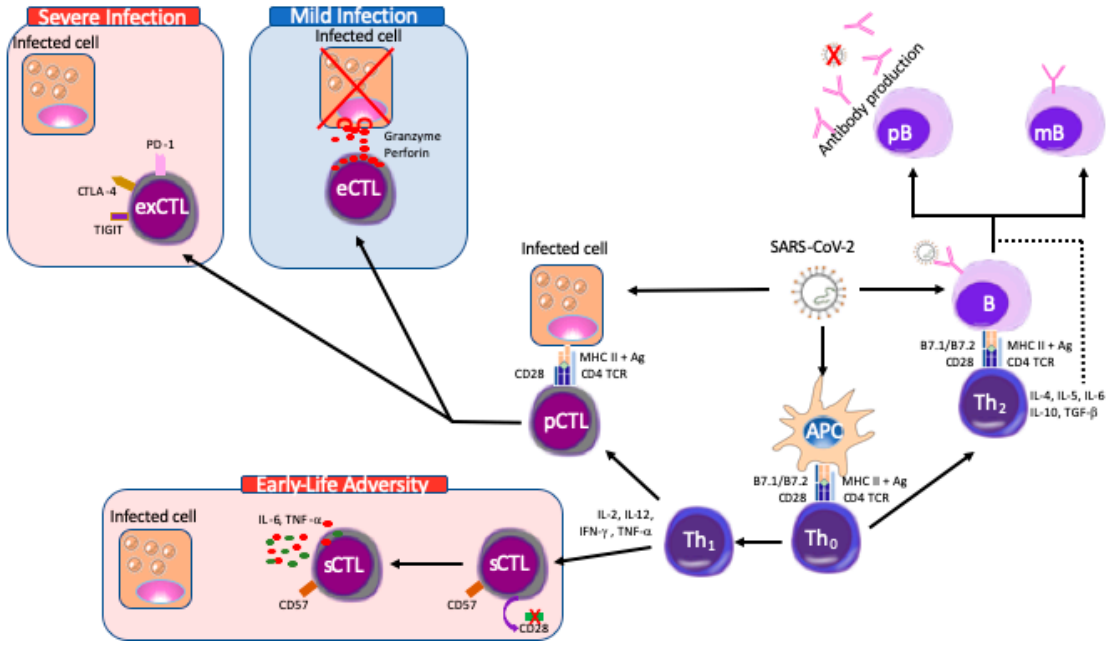


**Figure 2.** The immune reaction to coronavirus disease (COVID-19). The adaptive response to SARS-CoV-2 is a classical anti-viral response. On the right side, once recognized by antigen presenting cell (APC), $Th_2$ response is activated and induced maturation of B cell. After maturation precursor B cell produces a specific antibody against SARS-cov-2 while mature B cell retain memory of SAR-COV-2 to produce antibodies in case of new infection. Once the Th1 system is activated it induces activation of precursor cytotoxic lymphocyte T (pCTL) due to expression of many cytokines (IL-12, IL2). In one hand, effector (eCTL) can release proteins as granzyme to destroy infected cell in case of mild infection. In case of severe infection, CTL become exhausted (exCTL) and express PD-1, TIGIT and CTLA-4. In patients with having experienced ELA, the increased relative number of sCTL having lost CD28 expression will produce a less efficient lysis of SARS-CoV-2 infected cells. The recognition and clearance by NK cells and the initial role if Interferons is omitted for clarity. Cell images were from http://www.clker.com with the right to re-use them.

As the COVID-19 pandemic has progressed, there have been several reports of the anti-SARS-Cov-2 immune response. To date, the data suggests that the response is a classical anti-viral response with activation of Type-1 interferons and CD8+ CTLs. Although Thevarajan et al., analyzed a single patient, they nicely demonstrated the kinetics of the anti-SARS-CoV-2 immune response [112]. In a manner similar to both Influenza infection and a previous SARS-CoV-2 report [113] which showed that the numbers of $CD38^{+}HLA\text{-}DR^{+}$ $CD8^{+}$ T cells were higher in infected patients than in healthy controls, and rapidly increased from 3.57% (day 7), 5.32% (day 8) to a peak at 11.8% 9-days later. By day 20 they had decreased slightly to 7.05%. As would be expected, $CD38^{+}HLA\text{-}DR^{+}CD8^{+}$ CTLs, produced significant quantities of the lytic moieties—perforin, granzyme A and granzyme B—necessary to lyse virus-infected cells (Figure 2). Their kinetic data showed that this occurred at days 7–9, preceding symptom resolution, suggesting an important role in the resolution of the SARS-CoV-2 immune response [112].

*The anti SARS-CoV-2 immune response in severe/critical patients:* COVID-19 patients are generally considered either mild, severe, or critical. There are now data on the differences in the immune response in these different categories, although the categories are not always the same, complicating comparisons between studies. When Zheng et al. investigated T-cell derived functional molecules, they highlighted lower levels of interferon-γ (IFN-γ) and TNF-α in CD4+ T cells in severely affected patients than those mildly affected, although in the latter, they were considerably higher than expected in health controls [114]. Levels of perforin and granzyme B cells were increased in CD8+TIGIT- CTLs, and the numbers of senescent HLA-DR+ TIGIT+ CD8+ cells were increased in severely affected patients than those with a mild infection. The authors proposed that their data suggests COVID-19, like many chronic viral infections, reduces CD4-Tcell functionality, skewing the immune response towards a CD8+ response, with excessive activation leading to exhaustion of the CD8+ cells, diminishing the anti-viral immune reaction. Furthermore, upon deeper examination, they found differences in PD-1, CTLA-4, and TIGIT–markers of immune exhaustion. In severely affected patients, exhausted PD1+CTLA-4+TIGIT+ cells were significantly more frequent than in patients with a milder infection. This excessive CTL exhaustion may reduce the effectiveness of the immune response to SARS-CoV-2, explaining case severity [114]. Furthermore, in an independent study, it was also reported that as disease severity increases, the numbers of naïve, effector and memory classes of CD8+ T cells diminish, while B-cell, and CD4+ T cell numbers generally increase [115,116]. Overall, we interpret these data as showing that a functional CD8 response is required to clear SARS-CoV-2 infection, and COVID-19 severity is increased as the CD8+ response becomes somehow diminished (Figure 2). Indeed, Omarjee et al. have also come to a similar conclusion, that "Severe COVID-19 can therefore mimic a state of immune senescence" [117,118]. From the start of the pandemic, the involvement of the cytokine system was clear [119]. Initially described in January 2020, levels of CXCL8 and IFNγ, were increased in all COVID-19 patients, and severe cases had significantly higher levels CXCL10, CCL2 and TNFα than milder cases [120] reproduced in a more recent study that also observed increased levels of IL6, and IL 10 in the most severe cases [121].

*Does Immunosenescence link ELA to COVID-19 outcomes?* We have outlined above the ELA-induced long term immunophenotype. Although the origins are multifactorial, it would appear, from the work of Elwenspoek [27,28] and Reid [29], that an adverse social environment in early life drive T-cells, in particular CD8+ CTLs, towards a senescent state. When the different aspects of ELA are considered separately, immunosenescence would appear to be a common aspect. Senescence and exhaustion may have similar outcomes, a reduced immune reaction, but are distinct processes [122]. Senescent cells have a significantly reduced capacity to proliferate, however, they have a strong pro-inflammatory action. In a manner reminiscent of the senescence associated secretory phenotype (SASP) initially established in fibroblasts [123] senescent CD8+ CTLs aggregate in the highly differentiated states (effector memory and TEMRA), are highly resistant to apoptosis, and produce significant quantities of pro-inflammatory cytokines such as IL6 and TNFα upon stimulation [124]. Exhausted CD8+ CTLs

however, are not only unable to proliferate, but they no longer secrete cytokines after stimulation and are programed to undergo apoptosis.

The data currently available suggests that the aggregation of senescent CTLs will negatively impact the progression of COVID-19, and patients with the most senescent CTLs will have the poorest prognosis as they are less capable of mounting an effective CD8+ response, and they will have an exaggerated cytokine secretion from the senescent cells. This is further supported by the recent initiation of the SCOPE trial, "Sirolimus Treatment in Hospitalized Patients With COVID-19 Pneumonia" (NCT04341675). In this trial, the investigators propose administering rapamycin to down-regulate the IL-6 pathway through the mTOR pathway to not only reduce IL-β levels, but reduce the number of senescent T-cells as well [117]. This also raises the question about what happens to COVID-19 when ELA-induced senescence and COVID-induced exhaustion converge. It would seem logical to hypothesize that this would represent the worst-case scenario, and would produce the least-effective cytotoxic T cell response.

*The Conserved Transcriptional Response to Adversity (CTRA):* Studies have demonstrated that early life social adversity can act mechanistically through modifications of gene expression patterns. Gene expression implicated in the activation of T-lymphocyte and inflammation was enhanced while gene expression implicated in innate antiviral responses induced by type I IFN and innate antimicrobial responses of pathogen-specific was reduced [125]. These patterns of altered gene expression remain lifelong [125]. The pattern has been termed the conserved transcriptional response to adversity (CTRA), and has been noticed in many correlational studies regarding humans encountering with adverse life circumstances. [126–133]. CTRA dynamics are most strongly induced by social conditions in early life, at the first step of the development of postnatal immune system [125]. To the extent that transcriptome remodeling induced environmentally continue to affect immune responses of implicated pathogen, many, many years later in life (e.g., inhibiting immune responses to viral infections [134], or amplifying allergic inflammation [133,135]).

*Essential co-variates:* ELA is, however, associated with a range of negative health behaviors (reviewed in [136]) including an increased risk of smoking as well as increased smoking levels, levels of alcohol consumption, and poor diet leading to either malnourishment or obesity. The psychobiological and neurodevelopmental mechanisms linking ELA and risky health behaviors are starting to be dissected [137]. However, in the context of the COVID-19 pandemic, it would appear from the numerous studies that are becoming available that smoking increases the risk not only of hospitalization with COVID-19, but with ICU admission and death (odds ratio from 2.0 to 16 [138,139]) and was confirmed in recent meta analyses of the available studies [140–143]. On the other hand, there is little evidence available on the role of prior alcohol intake on the course of SARS-CoV-2 infection, however, considerable public health efforts are being made to combat alcohol abuse during the confinement period, and a prior history of ELA exposure may increase the risk of excessive alcohol consumption during this period.

Biological sex is one of the strongest drivers of the heterogeneity in COVID-19 disease severity. There is a clearly more favorable outcome for women across all age categories. The data available so far suggests that sexual dimorphism in the immune system may play a role in determining disease outcome. Sex impacts not only the development of $T_{reg}$ cells, but the distribution of lymphocyte subsets and the overall T-lymphocyte response to challenge [144]. Many immunologically important genes are found on the X-chromosome including CD40L and CXCR3. Incomplete X-inactivation or epigenetic modifications will induce sex-specific effects on T-cells [145,146]. There is also evidence that there is a stronger lymphopenia in males than females in severe COVID-19 disease [147,148]

There is also growing evidence for the role of vitamins D and K in the outcome of COVID -19 disease. Beyond its classical role in bone metabolism [149], vitamin D plays a role in the functioning of the immune system and in the regulation of inflammatory cytokines [150] and CRP [149] which reduces the risk of infection and cardiovascular disease [149]. Indeed, immune cells like T-cells, B-cells or antigen presenting cells can directly interact with vitamin D receptors. In this way, increased vitamin

D levels enhance the innate system and suppress the adaptive immune system, which demonstrates its role in immune regulation [151]. Vitamin D deficiency is also linked to comorbidities such as diabetes [152] and upper respiratory disease susceptibility, including common viral infections, allergies and airway inflammatory conditions (REF6). The logical assumption is that a possible explanation on the susceptibility of the elderly population is the fact that they naturally produce less vitamin D while they are exposed to less sunlight as many stay indoors. Considering also that the pandemic first made its global appearance during winter season increases the possibility for this correlative association [152]. Panfili et al. highlighted the potential that vitamin D supplementation has shown to be a successful cost-effective therapeutic for acute respiratory tract infections (ARTIs) in low socio-economic characterized countries [153]. In addition, studies have shown that vitamin D can help to reduce the risk of an activated renin-angiotensin system in the lung [154] in cases of severe COVID-19 disease in patients with hypertension and high expression of ACE2 receptors [155]. On the other hand, patients with comorbidities such as diabetes present a lack in vitamin K which is involved in blood coagulation or bone calcification mechanisms. In case of COVID-19 patients, insufficient levels of vitamin K could be associated with a risk of complications due to elastic fiber pathologies such as idiopathic pulmonary fibrosis (IPF) [156]. Coagulation has been reported as a common comorbidity linked to COVID-19 severity and mortality.

## 6. COVID-19 as a Natural Experiment

Given the obvious ethical objections to experimental studies manipulating the early life environment, there is a long history of using natural experiments. There are two classical natural experiments looking at the early life social environment, Project Ice Storm in Canada, and the Dutch Hunger Winter. When we look at these natural experiments in the light of the three-hit model, these examined the role of the second hit, the early life environment.

Project Ice storm is based on the 1998 Quebec ice storm and examines the impact of prenatal stress on adult outcomes. This particularly harsh meteorological event affected, residents of a well delineated area covering Nova Scotia, New Brunswick, Southern Quebec and eastern Ontario. These populations had to deal with a situation where they were deprived of electricity for weeks, and in certain cases months, as well as the shutdown of all activities in major cities (Montreal, Ottawa) as well as military deployment and several deaths. Project Ice Storm went on to examine the effects over the following 20 years on the children and now young adults that were exposed to the storm in utero [157,158]. They concluded that prenatal glucocorticoid exposure impacted a variety of outcomes in the next generation throughout childhood and persisting into adulthood, dysregulating metabolic pathways and the HPA axis [157,159] This was mediated through epigenetic (DNA methylation) encoding of the storm's effect [158]. Project Ice Storm demonstrated that an environmental stressor can have long-term effects and inducing numerous outcomes although there were additional mechanisms linked to socioeconomic factors that are still to be identified.

The Dutch Hunger Winter was the consequence of a food embargo placed on the Dutch population by the Germans at the end of world war II [160]. Here, the importance of timing of the adversity in the programing of adult disease was established [161]. Working on same-sex sibling pairs of which only one was exposed to famine they demonstrated that in utero exposure induced an adverse metabolic [162] or mental phenotype [163], depending on the time of exposure and fetal sex, and that this was mediated by DNA methylation [164].

As Project Ice Storm disaster and the Dutch hunger winter, the current COVID-19 pandemic must be considered as a relevant natural experiment to reveal the effects of socioeconomic factors on health and disease. In the context of the three-hit model, here we have an exquisite and unique opportunity to investigate the third hit. As outlined above, the early life period acts through underlying mechanisms such as DNA methylation and programing of the immune system to influence disease progression and severity later in life. These prior studies have provided unexpected mechanistic insight into the immunological consequences of early life stress exposure. Drawing parallels with COVID, if we

can collect the correct data, we can start to unpick the role of the whole life trajectory and how this contributes to disease risk through a pro-inflammatory immune bias.

COVID-19 may also be a form of early life adversity. It is yet to be discovered whether SARS-Cov-2 could have any immune programing capacity after an early life infection and what consequences could appear years later. Its strong association and impact on the early life microbiome is unknown. Pregnant women who tested positive for SARS-CoV-2 infection showed evidence of placental injury which impeded blood flow to the fetus [165]. Placental development is the first step in embryogenesis and may determine the quality of the intra-uterine environment [165,166]. Individuals who were exposed (intra-uterine) to the Spanish flu of 1918 have been reported to face lifelong low SES and cardiovascular diseases [167] which may be indicative of a bidirectional risk that has crossed over from the placenta jeopardizing their lifelong health profile. It is quite possible that the COVID-19 positive mothers pass on a similar risk to subsequent generations, serving as an ELA event, which ultimately makes them highly susceptible. Thus, these cases need strict follow up studies to validate this hypothesis.

## 7. Data that Should Be Collected

In light of the data presented here, it is clear that there are many types of data that should be collected in addition to the studies that are currently ongoing addressing the epidemiology and biology of COVID-19. As recently highlighted, it is essential to collect as much socioeconomic data as possible during the ongoing pandemic [16]. Data collection should be expanded to include retrospective data on life-trajectories and both exposure to adverse life events and how importantly they were perceived. There are well-recognized difficulties in retrospectively assessing adversity or the overall life-course, however, there are tools available that can measure the prior traumatic experiences. Recent adult trauma can be addressed by a brief questionnaire that covers the perceived importance (salience) of a range of stressful life events including "separation, relationship and money worries, accidents, illness and death, job loss, and violence" [168] that any future study participants may have experienced. To address traumatic experiences earlier in life, there are also validated questionnaires such as the Childhood Trauma Questionnaire CTQ or the Early Trauma Index that are available [169]. However, as with any retrospective study there is a risk of recall bias, although the validated questionnaires have questions within them to ensure internal consistency. Furthermore, in the context of a fast-moving pandemic, the ability to transpose such questionnaires to an online system is known to improve the accuracy of responses as the anonymity of the online process has been shown to reduce both social desirability and central coherence biases, although there is a potential risk of questions being mis-interpreted by participants [170]. All such tools are limited by what was thought of as being traumatic when they were developed, however, they remain the standard tool for assessing traumatic events during childhood as well as a poor social and familial environment [169]. The use of such questionnaires has already proven useful. Adverse social conditions, as measured by the CTQ have been shown to become embedded as functional changes in the immune system that are visible lifelong. Studies have shown adversity measured by the CTQ over a period of as little as 4 months changes the immune response up to 24 years later, the longest time-point investigated so far [27,28,34,125]. Tools such as the CTQ should play a role in studies addressing the overall disease severity if participants go on to develop COVID-19 rather than whether ELA plays a role in the overall prevalence of infection. Furthermore, health related behaviors such as smoking and alcohol consumption which are known to be elevated after ELA and may also play a role in the clinical evolution or susceptibility to SARS-CoV-2 infection must be recorded. All data should be analyzed with a sex-informed approach, taking differences in the immune system into account.

The collection of life-event meta-data must be complemented by the collection of the correct biological samples. We have highlighted the role of the immune system, the microbiome and pollution levels. It would seem logical to obtain stool and blood samples, and the markers to be investigated such as TIGIT, PD-1, CD28 and CD57 are now becoming clear. Furthermore, such biosampling would allow the analysis of vitamin levels, as they may be a key link in the pathophysiological chain. It would

also appear to be appropriate to rapidly collect measures of pollutants, determine how indoor and outdoor pollution levels have changes, how, with the strict confinement measures imposed, nutrition has changes. All of these will play into the susceptibility and immune response.

The data reviewed here highlights the role that the social environment will play in determining morbidity and mortality during the COVID-19 pandemic. In the future, such socioeconomic and lifestyle data must be considered as essential clinical data that is then analyzed concurrently with biological material to tease out the effects of the environment in health and disease.

## 8. Conclusions

The developmental origins of health and disease is firmly established for many non-communicable diseases. The current COVID-19 pandemic has shown that there are many health disparities, and the available (preliminary) data suggests that there is a strong socioeconomic impact on morbidity, and potentially mortality. Although there are no data so-far available to link the early life period to the morbidity and mortality of an infectious disease, an adverse early life environment would appear to impact the immune system and make it less efficient in fighting subsequent viral infections. Early-life researchers have a long history of taking advantage of natural experiments, teasing out the long-term consequences of ELA to produce a measurable phenotype many years, or even generations, later. The current pandemic can turn this paradigm on its head. Many discrepancies and inequalities in COVID-19 morbidity and mortality have been reported, and if the correct data is collected it may be possible to separate the early life elements that have made people particularly vulnerable to COVID-19 many years later. This will, naturally, then help us identify those that are most at risk from developing the severest forms of COVID-19. In order to do this, we need to recognize socioeconomic and early-life factors as genuine medically and clinically relevant data that urgently need to be collected. Finally, many biological samples have been collected in the ongoing studies. The mechanisms linking the early life environment with a defined later-life phenotype are starting to be elucidated, and perhaps hold the key to understanding inequalities and differences in the severity of COVID-19.

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# *Chapter 2*

# *Transgenerational impacts of early life adversity: from health determinants, implications to epigenetic consequences*

**My contribution to this Chapter:**

Conceptualisation, Literature review, Data integration, Making of all figures, and Writing of the article.

**This Chapter has been accepted for publication as:**

**Cyrielle Holuka**, Nathalie Grova, Eleftheria G. Charalambous, Jeanne Le Cleac'H, Jonathan D. Turner, Archibold Mposhi

Chapter 1 demonstrated the importance of taking into consideration the early life environment as well as how later exposure to external stressors shape individual phenotypes during the following decades of life. However, not only lifestyle and life experiences themselves can define this specific health trajectories. In the following Chapter we questioned the implications and role of (epi) genetic determinants in addition to environmental factors exposure leading to the transmission of genetics marks to the next generation.

The inheritance process is already well described in the literature and intervenes in many biological processes, however its exact underlying mechanism remains misunderstood (Xavier et al., 2019). To date, many articles demonstrated how this process occurs regarding the involvement of various genetic and biological factors (Legoff et al., 2019; van Otterdijk & Michels, 2016). Indeed, the biological and cellular processes as the germline reprogramming to the foetus development have been well described during the past decades. However, the precise demethylation and re-methylation step remains an actual a mystery (Singh et al., 2023).

Here we proposed a new theory to describe how the embryonic epigenetic reprogramming occurs during the development process and leads to demethylation errors that will be transmitted to the offspring later on. As we previously highlighted, there is a clear epigenetic imprint on the child's genome after early maternal trauma exposure. Indeed, we recently demonstrated how maternal directing CpGs can induce child's gene imprinting remaining and thus over at least seven years.

More precisely, we hypothesised that ELA plays a determinant role in this transmission process and can represent one of its numerous unknown causes. Here, we gathered information regarding various sources of exposure such as pollution, socioeconomic status (i.e. economic hardship), microbiome, etc. More particularly, we underlined the potential role of mitochondrial imprinting. Indeed, many studies reported the importance of genetic and epigenetic background in the inheritance process, however other factors such as mitochondria remain poorly described. In the same idea as mitochondria, we investigated the non-coding RNA implication. As well as all other indispensable biological components, ncRNA such as small interfering RNA or microRNA are known to be transmitted over many generations due to exposure to stress (Beck et al., 2021).

So far, almost all the studies described in the literature were based on non-human models due to the complexity of participant's recruitment and ethics. Nevertheless, it is now essential to transpose those findings on human models (Beck et al., 2022; Van Cauwenbergh et al., 2020). In order to develop better theoretical models and recruit human cohort to examine them, it is necessary to fully understand how every mechanisms work together. As we already suggested in

our researches, there is not only one factor influencing the epigenetic inheritance process, but rather an ensemble of factors all acting together during specific moments of development as well as on precise genomic positions.

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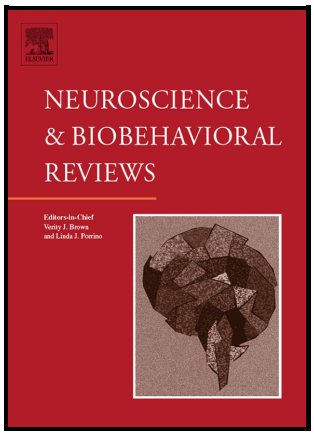


# Transgenerational impacts of early life adversity: from health determinants, implications to epigenetic consequencesRunning head: Transgenerational impacts of early life adversity

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# Transgenerational impacts of early life adversity: from health determinants, implications to epigenetic consequences

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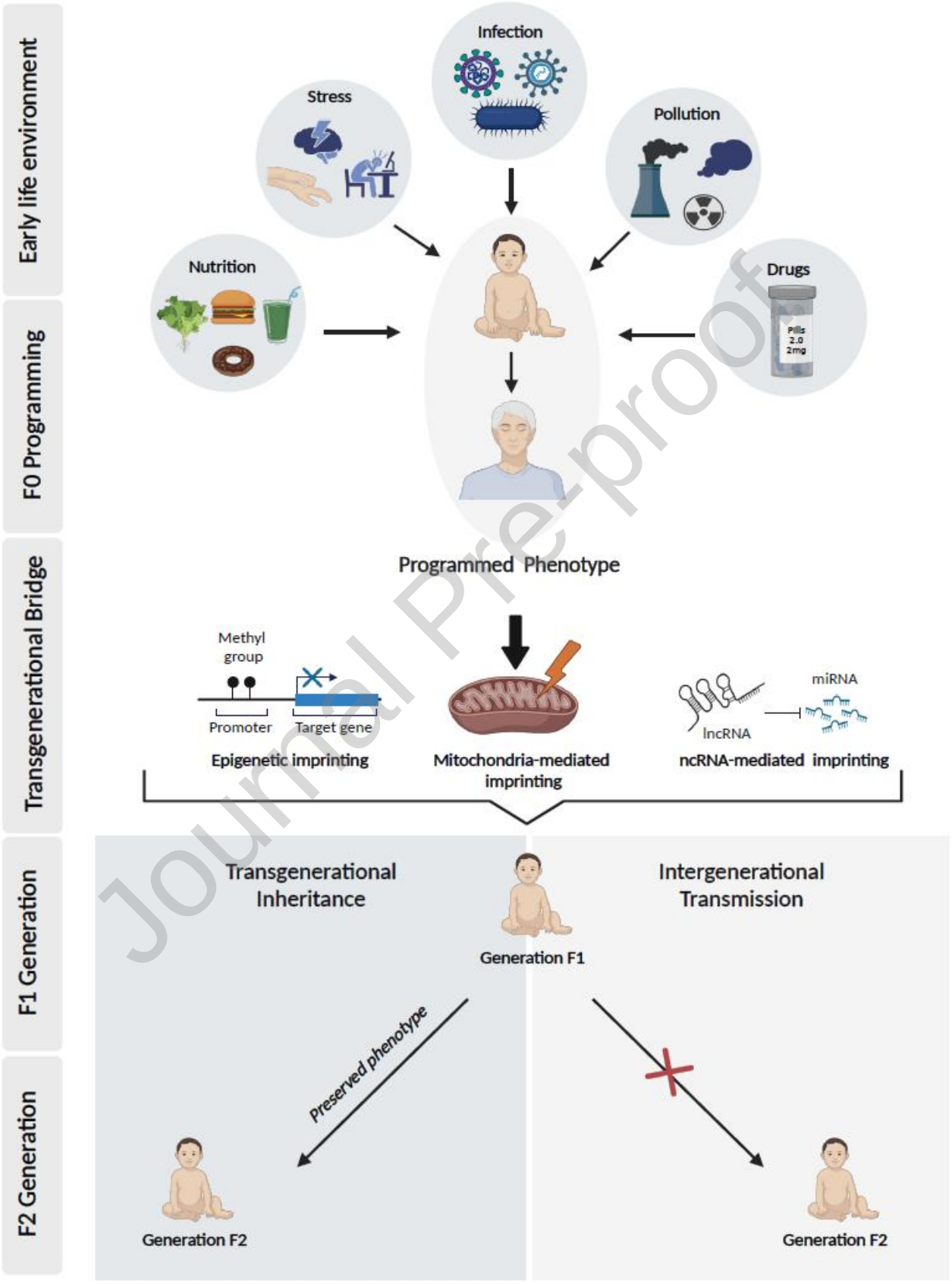
**Graphical abstract**



**Abstract**

Exposure to different environmental factors, social and socioeconomic factors promotes development of the early-life adversity (ELA) phenotype. The persistence of this phenotype across generations is an interesting phenomenon that remains unexplored. Of late many studies have focused on disease-associated outcomes of ELA following exposure during childhood but the persistence of epigenetic imprints transmitted by ELA exposed parents to their offspring remains poorly described. It is possible that both parents are able to transmit ELA-associated genetic imprints to their offspring via transgenerational inheritance mechanisms. Here, we highlight the role of the mother and father in the biological process of conception, from epigenetic reprogramming cycles to later environmental exposures. We explain some of the known determinants of ELA (pollution, socioeconomic challenges, infections, etc.) and their disease-associated outcomes. Finally, we highlight the role of epigenetics, mitochondria and ncRNAs as mechanisms mediating transgenerational inheritance. Whether these transgenerational inheritance mechanisms occur in the human context remains unclear but there is a large body of suggestive evidence in non-human models that points out to its existence.

**Keywords:** Early-life adversity, DNA methylation, Epigenetics, Transgenerational inheritance, Mitochondria, non-coding RNAs

## 1.0 Introduction

Early-life adversity (ELA) is associated with a higher risk for chronic diseases later in life such as autoimmune diseases, allergy or asthma [1]. Additionally, mental disorders such as depression and anxiety are associated with ELA [2]. It is known that early-life adversities play a role in the onset of disease later in life, and therefore influence the entire health trajectory. The persistence of certain disease-associated traits in the germ line and their inheritance across generations after exposure to ELA suggests that the phenotype can be transmitted over several generations. Over the last decade, transgenerational inheritance has gained prominence as more studies are starting to investigate its role in transmitting the ELA phenotype across generations. Transgenerational inheritance represents a series of altered phenotypes occurring during the second or third generation without re-exposure to environmental stressors [3]. Conversely, when the altered phenotypes are only observed for a shorter timescale in the first generation, we refer to them as intergenerational transmissions. Intergenerational transmissions frequently

occur when a generation is re-exposed to the same environmental conditions as its preceding generation [4].

Transgenerational inheritance depends of the epigenetic reprogramming which occurs during embryogenesis as the fertilised oocyte develops into a zygote and later becomes a foetus. During this process, both maternal and paternal genomes undergo two rounds of DNA methylation reprogramming [5]. However, epi-mutations can also occur during this step leading to disease development. The first reprogramming round corresponds to the demethylation of genomic DNA within primordial germ cells during early embryogenesis, followed by the restoration of methylation patterns during somatic development.

Embryonic epigenetic reprogramming allows for the removal of parental epigenetic marks or signatures acquired during their developmental exposure to environmental stressors. However, if the germline reprogramming fails, these marks can be conserved and transmitted from one generation to the next [6]. Even though the process remains poorly described, there are a handful of studies that highlight the potential role of biological processes involved in transgenerational inheritance. Indeed, Xavier et al. suggested that oxidative stress could affect the transgenerational-inherited information [7]. Oxidative stress results in the chemical modification of guanine bases leading to the formation of the mutagenic base, 8-hydroxydeoxyguanosine (8OHdG). In spermatozoa cells exposed to oxidative stress, the formation of 8OHdG is associated with birth defects and miscarriages [8]. This DNA damage associated DNA modification, in addition to incomplete or inefficient DNA repair by the oocyte, directly impacts the health of the next generation. Oxidative stress also plays a pivotal role in mediating epigenetic gene silencing by prompting hypermethylation of promoter regions [9]. From these previous studies, it is indeed evident that oxidative stress occurring in the germline induces “variability” which can potentially be transmitted across generations [7].

As opposed to the general notion that the maternal germline forms the bedrock of transmittable traits across generations, evidence from previous studies suggests that differential methylation in the paternal germline is also associated with *in utero* malnutrition or obesity in mice models [10]. Furthermore, it has been demonstrated that after exposure to a specific diet, metabolic changes are induced in the offspring via the paternal germline [11]. Indeed, in murine models paternal low-protein or high fat diet leads to a differential gene regulation and metabolic disorders in the offspring [12]. Moreover, an epigenetic asymmetry between parental genomes have been observed. For example, the foetal insulin-like growth factor (IGF2) is transmitted by

the paternal genome and the offspring phenotype shows growth deficiency. However, when transmitted by the maternal genome, the phenotype of the offspring remains normal [13].

The presence of transgenerational epigenetic inheritance (TEI) in man has been convincingly demonstrated in descendants of both holocaust and Dutch hunger winter survivors. The children of the Dutch hunger winter survivors that were exposed in-utero to famine conditions during their third trimester of development were not only lighter and, smaller at birth than those immediately before the famine, but these babies and their children went on to have a higher risk of cardiovascular and, metabolic diseases, with notably, impaired glucose tolerance and a propensity towards obesity and type 2 diabetes when they were adults [14]. Similarly, offspring of parents exposed to the holocaust had altered methylation levels within the *FKBP5* gene which correlated with their parents, showing evidence of possible transgenerational inheritance mechanisms at play [15]. In essence, the Dutch Hunger winter and the holocaust presented an opportunity to study the occurrence and persistence of epigenetic traits throughout life from the pre-/periconceptual period up to adult life.

The underlying mechanisms that drive transgenerational inheritance remain obscure but there are suggestive studies where different mechanisms have been explored in human and non-human models. These mechanisms, which include, DNA methylation, histone post-translational modifications and non-coding RNAs have been extensively reviewed [16-19]. In the current summative review, we focus on how environmental factors (i.e. pollutant exposure), social and socioeconomic factors (i.e. early infection, nutrition and microbiome contribution) during the early-life phase contribute to the development of chronic diseases later in life. In addition to the other mechanisms that have been previously reviewed, we introduce mitochondrial (DNA) mediated imprinting and explore maternal zygotic factor mediated epigenetic imprinting as potential mechanisms to consider in the context of ELA.

## 2.0 Connecting the Transgenerational Bridge

The maintenance of the "molecular memory" over generations does not only depend on alterations or depletions of DNA sequences but also on epigenetic mechanisms and the persistence of "yet-unknown" factors. Unlike DNA sequence depletions, epigenetic mechanisms are versatile and they allow dynamic gene expression transmission across generations, which is vital for adaptation [20]. In addition, every genome possesses inherited maternal and paternal imprints after the fertilization step, which are conserved into adulthood [21, 22].

### 2.1 Epigenetic reprogramming cycles

Epigenetic information is transmitted by patterns of DNA methylation and histones modifications. DNA methylation is the covalent attachment of a methyl group on CpG site. It has been associated with regulation of gene expression. DNA methylation within gene promoters can inhibit transcriptional activity (i.e. gene silencing) [23]. Histones are epigenetic regulators responsible of chromatin structure and gene expression. As DNA methylation, histones can also play a role modifying gene expression levels and patterns [24]. Histone acetylation leads to a better access to DNA for transcription while histone methylation influences transcriptional repression/activation of amino residues [7].

Genomic reprogramming occurs at different stages within germline cells. This process starts with the demethylation of female and male primordial germ cells (PGCs) which erase the parental genomic imprints [25]. The reactivation of the inactive X chromosome in female germ cell follows the demethylation step [26]. This step allows the removal/erasing of any aberrant epigenetic modifications, avoiding the transmission of epimutations [27]. Following germ line reprogramming, the zygote-reprogramming step includes the interaction of oocyte cytoplasmic factors with both parental genomes [28]. Maternal genome will be *de novo* methylated while paternal genome remains unmethylated [29]. Specific maternal oocyte cytoplasmic factor as the heterochromatin protein 1 (HP1) can interact/ bind with methylated histones HE (meH3) leading to potential *de novo* DNA methylation [30]. HP1 plays a crucial role in nuclear organization, chromatin assembly and gene regulation [31]. DNA methyltransferase 1 (DMNT1) is a large protein with multiple domains and responsible of intramolecular regulations [32]. DNMT1- dependant regions conserve specific human genome sequence and enriched genes playing a major role in cell to cell interaction. An enhanced expression of those genes will lead to neurological dysfunction due to environmental stress exposure [33]. In addition, Li et al. demonstrated that DNMT1 is essential in the maintenance of methylation imprints [34]. Lastly, research has shown that accurate histone methylation during spermatogenesis is crucial for the development and survival of offspring across several generations. These findings illustrate the significance of histone methylation as a molecular mechanism responsible for paternal epigenetic inheritance. Environmental factors that modify this process could impact embryo development and the transmission of complex diseases across generations [35].

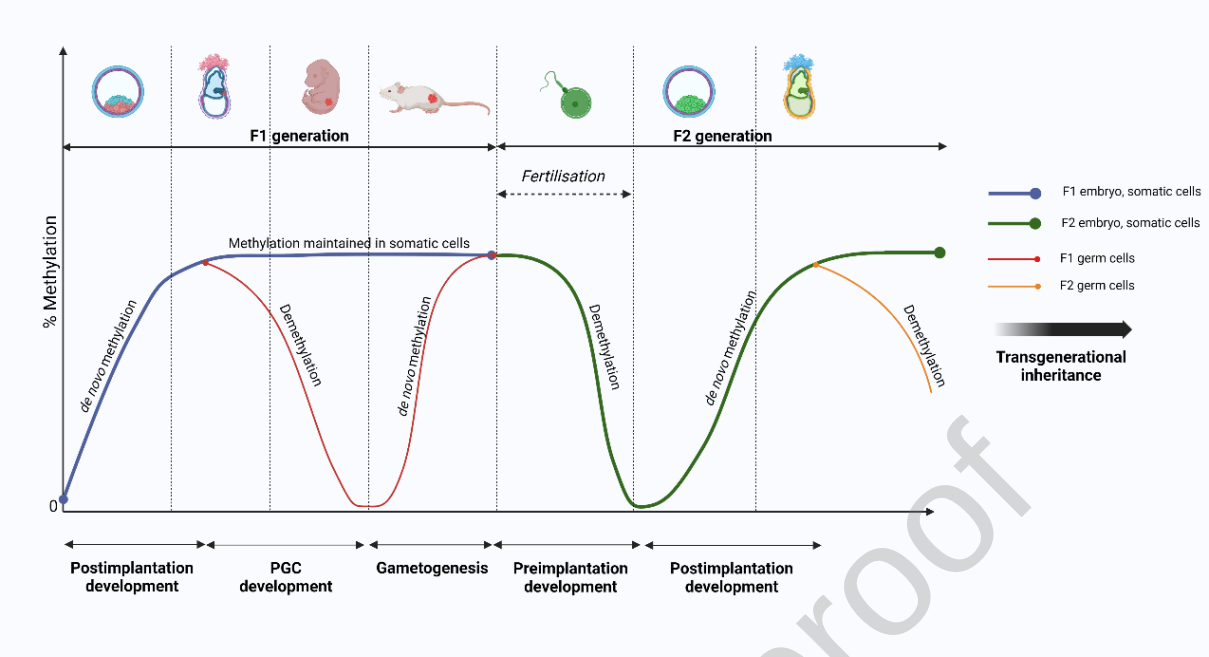



**Figure 1: DNA methylation is a conduit for transgenerational inheritance**. During development, cells undergo epigenetic reprogramming cycles to establish lineage-specific gene expression patterns. These cycles involve the erasure of existing DNA methylation marks, followed by the establishment of new marks that are specific to the cell type or developmental stage. After fertilization, the zygote undergoes a series of reprogramming events to remove DNA methylation inherited from the spermatozoa and oocyte. This erasure of epigenetic marks is necessary for embryonic cells to regain pluripotency. Subsequent reprogramming events then establish new methylation marks that direct the cells to follow specific developmental pathways.

## 3.0 Environmental pressures and factors associated with ELA

Early-life adversity is a somewhat "catch-all" term for negative aspects of the early-life exposome. It is all-encompassing and includes the social, psychological, financial, physical and emotional environment, categories that are often intertwined. Although it isn't a definitive categorisation, we have previously found it interesting to divide ELA into four categories that we maintain here: the socioeconomic and psychosocial environment, the microbial environment (microbiome and infection), the nutritional and metabolic environment, and the physical environment (e.g. pollution or pharmaceutical exposure) [36]. However, any form of classification is limited by the overlap between the categories as observed during the COVID-19 pandemic period. Indeed, a lack of social interaction, financial burdens and overall additional societal stress that further affect primary medical, nutritional and educational needs [37-40], together with changes in the exposure to pathogens, medication use and the richness

of early life nutrition significantly altered the establishment of the microbiome in this early life period [41, 42].

### 3.1 Socio-economic factors and stress

ELA is a series of environmental experiences and stressful events from conception to adulthood influencing development and health. ELA exposure induces long-term endocrine and pathologies risk later in life [43]. Indeed, ELA can lead to gene expression alteration and can be associated with an increased risk for alcohol disorders, suicide and mental health conditions [44, 45]. ELA is also associated with morbidity and poor socioeconomic outcomes in adulthood [46]. Socioeconomic factors (SES) such as education, incomes or occupation events are also considered as crucial determinants in human health and well-being [47]. SES regroup different factors as stressful jobs, pollution that can influence human health [48]. They are well known to be associated with cardiovascular, respiratory or intestinal diseases [49]. Additionally, literature has shown that maternal behaviour can play a crucial role in determining the health outcomes of children later in life [50]. Studies demonstrated that maternal behaviour induces stable alterations of DNA methylation and chromatin structure [51] demonstrating the long-term care maternal effects on gene expression [52]. More recently, economic hardships such as material deprivation or major financial problem are now considered as predictors of low socio-economic status. When occurring during the developmental period of life (pregnancy of the mother (F0)), these factors can directly affect DNA methylation levels. Recently, Clark et al. demonstrated that mothers who experienced maternal economic hardship during pregnancy, gave birth to children with smaller head circumference [53]. Here, parents represent generation F0 while their children are generation F1. It is now well recognized that financial issues induce worse cognitive (i.e. memory problem or fatigue) and non-cognitive outcomes (i.e. behaviour or intellectual abilities) during offspring's adulthood [54]. Studies demonstrated that developmental periods (i.e. early postnatal) in the F1 generation are the most sensitive periods in term of exposure to ELA in addition to the F0 generation exposure to ELA [55]. This is unsurprising, as during the perinatal period of children, neurogenesis and neuronal differentiation processes continue together with the establishment of synapses and neural circuits [56], processes that are sensitive to environmental and epigenetic changes [57]. Maternal stress can directly or indirectly influence foetal development through placenta [58]. The detailed underlying mechanism of how maternal stress can alter children development remains unclear. However, as germ cells are sensitive to stress, we can now potentially consider

that it is the result of transgenerational transmission [59]. Indeed we suggest that the maternal environment influenced by adversity exposure play a role in the epigenetic remodelling of the children epigenome such as DNA marks. As the conservation of those marks, supposedly through stem cell, can persist over years it is possible to speculate that without re exposition to additional adversity exposure, the F1 generation can transmit those mark to the generation F2. This mechanism of DNAm marks transmission corresponds to the transgenerational mechanism we highlighted above. So far, studies confirmed that traumatic events often occurring a long time before pregnancy such as childhood maltreatment (CM) lead to neuropsychiatric disorders in descendants [60]. Childhood sexual abuse (CSA) is one of the most common CM leading to offspring developmental alterations. Indeed, CSA is mostly associated with mental health diseases such as schizophrenia, suicide, depression, anxiety, etc. [61]. CSA trauma affects the hypothalamic-pituitary- axis (HPA) leading to an unbalanced stress process [62]. Additionally, when mother experienced trauma during their pregnancies, pro-inflammatory cytokines (i.e. IL-6) and C-reactive protein (CRP) levels were increased in comparison with non-exposed mothers. In a similar manner, SES play a crucial role in terms of health determinant exposure, however, many others external factors such as nutrition need to be considered. Chronic inflammation during pregnancy, triggered by high levels of pro-inflammatory cytokines, can harm the developing foetus, potentially leading to issues with brain development, cognitive function, and even future mental health [63]. Maternal stress studies are well described in the literature; however, paternal stress consequences remains unclear. In 2014, McGhee K.E et al. demonstrated that paternal care could also reduce DNA methyltransferase 3a (Dnmt3a) expression in the brain of their descendants [56]. The DNMT3 family plays a crucial role in the establishment of *de novo* DNA methylation marks during differentiation and embryogenesis [64] .

### 3.2 Early-life microbiome and nutrition

The ELA exposome includes the developing Early Life Microbiome (ELM) and the nutritional intake that the new-born receives. Individuals that spend up to their first 24 months of life within an institutional setting end up having taxonomic imprints of ELA experiences in their microbial profiles [65, 66]. The composition of the first ELM depends on the mother`s microbiota and the mode of birth, defining the primary colonisers [67, 68]. Subsequently, the establishment of this first microbial community depends on the nutrients available and in particular the diversity of human milk oligosaccharides (HMOs) and short-chain fatty acids (SCFAs), received during the

first hours and months [69, 70]. Simultaneously other physiological systems including nervous and immune systems are further developing. Evidence on microbiome-immune interaction exist since the prenatal period between maternal microbial metabolites and foetal thymus [71]. Whilst in early post-natal period the ELM aides in immune tolerance and maturation where the microbial metabolites are key moderators of immune signalling, differentiation and maturation [72, 73]. The microbiome-immune axis is closely related to the direct activation and differentiation of immune cell progenitors demonstrating the existence of the microbiome-bone marrow axis [74, 75]. The underlying mechanism is believed to act through direct methylation-demethylation on bone marrow derived immune progenitors and/or depended on INF-I Interferon type I and STAT1 signalling [76, 77]. There is now clear evidence for gut-brain, gut-liver, oral-gut-liver, oral-gut-brain and oral-brain axes as well as the gut-lung, and gut-bone marrow axis [78-80]. All of these interactions dependent to a certain extent on communication through nerves such as vagus nerve, which the gut-brain axis uses. Some other understudied routes are via the trigeminal nerve, or arising evidence from SARS-CoV-2 viral infection that suggest taste and olfactory receptors and their nerves [80, 81]. At the centre of this unintelligible net of interaction axes lays the immune system. Either as a reaction to a "threat" stimulus or as part of the typical physiological process for immune maturation, the microbiome-immune crosstalk is somehow always involved in health trajectories.

Epigenetic modifications are the core strings of how the early-life microbiome and its metabolic competency are able to influence developmental trajectories of health and disease [82, 83]. Microbial metabolites are essential for proper epigenetic processes, and their deficiency can disrupt and alter normal epigenetic programming [82]. Nonetheless, all the above examples highlight how this close interaction of the early life exposome the ELM and the host imprint the epigenome and define the health trajectory. What is worth noting is the ELM is given by the mother and its influence is likely adjusted to the mother`s physiology and including the maternal epigenome. The aforementioned interactions or crosstalk's and how they unfold during the early life phase could generate potent epigenetic imprints. Whether these imprints can be transferred onto the next generations remains an open question.

### 3.3 Early life-infections

Early-life infection (ELI) can have devastating short- and long-term effects. For example neonatal bacterial or viral sepsis increases the risk of death within the first 12 days of life threefold. While short-term risks are obvious, serious long-term complications often arise. For

example, neurodevelopment in survivors of neonatal sepsis is significantly poorer than usual [84], and longitudinal birth cohort analyses have shown that early-life infections associate with both measures of chronic inflammation and overall morbidity and mortality from cardiovascular disease later in life [85, 86]. Early-life respiratory infections, particularly of viral origin, increase the subsequent risk of developing asthma and/or allergies in childhood [87-89] and hint to a long-term viral-induced type 1 diabetes [90]. However, although ELI may represent a specific type of ELA, its' intimate link to social behaviour and the social environment means that there may be many other actors in play [91]. Initially, inflammatory elements of the ELI immune responses were thought to be responsible for later-life disease [92]. In particular, dysregulated inflammation was proposed to increase the risk of mortality from cardiovascular disease and stroke due to their association with atherosclerosis [86, 92]. Indeed, chronic exposure to infection, particularly in early life, induces lifelong increased CRP levels [93] as well as eosinophilia, and increased immunoglobulin E, interleukin-6, and erythrocyte sedimentation rate (ESR) [94]. Recent studies in male mice exposed to polyinosinic:polycytidylic acid (Poly I:C), a viral infection mimetic and liposaccharide (LPS), a bacterial infection mimetic have shown that the F2 offspring display behavioural changes which are mediated by miRNAs derived from spermatozoa [95, 96]. While this phenomenon is evident in mice models of viral and bacterial infection, it remains to be seen whether similar mechanisms are also present in humans.

In the human context ELI occurs in a specific context: the new-born immune system is immature and functional populations of both adaptive and innate immune cells have not been fully established [91], and the naïve T-helper (Th) cells present are epigenetically biased toward a Th2 phenotype [97]. This "blank slate" was proposed to be a period of plasticity during which the immune system can adapt and prepare for life in an environment similar to that in which the individual was born [98-100], shaping the long-term immune trajectory [91]. This adaptation of the immune system is exemplified by the hygiene hypothesis. At its most simple level, the hygiene hypothesis states that contact with as broad a range of non-pathogenic microorganisms as possible during childhood is a pre-requisite to successfully establishing immune tolerance, and reduced or absent exposure increases the risk of immune-mediated diseases such as auto-immune disease and allergy [101-103]. However, exposure to pathogens, allergens, and air pollution have the opposite effect, increasing the risk of allergy [91, 104, 105]. It remains to be investigated whether this increased risk of allergy persists in the subsequent generations via transgenerational inheritance mechanisms.

One of the key intermediates linking ELI to the eventual phenotype may be stress reactivity and the stress-cytokine axis [106]. ELI, while the immune system is not fully developped, leads to exaggerated cytokine responses, principally IL-1β, IL-6, and TNFα, all of which pass rapidly from the blood to the cerebrospinal fluid [107], impairing long-term memory and brain plasticity [108]. In a zebrafish model of ELI infection timing was the key element determining increased baseline pro-inflammatory gene expression in adult animals [109]. In human ELA paradigms such as institutionalisation-adoption where increased rates of infections are an integral element of the stressor, we and others have reported a blunted cortisol stress response that is accompanied by immune disturbances including higher numbers of exhausted Natural killer cells and both cytotoxic and helper T-lymphocytes [110-113].

While the molecular mechanisms underlying the long-term pathophysiological effects of ELI remain unclear, a recent observation gave a glimpse into how the phenotype may be maintained over many years and potentially across generations. Immune cells are perpetually renewed from a reservoirs of hematopoietic stem cells, and epigenetic modifications in this compartment led to long-term changes in levels of inflammation and immunometabolism after ELI, that induced functional difference upon re-infection later in life [114]. It is possible, however, that the mechanism may be somewhat simpler. ELA is associated with a higher rate of infection from *Herpesviridae* such as CMV, EBV and HSV. These chronic viral infections are regularly re-activated once acquired. Chronic CMV infection is associated with a reduction in naïve T-cells [115], and specific memory T-cell expansion [116, 117] reminiscent of the ELI and ageing phenotype [118].

### 3.4 Early-life drug and pollutant exposure

Direct exposure to medications, drugs and/or environmental toxicants may lead to epigenetic alterations and later contribute to the development of diseases in subsequent generations [36]. This concept is in line with the classic paradigm of genetic determinism, which emphasizes the role of genetics in the development of disease [119]. Therefore, by integrating environmental epigenetics into disease aetiology, we assume that xenobiotics not only impact the generation initially exposed but could also be transmitted to future generations through the germline. Their effects can manifest as a wide spectrum of health issues, including developmental abnormalities [120], reproductive disorders [121], increased susceptibility to neurodevelopmental and neurodegenerative diseases [36]. Anti-epileptic drugs (AEDs) have been widely prescribed to

pregnant women in developed countries [122, 123]. Several studies have suggested that children exposed *in utero* to AEDs present an increased risk of malformations and neurodevelopmental disorders [124]. This is particularly the case with valproate acid (VPA), whereas the level of evidence varies for other anti-epileptic drugs such as Topiramate, Carbamazépine, Phénobarbital, Primidone, (fos)phénytoïne [124]. In addition to the highest risk of childhood malformations, VPA also entails a high risk of neurodevelopmental disorders. An increased risk, by a factor of 4 to 10, of developing autism spectrum disorders (ASD) in children has been clearly established in women treated with VPA during pregnancy. [125]. VPA promotes histone acetylation, which in turn may impact DNA and histone methylation patterns, resulting in modifications in the expression of transcription factors. All these epigenetic modifications have been associated with chromatin remodelling effects [126]. It has been shown that the ASD phenotype induced by VPA in rodents is transmitted epigenetically, at least up to the third generation [126]. Tsuji et al (2022) recently investigated, in rodent model, behavioural deficit triggered by VPA on postnatal day 5 of the F2 generation to evaluate the onset of the ASD phenotype. The results pointed out that impaired locomotion as well as social deficit could be inherited by the subsequent generation and were apparent early in life [127]. Epigenetically-inherited adverse effects of valproate acid were recently demonstrated in 90 families comprising 85 women and 23 men, who experienced complications due to VPA exposure *in utero* and became parents in their turn. Among their children (n=187), 44% were suffering from neurodevelopmental disorders, 23% presented malformation(s), only 47% had neither developmental disorders nor malformation [120]. Although there have been reports of multigenerational inheritance across different types of AEDs, there is a scarcity of studies investigating transgenerational inheritance of traits following parental exposure to addictive substances [128]. To date, transgenerational phenotypes have also been observed following parental exposure to morphine, alcohol, and nicotine [121, 128]. It has been conclusively demonstrated that prenatal drug exposure has a significant influence on future generations, leading to consistent alterations in various aspects such as drug-taking behaviour, stress response and dopamine signalling in offspring [128]. Lastly, research demonstrates that exposing pregnant female mice to bisphenol A (BPA) leads to obesity in their F2 progeny, a trait that persists for up to the F6 generation even without further exposure. This obesity is linked to increased food intake and is inherited, with its mechanism associated with a chromatin-accessible site within a cis-regulatory element (CRE) located in an intron of the *Fto* gene. Importantly, exposure to BPA results in demethylation of this CRE [129].

A large number of environmental chemicals, such as dioxin, heavy metals, Polycyclic Aromatic Hydrocarbons (PAHs), pesticides, and even some plastics, are also likely to have transgenerational effects. In this context, Van Cauwenbergh et al. recently studied whether persistent epigenetic changes could occur in the male germline following exposure to synthetic endocrine disrupting chemicals (EDCs) [119]. To answer this question, they carried out a systematic search that resulted in the inclusion of 43 articles addressing the effects in mammals of commonly-used synthetic endocrine disruptors, including plasticizers (bisphenol A and phthalates), pesticides (methoxychlor, atrazine, dichlorodiphenyltrichloroethane and vinclozine), dioxins and PAHs. In most of these studies performed on animal models, transgenerational epigenetic effects have been highlighted, often associated with the appearance of metabolic disorders, behavioural testicular or prostatic abnormalities as well as tumour development [119, 130]. To date, there is a lack of evidence concerning the mechanisms of action of EDCs on the germline epigenome and the associated risk of disease in human offspring. In this context, longitudinal observational studies such as the Avon Longitudinal Study of Parents and Children (ALSPAC) have provided compelling evidence. One such study pointed out that adolescents whose fathers started smoking before puberty were at increased risk of developing obesity [131]. Although the specific biological mechanisms were not investigated by the authors, which finding led the authors to assume that the chemicals (such as PAH, heavy metal, nicotine) present in cigarette smoke could potentially induce epigenetic alterations in the production of spermatogonia in the testes before puberty, which would have an impact on the next generation [119, 132]. In parallel, World health Organisation (WHO) data highlight that almost the entire world population (99%) breathes air that exceeds WHO guidelines and contains high levels of environmental pollutants, with low- and middle-income countries being the most exposed. Once it has been demonstrated that airborne particles impair mitochondrial machinery and cause significant damage to the epigenome, the transgenerational effects of air pollution in human were the next logical area to investigate [133]. Thus, Shukla et al. recently pointed out that the alteration of DNA methyltransferases activity which in turn triggers changes in DNA methylation in human offspring that could be handed down from one generation to the next and therefore lead again to transgenerational epigenomic inheritance.

## 4.0 Molecular mechanisms of inheritance

Exposure to certain environmental pressures during the early life phase can have long lasting health implications which span generations. In our view, this can only be achieved through the

activation of robust mechanisms of inheritance which remain active in the offspring and their subsequent descendants. Several studies have proposed mechanisms to explain how transgenerational inheritance occurs in different species (reviewed in [7, 134, 135]). Using non-human models has made it possible to identify some overarching interspecies similarities, which provide potential mechanistic insights into the human situation with respect to transgenerational inheritance. It is important to note that these mechanisms do not necessarily occur in splendid isolation of each other but may occur sequentially or concurrently depending on the timing and severity of exposure to ELA.

### 4.1 Maternal zygotic factors mediated genomic imprinting

Genomic imprinting is a heritable epigenetic process, which results in the expression of a subset of autosomal genes in a parent of origin specific manner [136]. Basing on the evolutionary context, maintenance of genetic imprints across generations from parent to offspring is important because over time it enables species to pass down certain advantageous traits to their offspring. On the contrary, detrimental or diseases associated traits are also passed down in a similar fashion. Genomic reprogramming is an essential step during embryonic development because in resets the epigenetic landscape allowing for a “fresh start.” This reprogramming differs for the male and female germ lines in that the male genome undergoes reprogramming after fertilization, while reprogramming of the female genome occurs gradually during foetal development. During embryogenesis, maternal zygotic factors are known to facilitate demethylation of paternal alleles (Gse), protect maternal imprints from demethylation (Pgc7 and stella), maintain these imprints (Zfp57), and regulate epigenetic stability (Trim28) [137] (Figure 2). In essence, by providing protection for developing zygotic development of the next generation, the female germ line plays a pivotal role in maintaining transgenerational imprints.

In a recent study in mice, it was observed that even when methylation within CpG islands (CGIs) is erased in the parental germ line, the unmethylated gametes can still transmit DNA methylation memory of the CGIs to the zygote [138]. The same study suggests that DNA methylation of the CGIs in these zygotes occurs at the early post-implantation stage in the next generation. This brings up new and interesting perspectives with regard to genomic imprinting where other unknown factors are responsible for maintaining DNA methylation memory. The obvious question is, what is this epigenetic memory? Possibly there is another layer of

regulation above the epigenome which enables imprinted genes to be maintained across multiple generations.

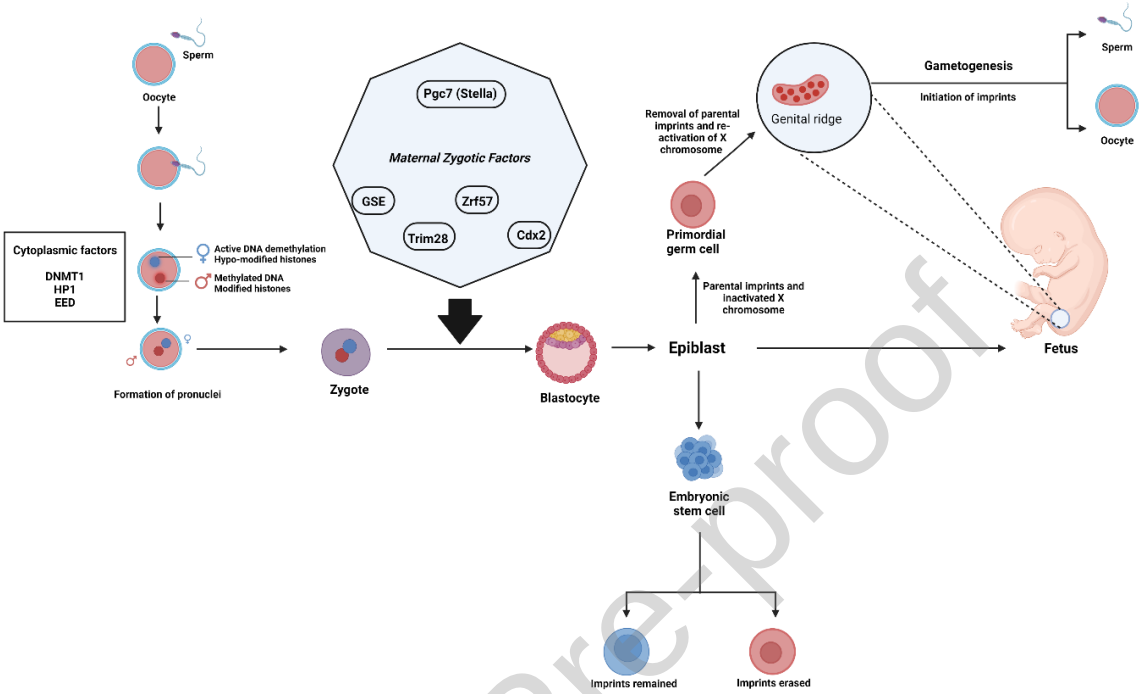



**Figure 2: Genomic imprinting.** DNA methylation, to specific regions of the DNA during gametogenesis results in epigenetic marks that are inherited by the offspring and persist throughout their development. This phenomena leads to parent-specific gene expression patterns in the offspring. Genomic imprinting primarily occurs in imprinted gene clusters in the spermatozoa and oocyte before fertilization. After fertilisation when both the spermatozoa and oocyte undergo sequential demethylation cycles, maternal zygotic factors play a critical role in preserving and maintaining genetic imprints. The offspring inherits two copies of each imprinted gene, that is, one from the mother and one from the father but only one copy is actively expressed, while the other copy is silenced or inactivated. During embryonic development some imprints are erased while some remain in place (Adapted from Surani, 2001) (Created with BioRender.com).



### 4.2. Mitochondrial mediated maternal imprinting

The mitochondria are largely considered as the powerhouse of the cell. Nevertheless, they are involved in many other cellular activities and processes besides energy production. The mitochondria produce reactive oxygen species (ROS), which serve as the main currency for intracellular signalling. Mitochondria are also involved in determining cell fate via ROS mediated signal transduction processes. The discovery of mitochondrial DNA (mtDNA) in the

1960s transformed our understanding of the role of mitochondria from being a mere energy factory. MtDNA is a circular double-stranded DNA molecule consisting of 13 protein-coding genes that encode some of the subunits involved in oxidative phosphorylation (OXPHOS). Each cell contains several copies of mtDNA, for example, mtDNA copy number is extremely high in oocytes ($\geq 10^5$ copies) as compared to the spermatozoa ($\leq 10^2$ copies) [139]. This phenomenon is particularly interesting when we consider the maternal inheritance of mtDNA. MtDNA is non-recombinant DNA and passes down virtually 'unchanged' through the direct maternal line over successive generations. In this regard, it is evident that mitochondria play an important role in transgenerational inheritance and are an interesting target for accessing the ELA across generations. From previous research, it is evident that the mtDNA undergoes epigenetic changes that potentially influence mitochondrial function [140, 141]. As described earlier in this review, there is a growing realisation that mitochondrial dysfunction may be one of the hallmark features characterising the ELA phenotype.

During embryogenesis, the nuclear genome undergoes extensive reprogramming which involves large-scale demethylation as mentioned earlier. After the spermatozoa enters the oocyte, both the maternal and paternal origin chromosomes undergo a sequential demethylation process. Paternal DNA undergoes TET3 mediated demethylation, which spares only imprinting control regions (ICRs) followed by a progressive demethylation of the maternal genome. Previous studies have shown that the maternally inherited factor, Stella, which is encoded by *DPPA3* protects the maternal genome and paternal ICRs from active demethylation. Stella is highly expressed in primordial germ cells and its expression is maintained throughout oocyte maturation right up to preimplantation of the embryo [142]. In essence, this indicates that the maternal genome, both nuclear and potentially mitochondrial are protected from active demethylation and that not all epigenetic imprints are erased during embryogenesis.

While it may appear logical to assume that all DNA in the cell including mtDNA undergoes demethylation, no studies that have been done to prove otherwise. It therefore, becomes a question of whether mtDNA undergoes similar reprogramming or maintains its epigenetic signature, which is then passed on to the next generation. On the other hand, if it undergoes demethylation, it remains elusive whether the mitochondria have efficient epigenetic machinery to reintroduce methylation. Moreover, it is plausible that mtDNA escapes the demethylation phase during embryogenesis, which enables mitochondrial information to pass down to the next generation unchanged. A recent study [143] demonstrated that bovine mtDNA methylation patterns were more highly conserved between oocytes and blastocysts compared to somatic

(granulosa cells). The maintenance of mtDNA methylation during embryogenesis suggests that maternal mtDNA does not undergo extensive epigenetic changes (demethylation) and therefore mtDNA may play an important role in genomic programming as well as genomic imprinting. Overall, it is evident that mitochondria play a critical role in biological embedding of ELA and provide potential mechanisms to explain transgenerational inheritance.

### 4.3 ncRNA mediated imprinting

The role of noncoding RNAs (ncRNA) such as small interfering RNA (siRNA), microRNAs (miRNA) and piwi-interacting RNA (piRNA) in transgenerational inheritance has been extensively studied in non-human models. The transmission of information by these ncRNAs across generations is another potential mechanism for transgenerational inheritance. Certain environmental conditions can evoke transgenerational gene regulation by endogenous siRNAs lasting several generations. For instance, in *C. elegans*, starvation conditions were shown to induce expression of a pool of endogenous siRNAs targeting several nutritional genes [144]. Expression of these siRNAs was maintained for at least three generations after returning the worms to nutritionally rich conditions, potentially transmitting an epigenetic memory for coping with food shortage. Consistent with this idea, these descendants had increased life span compared to control worms. Heat stress has similarly been found to alter gene expression through ncRNAs, and these expression patterns lasted for two to three generations after a return to normal temperature conditions [145]. All these lines of evidence point to an ncRNA-mediated mechanism that transfers epigenetic memory to the subsequent generations. Additionally, mouse models demonstrated the involvement of RNA-dependent mechanisms in the inheritance of acquired traits (Table 1). They emphasize the significance of small non-coding RNAs (sncRNAs) in germ cells and shed light on their susceptibility to early traumatic stress. Furthermore, they reveal the repercussions of exposure to such traumatic experiences in early life across multiple generations. Early exposure to toxicants such as DDT play a role in the epigenetic transgenerational inheritance of disease (e.g., obesity) through the germline. DDT induces sperm epigenetic alterations [146, 147].

However, the question still remains how these siRNA molecules manage to cross the transgenerational bridge from parent to offspring without encountering the reprogramming phase. Furthermore, it is debatable whether ncRNA mediated transgenerational inheritance occurs in humans as there have been no studies to date that prove the existence of such a

mechanism of inheritance. However, over the decades through the use of mice models, our understanding of these versatile ncRNA molecules has greatly improved. Previous studies have pointed out to the role of spermatozoa derived ncRNAs in murine transgenerational inheritance [148, 149]. The role of ncRNAs in regulating gene expression via chromatin remodelling, mediating epigenetic modification, transcriptional and post-transcriptional regulation has been extensively reviewed elsewhere [150]. Here, we hypothesize, that gametic ncRNAs could play a role in epigenetic regulation and therefore provide a potential conduit for transgenerational inheritance in humans.

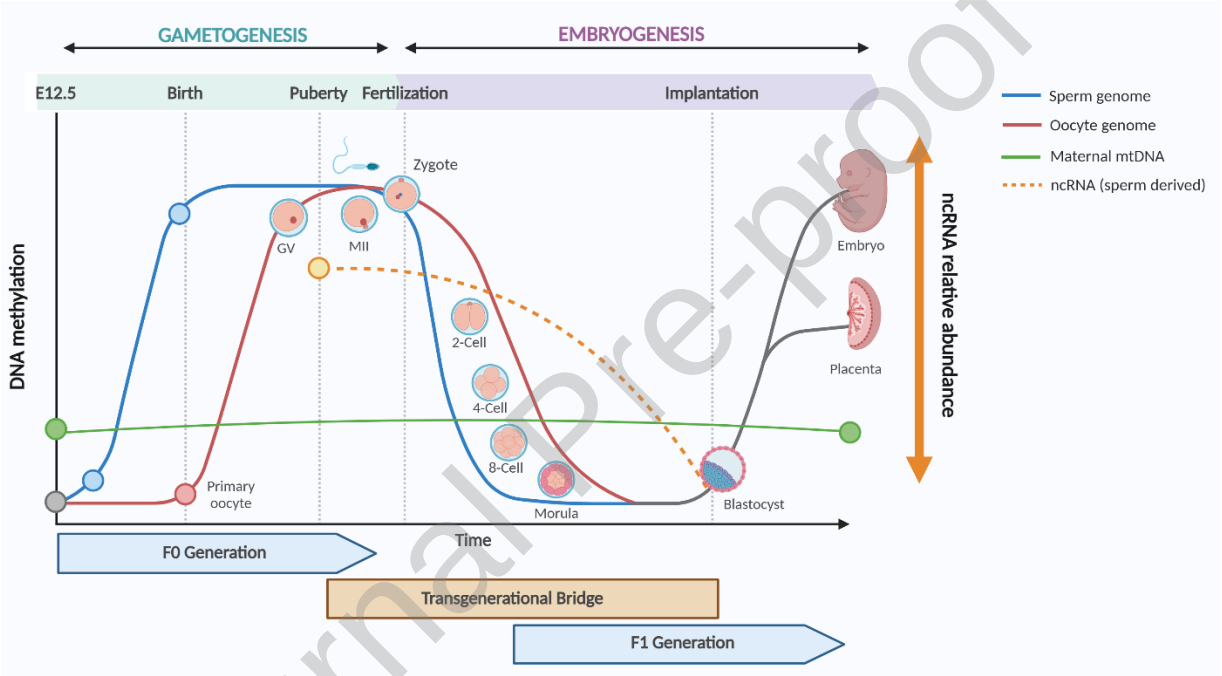



**Figure 3: Potential developmental mechanisms of transgenerational inheritance.** Diagram shows DNA methylation dynamics and spermatozoa derived ncRNA activity during embryogenesis. Hydroxymethylation of the paternal genome (blue) takes place soon after fertilisation followed by a passive demethylation of the maternal genome (red). *De novo* methylation of the fetal genome takes place at the blastocyst stage. We hypotheses that maternal mitochondrial DNA (mtDNA) (green) methylation remains relatively unchanged during embryogenesis and basically involves a transfer of the entire mitochondrial population from mother to offspring. We also hypothesise that spermatozoa-derived ncRNAs (yellow dashed) are present during the pre-implantation stage and they influence critical processes during fetal development. It is also during this stage that certain ELA traits associated with expression of these ncRNAs can be passed on to the F1 generation. Abbreviations: GV, germinal vesicle; MII, Metaphase II; mtDNA, mitochondrial DNA; ncRNA, non-coding RNA (Created with BioRender.com).

**Table 1: Summary of some key studies on intergeneration transmission (up till F1 generation) and transgenerational inheritance (F2 generation and beyond)**

| *Category* | *Stressor/ Exposure* | *Species* | *Exposed sex ($F_0$)* | *Offspring Phenotype* | *Proposed biological mechanism* | *Persistence (Generations)* | *Source* |
|---|---|---|---|---|---|---|---|
| *Physiological (Nutrition)* | Hunger/ Nutritional deprivation | Human | Female (during pregnancy) | -cardiovascular diseases<br>-impaired glucose tolerance<br>-obesity and type 2 diabetes | -DNA methylation (decrease in IGF2 gene methylation) | F2 | [14] |
| *Psychological* | Holocaust trauma | Human | Male and Female | - altered wake-up cortisol levels | -DNA methylation (FKBP5 intron 7) | F1 | [15] |
| *Psychological* | Maternal ELA (household chaos, disorganization, emotional abuse, and physical abuse) | Human | Female (5 – 15 years) | -decreased infant cortisol reactivity | None | F1 | [50] |
| *Psychological (Socio-economic)* | Economic hardships | Human | Female (during pregnancy) | -reduced birth weight<br>-reduced head circumference | None | F1 | [53] |
| *Physiological (Chemical)* | Valproate (VPA) | Human | Male and Female | -physical malformations<br>- neurodevelopmental disorders | -Histone acetylation<br>-histone methylation (based on [151]) | F2 | [120] |
| *Physiological (Chemical)* | VPA | Mouse | Female (during pregnancy) | - autistic-like impaired sociability<br>- increased seizure susceptibility<br>-hyperactivity<br>-decreased anxiety | -Histone acetylation<br>-histone methylation (based on [151]) | F3 | [126] |
| *Physiological (Chemical)* | Bisphenol A (BPA) | Mouse | Female (during pregnancy) | -obesity | -intronic DNA demethylation of Fto gene | F6 | [129] |
| *Physiological (Biological)* | Corticosterone | Mouse | Male: 10 weeks | -lower body weight<br>-hyperanxiety-like behaviour<br>-altered affective behavioural responses | -lncRNAs | F1 – F2 | [152, 153] |
| *Physiological (Biological)* | Parasitic Infection (*Toxoplasma gondii*) | Mouse | Male: 6 – 8 weeks | -anxiety-like<br>-depression-like<br>-impaired spatial working memory | -miRNAs | F2 | [154] |
| *Physiological (Biological)* | LPS (bacterial infection mimetic) | Mouse | Male: 8 weeks | Females:<br>-heightened social interaction<br>Males: | -miRNA, tRNAs, piRNAs | F2 | [96] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | -heightened activity<br>-only F2 had increased immune response (male and female) | | | |
| *Physiological (Biological)* | PolyI:C (viral infection mimetic) | Mouse | Male: | F1:<br>-depression-like behaviour<br>-altered stress response<br>-hippocampal changes<br>-increased immune responsivity<br>F2:<br>-mild behavioural changes | -miRNA | F2 | [95] |
| *Psychological* | -36h light exposure<br>-predator odor<br>-15 min restraint<br>-saturated bedding | Mouse | Male: 4 weeks | -altered neurodevelopment | -miRNAs | F1 | [155] |
| *Psychological* | -adverse childhood experiences<br>-stress and anxiety | Human | Male: 18 - 25 years | -altered neurodevelopment<br>- dysregulation in stress reactivity | -miRNAs | F1 | [155] |
| *Psychological* | Unpredictable maternal separation and stress | Mouse | Male: PND1 - 14 | - increased risk-taking behaviour | -lncRNAs | F2 | [156] |
| *Psychological* | -Chronic social defeat stress | Mouse | Male | -anxiety-like and depression-like symptoms (in both males and females) | -lncRNA | F1 | [157] |

## 5.0 Discussion / Perspectives

Over the past decades, many studies have become more focused on understanding the mechanism that drive epigenetic inheritance. Most of these studies have used animal models due the ethical complexity of carrying out such studies in humans. However, there are many gaps in knowledge as none of the animal studies has managed to reveal the “holy grail” mechanism of transgenerational inheritance that mimics the human situation. In this review, we highlighted and explored potential mechanisms that may drive transgenerational inheritance in humans following exposure to ELA. To describe the underlying mechanism of epigenetic transmission process we first considered the role of genetic and cellular components (i.e. germline cells) as a starting point transmitted by both parents.

Lately, many studies have pointed out the importance of maternal influence in the inheritance mechanism [3, 158, 159]. However, there is now growing evidence suggesting that not only maternal stress or maltreatment can directly influence offspring development. Indeed, paternal

stress elicits different mechanisms to induce epigenetic changes such as DNA methylation over time. It is now clear that parental pressures also lead the specific phenotype of offspring later in life "forcing" them to adapt to environmental pressures. Basing on all the studies we have highlighted, it becomes a question of whether maternal exposure to ELA is more important than paternal exposure in creating genomic imprints that have long lasting effects that transcends across generations. Indeed, from our previous studies we have demonstrated that maternal deprivation can induce epigenetic imprints on the offspring's genomes but no studies have been carried out to confirm the persistence of these imprints in succeeding generations. Nevertheless, the question remains whether, maternal imprints persist longer than paternal imprints.

Another aspect to consider when looking at the transgenerational inheritance of the ELA phenotype is the biological decline associated with ageing. Indeed, over time, diseases development becomes entangled with biological age and also with epigenetic age. Lately, the use of epigenetic clocks to measure epigenetic age demonstrated that ELA exposure occurring during the first stages of life left a specific reversible epigenetic signature on the genome. It therefore becomes a question of whether ageing is a determinant of ELA associated diseases or rather a parallel event whose characteristic features overlap with the ELA phenotype. Previous studies have suggested that ELA promotes accelerated ageing and also consequently promotes the development of age-related diseases such as diabetes. It is more likely to develop chronic diseases when ageing, however ELA can play a major part in accelerating their development. ELA exposure results in the genome modification by addition of specifics epigenetic marks. Some of these marks can persist over time while some will disappear few years after exposure. As demonstrated by Thorson et al., phenotypic and epigenetic consequences arise from ancestral exposure to a commonly encountered mixture of plastic-derived chemicals, even when administered at or near the no observable adverse effect level (NOAEL).Phenotypic effects assessed encompassed disease incidence within the transgenerational lineage, focusing on conditions such as testis and kidney diseases, as well as various other ailments [160]. The reasons of the DNA methylation conservation remains unknown. What are the mechanisms that determine which traits are conserved and which traits are removed? We hypothesised that age-associated canonical epigenetic marks will be erased while ELA-associated non-canonical epigenetic marks are carried over into the next generation. The conservation process is considered as a major element of the inheritance mechanism. Indeed, it appears that the position on the genome of methylation marks driven by ELA influence the time they remain present over time and brings into question whether cells have developed competent epigenetic

machinery to effect erasure of these non-canonical epigenetic marks. Furthermore, studies with fish models showed that exposure to cadmium (Cd) in one generation, along with elevated temperatures in subsequent generations, correlated with changes in the methylation levels of specific genes in female gonads and alterations in population sex ratios [161].

As previously alluded to in this review, another main aspect to consider is particular environmental factors and lifestyles enabling the development of imprints on the brain and immune system. Epigenetics marks such as DNA methylation are known to alter gene expression and by consequence affect the overall biological processes. Therefore, bringing together environmental and genetic factors might be the key point to elucidate the mechanisms behind the transgenerational inheritance process. So far, we have only scratched the surface in terms of understanding the roles of epigenetics, mitochondria and ncRNAs in transgenerational inheritance. We have highlighted these potential mechanisms because of the compelling evidence supporting their role in transgenerational inheritance in human and non-human models. As previously mentioned in this review, the study of transgenerational inheritance in murine models as well as other non-human models brings about the question of whether the same mechanisms of inheritance are present in humans. Although there is compelling evidence supporting meiotic epigenetic inheritance (MEI) in murine models, attributing the same mechanism to other animal models remains challenging due to the absence of clear causality [162, 163]. Given, the relative technical and ethical complexity of undertaking these type of studies in humans, animal models still provide us with the best platform to investigate potential mechanisms. It is evident that different types of adversity during the early life period elicit different inheritance mechanisms. The role of epigenetics in transmitting phenotypes across generations has long been a subject of debate. However, with the accumulation of examples of transgenerational epigenetic inheritance, its significance is becoming increasingly apparent, alongside a growing understanding of the underlying mechanisms. Common features include similar epigenetic signals and transmission methods, but variations exist both between and within organisms. The complex landscape of primary and secondary epigenetic signals reveals diverse regulatory pathways for TEI [164]. Here in, we further suggested that the magnitude of exposure determines which mechanisms will be activated. Overall, there is a rising need to construct well designed transgenerational cohorts that would allow to explore transgenerational inheritance in a multi-system approach. A promising starting example is the Lifelines NEXT cohort [165].

To conclude, the mechanisms by which transgenerational inheritance occur are still being studied. Based on our previous studies it is obvious that they involve epigenetic changes, alterations in gene expression or disruption of developmental processes (such as neurodevelopmental impairments or malformations). These changes may lead to lasting effects that are inherited by subsequent generations, even in the absence of continuous exposure to environmental pollutants, infection, stress and negative socio-economic factors. Mice, fish, and more recently, bird models have provided robust evidence of transgenerational inheritance through epigenetic modifications. While these animal models offer valuable insights into the mechanisms of transgenerational epigenetic inheritance , it remains to be seen whether these findings can be directly applied to the human context [166].

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**Abbreviations:** DNAmethylation (DNAm); Early life adversity (ELA); 8-hydroxydeoxyguanosine (8OHdG) ; Foetal insulin-like growth factor (IGF2); Non-coding RNAs (ncRNAs); Primordial germ cells (PGCs); Heterochromatin protein 1 (HP1); Methylated histones HE (meH3); DNA methyltransferase 1 (DMNT1); Socioeconomic factors (SES); DNA methyltransferase 3 alpha (Dnmt3a); Childhood maltreatment (CM); Childhood sexual abuse (CSA); Hypothalamic-pituitary- axis (HPA); C-reactive protein (CRP); Adenosine triphosphate (ATP); Early life microbiome (ELM); Human milk oligosaccharides (HMOs); Short-chain fatty acids (SCFAs); Signal transducer and activator of transcription 1 (STAT1); Early life infection (ELI); Erythrocyte sedimentation rate (ESR); T-helper (Th); Anti-epileptic drugs (AEDs); Valproate acid (VPA); Autism spectrum disorder (ASD); Polycyclic aromatic hydrocarbons (PAHs); Endocrine disrupting chemicals (EDCs); Avon longitudinal study of parents and children (ALSPAC); World health organization (WHO); CpG islands (CGIs); Reactive oxygen species (ROS); Mitochondrial DNA (mtDNA); Oxidative phosphorylation (OP); Imprinting control regions (ICRs); Non coding RNAs (mcRNA); Interfering RNA (siRNA); MicroRNAs (miRNAs); Piwi-interacting RNA (piRNA); meiotic epigenetic inheritance (MEI); cis-regulatory element (CRE)

## STATEMENTS AND DECLARATIONS

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Neither ethical approval nor consent to participate are applicable

**Consent for publication:**

Not applicable

**Availability of data and material:**

Not applicable

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Highlights

1. The persistence of the ELA phenotype across generations is a significant yet unexplored phenomenon, potentially involving the transmission of epigenetic imprints from ELA-exposed parents to their offspring.

2. While the occurrence of transgenerational inheritance mechanisms in humans remains uncertain, substantial evidence from non-human models suggests their existence.

3. Mechanisms such as epigenetics, mitochondrial mediated maternal imprinting and non-coding RNAs mediate transgenerational inheritance, potentially transmitting ELA-associated genetic imprints to offspring.

# *Chapter 3*

# *Adverse life trajectories are a risk factor for SARS-CoV-2 IgA seropositivity*

**My contribution to this Chapter:**

Conceptualisation, Literature review, Data generation, Data integration, Data visualisation, Final statistical analysis, Interpretation of results, Making of all figures, and Writing of the article.

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All the elements presented in Chapter 2 shed into the light into the potential mechanism behind the late phenotype shaping based on parental (epi) genetic determinants transmitted during conception. To confirm our preliminary hypothesis on the role played by socioeconomic status, we investigated how SES can determine individual immune ability to resist to SARS-cov2 infection.

The COVID-19 pandemic represents one the most important challenger worldwide since the Spanish flu pandemic a century ago due to the severity of symptoms and death rates. As the world was investigating the physiological causes of different individual's responses to SARS-coV2 infection, we questioned whether economic, social and mental status play a role in the SARS-coV2 susceptibility. Indeed, the DOHaD concept demonstrated that the overall life trajectory from conception to adulthood is a major risk in the development of a various range of diseases i.e. mental or cardiovascular (Wadhwa et al., 2009). Rothe et al. stated that people suffering from post-traumatic stress disorder (PTSD) or burn-out lead to extreme psychological trauma affecting the brain structure as well as stress hormone inducing "metabolic" changes (Rothe et al., 2020). Exposed populations to traumatic events are more vulnerable to infections, especially respiratory as they present severe infection forms with strong symptoms (Baumeister et al., 2016). We established that people carrying trauma since years tend to adapt their lifestyle based on the experiences that had. Following this hypothesis, we investigated if somehow, being exposed to a trauma while leading a behavioural change i.e. not respecting the lockdown, explain the increase infection rate after being more exposed to the virus.

Chapter 3 draws the infectious profile of early traumatised participants by investigating the association between psychological state of mind, immune responses and infection severity. Here, we used 5 psychological questionnaires characterising the adverse childhood experiences (ACE) and the IgA and IgG levels known to be the most relevant immune indicator during viral and respiratory infection such as the COVID-19 pandemic. The CON-VINCE cohort were we conducted the analysis confirmed that people with trauma history are more susceptible to develop SARS-coV2 seropositivity and develop severe forms of infections with later life complications. Here, we demonstrated that sex, smoking and ELA are important predictors in the SARS-coV2 seropositivity, however as traumatic experience are unique and differently experienced by individual, it remains complicated to draw conclusions between only ELA exposure and later life viral infections.

In other words, we can confirm that ELA is a risk factor for later life infection and will be responsible of individual phenotype shaping by altering immune, metabolic and physiological responses. Nevertheless it is now indispensable to better characterize SES type exposure i.e. time of exposure, period of occurrence and "intensity" to define which socioeconomic factor will represent the most important risk of infection and chronic disease devolvement. It is reasonable to hypothesise that some SES factors alone can't induce biological or epigenetic alterations while the accumulation of two or three SES stressors might.

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# Adverse Life Trajectories Are a Risk Factor for SARS-CoV-2 IgA Seropositivity

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**Abstract:** Asymptomatic individuals, called "silent spreaders" spread SARS-CoV-2 efficiently and have complicated control of the ongoing COVID-19 pandemic. As seen in previous influenza pandemics, socioeconomic and life-trajectory factors are important in disease progression and outcome. The demographics of the asymptomatic SARS-CoV-2 carriers are unknown. We used the CON-VINCE cohort of healthy, asymptomatic, and oligosymptomatic individuals that is statistically representative of the overall population of Luxembourg for age, gender, and residency to characterise this population. Gender (male), not smoking, and exposure to early-life or adult traumatic experiences increased the risk of IgA seropositivity, and the risk associated with early-life exposure was a dose-dependent metric, while some other known comorbidities of active COVID-19 do not impact it. As prior exposure to adversity is associated with negative psychobiological reactions to external stressors, we recorded psychological wellbeing during the study period. Exposure to traumatic events or concurrent autoimmune or rheumatic disease were associated with a worse evolution of anxiety and depressive symptoms throughout the lockdown period. The unique demographic profile of the "silent spreaders" highlights the role that the early-life period plays in determining our lifelong health trajectory and provides evidence that the developmental origins of health and disease is applicable to infectious diseases.

**Keywords:** SARS-CoV-2; COVID-19; early-life adversity; adult traumatic events; psychosocial adversity; relative risk; serology

## 1. Introduction

First reports of an outbreak of a novel coronavirus disease (COVID-19) in Wuhan, China, appeared in December 2019. This was rapidly attributed to a betacoronavirus principally affecting the respiratory system, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1]. This rapidly escalated, reaching the pandemic level in March 2020 [2]. As of now, more than 151 million cases of COVID-19 have been reported, and over 3 million deaths recorded worldwide [3]. COVID-19 symptoms appear between 2 and 14 days after

exposure. However, many SARS-CoV-2-infected individuals display no or only mild symptoms [4–8] even though they develop a clear, but weaker immune response to the virus than other COVID-19 patients [9]. It has become clear that there are many inequalities in severity and susceptibility to COVID-19 [10]. In a manner reminiscent of the influenza pandemics of 1918 and 2009 [11–13], initial data suggest that lower socioeconomic status (SES) is associated with increased mortality from COVID-19 [14], and this was observed in cohorts from the USA, the UK, and China. However, we currently know very little about the demographics of the asymptomatic SARS-CoV-2 carriers.

The overall life trajectory from conception, through early life, and towards adulthood plays a preponderant role in determining the risk of a wide range of non-communicable diseases including mental health, diabetes, cardiovascular disease, and obesity [15,16]. This produced the theory of the developmental origins of health and disease (DOHaD) [17]. This theory has been subsequently refined and can now be thought of as a "three hit model", incorporating genetic predisposition (hit one) and environmental insults during a sensitive period (hit 2). This produces a latent, quiescent phenotype. Many years later the risk is crystallised by a third hit in the later life environment [18,19]. The early-life period appears to be particularly sensitive to the external environment, with effects acting over many decades [17,20,21].

Although early-life adversity (ELA) covers an almost infinite range of stressors, psychosocial stress is predominant [22,23]. In the adverse childhood experiences (ACE) study [24], more than 50% of the study participants had experienced one (or more) forms of ELA, and 12% had experienced more than four forms of ELA. In the context of a viral pandemic this is particularly important, as ELA not only induces a clear, lifelong, pro-inflammatory immunophenotype [25,26], but also induces significant changes in antiviral cytotoxic T-cells (CTLs, CD8+ T-cells), rendering them largely senescent and reducing their cytotoxicity [27–30]. Consequently, ELA-exposed populations may have a higher risk of viral respiratory episodes compared to the general population. In the context of identifying exposed populations, it is therefore logical to include a history of traumatic life events.

In the case of infections that are asymptomatic or oligosymptomatic IgA is the most suitable immunoglobulin to analyse. In such mild cases, infection is limited to the upper respiratory tract where IgA is the predominant Ig [31] as the primary immune response originates from the mucosal immune system, particularly from the nasopharynx-associated lymphoid tissue (NALT) [32]. This parallels what is seen in HIV infection, where a mucosal IgA response is completely protective [33], something that is also seen for *Picornaviridae* (e.g., poliovirus), *Reoviridae* (e.g., rotavirus), or *Adenoviridae* and is essential in all cases to avoid infection [33–36].

There is now growing evidence that asymptomatic individuals can spread SARS-CoV-2 efficiently. The presence of these "silent spreaders" has complicated the control of the pandemic [4,6]. In this study, we describe the epidemiological, demographic, socioeconomic, and prior psychosocial life trajectory (including a history of ELA) in asymptomatic individuals within a cohort that is statistically representative of the entire Luxembourgish population. Furthermore, ELA has a major impact on mental health [37]. The CON-VINCE cohort of asymptomatic carriers, with a fixed disease severity, allowed us to examine both the socioeconomic factors underlying exposure to SARS-CoV-2, as well as factors that predispose individuals to a mild disease course, and the role that a negative life trajectory played in the subsequent response to the lockdown.

## 2. Materials and Methods

### 2.1. Cohort

This study used the previously reported CON-VINCE cohort [2]. Briefly, the CON-VINCE cohort is representative for the overall Luxemburgish adult population for age, sex, and residency [2]. All participants ($n$ = 1862) were recruited between 15 April and 5 May 2020 and underwent bi-weekly blood and pooled nasal and oropharyngeal swab sampling for 10 weeks, until 26 June 2020. SARS-CoV-2 rRT-PCR together with IgA and

IgG serology was performed at each bi-weekly sampling point together with a series of online questionnaires [2]. Inclusion criteria included people aged 18 and over and capable of providing informed consent that, at inclusion, were either (i) SARS-CoV-2 negative, (ii) SARS-CoV-2 positive but asymptomatic or oligosymptomatic (i.e., no fever, respiratory distress or cough not attributable to a pre-existing comorbidity), or (iii) post-infectious SARS-CoV-2 negative after a mild disease course. The CON-VINCE study was approved by the Comité National d'Ethique de Recherche (CNER, reference 202004/01) and the Ministry of Health (Luxembourg, reference 831x6ce0d), and is registered on ClinicalTrials.gov (NCT 04379297, accessed on 10 March 2021).

### 2.2. Data Collection

As previously reported [2] participants provided (i) demographic data including age, gender, origin, marital status, household composition, (ii) medical history, and (iii) socioeconomic data including educational attainment, employment status and category, annual income, and home-ownership through an online reporting system at inclusion.

### 2.3. Psychological Questionnaires

The baseline and bi-weekly follow-up questionnaires addressed the current medical and psychological states of the participants during the study period. As previously described, participants completed four psychological questionnaires: Center for Epidemiologic Studies Depression Scale (CES-D), Generalised Anxiety Disorder-7 (GAD-7), Perceived Social Stress (PSS), and UCLA loneliness scale (UCLA) in their unmodified forms [38–41]. In the final follow-up questionnaire, ELA exposure was measured with the 28-item Childhood Trauma Questionnaire (CTQ) [42] retrospectively assessing physical, emotional, and sexual abuse as well as physical and emotional neglect during childhood. The overall CTQ score (scale 0–4), and the five subscales were calculated as previously described [42]. Question replies were scored from 0–4 (never true, rarely true, sometimes true, often true, very often true). The three validity items (10, 16, 22) and questions 2, 3, 5, 7, 13, 19, 22, 26, and 28 were reverse scored (i.e., Q16 "I thought I had a perfect childhood": Very true = 0). The overall score and the three subscale scores were calculated as described by Bernstein et al. [42]. The mean of the three validity scores was compared to the overall CTQ score. Participants with differences $\geq$1.5 units difference between the overall and validity score were excluded from the dataset.

Exposure to traumatic events and principal psychosocial stressors in adulthood including death of a family member, job loss, financial difficulties, or divorce was assessed using the questions previously reported [43]. This questionnaire includes the salience (interpretation) of the event by the individual. A positive salience is exposure to a trauma e.g., divorce, but the overall experience was positive (such as escaping from a poor marriage), and a negative salience is undergoing the same event, but experiencing it is a negative, traumatic manner (such as the divorce being surprising and imposed).

### 2.4. IgA and IgG Serology

As previously reported [2], IgA and IgG levels specific to SARS-CoV-2 were determined by ELISA (Anti-SARS-CoV-2 ELISA IgA; Anti-SARS-CoV-2 ELISA IgG; Euroimmun, Lübeck, Germany) according to the manufacturer's instructions. Samples were considered positive with an OD ratio $\geq$1.1, borderline OD ratios (>0.8, <1.1) were considered positive for further analyses, and samples were considered negative with an OD ratio <0.8.

### 2.5. Statistical Analyses and Data Presentation

All data analyses were performed in R (version 3.6.3 R Core Team, 2019) running in R studio (version 1.3.959; R Core Team, 2019). Data cleaning, sorting, and dichotomisation was performed with the packages dplyr [44] and sjmisc [45]. Uncorrected relative risks were calculated for individual covariates independently from contingency tables using the package EpiTools [46]. Corrected relative risks were calculated using R base functions

through general logistic regression models. Initially, sex and smoking (univariate $p < 0.05$) were included as covariates in the corrected RR models. As sex was the only covariate that was significant in the adjusted RR model, the calculations were repeated and the data reported only for the sex-adjusted RR. Relative risks were subsequently extracted from the logistic regression model using the package epiDisplay [47]. K-means clustering of the Adult Traumatic Events questionnaire was performed using the package cluster [48]. In all analyses the covariates were included as explanatory variables for the IgA seropositivity outcome variable, and comparisons between covariates were not assessed. Figures were subsequently generated using SigmaPlot (version 12.5) and Adobe Illustrator CS6 (version 16.00). Data and statistical scripts are available upon reasonable request.

## 3. Results

### 3.1. Demographic Covariates

The CON-VINCE cohort recruited 1862 participants, and 1537 participants completed the study. Anti-SARS-CoV-2 IgA and IgG serology, together with complete medical, demographic, socioeconomic, lifestyle, and traumatic data were available for 1418 participants. As previously reported, this is above the threshold for a statistically representative sample of the entire Luxemburgish population [2]. In total, 199 participants had at any one point during the study a positive IgA serology test. IgG seroprevalence was significantly lower with only 41 participants having a positive serology result at any one point in the 12 weeks of the study, which is partly explained by the natural sequence of immune response to a recent exposure. Prior to calculating the relative risk of anti-SARS-CoV-2 IgA or IgG seropositivity associated with pre-existing comorbidities, or psychosocial and lifestyle parameters, we initially calculated crude uncorrected relative risks (RR) for the common demographic covariates, i.e., age, alcohol consumption, sex, smoking, and BMI (Figure 1A). RR were initially calculated for both IgA and IgG seropositivity. No significant associations were observed with IgG seropositivity due to the low number of positive participants. Subsequently, IgA seropositivity was used exclusively throughout the study. There were two significant demographic covariates that were associated with IgA seropositivity: sex (males) and smoking ($p = 0.001$ and 0.009 respectively). Having a family member with COVID-19 increased the relative risk 1.3-fold, but was not significant ($p > 0.1$). In the CON-VINCE cohort, age, BMI, and alcohol consumption were not significantly associated with IgA seropositivity. Categorical breakdowns of these key demographic covariates are provided in Table 1.

**Table 1.** Categorical breakdowns of key demographic covariates.

| Age Category | Total | Female/Male | IgA Positive | RR (95%CI; *p*-Value) |
|---|---|---|---|---|
| 18–29 | 150 | 90/59 | 21 (14%) | 1 (–) |
| 30–39 | 260 | 133/128 | 42 (16%) | 1.15 (0.7–1.9; 0.67) |
| 40–49 | 325 | 177/147 | 43 (13%) | 0.94 (0.58–1.53; 0.88) |
| 50–59 | 297 | 156/142 | 34 (13%) | 0.82 (0.49–1.36; 0.45) |
| 60–69 | 272 | 150/123 | 34 (11%) | 0.89 (0.54–1.48; 0.65) |
| 70–79 | 154 | 49/107 | 24 (15.5%) | 1.11 (0.65–1.91; 0.75) |
| BMI Category | | | | |
| Underweight | 32 | 26/6 | 5 | 1.12 (0.49–2.58; 0.79) |
| Normal | 614 | 333/281 | 85 | 1 (–) |
| Overweight | 493 | 228/265 | 60 | 0.87 (0.65–1.20; 0.42) |
| Obese | 334 | 170/167 | 49 | 1.05 (0.76–1.46; 0.77) |
| Smoking Category | | | | |
| Never smoked | 796 | 444/354 | 120 (15.1%) | 1 (–) |
| Live with smoker | 439 | 180/260 | 58 (13.2%) | 0.89 (0.66–1.17; 0.40) |
| Ex-smoker | 38 | 19/19 | 6 (15.7%) | 1.05 (0.49–2.22; 0.82) |
| Current smoker | 201 | 114/86 | 15 (7.4%) | 0.50 (0.30–0.83; 0.004) |

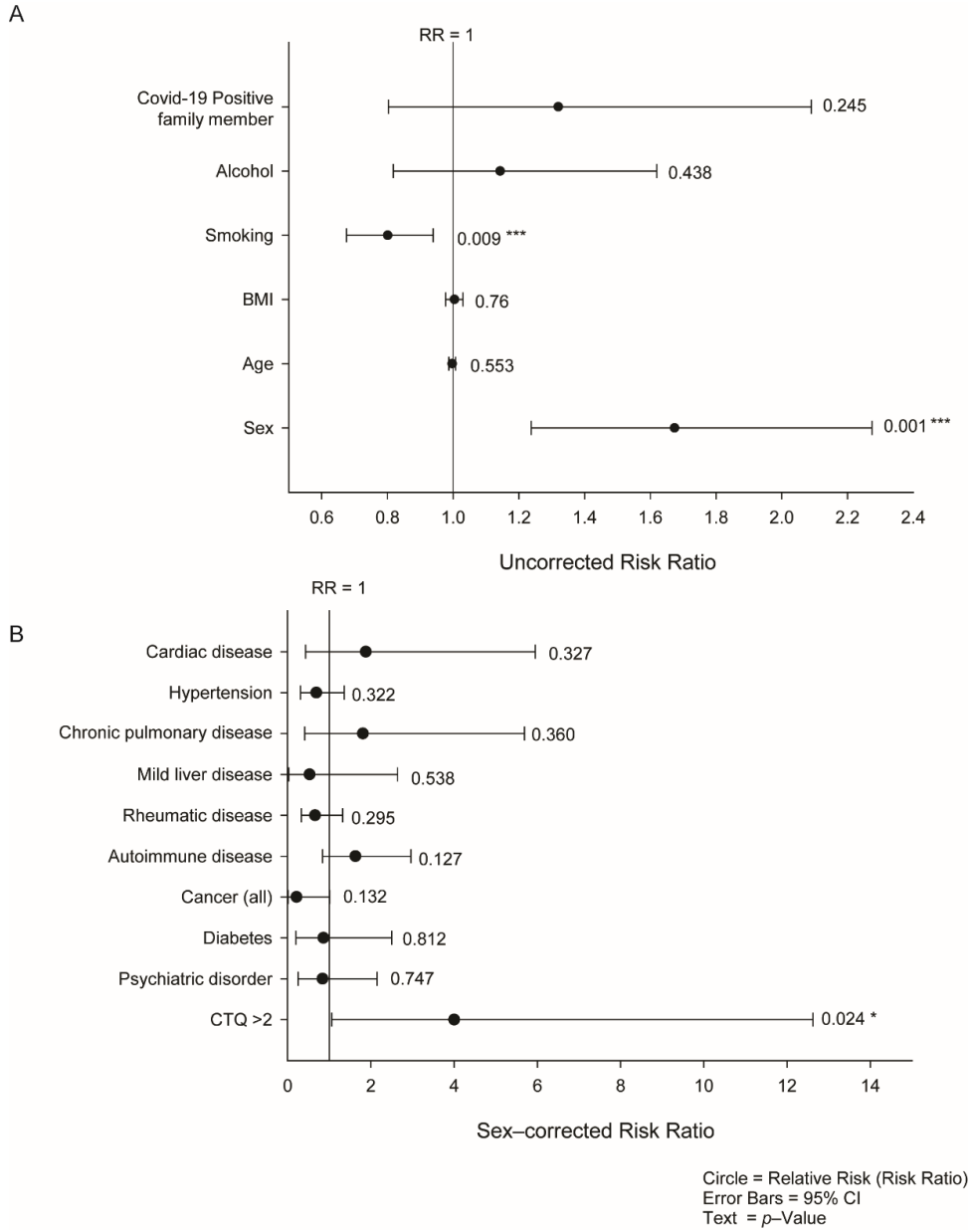


**Figure 1.** Demographics of SARS-CoV-2 IgA seropositivity in asymptomatic or oligosymptomatic individuals. (**A**) Crude relative risk estimates for SARS-CoV-2 IgA seropositivity for all members of the CON-VINCE cohort finishing the five experimental visits. (**B**) Sex-adjusted logistic regression relative risk of being SARS-CoV-2 IgA seropositive due to pre-existing comorbidities. Both panels: Circles represent the relative risk (RR); error bars: 95% confidence interval; text: $p$-value. *, $p < 0.05$; ***, $p < 0.005$.

Table 1 shows the covariates of age, BMI, and alcohol consumption associated with the IgA seropositivity percentage for categorical breakdowns.

### 3.2. Relative Risk Linked to Pre-Existing Comorbidities

The number of participants with each of the participant-reported comorbidities are given in Table 2. To examine the role of the pre-existing comorbidities we calculated sex, smoking, and having a COVID-positive household member adjusted RR models when the incidence of the comorbidity was >1% of the cohort (>14 participants with a certain comorbidity). In none of the models were smoking or having a COVID-positive household member significant covariates ($p > 0.1$), and neither had a significant interaction term be-

tween them or with sex. As such, smoking or having a COVID-positive household member were dropped from all reported models. None of the pre-existing comorbidities increased the RR significantly (Figure 1B). However, participants with a history of malignant disease (any type) had a non-significantly lower risk of IgA seropositivity (RR = 0.215; 95% CI from 0.012 to 1.01; $p = 0.012$). As ELA can directly impact the immune system, we calculated the RR when CTQ > 2. The risk of being IgA seropositive increased with exposure to ELA (RR = 4.00, 95% CI: 1.06 to 12.62, $p = 0.024$) (Figure 1B).

**Table 2.** Number of participants with each of the participant-reported comorbidities.

| Disease Categories | Case Numbers | Female/Male | $p$-Value (Chi$^2$) | IgA Positive |
|---|---|---|---|---|
| Cardiac | 53 | 17/36 | 0.009058 | 7 (13.2%) |
| Hypertension | 269 | 113/156 | 0.008748 | 36 (13.4%) |
| Pulmonary | 35 | 17/18 | 0.8658 | 6 (17%) |
| Liver | 36 | 17/19 | 0.7389 | 2 (5%) |
| Kidney | 13 | 7/6 | 0.7815 | 2 (15%) |
| Rheumatological | 199 | 121/78 | 0.002302 | 20 (10.1%) |
| Autoimmune | 115 | 90/25 | $1.35 \times 10^{-9}$ | 17 (14.7%) |
| HIV | 6 | 1/5 | 0.1025 | 1 (16%) |
| Cancer | 85 | 38/47 | 0.329 | 12 (14.1%) |
| Haematological | 19 | 11/8 | 0.4913 | 1 (5%) |
| Malnourished | 4 | 2/2 | 1 | 2 (50%) |
| Diabetes (I + II) | 76 | 32/44 | 0.1687 | 9 (11.8%) |
| Transplant | 7 | 3/4 | 0.7055 | 1 (14.3%) |
| Psychiatric | 69 | 45/24 | 0.01529 | 7 (10.1%) |
| Other | 0 | 0/0 | n/a | 0 (0%) |
| CTQ >2 | 18 | 17/1 | $4.56 \times 10^{-10}$ | 6 (33.3%) |

Table 2 shows exact number of participants per comorbidities with the ratio of female/male. The $p$-value and percentage of IgA positivity are given for each comorbidity.

### 3.3. Early-Life Adversity Incidence and the Associated Increases in Risk of IgA Seropositivity

In line with other recent studies [49], 69% of the total cohort reported no exposure to ELA, 19% of the participants were above the threshold in one category, 4% in two sub-categories, and <1% reported four or five different trauma types. We subsequently investigated the dose–response relationship of IgA seropositivity to the overall CTQ score. For all ELA exposure values CTQ >2, the RR was increased significantly, from 4.0- to 27-fold ($p$ from 0.06 to 0.005; Figure 2A). When ELA exposure increased, the risk of IgA seropositivity increased in a similar manner (Spearman Correlation: 0.829, $p = 0.058$; from a CTQ score of 1.25 to 2.5) showing a clear dose–response relationship (Figure 2A).

### 3.4. Physical Abuse Is the Predominant Driver of ELA

We examined the five subscales of the CTQ to identify the principal forms of ELA driving the association with IgA seropositivity. Using the subscales as continuous variables, physical abuse (PA) significantly raised the risk of IgA seropositivity by 4.38-fold ($p = 0.009$) (Figure 2B). The other CTQ subscales were not significant ($p$-value from 0.6 to 0.82). We concluded that the ELA plays a role in IgA seropositivity risk, and the largest contribution to this risk is from the physical abuse component.

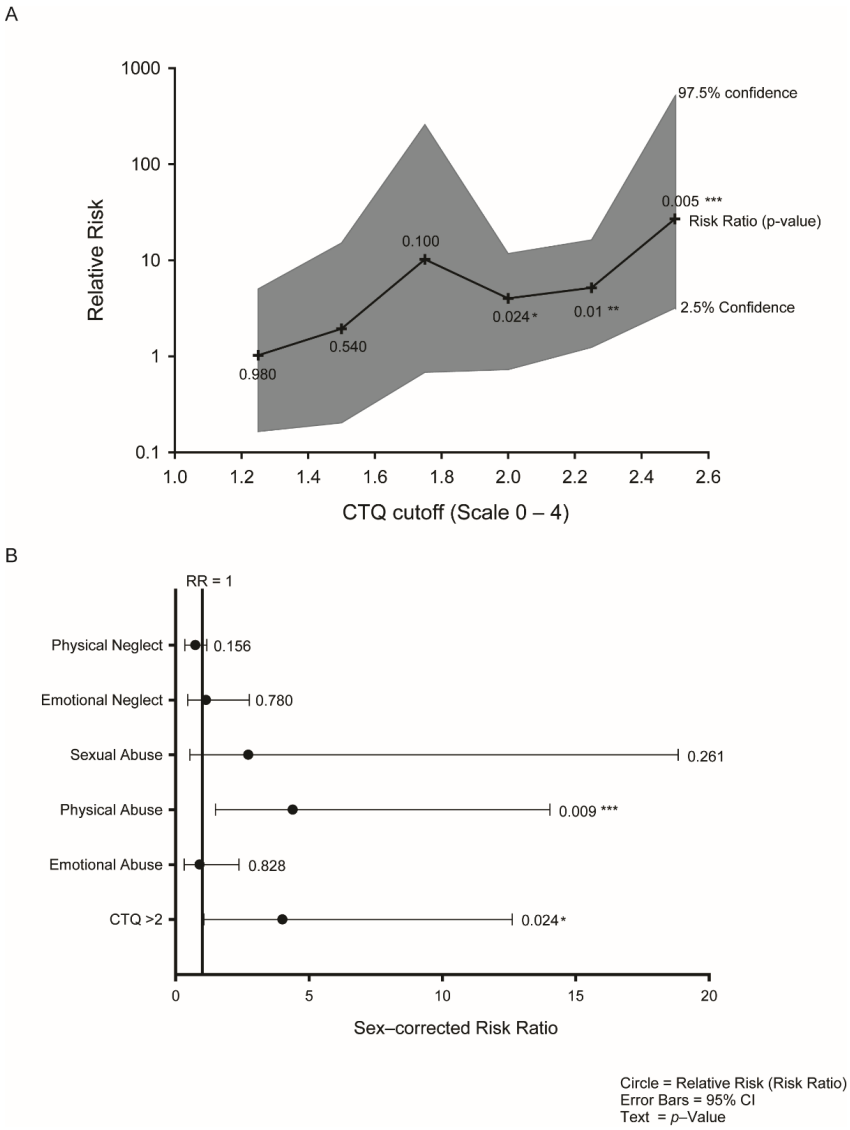


**Figure 2.** Exposure to early-life adversity is associated with SARS-CoV-2 seropositivity in asymptomatic or oligosymptomatic individuals. (**A**) ELA is linked to SARS-CoV-2 seropositivity in a dose-dependent manner as the cutoff for being considered subject to ELA increases. The central black line represents the sex-adjusted logistic regression relative risk (RR); the grey shaded area represents the 95% confidence interval; text: *p*-value. (**B**) Sex-adjusted logistic regression relative risk of IgA seropositivity for the CTQ subscales identifies physical abuse as a key element of the overall CTQ score contributing to the risk of IgA seropositivity. Circles represent the relative risk (RR); error bars: 95% confidence interval; text: *p*-value. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

### *3.5. Adult Trauma Is a Risk Factor for IgA Seropositivity*

Measurement of adult trauma is complicated by the "salience" or the importance attached to the event [43] and the ATE questionnaire scored salience from −3 to 0 to +3 (negative experience, irrelevant, positive experience, respectively). Consequently, raw questionnaire data underwent k-means clustering to find patterns in the data. Three clear clusters were identified (Figure 3). Mean responses to the individual questions for the three clusters are included in the Supplementary Table S1. By inspection, it is clear that the three clusters can be interpreted as follows: cluster 1 had the lowest trauma; cluster 2 experienced trauma and gave it a negative salience; cluster 3 experienced trauma and gave it a positive salience. The crude RR of IgA seropositivity was significantly increased in clusters 2 and 3 (RR = 1.48, 95% CI:1.05–2.05, $p = 0.023$ and RR = 1.48, 95% CI:1.07–2.07,

$p$ = 0.0216, respectively; Figure 3B). We examined the risk of IgA seropositivity when ELA and ATE were both present (Supplementary Table S2). Previous studies have already demonstrated the impact of childhood traumatic events on adult life [17,24]. When ELA was present, ATE cluster 1 (reference) and 2 ($p$ = 0.94 and 0.13) were not significant despite a high RR (from 1.07 to 7.53). Cluster 3 showed a significant result increase in RR (RR = 3; 95% CI 1.39–6.48; $p$ = 0.035) with 40% IgA seropositive (4/10 participants). However, the numbers of participants in all three subcategories were low (range 1–10) rendering their interpretation unreliable. It is, however, safe to conclude that people exposed to ATE had an increased risk of being IgA seropositive regardless of their ELA experience.

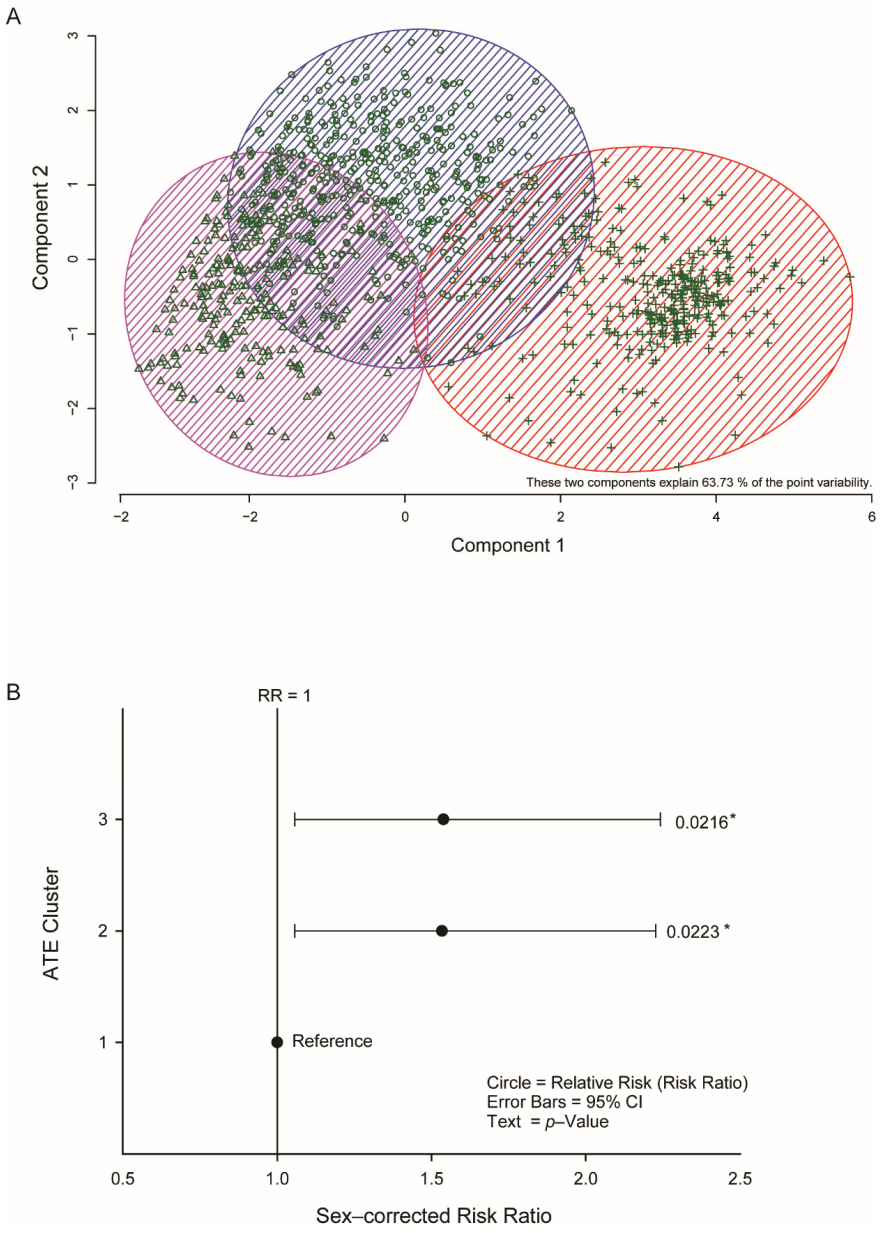


**Figure 3.** Exposure to adult traumatic events (ATE) is linked to IgA seropositivity in asymptomatic or oligosymptomatic individuals. (**A**) The two principal components after K-means clustering of the responses to the ATE questionnaire identified three clusters of responses. Circles (blue shaded area)—Cluster 1; Triangles (pink shaded area)—Cluster 2; Crosses (red shaded area)—Cluster 3. (**B**) Sex-adjusted relative risk CTQ subscales identified ATE clusters 2 and 3 (negative and positive salience, respectively) as having a similar effect on SARS-CoV-2 IgA seropositivity. Circles represent the relative risk (RR); error bars: 95% confidence interval; text: $p$-value. *, $p < 0.05$.

### 3.6. Socioeconomic, Employment, and Life Covariates Do Not Influence IgA Seropositivity

Given the important of asymptomatic carriers in viral transmission and their role in the COVID-19 pandemic, we examined the impact of socioeconomic parameters on IgA seropositivity (Figure 4) to see if any particular category had an increased exposure to SARS-CoV-2. Overall, there was no effect of any of the socioeconomic parameters including annual income, marital status, number of household members, employment category, or home ownership. In a secondary analysis these were dissected by category, confirming the numbers of participants in each category were sufficient and that there were no individual categories that were significantly associated with increased IgA seropositivity (Supplementary Table S3). Overall, these data highlight that in our study cohort the virus appeared to be circulating irrespective of socioeconomic context as recently suggested by [50], although this may be affected by the phase of the pandemic [51].

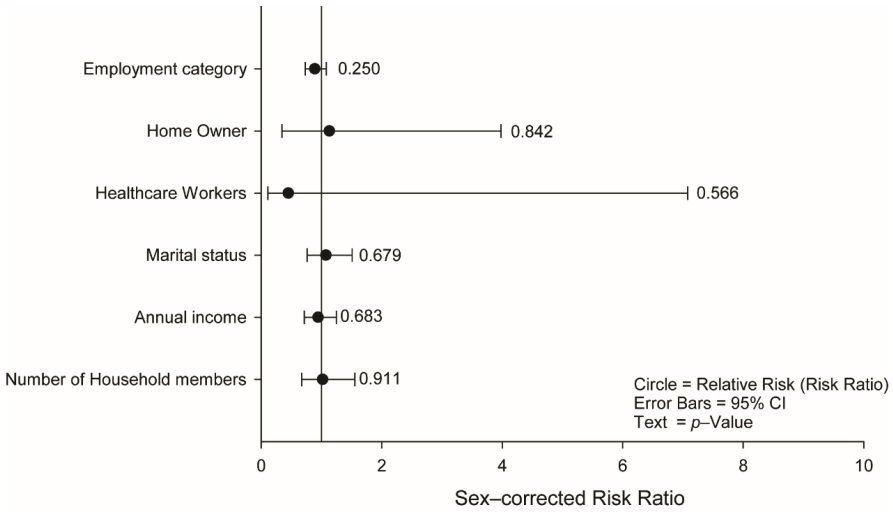


**Figure 4.** Socioeconomic covariates do not determine SARS-CoV-2 IgA seropositivity in asymptomatic or oligosymptomatic individuals. Sex-adjusted logistic regression relative risk of SARS-CoV-2 IgA seropositive due to current socioeconomic conditions. Circles represent the relative risk (RR); error bars: 95% confidence interval; text: *p*-value.

### 3.7. The Influence of ELA on Psychological States during Lockdown

Although psychiatric comorbidities do not constitute a significant risk factor for exposure to SARS-CoV-2, exposure to ELA may not only influence their subsequent development, but may also have a significant effect on the psychological reaction to the lockdown containment measures. Prior exposure to ELA increased the relative risk of developing psychiatric disorders in our cohort (crude RR = 16.91, 95% CI: 6.09–46.91, $p < 0.001$; sex-adjusted RR = 11.47, 95% CI: 3.89–33.85, $p < 0.001$). At the first study visit, this link was clearly visible in the psychological questionnaires.

*CES (Depression):* The mean CES score for the complete cohort declined from 10.00 +/− 8.01 to 8.01 +/− 8.32 during this period (Wilcoxson test $p < 2.2 \times 10^{-16}$; Figure 5A). In univariate analyses, the baseline CES score was strongly influenced by multiple covariates (*p*-values from 0.017 to $2 \times 10^{-16}$; Supplementary Table S3; Figure 5B). However, the change in CES score between inclusion and the end of the study period was dependent on the ATE cluster ($p = 0.039$) and ELA ($p = 0.0217$), with a trend towards significance for diabetes ($p = 0.0574$), sex ($p = 0.062$), and pre-existing rheumatic disease ($p = 0.072$) (Supplementary Table S4; Figure 5C). When these covariates were included in a multi-way-ANOVA (not shown), the change in CES score over the study period was significantly influenced by pre-existing rheumatic disease (main effect: $F(1,1418) = 6.145$, $p = 0.0133$), prior exposure to ELA (main effect: $F(1,1418) = 9.814$, $p = 0.0018$, and diabetes (main effect: $F(1,1418) = 4.990$, $p = 0.0257$). Confirming our prior hypothesis that diabetes may be linked to ELA [10], there was a significant interaction between comorbid diabetes

and ELA on the change in CES score (interaction: F(1,1418) = 2.454, $p$ = 0.117). Tukey post hoc analysis confirmed that prior exposure to ELA decreased the change in CES score by 12.2 points (95% CI: 2.10–22.35, $p$ = 0.01) in participants with diabetes. A similar interaction was seen for ELA exposure, rheumatic diseases, and the change in CES score (interaction: F(1,1418) = 5.35, $p$ = 0.0188). Tukey post hoc analysis confirmed that prior exposure to ELA increased the CES score by 9.04 points (95% CI: 1.83–16.27, $p$ = 0.007) in participants with rheumatic disorders compared to those without. Similarly, participants with rheumatic disease had an increase in CES score over the study period of 8.72 points (95% CI: 0.25–17.19; $p$ = 0.04).

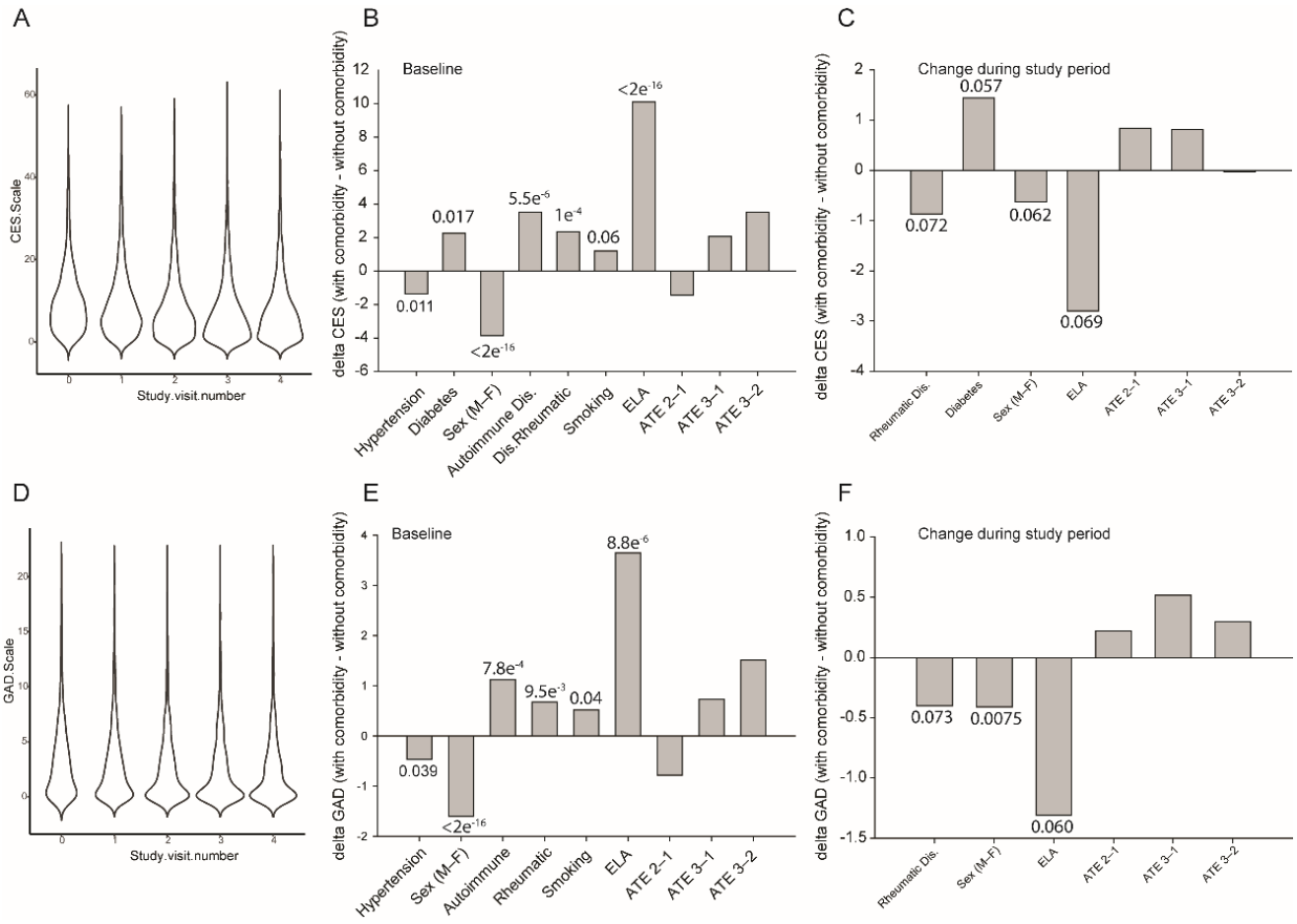


**Figure 5.** Psychological response to the containment measures during the study period in healthy, asymptomatic, and oligosymptomatic individuals. (**A**) Data density plot for the response to the Center for Epidemiologic Studies Depression Scale (CES-D) for the five experimental visits. (**B**) Significant group differences in CES-D score at the baseline CON-VINCE visit. Full statistical data are included in Supplementary Table S3. Data are from univariate ANOVA analysis, text above/below each bar is the ANOVA $p$-value. (**C**) Significant group differences in the change in CES-D score between the baseline and last CON-VINCE visit. Full statistical data are included in Supplementary Table S4. Data are from univariate ANOVA analysis, text above/below each bar is the ANOVA $p$-value. The full multiparameter ANOVA model is described in the Results Section. (**D**) Data density plot for the response to the Generalised Anxiety Disorder-7 (GAD-7) questionnaire for the five experimental visits. (**E**) Significant group differences in GAD-7 score at the baseline CON-VINCE visit. Full statistical data are included in Supplementary Table S4. Data are from univariate ANOVA analysis, text above/below each bar is the ANOVA $p$-value. (**F**) Significant group differences in the change in GAD-7 score between the baseline and last CON-VINCE visit. Full statistical data are included in Supplementary Table S4. Data are from univariate ANOVA analysis, text above/below each bar is the ANOVA $p$-value. The full multiparameter ANOVA model is described in the Results Section.

*GAD (Anxiety)*: As for the CES, the baseline GAD scores depended on nine covariates ($p$ values from 0.04 to $2 \times 10^{-16}$; Supplementary Table S3; Figure 5E). However, the change in GAD score between inclusion and the end of the study period was dependent on sex ($p = 0.0075$), ELA ($p = 0.061$), ATE cluster ($p = 0.062$), age ($p = 0.070$), and pre-existing rheumatic disease ($p = 0.073$; Supplementary Table S4; Figure 5F). In a multi-way-ANOVA, the change in GAD score was significantly influenced by ELA exposure (main effect: $F(1,1418) = 9.695$, $p = 0.0019$), pre-existing rheumatic disease (main effect: $F(1,1418) = 5.715$, $p = 0.016$), and age (main effect: $F(1,1418) = 1.328$, $p = 0.047$). The only significant interaction was between ELA exposure and ATE (interaction: $F(1,1418) = 5.705$, $p = 0.0035$) (Supplementary Table S4).

*UCLA (Loneliness)*: Baseline UCLA scores depended on nine covariates ($p$-values from $5.85 \times 10^{-13}$ to 0.061; Supplementary Table S3); however, in individual univariate analyses, the change in UCLA score over the study period was only dependent on exposure to ELA ($F(1,1418) = 6.724$, $p = 0.0096$).

*PSS (perceived stress)*: Baseline PSS scores depended on seven covariates ($p$-values from $5.5 \times 10^{-11}$ to 0.037; Supplementary Table S3); however, in individual univariate analyses, the change in PSS score was dependent on a concurrent autoimmune ($F(1,1436) = 9.072$, $p = 0.0026$) or rheumatic disease ($F(1,1400) = 3.856$, $p = 0.050$). In a two-way ANOVA, the change in PSS score was only influenced by pre-existing autoimmune disease (main effect: $F(1,14) = 9.399$, $p = 0.0022$) (Supplementary Table S4).

## 4. Discussion

Using the CON-VINCE cohort of healthy, asymptomatic, and oligosymptomatic individuals, we were able to demonstrate, at the population level, that exposure to a life history of traumatic events significantly increased the risk of SARS-CoV-2 IgA seropositivity, as did gender and smoking. Furthermore, a prior history of adversity was a key driver in the psychological reaction during the period of strict containment measures.

There is now a plethora of data available on the demographics of SARS-CoV-2 patients with active symptomatic disease in both the community and hospital situation [52–56]. Although we saw a clear sex bias in IgA seropositivity, an increase associated with other cases in the family home, and a decrease in seropositivity in active smokers, our data present a very different picture to COVID-19 patient cohorts. In our logistic regression relative risk models, only sex remained as a statistically significant covariate. Our initial statistical model suggested that neither age nor BMI were significant covariates. We confirmed both results in a secondary analysis, with all age and BMI categories having similar, statistically non-significant, RRs. Together with the socioeconomic data, these data indicate a generalised circulation of the SARS-CoV-2 throughout the population. A prior cancer diagnosis was a protective factor. Although this warrants further investigation, the most probably interpretation of this is a "conscientious phenotype". This may be true for household contacts too. We observed a non-significant 37% increase in RR from household contacts, higher than that which was recently reported, although we had less power to detect such associations [57]. As recently reported, there is a public under-appreciation of the importance of barriers. It is possible that behavioural modifications and awareness of the importance of health behaviours in this population may underlie stricter adherence to social distancing, facemask usage, and disinfectant gel usage, reducing the relative risk [58]. Similarly, the RR did not increase with the number of household members; however, in the context of significantly reduced social contact (i.e., lockdown) this result may not be surprising. This contrasts with the situation seen in COVID-19 patients. It is important to differentiate the risk of exposure from disease severity. As we have previously highlighted [10], data from Chicago clearly identified increased mortality in ethnic minorities [59]. They represented up to 70% of the overall COVID-19 deaths, and as the local population in lower socioeconomic classes increased, the local mortality rate increases significantly [14]. The authors ascribed this to both exposures from poverty and over-populated housing, as well as severity from pre-existing comorbidities such

as type 1 and 2 diabetes or cardiovascular disease [7]. We previously interpreted these reports as indicating the effect of current SES and environment conditions on SARS-CoV-2 morbidity and infection rates [10]. The present data suggest that it is not an effect of SES or the environment that leads to higher rates, but rather, these rates are representative of an underlying exposure to traumatic life events that changes the risk of exposure. Our prior interpretation may be partly correct, however, since SES and ELA correlate closely [60,61]. Our early-life data are almost unique in that they are from a cohort that is statistically representative of a national population. The rate of exposure to ELA concords with the only similar data available [49] confirm that our data will not be unique to Luxembourg, but representative of a wider European population. Indeed, recent data from the UK Biobank cohort confirmed the importance of this early-life period, as having been breastfed ~70 years ago still provided protection, whilst maternal smoking during gestation significantly increased the risk of SARS-CoV-2 infection during the pandemic [62].

Our data highlight two interesting risk factors: smoking and exposure to psychosocial adversity. Our observation that current smokers or their partners have a reduced risk of SARS-CoV-2 seropositivity agrees with clinical reports and a recent meta-analysis that smokers are underrepresented, by up to a factor of 10-fold in hospitalised cohorts [63]. This is, however, counterbalanced by reports that upon SARS-CoV-2 infection, smoking may increase the overall severity and progression of COVID-19 [64]. Mechanistically, this would appear to pass through changes in levels of ACE2, although the data are somewhat contradictory with both smoking-induced increases [65] and decreases [66] in ACE2 levels reported. This somewhat counter-intuitive result in smokers has to be treated with caution, as it may be due to a social desirability bias. Underreporting of smoking, alcohol, or drug use remains frequent, although internet-based self-reported data collection goes some way to alleviate this bias [67]. It is possible that this represents a similar "conscientious phenotype", with smokers taking more care as they perceive a higher risk, although there are no data to confirm this. Our observation that psychosocial adversity increases the risk of SARS-CoV-2 seropositivity concurs with the data from the 1970s [68] that social adversity is linked to more frequent infections as well as non-communicable diseases [69]. Furthermore, our data follow the same direction as a series of reports over the last few years that highlight the exaggerated effect of early-life adversity on adult immune function [70,71]. Adverse social conditions appear to be embedded as long-term functional changes in the immune system. The available data suggest that as little as 4 months of exposure to ELA can change the immune response up to ~24 years later [28,72–74]. Such exposure drives the accumulation of senescent immune cells that not only appear to have a decreased capacity to proliferate, but also, their responsiveness to subsequent bacterial or viral stimuli is reduced [28,30]. This association particularly strong for the senescent CD8+ CD57+ TEMRA cells that lose the ability to mount an effective immune response to a new infection, a finding that has been independently replicated [28,30,74]. Early-life social adversity also acts by enhancing the expression of inflammatory and T-lymphocyte activation genes, while concurrently reducing the expression of type I IFN-mediated innate antiviral response genes, as well as other pathogen-specific innate antimicrobial response genes [72]. These are patterns of altered gene expression that remain lifelong [72]. This specific gene expression pattern is termed the "conserved transcriptional response to adversity (CTRA)" and has been reported in many human observational studies of adversity [75–82]. The CTRA is most strongly induced by adversity in early life, corresponding to the postnatal period during which the immature immune system develops and starts maturing [72]. In a manner similar to the functional changes in the immune cells, this transcriptome remodelling persists, affecting the immune responses to pathogens or allergens encountered many years later [76,83,84].

Our data highlight the negative effect of ELA, ATE, and pre-existing autoimmune or rheumatic disorders on depression and anxiety levels during the lockdown period. Such containment measures have been associated with negative mental health outcomes, but a perception of performing essential work, receiving kindness, and community connect-

edness were associated with positive mental health outcomes [85]. There is a tight link between both autoimmune and rheumatic diseases and hypothalamus–pituitary–adrenal (HPA) axis functioning, with exaggerated responses to daily stressors [86,87]. Similarly, exposure to ELA or ATE affects HPA axis functioning [88]. This was seen after ELA in cohorts 10–12 years post ELA [89] or ~24 years after ELA [90]. Prior exposure to ELA has also been linked to stress-induced negative moods and emotions [89]. As such, the changes in the CES and GAD questionnaire appear to be consistent with the existing literature.

To evaluate the risk of exposure and seroconversion to SARS-CoV-2, we analysed IgA in preference to IgG or IgM, as levels of the latter are not only significantly lower in asymptomatic SARS-CoV-2-positive individuals than in COVID-19 patients [9], whilst IgA levels are higher and seroconversion occurs within 2 days of infection compared with up to 32 days for IgG and IgM [91]. Our data confirmed this, as we only had 39 IgG-positive cohort members giving a non-representative and non-significant conclusion, compared to 209 IgA-positive participants. Previously, we reported the specificity and sensitivity of the IgA and IgG ELISAs using hospitalised COVID-19 patients and a pre-pandemic cohort sera. The specificity of the IgA ELISA used was lower than for IgG (89.2% vs. 97.8%);however, it was more sensitive (92.9% vs. 85.7%) [2]. The large discrepancy between the number of IgA- and IgG-positive participants may be in part due to the lower specificity of the IgA ELISA; however, they are more likely to come from the sequential nature of the immune response, since IgA appears sooner than IgG [92], and is a stronger neutraliser of SARS-CoV-2 than either IgM or IgG [93]. Furthermore, IgA would also appear to be more relevant in mild infections as SARS-CoV-2 infection is, in principal, restricted to the upper respiratory tract, with the infection spreading to the lower respiratory tract only in more severe cases [31]. As reviewed by Russell et al. (2020), it would naturally be expected that in mild cases the primary immune response originates from the mucosal immune system, particularly from the nasopharynx-associated lymphoid tissue (NALT) [32]. The NALT is an inductive site for the mucosal immune system, and it includes the nasal epithelium as well as the adenoids and the tonsils [32]. It has been proposed that the bronchus-associated lymphoid tissue (BALT) that is normally found to form after infection, particularly in adolescents and children [94], may underlie the increased resistance of children and adolescents to the COVID-19 disease. The NALT and GALT generate almost exclusively an IgA response from mucosal B cells that locally differentiate into IgA-secreting plasma cells, although a small number of IgG-producing B cells are induced in NALT tissues such as the tonsils, producing detectable levels of IgG (and IgM) in the circulation [95]. Unfortunately, there has been a preponderance to study circulating IgG and IgM levels rather than the IgA levels [96–98]. However, in mild infections, IgM and IgG may only be effective if they can reach the infected upper respiratory tract mucosae, but they are not readily transported to mucosal surfaces [99]. Indeed, serious COVID-19 infection is associated with infections in the lower rather than the upper respiratory tract, particularly in the terminal airways. Here, IgG is the predominant class, and the intensely inflammatory nature of IgG induces severe COVID-19 infection through inflammation, complement activation, and induction of phagocytosis by, e.g., macrophages, neutrophils, and the activation of the cellular immune response, including CD4+ and cytotoxic CD8+ T cells that cannot, by their nature, prevent infection. Their role being to destroy infected cells to reduce the risk of the infection propagating, with a high cost. The best data available show that IgG and IgM levels do not accurately reflect prior PCR-confirmed mild infection, or patients did not seroconvert [100], and by ignoring IgA, the seroprevelance is significantly underestimated [101]. The most commonly used anti-nucleocapsid IgG ELISA identified 40/42 (95.2%) of severe hospitalized cases as seropositive, but in mild, non-hospitalised cases, only identified 539/1134 (47.5%) cases were seropositive; furthermore, the anti-nucleocapsid IgM assay failed to detect 95% of these milder cases [100]. Contrastingly, when the pan-Ig test, including IgA, was used, seroconversion was detected in >90% of the mild cases leading to the conclusion that measuring IgA is essential [93,100,102–104]. Based on these observations and on the lack of

sufficient number of IgM participants in our cohort, we conclude that IgA is more relevant for our population survey than either IgG or IgM.

We calculated the seropositivity risk using the relative risk (risk ratio). Although there are multiple models available (Cox regression, relative risk, odds ratio), we considered participants to be seropositive for either IgA or IgG if, during the study period, they had one or more positive serology results, and we did not consider the time at which they became positive during the study, negating the use of Cox's hazard ratio. As approximately 13% of the CON-VINCE cohort were IgA seropositive [2], the "rare disease assumption" that the odds ratio is similar to the relative risk when the outcome incidence is low did not hold true [92]. Furthermore, as the cohort is statistically representative of the whole country that was in lockdown during the study period, the 199 IgA seropositive participants was a genuine representation of the silent spreader population within the country at that time. As such, we calculated the RR, using sex-corrected logistic regression models, as the most appropriate measure in our cohort. Our cohort was recruited and repeatedly sampled over a 10-week period at the tail-end of the first epidemic wave in Luxembourg [2]. Although IgA has a plasma half-life of around 3–5 days, there is evidence that circulating antibodies to the related SARS-CoV-1 and MERS-CoV remain detectable for >12 months [105,106], and the more stable IgG remains detectable for 24–36 months after SARS-CoV-1 or MERS-CoV infection [107,108]. Recent data from Iceland suggests that >100 days post exposure, asymptomatic individuals still have detectable IgA and IgG levels [109].

As such, we are confident that the serology results obtained during the study period are reflective of the exposure during the entire period from the first case in Luxembourg on 29 February 2020, through the start of recruitment and sampling on 15 April 2020, to the end of the study on 5 May 2020. In the event of our increased IgA seropositivity being due to cross-reactivity with seasonal coronaviridae, the fundamental observation remains. A life history of traumatic events increases exposure to either SARS-CoV-2 or other coronaviridae.

Asymptomatic individuals represent a reservoir of virus that is proving to be an obstacle in managing the COVID-19 pandemic. In this study, we clearly identified the demographics of asymptomatic SARS-CoV-2-infected individuals. This profile was unique in that there were no underlying factors that predisposed individuals to being SARS-CoV-2 seropositive, nor were there factors that predisposed individuals to having a mild disease course. As recently reported, age was not a factor in mild SARS-CoV-2 infections [91,110]. Furthermore, there was no effect of any of the socioeconomic factors investigated; however, a prior exposure to traumatic life events appears to be one of the strongest predictors, along with sex, smoking, and having a SARS-CoV-2-positive family member, for being exposed to SARS-CoV-2 and becoming seropositive. Additionally, a life history of traumatic events or concurrent autoimmune or rheumatic disease were associated with a worse evolution of anxiety and depressive symptoms throughout the lockdown period.

The clear connection between SARS-CoV-2 seropositivity and a life history of adversity is particularly promising for future studies. There are, however, several areas that need to be investigated to take these results further. The role of the mucosal immune system, in particular IgA, needs to be clarified. Furthermore, our observations need to be expanded to more severe forms of COVID-19, where the well-established cellular immune deficiencies induced by ELA may also play an important role. While our data remain preliminary because they were taken over a very short period at the start of the pandemic, it will be essential to follow our CON-VINCE cohort over a longer period to see whether our epidemiological link between psychosocial adversity is retained with time, and whether over a longer period the IgA response has matured into an IgG response.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/jcm10102159/s1, Table S1: mean ATQ responses to the individual questions for the three clusters, Table S2: risk of IgA seropositivity when ELA and ATE are both present, Table S3: univariate analyses of the baseline for psychological scales, Table S4: univariate analyses of the delta between V0 and V4 for psychological scales.

**Author Contributions:** Conceptualization: J.D.T., R.K., and the CON-VINCE team; literature review: C.H. and J.D.T.; data collection: C.J.S., S.B.M., M.O., and the CON-VINCE team; data analysis: C.H., C.J.S., and J.D.T.; manuscript writing: C.H. and J.D.T.; manuscript editing: all authors. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Comité National d'Ethique de Recherche (CNER, reference 202004/01) and the Ministry of Health (Luxembourg, reference 831 × 6ce0d), and is registered on ClinicalTrials.gov (NCT 04379297).

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** Data from the CON-VINCE study are available upon reasonable request and after approval from the CON-VINCE study executive committee, chaired by R.K. The reduced dataset reported in this manuscript is also available upon reasonable request to either J.D.T. or R.K.

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# *Chapter 4*

# *Developmental epigenomic effects of maternal financial problems*

**My contribution to this Chapter:**

Conceptualisation, Literature review, Data generation, Data integration, Data visualisation, Final statistical analysis, Interpretation of results, Making of all figures, and Writing of the article.

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As described in the literature, ELA can be perceived and interpreted differently by an individual (Merrick et al., 2019). For example, the same severity of the exposure to a trauma can induce different consequences and also reflect individual tolerance. Additionally, exposure to stressors such as environmental pressures or low socio-economic status during the very early stages of life can induce a very unique ageing trajectory.

Environmental or economic stressors such economic hardship represent one of the major stressing factor a mother can experience during and right after pregnancy (Clark et al., 2021a). Indeed, the deprivation of items, clothes or even food for their child will induce stress, anxiety and insecurity. Those emotions are already well described in the literature as a considerable cause of the ageing process mostly associated with specific immune phenotype such as immuno-senescence (López-Otín et al., 2023). In addition to immune system dysregulation, epigenetic profile also takes part in the ageing process. Indeed, DNA methylation modifications act as a precursor to the up or down regulation of proteins levels leading through reduced or over expression of a gene (Angeloni & Bogdanovic, 2019).

Understanding how ELA influences both epigenetic and immune processes, at the same time or not, will allow us to correlate early life experiences to adulthood outcomes. By selecting two major economic hardship stressors such as maternal deprivation and major financial problem, we investigated if both are able to alter the ageing process by inducing DNAm modifications. In the first hand, epigenetic clocks, capturing the epigenetic age rather than chronological age, have been used to track the individual ageing of our ARIES participants (Ryan, 2021). In a second hand, we correlated the calculated epigenetic age of our participants with the number of maternal economic hardships reported by the mother.

As previously mentioned in the Chapter 2, we already questioned the implication of the *in utero* environment and how it can lead to children's epigenetic modifications. To confirm our hypothesis, we investigated if maternal exposure can directly or not influence the foetus (in the womb, during pregnancy) through the placenta and how long those modifications are conserved over time.

Here, we investigated the consequences of exposure to economic hardship such as MD and MFP during the prenatal period as well as the first years of life. In the following Chapter, we captured the change in DNA methylation profiles on children as well as differentially methylated regions that can induce functional and/or physiological differences.

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## Regular Article

# Developmental epigenomic effects of maternal financial problems

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**Abstract**

Early-life adversity as neglect or low socioeconomic status is associated with negative physical/mental health outcomes and plays an important role in health trajectories through life. The early-life environment has been shown to be encoded as changes in epigenetic markers that are retained for many years.

We investigated the effect of maternal major financial problems (MFP) and material deprivation (MD) on their children's epigenome in the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort. Epigenetic aging, measured with epigenetic clocks, was weakly accelerated with increased MFP. In subsequent EWAS, MFP, and MD showed strong, independent programing effects on children's genomes. MFP in the period from birth to age seven was associated with genome-wide epigenetic modifications on children's genome visible at age 7 and partially remaining at age 15.

These results support the hypothesis that physiological processes at least partially explain associations between early-life adversity and health problems later in life. Both maternal stressors (MFP/MD) had similar effects on biological pathways, providing preliminary evidence for the mechanisms underlying the effects of low socioeconomic status in early life and disease outcomes later in life. Understanding these associations is essential to explain disease susceptibility, overall life trajectories and the transition from health to disease.

**Keywords:** Aging; ALSPAC; biological pathways; DNA methylation; epigenome-wide association studies; financial issues; Pace of Ageing

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## Key messages

1. Exposure to economic hardship at any time period from conception to age 7 does not appear to accelerate epigenetic ageing in any of the common first or second-generation epigenetic clocks.
2. At an epigenome-wide level, exposure to economic hardship in-utero (from conception to birth had no effect.
3. There was a strong epigenomic effect of exposure to economic hardship from birth to age 7.
4. Subjective and objective measures of economic hardship affected methylation at different CpGs, however, these converged and affected identical biological pathways.
5. Targeting preventative measures in the postnatal period may have a stronger epigenetic effect than those put in place during pregnancy.

## Introduction

From conception onwards, the external environment molds development and well-being through epigenetic modifications that are retained for many decades (Naumova et al., 2012; Naumova et al., 2019). Three decades of epidemiological studies have provided solid evidence for the importance of sensitive periods e.g. the first 1000 days of life for the lifelong health trajectory (Clark et al., 2021; Lopez-Otin et al., 2013; Marini et al., 2020). An individual's experience of stressors, environmental pressures and low socioeconomic status (SES) during this early-life period is now accepted to set them on a unique aging trajectory and determining their health/disease profile later in life (D. W. Belsky et al., 2015; Hayward & Gorman, 2004; Raffington et al., 2021). Previous research has demonstrated that early-life exposure to psychological stressors from low SES increases the risk of developing e.g. Type 2 diabetes, cardiovascular disease, and cancer later in life as well as shortening life-span (Batten et al., 2004; Hayward & Gorman, 2004; Kaeberlein, 2013; Kirkwood, 2005; Lopez-Otin et al., 2013; McLaughlin et al., 2011; Rodriguez-Miguelez et al., 2022; van Lenthe et al., 2014). More precisely, early-life exposure is linked to stress-related disorders such as psychopathologies, metabolic diseases and long-term immune-mediated diseases. Studies reported the strong association between early trauma exposure and stress genes regulation modification leading to psychiatric disorders (Turecki et al., 2014).

As depicted in the literature, socioeconomic factors such as economic hardship are considered as determinants of health, as they play a major role in cardiovascular diseases due to their strong associations with adult risk factors (Alfano et al., 2019;

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Galobardes et al., 2006; Lynch et al., 1997) . Additionally, SES factors can influence offspring's genomes, with worse maternal socioeconomic outcomes being associated to delayed developmental age and worse anthropometric outcomes at birth (Girchenko et al., 2017; Knight et al., 2016).

Economic hardship, such as material deprivation (MD) or major financial problems (MFP), during the prenatal period and the first years of development have the potential to affect DNAm levels, accelerating or decelerating epigenetic clocks (EC). Recently, Clark et al., estimated the physical effects of major prenatal economic shocks occurring within the first eighteen weeks of gestation on the offspring (Clark et al., 2021): children who had experienced maternal MFP *in-utero* had a smaller head circumference (2–3 mm) and decreased birth weight (40–70g) (Clark et al., 2021). Similarly, MFP during early childhood was associated with worse cognitive and non-cognitive outcomes during adolescence, even when controlling for household income and wealth (Clark et al., 2021). Moreover, previous studies have demonstrated that poor birth outcomes are associated with poor developmental trajectories in infancy leading to long-term health issues in adulthood (Fiorito et al., 2019). As low SES are directly experienced by the mother and only indirectly by their child, we investigated whether the maternal epigenome can play an indirect mediating role in determining the child's epigenome. Here we considered the maternal epigenome as a proxy for her current and accumulated life experience as well as the developmental environment provided to her child both either *in-utero* or postnatally.

Early-life trauma is also strong implicated in the aging process, inducing a progressive decline in system integrity at the cellular level (i.e. telomere attrition or epigenetic modifications) (Girchenko et al., 2017). At the cellular level, telomere shortening is accelerated by early-life adversity. While the validity of telomere length as an index of cellular aging continues to be debated DNA methylation (DNAm) has been introduced as an alternative measure. DNAm is now considered a more powerful and precise biomarker of aging (Jylhava et al., 2017; Pearce et al., 2022). Using DNAm, a clear association between epigenetic age measured using the epigenetic clocks and the lifelong health/disease balance has been demonstrated (Fiorito et al., 2019; Horvath & Raj, 2018).

Epigenetic clocks were introduced in 2013 by Horvath et al. (2013) and Hannum et al. (2013). These first-generation EC were trained on chronological changes in DNAm. These clocks subsequently estimate the "epigenetic age" of the individual from their DNAm, and deviation from chronological age suggests accelerated or decelerated aging processes (reviewed in (Noroozi et al., 2021)). Second-generation clocks, such as the Dunedin Pace of Ageing (PoAm), were trained on longitudinal change in age-related biomarkers rather than chronological age (Belsky et al., 2015). The inclusion of eighteen markers reflecting biological aging and organ system integrity (e.g., BMI and creatinine clearance) makes PoAm particularly powerful. Eight biomarkers are common to PoAm and the Levine second-generation clock (Belsky & Shalev, 2016). PoAm and the other second-generation clocks are more sensitive to social determinants of health than the earlier clocks (Belsky et al., 2020; Lo & Lin, 2022; Raffington et al., 2021). Thus, PoAm reflects the underlying biological processes of aging. Additionally, experience suggests that PoAm can detect the epigenetic effect of social determinants of health in children, unlike first-generation clocks (Raffington et al., 2021).

Here, we investigated the effects of exposure to MFP or MD before the age of 7y on the aging process using EC in participants from the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort (Clark et al., 2021; Clark et al., 2021; Yan et al., 2020) (Fig. 1). The use of the first and second-generation clocks will provide information on age acceleration and SES associations. Additionally, an epigenome-wide association was conducted (EWAS) in order to investigate the impact of these socioeconomic parameters at the functional physiological level. We then extracted epigenetic information as differentially methylated chromosomal regions. Additionally, we examined their associated biological pathways to understand how MFP or MD may induce functional physiological differences.

## Materials and methods

### Cohort: ALSPAC

We used data from the ALSPAC cohort (Boyd et al., 2013; Fraser et al., 2013). ALSPAC was established to examine how both environmental and genetic factors influence health and development throughout the life course. The ALSPAC team recruited 14,541 pregnant women in the South West of England between April 1991 and December 1992. Both mothers and children have now been followed for more than 30 years. During this period, mothers were interviewed almost yearly about their lifestyle, socioeconomic positions and health situation. ARIES is a subset of 1022 ALSPAC mother-child dyads from whom DNA was collected from the children at birth, age 7, and age 15–17 for DNAm analysis. After data quality control, the number of participants dropped to 927.

The ALSPAC cohort was approved by the ALSPAC Ethics and Law Committee and the Local Research Ethics Committees. ALSPAC participants provided (i) demographic data including age, and gender, (ii) medical history, (iii) socioeconomic status including financial problems, income, and material deprivation items, and (iv) tissue samples (Fraser et al., 2013). These parameters were extracted from the ALSPAC cohort for the 927 ARIES participants with complete epigenetic profiles at ages 0, 7, and 15.

### DNA methylation

As described in (Relton et al., 2015), maternal peripheral blood samples (whole blood or buffy coat) were collected in the antenatal period (mean 25.7 gestation weeks). For children, blood samples were collected at birth (cord blood), age 7 and age 15–17 (peripheral blood). In all blood samples, DNA methylation was measured using the Illumina 450 K array. The detailed protocol of extraction, processing, and quality control of the DNA methylation data can be found in (Relton et al., 2015). Additional information are available in the Supplementary Methods.

### Major financial problems

Mothers who experienced MFP during a specific period reported it as one independent event. Mothers' reports of major financial problems were recorded first during a pregnancy assessment (18 weeks of gestation) and then ten additional times, when the study child was aged 8 weeks, 8 months, 21 months, 33 months, 47 months, 61 months, 73 months, 110 months, 134 months, and in May-September 2010 (around child age 18). From the beginning of the survey up to child age 20, they were asked eleven times whether they experienced a major financial problem. Due to the inconsistency in the way MFP were recorded over time (either focusing on the incidence or intensity of MFP), we coded MFP as a dummy variable taking the value "one" when the mother reported having experienced a major financial problem since the previous interview (regardless of the intensity reported) and "zero" otherwise.



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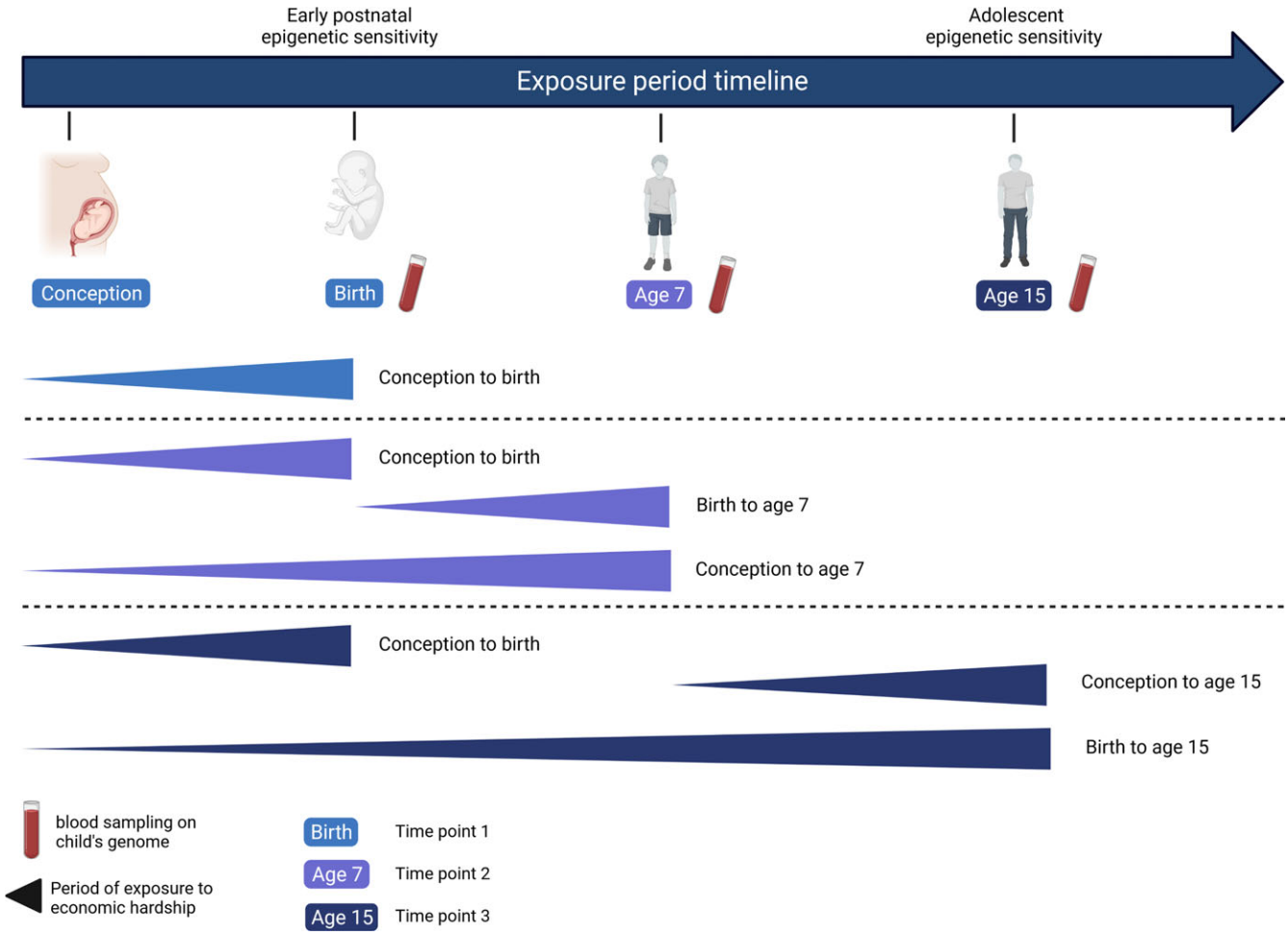


**Figure 1.** Exposure timelines, epigenetic sampling points, and EWAS analyses performed. The images (baby/child/adolescent) represents the different ALSPAC epigenetic time point when blood sampling was performed. The first time point is referred to as birth / age 0; the second: age 7; and the third: age 15. Underneath, each horizontal triangle represents the exposure period for a specific EWAS model with the colour of the triangle representing the age at which the DNA methylation was measured. The exposure periods are: (1) conception to birth, (2) birth to age 7, (3) conception to age 7, (4) conception to age 15 and (5) birth to age 15. Light blue = EWAS methylation at birth; purple = EWAS methylation at age 7; dark blue = EWAS methylation at age 15.

This was summed over all eleven incidences, providing a scale from 0 (never experienced) to 11 (reported at each interview). Different cutoff comprised between 0 and 8 or between 0 and 11, respectively at age 7 and 15, were assigned to MFP variable to investigate a potential dose response effect. A cutoff of 1 represents participants who experienced at least one financial problem while a cutoff of 2 represents at least 2 financial problem. In total 11 different cutoff were tested (from 0 to 11). Due to substantial attrition of mothers over time (26% of mothers who reported MFP consistently up to child age 7 stopped answering this question between child age 7 and 15), we do not consider MFP episodes after child age 7.

### *Material deprivation*

Mothers were asked from the beginning of the survey questions about the quality of the house where the child lives. These questions are the following: whether the house has an indoor toilet, a bath or shower for the sole use of the household, or a working phone; whether the house has a problem of mold, dampness or condensation; whether the child's bedroom or living room is adequately warm during the winter. We built MD as a count index, based on the presence (or absence) of the following items at home: indoor toilet, bath or shower, damp or mold, cold or very cold living room, working phone. These were measured during the pregnancy (at 8 weeks gestation) and when the study child was aged 8 months, 21 months, 33 months, 61 months, 85 months and 122 months .We considered as materially deprived the lack of at least two items out of the five listed above. As for MFP, mothers who experienced MD during a specific period reported it as one independent event. MD was coded in a similar manner as MFP. We defined three critical MD periods in a similar manner to MFP

### *Exposure period definition*

Here, we defined three exposure periods for MFP and MD: (i) from conception to birth, (ii) from conception to age 7 and (iii) from birth to age 7 respectively. These were termed (i) $MFP/MD_{(conception\ to\ birth)}$, (ii) $MFP/MD_{(conception\ to\ age\ 7)}$ and (iii) $MFP/MD_{(birth\ to\ age7)}$.

### *Epigenome-wide association studies (EWAS)*

To extract significantly associated CpGs and their epigenetic information, the DMRcate (version 2.6.0) package (Peters et al., 2015) was used. Probes or DMRs were considered significant when the Benjamini-Hochberg corrected $p$ value < .050.



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### High-dimensional mediation analysis (HIMA)

The "HIMA" package was used to investigate high-dimensional mediation effects between mothers' CpGs and child's CpGs found to be significant in the MFP or MD EWAS models. Here we set up three mediation models: the first model (Model 1) and the second model (Model 2) represent the period of exposure between birth and age 7 (Fig. 6). Model 1 shows the 3316 CpGs identified in the MFP EWAS while Model 2 represents the 254 CpGs of the MD EWAS. Model 3 highlights the 3 common CpGs to MFP and MD EWAS (Model 1 and Model 2).

### Data analysis

ALSPAC cohort data have been normalized by using the R "meffil" package to remove unwanted technical variation by regressing the variability due to the control probes on the array. We analyzed DNA methylation data from the ALSPAC/ARIES children at the three time points (at birth, $n = 927$; at age 7 years, $n = 925$; and at age 15 years, $n = 861$). A complete description of the cord and whole blood sample collection, data and transformations, and statistical analyses of the DNA methylation, high mediation model (HIMA) and data visualization protocols is included in the Supplementary Methods.

## Results

### Exposure to MFP is weakly linked to the child's epigenetic age

We calculated Horvath, Hannum and PoAm clocks at the second ALSPAC epigenetic time point (child age 7). Pearson correlations were used to examine the link MFP in the three time-periods: $MFP_{(conception\ to\ birth)}$, $MFP_{(conception\ to\ age\ 7)}$, and $MFP_{(birth\ to\ age\ 7)}$ and both first and second-generation EC (Fig. 2a–l). Recent literature suggests that the PoAm may be more suitable for younger populations (Caro et al., 2023; Raffington et al., 2021). In our child cohort, the first-generation clocks had non-significant correlations with MFP for each exposure period ($p > 0.1$ and $R^2 < 0.1$). The second-generation PoAm clock, thought to be more relevant to children, showed weak correlations with MFP exposure that were borderline significant at all time periods ($p = 0.04$–$0.07$ or $R^2 < 0.1$). Furthermore, we calculated the Levine clock but it did not associate with MFP exposure ($p > 0.1$, $R^2 < 0.1$; Supplementary Figure 1). Finally, age acceleration was calculated for the first-generation Hannum and Horvath clocks by using the following equation: Age accel = DNAm age – chronological age (Supplementary Figure 3). Results are similar to those for epigenetic age presented in Figure 2 and calculated Age.accel for both Horvath and Hannum clock (Supplementary Figure 3).

### MFP intensity is weakly associated with the rate of epigenetic aging

As we only observed a weak correlation between MFP and PoAm, we investigated whether MFP acted in a dose-dependent manner on the pace of epigenetic age. As MFP is a continuous variable (counting episodes of MFP over a given time interval) we dichotomized it with a series of cutoffs of increasing intensity from 1 to 4 episodes within the examined exposure period. The OR showed non-significant results for the three tested exposure periods (Fig. 2j–l). When the number of MFP events increased, the pace of epigenetic aging did not change significantly. We calculated BMI and sex-adjusted models, and neither covariate significantly altered the OR (data not shown).

### MFP is associated with epigenetic modifications on the children's genome at age 7

To determine the epigenetic implication of MFP beyond aging, we used a case-control (EWAS) approach to identify exposure-associated CpGs. Prenatal exposure, $MFP_{(conception\ to\ birth)}$, induced differential methylation at 5 CpGs (cg25857695, cg08462952, cg23840008, cg03737367, cg00414709) that do not overlap with the previously CpGs highlighted in (Laubach et al., 2019) and (Alfano et al., 2019). Exposure to MFP over a longer period, $MFP_{(birth\ to\ age\ 7)}$, induced differential methylation at 3316 CpGs from 445 differentially methylated regions, and $MFP_{(conception\ to\ age\ 7)}$ induced 3387 differentially methylated CpGs in 459 DMRs. Differentially methylated CpGs ($p$-value $< 0.05$) were distributed throughout the genome (Fig. 3ab). Differential methylation induced by $MFP_{(conception\ to\ age\ 7)}$ and $MFP_{(birth\ to\ age\ 7)}$, was largely identical, with 2916 common differentially methylated CpGs. Including the 5 differentially methylated CpGs in $MFP_{(conception\ to\ birth)}$, there were 2 CpGs (cg25857695 and cg03737367) common to all exposure periods considered (Fig. 3c).

### MFP is associated with epigenetic modifications on the children's genome at age 15

EWAS studies on MFP are somewhat complicated due to the lack of suitable validation cohorts. However, ALSPAC took a third series of biological samples at age 15 from which Infinium 450k array data is available. To confirm the data at age 7, we used this third series of samples from ALSPAC. As in the previous section, we ran a similar analysis for $MFP_{(conception\ to\ birth)}$, $MFP_{(birth\ to\ age\ 15)}$, and $MFP_{(conception\ to\ age\ 15)}$. We identified 536 significant CpGs for exposure from birth to age 15 (Supplementary Figure 4). Again, significant CpGs were distributed throughout the genome of children (Supplementary Figure 4A-B). We found 1 common CpG (cg20147595) between the two periods of exposure, $MFP_{(conception\ to\ age\ 15)}$ and $MFP_{(birth\ to\ age\ 15)}$, to economic shocks (Supplementary Figure 4C). We found 72 conserved CpGs from age 7 vs age 15 (Supplementary Figure 4D and Supplementary Table 1). Consequently, we conclude that at least part of the imprint was maintained at age 15 despite the potential epigenetic remodeling during adolescence.

### Exposure to MD is not linked to the child's epigenetic age

Similar to the procedure for MFP, we calculated the first-generation (Horvath and Hannum) and the second-generation (Levine and PoAm) EC at the second time point of ALSPAC (child age 7). Pearson correlations were used to examine the link with MD in the three time-periods: $MD_{(conception\ to\ birth)}$, $MD_{(conception\ to\ age\ 7)}$, and $MD_{(birth\ to\ age\ 7)}$ and both first and second-generation EC (Supplementary Figure 2, a–l). Both generation clocks returned non-significant correlations with MD and each time exposure ($p > 0.1$ and $R^2 < 0.1$).

### MD is associated with epigenetic modifications on the children's genome at age 7

As for MFP, we investigate the association between MD and epigenetic modifications (Fig. 3d–f). There were 254 significant CpGs and 45 DMRs $MD_{(birth\ to\ age\ 7)}$. As above, these CpGs were distributed throughout the children's' genome (Fig. 3d–e). We found 145 common CpGs between the two periods of exposition (birth to age 7 and conception to age 7) to MD. However, we did not find significant CpGs for MD exposure in the period conception to age 0



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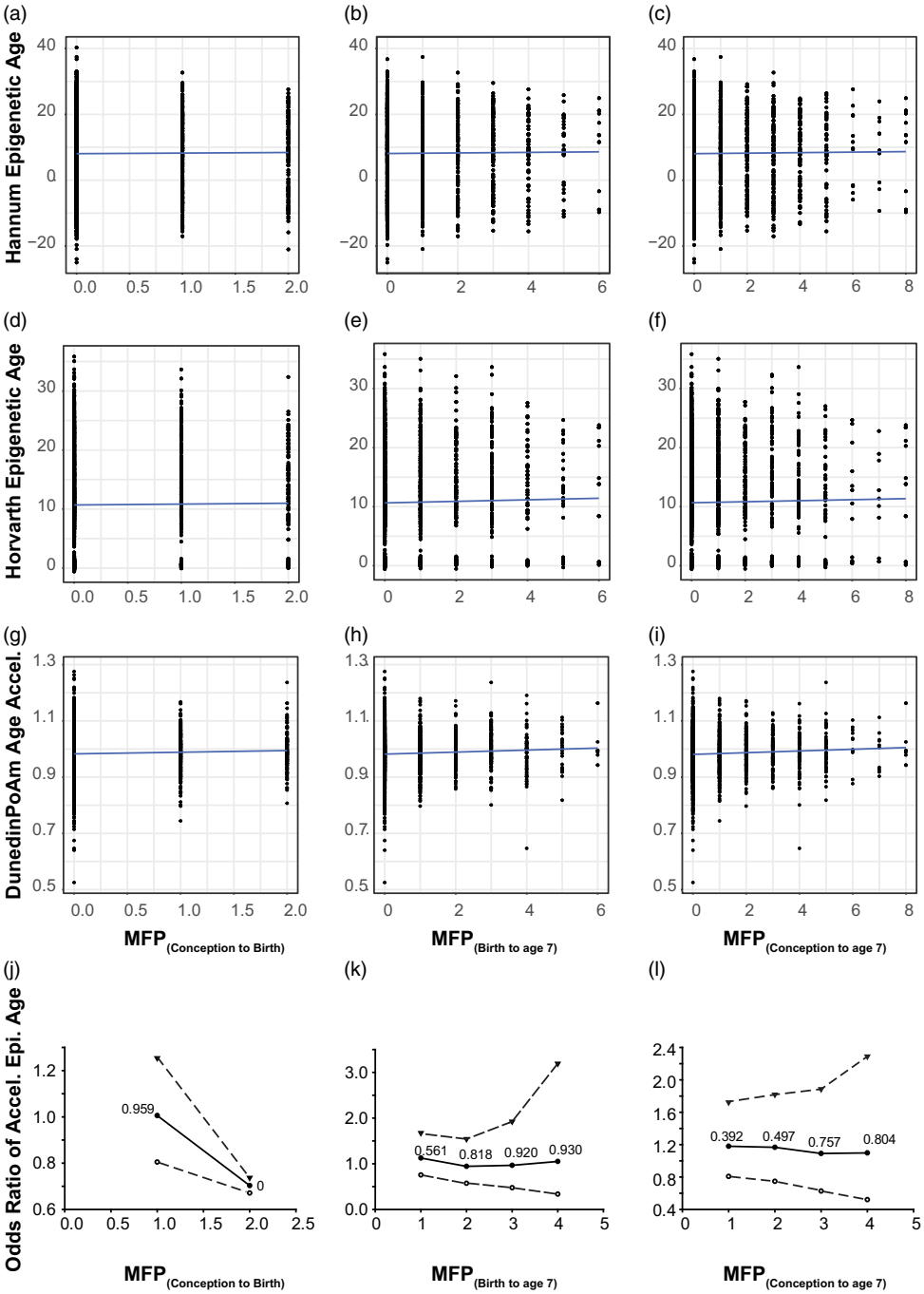


**Figure 2.** Time of MFP exposure is weakly correlated with poAm aging. Scatter plot represents correlation between major financial problem (MFP) and epigenetic age. Panel ***a–c*** represents the correlation between the number of MFP events and the hannum epigenetic age, ***d–f*** represents the same, but for the Horvath epigenetic age and ***g–i*** the poAm speedometer. Blue line is the linear regression line. Data points represent individual participants. Panel ***j–l*** represents odds ratio of accelerated epigenetic age of the children over the same time periods. Central solid line = OR; upper and lower dashed lines = 97.5 and 2.5% CI; text : *p*-value.

(Fig. 3f). The top 10 CpGs associated respectively with MFP and material deprivation are regrouped in Table 1. Thus, as for MFP, MD would appear to influence the epigenome during the period from birth to age 7 y.o. rather than *in-utero* (conception to birth).

### *MFP and MD have few common CpGs*

For the same period of exposure to socioeconomic parameters, birth to age 7, we investigate the number of common CpGs after experiencing major financial problems and material deprivation. We identified only 3 common CpGs (cg26217846, cg13819687 and cg25753631), suggesting that MFP and MD are independent stressors, affecting different genomic regions (Fig. 3g).

### *MFP and MD are involved in multiple, common, biological pathways*

After extracting the genes annotated to the differentially methylated CpGs, affected biological pathways (BP) were identified (Fig. 4). BP were found to be associated with epigenetic changes induced by both $MFP_{(\text{birth to age 7})}$ and $MD_{(\text{birth to age 7})}$ (Fig. 4a,b respectively). For both panels, the fold enrichment is comprised between 1.5 and 2.5. On the contrary, p-values are highly significant for most BP (50 < -log10 (p) < 100). Regarding the two periods found a total of 237 and 268 statistically significant biological pathways for $MFP_{(\text{birth to age 7})}$ and $MD_{(\text{birth to age 7})}$ respectively, with 229 common between the two datasets (Fig. 4c). Table 2 and Supplementary Table 2



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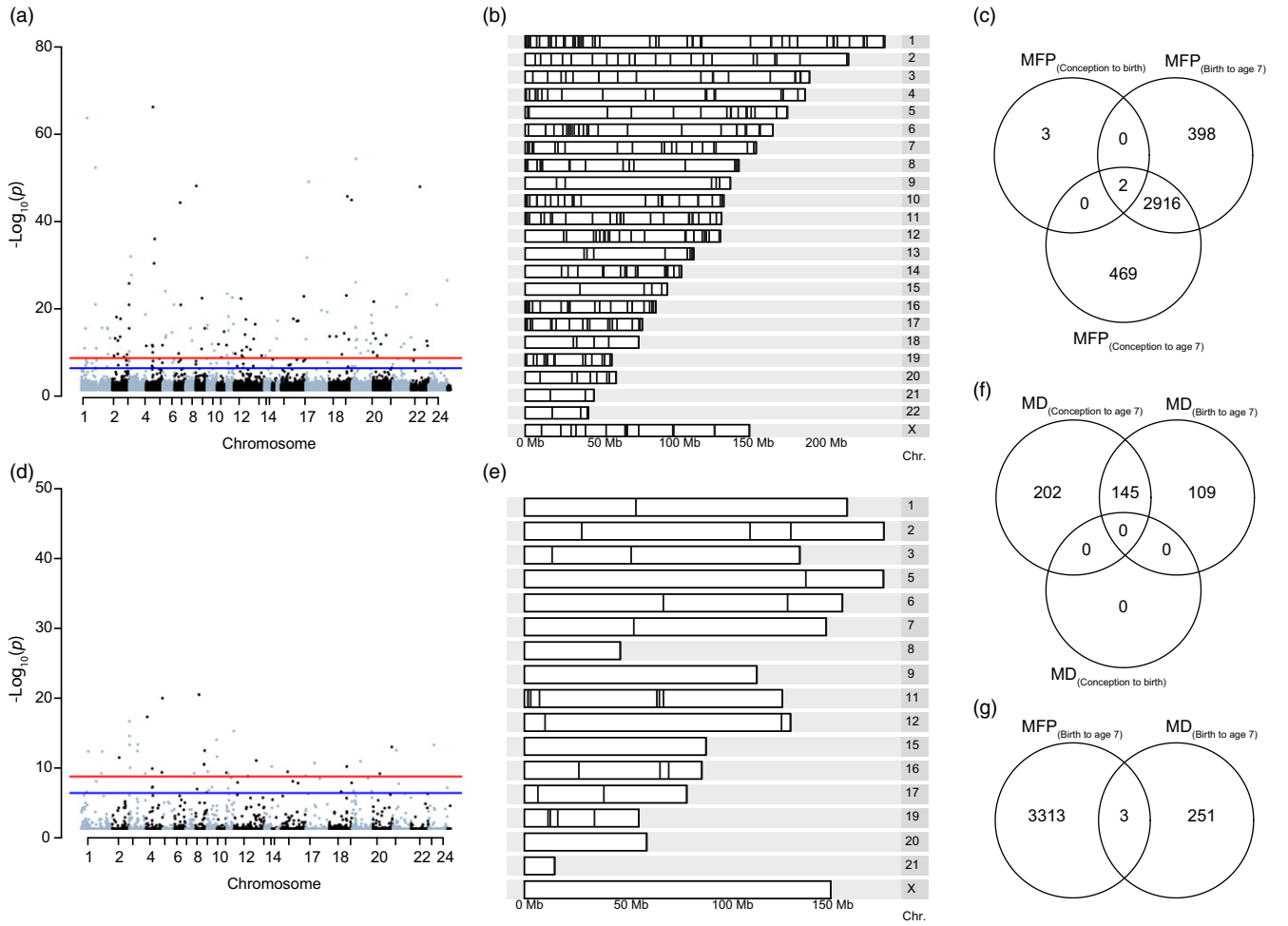


**Figure 3.** Differential methylation induced by exposure to MFP and MD between birth and age **7**. (***a***) Manhattan plot represents the repartition of the 3316 differentially methylated CpGs on each chromosome after exposure to MFP between birth and age 7. (***b***) Karyogram showing spatial distribution of the 445 DMRs. (***c***) Venn diagram summarizing the EWAS performed over the three exposure periods, highlighting the 2 common CpGs common to the three time-periods of exposure to MFP. (***d***) Manhattan plot represents the repartition of the 254 differentially methylated CpGs on each chromosome after exposure to MD between birth and age 7. (***e***) Karyogram showing the spatial distribution of the 45 DMRs. (***f***) Venn diagram summarizing the EWAS performed over the three exposure periods, highlighting the 145 common significant CpGs common to the three time-periods of exposure to MD. (***g***) Venn diagram shows the overlap (3 common CpGs) from the EWAS on DNA methylation at age 7 taking exposure to either MFP or MD from birth to age 7.

regroups respectively, the top 10 common pathways list and the full 229 common BP list.

From the top 10 common pathways, we selected "oxidative phosphorylation" (OP) and "Parkinson's disease" (PD), which are known to be associated with aging, for further investigation. We extracted information on the common up- and down-methylated genes in the OP and PD pathways. (Fig. 4d–e). We saw approximatively 200 hyper-methylated and 0 hypo-methylated genes, suggesting that the majority of the pathways will be down regulated as the predominant hyper-methylation observed will most probably reduce the expression of the associated genes (Table 2). The mitochondrial respiratory chain genes NADH ubiquinone oxidoreductase subunits 3 (NDUFB3) and cytochrome c oxidase subunits 7C (COX7C) were the most significantly differentially methylated extracted from the top 10 common pathways, and the probe locations and methylation are shown (Fig. 5a,b). For both genes, the DNAm modifications were situated in the promotor and coding region of the gene.

### *Maternal prenatal DNAm is a determinant of the impact of economic hardship on child DNAm*

As we were considering the effect of maternal socioeconomic conditions we wanted to investigate whether the mother's epigenome mediated changes in the child's epigenome. Unfortunately, within ALSPAC the maternal epigenome was sampled during gestational week 18, somewhat undermining the strict temporal relationship necessary for a mediation analysis. However, experience with epigenetic clocks suggest that 98% of CpGs do not change methylation levels with age (Unnikrishnan et al., 2019). As such, we used the maternal epigenetic data as the best approximation in our mediation model. To do this, we set up three mediation models (Fig. 6). Model 1 and Model 2 return 3316 and 254 CpGs respectively identified in the MFP and MD EWAS. Model 3 covers the differentially methylated CpGs commons between MFP and MD exposure. Figure 7 shows circos plot for these three mediation models. The 3316 tested CpGs in Model 1 returned 107628 links between maternal and child genome. Mediation Model 2 returns



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**Table 1.** Top 10 biological pathways differentially methylated after maternal exposure to either MFP (birth to age7) or MD (birth to age7). Pathways were calculated with pathfinder based on DNA methylation differences at child age 7 and maternal exposure to either MFP or MD from birth to age 7

| | ID | Term Description | Fold Enrichment | Lowest p–value | Highest p–value |
|---|---|---|---|---|---|
| Major financial problems | | | | | |
| 1 | hsa03010 | Ribosome | 1.24 | 1.67e–88 | 8.84e–13 |
| 2 | hsa05020 | Prion disease | 1.44 | 3.59e–69 | 1.70e–08 |
| 3 | hsa05012 | Parkinson disease | 1.41 | 2.45e–67 | 1.84e–10 |
| 4 | hsa05208 | Chemical carcinogenesis - reactive oxygen species | 1.44 | 1.15e–65 | 5.48e–08 |
| 5 | hsa05171 | Coronavirus disease - COVID-19 | 1.42 | 2.63e–63 | 4.22e–08 |
| 6 | hsa00190 | Oxidative phosphorylation | 1.45 | 8.07e–63 | 9.58e–10 |
| 7 | hsa05415 | Diabetic cardiomyopathy | 1.45 | 2.63e–62 | 2.82e–10 |
| 8 | hsa04932 | Non–alcoholic fatty liver disease | 1.45 | 1.87e–50 | 4.64e–08 |
| 9 | hsa04714 | Thermogenesis | 1.45 | 5.05–44 | 1.29e–08 |
| 10 | hsa03040 | Spliceosome | 1.39 | 1.29e–39 | 7.60e–05 |
| Material deprivation | | | | | |
| 1 | hsa05020 | Prion disease | 2.31 | 5.87e–77 | 1.79e–78 |
| 2 | hsa05012 | Parkinson disease | 2.17 | 1.14e–80 | 9.97e–75 |
| 3 | hsa05208 | Chemical carcinogenesis - reactive oxygen species | 2.34 | 4.58e–69 | 1.97e–66 |
| 4 | hsa00190 | Oxidative phosphorylation | 2.49 | 1.12e–67 | 4.38e–66 |
| 5 | hsa05415 | Diabetic cardiomyopathy | 2.31 | 3.27e–65 | 9.07e–63 |
| 6 | hsa03050 | Proteasome | 2.15 | 7.84e–56 | 8.71e–31 |
| 7 | hsa04932 | Non–alcoholic fatty liver disease | 2.47 | 8.36e–54 | 7.90e–52 |
| 8 | hsa04714 | Thermogenesis | 2.16 | 4.07e–49 | 4.50e–47 |
| 9 | hsa03010 | Ribosome | 0.90 | 6.93e–49 | 2.28e–39 |
| 10 | hsa05171 | Coronavirus disease – COVID-19 | 1.94 | 3.92e–41 | 2.61e–37 |

8901 mediating CpGs mediation model *p-value* < 0.05 significantly associated with maternal CpGs. Finally, Model 3 provides 151 common mediating CpGs between MFP and MD exposure.

## Discussion

Early-life adversity (ELA) and low SES are amongst the most powerful drivers of the aging process (Sun et al., 2020). Here, we initially demonstrated a weak association between early-life SES, proxied by the mother's MFP and MD, and the PoAm pace of epigenetic aging. Children exposed to MFP in the period from birth to age seven had epigenetic modifications genome-wide that were visible at age 7, that were partially retained at age 15.

The epigenetic aging analysis suggested that a child's PoAm pace of epigenetic aging was weakly associated with financial problems (coefficient of correlation $R = 0.003–0.06$) and explains a tiny percentage of their variance. This raises the hypothesis that the underlying mechanism linking exposure to the phenotype previously described could be a more specific biological process that is incompletely captured by PoAm rather than a simple change in the pace of epigenetic aging.

As MFP and MD only explained a tiny percentage of the variance in PoAm we performed a series of EWAS analyses. Both MFP and MD are independent epigenetic programing events. Extending this to the biological pathways, it became clear that despite the qualitative and quantitative differences in the nature of MFP and MD as maternal stressors the biological pathways affected were highly similar. Importantly, these biological pathways were centered on metabolism.

Overall, MFP data suggest that the child's epigenome is particularly sensitive to MFP in the postnatal period from birth to 7 years old. Indeed, as MFP appears to be significantly involved in the methylation level of these CpGs, we suggest that they will be associated with the eventual expression levels of these genes and potentially affect disease development or biological pathway functioning.

MFP and MD capture two different aspects of an individual's SES. Material deprivation is a multidimensional measure of poverty focusing exclusively on material living conditions. We followed the intermediate identification method of the poor (Bossert et al., 2013) and consider as poor someone experiencing at least two functioning failures out of the five material living conditions questioned. MFP is a measure of financial distress as perceived by the mother. This variable reflects both the economic resources available to the mother and the demand that is made on them. It captures financial insecurity over and above traditional income indicators: a major financial problem can be experienced by anyone, not only by those in poverty. In our differential methylation analyses at age 7 MFP and MD induced almost completely independent methylation signatures at the CpG level.

As the differential methylation patterns at age 7 were specific to either MFP or MD we focused on BP rather than on individual



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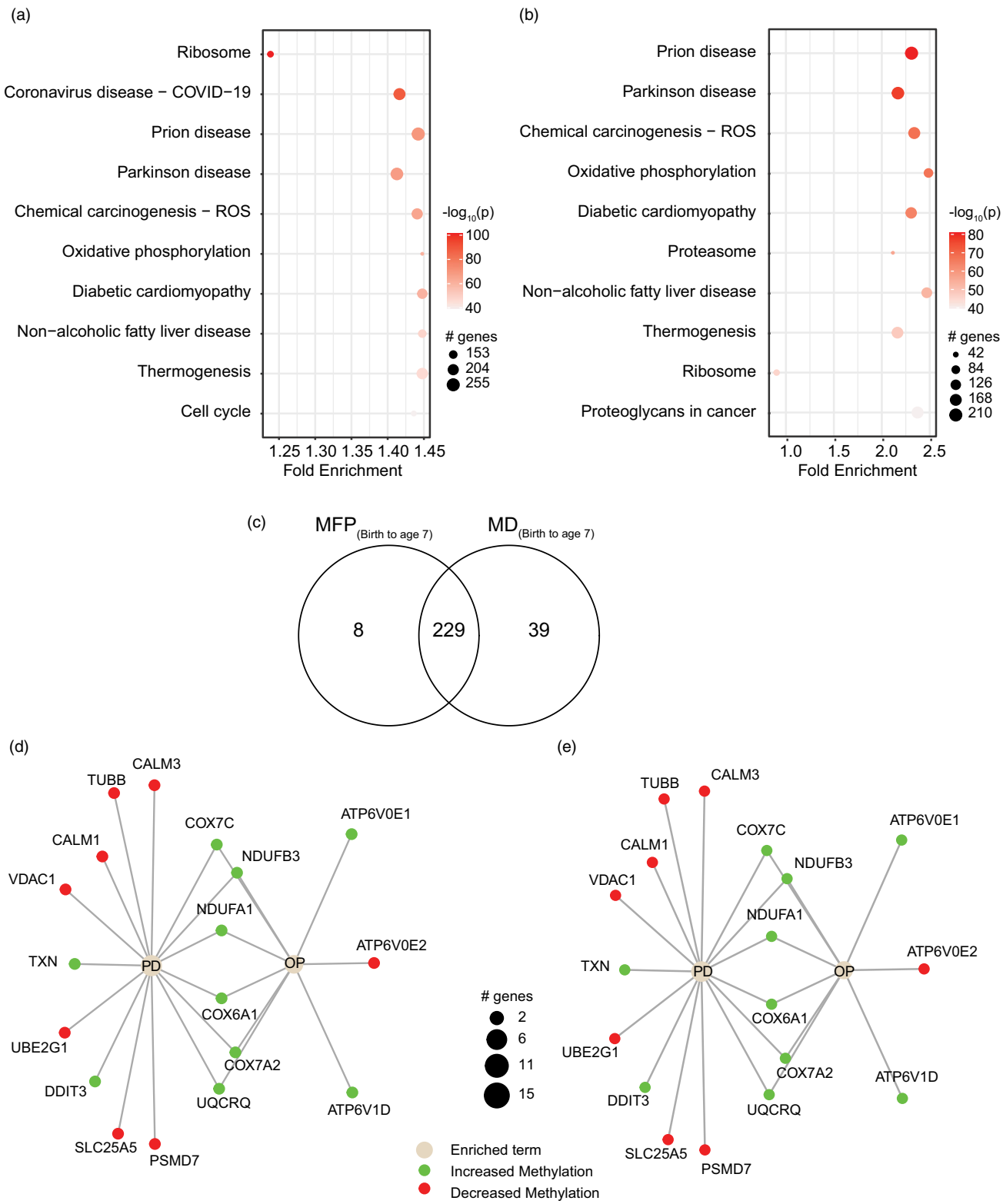


**Figure 4.** Biological pathways associated with differential methylation induced by MFP and MD. (***a***) Pathway summary showing the fold enrichment and number of genes involved in each of the 10 most significantly affected pathways at age 7 after exposure to MFP from birth to age 7. (***b***) Pathway summary showing the fold enrichment and number of genes involved in each of the 10 most significantly affected pathways at age 7 after exposure to MD from birth to age 7. Color code : -log(10) *p*-value. Black circle diameter: number of genes. (***c***) Venn diagram showing common BP extracted from the EWAS at age 7 after exposure to either MFP or MD from birth to age 7. (***d***–***e***) Graphs show genes involved in two example pathways: Parkinson disease (PD) and oxidative phosphorylation (OP). Black circle diameter: number of genes.



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**Table 2.** Top 10 biological pathways differentially methylated in common to both maternal exposure to MFP (birth to age7) as well as MD (birth to age7). Pathways were calculated with pathfinder based on DNA methylation differences at child age 7 and maternal exposure to either MFP or MD from birth to age 7

| | ID | Term Description | Fold Enrichment | Lowest p–value | Highest p–value |
|---|---|---|---|---|---|
| 1 | hsa03010 | Ribosome | 1.24 | 1.67E–88 | 8.84E–13 |
| 2 | hsa05020 | Prion disease | 1.44 | 3.59E–69 | 1.70E–08 |
| 3 | hsa05012 | Parkinson disease | 1.41 | 2.45E–67 | 1.84E–10 |
| 4 | hsa05208 | Chemical carcinogenesis - reactive oxygen species | 1.44 | 1.15E–65 | 5.48E–08 |
| 5 | hsa05171 | Coronavirus disease - COVID-19 | 1.42 | 2.63E–63 | 4.22E–08 |
| 6 | hsa00190 | Oxidative phosphorylation | 1.45 | 8.07E–63 | 9.58E–10 |
| 7 | hsa05415 | Diabetic cardiomyopathy | 1.45 | 2.63E–62 | 2.82E–10 |
| 8 | hsa04932 | Nonalcoholic fatty liver disease | 1.45 | 1.87E–50 | 4.64E–08 |
| 9 | hsa04714 | Thermogenesis | 1.45 | 5.05E–44 | 1.29E–08 |
| 10 | hsa03040 | Spliceosome | 1.40 | 1.29E–39 | 7.60E–05 |

CpG dinucleotides, genes, or differentially methylated regions. Gene ontology show relationships based on similar gene product functions (Dalmer & Clugston, 2019), while gene set enrichment analysis provided information on the enrichment of epigenetically modified genes with common molecular functions or biological processes. While the signatures of MFP and MD were unique at the CpG level, their effects converged at the gene and BP level, with almost identical functional and metabolic effects. Our data showed a clear effect of both MFP and MD on genes associated with OP and Parkinson's disease (PD). While superficially unrelated, there is a well-established link between ELA and OP-dependent processes such as immunosenescence, as well as between ELA, OP, and PD. The long-term immunosenescent phenotype after early-life adversity mainly involves T-cells (Elwenspoek et al., 2017; Reid et al., 2019) and was recently expanded to NK cells (Fernandes et al., 2021). As these cells become senescent they remain metabolically active, although in a more "glycolytic state" (reviewed in (Sabbatinelli et al., 2019)). This reprogramed metabolic activity is intimately linked to their pro-inflammatory senescence-associated secretory phenotype (Sabbatinelli et al., 2019).

In the ALSPAC cohort socioeconomic or psychosocial adversity clearly leads to an observable epigenomic imprint later in life. Here, we identified the specific influence of MFP and MD on methylation at age 7 that converge to common pathways. As described in the literature, biological systems like the HPA axis are recalibrated during adolescent and puberty (Gunnar et al., 2019). Despite this, there is a residual early-life SES signature at age 15. Our data suggest that these postnatal years are the predominant period of sensitivity, confirming the importance of early-life trajectories and negative environmental influences occurring during this period. However, DNAm induced by early adversity exposure affects many biological processes in different ways. As the Infinium 450k methylation arrays used are biased towards promoter regions of proven genes (Pidsley et al., 2016), we anticipate that the increased methylation levels seen would decrease the mRNA and protein levels from the associated genes.

As low SES was experienced by the mother directly, and only indirectly by their child we examined whether the maternal epigenome played an indirect mediating role in determining the child's epigenome. We considered the maternal epigenome as a potential determinant for her current and accumulated life experience as well as the developmental environment she provided her child both *in-utero* and postnatally. The mediation model demonstrated that the maternal epigenome is associated with children's CpGs which are sensitive to economic hardship such as MFP and MD. While epigenetic inheritance is currently debated, the mediation- model confirmed the existence of "maternal directing CpGs" that might explain an indirect epigenetic transmission mechanism. This will potentially explain the child's susceptibility to epigenetic modifications from maternal SES later in life.

In general, early trauma are associated to stress pathway dysregulation leading to physical and mental outcomes (Smith & Pollak, 2020). For example, alterations in the HPA axis play a direct role in the development of mental disorders. It has been suggested that chronicity, timing development but also the type of exposure are key factors shaping the reaction to adversity (Smith & Pollak, 2020; Turecki et al., 2014). As depicted in the literature, psychopathology seems to be associated with biomarkers such as genetic markers or cytokines, also present in biological systems. It is clear that mental health problems are linked to indicators of physical health (Goldsmith et al., 2023; Pollak, 2015).

To fully shed into light the underlying mechanisms it is essential to consider both biomarkers and their consequences and mental health outcomes. Indeed, studies demonstrated that genetic predisposition but also inflammatory actors such as cytokines are mainly responsible for the development of several psychopathologies later in life (Hassamal, 2023; Shin & Kim, 2023). Environmental and lifestyle factors can also directly contribute to chronic inflammation known to be a major risk factor for psychiatric disorders (Goldsmith et al., 2023). There is also evidence that when combined, there are metabolic alterations mainly influenced by a change in inflammatory signaling pathways and intracellular regulators (Alegría-Torres et al., 2011; Mposhi & Turner, 2023). For example those modifications include oxidative damage, mitochondrial stress or gene expression markers of cytokines alteration leading to depression, anxiety depression, bipolar disorder, etc (Bachmann et al., 2020; Butterfield & Halliwell, 2019).

Here, we demonstrated how financial crisis perceived as trauma can interfere with many metabolic pathways such as oxidative phosphorylation. Similar pathways, including mitochondrial respiration underlay the association with Parkinson's disease (Borsche et al., 2021). Our data, taken together with the well-established link between the early-life environment and



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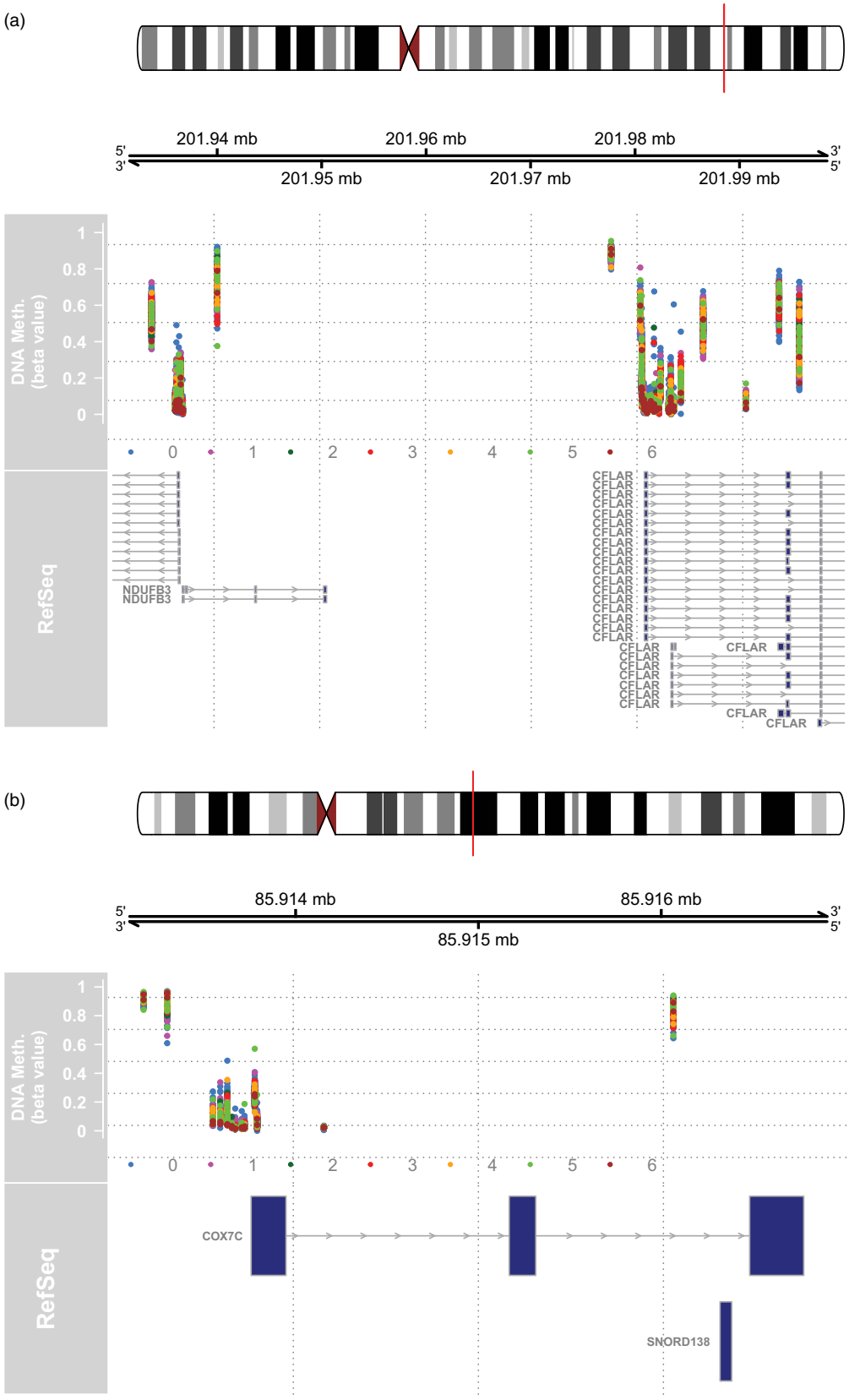


**Figure 5.** Two example differential methylated regions with genes of interest. (***a***) NDUFB3 is represented on chromosome 2. (***b***) COX7C gene is represented on chromosome 5. For both figures, top panel shows the position of the gene on the chromosome (red line). DNA methylation based on the number of financial problems are given for each participants. Relative exposure to MFP or MD (number of episodes) is shown by color.

development of psychopathology clearly points towards the need for future cohorts with specific biosamples to investigate the role of these metabolic pathways in the subsequent development of psychopathology. We confirm the potential importance of intracellular metabolic effects, and the importance of collecting suitable data and samples in future cohorts that would allow us to address intracellular metabolism adequately. We suggest that intracellular metabolic mechanisms may link early-life exposure



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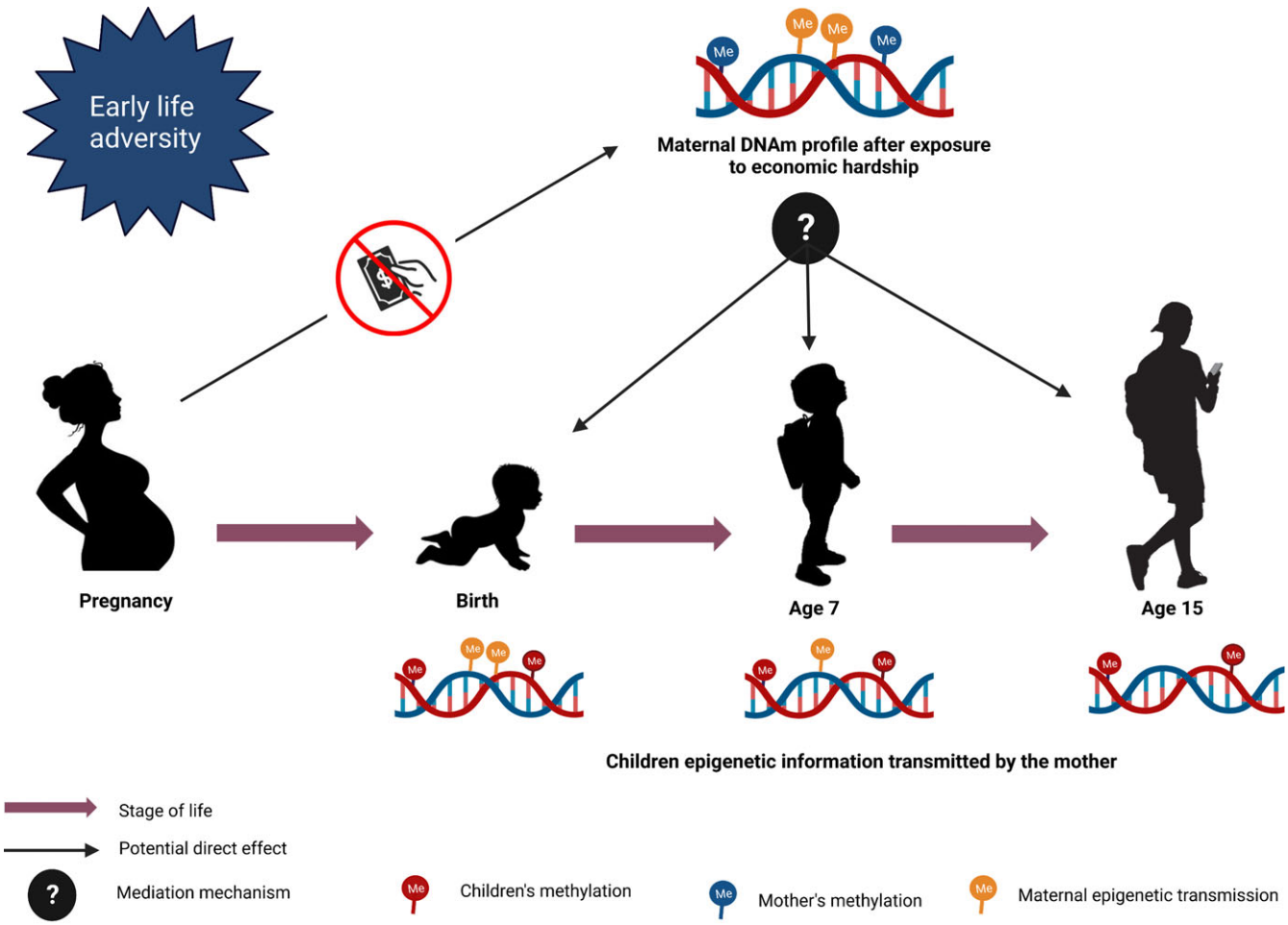


**Figure 6.** Mediation model design. The figure represents the mediation models to investigate the potential direct effect of maternal CpGs on children's CpGs after exposure to maternal economic hardship.

and the subsequent mental disorders development. There is a growing appreciation that there is a clear physical aspect to the development of psychopathologies (Spurrier et al., 2022), and we suggest that this may be due to the metabolic alterations leading to disease development or not when exposed to trauma.

There are several limitations to our study. Unfortunately we did not have enough power to test whether the MFP phenotype described by Clark *et al.*, (Clark et al., 2021) was mediated by DNA methylation. The ARIES subset of the ALSPAC cohort represents only 1022 of the > 14000 ALSPAC mother-child dyads in which the phenotype of reduced head circumference and birth weight was reported (Clark et al., 2021; Clark et al., 2021). Furthermore, this drops to less than 500 mother-child pairs at age 15-17 with complete MFP data (MD was only available up to child age 10). Additionally, the richness of the ALSPAC cohort and ARIES subset means that there is currently no suitable independent validation cohort in which the same socioeconomic parameters were collected over a similar time period, especially the repeated collection of socioeconomic data. Our mediation analysis was limited by the timing of the exposure measured and the measurement of the maternal genome. However, despite this, we saw significant associations and have provided a suitable methodology for future studies in which the data can be collected in the ideal temporal configuration. In addition, the access to data biomarkers measures at every time point is very limited ALSPAC. However, the final ARIES data-point at age 15–17, with separate biological samples and repeated analyses did allow us to identify the residual signature of the early-life exposure, despite the significant dropout for these variables at this collection time. Importantly, two independent measures of exposure to low SES, despite inducing differential methylation at completely different probe locations, identified almost identical biological pathways that are associated with previously reported immunological and physiological effects of exposure to ELA.

We initially hypothesized that the long-term effects of ELA were in part due to accelerated epigenetic aging. While poor maternal circumstances were very weakly associated with epigenetic age, they had much stronger effects on epigenome-wide DNAm levels. While there were no changes detected at birth, they became apparent at age 7 and a residual signature remained after puberty, at age 15–17. Despite MFP and MD having unique epigenomic signatures, they program common BP, showing that physiological processes underlie the long-term effects of ELA. We suggest that these epigenetic changes are the key determining the subsequent health trajectory and potentially the lifelong disease profile. The ALSPAC/ARIES data suggests that there is little influence of *in-utero* exposure to MFP or MD on the child's epigenome; the period from birth to age 7 appears to be more important. Our analysis suggests that preventative measures in the postnatal period may have a stronger epigenetic effect than those put in place during pregnancy.



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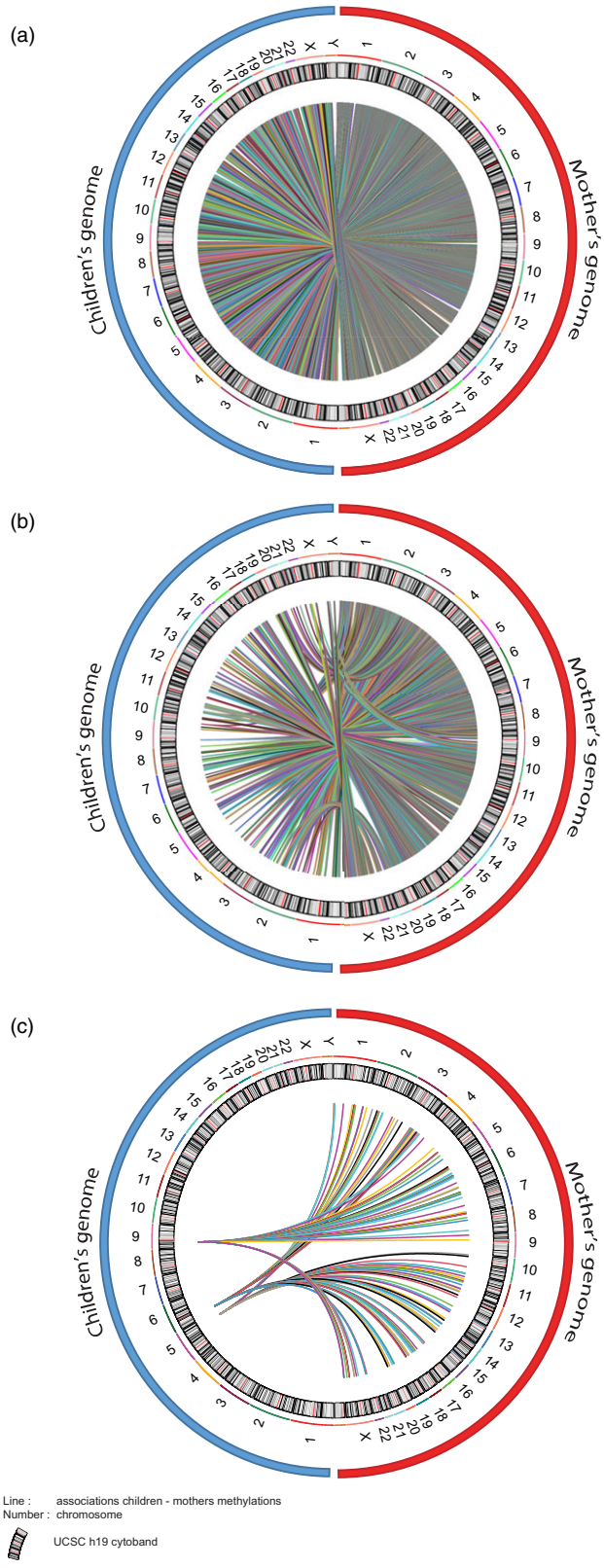


**Figure 7.** Circos plot of the associations between maternal and child CpG methylation from the mediation models. (***a***) 107628 CpGs of children found at age 7 associated with maternal CpGs after MFP exposure. (***b***) 8901 children's CpGs at 7 associated with maternal CpGs after MD exposure. (***c***) 151 children's CpGs found at age 7 associated with maternal CpGs in common between MFP and MD exposure. Blue and red circles respectively represent children's and mother's genomes. Number represents the chromosome. Color lines represent link (associations) between children and maternal CpGs.

**Supplementary material.** The supplementary material for this article can be found at https://doi.org/10.1017/S095457942400083X.

**Availability of data and material.** All data are available commercially from the ALSPAC consortium. Link: Access data and samples | Avon Longitudinal Study of Parents and Children | University of Bristol.

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**Author contributions.** Conceptualization. J.D.T., CH; literature review: C.H. and J.D.T.; data collection: C.D.A, and the ALSPAC team; data analysis: C.H., G.M, and J.D.T.; manuscript writing: C.H. and J.D.T.; manuscript editing: all authors. All authors have read and agreed to the published version of the manuscript.

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**Competing interests.** None.

**Ethics standards.** We are extremely grateful to all the families who took part in this study, the midwives for their help in recruiting them, and the whole ALSPAC team, which includes interviewers, computer and laboratory technicians, clerical workers, research scientists, volunteers, managers, receptionists and nurses. Please note that the study website contains details of all the data that is available through a fully searchable data dictionary and variable search tool: http://www.bristol.ac.uk/alspac/researchers/our-data/. The UK Medical Research Council and Wellcome (Grant ref: 217065/Z/19/Z) and the University of Bristol provide core support for ALSPAC. This publication is the work of the authors and Holuka, Menta, Caro, Vögele, D'Ambrosio and Turner will serve as guarantors for the contents of this paper. A comprehensive list of grants funding is available on the ALSPAC website (http://www.bristol.ac.uk/alspac/external/documents/grant-acknowledgements.pdf). Ethical approval for the study was obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics Committees. Consent for biological samples has been collected in accordance with the Human Tissue Act (2004). Informed consent for the use of data collected via questionnaires and clinics was obtained from participants following the recommendations of the ALSPAC Ethics and Law Committee at the time.

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# *Chapter 5*

# *The maternal epigenome as a window into the in-utero environment that the foetus experiences*

**My contribution to this Chapter:**

Conceptualisation, Literature review, Making of all figures, and Writing of the article.

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Early life adversity regroups many types of life experience although they may never affect individuals in exactly the same way (Merrick et al., 2019). Over the past decades we and others demonstrated how early life experience can lead to the development of chronic diseases and epigenetically changes altering the metabolism. Indeed, epigenetic modification such as DNAm can directly affect the expression of proteins and lead to the dysregulation of immune, cellular, molecular and metabolic systems (Moore et al., 2013). We can now affirm that DNAm are reversible and can appear all over the genome and not necessarily on clearly targeted position. It is clear that those reversible modifications can also disappear over time, however is it still very unclear whether those marks can directly be inherited from the parents.

We always have considered that these epigenetic adaptations appear during the first one thousand days life, representing the most critical period of life, as described in the Barker theory. Through the ALAC project, we demonstrated that some children were already epigenetically imprinted at birth (i.e. day 1 of life). This first element raised the question whether or not a maternal transmission can be possible. Indeed, we demonstrated that a traumatic maternal experience can be differently perceived by parents and the exposure consequences could also be in a first-hand imprinted on the maternal genome. The hypothesis we raised in Chapter 4 questioned the capability of the mother to vertically transmit epigenetic marks to their child.

The *in utero* environment represents the starting point of life were epigenetic, genetic, metabolic, etc. set ups take place prior to birth. Indeed, over time the human body will evolve and adapt to his environment to be able to survive to the environmental pressures it faces. Nevertheless, the initial set up of those systems takes place upstream in the development process. As we described in this Chapter, the embryogenesis depicted as an incredibly complicated process, and leading to the development of a unique organism, induces a de-methylation and re-methylation step. We considered that this particular step might fail leading to the conservation of parental methylation that will vertically be transmitted to the next generation.

The Chapter 4 covers a sum up of the key elements of the developmental process necessary to precisely understand how methylation marks can be conserved through multiple generations. Indeed, maternal exposure to a stressors (i.e. financial) and perceived and as a traumatic event can lead to a maternal imprint that will be pass from one generation to another. However, here, we highlighted the importance of considering some parameters associated to the stressor such as the timing (i.e. when the event occurred) or the efficiency of the placental barrier. As the stressor itself depends on those parameters, it is now necessary to consider their consequences and include them in a bigger picture as they can accentuate the imprinting process.

It is still very unclear how this early maternal exposition affects the offspring, however it is evident that to fully understand how epigenetic marks imprinting work, we need to take into consideration elements involved in the process. Here and before going further in our analysis, we took a step back to sum up what key elements need to be investigated. The Chapter 5 will provide a more precise picture of the maternal genome implication in the process of vertical transmission to their child in the *in utero* environment.

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# The maternal epigenome as a window into the *in utero* environment that the foetus experiences

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ABSTRACT

Maternal stress pre-pregnancy and exposure to stress *in utero* has life-long negative consequences for the developing foetus. There is growing evidence that this passes through changes in foetal epigenetic markers such as DNA methylation. We hypothesize that the mother's prior life experience and changes in her external environment will change the *in utero* environment she provides to the developing foetus, and both will be reflected in changes to the mother's epigenome. As classical dogma states that during embryo all DNA methylation marks are removed and replaced *de novo*, this raises the question as to how to assess the *in utero* environment, examining the role it plays in the transmission of environmental cues. We suggest that the maternal epigenome can act as a proxy for the developmental environment she provided to her offspring *in utero*; this developmental environment determines the child's epigenome and lifelong health trajectory. Furthermore, we suggest that the maternal origin of the placental decidua make this the perfect sample for assessing the *in utero* environment in the context of the mothers' prior life experience, mediating maternal exposure to infant phenotype.



## Introduction

The Developmental Origins of Health and Disease (DOHaD) also described as the "Barker theory" was established in the 1980 s byDavid Barker. In his seminal work, he linked birthweight to cardiovascular many decades later. This led to the hypothesis that an "inadequate or adverse" gestational environment programmed the significantly increased risk of cardiovascular disease [1–2]. The original epidemiological link to hypertension has been extended to an increased risk of developing multiple psychopathologies [3], diabetes; metabolic diseases [4], immune-mediated diseases [5] and cardiovascular diseases [6]..

During this early-life period, covering the first 1000 days from conception to age 2 years, many biological systems are put in place and set-points adapted to the developmental environment [7]. While early life adversity (ELA) is, in general, a clearly delineated and quantifiable negative entity in the post-natal period, it is not so easy to measure the *in utero* environment that the foetus experienced during development. A further complicating factor is the extent to which the environment experienced by the mother before and during pregnancy is transferred to the foetus. However, data from the 1944 German-imposed food embargo in the Netherlands, the Dutch Hunger Winter [8] and other natural disasters [9–10], including the most current findings from the 1998 Quebec Ice Storm [11–14], have shown the severe impact of external factors during the *in utero* period on the children's long-term cognitive and physical development. Although natural disasters present a very clear and often well-defined form of ELA, by far the most common stressors occur post-natally, and include dysfunctional households as well as emotional, physical and sexual abuse [15–16]. The Adverse Childhood Experiences (ACE) study [17] integrated health, social and medical data linking high post-natal adversity–scores and long-term negative health outcomes [16], mental disease [18] and social misfortune [19].

During embryogenesis a large proportion of the DNA methylation marks are removed, and then re-established *de novo*. When this was studied at the single cell-single nucleotide resolution with a "dynamic balance between strong global demethylation and drastic focused remethylation" [20]. Throughout the subsequent developmental period there is continued epigenetic remodelling as cells differentiate and tissues develop and mature, resulting in significant phenotypic plasticity,

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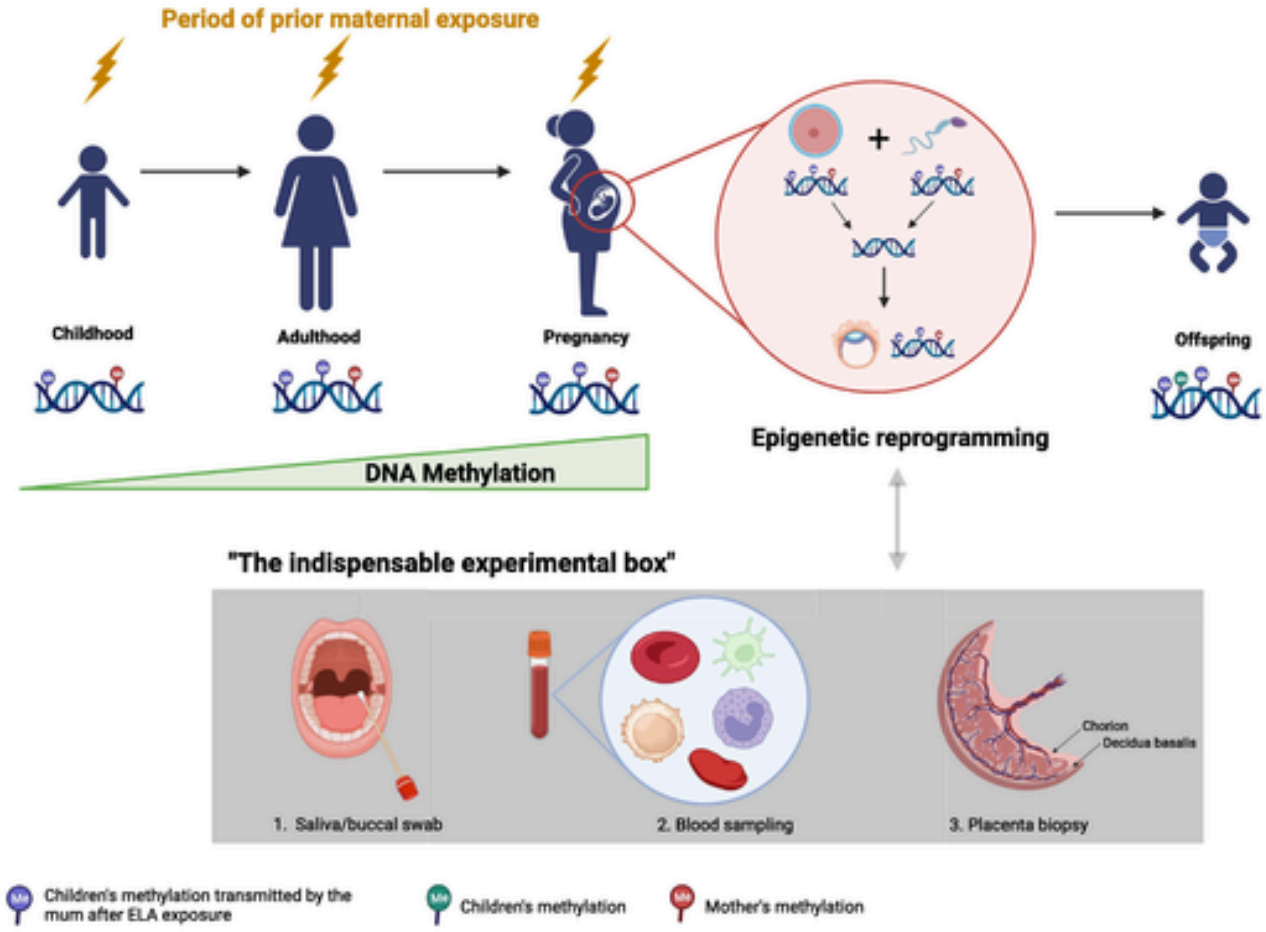


**Fig. 1.** Graphical representation of the "proxy maternal methylome" hypothesis of transgenerational phenotype transmission. We hypothesize that changes in the mother's external environment will change the *in utero* environment she provides to the developing foetus. Furthermore, both the mothers' history of exposure and the *in utero* environment will be reflected in changes to the mother's epigenome. We suggest that the maternal epigenome can act as a proxy for the developmental environment she previously provided to her offspring *in utero*; this developmental environment determines the child's epigenome and lifelong health trajectory. Furthermore, we suggest that the maternal origin of the decidua make this the perfect sample for assessing the *in utero* environment in the context of the mothers' prior life experience, mediating maternal exposure to infant phenotype, although both swabs from the oral cavity and peripheral blood may also be considered.

or inter-individual differences at birth. Differences in the external environment during this period are thought to alter these epigenetic programs, inducing life-long changes in gene expression of fully differentiated cells [21–22], retaining a "memory" of the developmental conditions [23].This demethylation-remethylation-adaptation process raises the question of whether (or not) epigenetic marks can be passed from one generation to the next, and if they are, how are they re-established in a potentially identical manner during the *in utero* development of their offspring? This complete demethylation and re-methylation suggests that direct inheritance of DNA methylation marks should not be possible, although mechanisms are in place for e.g. X-chromosome inactivation. Any hypothesis has to fully address the role the *in utero* environment plays in the DOHaD theory during embryonic and foetal development; we have to take into consideration several elements:

*The exposome/stressor*

External environmental elements experienced by the mother will cross the placenta to the developing foetus. The nature of those stressors need to be fully investigated as some will be able to directly affect gene expression by inducing epimutations that can impact proteins reading function for example [24]. Furthermore, all types of stressors (i.e. environmental, financial or emotional/physical) are consider "equal", however, there is little data on whether the consequences are equal [9]. This is further complicated by how the mother perceives psychosocial stressors as well as her potential tolerance or resilience to stressors.

*The timing and duration of exposure*

As epigenetic marks are put in place at specific points during development, the timing of the exposure will be particularly important, with potentially different effects at different times [23]. For example, maternal stress in the earlier stages of pregnancy has a stronger effect than later on [14], and maternal depression in the second trimester has a much stronger effect than during the third trimester [25]. Similarly, short-term exposure to air pollutant can, like long-term exposure, induce epigenetic modification, although the consequences may disappear more rapidly [26]. Current cohorts lack data on stress episode duration, rather concentrating on absolute numbers of episodes [9]. In addition there is now evidence that stress prior to pregnancy leaves a lifelong imprint in later children [27].

*The biological or behavioural effect on the mother*

If maternal exposure is to affect the developing foetus it has to have a measurable effect on the mother. This effect has to then affect the *in utero* environment. Since epigenetic processes are tissue-specific, tissues

will be unequally affected in any given exposure scenario. For there to be a *trans*-generational effect, any hypothesis has to consider this tissue-specificity and the possibility of the stressor generating either soluble signalling markers (proteins, hormones, metabolites….) or other physiological changes (blood pressure, heart rate, fever, hypoxia, hypercapnia…) that the developing foetus will be exposed to [9]. The placenta is one of the organs that is sensitive to its environment, with structural and vascular changes in, for example, gestational diabetes [28].

*The ability of the placental barrier to "filter" the stressor*

In the DOHaD theory, the placenta may be the most essential organ [29]. It is responsible for many processes that will be taken on by individual organs such as the liver, kidneys, lungs, endocrine glands and gut, supplying the foetus with nutrient and oxygen while passing waste to the maternal circulation. It ensures the development of neuroendocrine and steroid hormone systems, acting as a barrier to both maternal stress and stress hormones as well as environmental pollutants. For this, syncytiotrophoblasts are equipped with an array of transporter proteins and detoxifying enzymes in a manner similar to adult kidney cells [29]. Amongst these is 11-β-hydroxysteroid dehydrogenase 2 (11-βHSD2) which inactivates maternal cortisol, limiting foetal exposure. At the same time, P-glycoprotein and multi-drug resistance proteins are present both in the syncytiotrophoblasts and in endothelial cells in the villous capillaries, reducing the transfer of organic compounds, and potentially noxious xenobiotics into the foetal circulation [30]. Furthermore, the psychological and social elements of the exposome will influence maternal behaviour and health-behaviours. Many seemingly innocuous behaviours will affect the *in utero* environment with e.g. vitamins from OTC vitamin pills crossing the placenta, as will molecules from noxious behaviours (e.g. smoking) [23].

*Immune alterations during pregnancy*

While the maternal immune system naturally changes during pregnancy, dysregulation has been associated with long-term health consequences. Increased maternal inflammatory markers (e.g. CRP, TNFα, IL-4, −5, −6, and −8) have been associated with neurodevelopmental and cognitive delays [31–33]. During this period the maternal immune system finds a new homeostasis, balancing foetal tolerance through anti-inflammatory actions within the placenta against maintaining pro-inflammatory responses at mucosal surfaces to protect the mother and child [34–35]. Consequently, any maternal epigenome taken from samples rich in immune cells (e.g. blood, or placenta) may not truly represent all her prior experience due to changes in its composition, although judicious choice of a purified immune cell type, if we knew which one was most representative, may partially overcome this.

*The biological relevance of small methylation changes and the epigenome-phenotype association*

Small changes in methylation have been reported in psychobiological paradigms [36]. Indeed, demonstrating a clear mechanistic or functional link between individual epigenetic marks, whether they are from DNA methylation or histone modifications, and a later-life phenotype remains an unconquered challenge. It has been suggested that cell type or tissue specific epigenetic mechanisms underlie this environmental adaptation [37]. The affinity between exposure and tissues might play a major in the long-term "conservation" of those epigenetic marks. Indeed, the maternal environment influences both the epigenome and the development of the placenta [38].

*Gene-Environment interactions*

Epigenetic changes do not occur in isolation, and there will be a link between maternal genome, genetic (inheritable) traits the maternal epigenome and their child's phenotype. Indeed in many tissues, including the placenta, methylation is linked to the underlying DNA sequence [39].

This raises the fundamental question of what parts of the maternal environment are transmitted to the next generation, and how this can occur.

## The hypothesis

We hypothesize that changes in the mother's external environment will change the *in utero* environment she provides to the developing foetus. Furthermore, both the mothers' history of exposure and the *in utero* environment will be reflected in changes to the mother's epigenome. We suggest that the maternal epigenome can act as a proxy for the developmental environment she previously provided to her offspring *in utero*; this developmental environment determines the child's epigenome and lifelong health trajectory. Furthermore, we suggest that the maternal origin of the decidua make this the perfect sample for assessing the *in utero* environment in the context of the mothers' prior life experience, mediating maternal exposure to infant phenotype (Fig.1).

Our hypothesis makes several basic assumptions including:

1. The maternal epigenome encodes both her cumulative life experience and her current physiological state, often adapted to this prior life experience.
2. One of the tissue-specific maternal epigenomes will be a suitable proxy for the *in utero* environment that she provides the developing foetus.
3. Any external factors that induce biological or biochemical differences in the mother, either from prior exposure or her current physiological state, will be detectable as epigenetic modifications in accessible maternal tissues (buccal cells, blood, placenta).
4. Any external factors capable of inducing biological or biochemical differences in the mother will also have some impact, albeit potentially reduced by the placental barrier, on the developing foetus.
5. That the maternal and paternal gametes are fully demethylated (as classical dogma says) and that the re-establishment of the offspring methylation during gestation is dependent on the *in utero* environment that the mother provides.

*Implications of the hypothesis*

The most direct consequence of our hypothesis is that it provides a clear explanation of how the epigenome and phenotype of the developing foetus are shaped during development in the absence of a direct epigenetic inheritance due to the complete demethylation of the parental genomes during embryogenesis. The most recent natural experiments investigating the long-term effects of the *in utero* environment (Dutch Hunger Winter, Quebec Ice Storm) clearly demonstrated that unlike other life periods, the gestational period is crucial. Here, both the mothers and their neonates are affected, potentially setting them both on a lifelong negative health trajectory; furthermore, gamete-producing germ cells present in the foetus will also be exposed, affecting subsequent generations. Importantly, a single highly-stressful event during this period that lasting for a matter of days or weeks is enough to set an individual on a lifelong negative health and developmental trajectory [40]. Furthermore, ours hypothesis changes where DNA methylation is placed within the statistical analyses of any future cohort. The logical extension of our hypothesis is that the maternal DNA methylation levels (proxying the *in utero* environment) will be a mediating variable, link-

ing maternal exposure to the epigenetic or phenotypic changes in their offspring. Indeed, in such paradigms, a limited number of mediation models have so far been reported, most notably from the Canadian "Project Ice Storm" [13,41–42], while most studies appear to limit their use of DNA methylation to being a direct consequence of adversity that can be measured in the offspring, potentially predicting long-term health trajectories [14]. Additionally, if the correct data are collected, we should be able to identify both the vertically transmitted marks and those that are representative of the *in-utero* environment provided. Direct correlations of methylation levels at the same epiallele within the parental/child epigenomes would represent vertically inherited marks. Those that represent the *in-utero* environment provided would not have a direct correlation, rather being a statistical mediator between methylation levels at different epialleles.

*Limitations and testing the hypothesis*

For our hypothesis, the principal limitation is our lack of knowledge as to which external environmental elements will have the largest or longest effect on the in-utero environment and the developing foetus. Furthermore, we have very little knowledge as to which accessible maternal epigenome may proxy the *in utero* environment, nor which elements of the *in utero* environment may be reliably estimated from the maternal epigenome. In pre-clinical studies it is relatively easy to assess the epigenome in a wide range of tissues, however, in observational (human) studies we are limited to accessible tissues [23]. The placenta should be widely available, however, is rarely collected. Furthermore, there is little data on the epigenetic changes in the available samples, and it is not really known how they reflect either prior maternal exposure or her state when samples were taken. This is compounded by a lack of studies that address how readily available human samples (blood, saliva) represent other tissues epigenetically, and whether they may be useful markers of dysfunctional distal tissues, disease or phenotypic differences.

This leads us to the second limitation, the statistical power and the study design necessary to test the hypothesis. Pregnancy will have epigenetic effects on the mother so any study would need to start pre- conception. Then, it would be necessary to obtain DNA methylation profiles from several tissues pre-pregnancy, at several time points during pregnancy, and post-partum to identify which maternal sample would provide the best proxy for the *in utero* environment. It could be possible if we know maternal DNAm profile before, during, and after birth to see evolution of epigenetic imprints. This can then be compared with their offspring to identify transmitted elements. Maternal methylation patterns will most certainly change during pregnancy, making the choice of sample media and collection timing primordial. Buccal and blood samples can be collected repeatedly, allowing access to pre-pregnancy, gestational, and post-partum epigenomes. Placenta samples, however, are limited to one point in time, although the presence of both a maternal and foetal compartment is a significant advantage. The maternal-derived decidua has the potential advantage that it is the closest maternal-derived tissue to the foetus, although how much of the previous adaptation of the maternal epigenome to her life-environment is accessible through placental samples remains to be investigated. Consequently, to fully establish what is actually happening requires the identification of a suitable natural experiment, the collection of suitable maternal samples, and a long-term follow-up of the children to determine their long-term phenotype. This is currently not available, and an adequately powered cohort, including gene-environment and epigenetic effects would be a mammoth undertaking. Furthermore, the decidua basalis that we think most likely represents the maternal *in utero* environment is conspicuously absent from the current cohorts.

The third limitation is that we do not address the role of the father. As paternal DNA is thought to be completely demethylated during early embryogenesis it is assumed that no epigenetic information will be transferred from the father to the next generation, however this may not be true. There are many studies addressing the role of the mother in transmitting epigenetic information, however, the father's contribution remains poorly described, although a few rodent studies examined transmission of anxiety and depression-related phenotypes [43]. It was clear that the timing of paternal exposure affected the transmission to their offspring. Paternal early-life stress reduced the baseline levels of anxiety and depression in their offspring over several generations, but this effect was lost when the fathers were exposed to stress during either adolescence or later life [44–45]. Rodent work has the advantage over human studies that the fathers are not involved in rearing the pups, excluding any form of behavioural transmission of the phenotype. Ex-vivo manipulation of paternal gametes demonstrated that DNA methylation was not involved in the transmission of paternal phenotypes, but microinjection of specific miRNA and lncRNAs induced stress-associated phenotypes in their offspring [43,45]. Consequently, this limitation may be sidelined during this development and testing phase, to be re-integrated once the basic hypothesis has been confirmed.

The largest challenge to testing our hypothesis is the lack of data on exactly which maternal sampling matrix would provide an epigenome that best reflects the *in utero* environment. Maternal epigenomes from saliva or blood are readily available, while few studies examined placental epigenomes. In the absence of such data it is impossible to say whether the placental maternal epigenome represents her accumulated "life experience" and allostatic load, or the current physiological status. However, the placenta is interesting as a candidate maternal epigenome because there is detailed evidence on the role of the placenta in eliminating noxious specific stimuli such as the inactivation of maternal cortisol by steroid dehydrogenases. The placenta has both foetal and maternal origins, forming the *chorion frondosum* and *decidua basalis* respectively, and disection and examination of the *decidua basalis* specifically has been reported in previous studies [46]. Furthermore, our knowledge of the epigenetic and transcriptional regulation of enzymes such as 11β-HSD within the placenta is also limited. Indeed, many stressors induce only small changes in maternal DNA methylation in peripheral blood, and their effect on placental methylation levels is unknown, although most probably of similar magnitude to that observed in the blood [38]. Indeed, growth-restricted pregnancies have lower placental 11-βHSD2 [47] with the consequential increases in foetal cortisol levels that are associated with increased risk of developing autism later in life [29,48]. One additional point that blurs the distinction between placenta and peripheral blood epigenomes, is the high percentage of immune cells in the placenta, particularly in the decidua basalis. One of the placenta's most fundamental roles is to protect the foetus from infections. Consequently, the healthy decidua contains a disproportionately high number of immune cells, with upto 40 % of all cells in the decidua are immune cells [49–50] These are mainly regulatory T ($T_{reg}$) and natural killer cells as well as macrophages [35,51], although B lymphocytes are notably absent [35].

Recently, placental epigenetic clocks have been develop to calculate an "epigenetic age" of children from DNA methylation. This suggests that there are epigenetic differences and changes within the placental tissue may reflect some aspects of the *in utero* environment or the natural development of the placenta. However, currently such clocks are not fully developed and their link to the subsequent development and eventual ageing of the individual at the phenotype level remain uninvestigated. These represent very small changes in methylation levels, and this has a detrimental effect on testing the hypothesis: small differences in means require large numbers of individuals to test the hypothesis with adequate statistical power. When this is combined with the heterogeneous nature of all the proposed sampling matrices and the potential that the inheritance is not a direct 1-for-1 maternal CpG to child CpG further reduces the statistical power, and increases the size and cost of any potential study.

The human exposome is highly complex, and in even the most unique natural experiment is confounded by the overall life experience, that will be different for each individual, making it hard to identify the effects of each specific stressor. Furthermore, the combination of different stressors will potentially have additive, contradictory, multiplicative, or many other potential interaction effects further complicating the dissection of the overall effects observed to individual exposures.

## Conclusions

There is now a large volume of evidence that pre-pregnancy, and pre-natal maternal stress leads to an exposure-associated phenotype in her offspring. Building on the work of David Barker and many others that has evolved into the current DOHaD theory,Here we propose that the *in utero* environment a mother provides to her developing foetus will reflect not only her prior life history, but also her current state throughout pregnancy. Furthermore, we propose that the mothers' life history and current state will be encoded in her epigenome and that this will also reflect the *in utero* environment she provides to her foetus, mediating the development of her offspring's phenotype. There are numerous challenge to test this hypothesis, notably identifying the maternal epigenome that best reflects her prior life experience and current state throughout gestation, although we propose that the decidua basalis would potentially meet this requirement. Expanded data collection including perceived and actual stressors, in addition to the correct biological sampling (tissues and cells) are now necessary for further investigations [52]. Unfortunately, our previous suggestion of "appropriate samples" missed the importance of the placenta. Consequently, the challenge remains to find a quasi-randomly assigned natural experiment similar to the Quebec Ice Storm, or the Dutch Hunger Winter, in which adequate placenta samples can be collected with the appropriate ethical approvals, as well as detailed pre-stress retrospective data collection, and a suitable infant development follow-up.

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## Consent statement/Ethical approval.

Not required

## Authors' contributions

CH, MM, NG and JDT all participated in conceiving the hypothesis, literature search, manuscript writing and reviewing the final draft. All the authors approved the final version of the manuscript.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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# Chapter 6

# Maternal DNA Methylation Mediates Intergenerational Transmission of the Impact of Early Life Adversity

**My contribution to this Chapter:**

Conceptualisation, Literature review, Data generation, Data integration, Data visualisation, Final statistical analysis, Interpretation of results, Making of all figures, and Writing of the article.

**This Chapter is ready to be submitted as:**

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Chapter 5 investigates the implications in maternal epigenome changes. Here, we showed that not only maternal lifestyle can influence offspring epigenetic profiles, but refers to an addition to pre-existing maternal epigenetic marks. We then suggested that maternal experience will let an imprint on mother's genome. We hypothesised that this specific maternal profile including differentially methylated CpGs can somehow induce similar imprinting in the genome of their offspring.

In this Chapter, we considered a new form of adversity, childhood sexual experience, with or without consent consequence, in the context of heath determinants later in life as consequences of such trauma can be observed on the next generation. So far, transgenerational mechanisms are still unclear, however here we started to address potential hypothesis to describe this key concept. Studies reported that mother who experienced trauma could be able to "break the intergenerational transmission", however maternal stress exposure remains a considerable risk factor for child maltreatment taking place during the first 2 years of life and thus until the preschool period (Briere et al., 2008). Indeed, one of the most plausible explanation of child development is the maternal epigenetic material transmission (Alhassen et al., 2021). Literature mentioned many times that intergenerational transmission could be responsible for one particular child developmental trajectory. Intergenerational trauma can increase lifetime susceptibility to depression and mental health diseases. As described in the literature, early maternal ELA impact genes associated to stress but can as well modify some locus in epigenetic marks responsible for health's outcome alteration (Mulligan et al., 2012; Ramo-Fernández et al., 2019; Ramo-Fernández et al., 2021).

The overall hypothesis around the implication of the maternal epigenome on child's imprinted epigenome have been confirmed in the present Chapter. Our analysis showed that maternal associated CpGs with CSE encountered during adolescence act as "directing" CpGs inducing specific imprinted epigenetic marks on their offspring's genomes.

Those results suggest that ELA can let a specific signature on the genome that will be conserved for years before being transmitted to the next generation. Here, we also reinforced the hypothesis regarding the fail of maternal epigenetic marks during embryonic reprogramming as already mentioned in the literature.

The question of transgenerational inheritance remains poorly described and needs further investigations, especially regarding paternal implications as so far we exclusively focused on the role of maternal implication. Additionally, maternal behaviour such as maternal bonding as well as breastfeeding represent two major steps in mother life and can also trigger children's genome

shaping. Based on our findings we reached the “intellectual breakthrough” that epi- inheritance mechanism is not 1:1 on the same CpG position.

# Maternal DNA Methylation Mediates Intergenerational Transmission of the Impact of Early Life Adversity

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*Keywords*: early life adversity, epigenetic programming, intergenerational transmission, childhood sexual experience, DNA methylation; EWAS

**Abstract**

Early-life adversity (ELA) such as abuse or neglect is associated with poor mental/physical health later in life, and can define health trajectories from early life to adulthood. There is epidemiological evidence that this effect persists into subsequent generations. In order to demonstrate and elucidate mechanisms for epigenetic inheritance, we examined whether maternal prenatal DNA methylation levels mediate methylation in their child's epigenome. Here, we used the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort for which there is a wide range of longitudinal data available. We used the mother's prior history of sexual experience (CSE) as a form of ELA. In a two-step analysis we initially demonstrated that maternal CSE decades before pregnancy induced epigenetic programming on their child's genomes, with differential methylation maintained at 11 CpGs 7 years later. Subsequently we used a high-dimensional mediation model to identify regions of the maternal epigenome in the perinatal period that drive the changes in their child's DNA methylation. At age 0 and age 7, our mediation models identified 4121 and 16417 maternal CpGs that strongly influenced their child's methylation. 412 maternal prenatal CpGs mediated the 11 differentially methylated CpGs common between age 0 and 7y. There was an interesting population of maternal CpGs that mediate a high number of their child's differentially methylated CpGs. We term these "directing methylation sites" because of their role directing their child's methylation levels. How these directing methylation sites pass information on to the next generation remains to be examined. We interpret this as the mother's epigenome representing both her accumulated exposome and life experience and may, at the same time, be a proxy for the developmental environment she provides to her offspring *in utero*, that will then determine the epigenomic profile of her child.

**Introduction**

Early life adversity (ELA) is a well-established risk factor for lifelong developmental, behavioural and health problems [1, 2]. The detrimental impact of ELA is exerted through changes in multiple biological pathways (BP), including stress and immune systems among others [3-5]. Research in humans and non-human animals suggest that this biological embedding of ELA is mediated by epigenetic mechanisms, particularly DNA methylation (DNAm) [6-10]. A growing number of studies show associations between ELA and gene-specific and genome-wide DNAm leading to adverse outcomes across the lifespan, as well as in the next generation [11-17].

In humans, potential intergenerational transmission of ELA has mainly been investigated in the context of the prenatal period, examining the associations between maternal prenatal exposure and infant DNAm [18-20]. A majority of these studies prenatal exposure, such as maternal mood or stress, was associated with infant methylation of either stress-related candidate genes (e.g. *NR3C1*) [21-23] or genome-wide [24-28]. While these studies are important in establishing the associations between exposure and epigenome across generations, further research is necessary to provide a mechanistic insight. In addition, since the timing of measured infant outcomes varies drastically between studies, from perinatal period to adulthood, it is somewhat challenging to understand how stable these effects are over time, together with the contribution of postnatal factors.

In an attempt to explain a potential mechanism, recent studies examined whether gene-specific or genome-wide methylation may serve as a mediator between exposure and outcome (reviewed in [18, 29]). These studies considered maternal prenatal factors, such as exposure to stress, obesity and toxins, as an early stressor for the foetus, leading to changes in infant birth, development and health outcomes through altered infant DNAm. While these findings are important in showing how DNAm mediates the impact of prenatal environment on the offspring, they have not yet demonstrated intergenerational transmission of epigenetic information. Furthermore, in case of maternal exposure during the pregnancy, it becomes challenging to dissociate the impact of the exposure on the maternal vs. foetal epigenome in addition to the effects of the pregnancy itself [30].

In this respect, examining the impact of maternal ELA on the offspring may provide unique insights on epigenetic intergenerational transmission. In recent years related evidence started to accumulate on this topic, with maternal ELA associated with infant birth outcomes, neurodevelopment and mental health, possibly through changes in maternal prenatal physiology [31-35]. These findings establish the intergenerational effect of maternal ELA on the offspring starting from birth, paving the way for examining underlying epigenetic mechanisms. To our knowledge, there has been no human studies so far investigating an epigenetic intergenerational

transmission of the impact of ELA. Two recent studies from the same cohort [36, 37] examined the effect of maternal ELA on maternal and infant DNAm after birth at stress-related candidate genes. In the first study, maternal ELA was associated with maternal postnatal DNAm of 3 candidate genes (*FKBP5, NR3C1, CRHR1*) in blood, but not with newborn DNAm in cord blood. Moreover, there was no correlation between maternal and newborn DNAm at the investigated CpG sites. In the second study, maternal ELA was associated with differential maternal DNAm, but not newborn DNAm, at specific CpG sites of the oxytocin receptor gene (*OXTR*). Furthermore, mother-newborn DNAm was not correlated for the maternal ELA group, but was for the non-ELA group for some CpGs. These results may in part suggest an epigenetic intergenerational transmission, however remain limited in explaining an epigenetic mediation due to focusing on a number of candidate genes and postnatal maternal DNAm. On the other hand, lack of correlations between maternal and newborn DNAm raises the question of whether there is a *direct epigenetic inheritance* from the mother to the infant such that the DNAm pattern at specific CpG sites would be directly inherited, or an *indirect epigenetic transmission* that mother's methylation by ELA would be associated with infant DNAm at different CpG sites.

In order to demonstrate and elucidate mechanisms for epigenetic inheritance, the aim of this study was to examine whether maternal prenatal DNAm mediates the impact of maternal ELA on child DNAm. Here, we used the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort for which there is a wide range of longitudinal data available, including: a) maternal ELA with subtypes of adversity, b) maternal genome-wide DNAm during pregnancy reflective of maternal early life programming during foetal development, c) longitudinal infant genome-wide DNAm (at birth and age 7) allowing to test for persistent intergenerational effects, and d) large sample size to test mediation effects. As recent literature reports differential associations of BPs by the type of adversity experienced [38, 39] and this was confirmed by epigenome-wide association studies (EWAS) [17, 40], we focused on one specific type of ELA rather than using an overall ELA measure. Considering the rich and consistent prior literature emphasizing the strength, uniqueness, and long-term impact of childhood sexual experience (CSE) on neurodevelopment, behaviour, and epigenetics (including findings from the ALSPAC cohort [12, 41, 42]), we chose CSE as the maternal early life stressor and investigated how this is associated with offspring methylation decades later.

## Methods and Materials

### Participants and measures

ALSPAC was established in the 1990s to examine how genetic and environmental factors influence health and development across the lifespan starting with the prenatal period (http://www.bristol.ac.uk/alspac/). ALSPAC recruited 14,541 pregnant women in the Southwest of England recruited between April 1991 and December 1992. Mothers and their children were followed up over 30 years. ALSPAC participants provided a wide range of data throughout the study, demographics, medical history, ELA, as well as tissue samples [43]. In this study we used a subset of data from the ALSPAC cohort [44, 45].We restricted our analysis to the 1022 mother-child dyads in the Accessible Resource for Integrated Epigenomics Studies (ARIES) sub-section of the ALSPAC cohort that focuses on mother and child genome-wide DNAm analyses [46]. The ARIES sub-section of the cohort was approved by the ALSPAC Ethics and Law Committee and the Local Research Ethics Committees (http://www.bristol.ac.uk/alspac/researchers/research-ethics/). In this study, we consider mother-child dyads from ARIES that have the following data available: a) maternal CSE and b) maternal prenatal genome-wide DNAm, and c) child genome-wide DNAm at birth and age 7y. Once all data were extracted, there were 718 participants with complete datasets at birth and 776 with complete datasets at age 7y.

At the 32$^{nd}$ of week of pregnancy, mothers completed retrospective self-reports related to the occurrence of childhood traumatic experiences before they were 16 years old, including CSE. CSE was measured by 5 items that were coded as 0: *No, did not happen*, 1: *Yes, happened once only,* 2: *Yes, happened more than once*. As a result, a total maternal CSE score ranging from 0 to 10 was calculated. CSE items and their frequencies in the ALSPAC cohort were shown in Table 1. Our observation of up to 36% of individuals in the ARIES subset experiencing each of the individual forms of trauma agrees with the overall rate in ALSPAC of 55% (5864/10726 participants) of mothers experienced at least one CSE items in their life, representative of the overall ALSPAC cohort.

| Variable name | Item | 1: Yes, happened once only (%) | 2: Yes, happened more than once (%) |
|---|---|---|---|
| c830 | Did anyone ever purposefully expose/flash themselves to you before you were 16? | 16.6 | 9.7 |
| c850 | Did anyone masturbate in front of you before you were 16? | 3.8 | 4.7 |
| c870 | Did anyone ever touch or fondle your body, including your breast or genitals, or attempt to arouse you sexually before you were 16? | 7.5 | 35.9 |
| c910 | Did anyone rub their genitals against your body in a sexual way before you were 16? | 4.9 | 26.9 |
| c930 | Did anyone have sexual intercourse with you before you were 16? | 4 | 15.9 |

Genome-wide DNAm was measured from DNA extracted from whole cord and peripheral blood samples at birth and age 7y respectively. The Zymo EZ DNA methylation™ kit (Irvine, CA) was utilized for bisulfite conversion [45, 47] and genome-wide DNAm was assessed with the Illumina Infinium 450K platform (Illumina HT-12 V3 BeadChip, Illumina Inc, CA) as previously described [44]. Genome studio (version 2011.1) and R-platform were used to analyse data after scanning on the Illumina iScan [48]. We used the ARIES data as provided by ALSPAC after their considerable pre-processing and normalisation in a manner similar to our recent work [49, 50]. Briefly, batch-effects were controlled in the ARIES data by using a semi-randomization of each samples [40, 46], furthermore, quality control was assessed as described by Min et al. [51], and data were normalized using the Meffil package in R to remove unwanted technical variation. Finally, the Houseman method was used to control batch effect and cell counts during the normalization process [46].

**Data analysis**

***Transformation and dichotomization***

We used a base-10 exponential function in R to transform total maternal CSE for the data to meet statistical assumptions [52]. After the transformation, we tested the correction of *p*-value inflation in our data with the R/Bioconductor BACON package (version 1.22.0). As in previous studies [53], this did not remove inflation from our data. Consequently, all further analyses were performed on uncorrected data.

Where necessary, data were dichotomized to 0, 1 and 2 using the sjmisc package (version 2.8.7) in R. Cut-off strategies are explained in the results section. CSE was originally coded as a series of dummy variables taking the value “1” if mothers experienced at least one time any of the traumata outlined above. However, the value “2” is if they experienced more than once a trauma.

We then summed answers of the five questions giving us one unique CSE value with a ranking comprised between 0 and 10.

***Statistical analysis***

All data analysis was performed in R (version 4.1.1 R Core Team, 2020) on R studio (version 1.3.959 R Core Team, 2019). For data cleaning and dichotomization, we used "dplyr" packages (version 1.0.7) [54] and "sjmisc" (version 2.8.7) [55].

To investigate association between maternal CSE exposure and DNAm we calculated Odds Ratio (OR), the chance of an outcome to occur due to exposure in comparison to unexposed individuals. OR were calculated and extracted with the epiDisplay package (version 3.5.0) to calculate general logistic regression models [56]. As none of the tested covariates (child age and maternal smoking) (Supplementary Figure 1) were significant, the final model was not corrected.

***Case-control EWAS***

In the analysis we removed siblings and ARIES beta values were used with a detection *p-value* < 0.01. In addition, technical replicates and low-quality probes were removed as recommended by ALSPAC [46]. To extract significantly associated CpGs and related epigenetic information as genes positions from differential methylated regions (DMRs) we used the R (version 4.1.1) packages "minfi" (version 1.38.0) and "DMRcate" (version 2.6.0) [57]. "Dplyr" (version 1.0.7), "DMRcate" (version 2.6.0) and the Bioconductor "missMethyl" packages (version 1.26.1) were used to sort out probe statistics. Finally, "GOfuncR" (version 1.12.0), "pathfindR" (version 1.6.2) packages were used to extract plausible biological pathways associated with maternal CSE [58]. We considered the probes or DMR significant when the BH corrected value $p < 0.05$.

***High-dimensional Mediation Analysis (HIMA)***

To investigate high-dimensional mediation effects between mother's CpGs after exposure to CSE and child's epigenome, we used the "HIMA" package on R (version 2.0.0) [59]. Here, we considered the 450K CpGs of the mothers as potential mediators between maternal CSE (exposure) and epigenetic modification on child's epigenome later in life (outcome). Briefly, HIMA uses a three steps approach to identify significant CpGs through the genome. First, it identifies the CpGs with the largest effect sizes for the response variable based on a "sure independence screening" [60]. The second step is to estimate the mediation effect following a minimax concave penalty. The final step is the joint significance testing step [61]. In the end, HIMA returns a list of association and *p-value* between mother's CpGs and children's CpGs that were extracted for visualization. We made

three mediation models: the first model (Model 1), based on the 412 CpGs common to the birth and age 7y EWAS; Model 2, CpGs specific to the birth EWAS; and Model 3 specific to age 7y EWAS.

***Data visualization***

The R package "VennDiagram" (R version 4.0.2) and "ggplot2" (version 3.3.5) were used to respectively generate Venn Diagrams and Karyogram/Manhattan and Heatmap plots. Corplots were generated with "Psych" package (version 2.2.9 [62]). The "pathfindR" package (version 1.6.3) was used to generate biological pathways plots. We used SigmaPlot (version 14) and Adobe Illustrator CS6 (version 16.0.0) to produce graphical figures.

## Results

### Maternal CSE is associated with differential child DNAm in multiple BPs

We used a case-control EWAS approach to identify differentially methylated CpGs in children decades after their mothers' trauma. Initially, we adjusted our model for maternal age and smoking, which are known to influence DNAm [63-66]. In our model, we found 418 and 452 differentially methylated CpGs (*p-value* < 0.05) on children's DNAm that were from 80 and 85 DMRs at birth and at age 7, respectively (Figure 1 A-D). Among these CpGs, 11 were common at birth and age 7 (Figure 1E), suggesting potentially conserved sites after birth. Adjusted models for age and smoking did not differ from uncorrected models (Supplementary Figure 1 A-B).

We then investigated the BPs within the children's methylation profile associated with maternal CSE (Figure 2). Maternal CSE was associated with BPs of a fold enrichment between 1.5 and 4 (*p-values* < 0.05). The affected pathways contained 117 to 156 differentially methylated genes at birth (Figure 2A) and 49 to 245 genes at age 7 (Figure 2B). While multiple BPs were influenced, we selected "oxidative phosphorylation" (OP) and "Parkinson's disease" (PD) pathways for visualization and further analysis from the 10 most significant BPs due to higher fold enrichment and number of genes, as well as the association of these pathways with stress, nervous system and inflammation [67-69]. We extracted further information on the up and down regulated genes in these two pathways, and almost all of the genes were hypermethylated leading to an assumed generalized down regulation of both BPs (Figure 2 C).

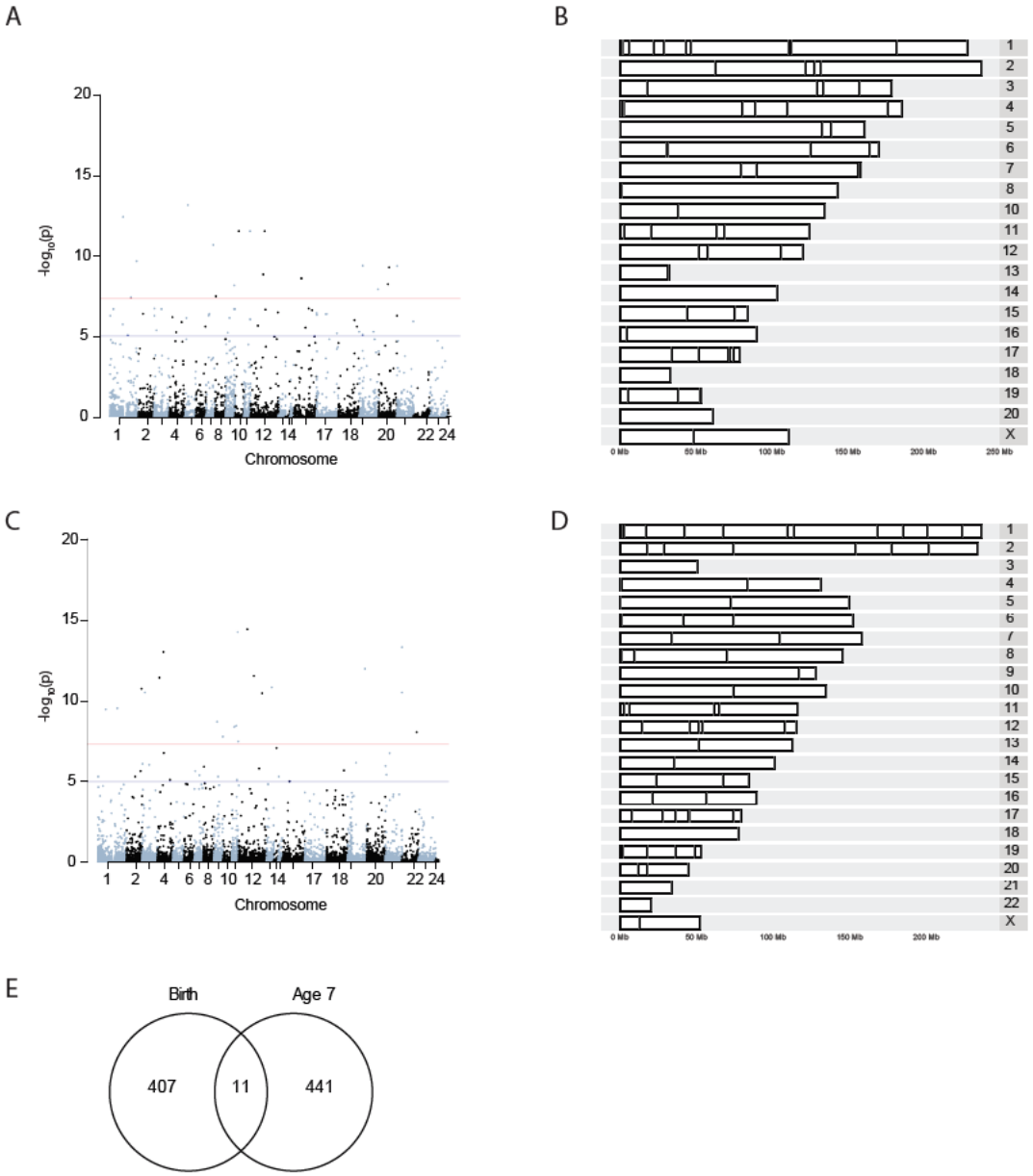


**Figure 1: Maternal CSE-associated child DNAm at birth and age 7.** Panels A-B) Plots represent results at birth. A) Manhattan plot represents the repartition of 418 CpGs on chromosome after exposure to maternal CSE at birth. B) Karyogram shows 80 DMRs after exposure to maternal CSE. Panels C-D) Plots represent age 7. C) Manhattan plot represents the repartition of 452 CpGs on chromosome after exposure to maternal CSE at age 7. D) Karyogram shows 85 DMRs after exposure to maternal CSE at age 7. E) Venn diagram represents the 11 CpGs common found between birth and age 7.

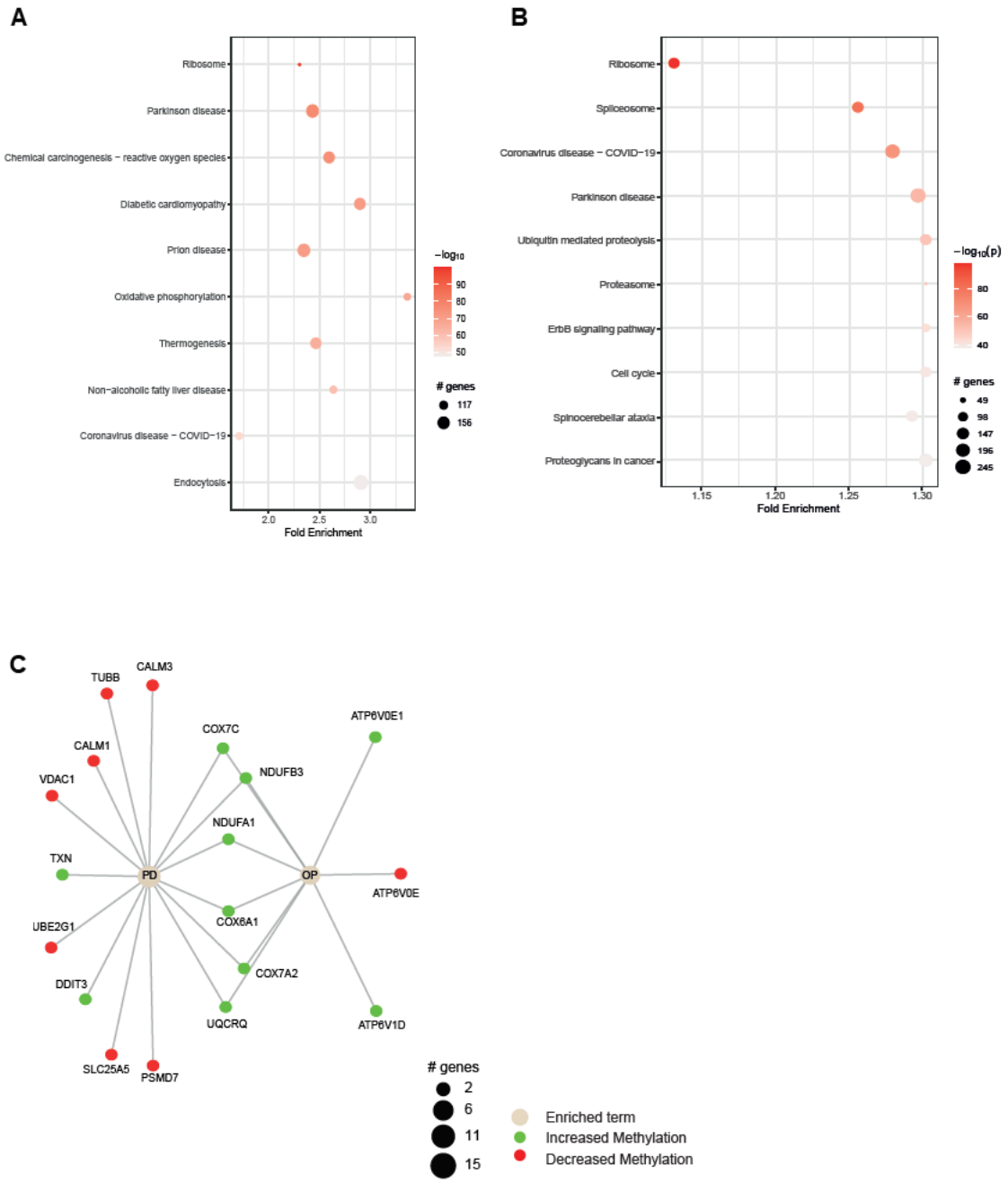


**Figure 2: Biological pathways associated to CpGs after maternal exposure to CSE.** A) Graph shows fold enrichment of the most 10 significant BPs associated to maternal CSE exposure at birth. B) Graph shows fold enrichment of the most 10 significant BPs associated to maternal CSE exposure at age 7. Colour code: -log(10) *p-value*. Black circle: number of genes. C) Graph shows genes involved on Parkinson Disease (PD) and Oxidative Phosphorylation (OP) pathways.

### Maternal prenatal DNAm mediates the impact of CSE on child DNAm

To test whether maternal prenatal DNAm mediates the impact of maternal CSE on child DNAm, we developed three mediation models (Figure 3). The three models covered the 11 common CpGs that were differentially methylated in the EWAS at birth and age 7. Two age-specific models

explained the 407 differentially methylated CpGs identified in the EWAS at birth (Model 2) and 441 specific CpGs at age 7 (Model 3). Figure 4 shows circos plot for these three mediation models. Model 1 returned 412 CpGs that mediated the differential methylation of the 11 CpGs common to the EWAS at birth and age 7. Mediation model 2 returned respectively 4121 mediating CpGs (mediation model *p-value* < 0.05) significantly associated with maternal CpGs at birth while mediation model 3 returned 16417 CpGs. We filtered the most significant maternal CpGs from each model on the mediation effect (a/b < 0.01 and Benjamini-Hochberg corrected *p-value* < 0.05).

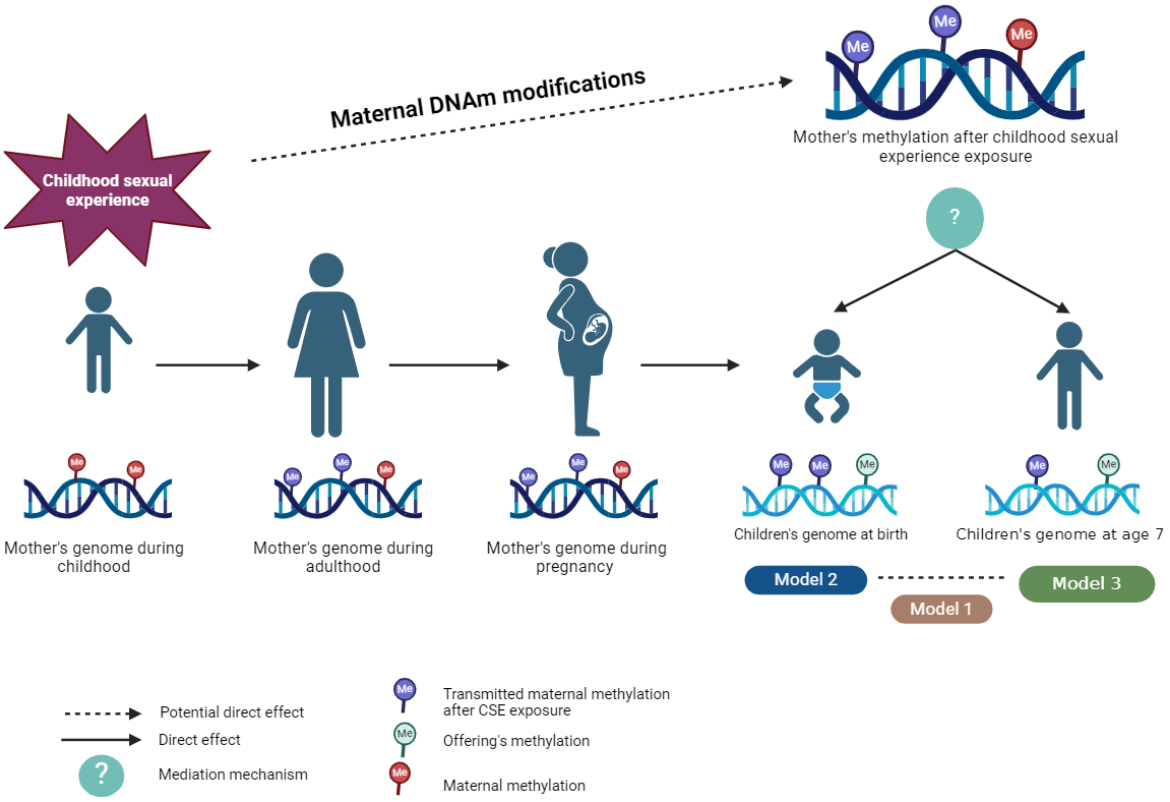


**Figure 3: Mediation model design**. The figure represents the 3 mediation models to investigate the potential direct effect of maternal CpGs on children's CpGs after exposure to maternal CSE. The first mediation model has been performed on children's CpGs found at birth. Model 1 (brown box) is based on CpGs common to the birth and age 7y EWAS; Model 2 (blue box), CpGs specific to the birth EWAS; and Model 3 (green box) specific to age 7y EWAS.

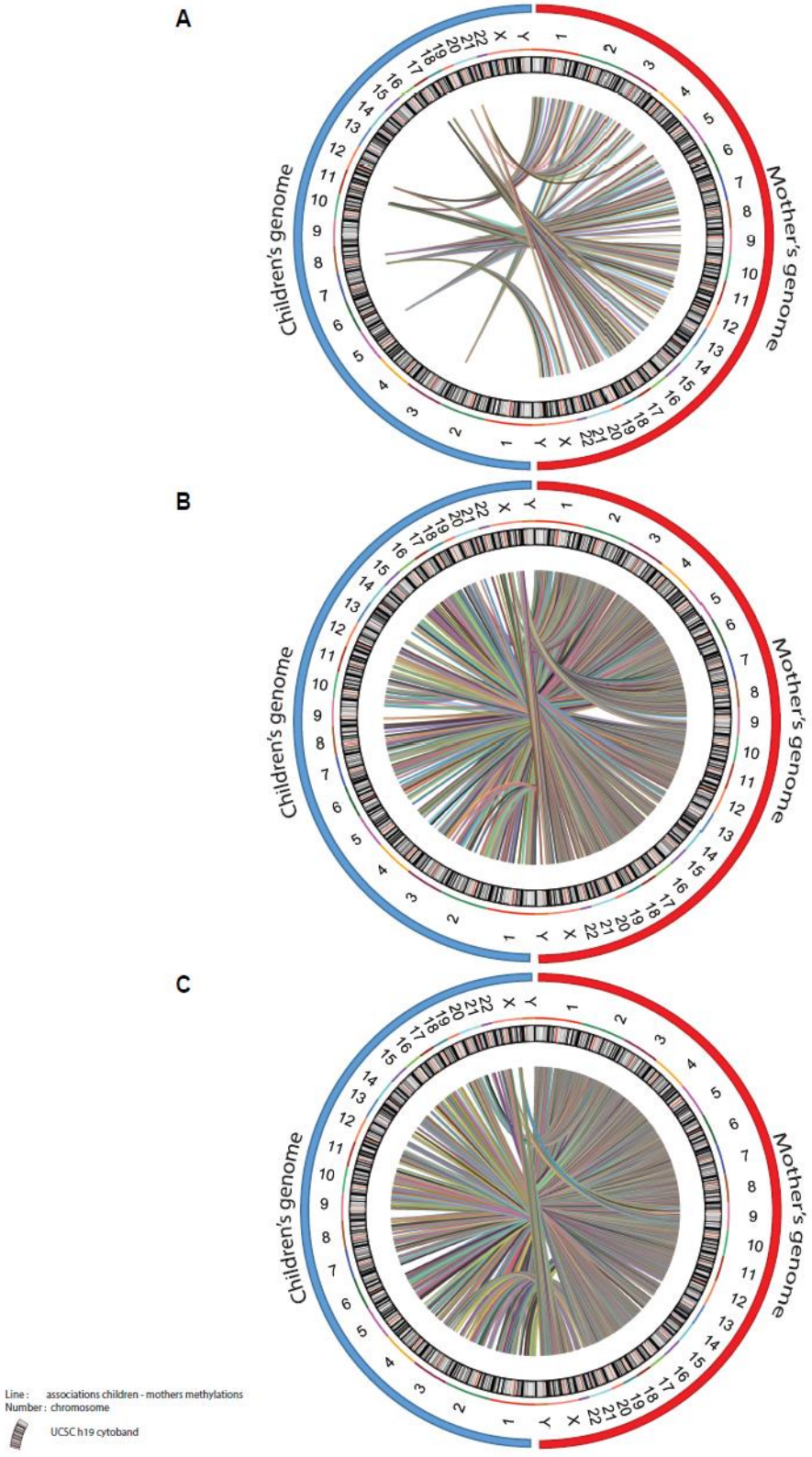


**Figure 4: Circos plot of the associations between maternal and child CpG methylation from the mediation models.** A) 412 CpGs of children found at birth and age 7 associated with maternal CpGs. B) 4121 children's CpGs at birth associated with maternal CpGs. C) 16417 children's CpGs found at age 7 associated with maternal CpGs. Blue and red circles respectively represent children's and mother's genomes. Number represents the chromosome. Colour lines represent link (associations) between children and maternal CpGs.

Additionally, we calculated the number of child CpGs that each maternal CpG mediated, termed frequency of Mediation. After filtering (Figure 5), we extracted the top 19 maternal mediating CpGs for Models 2 and 3. We suggest that these play a role directing methylation in the child. Within this restricted selection of maternal directing CpGs, we focused on those conserved at birth and age 7. To filter them and keep the most significant, we used the same approach previously described (BH-FDR adjusted *p-value* < 0.05). Between the 2 epigenetic time points, and after the last step of filtering, we found that cg08566437 and cg05806180 were associated with 3 maternal CpGs (cg00023351, cg03606954, cg18681375; Figure 6). General linear model (GLM) did not show a significant correlation between maternal CpGs and age or maternal smoking covariates (data not shown).

The 3 significant maternal CpGs found in the mediation model share key roles in biological processes. The first CpG (cg15011041) is associated with *HSPG2* (heparan sulfate proteoglycan 2) that encodes the perlecan protein involved in the maintenance of the endothelial barrier function by promoting different growth factors [70]. The second CpG (cg03606954) is associated with *HSD17B12* (hydroxysteroid 17-beta dehydrogenase 12) that encodes the enzyme 17-beta-hydroxysteroid dehydrogenase (17-beta-HSD). This enzyme is responsible for catalysing the conversion of estrone into estradiol and involved in the elongation of fatty acids [71]. Finally, the third CpG (cg18681375) is associated with *CACNA1G* (calcium voltage-gated channel subunit alpha1 G) that encodes low voltage-activated calcium channel subunit alpha-1G. These channels mediate the passage of calcium ions into the cell facilitating cellular communication as well as calcium-dependent processes including neurotransmitter release, muscle contraction and cell division [72].

The 2 significant CpGs of children, cg08566437 and cg05806180, are associated with *GABRG2* (gamma-aminobutyric acid type A receptor subunit gamma2) and *SULF1* (sulfatase 1), respectively. *GABRG2* encodes for the GABA receptor that binds GABA, a major inhibitory neurotransmitter in the mammalian brain. Mutations in this gene are associated with epilepsy and febrile seizures [69]. *SULF1* encodes for extracellular sulfatase that catalyse the removal of 6*O*-sulfate groups from heparan sulfate proteoglycans (HSPGs) and thus play an important role in development and cellular growth [73]. Relatedly, dysregulation of sulfates were associated with different types of cancer, including ovarian cancer [74].

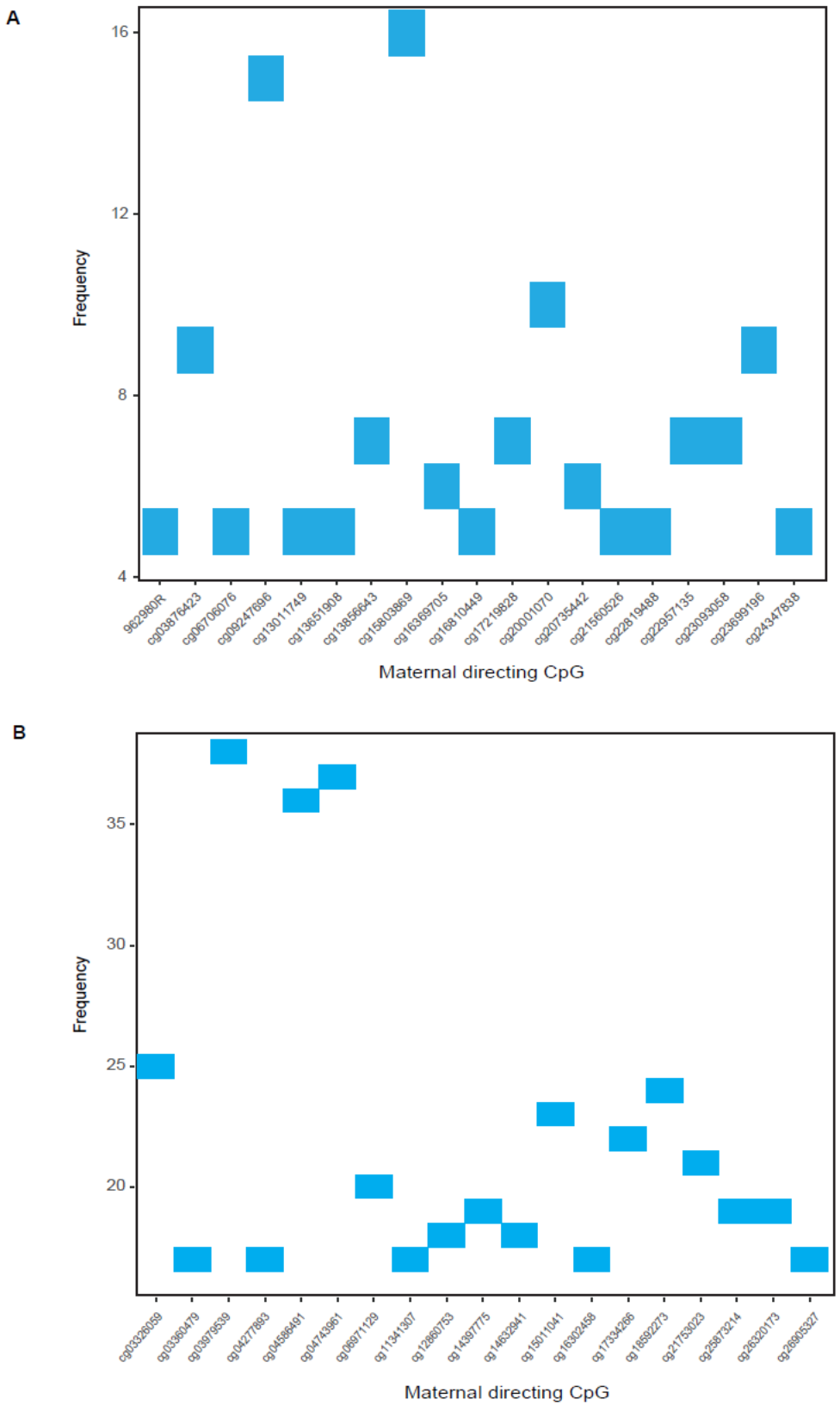


**Figure 5: Heatmap of the frequency of association between maternal directing methylation sites and children's CpGs.** Panel A shows the frequency of association between maternal and child CpGs at birth. Panel B shows the frequency of association between maternal and child CpGs at age 7.

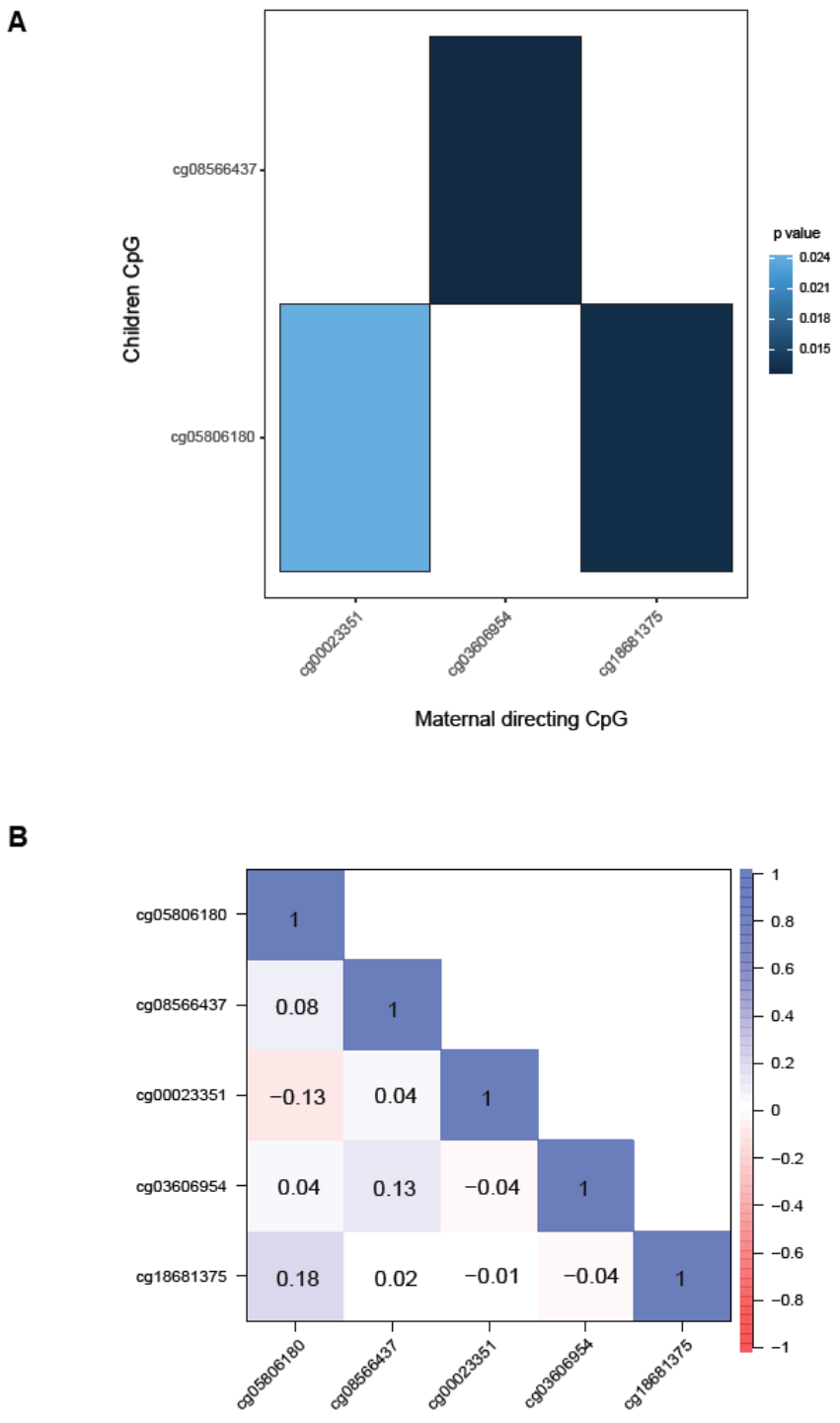


**Figure 6: Mediation model: associated maternal CpGs to children's CpG**. Panel A shows the 3 maternal CpGs associated with children's CpG found at birth and age 7. Panel B shows the correlation plot of children and maternal CpGs found at birth and age 7. Text: correlation coefficient.

## Discussion

To our knowledge, this is the first study to report on intergenerational transmission of the impact of maternal CSE on offspring epigenome, together with providing a mechanism through the

maternal prenatal epigenome. The findings would contribute to our understanding of the epigenetic mechanisms underlying the intergenerational transmission of ELA in multiple ways. First of all, maternal ELA was associated with differential methylation of hundreds of CpGs associated with multiple BPs both at birth and age 7, suggesting a potential programming of offspring epigenome by maternal early life exposure. Among the BPs, we investigated OP and PD pathways in detail due to increased fold enrichment and their associations with neuroendocrine and immune pathways [67-69]. These changes may in part explain studies associating ELA with health problems in relation to oxidative stress and inflammation, such as mood, cardiovascular, neurodegenerative disorders [75-78]. (As shown in a recent study, similar mechanisms may play into the intergenerational transmission of the effects of maternal ELA in terms of increased risk of children for asthma, obesity, and mood and neurodevelopmental disorders [34, 79] Since these disorders arise across the childhood, it is likely that some of the underlying epigenomic changes are stable over time. Indeed, among these hundreds of CpGs associated with maternal ELA, 11 CpGs were differentially methylated at both time points that may signify persistent epigenomic changes by maternal ELA despite the effects of postnatal factors over 7 years. Further focus on these pathways and genes may provide additional information about the impact on children's development and health across the lifespan.

In addition to associations between maternal ELA and child DNAm, testing mediation by maternal prenatal DNAm also contributed to our understanding of the mechanisms of intergenerational transmission. Focusing on maternal DNAm during pregnancy as a mediator was crucial due to previous literature reporting prenatal mood, behavioural and physiological pathways influenced by maternal CSE, such as in pregnancy-related worries, smoking, and HPA-axis activity [80, 81]. The changes in these pathways may in part be explained by changes in maternal epigenome during pregnancy, which may further influence programming of the offspring's epigenome. At this point, the mediation models we ran were critical in understanding the mode of this epigenetic transmission. Our results suggest that an *indirect* epigenetic inheritance might be possible, in which methylation at certain CpG sites in the offspring by maternal ELA are mediated through changes in maternal DNAm, although at different genomic locations. Here, the mother's epigenome, represents both her prior accumulated life experience and may, at the same time, be a proxy for the developmental environment she provides to her offspring *in-utero*, although our data do not allow us to draw any definitive conclusions.

When we identified the genes associated with the differentially methylated CpGs in mediation models (*HSPG2, SULF1, GABRG2, HSD17B12*, *CACNA1G*), many of them were associated with pregnancy and stress and influence each other's' function. For instance, maternal serum perlecan levels, encoded by *HSPG2*, was previously suggested as a biomarker for preeclampsia

severity [82, 83]. Given research associating CSE with increased risk of preeclampsia, epigenetic changes in this gene by CSE should be investigated in detail to understand its impact on the mother and the child. Interestingly, another gene that includes one of the CpGs is *SULF1* that encodes sulfatase involved in the metabolism of HSPGs and thus regulating development and cellular growth and differentiation critical during pregnancy [73]. In support of this, a recent study suggested sulfatase deficiency as a risk factor for postpartum mood disorders [84]. Furthermore, the effects of sulfatases on steroids were reported to influence their interaction with membrane receptors, such as GABA receptors [85]. The steroid sulfate axis and its relationship to maternal behaviour and mental health [85]. In relation to *HSD17B12,* the enzyme it encodes was associated with influencing oestrogen metabolism as well as production of precursors of prostaglandins, such as arachidonic acid, influencing ovarian function, embryonic survival and differentiation and fertility [86]. Finally, low voltage-activated calcium channels encoded by *CACNA1G* were reported to influence uterine physiology and interact with the steroid beta-estradiol and contribute to changes in uterine during pregnancy, leading to potential risks such as miscarriage and preterm birth [87]. The interactions between these proteins may serve as a potential mechanism by which maternal CSE shapes the intra-uterine physiology during pregnancy, which may influence offspring birth outcomes and development. If replicated in other cohorts, these differential epigenetic patterns may also serve as a way CSE leaves an epigenetic imprint on the reproductive system that alters prenatal physiology and thus foetal development.

Our study, like all others, is not without limitations. Firstly, few cohorts are as rich as ALSPAC, and we were not able to replicate our data in an independent cohort. This is something that needs to be done in the future, when such data becomes available. Furthermore, our EWAS-mediation strategy is novel. We were able to demonstrate its utility in this particular model. The question is now whether it will work on models where the trauma is less intense, or in other paradigms where there is a possibility of epigenetic transmission. Despite providing insight into the mechanism, this comes at the cost of inflating the number of CpGs that are involved in the process, since each of the child's differentially methylated CpGs in the initial EWAS is mediated by numerous maternal CpGs. There is, however, an interesting population of maternal CpGs that mediate a high number of their child's differentially methylated CpGs. We term these "directing methylation sites" because of their role directing their child's methylation levels. How these directing methylation sites pass information on to the next generation remains to be examined. In our follow up studies, we plan to focus on the CpGs that were differentially methylated by maternal CSE from birth to age 7 and use the richness of the ALSPAC dataset to identify potential moderating postnatal factors, such as stress and breastfeeding [17, 88, 89] that can be tested experimentally. As reported by Dunn et al. in the ALSPAC cohort [17], the timing of postnatal exposure may play an important role

in these associations, and our EWAS-mediation methodology should also be tried to examine the effect of the age of trauma-exposure. Considering the genes that house the differentially methylated CpGs by maternal CSE, future studies focusing specifically on these genes and their expression may provide further insights into impact of maternal CSE on perinatal physiology and risk of complications [73]. In addition, considering the potentially ameliorating effects of additional factors in the face of ELA, it is important to identify such protective factors, such as emotional support [90], in relation to epigenetic changes. In terms of BPs, considering the role of OP pathway in energy metabolism, focusing on mitochondrial function and DNA as potential targets related to ELA is promising [91, 92]. Finally, while this study focused on DNAm as an epigenetic mechanism of intergenerational transmission, other studies, mostly in non-human animals, emphasize the importance of RNA-mediated epigenetic mechanisms in this transmission [93], which would extend these findings in terms of alternative routes of epigenetic inheritance.

The results of this study have important implications for the way we think about inheritance and evolution [94], as well as for public health and social policies by providing an epigenetic mechanism underlying intergenerational transmission of the effect of trauma across generations. While some of these intergenerational effects may be related to social learning and persistent environmental and contextual factors, such as poverty and family environment [95], epigenetic programming may also contribute with potential interactions with the aforementioned factors [96]. Therefore, development of prevention programs in relation to ELA starting from the prenatal period is of utmost importance, along with identification of protective factors and optimizing and providing access for different intervention programs across the lifespan [97]). These advances in turn could assist in our individual and societal ability to break the intergenerational cycles of trauma.

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**Disclosures**

The authors declare no conflict of interest.

**Ethics approval and consent to participate**

We are extremely grateful to all the families who took part in this study, the midwives for their help in recruiting them, and the whole ALSPAC team, which includes interviewers, computer and laboratory technicians, clerical workers, research scientists, volunteers, managers, receptionists and nurses. Please note that the study website contains details of all the data that is available through a fully searchable data dictionary and variable search tool and reference the following webpage: http://www.bristol.ac.uk/alspac/researchers/our-data/. The UK Medical Research Council and Wellcome (Grant ref: 217065/Z/19/Z) and the University of Bristol provide core support for ALSPAC. This publication is the work of the authors and Duman, Holuka, Menta, D'Ambrosio, and Turner, will serve as guarantors for the contents of this paper. A comprehensive list of grants funding is available on the ALSPAC website (http://www.bristol.ac.uk/alspac/external/documents/grant-acknowledgements.pdf). Ethical approval for the study was obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics Committees. Consent for biological samples has been collected in accordance with the Human Tissue Act (2004). Informed consent for the use of data collected via questionnaires and clinics was obtained from participants following the recommendations of the ALSPAC Ethics and Law Committee at the time.

**Consent for publication**

Not applicable.

**Availability of data and material**

All data are available commercially from the ALSPAC consortium. Link: Access data and samples | Avon Longitudinal Study of Parents and Children | University of Bristol

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**Author Contributions**: Conceptualization: CH, EAD, and JDT; literature review: EAD, CH, and JDT; data: CD, GM and the ALSPAC team; data analysis: CH and JDT; manuscript first draft: EAD, CH, and JDT, manuscript editing: all authors. All authors have read and agreed to the published version of the manuscript.

**Abbreviations:** Childhood Sexual Experience (CSE); Avon Longitudinal Study of Parents and Children (ALSPAC); DNA methylation (DNAm); Differentially-methylated regions (DMR); Odds Ratio (OR); Epigenome-wide Association Study (EWAS); Biological pathways (BP); Oxidative phosphorylation (OP); Parkinson's disease (PD); Early life adversity (ELA); High-dimensional mediation analysis (HIMA); General linear model (GLM); Heparan Sulfate Proteoglycan 2 (HSPG 2); Calcium Voltage-Gated Channel Subunit Alpha1 G (CACNA1G); Sulfatase 1 (SULF1); Accessible Resource for Integrated Epigenomics Studies (ARIES); Ocytocin Receptor Gene (OXTR); *HSD17B12* (hydroxysteroid 17-beta dehydrogenase 12); 17-beta-hydroxysteroid dehydrogenase (17-beta-HSD); *GABRG2* (gamma-aminobutyric acid type A receptor subunit gamma2.

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# Chapter 7

# Mode of birth and DNA methylation at birth, in childhood, and in adolescence: uncovering the relationship using ALSPAC data

**My contribution to this Chapter:**

Conceptualisation, Literature review, Data generation, Data integration, Data visualisation, Final statistical analysis, Interpretation of results, Making of all figures, and Writing of the article (80%).

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As often depicted in the literature, the maternal environment represents a major component involved in foetal development as well as in the post-natal period, more precisely in the first thousand days of life (DOHaD). If many aspects of maternal interventions have been already studied over the past years, there is an important lack of data regarding the mode of birth. Indeed, it has recently been suggested that mode of birth can contribute to the offspring's overall health later in life. Over the past 30 years, opting for a C-section rather than a vaginal birth has become much more accessible to every woman in most developed countries.

For multiple reasons, many woman request a caesarean section birth when possible. From the outside, this mode of birth represents an easier and less scary event, however it involves important metabolic changes. Indeed, the birthing process is accompanied by a strong activation of both the HPA axis and the "anti-stress" oxytocinergic system. Exposure to these exceptionally strong physiological stimuli are indispensables for the normal development of the immune system as well as the hypothalamus-pituitary-adrenal (HPA-) axis. This led to the hypothesis that, epigenetic mechanisms such as DNAm can be altered due to elective or emergency C-section.

Although the consequences of the mode of birth on child's health remains poorly described, nevertheless some studies reported that children born with C-section had a higher level of DNAm in leucocytes at birth. Here, we investigated whether C-section induces more DNAm than vaginal birth and what would be the biological and physiological consequences for the offspring.

By conducting an EWAS analysis, we tried to demonstrate that not only the immune system can be affected by birth mode, but also epigenetic reprogramming leading to important health consequences that will be conserve over time.

Barker et al. highlighted how important the first thousand days of life are as they represent the most critical period of devolvement for a human being. Nevertheless the first stressful event experienced by a child, which is the birth itself is rarely recognised as important and worthy of being studied. Indeed, the birth event experienced by every human being is underestimated and most of the time forgotten in the list of maternal stressors affecting epigenetic profiles of children. As those epigenetic modifications i.e. DNAm are now known to be conserved over time and/or transmitted to the next generation, and can exacerbate the biological consequences (i.e. chronic diseases development), it is indispensable to investigate their consequences.

Developmental Psychology

# Mode of Birth and DNA Methylation at Birth, in Childhood, and in Adolescence: Uncovering the Relationship Using ALSPAC Data

Isabel Jaramillo, Luisa Bergunde, Cyrielle Holuka, Carlo Schuengel, Jasminka Štefulj, Susann Steudte-Schmiedgen, Maria Kaźmierczak, Giorgia Menta, Conchita D'Ambrosio, Joan G. Lalor, Jonathan D. Turner, and Susan Garthus-Niegel

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# Mode of Birth and DNA Methylation at Birth, in Childhood, and in Adolescence: Uncovering the Relationship Using ALSPAC Data

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Mode of birth has been linked to offspring health. Changes in DNA methylation (DNAm) may represent a potential mechanism; however, findings are heterogeneous and limited to early infancy. This preregistered study examined whether mode of birth (vaginal birth compared with elective or emergency cesarean section) affects DNAm at birth, in childhood, and adolescence and whether these effects are modified by the postnatal care environment, specifically by breastfeeding and mother–infant bonding. Using data from 876 mother–infant dyads from the U.K. Avon Longitudinal Study of Parents and Children, we examined differentially



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Isabel Jaramillo served as lead for conceptualization, formal analysis, methodology, visualization, writing–original draft, and writing–review and editing. Luisa Bergunde served as lead for conceptualization, formal analysis, methodology, visualization, writing–original draft, and writing–review and editing. Cyrielle Holuka served as lead for data curation, formal analysis, funding acquisition, investigation, resources, and software, contributed equally to visualization, and served in a supporting role for conceptualization. Jasminka Štefulj served as lead for writing–review and editing. Giorgia Menta served as lead for funding acquisition, project administration, and resources. Conchita D'Ambrosio served as lead for funding acquisition, project administration, and resources. Joan G. Lalor served as lead for funding acquisition and project administration. Jonathan D. Turner served as lead for funding acquisition, methodology, project administration, resources, and supervision. Susan Garthus-Niegel served as lead for funding acquisition, project administration, and supervision. Cyrielle Holuka, Carlo Schuengel, Jasminka Štefulj, and Susan Garthus-Niegel contributed equally to methodology. Carlo Schuengel, Jasminka Štefulj, Susann Steudte-Schmiedgen, Maria Kaźmierczak, Joan G. Lalor, Jonathan D. Turner, and Susan Garthus-Niegel contributed equally to conceptualization. Carlo Schuengel and Jasminka Štefulj contributed equally to supervision. Cyrielle Holuka, Carlo Schuengel, Susann Steudte-Schmiedgen, Maria Kaźmierczak, Joan G. Lalor, Jonathan D. Turner, and Susan Garthus-Niegel contributed equally to writing–review and editing.

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methylated cytosine-phosphate-guanine dinucleotides and regions associated with mode of birth. DNAm was quantified using Illumina Infinium Human Methylation 450 K BeadChip in cord blood (at birth) and in peripheral blood (at 7 and 15–17 years). Analyses controlled for maternal age, education, smoking during pregnancy, child sex, gestational week at birth, and batch effects. We also examined interactions of mode of birth with breastfeeding practices and mother–infant bonding. In cord blood, two cytosine-phosphate-guanine dinucleotides (cg05230316; cg13230077) were linked to mode of birth ($p_{FDR} < .050$). DNAm in childhood or adolescence was not statistically associated with mode of birth ($p_{FDR} > .050$), and breastfeeding and mother–infant bonding were not moderators ($p > .050$). Overall, findings suggest mode of birth may have a small effect on cord blood DNAm, but these effects may not persist into later developmental stages. Other postnatal influences should be considered, and further investigation is needed to address study limitations.

***Public Significance Statement***

This study suggests that the mode of birth, whether a child is born vaginally or by emergency or elective cesarean section, has a small effect on how the child's genes are methylated in cord blood cells at birth. DNA methylation in peripheral blood cells during childhood and adolescence was not predicted by mode of birth. Breastfeeding and mother–infant bonding did not buffer the effects of mode of birth, highlighting the necessity to further investigate other postnatal factors as potential determinants of the offspring methylome across development.

*Keywords:* labor, epigenome-wide association studies (EWAS), breastfeeding, mother–infant bonding, Accessible Resource for Integrated Epigenomic Studies

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The last 30 years have seen a sharp increase in the frequency with which elective cesarean sections (ELCS) are requested and conducted (Mylonas & Friese, 2015). Especially upper middle-income countries show an 11-fold increase in ELCS compared with high- or low-income countries (Begum et al., 2021). Epidemiological studies indicate that, compared with vaginal birth (VB), cesarean section (CS) might be associated with short- and long-term physiological and psychological developmental outcomes. There is well-documented evidence for altered immune responses (Cho & Norman, 2013), an increased risk for obesity, allergy, and asthma, as well as reduced microbiome diversity (Sandall et al., 2018), in offspring born by CS as compared to those born by VB. Findings on the association between mode of birth and psychological outcomes are inconclusive to date (e.g., Blake et al., 2021; Cohen et al., 2022). CS might be linked to poorer cognitive development (Blake et al., 2021; Polidano et al., 2017), delayed motor development (Grace et al., 2015; Khalaf et al., 2015), delayed personal social development (Khalaf et al., 2015), and an increased risk for autism spectrum disorders and attention-deficit/hyperactivity disorders (ADHD; Zhang et al., 2019). It follows that a better understanding of how mode of birth may impact offspring development is clearly warranted. Mode of birth can be categorized as VB, including noninstrumental as well as instrumental VB (i.e., VB assisted by instruments such as forceps or a vacuum extractor), emergency cesarean section (EMCS; i.e., emergency surgical procedure initiated during the first or second stage of labor), and ELCS (i.e., elective surgical procedure initiated before labor began).

Several biological mechanisms have been suggested to link mode of birth and children's development. For example, the maternal vaginal, fecal, and skin microbiome might not transfer to the infant during CS as the infant does not pass through the birth canal (Dominguez-Bello et al., 2010; Mueller et al., 2015), resulting in altered bacterial colonization being instead mainly influenced by environmental microflora (Biasucci et al., 2010), which might in turn influence subsequent behavioral outcomes (Loughman et al., 2020; Repetti et al., 2011; Zhu et al., 2020). Furthermore, CS, in particular ELCS, bypasses key physiological stimuli (e.g., uterine contractions) assumed to be necessary for immune system and hypothalamus–pituitary–adrenal axis development, systems that are involved in behavioral adaptation (Lagercrantz, 2016; McCallie et al., 2017). Alterations of the body's central stress system can impact gene regulatory systems and developmental programming of future diseases (Matthews & McGowan, 2019). It has been suggested that childbirth could represent a critical formative event that may impact the infant's regulation of gene expression through epigenetic mechanisms such as DNA methylation (DNAm) of cytosine-phosphate-guanine (CpG) dinucleotides (Chen et al., 2021; Cho & Norman, 2013; Dahlen et al., 2013). Epigenetic alterations may, in turn, influence offspring's psychological, neurological, and hormonal development and behavior (Mulder et al., 2021; Serdarevic et al., 2023).

The Epigenetic Impact of Childbirth (EPIIC) hypothesis (Dahlen et al., 2013) postulates that childbirth represents a healthy type of stress (eustress) which might be critical for adaptation of the fetal genome to the extrauterine environment. Physiological labor during VB and EMCS involves massive activation of the sympathoadrenal and inflammatory defense systems, leading to changes in various hormone levels, including cortisol, adrenalin, and oxytocin levels (Lagercrantz & Bistoletti, 1977; Yektaei-Karin et al., 2007). These physiological processes may adaptively program development through genes relating to the immune response, body weight regulation, and tumor suppression and preparing the infant for extrauterine transition. In contrast to the healthy "eustress" of physiological labor (i.e., during VB and EMCS), "distress" (i.e., during an ELCS) is postulated as an altered stress response above or below the norm and has been suggested to trigger epigenetic remodeling that differentially affects gene expression (Dahlen et al., 2013), which may in turn influence disease and biobehavioral outcomes later in life (Sandall et al., 2018; Schlinzig et al., 2009).

Studies on the association between mode of birth and epigenetic changes in offspring have been few and findings mixed. Almgren

et al. (2014) assessed DNAm in cord blood and showed that compared with VB, ELCS was associated with a 2% higher global DNAm in hematopoietic stem cells (CD34+). Similarly, Schlinzig et al. (2009) found that global DNAm in cord blood was higher in infants born by CS compared to those born vaginally. However, global DNAm measured in peripheral blood 3–5 days after birth decreased in the CS group but remained stable in VB-born infants, suggesting that events around birth can rapidly influence the dynamic of DNAm.

Other studies did not find statistically significant associations between mode of birth and global methylation in cord blood, whether or not adjusting for relevant confounders (Franz et al., 2014; Virani et al., 2012). Epigenome-wide association studies (EWAS) are conflicting as well. While Almgren et al. (2014) identified 343 differentially methylated positions (DMPs), of which most (76%) showed lower levels of DNAm in infants born by ELCS compared with VB ($n = 64$), Chen et al. (2021) identified 165 DMPs measured in DNAm in cord blood, of which most (61.2%) presented higher DNAm levels in ELCS compared with VB ($n = 140$). Both EWAS published to date identified gene-specific methylation with potential impact on gene expression and functional outcomes. However, small sample sizes limit generalizability. Furthermore, to the best of our knowledge, there are no studies investigating the effect of mode of birth on differentially methylated regions (DMRs). DMRs are genomic regions that differ regarding methylation pattern between phenotypes and may occur throughout the genome but have frequently been identified at prominent regulatory positions, including promoter regions of genes, within the gene body, and at intergenic regulatory regions. Compared to investigating methylation status at single-base resolution (i.e., individual CpG sites), analyzing multiple cytosine-phosphate-guanine dinucleotides (CpGs) in DMRs results in fewer tests being conducted, less adjustment for multiple testing, and therefore a higher power to detect significant regulated methylation targets (T. J. Peters et al., 2015).

Until now, epigenetic research has mainly focused on ELCS compared with VB. Yet, EMCS has been previously linked to a higher odds ratio for respiratory and other system infections, gastrointestinal disorders, metabolic disorders, and eczema in children (L. L. Peters et al., 2018). Therefore, potential alterations of the epigenome in relation to EMCS need to be further investigated. Also, by looking primarily at DNAm in cord blood taken directly after childbirth, previous studies have not provided an understanding of epigenetic changes associated with mode of birth across the life span. A better understanding of the short- and long-term epigenetic changes in response to mode of birth is necessary to inform perinatal medical care and disease prevention efforts. Therefore, EWAS studies investigating different modes of birth with large sample sizes are needed to identify gene-specific methylation in or near promoter regions with implications for functional outcomes and gene expression (Dahlen et al., 2013; see Figure 1).

In addition to childbirth as the last prenatal experience for the fetus, exposure to specific postnatal environmental influences during critical or sensitive developmental periods have also been shown to affect an individual's short- and long-term health (e.g., Burggren & Mueller, 2015). From animal and human studies, it is known that maternal care and sensitivity during early life play a role in behavioral outcomes and epigenetics (e.g., Champagne & Curley, 2009; Lester et al., 2018). Hence, it has been suggested that maternal caregiving might not only directly affect the infant's epigenome but might also have a buffering effect on the DNAm of genes impacted by earlier adverse events (Provenzi et al., 2020).

The social buffering hypothesis postulates that the presence of an intimate conspecific plays a central role in modulating the effects of adverse experiences on health (Gunnar & Hostinar, 2015; Peen et al., 2021). In line with this hypothesis, breastfeeding, as an important aspect of early postnatal care, has been shown to have short-term health benefits and is increasingly implicated in long-term health outcomes of the offspring (Dogaru et al., 2014; Hernández-Luengo et al., 2022; Horta et al., 2015). Studies investigating the biological effects of breastfeeding have shown that lactation is associated with distinct DNAm patterns in offspring (Hartwig et al., 2020) and that microbial transfer also occurs via breast milk (Pannaraj et al., 2017). Epigenetic analyses conducted on the Avon Longitudinal Study of Parents and Children (ALSPAC) suggest that two differentially methylated sites and 12 DMRs are associated with having ever breastfed 7 and 15–17 years after childbirth with three DMRs showing stable associations longitudinally (Hartwig et al., 2020). Consequently, it is important to explore whether breastfeeding can buffer the potential association between mode of birth and child DNAm across the developmental span.

Mother–infant bonding is defined as maternal feelings about her child and may indirectly influence offspring outcomes, including social–emotional problems (Fuchs et al., 2016; Le Bas et al., 2020; Rusanen et al., 2022), neurodevelopment (Faisal-Cury et al., 2021), and social-cognitive skills (Joas & Möhler, 2021). At the neural level, bonding enhances the attraction to infant stimuli and stimulates maternal caregiving practices, while reducing rejecting behaviors (Numan & Young, 2016). At the physiological level, the oxytocin system, the endogenous opioids, and their receptors are strongly intertwined in the context of mother–infant bonding (Magon & Kalra, 2011). Until now, studies have not investigated associations between mother–infant bonding and child DNAm. However, the buffering effect of mother–infant bonding on the relationship between early stressful life events (such as stress during a nonphysiological birth) and offspring DNAm is plausible and needs to be investigated.

Therefore, this study investigated whether and to what extent mode of birth (i.e., VB, EMCS, and ELCS) is associated with epigenetic alterations in the offspring immediately after birth and at 7 and 15–17 years of age. We conducted one of the first EWAS in this research area extending beyond the immediate postnatal period, thereby contributing to an innovative exploration of the developmental effects of mode of birth. Secondly, we focused on specific CpG sites that were identified in the EWAS as associated with mode of birth to conduct moderation analyses. Considering that a nurturing early postnatal care environment has positive psychological and physiological benefits for both mother and child (e.g., Lester et al., 2018), we hypothesized that breastfeeding and mother–infant bonding would moderate the association of mode of birth with offspring DNAm at ages 7 and 15–17 years.

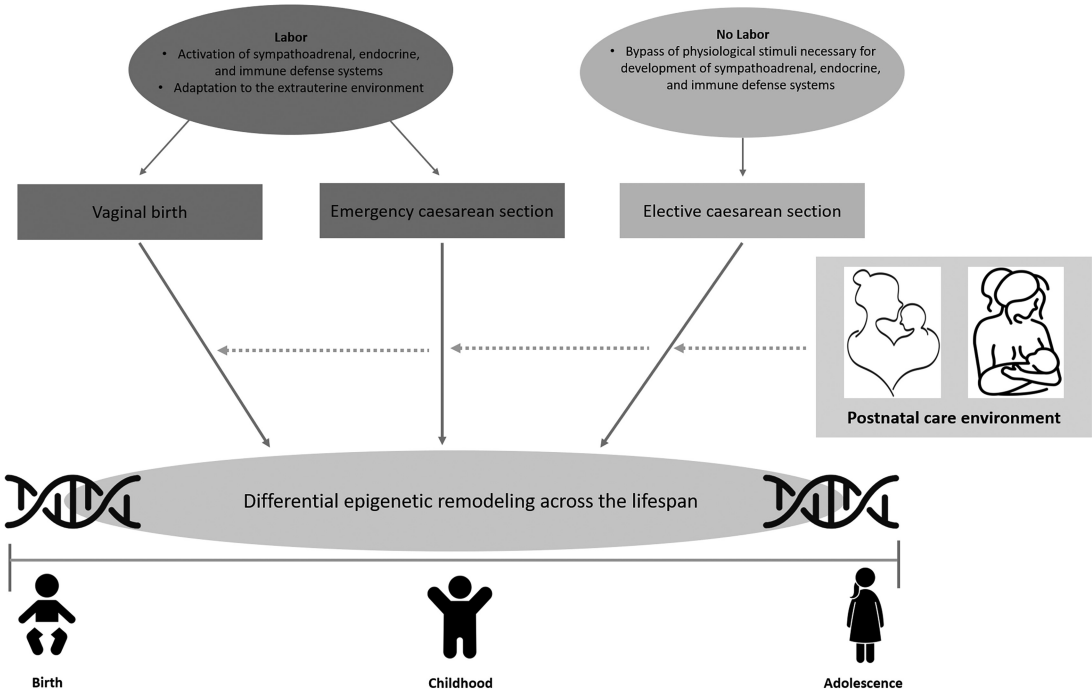
## Material and Method

### Description of ALSPAC and Participants Included in the Study

The present study focuses on data derived from the Accessible Resource for Integrated Epigenomic Studies (ARIES; Relton et al., 2015), a subsample of the larger prospective birth-cohort ALSPAC ((Boyd et al., 2013; Fraser et al., 2013). ALSPAC

**Figure 1**

*Theoretical Model for the Epigenetic Effects of Mode of Birth Across the Life Span*






comprises data from 14,541 pregnant female residents in Avon, UK, with expected due dates between April 1, 1991, and December 31, 1992. The initial pregnancies resulted into 13,988 children being alive at 1 year of age. Among these participants, some mother–infant dyads also provided DNA samples for genome-wide DNAm profiling as part of ARIES. Consent for biological samples was collected in accordance with the Human Tissue Act (2004). Information regarding (epi)genetics, psychobiological factors, social, and other environmental exposures has been collected during pregnancy and follow-up assessments up to 18 years of age. Ethical approval was obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics Committees and include appropriate permissions from the Human Development Biology Resource, Newcastle Brain Tissue Resource and Leiden University Medical Center (Relton et al., 2015). Some mother–infant dyads who participated in ARIES did not provide epigenetic data at all time points and some did not specify their mode of birth; hence, sample size varied slightly: at birth ($n = 813$), at 7 years ($n = 876$), and at 15–17 years ($n = 874$). For the present study, we only include singletons to ensure a more homogenous sample.

## Measures

### *Mode of Birth (Predictor)*

Women were asked whether they had had any CS (ALSPAC variable name: e040), including the following response categories "no," "yes after being in labour," and "yes and never had any labour." Mothers who reported having had a CS but not having had any labor were categorized as having had an ELCS, whereas those with labor experience were categorized as having had an EMCS, whereas the remainder was grouped as VB. In regression analyses, these three groups were treated as dummy variables to compare the effects of (a) VB versus ELCS, (b) VB versus EMCS, and (c) EMCS versus ELCS, respectively.

### *DNAm (Outcome)*

Genome-wide methylation profiles in children were assessed using the Illumina Infinium Human Methylation 450 K BeadChip. DNAm was quantified in cord blood drawn from the umbilical cord upon childbirth and from peripheral blood drawn at age 7 and 15–17 years. Methylation data preprocessing was conducted as part of the ARIES project at the University of Bristol (Touleimat & Tost, 2012). A post hoc Houseman correction for cellular heterogeneity for DNAm was generated for each time point (Richmond et al., 2015). For the present study, we used cell-type corrected postnormalization data. Prior to normalization, samples had to be removed because of (a) failure of genotype or normalization quality controls, (b) mismatches using genome-wide association concordance (concordance $< 80\%$), (c) ratio of methylated/unmethylated signal, (d) dye bias, (e) a high proportion of undetected probes using a threshold of 0.01, and (f) probes with low bead numbers and low detection scores were also excluded (Briollais et al., 2021). Methylation data were transformed into beta values, which measure the ratio of methylated probe intensity to total intensity. These were used as our outcome variables.

### *Breastfeeding (Moderator)*

A dichotomous breastfeeding variable assessing whether children were ever or never breastfed was created based on mothers' answers provided when children were 15 months old in the questionnaire "My infant Daughter/Son." If children were never breastfed, they

were coded as "never breastfed," whereas if children were still being breastfed or breastfeeding had taken place but stopped, these were coded as "ever breastfed."

### *Mother–Infant Bonding (Moderator)*

Mother–infant bonding was obtained at 8 months postpartum from the "Looking after a baby" questionnaire developed by the ALSPAC team. The questionnaire consists of two subscales assessing maternal enjoyment of baby ("I really enjoy my baby") and maternal confidence ("I feel confident with my baby"). Mothers indicated their agreement with 11 items as "This is exactly how I feel," "This is often how I feel," "This is how I sometimes feel," or "I never feel this way." A total score of maximum 44 was generated, with higher scores indicating higher mother–infant bonding. The questionnaire has shown positive predictive value for maternal positive parenting interactions (Thomson et al., 2014), carefully indicating its convergent validity. In the present sample, the scale demonstrated acceptable internal consistency ($\alpha = .78$).

### *Covariates*

To correct our statistical model, we included different covariates that have been shown to affect both mode of birth and the offspring's DNAm: maternal age at childbirth (calculated from entries at 8 weeks postpartum, Dunn et al., 2017; Sharami et al., 2022), maternal education (Alfano et al., 2019; Ryding et al., 2016), maternal smoking (Richmond et al., 2015) during the last 2 months of pregnancy (informed by mothers 8 weeks postpartum), child sex (Yousefi et al., 2015), and declared child gestational week at birth (Merid et al., 2020). Maternal ethnicity and prepregnancy body mass index (BMI; calculated from height and weight measures at 12 weeks gestation) were not included as covariates because of a lack of variability in ethnicity (98.74% White) and a large number of missing values ($n = 168$) regarding BMI. We also controlled for batch effect as recommended in the literature.

## Statistical Analysis

All analyses were performed in R (Version 4.1.1; R Core Team, 2020) on R studio (Version 4.2.2; R Core Team, 2022). "Dplyr" (Version 1.0.7; Wickham et al., 2020) and "smjics" (Version 2.8.7; Lüdecke, 2018) were used for data cleaning. This study was preregistered (https://osf.io/scxh6/). Any deviations from the preregistered plan can be found in the online supplemental materials. Data cannot be made available to third parties without permission by the ALSPAC committee.

### *Case–Control EWAS*

To extract significantly associated CpGs and their epigenetic information (i.e., differential methylated regions), we used the "DMRcate" (Version 2.6.0) package (T. J. Peters et al., 2015). In addition, probe statistics were extracted with "DMRcate" (Version 2.6.0) and the Bioconductor "missMethyl" packages (Version 1.26.1). After false discovery rate (FDR) correction, regions are agglomerated from groups of postsmoothed significant probes where the distance to the next consecutive probe (lambda) was set at less than 1,000 nucleotides. Finally, we used the "GOfuncR" (Version 1.12.0) and "pathfindR" (Version 1.6.2) packages to highlight biological pathways associated with mode of birth (Cabrera et al., 2019; Ulgen et al., 2019). We examined gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for the differentially methylated genes identified in our analyses. In a second step, to investigate the functional relevance of the selected differentially methylated CpGs, we consulted the EWAS catalog (EWAS Catalog, 2023; https://www.ewascatalog.org) and Gene Cards (Gene Cards, 2023; https://www.genecards.org). Probes or DMRs were considered significant when the Benjamini–Hochberg corrected $p$ value $< .050$.

### *Follow-Up Analysis: Linear Regression Analyses for Single CpG Sites*

To examine in greater detail the direction of the effects of modes of birth on statistically significant CpG sites identified in EWAS and examine the aspects of the postnatal environment, linear regression analyses were conducted with the "lm" function in the "MASS" package in R (Venables et al., 2002). Here, results with $p$ values between .050 and .100 were discussed as moderately suggestive of a significant association, but further investigation on a larger sample is needed to obtain a higher level of confidence. First, we estimated multiple linear regression analyses with the beta values in cord blood of the significant CpGs from the EWAS as the respective outcome variables and with mode of birth as a dummy-coded predictor as well as the covariates specified above included in the model. Second, we ran multiple linear regression analyses with beta values at age 7 and 15–17 as the outcome for the significant CpGs in cord blood from the EWAS that were significantly associated or moderately suggestive of being significantly associated with mode of birth in the initial linear regression analyses. The first model included the dummy-coded mode of birth variables, the covariates, and either breastfeeding or mother–infant bonding as predictors, whereas the second model further also included the interaction terms between breastfeeding or mother–infant bonding and mode of birth.

# Results

## Descriptive Statistics

Mothers and children who only participated in ALSPAC versus mothers and children who participated in both ALSPAC and ARIES and whose data were used to investigate the present research questions differed significantly regarding several sociodemographic characteristics (see Table 1). While the sex of the child was similarly distributed in both samples, birth weight and gestational week at birth were significantly higher in the ARIES subset. Mothers in ARIES were older on average than mothers partaking in ALSPAC only as well as more highly educated (i.e., higher percentage of mothers with A level and degree qualifications). Furthermore, while the majority of mothers in both ALSPAC and ARIES was White (97.3% and 98.7%, respectively), significantly fewer mothers in ARIES reported non-White ethnic background. Also, mothers in ARIES were less likely to have smoked during pregnancy and never having breastfed than mothers in ALSPAC. No significant differences in mother–infant bonding and prepregnancy BMI emerged between mothers in ALSPAC and ARIES. The following results presented comprise only data from the ARIES subsample.

**Table 1**
*Descriptive Characteristics of the Only ALSPAC Mother–Infant Cohort and the ARIES Subset at Birth*

| Variable | ALSPAC $n$ (%) or $M \pm SD$ | ARIES $n$ (%) or $M \pm SD$ | Differences in samples $\Delta\chi^2$ or $t(df)$, $p$ |
|---|---|---|---|
| Child characteristics | | | |
| Sex ($n$, %) | | | 2.44, $p = .118$ |
| Male | 7,248 (51.3) | 439 (48.6) | |
| Female | 6,881 (48.7) | 465 (51.4) | |
| Birth weight (g; $M \pm SD$) | 3,373 ± 586 | 3,487 ± 487 | −6.62(1064.2), $p < .001$ |
| Gestational week at birth ($M$, $SD$, Range) | 38.3 ± 5.7 | 39.6 ± 1.5 | 2.66(83), $p = .009$ |
| Maternal characteristics | | | |
| Age at childbirth ($M \pm SD$) | 27.88 ± 4.94 | 29.56 ± 4.49 | 5.60(63), $p < .001$ |
| Education ($n$, %) | | | 338.86, $p < .001$ |
| CSE | 2,445 (21.1) | 78 (8.8) | |
| O-level | 4,021 (34.7) | 298 (33.7) | |
| Vocational | 1,160 (10.0) | 69 (7.8) | |
| A-level | 2,530 (21.8) | 263 (29.8) | |
| Degree | 1,433 (12.4) | 175 (19.8) | |
| Ethnicity ($n$, %) | | | 197.34, $p < .001$ |
| White | 11,191 (97.3) | 865 (98.7) | |
| Other | 315 (2.7) | 11 (1.3) | |
| Smoking during pregnancy ($n$, %) | | | 50.84, $p < .001$ |
| Yes | 2,214 (20.3) | 89 (10.2) | |
| No | 8,711 (79.7) | 780 (89.8) | |
| Prepregnancy BMI (kg/m$^2$; $M \pm SD$) | 24.78 ± 4.67 | 24.73 ± 4.62 | 0.27(879.75), $p = .791$ |
| Mode of birth ($n$, %) | | | 0.163(2), $p = .922$ |
| VB | 9,539 (91.8) | 780 (91.5) | |
| EMCS | 382 (3.7) | 31 (3.6) | |
| ELCS | 469 (4.5) | 41 (4.8) | |
| Breastfeeding ($n$, %) | | | 57.55, $p < .001$ |
| Ever breastfed | 7,344 (72.8) | 730 (84.5) | |
| Never breastfed | 2,745 (27.2) | 134 (15.5) | |
| Mother–infant bonding ($M \pm SD$) | 28.27 ± 3.69 | 28.14 ± 3.53 | 1.05(1,018.8), $p < .296$ |

*Note.* $n$ varied slightly because of missing values. CSE = Certificate of Secondary Education; O-level = ordinary level; A-level = advanced level; BMI = body mass index; VB = vaginal birth; EMCS = emergency cesarean section; ELCS = elective cesarean section; ALSPAC = Avon Longitudinal Study of Parents and Children; ARIES = Accessible Resource for Integrated Epigenomic Studies.

## EWAS Results

### *Association of Mode of Birth With DNAm in Cord Blood*

Below the FDR level of $p < .050$, we identified two differentially methylated sites in relation to mode of birth in cord blood in EWAS, showing that mode of birth was associated with differentially methylated CpGs in cord blood (see Table 2). EWAS analyses to identify DMRs did not reveal any statistically significant findings with mode of birth ($p_{FDR} > .050$).

Graphical depiction of beta values according to mode of birth for the two CpG sites identified in EWAS (Figure 2) illustrates very small differences in methylation between modes of birth.

A more detailed examination of the two differentially methylated CpGs was performed using linear regression models (see Table 3). Results were moderately suggestive of individuals with ELCS showing lower methylation at cg05230316 compared with VB ($p = .079$), whereas no effects of mode of birth were significant for cg13230077 ($p > .432$).

**Table 2**
*DMPs (FDR $p < .05$) in Cord Blood at Birth*

| CpG name | Chr | Nearest gene | Position | statf. | Diff. | $p_{FDR}$ |
|---|---|---|---|---|---|---|
| cg05230316 | 8 | — | 63056032 | 5.31 | 0.015 | .03 |
| cg13230077 | 9 | *ZNG1E* | 70490060 | −5.76 | −0.0007 | .006 |

*Note.* Model was adjusted for maternal age, education, smoking during pregnancy, child sex, child gestational week at birth, and batch effects. Chr = chromosome; Position = genomic location; statf. = change in methylation; diff. = methylation of difference; $p_{FDR}$ = FDR corrected $p$ value; CpG = cytosine-phosphate-guanine; FDR = False discovery rate; DMPs = differentially methylated positions.

### *Associations in Childhood and Adolescence*

We did not identify DMPs or regions in relation to mode of birth in 7-year-olds and 15- to 17-year-olds ($p_{FDR} > .050$). When considering the effects of variables representing the postnatal care environment (i.e., breastfeeding and mother–infant bonding), no significantly differentially methylated sites emerged ($p_{FDR} > .050$) and no significant DMRs were found ($p_{FDR} > .050$).

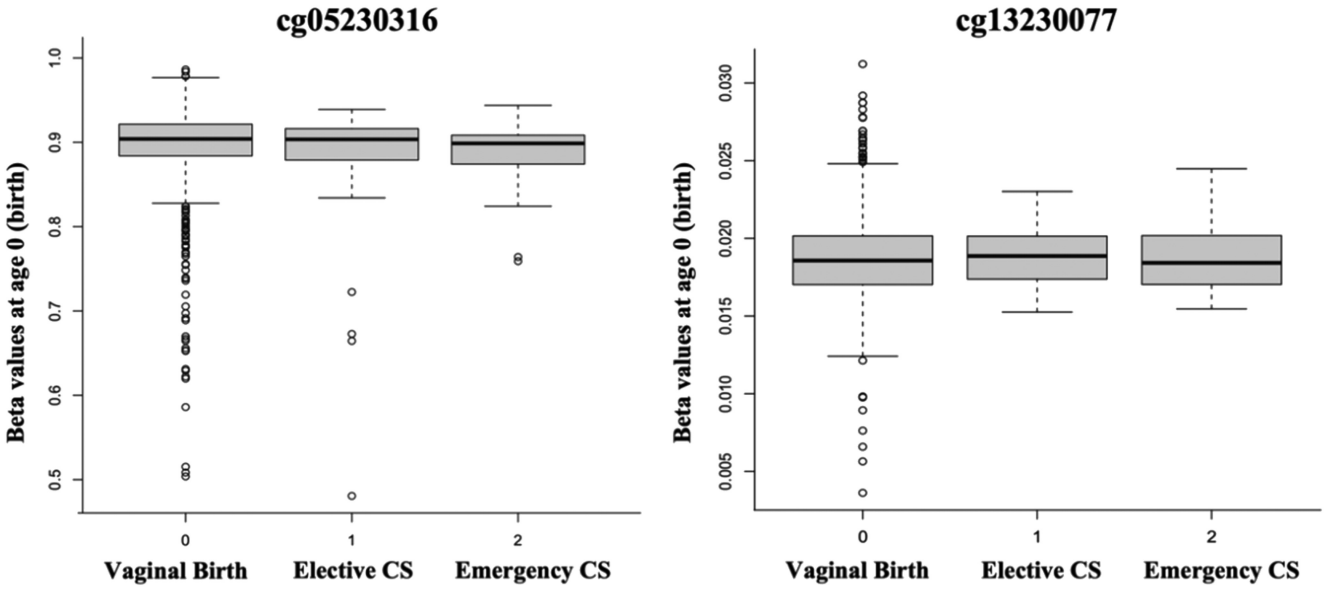
Follow-up analyses examining the predictive effects of mode of birth and the postnatal care environment as well as interactions between the mode of birth and postnatal care environment for individual CpG site DNAm at age 7 and 15–17 were conducted for the CpGs identified as significantly differentially methylated at birth in the EWAS. Neither breastfeeding or mother–infant bonding nor the interaction between mode of birth and the postulated moderators predicted DNAm at the CpG sites examined ($p > .050$; Table 4).

## Genomic Location of the Differentially Methylated Sites

Results showed that zero GO terms were enriched based on the 450K analysis and the differentially methylated sites did not overlap with any KEGG pathways. Examination of the significant CpGs in

**Figure 2**

*Box Plots of Beta Values at the cg05230316 and cg13230077 According to Mode of Birth*




*Note.* DNA methylation at birth at cg05230316 (left plot) and cg13230077 (right plot). For cg05230316, linear regression results suggested the ELCS group differed marginally significantly from the VB group ($p = .079$). No other group differences emerged for cg05230316 and cg13230077 in linear regression analyses ($p > .432$). CS = cesarean section; ELCS = elective cesarean section; VB = vaginal birth.

the EWAS catalog showed that while cg05230316 was not related to a specific gene, DNAm changes at this site have previously been linked to age (Mulder et al., 2021). The CpG site cg13230077 was related to the *Zn-regulated GTPase metalloprotein activator gene 1E (ZNG1E)* and *1C* (*ZNG1C*) and DNAm changes here have also been related to age (Mulder et al., 2021). *ZNG1E/C* encodes the Zinc Regulated GTPase Metalloprotein Activator 1E/1C, a protein which might enable adenosine triphosphate binding activity and plays an important role in regulating cellular zinc (Zn) homeostasis, with potential implications in various physiological and pathological processes. Diseases that have been associated with *ZNG1E* include intellectual developmental disorder, autosomal dominant 2, and hereditary spastic paraplegia 51 (EWAS Catalog, 2023; Gene Cards, 2023).

**Table 3**

*Linear Regression Results for Methylation at Individual CpG Sites in Cord Blood as the Outcome*

| CpGs from EWAS | Predictor | β | *SE* | *p* |
|---|---|---|---|---|
| cg05230316 | VB versus ELCS | −.017 | 0.010 | .079 |
| | VB versus EMCS | −.007 | 0.011 | .552 |
| | EMCS versus ELCS | −.010 | 0.014 | .467 |
| cg13230077 | VB versus ELCS | .0004 | 0.0005 | .432 |
| | VB versus EMCS | .00005 | 0.0005 | .931 |
| | EMCS versus ELCS | .0003 | 0.0007 | .644 |

*Note.* Model was adjusted for maternal age, education, smoking during pregnancy, child sex, child gestational week at birth, and batch effects. Two individual covariates (sex and batch) significantly predicted DNAm ($p < .050$) in both regressions. VB = vaginal birth; ELCS = elective cesarean section; EMCS = emergency cesarean section; CpG = cytosine-phosphate-guanine; CpGs = cytosine-phosphate-guanine dinucleotides; EWAS = epigenome-wide association studies; DNAm = DNA methylation.

## Discussion

The goal of this study was to investigate the effect of different modes of birth (VB, EMCS, ELCS) on the offspring's DNAm (CpGs and DMRs) at birth and at 7 and 15–17 years of age. Furthermore, we aimed to examine possible buffering effects of breastfeeding and mother–infant bonding on the association between mode of birth and the offspring's epigenome in childhood and adolescence. Based on analysis of the ALSPAC cohort, our findings suggest that mode of birth has a very small effect on offspring DNAm in the immediate postpartum period (i.e., in cord blood), yet we found no evidence for long-lasting effects at age 7 and 15–17 years. Results also provided no support for the contention that postnatal environmental nurturing factors might moderate the impact of mode of birth on offspring DNAm in childhood or adolescence.

### Epigenetic Effects of Mode of Birth in Cord Blood

Firstly, EWAS results showed that two CpGs (cg05230316; cg13230077) were differentially methylated in cord blood in relation to mode of birth. Subsequent regression analyses comparing (a) VB versus ELCS, (b) VB versus EMCS, and (c) EMCS versus ELCS revealed no evidence for a significant difference in DNAm. Regarding cg05230316, while only marginally significant, the effect was moderately suggestive of DNAm being lower in children born by ELCS compared with VB. Furthermore, to uncover the functional and biological relevance of the epigenetic effects associated with mode of birth, the present study was the first that also examined DMRs in the context of mode of birth. Results did not provide evidence for an association between mode of birth and DMRs in cord blood.

**Table 4**
*Linear Regression Results for the Predictive Effects of Mode of Birth, Breastfeeding, and Mother–Infant Bonding*

| | | Age 7 | | | Age 15–17 | | |
|---|---|---|---|---|---|---|---|
| CpGs from EWAS | Predictor | β | *SE* | *p* | β | *SE* | *p* |
| cg05230316 | VB versus ELCS | .010 | 0.007 | .156 | .012 | 0.010 | .230 |
| | VB versus EMCS | .001 | 0.008 | .901 | .004 | 0.012 | .757 |
| | EMCS versus ELCS | .009 | 0.010 | .390 | .009 | 0.015 | .567 |
| | Breastfeeding | .002 | 0.004 | .619 | .006 | 0.006 | .352 |
| | Mother–infant bonding | −.0001 | 0.0004 | .727 | −.0006 | 0.0006 | .367 |
| cg13230077 | VB versus ELCS | −.001 | 0.001 | .100 | .0004 | 0.0004 | .425 |
| | VB versus EMCS | −.0002 | 0.001 | .791 | −.00004 | 0.0005 | .931 |
| | EMCS versus ELCS | −.0007 | 0.0008 | .365 | .0004 | 0.0007 | .547 |
| | Breastfeeding | .00001 | 0.0003 | .973 | .0002 | 0.0003 | .391 |
| | Mother–infant bonding | .00002 | 0.00003 | .627 | .00003 | 0.00003 | .320 |

*Note.* VB = vaginal birth; ELCS = elective cesarean section; EMCS = emergency cesarean section; CpGs = cytosine-phosphate-guanine dinucleotides; EWAS = epigenome-wide association studies.

Previous studies have been inconsistent regarding the epigenetic effects associated with mode of birth in cord blood. The only other study to date investigating the three modes of birth simultaneously examined global DNAm of cord blood-derived leukocytes and found no differential associations with mode of birth when adjusting for maternal age, maternal smoking, and infant gender (Virani et al., 2012). Similarly, Franz et al. (2014) also found no differences in global DNAm associated with mode of birth (VB vs. ELCS). The authors then examined 96 single candidate genes related to the immune system and found *ELA2* and *IRF1* to be hypermethylated in infants born by ELCS compared to those born by VB. This finding is consistent with the results from an EWAS study by Chen et al. (2021), who found that infants born by ELCS had more locus-specific hypermethylation in genes related to the immune system (e.g., maturation and normal B cell function) compared with VB. Another EWAS study found 343 DMPs because of mode of birth (Almgren et al., 2014) and in contrast to Chen et al. (2021), the majority showed reduced DNAm in infants born to ELCS compared with VB. Taken together, available evidence is very heterogeneous as to whether mode of birth affects offspring DNAm, and if so, in which direction (i.e., lower or higher levels) methylation differences occur.

Our results extend these previous investigations, all of which, with the exception of Virani et al. (2012), compared only VB and ELCS, by also including EMCS. Our finding that differences in methylation occurred in ELCS compared with VB, but not in EMCS, underscores previous research (e.g., Virani et al., 2012) and further provides a first indication that the experience of physiological labor, which is lacking in ELCS compared with EMCS (as defined in the present study) and VB, may be a crucial factor for epigenetic remodeling at birth (Dahlen et al., 2013; Tribe et al., 2018).

This investigation did not find any GO terms associated with specific genes or regions, and there was a lack of overlap with KEGG pathways, possibly because of the small number of CpGs identified in EWAS. However, it is also possible that the differentially methylated CpGs found in this study have not yet been extensively studied for their biological function and are therefore not yet covered by the currently annotated GO terms and KEGG pathways. Furthermore, it is possible that differentially methylated sites have diverse functions and therefore may not be easily assigned to a single KEGG pathway. In addition, the screening tool used to quantify DNAm (Illumina Infinium Human Methylation 450 K) has limited coverage and resolution of the epigenome compared to newer technologies that provide a more accurate representation of differentially methylated sites with known biological relevance. Therefore, it would be interesting to investigate current research questions using DNAm data that provide an even higher methylation coverage, such as with the 850 K (Moran et al., 2016).

Results for differentially methylated CpGs differed in terms of statistical significance between EWAS and linear regression analyses. The DMRcate package used for EWAS is computationally efficient but does not take into account the correlation between neighboring sites (Lent et al., 2021), whereas the "lm" function in the "MASS" package applies different corrections, which may explain the discrepant results (Venables et al., 2002). Although our study found very small effects of mode of birth on DNAm, the fact that cg05230316 was moderately suggestive of being differentially methylated in infants born by ELCS compared with infants born by VB aligns with the EPIIC hypothesis, which postulates that labor may exert a physiologically optimal form of stress on the offspring. Alterations in cortisol and oxytocin levels and a less pronounced catecholamine surge present in ELCS could manifest in a reprogramming of the offspring's genome at birth (Dahlen et al., 2013; van den Berg et al., 2001). The two significant CpGs in the EWAS catalog were related to age and cg13230077 was additionally related to a relevant protein coding gene which modulates adenosine triphosphate binding activity.

Within the scope of the EPIIC hypothesis, Dahlen et al. (2016) assume that labor has an epigenomic effect on genes related to the immune system, weight regulation, and distinct tumor-suppressor genes. Previous literature reveals that alterations of the immune system in the perinatal period, especially cytokine production, act as a vulnerability factor for neurodevelopmental and psychological disorders throughout the life span (Bilbo & Schwarz, 2009; Tanabe & Yamashita, 2018). Our study found limited effects of mode of birth on DNAm and does not replicate previous findings in genes relevant to the immune system. While earlier studies show a link between DNAm in cord blood and emotional regulation and neurodevelopment (e.g., ADHD; Barker et al., 2018; Cecil et al., 2018), there is currently insufficient evidence to postulate a role for our differentially methylated CpGs in later psychological development.

## Epigenetic Effects of Mode of Birth in Childhood and Adolescence and the Postnatal Care Environment

The second major aim of this study was to examine for the first time whether mode of birth is associated with epigenetic alterations beyond infancy, in childhood and adolescence. Our EWAS results revealed no differentially methylated CpGs and no DMRs linked with mode of birth at ages 7 and 15–17 years. Regression analyses confirmed that the CpGs significantly differentially methylated immediately upon birth did not continue to be statistically associated with mode of birth in childhood and adolescence, thereby indicating a lack of persistence beyond infancy. It must be noted that we analyzed the association between mode of birth and DNAm only immediately after birth and then in childhood and adolescence, leaving a considerable developmental period unobserved. Our results also imply that while differential DNAm patterns associated with ELCS may occur directly after birth, these appear to be compensated to the extent that DNAm differences are no longer statistically significant in childhood and adolescence. Following this rationale, Schlinzig et al. (2009) measured DNAm in cord blood and in peripheral blood 3–5 days after birth. They found higher global leukocyte DNAm in cord blood of infants born by ELCS versus VB. However, DNAm levels had decreased at 3–5 days after birth in ELCS-born infants, whereas DNAm patterns remained stable in VB-born infants, showing that DNAm can be very dynamic in the first days of life. Therefore, it would be valuable to investigate the effects of mode of birth in infancy and toddlerhood, as the rate of change of DNAm is higher in early life compared to later in life (Jones et al., 2015).

When interpreting our results, it is important to consider that the methylation pattern in cord blood differs from that in peripheral blood at 7 and 15–17 years of age not only in terms of developmental stage but also in terms of cell composition and exposure to environmental factors. Cord blood has a higher proportion of hematopoietic stem cells compared to a more differentiated population of blood cells in peripheral blood (Yasui et al., 2003). Also, cord blood samples may be contaminated in 2%–20% of cases with maternal white blood cells that cross the placenta during pregnancy or are mixed at the time of collection (Morin et al., 2017). As we cannot exclude that some of the effects found in our study may be influenced by possible contamination of cord blood samples with maternal blood cells, future research would benefit from using a panel of markers to identify and remove contaminated samples (Morin et al., 2017).

Our investigation did not support evidence that aspects of the postnatal care environment, namely breastfeeding and mother–infant bonding, might impact the effect of mode of birth on offspring DNAm at ages 7 and 15–17 years. Neither including these variables as covariates in the EWAS nor examining them directly in linear regressions as predictors and moderators for differentially methylated sites identified in cord blood showed the buffering effect we hypothesized. These results suggest that whether or not children had ever been breastfed and regardless of the level of bonding their mother felt at 8 months postpartum, this did not alter the predictive effects of mode of birth on offspring DNAm. Our results thereby do not support the social buffering hypothesis, which postulates that the presence of a conspecific can buffer the negative impact from potential adverse life events on physiological aspects (Gunnar & Hostinar, 2015). A point to consider when interpreting our results is the considerable interval between the measurement of moderators and DNAm in childhood and adolescence. Although previous research has shown that aspects of the early mother–infant relationship and breastfeeding have far-reaching physiological and psychological effects beyond infancy (de Cock et al., 2016; Krol & Grossmann, 2018), it is possible that early mother–infant bonding or breastfeeding significantly predict DNAm and moderate its association with mode of birth when DNAm is measured at earlier developmental stages.

Research documenting the impact of ELCS on public health is accumulating. When medically necessary, for instance in case of breech presentation, having had previous CS, placenta or vasa previa, ELCS should be indicated, as it can reduce maternal and neonatal mortality and morbidity (Coates et al., 2020; World Health Organisation, 2015). However, the number of ELCS performed upon maternal request without indication has increased in developed and emerging countries (Mylonas & Friese, 2015). The available evidence points to several negative effects that ELCS can have on children. Compared with VB, ELCS might be linked to health problems, for example, childhood-onset Type 1 diabetes (Cardwell et al., 2008); overweight and obesity (H. Li et al., 2013), a higher risk for delayed motor development (Grace et al., 2015), autism spectrum disorder, and ADHD (Zhang et al., 2019). Based on our findings, it seems that after ELCS very subtle alterations in DNAm may occur, which however do not persist into childhood and adolescence and do not interact with factors of the postnatal care environment investigated in this study (i.e., breastfeeding and mother–infant bonding). Given the small effects found in our study, future research should investigate other mediating mechanisms besides DNAm for the association between mode of birth and later developmental consequences.

However, based on this investigation, we cannot rule out the possibility that early epigenetic effects may trigger developmental cascades, that is, that the effects of mode of birth on the epigenome are short-lived but might indirectly affect other developmental outcomes in the long term. CS remains a factor that may increase risk for suboptimal child health and development. Hence, our study highlights the need to investigate other possible epigenetic and nonepigenetic mechanisms responsible for long-lasting developmental effects. For example, it is possible that the microbial gut colonization is altered in neonates born by CS compared with VB (Jakobsson et al., 2014). Future research should focus on investigating differences in the microbiome related to mode of birth and the interplay with the presence/duration of labor itself on epigenome anomalies and later developmental outcomes (Dahlen et al., 2016). Furthermore, it should be considered whether the labor was spontaneous or induced and whether the birth is premature, term, or postterm. Also, other epigenetic factors such as histone posttranscriptional modification, chromatin remodeling, or microRNA differences should be investigated additionally or simultaneously as possible mediating factors between CS and pathological phenotypes to enable a more complete understanding of how these modifications and their interplay may contribute to later developmental outcomes (Y. Li, 2021).

## Strengths, Limitations, and Ideas for Future Research

Strengths of this investigation include the fact that based on a detailed literature search, factors likely to affect both mode of birth and offspring DNAm were controlled for in all analyses and that we also controlled for factors of the postnatal care environment in the EWAS for ages 7 and 15–17 years. While EWAS results did not

change when adjusted for these variables, this approach increases confidence that the effects detected represent a true effect of mode of birth (Skelly et al., 2012). Further strengths include that we differentiated between different types of mode of birth and were the first to systematically examine DMRs as more biologically informative outcomes in relation to mode of birth (T. J. Peters et al., 2015). The large sample size of ARIES also represents a key strength in comparison to previous research; however, the composition of groups according to mode of birth was very uneven, highlighting the fact that the present study was not originally designed to explicitly test these research questions.

We note that the 8.4% CS in the ARIES cohort was below the UK average of 12.5% CS in 1990 (Black et al., 2005). Furthermore, the ARIES subsample differed from the larger ALSPAC sample regarding neonatal health (higher birth weight and gestational age in ARIES) and health-related behavior (e.g., lower percentage of smoking and higher breastfeeding rates in ARIES). Therefore, the specificity of the sample regarding these variables should be considered when extrapolating findings to the broader population. Further limitations include the fact that we were restricted to using cord blood and peripheral blood to examine DNAm, as is common in human epigenetic studies. However, blood is a complex tissue composed of different cell types with individual and distinct DNAm patterns. This heterogeneity was taken into account using the Houseman correction (Houseman et al., 2012). However, whole blood does not necessarily reflect DNAm patterns and changes in individual cell types and other tissues and organs, although it remains the most commonly used proxy (Husby, 2020; Reinius et al., 2012). Hence, the effects of such early stress may manifest in a cell type- or tissue-specific manner (e.g., Alt et al., 2010). It could be that mode of birth does affect DNAm in other relevant tissues across development; however, because of methodological and ethical constraints, we were limited to using human cord/peripheral blood and were not able to detect these. Finally, it should be considered that we did not consider several factors that may play an important role for distinguishing the epigenetic impact of mode of birth. These include not differentiating between unassisted and instrumental VBs, use of synthetic oxytocin for labor induction and augmentation (Kernberg & Caughey, 2017), and use of intrapartum antibiotics (Stokholm et al., 2013), which have been suggested to impact offspring DNAm (Kenkel et al., 2019; Vidal et al., 2013).

As this investigation used secondary data to examine the effects of mode of birth on offspring DNAm, future research should endeavor to investigate these relationships in a study designed specifically for this purpose. In detail, we recommend specific recruitment of individuals with different mode of births, as conducted by Chen et al. (2021), to ensure comparable number of individuals per mode of birth group. Furthermore, a study that considers the different modes of birth, also in the realm of VB, is needed to differentiate between instrumental and noninstrumental VB (Simms & Hayman, 2011), but also whether birth was induced or not and whether antibiotics were administered. Finally, a detailed assessment of maternal health status should be conducted to account for this factor in determining the mode of birth as well as later health outcomes of the offspring (Sandall et al., 2018).

## Conclusion

Our results suggest that whole-blood DNAm may be minimally altered in the immediate postnatal period in offspring born by different modes, although these effects do not persist into childhood or adolescence. These findings suggest that the experience of different modes of birth may not have an enduring effect on the epigenome across development, and that other pre- and postnatal influences and early life factors, as recently suggested (S. Li et al., 2022), may be of greater relevance for DNAm. While no enduring effect on DNAm in blood cells was found, we cannot rule out the possibility that mode of birth has long-lasting effects on developmental trajectories via other mechanisms. Future research should validate these findings in a sample with a higher proportion of CS and a distinction between instrumental and unassisted VB. Additionally, it should investigate alternative mechanisms other than epigenetics that may be responsible for the long-term health consequences of different modes of birth (Sandall et al., 2018), in particular prelabor CS.

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# Chapter 8

# Children's internalizing behaviour development is heterogeneously associated with the pace of epigenetic ageing

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As depicted in the previous Chapters, early life adversity regroups different types of external stressors exposure such as economic, environmental, social and even mental. As we have demonstrated, both parental implications either behavioural or genetic will influence the overall health status of their offspring. Nevertheless, a specific attention to the maternal influences showed considerable consequences i.e. epigenetic imprinting via DNA methylations. So far, we confirmed that maternal early life exposure either to economic hardship or early sexual experiences led to changes in the child's epigenetic marks, which then play a role in the regulation of genes from major BP such as oxidative phosphorylation.

DNAm is one of the consequence of ELA exposure and results in a gene transcription able to alter and shape the later health outcomes in life (Szyf & Bick, 2013). At birth, the DNAm variability depends on both genotype and prenatal environment, nevertheless external factors might play a major role as well. In addition to biological consequences, it has been demonstrated in the literature that DNAm can also induce mental emotional development alteration i.e. anti-social behaviour (Czamara et al., 2021). Worldwide, approximatively 14% of diseases are covered by neuropsychiatric disorders mostly due to mental disorders, depression or use of alcohol (Prince et al., 2007). Today, 1 out of 10 young adults reported suffering from at least one of mental disorders (Kieling et al., 2024). It is now recognised that mental disorders are susceptible to specific health conditions that can facilitate their development. Indeed, the WHO organisation claimed that there is "*no health without mental health*" as for example depression can induce both physical and social disability mediating physical health conditions. In addition, neuropsychiatric disorders lead to millions of deaths per year due to diseases complications i.e. suicide, infection or chronic diseases (Walker et al., 2015). We here refer to internalizing behaviours also known as internalizing symptoms such as physical problems (stomach-aches, headaches), depression or even anxiety. In adolescents they are associated with lower well-being levels where relationship's with parents is a major determinant (Guo et al., 2018; Luijten et al., 2021). Additionally, it has been demonstrated that those behaviours, consequences of early life adversity, can be associated with advanced physical ageing characterized as epigenetic age acceleration (EAA) (Copeland et al., 2019). EAA is associated with cancer and cardiovascular diseases, and can be predicted by life stressing events. In 2021, Tollenaar et al. confirmed associations between epigenetic ageing and psychiatric symptoms when investigating these associations at age 6 (Tollenaar et al., 2021). They highlighted that some children might be more susceptible to develop internalizing symptoms and present accelerated ageing, due to pre-existing genetic and environmental factors.

To better understand whether internalizing behaviours can trigger children's psychological and emotional development and consequently predict future mental disorders, we conducted a

Poisson quantile regression analysis. The following Chapter will investigate the heterogeneity in the trajectories of internalizing behaviours based on a longitudinal latent class analysis using at least two time points of measure.

Indeed, as we already suspected in the previous Chapters, it is possible than later life trajectories can be anticipated following the overall early life adversity experience as well the parental genetic predispositions. This Chapter will demonstrate the dynamic relationship between biological age acceleration and internalizing behaviours confirming the DOHaD theory linking early life adversity and later life consequences.

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# Children's internalizing behavior development is heterogeneously associated with the pace of epigenetic aging

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ABSTRACT

*Background:* Internalizing behaviors are an indicator of children's psychological and emotional development, predicting future mental disorders. Recent studies have identified associations between DNA methylation (DNAm) and internalizing behaviors. This prospective study aimed at exploring the associations between pace of biological aging and the developmental trajectories of internalizing behaviors.

*Methods:* Participants were children from the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort (N = 974). Measures of DNA methylation were collected at birth, age 7 and ages 15–17. The pace of aging was estimated using the DunedinPoAm algorithm (PoAm). Internalizing behaviors reported by caregivers between ages 4 and 16 using the Strengths and Difficulties Questionnaire. To explore heterogeneity in the association between PoAm and internalizing behaviors we use Poisson quantile regression in cross-section heterogeneity and longitudinal latent class analysis over the childhood and adolescence.

*Results:* Internalizing behavior trajectories were identified: low-risk, childhood limited, late onset and early onset (persistent). Accelerated aging at birth was negatively associated with internalizing behaviors in early childhood but positively correlated during adolescence. Higher PoAm at birth increased chance of low-risk profile, while decreasing likelihood of childhood limited trajectory. PoAm at age 15 was negatively associated with childhood limited profile and positively linked to late onset trajectories. Associations were larger at higher values of internalizing symptoms.

*Conclusions:* The heterogeneity in the association between biological age acceleration and internalizing behaviors suggests a complex dynamic relationship, particularly in children with high or increased risk of adverse mental health outcomes.

## 1. Introduction

Mental disorders are among the leading causes of non-transmissible, chronic diseases and health disparities in adults (Prince et al., 2007), children, and adolescents worldwide (Baranne and Falissard, 2018; Kieling et al., 2011). Internalizing behaviors (or internalizing symptoms) are an early indicator of children's psychological and emotional development, predicting future mental disorders such as anxiety and depression (Liu et al., 2011). Adverse life experiences during childhood increase the risk for internalizing symptoms, although the precise biological and psychological pathways remain unclear (Barker, Walton et al., 2018). Evidence from longitudinal studies suggests that internalizing behaviors in 11-year-old children are prospectively associated with early substance use (age 14 years), and anti-social behavior and mental disorders in adulthood (Althoff et al., 2010; King et al., 2004).

Recent advances in epigenetics helped identified associations between children's emotional development and biological developmental pathways, particularly DNA methylation, an epigenetic process that regulates gene expression (Czamara et al., 2021; Khulan et al., 2014; Parade et al., 2021). For example, Barker, Cecil et al. (2018) showed that a methylation index for low-grade inflammation risk is correlated with internalizing behaviors during childhood.

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In the last decades, epigenetic clocks have been developed as global indices of DNA methylation to estimate biological age acceleration (AA) from chronological age (Horvath and Raj, 2018; Marioni et al., 2019). We define AA as individuals who are biologically *older,* relative to their chronological age. To address the biological mechanisms underpinning AA, a new generation of epigenetic clocks have been designed involving multiple biological and clinical biomarkers of ageing rather than chronological age alone (Belsky et al., 2018; Levine et al., 2018). Belsky et al. (2020) proposed a novel method for quantifying accelerated aging (hereafter, the PoAm algorithm standing for Dunedin (P)ace (o)f (A)ging (m)ethylation) using longitudinal data over a wide array of biological markers tracking organ integrity in young adults (from age 26–38 years). Unlike the earlier epigenetic clocks measuring biological age in years, PoAm calculates relative age acceleration (or deceleration), with respect to the reference population. PoAm is strongly associated with cognitive functioning and physical decline in adults from the Dunedin cohort at age 45. PoAm has been recently validated in a pediatric sample, showing positive associations between economic disadvantage and accelerated aging, in a sample of U.S. children and adolescents (Raffington et al., 2021). Moreover, in E-Risk longitudinal data, Belsky et al. (2020) found significant associations between early childhood adversity (i.e. poverty and victimization) and AA at age 18.

Based on the evidence from Simpkin et al. (2017), Barker, Cecil et al. (2018) and Ellis et al. (2019), DNAm is associated to multiple biological processes, including cortisol levels and the timing of physical development, both associated with the emergence of internalizing behaviors. Moreover, the potential effects of DNAm on psychopathology risks can be both cumulative and time-sensitive, varying significantly in the population (Barker, Walton et al., 2018). Therefore, as noted by Barker, Walton et al. (2018), environmental exposures imprinted in the pace of epigenetic aging are likely to be linked dynamically with the developmental trajectories of internalizing behaviors, due to both the timing of exposure and cumulative hazard. Recent studies have established direct associations between AA and higher internalizing symptoms in children, using the first-generation clocks in cross-sectional data (Sumner et al., 2019; Tollenaar et al., 2021). Using Horvath's (2013) clock at a single time point (age 6), Tollenaar et al. (2021) showed that epigenetically older children were more likely to exhibit high internalizing symptoms at age 30 months. Also, AA was positively associated with internalizing behaviors between the ages of 8 and 10 years. Using the same epigenetic clock, Sumner et al. (2019) found that early experiences of high stress (e.g. violence) were associated with accelerated epigenetic aging in children, which in turn was positively linked to depressive behaviors and early pubertal stage during adolescence. Recently, emotion regulation skills, such as self-control, have been shown to be prospectively associated in a similar manner with a deceleration in ageing, using the PoAm clock (Richmond-Rakerd et al., 2021). While prior research had established associations between DNAm and internalizing symptoms during childhood, these studies do not account for developmental trajectories. As shown in Barker and Maughan (2009) and Barker, Walton et al. (2018), the developmental trajectory of internalizing behaviors in childhood and adolescence is both highly dynamic and heterogeneous in any given population. Similarly, environmental exposures are imprinted in the DNAm along the life course, which in turn can shape developmental trajectories, increasing (or decreasing) psychopathology risks (Barker, Walton et al., 2018). Therefore, relying in a longitudinal cohort with repeated measurements is key to understand the dynamic heterogeneity between DNAm and internalizing behaviors.

The current study extends the literature by investigating the heterogeneity of cross-sectional and longitudinal associations between the epigenetic pace of aging and internalizing behavior trajectories from birth to age 17 years, accounting for concurrent environmental stressors and demographic characteristics. We believe understanding the range of associations between AA and internalizing behaviors in the population is key to determine at-risk groups and the severity of environmental risks that are imprinted in DNAm. We explore the degree of heterogeneity in the association between PoAm and internalizing symptoms in cross-section at different quantiles of the internalizing sub-scale score for given ages, as well as the impact of PoAm on latent trajectories of internalizing behaviors during childhood and adolescence.

## 2. Methods

### 2.1. Participants

The Avon Longitudinal Study of Parents and Children (ALSPAC) survey, also known as "The Children of the 90 s", is an ongoing population study to understand the environmental influences on different aspects of childhood development. The study website contains details of all the data that is available through a fully searchable data dictionary and variable search tool. (http://www.bristol.ac.uk/alspac/). For detailed cohort information see (Fraser et al., 2013). A brief cohort description is provided in Supplemental Appendix A, Section A. Informed consent was obtained from all participants. Consent for biological samples was collected in accordance with the Human Tissue Act (2004).

We restricted our analytical sample to the ALSPAC subgroup of families that provided epigenetic data (1018 mother-child pairs) at birth for mothers and newborns, for the children only at age 7, and then again for mother-child dyads at child age 15–17. We excluded observations where it was not possible to compute PoAm, or those where caregivers did not provide information in the behavioral questionnaire to build measures for internalizing behaviors. Our final analytic dataset, excluding 44 removed samples, consisted of 974 participants with epigenetic profiles and behavioral questionnaire data from at least one time point.

### 2.2. DNA methylation

DNA methylation values were obtained from the ALSPAC consortium. The procedure for collecting and storing biological samples has been described elsewhere (Alfano et al., 2019; Sharp et al., 2015). Briefly, following DNA extraction from peripheral venous blood, bisulphite modification was performed with the Zymo EZ DNA MethylationTM kit (Zymo, Irvine, CA, USA). DNA methylation levels were measured using the Infinium HumanMethylation450 BeadChip (Relton et al., 2015). Initial data processing by the ALSPAC consortium was performed in R (version 3.2.4) with the *meffil* library (https://github.com/perishky/meffil). Quality control included functional normalization and correction for cellular heterogeneity using the Houseman procedure, leaving 4854 individual samples for subsequent analysis. Full description to the quality control procedure is available in Supplemental Appendix A. In our analytical sample, we used the ALSPAC technical guide to account for population stratification, excluding duplicates and individuals with non-Caucasian or missing ethnicity (261 observations).

### 2.3. Internalizing behaviors

The child mental health measure used in the present study was derived from the internalizing sub-scale of the Strengths and Difficulties Questionnaire (SDQ). The SDQ is a behavioral-screening questionnaire for children, consisting of 25 questions capturing dimensions of social and emotional development (Goodman, 1997). A full list of the items included in the SDQ questionnaire appears in Supplemental Appendix A, Section B. Ratings of the child's SDQ were provided by the main caregiver (typically the mother) when children were approximately 4, 6, 8, 9, 11, 13 and 16 years old. The 25 items in the SDQ questionnaire correspond to five sub-scales addressing emotional symptoms, peer symptoms, conduct symptoms, hyperactivity/inattention and pro-social behavior. For each sub-scale (ranging from 0 to 10 points), a reference cut-off point of five has been suggested for at-risk groups (Durbeej et al., 2019). The internalizing sub-scale is the sum of the questions related to

the emotional and peer symptoms sub-domains, as defined by (Goodman, 2001) and (Goodman et al., 2010). We only focus on internalizing (and not externalizing) symptoms, as they provide a proxy for psychopathology risks in adult life (Barker, Walton et al., 2018).

### 2.4. Data analyses

Pre-processed Infinium array methylation beta values were used to calculate the Dunedin PoAm pace of biological aging (Belsky et al. 2020) using the library DunedinPoAm38 (https://github.com/danbelsky/DunedinPoAm38) in R (version 4.0.2.). The PoAm algorithm is based on 46 CpG sites, obtained from fitting DNAm data with elastic-net over a standardized index which summarizes 18 different biomarkers at multiple time points during adulthood (ages 26–38). The PoAm epigenetic clock tracks multiple biological processes in a longitudinal sample to estimate acceleration (or deceleration) in organ systems integrity, while most available clocks are based in cross-section comparisons, focusing mostly on predicting chronological age and mortality. The PoAm has been recently contrasted in a sample of children, showing significant associations with several risk factors and biological markers (Raffington et al., 2021).

For comparison, we also calculated the clocks from Horvath (2013), Hannum et al. (2013) and Levine et al. (2018), using the R libraries wateRmelon, limma and minfi (Pidsley et al., 2013). We also estimated competing gestational DNAm clocks (Knight, Lee, Bohlin, Mayne) at birth using the *methylclock* library in R (Pelegí-Sisó et al., 2021).

To examine heterogeneity in the trajectories of internalizing behaviors over time, we conducted a longitudinal latent class analysis of the internalizing symptoms score. Longitudinal latent class models allow to infer multiple subpopulation trajectories from a sample of longitudinal data. For a detailed description of the estimation methods see Marcoulides and Schumacker (2001). The adjusted Bayesian information criteria (BIC) and entropy index were used to determine the number of subgroups (van der Nest et al., 2020).

We estimated mean associations and heterogeneity in cross-section using Poisson regression, as shown to be consistent even when the model is misspecified, providing reliable estimates without imposing additional distribution assumptions (Wooldridge, 1999). Model selection included a wide set of covariates that could be expected to be linked to internalizing behaviors, including family history, household demographics and socio-economic characteristics, child stimulation and diet, and other aspects of birth and early life. We distinguished between baseline covariates determined at or before birth (parental education, gestational age, parity, sex, maternal age at birth, tobacco exposure, and diet quality index), environmental covariates associated with exposure before age 4 (parenting score and breastfeeding), and longitudinal covariates that were measured prior to the SDQ subscale in each period (financial difficulties, income and maternal depression). In our sensitivity analysis we also included polygenic risk scores (PGS) for depression and non-cognitive skills based on previous work by Demange et al., 2021, Turley et al., (2018), and computed as described in Menta et al. (2021). Supplemental Appendix A, Sections C and D contain the description of the covariate selection process and model specification.

Attrition in the sample reporting SDQ questionnaires between ages 4 and 16 was less than 17 %. To account for attrition in the sample over time, we used the Inverse Propensity Weighting (IPW) method, both for latent models and mean associations (Wooldridge, 2007). After estimating the regression parameters, partial effects were calculated at the means of the covariates. Standard errors were estimated using the bootstrap method, with 1000 repetitions.

We estimated quantile partial effects across the distribution of internalizing symptoms for PoAm at birth, age 11 and age 16, using the method proposed by Machado and Silva (2005) to address count data (such as discrete scales). Each discrete point in the distribution is jittered randomly to obtain a continuous cumulative distribution. In each regression, we set the number of jittered samples to 1000 repetitions, and then estimate the partial effects at each given quantile. Similarly, we explored the heterogeneity over time by modelling class membership for each latent trajectory of internalizing behaviors as a function of PoAm at birth, age 7 and age 15, using a multinomial Logit model, after correcting for potential class misclassification (Asparouhov and Muthén, 2014).

A detailed description of the all methods and formulas used in our analyses is available in Supplemental Appendix A, Section D. All statistical analyses were conducted on Stata, version 16 and R v4.1.1. Code scripts are available upon reasonable request.

## 3. Results

### 3.1. Latent trajectories on internalizing behaviors

Table 1 summarizes the distribution of internalizing behaviors at every period measured. Mean values and dispersion decrease as children reach adolescence. Fig. 1 shows the results from the estimated trajectories based on longitudinal latent class analysis. The model with four classes was considered the best fit based on adjusted BIC criteria and likelihood ratio tests (fit indices are reported in Supplemental Appendix A, Section D). Over a quarter of all children showed minimal internalizing behaviors over time (low-risk). A similar proportion of children showed increasing internalizing behaviors during childhood and adolescence (late onset). Similarly, one of every four children showed higher values of internalizing symptoms at age 4, but these behaviors decreased sharply in early childhood (limited). Finally, almost 20 % of participants exhibited large and stable values of internalizing behaviors, only decreasing slightly in late adolescence (early onset).

### 3.2. Epigenetic age

PoAm does not predict biological age in years, but rather provides a continuous measure of acceleration, relative to the overall population of children observed at the same time point. It is important to note, however, that PoAm in each period reflects the population heterogeneity in accumulated DNAm up to the measurement point. A value of one in the PoAm indicates neither age acceleration nor deceleration relative to the same-age peers, with values lower than unity indicating deceleration, and values greater reflecting acceleration (see Table 1). In our subset of the ALSPAC cohort, PoAm was normally distributed for each timepoint, albeit the variance decreased significantly after birth (see Fig. 2). Preliminary analyses showed differential associations between PoAm and internalizing behaviors, across time points, with higher significance, relative to other DNAm clocks (Knight, Lee, Horvath, Hannum and Levine). However, we note that Levine and Hannum clocks provide

**Table 1**
Descriptive statistics for the estimation sample.

| Internalizing behaviors (score 0–20) | | | | | |
|---|---|---|---|---|---|
| Age | Mean | SD | p10 | p90 | N |
| 4 | 2.66 | 2.95 | 0 | 6 | 913 |
| 6 | 2.23 | 2.15 | 0 | 5 | 902 |
| 8 | 2.60 | 2.46 | 0 | 6 | 874 |
| 9 | 2.32 | 2.40 | 0 | 6 | 909 |
| 11 | 2.32 | 2.61 | 0 | 6 | 875 |
| 13 | 2.27 | 2.20 | 0 | 5 | 843 |
| 16 | 2.12 | 2.23 | 0 | 5 | 753 |

| Dunedin Pace of Aging | | | | | |
|---|---|---|---|---|---|
| **Age** | **Mean** | **SD** | **Min** | **Max** | **N** |
| birth | 0.97 | 0.09 | 0.52 | 1.27 | 906 |
| 7 | 0.99 | 0.06 | 0.72 | 1.23 | 972 |
| 15 | 0.99 | 0.07 | 0.67 | 1.29 | 970 |

Notes: Statistics include all children with observations in each period. SD stands for standard deviation, p10 and p90 indicate percentiles 10 and 90, respectively.

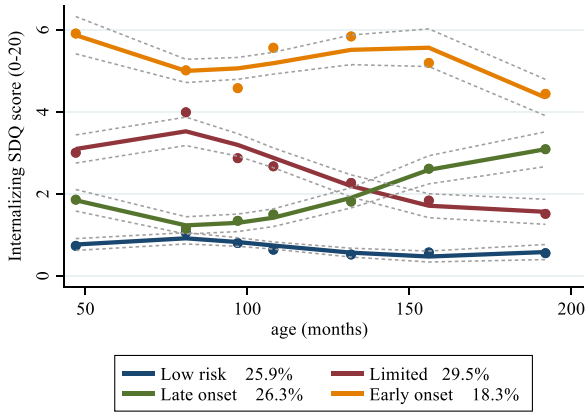


**Fig. 1.** Trajectories of internalized behaviors based on longitudinal latent class analysis, Notes: Estimated means (dots) and latent classes with longitudinal latent class analysis (with 95% confidence intervals in dashed lines).

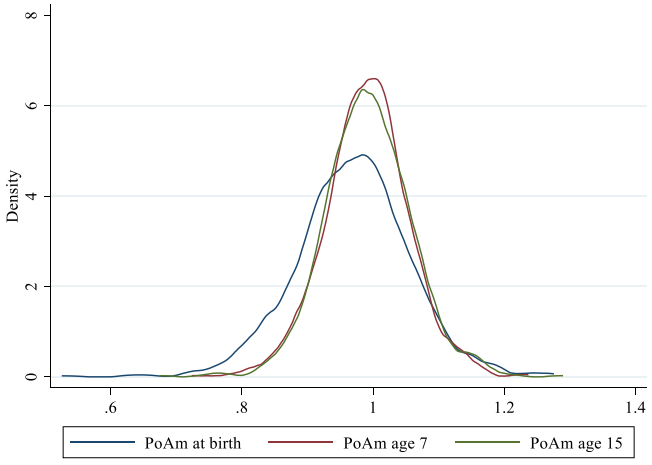


**Fig. 2.** Estimated kernel densities for PoAm at different ages, Notes: Kernel density estimated for each age at measurement using Epanechnikov method, with bandwidth 0.02.

similar information regarding internalizing behaviors at age 4. Supplemental Appendix A, Section E contains a summary of the comparison across different epigenetic clocks.

### 3.3. Mean associations between PoAm and internalizing behaviors

We conducted extensive model selection based on the environmental factors that could potentially affect the association between DNAm and internalizing behaviors (see Supplemental Appendix A, Sections C and D). After excluding variables that did not improve model fit, our preferred specification included parity, parental education, gestational age, breastfeeding, home stimulation index at age 3, financial difficulties, income and maternal depression. For more details on the variables used in each regression and the full estimates see Supplemental Appendix B.

Fig. 3 shows the estimated mean partial effects between PoAm and internalizing symptoms, based on the results from the Poisson regressions, with one panel for each measure of PoAm (values reported in Table 2). Since PoAm is standardized, our results show the potential impact of accelerating one SD above the mean on the outcome. Accelerated aging at birth reduced the frequency of internalizing behaviors at age 4, on average by 0.31 units (95 % CI −0.55, −0.12; Table 2). PoAm at birth correlated positively with internalizing behavior scores at age 11 (0.19, 95 % CI −0.02, 0.40; Table 2). At age 7, there was no association

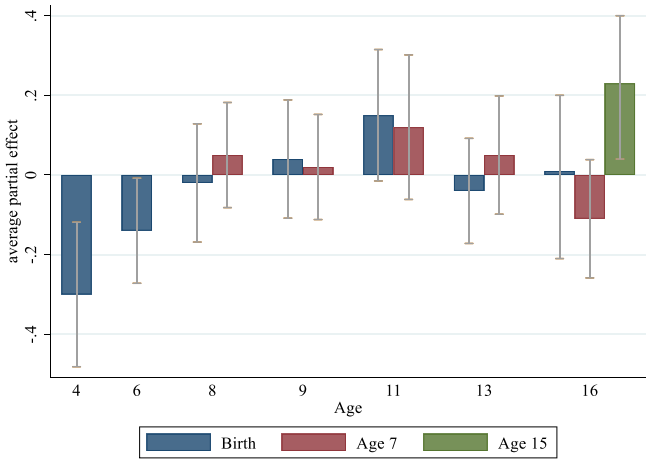


**Fig. 3.** Estimated partial effects of PoAm on internalizing behaviors, Notes: Estimated partial effects based on Poisson models adjusted by parental education, gestational age, mother's age at birth, parity, gender, diet score during pregnancy, breastfeeding, tobacco exposure at birth, home stimulation index at age 3, income, financial difficulties, and maternal depression. 90 % bootstrap confidence intervals with 1000 repetitions.

between PoAm and internalizing behaviors. Finally, accelerated aging up to age 15 positively correlated with internalizing behavior scores at age 16 (0.22, 95 % CI 0.04, 0.40; Table 2). We conducted extensive sensitivity analysis to validate the robustness of our results (details available in Supplemental Appendix A, Section G). Our estimates remained robust to model selection over a wide range of covariates, and the inclusion of polygenic risks scores as controls.

### 3.4. Heterogeneity analysis

Fig. 4 shows heterogeneity based on the severity of internalizing behaviors, by estimating the quantile partial effects for PoAm at different deciles of the cumulative distribution function. A low quantile indicates smaller scores of internalizing behaviors, while a high quantile refers to higher scores. In all cases (ages 4, 11 or 16), effect sizes are small and non-significant for children with few or no internalizing behaviors. For those individuals at the top half of the distribution (thus higher values of internalizing symptoms), effects sizes can be almost double, compared to the average partial effects. Additional analyses using logistic regression showed that PoAm did not predict the likelihood of having zero versus any degree of internalizing behaviors (incidence), but rather symptom intensity, which is consistent with the quantile analysis.

Table 3 shows the predicted change in the probability (percent points) to belong to each latent trajectory of internalizing behaviors due to an increase of PoAm of one SD, as well as the relative risk ratios, using the low-risk group as reference. At birth, higher PoAm increases the probability to be classified as low-risk (0.03, 95 % CI 0.00, 0.06), while decreasing the probability to be in the limited group (−0.05, 95 % CI −0.08, −0.02). Higher PoAm, however, does not change the odds to be assigned to the early onset class, relative to the low-risk group. Using the PoAm at age 7, there was no correlation between AA and class membership. At age 15, age accelerated children were more likely to be in the early onset group (0.02, 95 % CI 0.00, 0.05), but less likely to belong to the limited group (−0.03, 95 % CI −0.06, −0.01). Results of the multinomial logistic regressions are included in Supplemental Appendix A, Section H.

## 4. Discussion

Our results, based on prospective analysis of the ALSPAC cohort are consistent with a dynamic and complex longitudinal relationship

**Table 2**
Average partial effects of PoAm on internalizing behaviors.

| | PoAm at birth | | | PoAm age 7 | | | PoAm age 15 | | |
|---|---|---|---|---|---|---|---|---|---|
| Age | Partial effect | 95 % CI | p-value | Partial effect | 95 % CI | p-value | Partial effect | 95 % CI | p-value |
| 4 | -0.31 | [−0.55 −0.12] | *0.02* | | | | | | |
| 6 | -0.16 | [−0.35 0.04] | *0.11* | | | | | | |
| 8 | -0.07 | [−0.26 0.12] | *0.48* | 0.05 | [−0.11 0.21] | *0.54* | | | |
| 9 | 0.01 | [−0.20 0.21] | *0.95* | 0.02 | [−0.17 0.21] | *0.81* | | | |
| 11 | 0.19 | [−0.02 0.40] | *0.07* | 0.12 | [−0.11 0.34] | *0.33* | | | |
| 13 | -0.03 | [−0.19 0.14] | *0.76* | 0.05 | [−0.14 0.24] | *0.59* | | | |
| 16 | 0.01 | [−0.20 0.21] | *0.95* | 0.11 | [−0.30 0.07] | *0.24* | 0.22 | [0.04 0.40] | *0.02* |

Notes: Poisson models adjusted by parental education, gestational age, mother's age at birth, parity, gender, diet score during pregnancy, breastfeeding, tobacco exposure at birth, home stimulation index at age 3, income, financial difficulties, and maternal depression. Bootstrap confidence intervals with 1000 repetitions. P-values corrected for multiple hypothesis testing with the Sidak method.

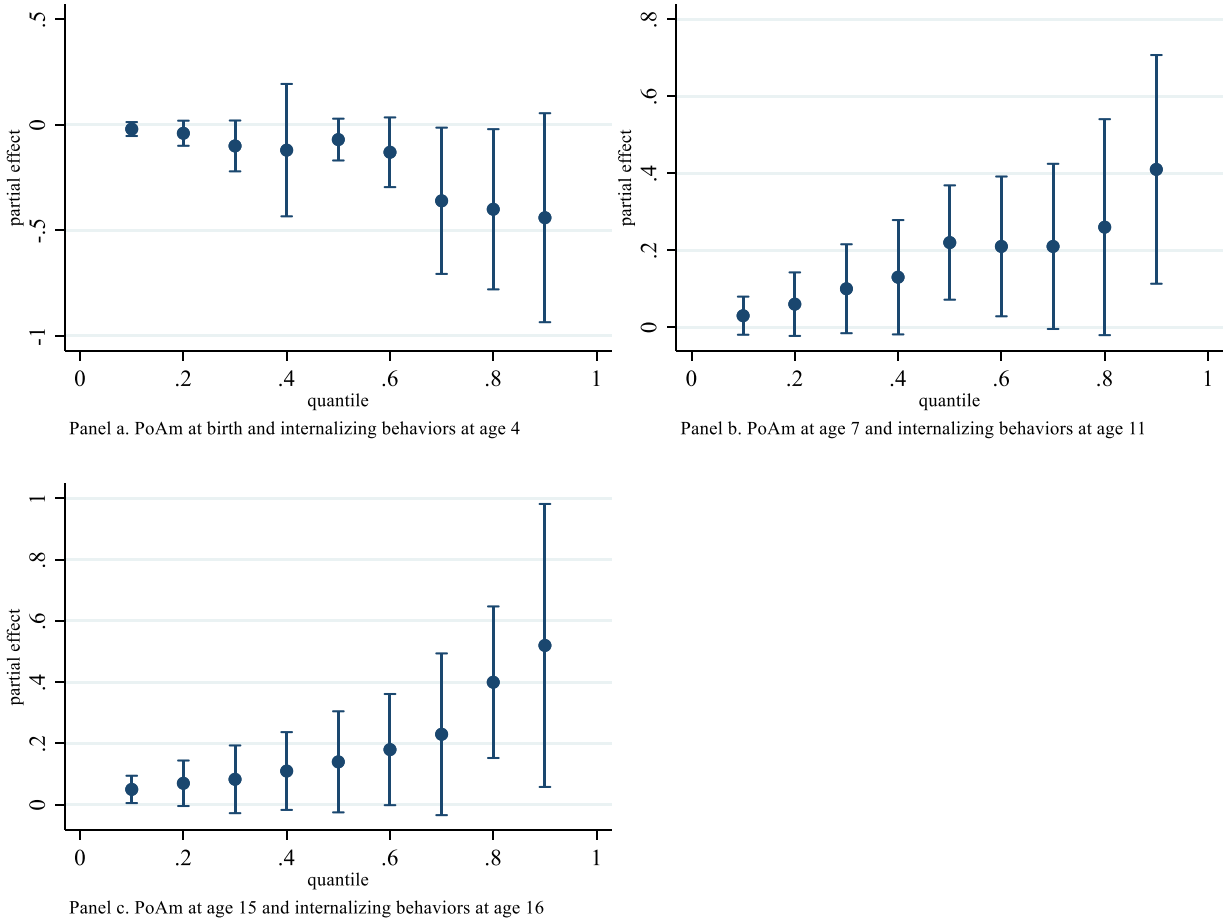


**Fig. 4.** Quantile partial effects, Notes: Estimated partial effects at each decile calculated with the method proposed by Machando and Santos Silva (2005), using same covariates as in Table 2. PoAm at birth and internalizing behaviors at age 4 (panel a), PoAm at age 7 and internalizing behaviors at age 11 (panel b), and PoAm at age 15 and internalizing behaviors at age 16 (panel c). 90 % bootstrap confidence intervals with 1000 repetitions.

between the biologically-based PoAm marker of epigenetic age acceleration and the development of internalizing behaviors. We found that AA at birth is associated with lower internalizing symptoms in early years for some children, while potentially higher internalizing behaviors at the beginning of adolescence, for others. AA at the end of adolescence was significantly correlated with internalizing symptoms at late adolescence (age 15–17).

In line with previous work (Barker and Maughan, 2009), we found different developmental trajectories of internalizing behaviors across individuals. A large proportion of children showed either persistent absence or presence of internalizing symptoms, which were associated with AA both at birth and during late adolescence. There was also a sizeable proportion of children who showed either decreasing or increasing internalizing symptoms between early childhood and late adolescence.

Our estimates of the associations with PoAm at birth most likely reflect heterogeneity on environmental (adverse or favorable) factors during fetal development and birth. Therefore, while PoAm was not trained on birth data, it still indexes a measure of epigenetic gestational age (GA) acceleration at birth. Similar epigenetic GA clocks have shown to be highly correlated with other markers of biological (e.g., ultrasound imaging) and chronological gestational age (Knight et al., 2016; Suarez et al., 2018). In the ALSPAC sample, the correlation between PoAm at birth and estimated gestational period is 0.13 (comparative results of

**Table 3**
Estimated partial effects and relative risk ratios of PoAm on class membership (relative to low-risk group).

| | PoAm at birth | | | PoAm age 7 | | | PoAm age 15 | | |
|---|---|---|---|---|---|---|---|---|---|
| **Class** | Partial effect | 95 % CI | p-value | Partial effect | 95 % CI | p-value | Partial effect | 95 % CI | p-value |
| Low risk | 0.03 | [0.00 0.06] | *0.05* | 0.00 | [−0.03 0.03] | *0.88* | -0.02 | [−0.05 0.01] | *0.26* |
| Late onset | -0.05 | [−0.08 − 0.02] | *0.01* | 0.01 | [−0.02 0.04] | *0.60* | -0.03 | [−0.06 − 0.01] | *0.03* |
| Limited | -0.01 | [−0.03 0.02] | *0.77* | -0.01 | [−0.04 0.02] | *0.46* | 0.03 | [0.01 0.06] | *0.02* |
| Early onset | 0.02 | [−0.01 0.05] | *0.20* | 0.00 | [−0.01 0.04] | *0.99* | 0.02 | [−0.01 0.04] | *0.16* |
| | PoAm at birth | | | PoAm age 7 | | | PoAm age 15 | | |
| **Class** | RRR | 95 % CI | p-value | RRR | 95 % CI | p-value | RRR | 95 % CI | p-value |
| Late onset | 0.75 | [0.61 0.91] | *0.01* | 1.02 | [0.87 1.24] | *0.83* | 0.95 | [0.78 1.15] | *0.60* |
| Limited | 0.87 | [0.71 1.05] | *0.16* | 0.95 | [0.91 1.33] | *0.59* | 1.24 | [1.02 1.49] | *0.03* |
| Early onset | 0.98 | [0.76 1.25] | *0.88* | 0.99 | [0.79 1.23] | *0.95* | 1.18 | [0.96 1.46] | *0.12* |

Notes: Multinomial models adjusted by parental education, gestational age, mother's age at birth, parity, gender, diet score during pregnancy, breastfeeding, tobacco exposure at birth, home stimulation index at age 3, income, financial difficulties, and maternal depression. Bootstrap confidence intervals with 1000 repetitions. P-values corrected for multiple hypothesis testing with the Sidak method.

PoAm and other GA epigenetic clocks available in Supplemental Appendix A, Section E). Results suggest that for some children with high AA at birth, the likelihood to develop internalizing symptoms during early childhood is significantly lower. This potential early advantage at birth seems to become less important as children grow up, potentially due to the accumulation of diverse experiences during childhood and adolescence. Moreover, PoAm at birth is also associated with lower probability to have a trajectory of decreasing symptoms as children grow up. In other words, for some children, low AA at birth increases the chances to mitigate initial risk of internalizing behaviors during later development. In addition, we note that accelerated GA potentially plays a larger role in preventing internalizing behaviors in children with higher intensity of symptoms, consistent with previous literature connecting mental health risk factors and DNA methylation in children (Starnawska et al., 2017; Szyf and Bick, 2013).

Epigenetic aging during early childhood (up to age 7 in the ALSPAC cohort) might not track long-term environmental incidents as they relate to internalizing symptoms beyond puberty. Recent evidence suggests that during the first five years of life, over 100,000 CpG sites show within-individual variation or re-modelling, while less than 460 CpG sites show changes in methylation between ages 5 and 10 years old (Pérez et al., 2019). Only 220 CpG sites exhibit changes in DNA methylation in the 5–10 years period but no changes between birth and age 5. During this first period of rapid growth, it is possible that changes in PoAm during early childhood might only weakly reflect potential mediation between environmental factors in early years and mental health during middle childhood and adolescence.

Finally, we found that the accumulated DNA methylation changes tracked by the PoAm algorithm up to late adolescence are strongly associated with the trajectories of increasing internalizing symptoms up to age 16, particularly for individuals developing symptoms during adolescence. Our results are consistent with recent evidence on the association between epigenetic aging and brain structures in adolescents from low-income families, suggesting a connection between AA, neurocognitive impairment and the emergence of internalizing symptoms (Blanken et al., 2017; Hoare et al., 2020). Moreover, several studies have identified links from stress and adversity in early years to DNA methylation and mental health in adolescence (Barker, Cecil et al., 2018; Cam et al., 2017; Essex et al., 2013; Suarez et al., 2018; Sumner et al., 2019). Given the observational nature of our study and data availability, we cannot determine whether these associations reflect contextual or cumulative effects of internalizing symptoms on DNA methylation, or concurrent changes in DNA methylation due to environmental factors.

Taken together, our results suggest that epigenetic age acceleration at birth correlates with trajectories of low (but not decreasing) internalizing behaviors during early life. In turn, trajectories of increasing internalizing symptoms were associated with increased AA at adolescence. In contrast, children with decreasing internalizing behavior profiles were more likely to show epigenetic age deceleration at ages 15–17. Unfortunately, while evidence suggests the existence of two critical periods for concurrent changes in emotional development and DNAm aging, it is not possible to test potential causality or bidirectionality in these associations without exogenous changes in adverse events that can uniquely lead to higher internalizing behaviors alone (Barker, Walton et al., 2018).

### 4.1. Strengths and limitations

To our knowledge, this is the first study to unveil heterogeneous longitudinal and cross-sectional associations between biological AA and internalizing behaviors, from birth to adolescence. Moreover, unlike previous studies, we rely on multiple measures of both internalizing behaviors and DNA methylation, thus being able to construct dynamic profiles over childhood and adolescence. We conducted multiple sensitivity analyses that provide confidence in the robustness of the estimated associations. Still, our work has several limitations, particularly due to the small sample size. The SDQ sub-scale of internalizing behaviors is ordinal by design, which violates the basic assumptions of traditional statistical methods. In addition, attrition is a serious threat to internal validity. To overcome these challenges, we used statistical approaches that allowed us to maintain the data on its natural scale, avoiding additional assumptions. To address attrition and the small sample size, we balanced the sample using propensity score weights and bootstrapped standard errors whenever possible. We acknowledge that we are not able to replicate the results in this analysis in an independent sample at this time, due to the unique characteristics of the ALSPAC cohort. Moreover, the ALSPAC cohort is representative of a predominantly White and high-income population, thus findings should be extrapolated in context.

## 5. Conclusions

We present a novel analysis of heterogeneity on the longitudinal associations between biological accelerated aging and internalizing behaviors from birth to adolescence, relying on multiple measures of DNA methylation over time. Our results suggest that accumulated DNA methylation due to adverse experiences in utero and during adolescence have divergent effects on the development of internalizing behaviors, in a representative high-income population. Accelerated aging during adolescence significantly correlates with higher internalizing symptoms in the same period, suggesting common pathways between environmental factors, biological aging and mental health at this age.

From a policy perspective, while DNA methylation markers at birth are useful to determine gestational age, they could also help to identify children that hold the potential to mitigate early developmental risks during adolescence. In a similar vein, prevention strategies to mitigate

the risk of chronic internalizing behaviors during late childhood and adolescence could lead to a slower pace of biological aging in adulthood (Brody et al., 2016; Miller et al., 2015; Richmond-Rakerd et al., 2021).

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationshipsthat could have appeared to influence the work reported in this paper.

## Data availability

The authors do not have permission to share data.

## Acknowledgements

We are extremely grateful to all the families who took part in this study, the midwives for their help in recruiting them, and the whole ALSPAC team, which includes interviewers, computer and laboratory technicians, clerical workers, research scientists, volunteers, managers, receptionists and nurses. The UK Medical Research Council and Wellcome (Grant ref: 217065/Z/19/Z) and the University of Bristol provide core support for ALSPAC. This publication is the work of the authors and will serve as guarantors for the contents of this paper. Financial support from the Fonds National de la Recherche Luxembourg (Grant C19/SC/13650569/ALAC) is gratefully acknowledged.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopsycho.2022.108463.

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# Conclusions

The ALAC project allowed us, during the past four years, to investigate the role of DNA methylation in the context of ageing as well as in the transgenerational phenotype inheritance. Along all conducted analysis, we focused on maternal exposure and how this can be epigenetically integrated by their child. As we know from the literature and based on our findings, it is evident that mother-child bond is unique and by consequence could play a determinant role in shaping the child's phenotype later in life. David Barker claimed that the very early period of life (i.e. first two years of life) are crucial for child development, nevertheless we demonstrated how maternal pre-natal exposure can affect child health outcomes. One of the first major bonds between mother and child is the placenta. The placenta acts as a filtering barrier transporting necessary nutriments to the foetus while removing waste produced by the foetus. During pregnancy, it can change its composition and function following the foetus development even if it mostly remains influenced by the mother environment. Indeed the placenta can mediate the gestational environment that will influence the foetal programming of health outcomes later in life (Novakovic & Saffery, 2012). As we demonstrated, every positive or negative experience in life can be directly imprinted in the genome of the exposed person. This imprinting mechanism is similar to the cellular memory mechanism allowing the body to easily face a similar stressor exposure later in life. Obviously, the time of exposure as well as the severity of the exposure will induce or not the imprinting mechanism through epigenetic marks such as DNAm. Nevertheless, those epigenetic marks are reversible and can then disappear over time without any known precise reason. As mentioned in the introduction, the reversibility of DNAm remains unclear, however it is well established that they can last for decades. In our analysis, we suggested that those marks are mostly sensitive during the most sensitive period of development (first thousand days of life) but also during the following period and thus until the puberty. Indeed, between 2 years old and approximatively 12 and 14 years old respectively for female and male, children will in one hand carry their parental epigenetic material and in another develop their own genetic material. At birth, every child possesses 50% of both maternal and paternal genetic material that can contain DNAm, beforehand encoded in their genome due to personal experiences in the past if the DNA demethylation step fails during reprogramming. Additionally, they also possess "a blank slate" that they will shape with their own experiences and based on their lifestyle. As children usually grow up in the same environment as their parents and siblings, the parental exposures to environmental stressors can persist and feed through to their genomes. As we demonstrated in our analysis, when the maternal trauma occurs before pregnancy, it can let an imprinting in child's genome that will be directly transmitted during the foetal development. However, when occurring after pregnancy, it will not directly imprint the child's

genome, however, we hypothesised that it will influence the environment in which the child develops, leading to the imprinting of their epigenome. Here, we started to answer questions vertical transmission and have demonstrated using ALSPAC data our "intellectual breakthrough" about the epi-inheritance mechanism is not 1:1 on the same CpG position. Indeed, the mediation models highlighted that one maternal CpG can be associated with up to 30 different CpGs in their child. These results shed on to light the complexity of inter-generational epigenetic transmission. Additionally, as reported in the literature, DNAm will not share to same consequences between male and female. The sex question has been mainly investigated during the past decades however it remains impossible to confirm whether this sex differentiation can induce epigenetic differences. Nevertheless, what remains clear is that the transmitted maternal DNAm to the next generation can contribute to the inheritance of child's diseases susceptibility.

Sex chromosomes are the main difference between males and females. Studies have demonstrated that DNAm sites were differently methylated due to sex that can contribute to sex-biased gene expression (van Dongen et al., 2016). To date, one of the most plausible explanations is the inactivation of the X chromosome during embryonic development in females, leading to its complete methylation. Due to the high number of genes on the X chromosome (~900-1500) against ~70 genes on the Y chromosome, compensation mechanisms such as X chromosome upregulation or X chromosome inactivation (XCI) are necessary to balance the genes differences between males and females (Pereira & Dória, 2021). XCI happens during female embryonic development and has been associated with the pathogenesis of several diseases and health problems such as mental impairment diseases. There is plethora of studies investigating the differences between males and females as well as monozygotic and dizygotic twins, demonstrated the importance of sex in diseases susceptibility.

The ALSPAC cohort offers great potential as there is a large number of participants and there is a plethora of personal information such as lifestyle, SES or environmental conditions. Nevertheless, this cohort has many limitations that we had to take into consideration over the past 4 years. As all participants have been recruited in the same area of England, the cohort is homogenous and do not present a lot of diversity, particularly when the question of ethnicity occurs. The cohort possesses very few pair of siblings or twins, and it does not allow us to investigate the "intra-house environment" as well as inter genetic differences. Additionally, the number of participants in the sub cohort ARIES (including ~1022 dyads of mother and children) providing epigenetic information represents a major difference compared to the entire ALSPAC cohort (~15 000 participants). Indeed, the participation rate for lifestyle questionnaires significantly decreased at the 3$^{rd}$ time point when children were 15-17 years old. This drop out leads to a weaker

statistical power necessary to demonstrate how socioeconomic determinant i.e. MFP are associated the particular phenotype at birth such as a decrease of head circumference as highlighted by Clark et al. (Clark et al., 2021b). It is important to highlight that the adolescent period is critical in term of hormonal and physiological changes. During this period, there are many parameters that we could not control and track making the investigation of this period trickier that birth or age 7.

ALSPAC is one of the best characterised cohorts in the world and provides ~90 000 variables covering the mother and child's life in details. Nevertheless, there are many missing variables and biological markers that were not considered in the 90's when participant recruitment occurred. Indeed, lifestyles habits evolved and dramatically have changed over the past 30 years leading to the necessity of collecting even more data than expected. Up to now, the ALSPAC cohort is not considered as "terminated" as they are still collecting data. It is important to highlight that over 30 years, the focus and perspectives have evolved and what was considered not relevant in the 90's is now indispensable. Indeed, we have been confronted to the lack of biological data such as cortisol level during pregnancy that would have been ideal to interpret how maternal stress level and SES were correlated. There is now considerable literature that cortisol and oxytocin, both measured during birth, are major biological markers that were not taken due to their long term effect and biological consequences on the children.

In addition, due to the richness of the ALSPAC cohort, it is complicated to find a suitable cohort of validation that regroups as many variables as ALSPAC and covers longitudinal analysis from conception to age 15-17. So far, only the Generation R (GenR) cohort is considered as potential cohort of validation as it investigates five similar areas of research to ALSPAC. Those area are 1. Growth and physical development, 2. Behavioural and cognitive development, 3. Asthma atopy, 4. Diseases in childhood and 5. Health and healthcare (Jaddoe et al., 2006). However GenR do not provide the identical economic variables to ALSPAC, making comparison between them complicated to interpret. Yet, GenR can be used as validation cohort for the first two ALSPAC time points (age 0 and age 7) where the data is now accessible. Like ALSPAC, GenR is still ongoing and the data collection will only be finalised in the next coming years. Until then, it remains complicated to fully reproduce our models and confirm our findings in the GenR cohort. The use of at least two major cohorts such as ALSPAC and GenR in the future will be necessary and indispensable as there is growing evidence that pre and post-natal SES do influence the foetus and will impact the overall health life trajectories. However, one of the main limit between those two cohorts could be the period of data collection, more precisely the decades in child recruitment occurred. For example, in the 90's C-section delivery was less accessible than nowadays. It is more common that expectant

mother will have access to a longer range of options regarding birth preparation and delivery locations including medical staff and material availability. Unfortunately, such factors between the two cohorts can only be considered if the relevant data was recorded back in the 90's.

The ALSPAC cohort was the most suitable cohort for the ALAC project, however the lack of specific data limited our investigations and do not allowed us to fully provide an explanation on parental exposure and children diseases susceptibility later in life. Indeed, in addition to the lack of biological measures such as hormones, most of the paternal data were missing. ALSPAC mainly focused on the link between the mother and her child without really considering the father's role. As explained in the study documentation, there were a large number majority of single mothers taking part in the study as well as an important number of fathers and/or partners who preferred to not participate. The lack of paternal data did not allow us to draw conclusion on their involvement and what role are they exactly playing in their child health trajectories. As mentioned in the Barker theory, both parents play a role in their child's life but especially in their genetics as each parent provides 50% of genetic material.

In the ALAC project, we demonstrated that early maternal exposure is associated with a child's epigenetic imprint, influencing the regulation of genes. The mediation model confirmed that methylation of maternal CpGs are associated with the methylation of their child's CpGs. However some of the child's epigenetic modifications were independent of the maternal exposure. It then became obvious that paternal involvement need to be investigated to confirm our hypothesis on the role played by the father. In our analysis, we always considered the maternal (epi) genetic profile as well as emotional environment provided by the mother during important life experiences such as birth. We know that most of the epigenetic imprints found on children's genomes are the consequences of the maternal epigenetic imprinting itself, however we also took into consideration the maternal behaviour towards children. We raised the hypothesis that regarding the outcome of the trauma, the mother will differently act and adapt their behaviour with child either to protect them either to have an involuntary "payback". These two behaviours can respectively be characterized as "over protection" and "pseudo-baby blues". In ALSPAC, we observed two different maternal reactions due to traumatic birth delivery for example. In one hand, the mother will not change her behaviour towards their child and will completely forget what happened while some won't be able to go over the trauma and will adapt their behaviour to protect themselves as a self defence mechanism.

Despite the lack of paternal data and hormonal sampling, the ALSPAC cohort offered enough material to start elucidate the epigenetic bond between mother and child. Our findings

shed into light the clear “epigenetic imprinting” mechanism leading to a trauma memory system. We also confirmed that somehow those maternal epigenetic marks can influence or be associated with child epigenetic profiles. Those specific epigenomic signatures, using an unknown mechanism, play a role in the gene regulation confirming that physiological processes underlie the long-term effect of ELA. As those marks can play a determinant role in lifelong disease profile, further investigations including hormonals measures and paternal implications are necessary.

# Perspectives

## 1. ALAC 2.0

Following on from our primary results, the continuation into an "ALAC 2" project is considered. ALSPAC is currently collecting similar data as previously on the next generation (G2) of participants, more precisely on children of those ALSPAC children born in the 90's. Thanks to the extension of the cohort into G2, it will be possible to reproduce our analysis and to confirm if the existing epigenetic imprint remains and if it has been transmitted from one generation to another. As for the ALSPAC G1, various series of biological and lifestyles data will be collected (Figure 1).

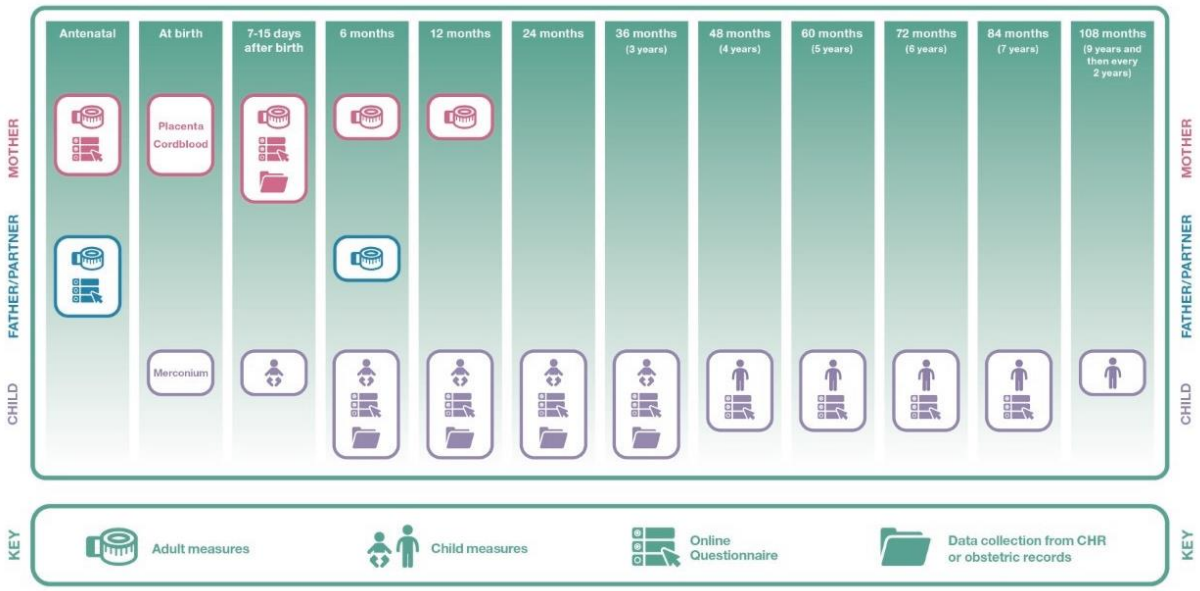


**Figure 9. Children of the Children of the 90s (G2).**

The figure represents the timeline of data ongoing sampling on the G2 from the ALSPAC cohort. (Source: https://www.bristol.ac.uk/alspac/researchers/our-data/alspac-g2/).

Additionally to the G2, the use of the adequate cohort of validation from the beginning of our investigations will be necessary to highlight the mechanism of epigenetic maternal transmission. As mentioned in the ALSPAC documentation, the G2 will provide as much data as the first ALSPAC generation, however will provide more details regarding the parents and what they experienced as the mother (at least) already filled all questionnaires in the 90's. Finally, it would be beneficial to also use a different cohort than ALSPAC G2 or GenR, and to focus on e.g. monozygotic twin cohort that will allow us to screen the divergence between each twin pair. The existing TwinLife cohort and more recently the ImmunoTwin cohort investigating the twin divergence based on ELA exposure will provide more input on how epigenetic marks occur and evolve through time based on personal experiences. In theory, monozygotic twins share the same placenta and possess similar

(epi) genetic background, however if they were not sharing the same sac during pregnancy, it is possible that they will not be identical anymore and present specific traits making the interpretation of the results trickier.

As it can be difficult to have access to cohorts presenting all the key elements we need to further investigate our hypothesis, a mice model might be a better solution in the first hand to at least corroborate our findings.

## 2. Mouse models

The use of the human cohort allowed us to study a large collection of data and to screen what type of ELA can mediate the child phenotype later in life and by consequence lead to chronic disease development. As we already shed into light how genes regulation will be influenced by a maternal economic hardship, it would now be interesting to narrow our investigation and to see how this regulation happens. Nevertheless, in the future only a mice model would allow us such investigations.

The mice model allows a controlled environment and a manipulation of each parameter in a manner that isn't possible in a human because of our natural heterogeneity and incontrollable external stressors. Here, a knock out model can be considered to investigate the biological pathways we have identified here using the ALSPAC cohort.

In the knock out model, we will target several genes of interest, shutting them down to observe the eventual phenotypic and physiological outcomes. As highlighted in our analyses, oxidative phosphorylation was one of the main affected pathways by ELA and most of its genes were up-regulated (i.e. *COXC7, NDUFB3, NDUFA1, COX6A1, COX7A2, UQCRQ, ATP6V0E1, ATP66V1D*) although some were down-regulated (i.e. *ATP6V0E2*). Additionally, most of those genes were also up-regulated in other BP such as Parkinson disease, making them ideal gene candidates to target in a mouse model. To start our analysis, we can select genes that are also associated with ageing such as *COXC7* and *NDUFB3.*

To target the regulation of those genes, it is possible to create a RNA interference (RNAi) model in the mice that will silence the gene expression or an inducible (LoxP Cre) knockout model inducing the complete deletion of the gene. Briefly, the LoxP Cre model targeting genes expressed in bone marrow cells will be introduced into suitable mice. It will then be activated with the addition of tamoxifen directly in the mice. A genotyping step will be necessary to confirm the deletion of the

gene of interest in the G2 group. After breeding and genotyping, the use of epigenetic clocks dedicated to mice model will provide information on the pace of ageing after being exposed to ELA. Every day, different types of ELA such as maze experience, maternal separation and confrontation with a dominant mice will be applied to the pups in order to investigate their reactions to ELA. Additionally, their cortisol levels will be measured to evaluate the stress body response (Figure 10). The controlled conditions of the *in vivo* mice model provides us more flexibility regarding the ELA type exposure as well as the timing of exposure. However, the "unpredictable ELA" found on daily basis in the environment can't be included in our model as the mice model is too "clean" compared to the natural environment we are leaving in.

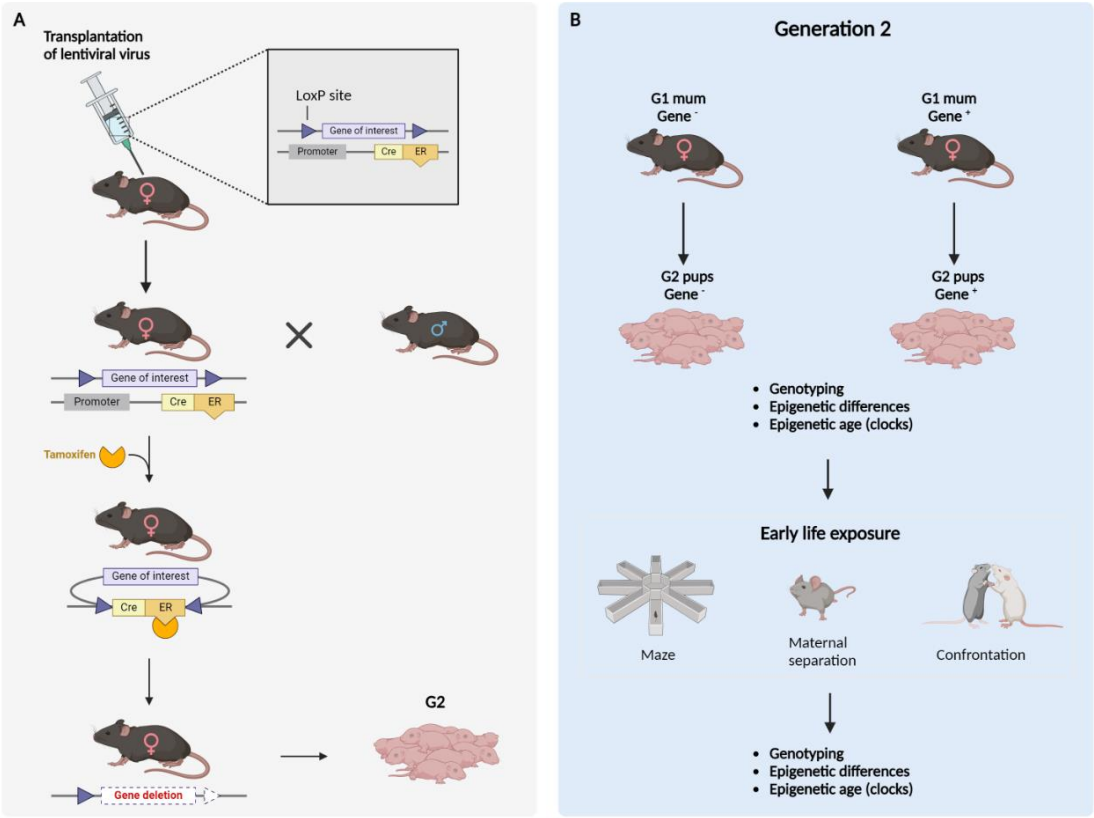


**Figure 10. Proposed experimental model.**

The figure represents the designed experimental model to confirm our findings and to highlight the consequences of the gene interest knockout. A) The left part of the figure represents the development of a maternal knockout model. Briefly, an inducible LoxP Cre system targeting the gene of interest will be transplanted to the oocyte of the female mice. After breading with male, the LoxP Cre system will be activated by adding the tamoxifen to knockout the gene of interest. B) The right side represents generation 2. The pups will then be exposed to 3 types of adversity: maze, maternal separation and confrontation. Different biological measures will be done before and after ELA exposure: genotyping, cortisol measure, epigenetic age. (Created with BioRender.com).

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