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by

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DNA METHYLATION: CONDUCTING THE ORCHESTRA FROM EXPOSURE TO PHENOTYPES

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Supplementary figure 11 - After demonstrating the steadily increase of 6mA throughout development, we decided to evaluate how values of 6mA change in the brain of rats followed by exposure to α-HBCDD through the dams in a known neurodevelopmental toxicity model. At PND270, the statistical analysis of 6mA levels in cerebellum showed an almost significant difference between the control and the animals treated with α-HBCDD, for both males and females (Supp. Fig 4A; ANOVA main effect $p>0.05$). Additionally, a significant interaction between sex and treatment (ANOVA interaction effect $p=0.024$) was observed, which can be explained by the decrease in 6mA observed at 22 ng/kg/day dose, only in females (Supp. Fig 4A). Upon measuring the

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Abbreviations

6mA	6-methyladenine
ACE	Adverse Childhood Adversities
ACTH	Adrenocorticotropic Hormone
ADC	Apparent Diffusion Coefficient
ANOVA	Analysis of Variance
CBG	Corticosteroid-Binding Globulin
CMV	Cytomegalovirus
CO	Cytochrome Oxidase
CORT	Corticosterone
CRH	Corticotropin-Releasing Hormone
CRP	C-Reactive Protein
CTR	Control
DOHaD	Developmental Origins of Health and Disease
ELA	Early Life Adversities
ELS	Early Life Stress
ERP	Ethics Review Panel
FA	Fractional Anisotropy
FNR	Fonds National de la Reserche
GC	Glucocorticoids
GR	Glucocorticoid Receptor
HBCDD	Hexabromocyclododecane
HPA	Hypothalamic-Pituitary-Adrenal
HPF	Hours Post Fertilization
IFNγ	Interferon Gama
LC-MS/MS	Liquid chromatography–mass spectrometry
MD	Maternal Deprivation
MR	Mineraloid receptor
NK Cell	Natural Killer Cell
NKT Cell	Natural Killer like T cell

PBMCs	Peripheral Blood mononuclear Cells
PND	Post Natal Day
PTSD	Post-Traumatic Stress Disorder
SD	Standard Deviation
SEM	Standard Error of Mean
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
WHO	World Health Organization

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Summary

According to World Health Organization (WHO), in 2020, around 300 million children suffered from physical and/or psychological violence and 264 million people suffer from depression. Albeit constant advances made towards the development of new therapies, stress related disorders are still leading causes of burden of disease worldwide affecting over 350 million of people (WHO), highlighting the need for novel and innovative therapeutics and a deeper understanding of the mechanisms underlying these events.

My PhD project (MetCOEPs) aimed to study and understand the mechanisms linking early life stress (ELS), in the form of maternal separation, and the consequences it brings in adulthood. This early period is already known to have a great impact in the development of diseases such as depression, anxiety, diabetes and cardiovascular diseases. Particularly, maternal separation is widely associated with neurodevelopment impairment, with brain regions such as the cortex, amygdala and hippocampus being highly susceptible. Although some studies also associate ELS and poor immune system functionality, little is known about the mechanisms underlying such events. The first part of my PhD aimed at assessing the immune phenotype of animals that were maternal separated in the first days of life. Particularly, I studied how the immune system would behave when facing exposure to external stressors, and if the response would be similar in rodents and humans.

In a second phase, I also studied the impact on ELA on the brain development. Through magnetic resonance imaging, I was able to determine the different impacted regions and follow those changes through RNA analysis. Genes involved in such changes were identified and associated with pathways that can be manipulated in the future, in order to prevent further damages.

Finally, in the last year of my PhD I investigated the role of DNA methylation in neurodevelopment. 5-methylcytosine was already described to have a great impact on this important period of life but not so much was known about 6-methyladenine. In a collaboration project, I was able to identify this

modification and set a base-calling software to be able to read targeted sequencing results from specific brain regions.

Chapter I: General Introduction

My contribution to this chapter:
Literature research and writing

1. INTRODUCTION

Responses to external stimuli were first studied by Walter B. Cannon in the beginning of the 20th century. During his early years as a physiologist, Canon studied the movement of digestive organs, through x-rays, and discovered that when suffering any type of distress, the peristalsis of lab rats would stop (Traube 1987, Brown and Fee 2002). He then turned his focus to the physiology of emotions and studied the impact of stress in physiological responses but it wasn't until 1936 that the term "stress" was coined and defined as "the non-specific response of the body to any demand for change" by Hans Selye (Szabo, Tache Y Fau - Somogyi et al.). After studying animals and human reactions to different diseases, Selye realized that all displayed the same signs and symptoms: weight loss, loss of appetite, fatigue and general anhedonia. Together with findings of the contribution of the adrenal and pituitary glands in the stress response, Selye carved the way to the conceptualization of stress as we know it (Perdrizet 1997, Tan and Yip 2018).

Stress is a defense mechanism that allows the body, as a system, to adapt to different stimulus, whether internal or external, as a way of keeping homeostasis. This response affects individuals differently and at different time points. Early life development, from *in utero* to the first years of life, is said to be one of the most crucial periods for triggering the development of disease phenotypes later in life, if associated with exposure to stressful events (Barker 1995). These events can occur in various forms such as physical and psychological violence, sexual abuse, abandonment or parent neglect, low social-economic status, lack of medical care and education (Felitti 2002, Arias, Leeb et al. 2008), and are related with the development of cardiovascular diseases (Lehman, Taylor Se Fau - Kiefe et al. , Carroll, Gruenewald et al. 2013), cancer (Kelly-Irving, Lepage B Fau - Dedieu et al.), mental and neurological disorders (Repetti, Taylor Se Fau - Seeman et al. , Varese, Smeets F Fau - Drukker et al. , Aas, Henry et al. 2016, Krugers, Arp et al. 2017) and an impairment of the immune system (Baumeister, Akhtar et al. , Elwenspoek, Hengesch et al. , Elwenspoek, Sias et al. 2017). Likewise, traumatic

circumstances such as war, terrorism or natural disasters are also stress-related events that, together with childhood abuse, can trigger the development of post-traumatic stress disorder (PTSD) (Yehuda, Hoge et al. 2015, Bryant 2019) and comorbid depression and anxiety (Flory and Yehuda 2015, Negele, Kaufhold et al. 2015, Rehan, Antfolk et al. 2017, Brown, Fiori et al. 2019) that can ultimately lead to suicide (Ramsawh, Fullerton et al., Campbell, Felker et al. 2007).

Physiologic deviations also follow the development of these diseases. Dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis (Maniam, Antoniadis et al. 2014, Krugers, Arp et al. 2016, Brown, Fiori et al. 2019), changes in brain circuits that control the stress response (Nutt and Malizia , Shin, Rauch Sl Fau - Pitman et al. , Krugers, Arp et al. 2016), abnormal regulation of neurotransmitters (Bremner 2006, Sherin and Nemeroff 2011) and changes in the volume of some particular brain regions, such as amygdala (Gupta, Mayer et al. 2017), pre-frontal cortex and hippocampus (Shin, Rauch Sl Fau - Pitman et al.). Furthermore, malfunction of the immune system has also been reported in human and animal studies of stress (Elwenspoek, Hengesch et al., Roque, Mesquita et al. 2014), with shortening of the telomeres and increased immunosenescence being the most prevalent changes (Lohr, Palmer et al., Neigh and Ali 2016, Elwenspoek, Sias et al. 2017).

My PhD focused on the study of the impact of early life stress in adulthood, particularly in the immune and neuronal systems, and how DNA methylation plays a role in such modifications. The introduction that follows will give an overview of early life stress and its impact in the different body systems. Literature content on the impact of these events in both humans and rodents is reviewed as well as the role of DNA methylation.

1.1 Early life stress and consequences in adulthood

Early life stress (ELS) is defined as stressful events occurring in the first years of life (Barker Dj Fau - Osmond and Osmond 1986). In 2020, it was estimated that around 300 million children suffered from physical or psychological violence from the parents, putting them at risk for mental and physical problems in adulthood (WHO). ELA can occur in the most varied ways, from low social-economic status, physical and psychological violence, sexual abuse, parental illness and domestic violence that, consequently, influence the organism in different ways ((Felitti, Anda Rf Fau - Nordenberg et al. 1998).

In the postnatal (PN) period, mammals are subjected to a series of adaptations to the surrounding environment that are essential for the normal development of the organism. Deviations to this adaptation can trigger the development of certain diseases in adulthood, such as depression, anxiety and PTSD (McCrory, Dooley et al., Short and Baram 2019). Both clinical and animal studies report that childhood trauma, in the form of infections, malnutrition, low social-economic status or paternal/maternal separation has been clearly linked to a variety of diseases such as asthma, depression, anxiety, auto-immune diseases and neurodegenerative disorders (Kendler, Thornton et al. 2000, Mock and Arai 2011, Kelly-Irving, Lepage et al. 2013, Arrieta, Stiemsma et al. 2015, Krugers, Arp et al. 2016). However, the mechanisms behind such events are not always clear.

One of the first studies describing the link between these events was the ACE (Adverse childhood experiences) study (Felitti, Anda Rf Fau - Nordenberg et al. 1998) in which the authors aimed at determine different levels of childhood adversity and the adulthood consequences that each one would bring (Figure 1). In order to determine early life experiences, each participant was questioned about childhood exposure to physical and/or sexual violence; emotional neglect; familiar with mental illness or substance dependence; and domestic violence between householders. More than 50% of the participants had experienced at least one type of adversity, with 7% having had four, which strongly correlated with the later engagement in risky behaviors such as smoking and substance abuse and correlation with the development of deathly

diseases. Since then, there is a plethora of literature on ELA and adulthood consequences.

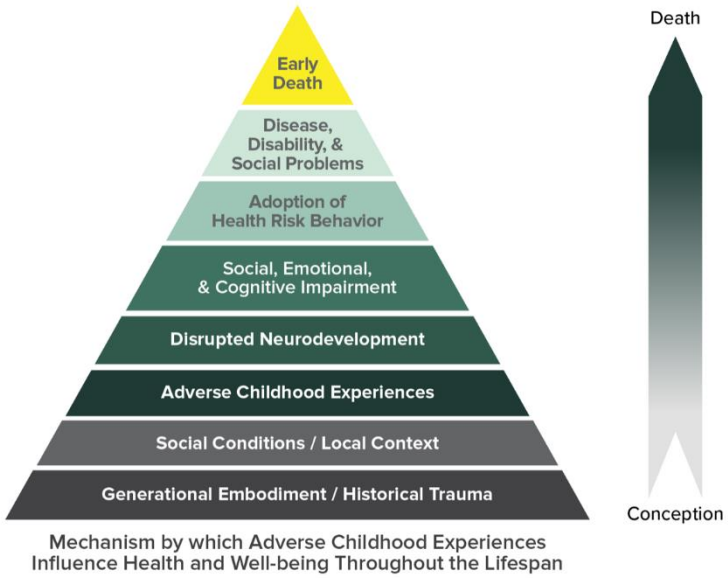

Figure 1 - Pyramid scheme of the effect of early life adversities in the development of diseases later in life. Image obtained from: center for disease control and prevention.

Leading causes of diseases worldwide, such as cancer, diabetes, neurodegenerative disorders and depression, are often linked to traumatic experiences in childhood. A retrospective study from the ACE cohort demonstrated that people that suffered from ELAs were more prone to be hospitalized for autoimmune diseases in adulthood (Dube, Fairweather et al. 2009), emphasizing the importance and impact that these early life events might have on the correct development of the immune system. Moreover, there is increasing evidence that development of Type 1 and Type 2 diabetes (T1D and T2D), although influenced by genetics, obesity, life style and diet, might also be due to early traumatic events (Huffhines, Noser et al. 2016). Around 422 million people worldwide suffer from diabetes and its complications, from obesity to blindness and heart attacks (WHO). Although the literature casts some doubts on whether childhood adversities is linked to the development of diabetes (Cosgrove 2004), recent clinical studies clearly associate psychological stress, early life parental adversity and social-economic status to the induction and progression of T1D and T2D (Sepa, Wahlberg J Fau - Vaarala et al. 2005, Tamayo, Christian et al. 2010, Lundgren, Ellström et al. 2018). Similar to diabetes, and as a consequence, the chances of developing cardiovascular

diseases is also increased after ELA. After the ACE study, where it was determined that one traumatic experience is enough to induce cardiovascular problems, several other clinical studies have been associating ELA with heart attacks, strokes and heart failure (Jakubowski, Cundiff et al. 2018, Pierce, Kershaw et al. 2020). On top of these events, the engagement in risky behaviors, such as smoking and alcoholism, also plays a fundamental role in the impact and development of such diseases. As previously published (Felitti, Anda Rf Fau - Nordenberg et al. 1998), childhood trauma is highly associated with these behaviors, and the reasons behind the appearance of such behaviors might be the coping with the distress of ELAs. Clinical studies showed that people that suffered from ACEs are more likely to smoke and consequently develop lung cancer (Brown, Anda et al. 2010). Furthermore, excessive alcohol consumption has been strongly linked to ACEs and associated with a higher probability of disease development (Felitti, Anda Rf Fau - Nordenberg et al. 1998, Brady and Back 2012, Eames, Businelle et al. 2014, Rehan, Antfolk et al. 2017). Food comforting is also strongly associated to coping mechanisms after stress, linking ELAs to later obesity (Greenfield and Marks 2009, Danese and Tan 2014).

The brain, and its functionality, is one of the most affected regions after early life adversities, since it is still not fully developed and a small deviation to this process might trigger the development of diseases (Krugers, Arp et al. 2016, Kraaijenvanger, Pollok et al. 2020). One such disease is depression, known to affect over 260 million people and to be one of the leading causes of disability, worldwide (WHO). Brown J. and colleagues stated that suffering from early life adversities would increase 3 to 4 times the chances of developing depression and suicidal thoughts (Brown, Cohen P Fau - Johnson et al. 1999). People suffering from sexual abuse in childhood are reportedly associated with the development of major depressive disorders, with women being more affected than men (Erica L. Weiss, James G. Longhurst et al. 1999). Furthermore, factors such as low social-economic status, maternal mental illness and marital relationship of the parents strongly contribute to the development of anxious-depressive related disorders in adulthood (Spence, Najman et al. 2002, Gilman, Kawachi I Fau - Fitzmaurice et al. 2003).

Development of cancer, although not directly associated, has also been hold as a consequence from ELA. Results from the ACE study (Felitti, Anda Rf Fau - Nordenberg et al. 1998) state that there is an odd ratio of 1.9 for the development of cancer after any of the studied adversities (physical and/or psychological violence, sexual abuse, substance abuse or mental illness from one of the householders, violence against the mother). Furthermore, a clinical study from England showed that individuals who suffered from ELAs had 2.38 times higher rate to develop cancer than control individuals (Bellis, Hughes et al. 2014). Fatigue in patients that survived breast cancer was also shown to be significantly increased in patients with history of ELA (Bower, Crosswell et al. 2014).

All these consequences have in common, most of times, a dysregulated secretion of glucocorticoids (GCs), which in turn will have a negative impact on the HPA axis-maintained homeostasis. High levels of circulating GCs due to chronic stress are often associated with development of diseases, specifically cardiovascular and neuronal-related (Kadmiel and Cidlowski 2013).

1.2 Early life stress and the HPA axis

Several factors, such as stress, illness and high levels of glucocorticoids, like cortisol and corticosterone, in the blood, lead to the activation of the hypothalamic–pituitary–adrenal (HPA) axis, a complex system that bridges the neuro- and endocrine systems. It is mainly known for controlling the body response to stress but it was also proved to be involved in other body processes such as digestion, mood, emotions and regulation of the immune system (Bellavance and Rivest 2014, Herman, McKlveen et al. 2016, Bao and Swaab 2018).

When activation of the HPA axis happens, there is the release of corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) from the parvocellular neurons of the hypothalamus into specialized channels that lead to the pituitary gland. This brain region is an endocrine gland located at the bottom of the hypothalamus and when triggered by the release of hormones from the hypothalamus, secretes adrenocorticotrophic hormone (ACTH) into the bloodstream. This in turn, will stimulate the release of GCs, namely cortisol in humans and corticosterone in rodents, which will call for a metabolic adaptation of the body, when the axis is not activated under normal physiological conditions (Aguilera 2016, Arnett, Muglia et al. 2016) (Figure 2). The daily regulation of the HPA axis is conditioned by several factors such as the circadian rhythm. In 1971, Weitzman and colleagues determined the daily pattern of cortisol secretion in humans and showed that it reaches the peak of secretion in early morning, after building up during sleep and reach minimum levels around midnight (Weitzman Ed Fau - Fukushima, Fukushima D Fau - Nogeire et al. 1971). In rodents, on the other hand, the maximum peak of secretion is reached at night and the minimum early in the morning, according to their sleep and activity patterns (Krieger 1973). In response to stress, GCs act in a negative feedback way through the hippocampus and hypothalamus, in order

to suppress the production of CRH and, consequently, ACTH, which will terminate the body adaptation to stress (Kadmiel and Cidlowski 2013).

However, when the negative feedback mechanism fails, normally due to chronic stress conditions, and fails to control and cease the physiological response to a threat, the concentration of GCs starts increasing over time and reaches levels that are no longer physiologically accepted. These high levels will activate a cascade of events that might later end up in disease, such as Cushing's disease but also cancer, diabetes, autoimmune diseases and depression (Kadmiel and Cidlowski 2013, Oakley and Cidlowski 2013, Bellavance and Rivest 2014, Cruz-Topete, Oakley et al. 2020). Development of such diseases is often a result of a downstream effect of GCs that act through binding to the glucocorticoid receptors (GRs) or mineraloid receptors (MRs) (Joëls and de Kloet 1994).

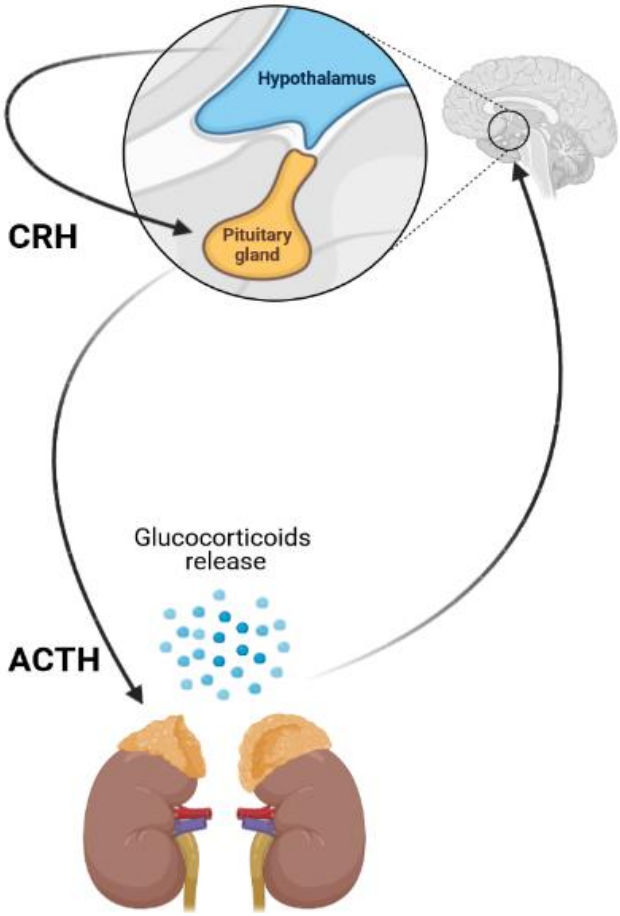

Figure 2 - Representation of the HPA-axis. Image created in Biorender.

Glucocorticoids are steroid hormones and, once in circulation, the majority will bind to carrier proteins such as corticosteroid binding globulin (CBG) and albumin. The remaining GCs (around 5% in humans and 35% in rodents) are free and the ones exerting the biological effects by binding to the GC receptors. These cytoplasmic proteins form complexes with chaperone proteins such as heat shock protein 90, 70 and 23 (hsp90, hsp70 and hsp23) when not in the form of ligand-receptor complex with GCs (Erhuma 2012, Aguilera 2016). Upon binding of GCs, the GC-GR complex will suffer a

conformational change that will allow the binding to DNA or to glucocorticoid responsive elements (GREs), which are located in the promoter region of GC-associated genes. This binding will lead to the recruitment of co-activators or co-repressors that will influence gene transcription (Oakley and Cidlowski 2013, Aguilera 2016, Cruz-Topete, Oakley et al. 2020). GRs are present throughout the body and help controlling development, the neuroendocrine and immune systems' functioning (Carpenter, Shattuck et al. 2011, Erhuma 2012, Bellavance and Rivest 2014, Maniam, Antoniadis et al. 2014).

Early life adversities are known to be a risk factor for the development of diseases but little is known about how it affects the HPA responsiveness to a stressor. Glucocorticoid receptors have low affinity for cortisol and only bind to it when present in high amounts, which corresponds to a response to a stressful situation or chronic exposure to stress (Cintra, Bhatnagar et al. 1994). Often, upon prolonged exposure to stress, the GRs are epigenetically downregulated, which creates difficulties in the regulatory mechanisms (Tyrka, Parade et al. 2016). The gene that encodes for GRs is *NR3C1,* located in chromosome 5 throughout the body (Gehring U Fau - Segnitz, Segnitz B Fau - Foellmer et al. 1985, Hollenberg Sm Fau - Weinberger, Weinberger C Fau - Ong et al. 1985) and it is composed of eight coding exons (2-9) that either translate into the receptors or interfere with their expression plus eight alternative exons (1A-1F, 1H-1J) (Figure 3). A growing body of evidence strongly suggests that ELA induces differential DNA methylation of different exons' promoters of the *NR3C1* gene, which might explain the impaired response to stress in adulthood. Exon $1_{F,}$ the human homologue the rat exon 1_{7} (Turner and Muller 2005), appears to be the one more affected by ELA: Vukojevic and colleagues showed that DNA increased methylation at this region leads to decreased expression of the *NR3C1* gene and is strongly associated with the risk of developing post-traumatic stress disorder (PTSD) in men (Vukojevic, Kolassa et al. 2014). Furthermore, hippocampal decreased *NR3C1* gene expression, equally due to increased DNA methylation, was also shown to be significantly associated with suicidal victims who suffered from childhood adversity, when compared with victims of sudden accidents (McGowan, Sasaki et al. 2009). When taking a step forward and evaluating the peripheral blood of individuals with a history of

childhood sexual abuse, Perroud and his team were able to strength the hypothesis that ELA affects the HPA axis through epigenetic regulation of the *NR3C1* gene, also in blood cells. Furthermore, they were able to show that repetitive abuse, either physical or emotional, was positively correlated with an increase in the methylation levels of the GR gene (Perroud, Paoloni-Giacobino et al. 2011). Although these findings were later corroborated by other groups (Martín-Blanco, Ferrer et al. 2014, Cicchetti and Handley 2017), others strongly state that ELA is negatively correlated with DNA methylation of the GR receptor (Heinrich, Buchmann Af Fau - Zohsel et al. 2015, Tyrka, Parade et al. 2016, Vangeel, Kempke et al. 2018) or that there are no changes following childhood adversity, raising the hypothesis that diseases that develop in adulthood and after ELA do not derive from an impaired HPA axis, via changes in the expression of glucocorticoids (Vangeel, Van Den Eede F Fau - Hompes et al. 2015, Elwenspoek, Hengesch et al. 2020). The same trend can be observed in

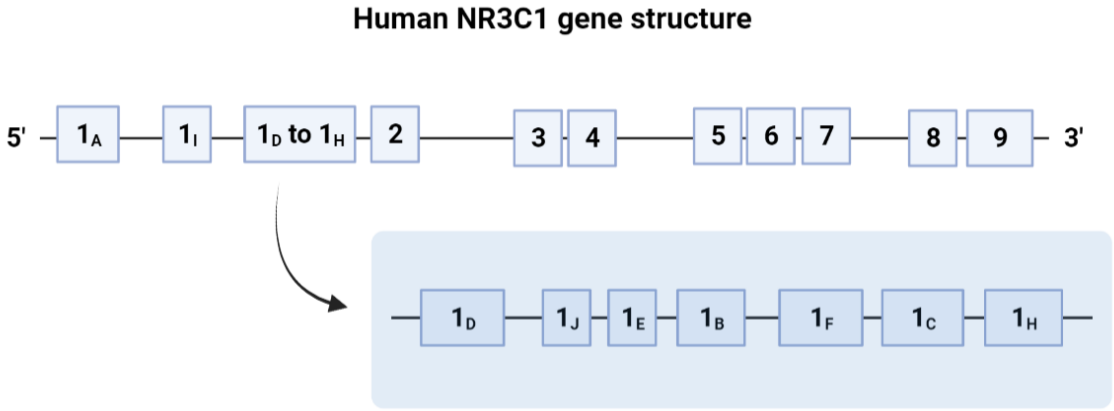

Figure 3 - Human glucocorticoid receptor (NR3C1) gene structure. Image created in Biorender.

rodents, with ELA in the form of lack of maternal care leading to either high methylation levels of the GR (Weaver, Cervoni et al. 2004) or no apparent changes (Daniels, Fairbairn et al. 2009, Breivik, Gundersen et al. 2015).

Particularly in the framework of my PhD, where we assessed the consequences of early life adversity in rats, through maternal separation, the methylation of the glucocorticoid receptor and consequent influence in disease phenotype through GR expression does not appear to be the mechanism. Although we did not evaluate the levels of GR methylation, we did not find significant changes in the corticosterone levels in the maternal deprived

animals, after an acute stress (Fernandes et al., 2021; Chapter 3 of this thesis). Furthermore, we were able to reproduce our findings in a human cohort from a previous study of our group, where we also did not see differences in the methylation of the glucocorticoid receptor (Elwenspoek, Hengesch et al. 2020). We then hypothesize that changes after ELA are not exclusively happening through the HPA axis.

1.3 Early life stress and the immune system

One of the systems known to be the most affected by stress is the immune system, which is divided into innate and adaptive immune systems, that differ in their mode of action. The first, called like so as it provides the first naturally developed line of defense against infections, is composed by macrophages, dendritic cells (DCs), neutrophils and Natural Killer (NK) cells (Figure 4). When infected by bacteria, macrophages and neutrophils act through phagocytosis, a process by which the target particle is engulfed and taken inside the macrophage. These particles are then fused with lysosomes that release destructive chemicals that eliminate them. NK cells, on the other hand, act differently (Sompayrac 2019). Similar to what happens in the adaptive immune responses, NK cells have cytotoxic capacities and can destroy infected cells by inducing them to kill themselves. This happens as NK cells express cell surface receptors that are able to recognize "self" (healthy cells) from "non-self" (infected/tumor cells) and once the non-self are recognized, NKs interact and activate other cells from the innate immune system, inducing a strong and fatal response against infected cells (Yoon, Kim et al. 2015, Paul and Lal 2017).

On the other hand, when the innate immune response is not enough to surpass the invaders, the adaptive response comes in. This part of immune system is composed by specialized cells, such as T and B-lymphocytes that adapt the system's response to almost all stressors that it encounters (Figure 4). B cells originate in the bone marrow and develop in secondary lymphoid organs such as lymph nodes and spleen. These cells are responsible for the production of antibodies that will bind to specific antigens in the surface of the invaders (such as virus or bacteria). This will tag them as "non-self" and signal other immune cells for elimination. Similarly, T cells are also originated from the bone marrow but mature in the thymus. There are three different types of T cells, with different functions: cytotoxic T cells ($CD8^+$), responsible for the elimination of infected cells, by contacting the target cell and inducing it to commit "suicide"; T helper cells ($CD4^+$), responsible for the secretion of cytokines that signal to other immune cells, helping recognizing and eliminating

invaders; and regulatory T cells that help balancing the T cell response, in order to not overreact or underreact (Sompayrac 2019). Altogether, these cells are

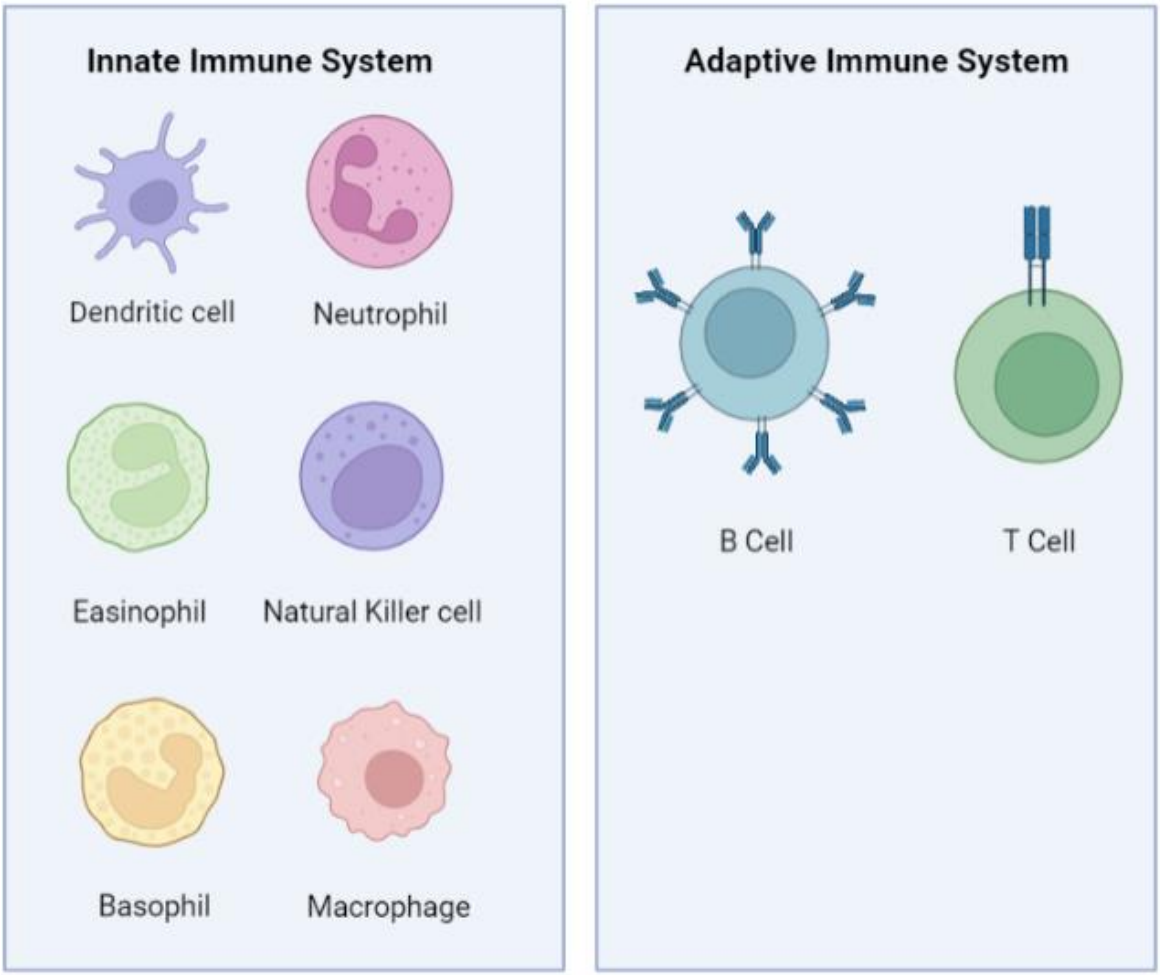

Figure 4 - Cells from the innate (left) and adaptive (right) immune system. Image created in Biorender.

part of a series of complex immune responses that if weakened by some reason, could endanger the whole system and lead to disease or death.

The innate and adaptive cells start to develop already in the first and second trimester of pregnancy (Palmer 2011, Simon, Hollander et al. 2015) and the continuous development is highly affected by the surrounding environment (Noakes, Hale J Fau - Thomas et al. 2006, Nielsen, Hansen et al. 2011, O'Connor, Winter et al. 2013). Several clinical and animal studies suggest that stress in an early period of life significantly affects the immune system development and consequent action or response towards target cells. Strong associations between ELA and high levels of white blood cell count and

circulating cytokines in adulthood were found in a large cohort study of around 12.000 people (EPIC-Norfolk) (Surtees, Wainwright N Fau - Day et al. 2003). The same findings were confirmed some years later in a meta-analysis study, where they found significantly increased levels of inflammatory markers in people who suffered from ELA when compared to control individuals (Baumeister, Akhtar et al. 2016). Available immune data after ELA is mainly related with the adaptive response as studies focusing on the innate system are scarce. Nevertheless, some groups have focused their research on ELA and NK cells, providing evidence for decreased NK activity in adolescents suffering from depression (Birmaher, Rabin Bs Fau - Garcia et al. 1994). A study with rhesus monkeys that were separated from their mothers early in life showed increased levels of circulating NK cells (Lewis, Gluck Jp Fau - Petitto et al. 2000), which was later confirmed in a clinical study with adolescent victims of sexual abuse (do Prado, Grassi-Oliveira et al. 2017).

In regards to the adaptive response, the numbers of studies linking ELA and the consequences in the immune cell's performance are steadily increasing. Initial studies started by showing that children who were maltreated had an increased involution of the thymus, the organ where T cells develop and mature, which significantly contributes to lower immune function (Fukunaga, Mizoi Y Fau - Yamashita et al. 1992). Additionally, young adults that were early separated from their parents and adopted into new families a significant activation of the CD8+ T cells (pro-inflammatory) as well as an increased senescent state (Elwenspoek, Hengesch et al. 2017, Elwenspoek, Sias et al. 2017). Another independent cohort (Reid, Coe et al. 2019) later confirmed these findings. Aging of the immune system, immunosenescence, can be defined by the shortening of the telomeres but also by increase in the expression of the CD57 marker in the cells' surface (Blackburn 1991, Strioga, Pasukoniene et al. 2011). ELA has been shown to decrease telomere length over time (Cohen, Janicki-Deverts et al. 2013, Ridout, Levandowski et al. 2018) and also to increase the expression of CD57 receptor (Elwenspoek, Sias et al. 2017). Although some mechanisms for the occurrence of these events have already been suggested, there is still some doubts on how ELA has such an impact on the development of the immune system.

1.4 Epigenetic modifications upon early life adversity

Epigenetics refers to changes in the gene expression without alterations to the original DNA sequence. Types of epigenetic changes have been identified and include methylation, phosphorylation, acetylation and ubiquitination (Weinhold 2006). Such processes are constantly occurring and are essential for many biological processes. However, if any of them occurs improperly, it can lead to the development of diseases such as cancer and neurodevelopment diseases (Sharma, Kelly et al. 2010, Mastrototaro, Zaghi et al. 2017).

One of the most important and studied epigenetic modifications is DNA methylation and the majority of the available data is related with 5-methylcytosine (5mC) (Figure 5). This modification is present in both prokaryotes and eukaryotes and involved in several biological processes such as regulation of gene transcription, neurogenesis, normal development and disease (Smith, Chan et al. 2012, Moore, Le et al. 2013, Greenberg and Bourc'his 2019). Studies related with stressful events, such as war and famine strongly suggest the passage of this modification to further generations, which might partially explain the heritability of certain phenotypes (Heijmans, Tobi et al. 2008, Tobi, Goeman et al. 2014). Furthermore, 5mC has been linked to early life stress (Taylor 2010, Mitchell, Schneper et al. 2016, Fernandes 2021) and published data provided information on the link between early stressful events

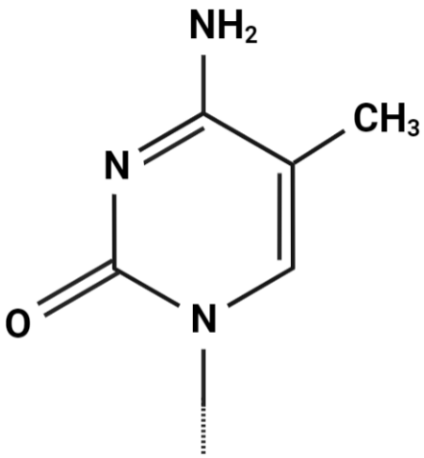

Figure 5 - Chemical structure of 5-methyl-cytosine. Image from Biorender.

and development of disease phenotypes later in life (Murgatroyd, Patchev et al. 2009, Mitchell, Schneper et al. 2016, Tyrka, Parade et al. 2016, Kundakovic

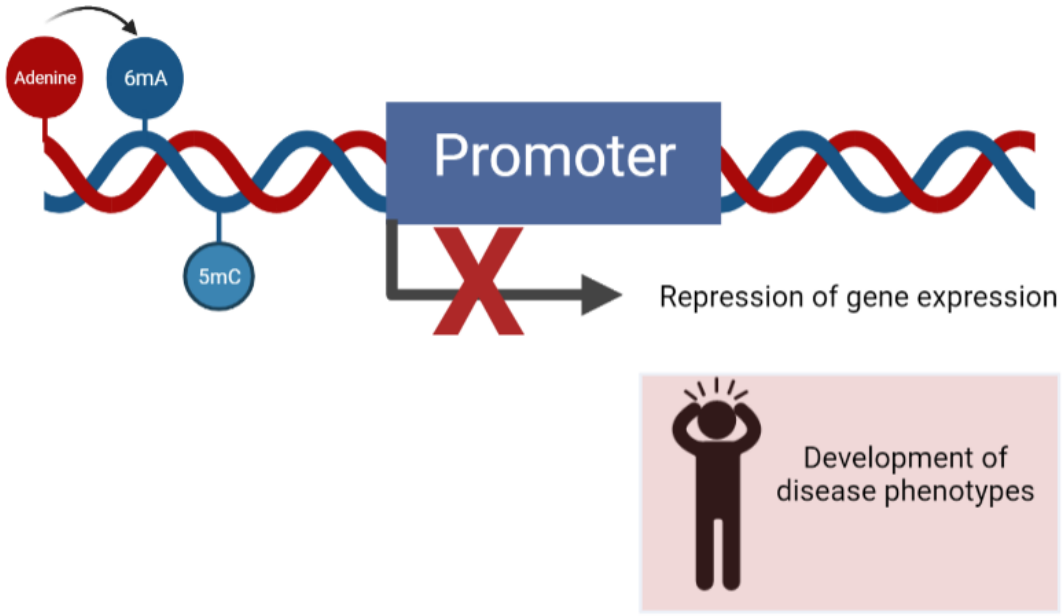

Figure 6 - Methylation of cytosine or adenine translates into the repression of gene transcription which is often linked to the development of disease phenotype. Image created on Biorender.

and Jaric 2017, Raineki, Bodnar et al. 2017, Vidrascu, Bashore et al. 2019). Anxiety and depression disorders derived from early life events have been strongly linked to DNA methylation. Adversities occurring in the early period are suggested to induce DNA methylation, which in turn leads to the repression or activation of genes that play major roles in the development of such diseases (Vaiserman and Koliada 2017, Wiegand, Kreifelts et al. 2021) (Figure 6).

Recently, 6-methyladenine (6mA), a DNA modification known for years to occur in prokaryotes (Dunn and Smith 1955, Dunn and Smith 1958), has been shown to be present in eukaryotes, such as plants (Zhou, Wang et al. 2018) and flies (Zhang, Huang et al. 2015) but also in mammals (Yao, Cheng et al. 2017, Xiao, Zhu et al. 2018). Interestingly, this modification was equally shown to influence and contribute to important biological processes, such as repression and silencing of genes, particularly in the X-chromosomes (Wu, Wang et al. 2016, Alderman and Xiao 2019), and tumorigenesis (Xie, Wu et al. 2018). Furthermore, stress, both in early life or adulthood, was shown to induce changes in the levels this modification, in different brain regions, which in turn

led to transcriptional changes (Kigar, Chang et al. 2017, Yao, Cheng et al. 2017).

Although there is still some controversy on whether 6mA is a genuine DNA modification or a simple bacterial contamination or artefact (O'Brown, Boulias et al. 2019, Douvlataniotis, Bensberg et al. 2020), accumulating data strongly indicates otherwise. Detection of 6mA by different techniques, from sequencing to mass spectrometry and dot blot (Wu, Wang et al. 2016, Ye, Luan et al. 2017), together with data showing that manipulation of the enzymes that accumulate or remove this modification from DNA significantly change the outcome (Xiao, Zhu et al. 2018), are starting to overcome and eliminate the initial doubts.

As evidence started to accumulate on the impact of 6mA during development (Liu, Zhu et al. 2016), we decided to investigate its presence and potential role in vertebrates and mammals such as zebrafish, mice and human. We were able to detect this modification in different mice tissues and, more interestingly, in twenty-eight human brain regions (Fernandes, Grova et al. 2021). Furthermore, we were able to show that, from a pluripotent cell to an almost fully formed individual, 6mA levels steadily increase (Fernandes, Grova et al. 2021). Such findings lead us to believe that 6mA may be associated with developmental processes and implicated in the development of diseases upon early life adversity (Chapter 5).

1.5 Animal models of stress

Modelling human diseases, in particular the ones linked to psychological stress, is a hard task due to the complexity of the different existent diseases and subjective natures of their symptoms. Animal models are widely used in order to better understand the mechanisms behind disorders and possible ways to treat them; however, they often fail to fully reproduce the human characteristics. Additionally, the limited knowledge on the physiological and molecular mechanisms underlying the triggering events of most disorders highly contributes to the struggle that is mimicking human disorders.

Early life adversity in humans has been shown to have a significant impact in the development of diseases, increasing the need for an animal model that would allow an in-depth study on the origins of such events. Most popular and commonly used animal models of stress are based on the exposure of the animals to a set of environmental stressors (Vetulani 2013, Iñiguez, Riggs et al. 2014, Monteiro, Roque et al. 2015), which will ultimately induce depressive and anxious-like behaviors, but also produce more severe disease phenotypes (Lewis, Gluck Jp Fau - Petitto et al. 2000, Roque, Mesquita et al. 2014, Breivik, Gundersen et al. 2015). Behavioral assays merged with latest technological advances in molecular biology and automated video tracking are what allow researchers to easily quantify representative behaviors characteristic of human diseases in rodents.

As defined by Nestler and Hyman, animal models are considered good if they respect some requirements such as construct, face validity and predictive validity. Face validity indicates the model good mimicking of the different human characteristics such as anatomical, biochemical and behavioral. Although it is difficult to have them all in the same model, the more the model replicates, the better it resembles the human features. Construct refers to the capacity of a model to replicate the etiological features underlying a specific human disease, such as the neuronal network impairment happening in depression and anxiety. Finally, predictive validity means that an animal model should, ideally, respond to certain treatments in a way that predicts the human response to those same treatments (Nestler and Hyman 2010). There are

several types of stress that can be induced in rodents that trigger different behavioral and neurological phenotypes that resemble mood-related disorders in humans, such as maternal deprivation, restraint stress, fear conditioning and social defeat stress. All of them provide valuable tools for the identification and characterization of biological processes involved in the stress response.

Maternal separation is the closest animal model to one of the human types of early life adversity, adoption and/or abandonment. Animals are deprived of maternal care for some time, every day, usually lasting for two weeks. The period of separation varies from a single 24-hour separation or repeated 3 to 12-hours a day, from post-natal day (PND) 1 to PND14-21, depending on the type of stress and consequences to be studied (Lehmann, Logeay et al. 2000, Fabricius, Wörtwein et al. 2008, Roque, Mesquita et al. 2014, Figueiredo, Frota et al. 2016). In addition, some studies also add a 15 minutes separation group in order to mimic the natural mother-pup interactions, which normally do not induce any significant changes or decrease in maternal care (Meaney 2001).

The maternal separation protocol can be done in different ways, which will induce different degrees of stress in the dam and the pups. Pups can be separated from the mother by removal of the mother from the cage, leaving the pups in a familiar environment without maternal care but presence of the siblings or all pups can be transferred into a new cage, outside the familiar environment, deprived of maternal care. The first was shown to be more stressful for the mother while the later was proven to be more stressful for the dam. Increased stress can also be induced by separating the dam individually, into a new environment (Bailoo, Jordan Rl Fau - Garza et al. 2014). Independently of the length of the protocol, maternal separation is proved to induce several changes at the endocrine, neuronal and immune systems, making it a good model to mimic long term ELA in humans (Daniels, Pietersen Cy Fau - Carstens et al. 2004, Meagher, Sieve et al. 2010, Roque, Mesquita et al. 2014, Bölükbas, Mundorf et al. 2020, Gehrand, Phillips et al. 2020).

Repeated stress can also be induced by other ways such as with social defeat and restrain stress protocols. Exposure of animals to a larger and more aggressive animal (Golden, Covington et al. 2011) has been shown to lead to

the development of anxious and depressive-like behaviors, as well as clear changes in the neuronal network (Iñiguez, Riggs et al. 2014, Tomas-Roig, Piscitelli et al. 2016, Weber, Godbout et al. 2017, Abe, Okada et al. 2019). Similarly, the restraint stress model, in which the animal is immobilized for a period of time, combining both psychological and physical stress, also results in behavioral and biochemical changes in the brain, providing insights on the neurobiology of stress (Sántha, Veszelka et al. 2016, Wei, Bao et al. 2018, Woo, Hong et al. 2018).

All these models allow researchers to investigate the behavioral and physiological changes underlying stress and its consequences, but they still fail to fully mimic the human condition. Although providing important insights, it is also important to combine results from animal studies with available clinical data.

2. Objectives

The first chapter of this thesis gave an overview of the accumulated data on the consequences of suffering from different types of stress in an early period of life. A considerable amount of research has clearly established a link between ELA and diseases in adulthood and it is not debatable that adoption or separation from parents contributes greatly to those outcomes. However, the mechanisms behind such events are still not completely clear. ELA in humans can be in some cases an accumulation of different stressors, which does not allow to clearly specify the role of each adversity. For this purpose, in this thesis, we took advantage of the animal model of maternal separation to dissect how such this specific stress impacts development and leads to disease.

A previous study from our group, on which this thesis was grounded, showed that early parental separation with consequent adoption significantly influences the immune system, leading to a pro-inflammatory status and immunosenescence (Elwenspoek, Hengesch et al. 2017, Elwenspoek, Sias et al. 2017). Additionally, in the same study, it was shown that the cortisol levels (Hengesch, Elwenspoek et al. 2018) and glucocorticoid receptor activation (chapter 2 of this thesis, (Elwenspoek, Hengesch et al. 2020)) was not impaired, increasing the evidences that ELA might not always act through the HPA axis. Following those results, we aimed at researching the immune phenotype of the maternal separated animals and mechanisms behind such changes, using flow cytometry and cell culture assays. Chapter 3 reports that data, which has been submitted for publication in Frontiers in immunology.

The second objective of this thesis was to understand the changes that are occurring in the brain. Clinical and experimental studies clearly state that ELA affects the brain greatly and in different ways, as already exposed in chapter 1 but, to this date, very little was known about the effects of maternal separation on the brain structural integrity and connectivity. Using MRI, we were able to identify cerebellum as the brain region most affected after maternal separation, in terms of connectivity. Furthermore, we were able to demonstrate that acute stress is enough to induce such changes. Obtained results were accompanied by changes in gene expression, which consequently

impacts brain pathways that are associated with specific neurodegenerative diseases. This data is reported in chapter 4 as preliminary data.

Finally, the third objective of the thesis was to determine the possible mechanisms linking ELA and the observed phenotypes. DNA methylation has been proposed to be one such mechanism. In order to determine the role of DNA modifications in early life adversity, we explored the abundance of 6-methyladenine during embryonic development and tested how a deviation to normal development, in the form of stress, would contribute to changes in 6mA. Chapter 5 reports these results, which were published in *Frontiers in genetics.*

Chapter 6 is the general discussion of this thesis, which aims to bring together all these findings and put them in context with the current knowledge on ELA.

Chapter 7 includes a review on early life adversity and COVID-19, a literature review that addresses and proposes that different types of early life stressors might contribute to a higher vulnerability to a virus, such COVID-19.

Chapter II: Early life adversity and GR signaling

My contribution to this chapter:
Performing the GR pyrosequencing and mRNA expression experiments. Participated in the writing of the manuscript.
Manuscript published in *Development and Psychopathology*, in August 2020.

2. GLUCOCORTICOID RECEPTOR SIGNALING IN LEUKOCYTES AFTER EARLY LIFE ADVERSITY

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2.1 Abstract

Early life adversity (ELA) has been associated with a clear immune phenotype of higher levels of inflammation and accelerated immunosenescence, as well as hypo reactivity of the HPA-axis. However, it is still unclear how these two findings are linked and whether the immune phenotype is a result of dysregulated peripheral glucocorticoid signaling.

We, therefore, examined glucocorticoid receptor functionality in immune cells in our EpiPath cohort, where we have previously observed higher immune activation and senescence. Participants had either been exposed to ELA in the form of separation from parents and/or institutionalization followed by adoption in early childhood (ELA, n=40) or had been reared by their biological parents (Ctrl, n=72).

Although we did not find differences in methylation at the *GR* 1F exon or promoter region, we identified a region of the *GR* 1H promoter (CpG 1-9) that showed lower methylation levels in ELA. Nevertheless, mRNA expression levels of first exon-specific *GR* transcripts as well as expression of the *GR* target genes *FKBP5* and *GILZ* were similar between groups. We also did not observe group differences in GR sensitivity of immune cells.

In sum, overall peripheral GR signaling was unperturbed in our cohort and the observed immune phenotype does not appear to be secondary to an altered glucocorticoid receptor response to glucocorticoids. To identify signaling pathways that may underlie the ELA immune phenotype, future research should focus on unbiased approaches, such as investigating whole genome methylation profiles.

Keywords

Early life adversity, DNA methylation, *NR3C1*, glucocorticoid receptor, *FKBP5*, *GILZ*.

2.2 Introduction

Early life adversity (ELA) has been associated with long-term endocrine, immune, and behavioral effects, and an increased risk of pathologies in adult life. We and others have identified an ELA immune phenotype, characterized by inflammation and immunosenescence, which may mediate the association of ELA and the risk of diseases (Elwenspoek, Hengesch et al. 2017, Elwenspoek, Kuehn et al. 2017, Elwenspoek, Sias et al. 2017). ELA concurrently increases the incidence of a wide spectrum of stress-related disorders (McCauley, Kern et al. 1997, Batten, Aslan et al. 2004), implying a pathophysiological role for the hypothalamic-pituitary-adrenal (HPA) axis and potentially the autonomic nervous system (ANS). This has led to the suggestion that HPA axis functioning may link ELA to impaired adult health (Barton, Zakreski et al. 2016). This raises the question whether the immune phenotype is a direct result of ELA, or an indirect consequence of dysregulated GC levels and GR signaling.

Stress signals are processed by higher brain regions that stimulate the hypothalamus to release Corticotropin-Releasing Hormone (CRH). CRH, in turn, activates the pituitary gland to release Adrenocorticotropic Hormone (ATCH), which stimulates synthesis and release of GCs from the adrenal gland. This cascade of events is called the hypothalamic-pituitary-adrenal (HPA) axis, which closely interacts with the immune system. The glucocorticoid receptor (GR) is constitutively expressed and resides in the cytosol bound to a chaperone protein complex. Upon GC binding, the GR translocates to the nucleus, where it influences transcription (Oakley and Cidlowski 2013). HPA axis activation modulates immune function by affecting gene transcription and reducing immune cell trafficking to sites of inflammation, leading to an overall inhibition of the inflammatory response (Cain and Cidlowski 2017). ELA has been associated to both higher levels of inflammation (Baumeister, Akhtar et al. 2016) and HPA axis dysregulation, such as altered epigenetic regulation of the *GR* gene and GR signaling affecting GR sensitivity (Koss and Gunnar 2017).

The GR is encoded by the *NR3C1* gene, containing 8 coding exons (2-9) and 9 non-coding alternative first exons (1A-1J, excluding G). The first exons are located at the gene promoter and play an important role in *GR* regulation. A

growing body of data from animal and human studies suggests an association between ELA and epigenetic modulation of the *NR3C1* gene (Turecki and Meaney 2016). ELA has been associated with differential DNA methylation levels at the 1B, 1C, 1D, 1F, and 1H promoter regions in human studies (reviewed in (Palma-Gudiel, Cordova-Palomera et al. 2015). Although the numerous study are not fully concordant, one of the more consistent findings is hypermethylation of the 1F promoter and/or exon in association with ELA (Perroud, Paoloni-Giacobino et al. 2011, Tyrka, Price et al. 2012, Martin-Blanco, Ferrer et al. 2014, van der Knaap, Riese et al. 2014, Romens, McDonald et al. 2015). The human 1F promoter contains the orthologous NGFI-A binding to the region originally identified by Weaver et al. in the rat (Cao-Lei, Leija et al. 2011), who reported an association between methylation levels and maternal care (Weaver, Cervoni et al. 2004).

The first exons are transcribed to mRNA in a tissue specific manner, but are not translated into protein (Turner and Muller 2005, Palma-Gudiel, Cordova-Palomera et al. 2015). Although *1F* and *1H* are only intermediately expressed, they are of special interest because they are expressed in both hippocampus as well as in immune cells (Turner and Muller 2005). Increased methylation of the *GR* promoter has been associated with lower levels of total *GR* expression in hippocampal tissue (McGowan, Sasaki et al. 2009), peripheral blood (Labonte, Azoulay et al. 2014, Perroud, Rutembesa et al. 2014), and saliva (Vukojevic, Kolassa et al. 2014). However, few studies have investigated the relationship between methylation levels at specific first exons and the corresponding transcripts (Cao-Lei, Suwansirikul et al. 2013).

The GR plays a crucial role in the negative feedback of the HPA axis (Figure 2). The sensitivity of this feedback mechanism, which appears to be altered after ELA (Koss and Gunnar 2017), is largely determined by GR levels and further fine-tuned by the FK506 binding protein 51 (FKBP5), a co-chaperone of the GR that reduces the affinity of the receptor for GCs. Demethylation of *FKBP5* has been found in ELA (Klengel, Mehta et al. 2013), which – together with increased methylation of the *GR* – promotes GR resistance and impairs the negative feedback of the HPA axis. GR resistance in immune cells leads to

excessive immune activation and may exacerbate inflammation (Baumeister, Akhtar et al. 2016).

Another *GR* target gene is the glucocorticoid-induced leucine zipper (*GILZ*), which is thought to mediate the anti-inflammatory effects of GCs. GILZ antagonizes the NFκB pathway – a major cell signaling pathway involved in immune activation (Ronchetti, Migliorati et al. 2015). Induced NFkB activity has been found in ELA (Pace, Wingenfeld et al. 2012), although it is still unclear if this can be explained by upstream differences in *GILZ* expression levels.

We have previously reported higher activation and senescence of the immune system in association with ELA in the EpiPath cohort (Elwenspoek, Hengesch et al. 2017, Elwenspoek, Sias et al. 2017). This cohort consists of in young adults who experienced separation from their parents and/or institutionalization followed by adoption in early childhood and controls reared by their biological parents. To address whether GR dysregulation could underlie the observed immune phenotype, we have examined the sensitivity, functionality, and transcriptional regulation of the GR in immune cells in the EpiPath cohort.

2.3 Material and methods

2.3.1 Subject enrolment

The EpiPath cohort, as previously described (Elwenspoek, Hengesch et al. 2017, Elwenspoek, Sias et al. 2017), consists of participants between 18-35 years of age with or without ELA. ELA was defined as "separation from parents in early life and subsequent adoption". While the majority of the ELA participants had been exposed to the additional stress of institutionalization, four participants were adopted from foster or birth families. This study was approved by the National Research Ethics Committee (CNER) and the Ethics Review Panel (ERP, University of Luxembourg) and written informed consent was obtained from all participants, in compliance with the Declaration of Helsinki. The participants were compensated for the inconvenience and their time.

2.3.2 Study protocol

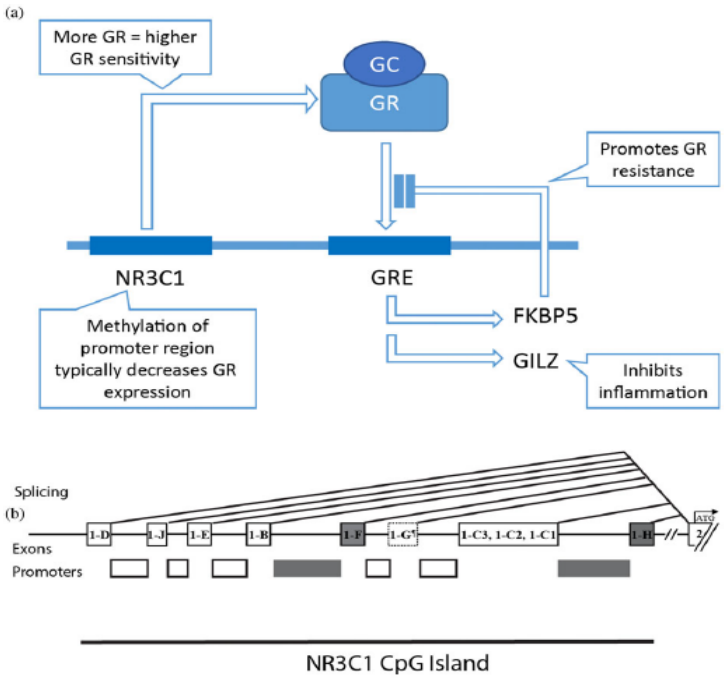

Figure 7 - The glucocorticoid receptor (GR): structure and signaling. (A) Schematic representation of transcription / translation of the NR3C1 gene producing active GR, its activation upon ligation of GC, and transcription of FKBP and GILZ after binding to genomic GREs. (B) Schematic representation of the NR3C1 gene and the promoter / first exon region studied here (dark boxes). Abbreviations: GC, Glucocorticoid; GR, Glucocorticoid Receptor; NR3C1, GR, gene; GRE, Glucocorticoid Response Elements; FKBP5, FK506 Binding Protein 51; GILZ, glucocorticoid-Induced Leucine Zipper.

Participants were invited to two laboratory visits. Participants were requested to refrain from smoking and physical exercise, avoid caffeinated drinks (>1h) or alcohol (>24h) prior to visit 1. All women were either using hormonal contraceptives or were in the luteal phase of the menstrual cycle. At approximately 11:30 am an indwelling cannula was inserted and the first blood samples were collected in sodium heparin and EDTA anti-coagulated tubes, for the *ex vivo* stimulating experiments and isolation of DNA and RNA, respectively. At visit 2, participants filled out the childhood trauma questionnaire (CTQ) and were asked about their age at adoption if applicable.

2.3.3 GR methylation

Automated genomic DNA (gDNA) extraction was performed on a QIAcube on 200µl of whole blood using the QIAamp DNA Mini and Blood Mini (Qiagen, Venlo, Netherlands). Subsequently, 500ng gDNA was bisulfite-converted using the EZ DNA Methylation-Gold™ Kit (Zymo, Freiburg, Germany) according to the manufacturer's instructions. *NR3C1* promoter regions were biotin-labelled in a 50µl amplification reaction containing 20mM Tris-HCl (pH 8.4), 50mM KCl, 200mM

deoxynucleoside triphosphates (dNTPs), 1x concentrated SYBR Green (Cambrex, Verviers, Belgium), 1x concentrated Platinum Taq DNA polymerase (Invitrogen, Erembodegem, Belgium) as well as primers and MgCl2 (Supplementary Table 1). Thermocycling was performed on a BioRad CFX96 thermal cycler for 45 cycles with initial denaturation of 95°C for 2 min, 95°C for 20s, annealing for 20s and 72°C for 20s with a final elongation for 10 min at 72°C. Biotin-labelled amplicons were pyrosequenced as previously described (Cao-Lei, Suwansirikul et al. 2013). All PCR and pyrosequencing primers are given in Supplementary Table 1.

2.3.4 GR and GR-target genes mRNA expression

Total RNA was isolated using the RiboPure™ RNA Purification – Blood kit according to the manufacturer's instruction (Fisher Scientific, Aalst, Belgium). First strand cDNA synthesis was carried out at 50° C for 60 min in a 20uL reaction containing 200ng/mL RNA, 250mM Tris-HCl (pH 8.3), 375mM KCl, 15mM MgCl2, 0.2mM of dithiothreitol (DTT), 10mM of deoxynucleoside triphosphate (dNTPs), 40U RNase OUT™ (Invitrogen, Carlsbald, USA), 200U SuperScript™ III Reverse Transcriptase (Invitrogen), and 0.50uM dT(20) primers.

To assess the mRNA levels of *FKBP5*, *GILZ*, total *GR* (Exon 3/4), *GR* transcript variants (1F and 1H), and of three housekeeping genes: β-actin, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and peptidylprolyl isomerase A (*PPIA*), cDNA was amplified by real time quantitative PCR (RT-qPCR) in 25uL reactions containing 200mM Tris-HCl (pH 8.4), 500mM KCl, 200 uM dNTPs, 1x concentrated SYBR Green (GelStar™ Nucleic Acid Gel Stain, Lonza, BioWhittaker, Versviers, Belgium), 1x Platinum Taq DNA polymerase (Invitrogen). Temperature, primer and MgCl2 concentrations were optimized per primer pair (for PCR conditions and primer sequences see Supplementary Table 2). Each plate included a cDNA pool

of thirteen individuals from the cohort as positive control and a no template control. All samples were assayed in duplicate.

Thermal cycling (CFX96, BioRad, Hercules, CA, USA) conditions were as follows: denaturation at 95°C for 2 min; 44 cycles of denaturation at 95°C for 20s, annealing at respective temperatures (see Supplementary Table 2) for 30s, and elongation at 72°C for 30s; final elongation at 72°C for 2.5 min. Ratios of all housekeeping genes were calculated to identify the most stable gene in our sample. The comparative cycle threshold (Ct) method was used to calculate the relative target gene expression ($2^{-\Delta\Delta Ct}$), using the positive control as reference sample and GAPDH as reference gene (Schote, Turner et al. 2007).

2.3.5 GR sensitivity assay

Whole blood was stimulated with either 0.5µg/mL pokeweed mitogen, 2 µg/mLPHA-M (PHA) or 5ng/ml LPS from *Escherichia* coli 0111:B4 (Sigma-Aldrich, Overijse, Belgium) in the presence of a 10^{-5}-10^{-10} M titration of dexamethasone (Sigma-Aldrich). After 19 h of incubation at 37°C, 5% CO2, 95% humidity, supernatants were harvested and immediately frozen at -80°C. IL-6 secretion was measured using the Human IL-6 ELISA Set in a 1:50 dilution (BD OptEIA, Erembodegem, Belgium). IC50 values of dexamethasone inhibition of IL-6 secretion – a measure of GR resistance – were calculated with the Hill-equation using SigmaPlot (version 12.3, Systat Software, San Jose, CA).

2.3.6 Statistical analysis

To investigate statistical group differences, Student's T-tests or Wilcoxon rank-sum tests were used for numerical variables and Chi-square tests for categorical variables. Whenever possible, variables were log-transformed to obtain a normal distribution. Correlations between data were estimated with the Spearman's rank correlation test. For all correlations, methylation data, and demographics, p-values were corrected with the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). All statistical tests were performed in R (version 3.3.2; (R Core Team 2016).

2.4 Results

2.4.1 Participant characteristics

Participant characteristics of the whole EpiPath cohort (n=112) that was used for the measurements of *GR* methylation and expression levels are depicted in Table 1. Ctrl and ELA groups were similar in age, sex, smoking status, body mass index, and CTQ scores (p>0.05).

Table 1 - Demographics of the EpiPath cohort.

	All (*n* = 112) median [IQR]	Ctrl (*n* = 72) median [IQR]	ELA (*n* = 40) median [IQR]	Corrected *p*-values
Age (years)	22 [20–24]	22 [21–23]	24 [20–26]	0.087
Sex (% female)	61%	58%	66%	0.626
Smoking (% yes)	21%	14%	32%	0.087
Body mass index	22.6 [20.8–25.1]	22.5 [20.5–24.3]	23.1 [21.1–26.2]	0.087
Childhood trauma (CTQ scores)	1.2 [1.1–1.4]	1.2 [1.1–1.4]	1.2 [1.1–1.4]	0.734
Age at adoption		0 [0–0]	4.3 [0–15]	<0.001

2.4.2 1F *and* 1H GR *DNA methylation levels*

We determined DNA methylation levels at 22 CpGs within the 1F exon and promoter and at 35 CpGs within the 1H promoter region (Figure 3). Overall methylation levels over the 1F and 1H regions (sum of single CpGs) were similar between groups. However, when examining methylation at the single CpG level, minor differences emerged in the *GR 1H* promoter region. At CpG position 1 and 53, ELA showed significant lower (Wilcoxon rank-sum test, p=0.023) and higher methylation levels (Wilcoxon rank-sum test, p=0.047), respectively. Furthermore, we identified a region at the start of the 1H promoter containing 9 CpGs that showed overall lower methylation levels (sum) in ELA (Wilcoxon rank-sum test, p=0.021).

2.4.3 Relative transcription levels of GR and GR target genes FKBP5 and GILZ

To investigate the impact of these methylation differences on *GR* signaling, relative expression levels of total *GR*, *1F*, and *1H* transcripts and two *GR* target genes were examined in whole blood. Messenger RNA levels of Total *GR,* exon 1F and 1H transcripts as well as the two target genes were similar between ELA

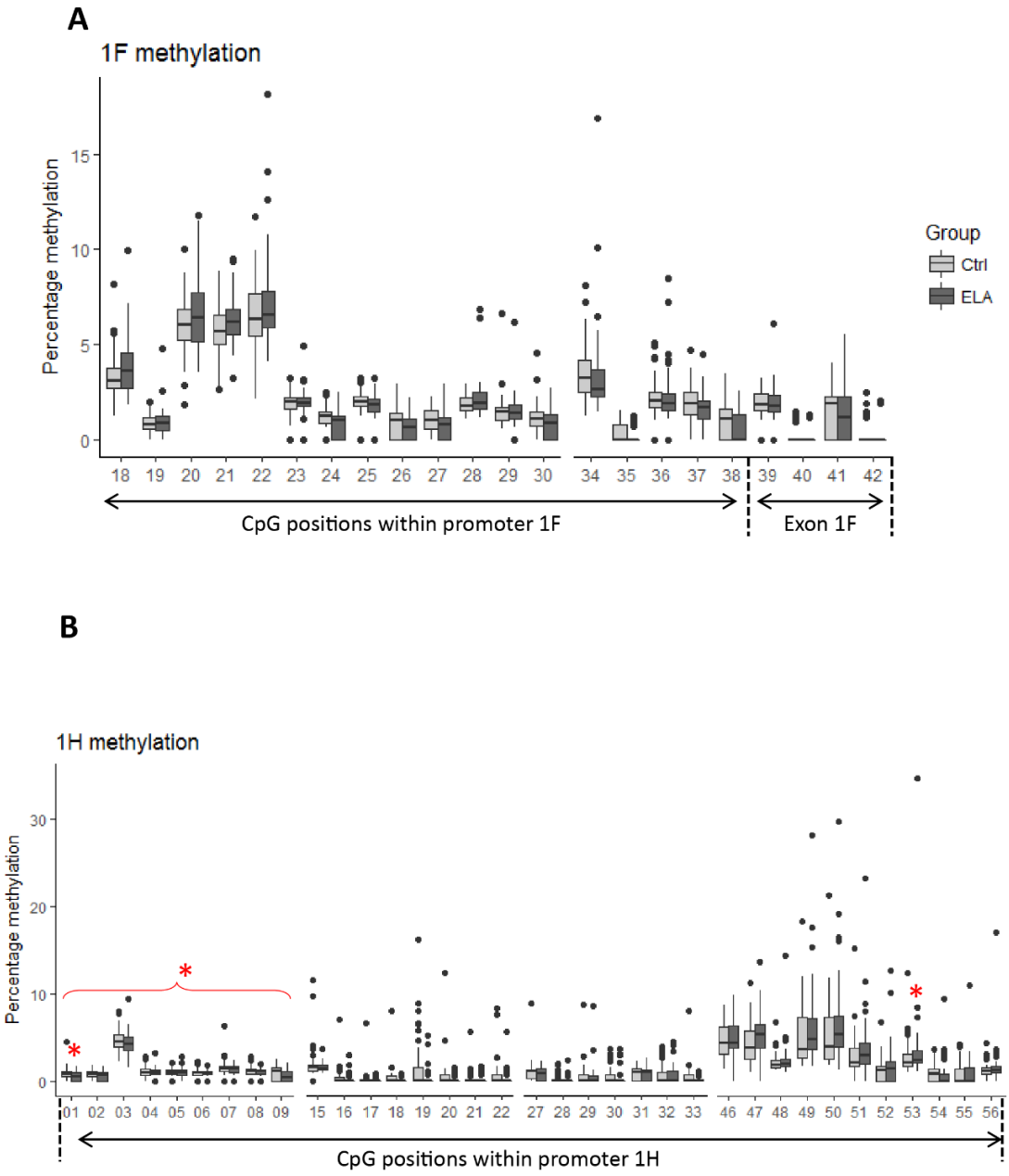

Figure 8 - GR DNA methylation levels measured by quantitative pyrosequencing in Control (light grey) and ELA (dark grey) participants in the two first exon promoter regions: 1F (A) and 1H (B). Data are the median (central line), 25th and 75th percentile (upper and lower box limits), 2.5th and 97.5th percentiles (whiskers), and outliers (individual points).

and controls (p>0.05, Figure 4a). Our data showed high inter-correlation between *1F* and *1H* transcripts (Figure 4), suggesting that first exon expression levels are co-regulated as previously reported (Cao-Lei, Suwansirikul et al. 2013). As *FKBP5* is one of the target genes of the GR, we expected a positive association between *FKBP5* expression and *GR* mRNA levels. Indeed, FKBP5 expression was significantly correlated with total *GR*, *1F*, and *1H* transcripts (Spearman's rank correlation, p<0.001), with the strongest correlation with 1F transcripts (rho=0.74, p<0.001; Figure 4b). Also, *GILZ*, another *GR* target gene, was significantly correlated with all three *GR* transcripts (Spearman's rank correlation, p<0.05), although the correlations were less strong (Figure 4b). *GILZ* was strongest related to *1H* transcripts (rho=0.26, p=0.010; Figure 4b).

2.4.4 Associations between mRNA expression and methylation levels at single CpGs

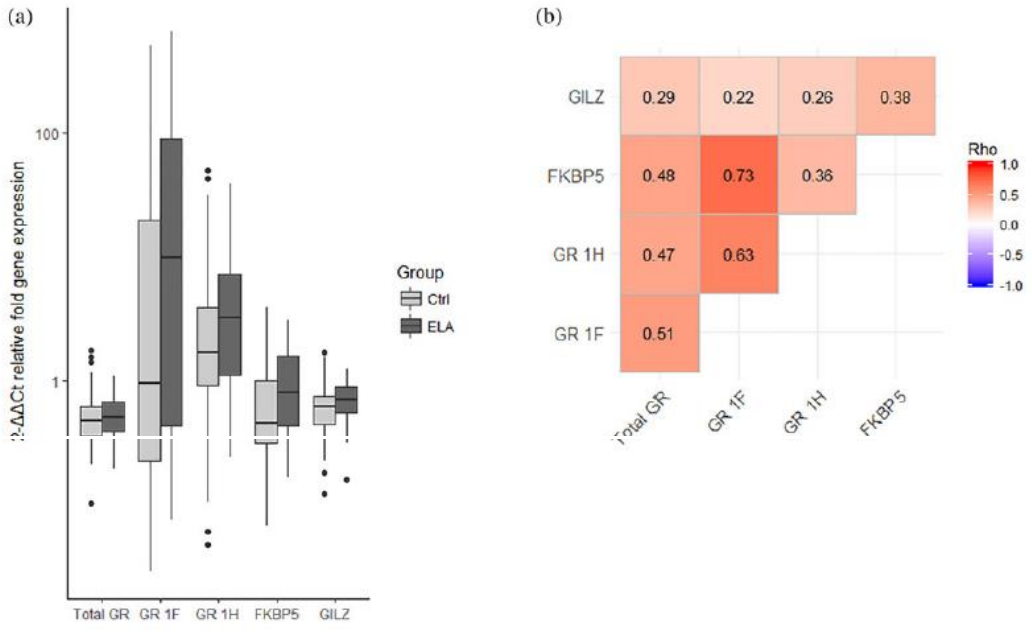

Figure 10 - Methylation-expression correlation matrix between methylation levels at single CpGs in the GR 1F (A) and GR 1H (B) promoter regions and relative mRNA expression of GR and GR target genes. Statistics: Spearman rank-order correlations. Rho values according to the color scale after Benjamini-Hochberg false discovery rate correction. *p < 0.05 **p < 0.01 *** p < 0.001.

Overall methylation levels over the *1F* and *1H* regions (sums) were not related to mRNA expression of the *GR* or target genes. However, when examined on single CpG level, weak correlations between *1F* methylation levels and relative expression of *1F, 1H, FKBP5*, and *GILZ* became apparent (Figure 5a). The first 5 CpGs of the *1F* region are positively correlated with transcription

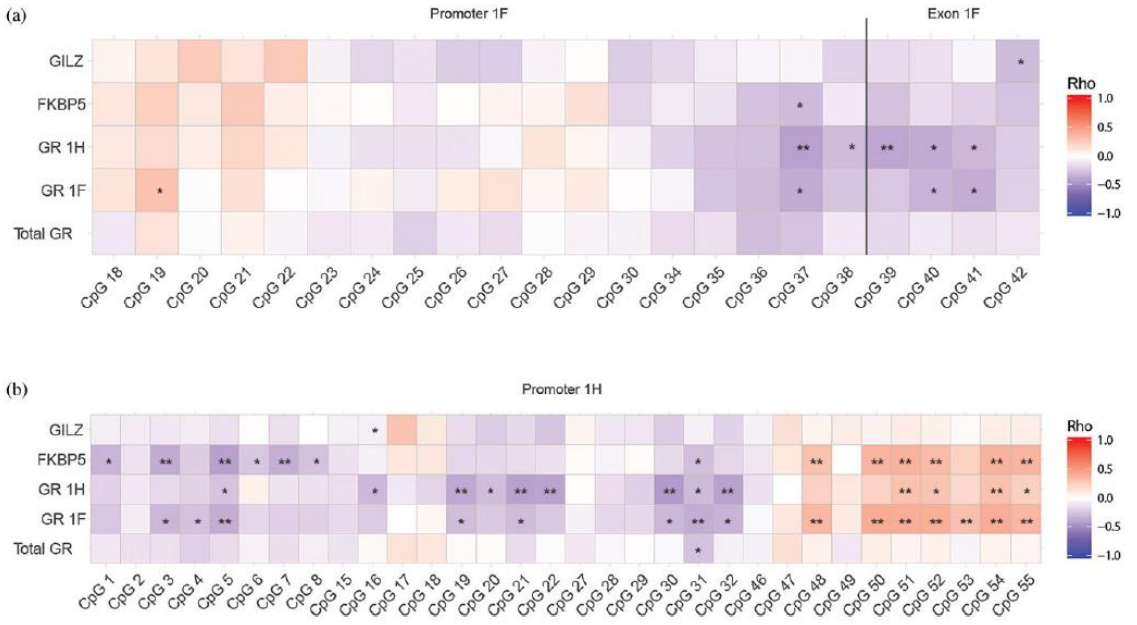

Figure 9 - Methylation-expression correlation matrix between methylation levels at single CpGs in the GR 1F (A) and GR 1H (B) promoter regions and relative mRNA expression of GR and GR target genes. Statistics: Spearman rank-order correlations. Rho values according to the color scale after Benjamini-Hochberg false discovery rate correction. *p < 0.05 **p < 0.01 *** p < 0.001.

of all four mRNA transcripts (sum of position 18-22: *1F* rho=0.20, p=0.053; *1H* rho=0.22, p=0.026; *FKBP5* rho=0.22, p=0.022; *GILZ* rho=0.22, p=0.025), whereas from position 36-42 methylation levels were negatively correlated with transcription (sum of positions 36-42: *1F* rho=-0.32, p=0.002; *1H* rho=-0.36, p<0.001; *FKBP5* rho=-0.24, p=0.012). Figure 5b shows correlations between *1H* methylation levels and relative expression of *1F*, *1H*, *FKBP5*, and *GILZ*. Surprisingly, our data show that the region at the end of *1H* (position 47-55) is *positively* correlated with the expression of *FKBP5* (rho=0.35, p<0.001), *1F* (rho=0.41, p<0.001), and *1H* (rho=0.30, p=0.002) transcripts. Positions 1-32 seem to be negatively correlated with all four mRNA transcripts (sum position

1-32: *1F* rho=-0.33, p=0.001; *1H* rho=-0.36, p<0.001; *FKBP5* rho=-0.26, p=0.007, *GILZ* rho=-0.21, p=0.038).

2.4.5 GR sensitivity

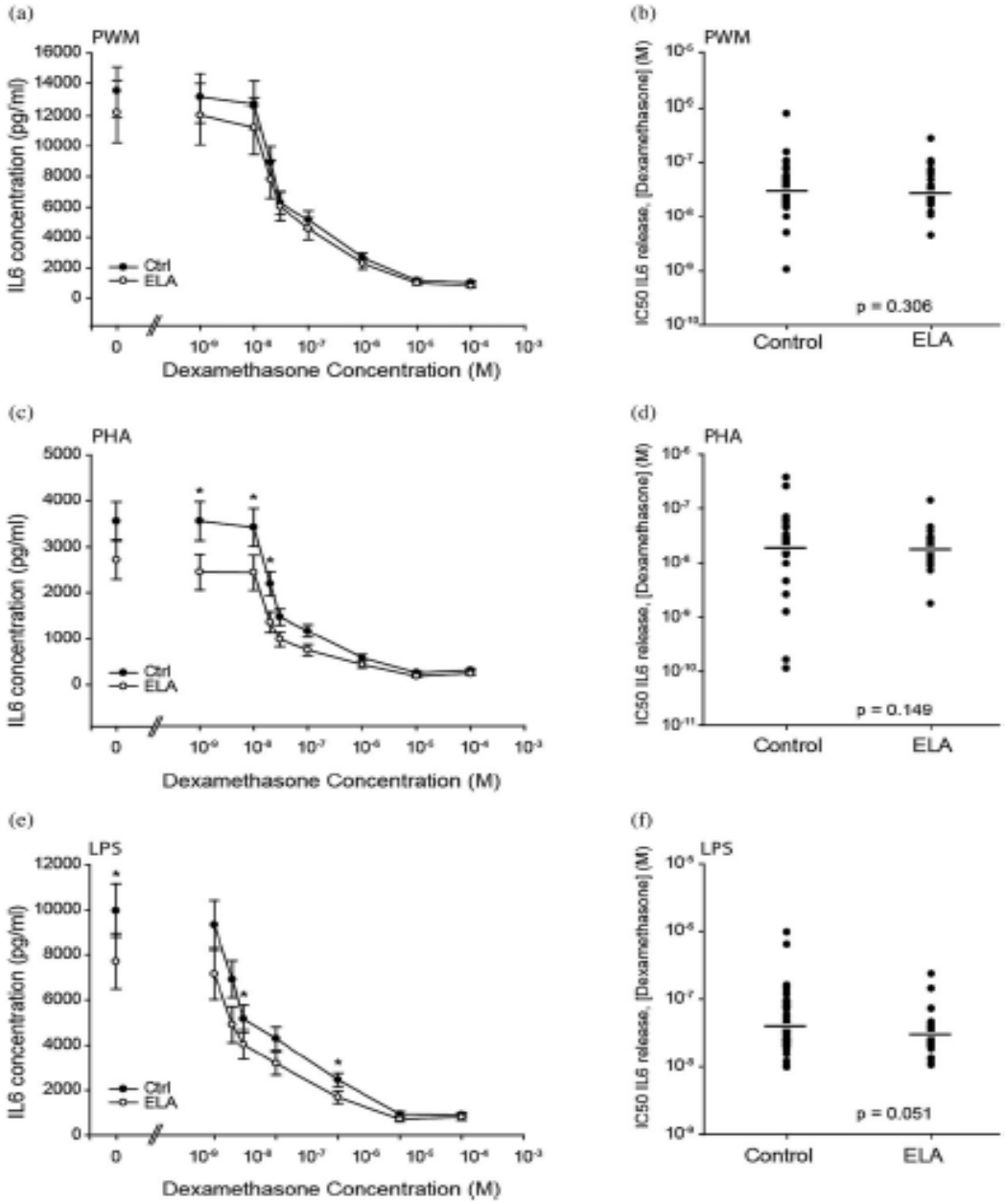

Figure 11 - . Inhibition of IL6 secretion by dexamethasone after PWM, PHA, or LPS stimulation. Supernatant concentration of IL6 after stimulation of whole blood with B-cell specific PWM in the presence of dexamethasone (A). IC50 was determined for Control and ELA participants (B). Inhibition curve for the T cell specific PHA (C) and IC50 values for Control and ELA participants (D). Inhibition curve for the B cell and monocyte stimulant LPS (E) and IC50 values for Control and ELA participants (F). A, C, E—open circles, early life adversity; closed circles, control participants. *p < 0.05 in a pairwise comparison of the specific concentration.

To determine GR sensitivity, whole blood from participants was stimulated with the cell-type specific stimuli phytohemagglutinin (PHA; T cells) pokeweed mitogen (PWM; B cells), and lipopolysaccharide (LPS; B cells and monocytes). The concentration of dexamethasone that was needed to achieve a 50% inhibition of IL-6 production was used as a measure of GR resistance (Figure 6). For all three stimuli IL6 levels were lower, irrespective of the dexamethasone concentration, in ELA than in controls, although only significant for a limited number of conditions. IC50s were calculated for all participants. There was no group effect on IC50, although there was a strong trend towards a lower IC50 after LPS stimulation (p = 0.051; Figure 6f), suggesting that GC/GR signaling remains unaffected in T cells, B cells, and potentially monocytes, after ELA. Although there was not a major effect of ELA on either

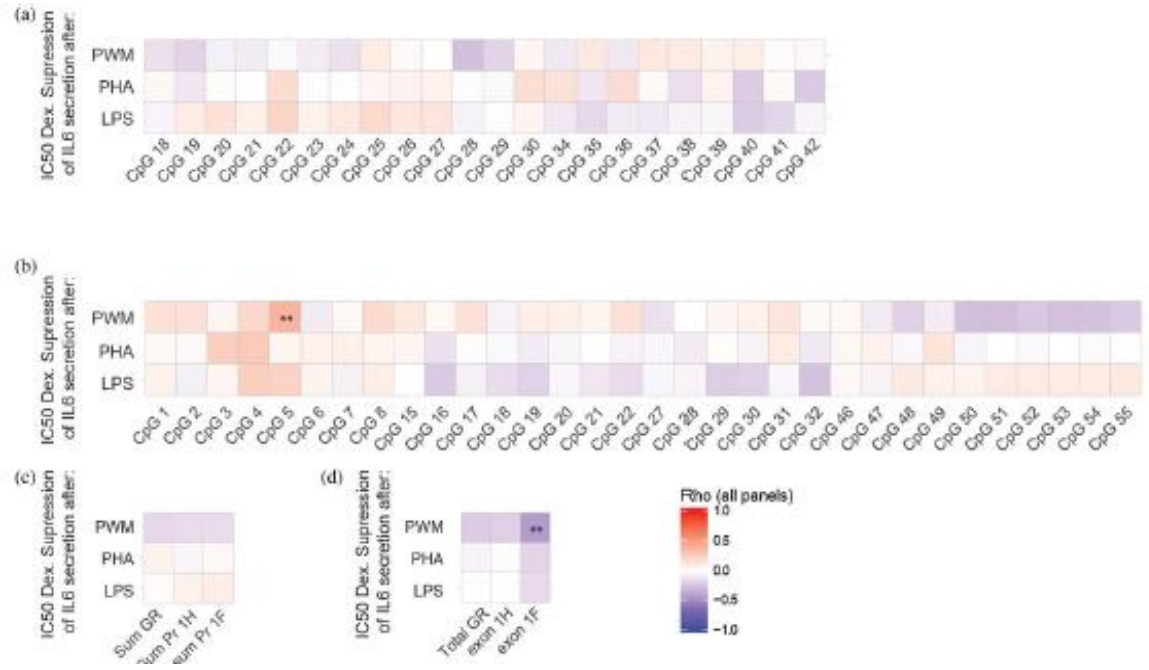

Figure 12 - Correlation matrix between the IC50 of the dexamethasone suppression of IL6 production by PWM, PHA, and LPS and the methylation levels at single CpGs in the GR 1F (A) and GR 1H (B) promoter regions, sum methylation levels throughout the promoters (C), and with GR transcript levels (D). Statistics: Spearman rank-order correlations. Rho values according to the color scale after Benjamini-Hochberg false discovery rate correction. **p < 0.01.

promoter 1F/H methylation or GR functioning, higher methylation levels at *GR* 1H CpG 5 were associated with a lower IC50 to PWM induced IL6 secretion, indicating GR resistance (rho=0.40, p<0.001; Figure 7b), although this was limited to a single CpG in one promoter. There was no correlation between GR functionality as measured by Dex inhibition of IL6 and the sum of methylation

throughout promoter 1F and 1H (Figure 7c). As expected, higher levels of *GR* 1F mRNA transcripts were related to a decreased IC50 after PWM stimulation (rho=-0.42, $p<0.001$). A similar relationship existed between PWM stimulated IC50 and *GR* 1H transcripts, albeit as a trend and to a lesser extent (rho=-0.20, $p=0.053$). There was no group difference in GR resistance between ELA and controls after the three different stimuli (Wilcoxon rank sum test, $p>0.1$), although there may be an increased sensitivity of T cells as dexamethasone suppression of PWM induced IL6 by T cells correlated with methylation at a CpG in promoter 1H and with exon 1F transcript levels.

2.5 Discussion

In the present study, we investigated GR functioning in the EpiPath cohort. We could demonstrate that methylation levels at certain CpGs at the *GR* promoter predicted expression levels of *GR* transcripts and target genes. Moreover, we showed that expression of *GR* transcripts correlated with *GR* target gene expression. Interestingly, in this cohort, ELA was associated with a higher prevalence of chronic diseases and psychological disorders, and we have demonstrated a clear immune phenotype (Elwenspoek, Hengesch et al. 2017, Elwenspoek, Sias et al. 2017). Here, we investigated whether this phenotype was related to disturbances in *GR* signaling. We found that DNA methylation levels of two first exon promoter regions of the *GR* gene, transcription levels of *GR* and *GR* target genes, and GR sensitivity in leukocytes were largely unaffected by the experience of ELA in our EpiPath cohort. These data suggest that peripheral GR signaling was unperturbed in our cohort and that the observed immunophenotype was not secondary to an altered GR response to GCs.

Here, ELA was characterized by the early separation from parents and institutionalization prior to adoption, which are known and well-established adverse childhood experiences (van Ijzendoorn, Palacios et al. 2011, Julian 2013, Phillips and Carver 2015). The adversity happened very early in life – median age of adoption was 4 months – so most participants had no recollection of these events. As expected, ELA participants had low scores of childhood maltreatment (CTQ), which is dependent on the participants' recollection of the adversity. In contrast, studies that have investigated adversity measured by high CTQ scores (recalled traumatic events that occurred before age 16) have found hypermethylation in the 1F region in both hippocampus tissue (McGowan, Sasaki et al. 2009) and circulating immune cells (Perroud, Paoloni-Giacobino et al. 2011, Tyrka, Price et al. 2012, Martin-Blanco, Ferrer et al. 2014, Romens, McDonald et al. 2015), others have not found such an association (Steiger, Labonte et al. 2013), or, inversely hypomethylation (Tyrka, Parade et al. 2016). The situation may, however, be more complex. Alexander et al recently observed that there was no direct association between ELA and *GR* methylation

but rather GR promoter 1F methylation may moderate whether an individual becomes hypo- or hyper-responsive to a subsequent psychosocial stressor (Alexander, Kirschbaum et al. 2018), although in a previous study we did not observe a link between *GR* promoter 1F and the stress response (Li-Tempel, Larra et al. 2016). To our knowledge, there is only one report that examined a form of ELA comparable to the EpiPath cohort. Melas et al. (2013) found higher 1F methylation in saliva in association with early parental death, similar to the results for maltreatment exposure (Melas, Wei et al. 2013). Although saliva can contain a mixture of cells, immune cells are the main source of DNA.

Limited overlap between CpGs investigated in the above-mentioned studies and differences in timing, duration, and severity of our model of ELA compared to other models may have caused these discrepant findings. We focused on CpGs anterior to and within exon 1F, which are most likely affecting transcription of the 1F exon, while others also included CpGs posterior to the 1F exon (Perroud, Paoloni-Giacobino et al. 2011, Martin-Blanco, Ferrer et al. 2014). In addition, we included CpGs within the NGFI-A binding region orthologous to the original site identified by Weaver et al. (2004), whereas other studies did not. Moreover, compared to individuals that were exposed to childhood maltreatment, participants in the EpiPath cohort were exposed to ELA for a relatively short time period.

We found hypomethylation in a small region within the 1H promoter in leukocytes in association with ELA. As there were no differences in the baseline levels of relative numbers of major cell subtypes (Elwenspoek, Hengesch et al. 2017), it is unlikely that these results were affected by differences in methylation across leukocyte types that co-varied with adversity. Interestingly, Labonté et al. (2012) examined methylation levels in hippocampus tissue of suicide completers with a history of childhood maltreatment and reported hypomethylation in the exact same region of the 1H promoter (Labonte, Yerko et al. 2012). In addition, Steiger et al. (2013) found hypomethylation at the 1H promoter in association with borderline personality disorder among bulimia nervosa patients. This is in line with the increased risk of borderline personality disorder found in our ELA cohort (Schaan et al., 2017, manuscript in preparation). However, 1H has been studied far less than 1F and there are also

contradictory reports showing hypermethylation at the 1H promoter in association with ELA (van der Knaap, Riese et al. 2014).

It has become increasingly clear that there is no one-to-one relationship between methylation and transcription (Leenen, Muller et al. 2016). Although studies have reported correlations between higher *GR* methylation and lower *GR* expression, other studies found no association (Hogg, Blair et al. 2013) or in the opposite direction (Labonte, Yerko et al. 2012). However, Labonté et al. (2012) found a positive relationship between methylation levels at *1H* CpG 1-13 and *GR* mRNA levels. In contrast, we found no relationship between methylation levels at *1H* CpG 1-8 (9-14 were not included) and total *GR* expression, and an inverse relationship with *1F* transcripts. Nevertheless, in *1H* CpG 47-55 specifically, we found positive correlations between methylation and transcription.

Differential methylation of other first exon promoters (such as *1C* and *1B*) in ELA have been reported (e.g. (Labonte, Yerko et al. 2012), which were not investigated in this study. However, although there may have been small methylation changes in these regions as well, we can exclude that they affected expression of *GR* transcripts in immune cells. Similarly, the observed differential methylation of one region in the *1H* promoter was not related to detectable changes in total *GR* mRNA, nor in *1H* transcripts specifically.

Not all immune cell types were equally affected by ELA and differences were only detectable when examining specific immune subsets (Elwenspoek, Hengesch et al. 2017, Elwenspoek, Sias et al. 2017). Therefore, a limitation of the present study is that like many other studies (Batten, Aslan et al. 2004, Perroud, Paoloni-Giacobino et al. 2011, Labonte, Yerko et al. 2012, Tyrka, Price et al. 2012, Klengel, Mehta et al. 2013, Labonte, Azoulay et al. 2014, Martin-Blanco, Ferrer et al. 2014, Perroud, Rutembesa et al. 2014, van der Knaap, Riese et al. 2014, Vukojevic, Kolassa et al. 2014, Palma-Gudiel, Cordova-Palomera et al. 2015, Romens, McDonald et al. 2015, Tyrka, Parade et al. 2016, Alexander, Kirschbaum et al. 2018) we used DNA and RNA isolated from whole blood, which contains multiple different cell types and may have obscured possible cell specific changes in GR transcription or methylation. However, we

found no evidence of GR dysregulation in peripheral immune cells using three specific stimuli, suggesting that, overall, GR signaling appeared to function normally in participants exposed to ELA in T cells, B cells, and monocytes. Thus, GR programming does not seem to underlie our observed ELA immune phenotype. Nevertheless, other molecular pathways may have been epigenetically programmed by ELA, such as the NFkB pathway that plays an important role in immune activation. Future research should focus on unbiased approaches, for instance investigating whole genome methylation profiles, preferably in single immune cell subsets. Finally, longitudinal studies will be necessary to differentiate between cause and effect, and to determine whether immune dysregulation precedes stress axis dysregulation or whether they develop concurrently.

2.6 Funding

Fonds National de Recherche (FNR) Luxembourg (C12/BM/3985792 "EpiPath" and an AFR PhD fellowship No 9825384), the Ministry of Higher Education and Research of Luxembourg, University of Luxembourg, and DFG funding (SCHA1067/3-1) funded this work. These funding bodies were not involved in the study design, nor in the collection, analysis, and interpretation of the data. JDT is a management board member of the EU-funded COST action CM1406.

2.7 Ethics approval and consent to participate

In accordance with the declaration of Helsinki, all participants provided written informed consent and the study protocol was approved by the Ethics Review Panel (ERP, University of Luxembourg, No 13-002) and the National Research Ethics Committee (CNER) of Luxembourg (No 201303/10 v1.4).

2.8 Authors' contributions

The article was written by MMCE, KS, and JDT. MMCE recruited the study population. XH, MMCE, FADL, and HS were involved in performing visit 1. SBF, FB, and SS performed the *GR* pyrosequencing. KS performed the mRNA expression experiments. SBM, SS, MMCE, and FADL performed the GR sensitivity assay and ELISAs. VKS and CV were involved in performing visit 2. The study was conceived by CPM and JDT with the support and contribution of HS and CV.

2.9 Conflicts of interest

None

2.10 Acknowledgements

We would like to thank Martyna Marynowska and Fei McMahon for laboratory assistance (LIH); Jérôme Graas, Jean-Yves Ferrand, Roxane Batutu, Myriam Alexandre and Graziella Ambroset from the Clinical and Epidemiological

Investigations Center (CIEC, LIH) and Johannes Finke (University of Trier) for assisting in sample collection during the visit 1.

2.11 Abbreviations

CpG, cytosine nucleotide followed by a guanine nucleotide; CTQ, childhood trauma questionnaire; ELA, early life adversity; GC, glucocorticoid; GR, glucocorticoid receptor; HPA, Hypothalamic-Pituitary-Adrenal; IQR, interquartile range; LPS, lipopolysaccharide; NK cell, Natural Killer cell.

2.12 Supplementary data

Supplementary table 1 - Supplementary table 1 - 1F and 1H GR PCR primers and conditions and sequencing primers for pyrosequencing

Promoter	1st round	Sequence	2nd round	Sequence	Sequencing primers
1F	F_B Tm=54°C	Fw: 5'-GGTYGAGAYGTTGYGGTATYGTTTTYGTG-3' Rev: 5'-CCTTAACRACAAACRCCRCCAATAC-3'	F_B Tm=51°C	Fw: Biotin 5'-GTTGYGGTATYGTTTTYGTGTAATTT-3' Rev: 5'-ACRAATAACAACRAACRAACCACAA-3'	aacRaacRaaccaca
	F_C Tm=54°C	Fw: 5'-TTGTGGTTYGTTYGTTGTTATTYGTAGG-3' Rev: 5'-CACCRAATTTCTCCAATTTCTTTTCTC-3'	F_C Tm=52°C	Fw: 5'-TTGTGGTTYGTTYGTTGTTATTYGTAGG-3' Rev: Biotin 5'-CAATTTCTTTTCTCRCTACCTCCTTCC-3'	GTtgtTaTTYGTaggggTaT
1H	H_A Tm=54°C	Fw: 5'-TTYGGTTGYGGYGGGAATTGYGGAYGGTG-3' Rev: 5'-AAACTAATAAAAATTTATAAACTCC-3'	H_A Tm=60°C	Fw: 5'-GGTGGYGGGYGAGYGGTTTTTTTGTTAGAG-3' Rev: Biotin 5'-ACTCCCRCRACRACCCCCRAATTATCTC-3'	TTtTtgTTagaggtaagaag
	H_B Tm=58°C	Fw: 5'-GGGYGYGTTYGTTTTTTYGAGGTGTYGTTG-3' Rev: 5'-CTCCCCCTCRACCCRACCAAA-3'	H_B Tm=52°C	Fw: 5'-GAGATAATTYGGGGGTYGTYGYGGGAG-3' Rev: Biotin 5'-ACCCRACCAAAAAACRCCTAC-3'	tgggggTtggTaag
	H_C Tm=51°C	Fw: 5'-TTTYGTAGGYGTTTTTTGGTYGGGTYGAG-3' Rev: 5'-AATTCAAACRCRACTTAACRTTCACCACRAA-3'	H_C Tm=54°C	Fw: 5'-TTTYGTAGGYGTTTTTTGGTYGGGTYGAG-3' Rev: Biotin 5'-CCAAAATTCCCRCRAAAAATAAAAAACTC-3'	gTYGaggggggaggaa
	H_D Tm=51°C	Fw: 5'-GGTYGTTYGATATTYGTTTTYGTGGTG-3' Rev: 5'-CCCRCTTATACACCCTCAC-3'	H_D Tm=56°C	Fw: Biotin 5'-GGTYGTTYGATATTYGTTTTYGTGGTG-3' Rev: 5'-CCRCACRCCCTCCTCAAACCA-3'	Rcccttctcaaacca

Supplementary table 2 - Supplementary table 2 - Sequences of forward and reverse primers and conditions used in real time qPCR analyses. All primers were purchased at Eurogentec, Liege, Belgium.

			PCR conditions			
Gene	Primers	Sequences	MgCl2	Primer concentration	Annealing temperature	Size product
GAPDH	GAPDH_fwd GAPDH_rev	FWD: 5'-GAAGGTGAAGGTCGGAGTC-3' REV: 5'-GAAGATGGTGATGGGATTTC-3'	2 mM	1.0 uM	60 °C	226 bp
β Actin	Bactin_fwd Bactin_rev	FWD: 5'-GGCCACGGCTGCTTC-3' REV: 5'-GTTGGCGTACAGGTCTTTGC-3'	2 mM	1.0 uM	60 °C	208 bp
PPIA	PPIA_fwd PPIA_rev	FWD: 5 -TCCTGGCATCTTGTCCATG-3' REV: 5 -CCATCCAACCACTCAGTCTTG-3	2 mM	1.0 uM	60 °C	90 bp
FKBP5	FKBP5_fwd FKBP5_rev	FWD: 5'-AAAAGGCCAAGGAGCACAAC-3' REV: 5'-TTGAGGAGGGGCCGAGTTC-3'	4 mM	0.50 uM	67 °C	236 bp
GILZ	GILZ_fwd GILZ_rev	FWD: 5'-GCACAATTTCTCCATCTCCTTCTT-3' REV: 5'-TCAGATGATTCTTCACCAGATCCA-3'	2 mM	0.50 uM	60 °C	146 bp
Total GR	Exon 3/4_fwd Exon 3/4_rev	FWD: 5'-CTCAACAGCAACAACAGGACCAC-3' REV: 5'-GATGCAATCATTCCTTCCAGCA-3'	2 mM	0.50 uM	56 °C	166 bp
GR H1	Exon 1H_fwd Exon 2_rev	5'-CTGACAGCCCGCAACTTGGA-3' 5'-CAGTGGATGCTGAACTCTTGG-3'	3 mM	1.0 uM	65 °C	531 bp
GR F1	Exon 1F_fwd Exon 2_rev	5'-GTAGCGAGAAAAGAAACTGG-3' 5'-CAGTGGATGCTGAACTCTTGG-3'	1.75 mM	0.50 uM	60 °C	511 bp

Chapter III: Early life adversity shapes the immune system in adulthood

My contribution to this chapter:

Plan and perform the animal experiments, including the maternal separation paradigm, stress test and behavioral tests. Performing the immunological measurements; Setting up and perform all flow cytometry analysis. Final statistical analysis. Literature research and writing of the manuscript.
Manuscript published in *Frontiers in Immunology.*

3. UNBIASED SCREENING IDENTIFIES FUNCTIONAL DIFFERENCES IN NK CELLS AFTER EARLY LIFE PSYCHO-SOCIAL STRESS

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3.1 Abstract

Early Life Adversity (ELA) is closely associated with the risk for developing diseases later in life, such as autoimmune diseases, type-2 diabetes and cardiovascular diseases. In humans, early parental separation, physical and sexual abuse or low social-economic status during childhood are known to have great impact on brain development, in the hormonal system and immune responses. Maternal deprivation (MD) is the closest animal model available to the human situation. This paradigm induces long lasting behavioral effects, causes changes in the HPA axis and affects the immune system. However, the mechanisms underlying changes in the immune response after ELA are still not fully understood.

In this study we investigated how ELA changes the immune system, through an unbiased analysis, viSNE, and addressed specially the NK immune cell population and its functionality. We have demonstrated that maternal separation, in both humans and rats, significantly affects the sensitivity of the immune system in adulthood. Particularly, NK cells' profile and response to target cell lines are significantly changed after ELA. These immune cells in rats are not only less cytotoxic towards YAC-1 cells, but also show a clear increase in the expression of maturation markers after 3h of maternal separation. Similarly, individuals who suffered from ELA display significant changes in the cytotoxic profile of NK cells together with decreased degranulation capacity. These results suggest that one of the key mechanisms by which the immune system becomes impaired after ELA might be due to a shift on the senescent state of the cells, specifically NK cells. Elucidation of such a mechanism highlights the importance of ELA prevention and how NK targeted immunotherapy might help attenuating ELA consequences.

Keywords:
Early life stress, maternal deprivation, Immune system, natural killer cells, NK cells

3.2 Introduction

Early life adversity (ELA), which means stressful events occurring in the first 1000 days of life (Barker 1997; Barker and Osmond 1986; Martyn, Barker, and Osmond 1996), plays a major role in adult-onset illness (Agorastos et al. 2019; Fogelman and Canli 2019; Taylor 2010). These stressful events include a series of negative situations such as poor socio-economic status, parental mental disease, abandonment and/or institutionalization. Exposure to ELA has long-lasting effects on both mental and physical health as well as having negative behavioural consequences. It is associated with an increased risk of developing cardiovascular diseases (Carroll et al. 2013), asthma (Arrieta et al. 2015), cancer (Kelly-Irving et al. 2013) and mental disorders such as depression and anxiety later in life in both humans and in rodent models (Diaz-Chavez et al. 2020; Donoso et al. 2020; Kendler, Thornton, and Gardner 2000; Krugers et al. 2016; Roque et al. 2014). There is now growing clinical and pre-clinical evidence that ELA and the associated negative health risk behaviours act through an altered immune system to induce the later-life disease risk (Ramiro, Madrid, and Brown 2010; Elwenspoek, Hengesch, Leenen, Schritz, Sias, Schaan, Mériaux, et al. 2017; Baumeister et al. 2016; Beijers et al. 2010; Brodin et al. 2015).

Development of the immune system starts in early gestation. Innate immune cells such as monocytes, neutrophils and NK cells appear in the first trimester of pregnancy. Adaptive immune cells (T and B-lymphocytes), emerge around the start of the second trimester (Palmer 2011; Gollwitzer and Marsland ; Simon, Hollander, and McMichael 2015). During the pre- and perinatal period, this development is highly affected by several maternal factors such as obesity (Odaka et al. 2010), malnutrition (van de Pavert et al. 2014; Fisher et al. 2019), anxiety (Beijers et al. 2010; O'Connor et al. 2013; Henriksen and Thuen 2015; Nielsen et al. 2011) and smoking (Herberth et al. 2014; Noakes et al. 2006), but also environmental factors such as parturition (Weinberger et al. 2007; Yektaei-Karin et al. 2007), breastfeeding (Belderbos et al. 2012; Field 2005) and antibiotic treatment (Culić, Eraković V Fau - Parnham, and Parnham 2001). Alterations in specific cellular subsets in the immune system resulting from ELA

have been widely documented in clinical studies. Individuals subjected to parental separation and subsequent adoption displayed a higher activation state of the immune system, with decreased levels of circulating central memory T cells and CD8+ T regulatory cells (Elwenspoek, Hengesch, Leenen, Schritz, Sias, Schaan, Mériaux, et al. 2017). Teenagers who suffered from childhood maltreatment, such as sexual and physical abuse, physical and emotional neglect showed increased circulating levels of NK and NKT cells after ELA (do Prado et al. 2017). In addition, blood levels of the inflammatory marker C - reactive protein (CRP) in teenagers who had a low social-economic status as children was found to be increased when compared to individuals not exposed to early stress (Schmeer and Yoon 2016). Individuals with poor maternal care and harsh discipline during childhood also presented increased levels of CRP in the blood (Danese et al. 2007). Studies with rhesus monkeys, early isolated from their mothers at early age, also show a significant decrease in the CD4+/CD8+ ratio and increase in the circulating levels of NK cells (Lewis et al. 2000). Even though some animal studies do not show any difference in the cell number after maternal separation (Kruschinski et al. 2008), others document a decrease of the CD8+ T cells with subsequent increase of the CD4+/CD8+ ratio (Roque et al. 2014), opposite to what was documented in monkeys. Furthermore, prenatal exposure to alcohol lead to an increase in the CRP serum levels, indicating an inflammatory state of the immune system (Raineki et al. 2017), similarly to what was previously observed in clinical studies (Schmeer and Yoon 2016; Danese et al. 2007). Despite these numerous investigations, the literature is still lacking an unbiased overview of the complete cellular immune system.

Although the mechanisms through which these events occur are still not fully understood, increasing evidence shows that the mechanism by which ELA influences the function of CD8+ T cells and, consequently, viral responses, may be through the HPA axis (Bailey et al. 2003). The neuro-endocrine axis plays an important role in the adaptive response to stress. When facing insults, corticotropin-releasing factor (CRF) is released from the hypothalamus, which in turn stimulates the production and release of adrenocorticotropin (ACTH). This hormone's main target is the adrenal cortex where the production and

release of glucocorticoids (GCs) happens. Release of GCs into the bloodstream will trigger the adaptive mechanisms, in a negative feedback manner (Aguilera 2016; Smith and Vale 2006). Stress events in an early period of life are known to have an impact in the HPA axis, programming its effects and responses in adulthood. This leads to decreasing levels of blood corticosterone and cortisol, which consequently affects the response of the peripheral immune system, leading to compromised viral responses (Silverman et al. 2005; Roque et al. 2014; Hong et al. 2020). Such dysregulation of the HPA axis is thought to occur through the glucocorticoid receptor (GR), by regulation of gene transcription and negative feedback on the HPA axis, which in turn decreases the expression of certain cytokines (Cain and Cidlowski). Clinical studies show an association between increased GR1F promoter methylation and ELA (van der Knaap et al. 2014; Romens et al. 2015). However GR/GC signalling remains undisturbed after ELA despite a slight increase in GR1F promoter methylation (Elwenspoek et al.), raising doubts as to the importance of single-digit changes in promoter methylation levels (Leenen, Muller, and Turner 2016).

ELA also accelerates immunosenescence, the natural aging process by which the immune cells begin to deteriorate and lead to weakened immune responses (DeWitt and Luebke 2015). Immunosenescence is accelerated not only after exposure to ELA (Elwenspoek, Sias, et al. 2017b), but also with depression after physical injury (Duggal et al. 2015). T cells are strongly affected. Naïve T cell numbers decrease while memory T cell and terminally differentiated effector T cell (TEMRA) numbers increase, with concurrent telomere shortening (Xu and Larbi 2017; Elwenspoek, Kuehn, et al. 2017; de Punder et al. 2019; Shalev et al. 2013). Furthermore, these cells have decreased expression of the co-stimulatory CD28 molecule and increased expression of the glycopeptide CD57, which leads to increased cytotoxicity and decreased proliferative capacity (Xu and Larbi 2017; Voehringer, Koschella, and Pircher 2002; Brenchley et al. ; Elwenspoek, Sias, et al. 2017b). This increase in T cell senescence after ELA has been reported to be influenced by the exposure to and subsequent reactivation of cytomegalovirus (CMV), as levels of CD57+ cells are increased in patients seropositive for CMV (Elwenspoek, Sias, et al. 2017b; Wertheimer et al. ; Weltevrede et al. ; Klenerman and Oxenius). Moreover, CMV in ELA

individuals was recently reported to be linked to the presence of certain gut bacteria and CD8+CD57+ cells (Reid et al. 2020), suggesting an impact of ELA through the immune-brain-gut axis.

Although it is not as well documented as for T cells, immunosenescence also occurs in other cell types, such as B (Frasca 2018; Ma et al. 2019) and NK cells (Judge, Murphy, and Canter 2020; Solana, Tarazona, and Solana 2018). The expression of CD57 in natural killer cells does not necessarily mean they are senescent but rather that they reached a higher maturation state, which is accompanied by functional changes similar to those observed in senescent T cells: less proliferation and higher cytotoxic capacity (Lopez-Vergès et al. 2010; Nielsen et al. 2013; Judge, Murphy, and Canter 2020). Moreover, NK cells are clearly involved in the response to CMV infections (Goodier et al. ; Béziat et al. 2013) and links between NK cells, CMV and immunosenescence are starting to emerge (Goodier et al. ; Lopez-Vergès et al. 2011; Della Chiesa et al. 2012).

Using an unbiased screening tool for flow cytometry data visualization, viSNE (Amir el et al. 2013), this study provides a detailed description of the overall immune changes induced in the rat maternal deprivation (MD) model of ELA, identifying unexpected, but clear changes in NK cell properties. Furthermore, we describe the functional profile of NK cells, showing a shift in the maturity and cytotoxic capacities. We validated the NK cell phenotype in samples from our EpiPath ELA cohort (Elwenspoek, Sias, et al. 2017b; Hengesch et al. 2018). This cohort consists of young adults (average age 24) institutionalized or otherwise separated from their biological parents at birth and adopted in early childhood (mean age of adoption 4.5 months) together with control participants in their natal families, all brought up in Luxembourg under similar societal and socioeconomic conditions.

3.3 Material and Methods

3.3.1 Human Samples

Peripheral blood mononuclear cells (PBMCs) from individuals that had experienced ELA in the form of institutionalization and subsequent adoption were obtained from our previously published EpiPath cohort (Hengesch, Elwenspoek et al. 2017; Elwenspoek, Hengesch et al. 2018). Briefly, participants aged between 18 and 35 years old with a prior history of ELA (institutionalization followed by adoption) or raised by their natural parents were recruited in Luxembourg between 2014 and 2016. Baseline EDTA anti-coagulated blood samples were drawn at a fixed time (11 am). Peripheral blood mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation as previously reported (Elwenspoek, Sias et al. 2017). and stored in liquid nitrogen until analysed. All participants provided written informed consent, and the study was performed in accordance with the Declaration of Helsinki. The study was approved by the Luxembourg National Research Ethics Committee (CNER, No 201303/10 v1.4) and the Ethics Review Panel (ERP, University of Luxembourg, No 13-002).

3.3.2 Animals

Ten to twelve week old 2-day timed-pregnant Wistar rats were obtained from Janvier Labs (Le Genest-Saint-Isle, France). Pregnant dams were housed in groups of 3 in 48 × 37.5 × 21 cm clear plastic isolator cages (Tecniplast, Varese, Italy) under a conventional 12-h light-dark cycle at 21°C and 49-54 % relative humidity with food and water provided ad libitum. During pregnancy only routine husbandry was performed. Nesting material was provided for all females from gestational day (GD) 16 onwards and the cage was not changed between GD17 and post-natal day (PND) 2. Litters were naturally delivered between days 21-23 of gestation and size was adjusted to 12 pups/dam. Dams were randomly assigned to give birth to pups for one of the following groups (one condition per litter; two litters per group): 3 hours Maternal Deprivation from PND2 to PND14 (MD180), 15 minutes Maternal Deprivation from PND2 to PND14 (MD15) and no separation (CTR). Study outcomes are thus from two

independent experiments. The experiments were carried out in accordance with the European Union directive 2010/63/EU as incorporated in Luxembourgish law for the care and use of laboratory animals. The study protocol was approved by the local Animal Welfare Structure (DII-2017-18).

3.3.3 Rat Maternal Deprivation (MD)

Pups from both MD groups underwent a separation from the dam at a fixed time every day (MD180: 9 am - 12 am, MD15: 9 am - 9:15 am) from PND 2 to PND 14. Separated pups were placed in a clean bedding-free cage and maintained at 33°C in a heated vented animal cabinet (Noroit, France). At the end of the daily separation period, pups were returned to their mothers in the original home cage. Control litters were only handled for regular husbandry (e.g. cage cleaning) and otherwise left undisturbed until weaning. All animals were weaned on PND21, and subsequently housed (2 to 3 per cage) by sex and experimental group, and only received regular husbandry until further experiments.

3.3.4 Rat Restraint Stress

All animals underwent a 1-hour restraint stress on PND49 +/- 1 day. Restraint stress was performed between 9 and 12 am during the inactive (light) phase. Animals were immobilised in a 50mm diameter dark grey PVC tube, closed at the front and with an adjustable lock in the back. Breathing of the animals was controlled during the whole procedure.

Rat Corticosterone and Glucose Levels

Blood samples were drawn from the tail vein using a SAFETY Blood Collection/Infusion Set (Greiner Bio-One, Germany), immediately on being placed in the restrainer and in the minutes preceding their release. At the same time, a single blood drop was used to measure glucose levels, using an electronic glucometer (Accu-Chek, Roche). All blood samples were centrifuged at 2000 x g for 5 minutes and the plasma collected and stored at -80°C, until further analysis. Plasma corticosterone levels were measured by ELISA (IBL International, Hamburg, Germany), according to the manufacturer's instructions. A 4-parameter curve was fitted to the calibrator sample OD values;

sample concentrations were calculated and, for glucose, presented as delta values (values after stress – values before stress).

3.3.5 Rat Immunophenotyping

At PND56 animals were euthanized by CO2 inhalation and cardiac puncture was performed post- mortem to collect blood. Post-mortem blood (100µL per animal) was used for immunophenotyping by flow cytometry (LSR Fortessa, BD Biosciences, NJ, USA). Cell surface specific antibodies (see Supplementary Table 3) were diluted in flow cytometry staining (FACS) buffer (1X PBS, 1% BSA, 2mM EDTA), added to each individual sample and incubated for 30 minutes, at 4°C in the dark. Subsequently, samples were washed three times (100µl, 4°C, 300 x g, 10 minutes, FACS buffer) and erythrocytes lysed with Lysis buffer (BD Biosciences) for 10 minutes at room temperature in the dark. Cells were fixed with fixation buffer (Invitrogen, CA, USA) for 1h, washed (100µl, 4°C, 300 x g, 10 minutes, FACS buffer) and permeabilized for 1 hour with permeabilization buffer (Invitrogen, CA, USA). Intracellular markers (Supplementary Table 3) were diluted in FACS buffer and added to the samples. After 30 minutes incubation (4°C, protected from light), the samples were washed three times (100µl, 4°C, 300 x g, 10 minutes) and re-suspended in FACS buffer for further analysis.

3.3.6 Natural Killer Cell Phenotyping

NK cell phenotyping was performed on both rat splenocytes and human PBMCs. Single-cell splenocyte suspensions were prepared on the day of the sacrifice and stored in liquid nitrogen in FBS (Sigma Aldrich, MO, USA) containing 10% DMSO (Sigma Aldrich) until analysed. On the day of the assay, vials were thawed at 37°C and washed with RPMI-1640 (Lonza, Basel, Switzerland) complemented with 10% FBS, 1% Penicillin/Streptomycin (Lonza), 1% Glutamine (Lonza) and 50µM of β-mercaptoethanol (Invitrogen). Cells were diluted to 106 cells/ml and 200µL aliquots distributed in 96 well plates, prior to incubation for 1 hour at 37°C, 95% humidity and 5% CO2. NK cell maturation state was assessed by flow cytometry (antibodies in Supplementary Table 3)

as described above. Cell viability was measured using CellTrace Violet (CTV, Life Technologies, Paisley, UK).

3.3.7 Natural Killer Cell Cytotoxicity Assays

The cytotoxic response of rat NK cells was determined against YAC-1, a murine lymphoma cell line. Target cells were thawed and cultured in suspension in flasks with complete RPMI medium (RPMI-1640, 10% FBS, 1% Pen/Strep, 1% Glutamine, 1 mM HEPES, 50μM β-mercaptoethanol). Only cells in the exponential growth phase were used in the assays. Single-cell splenocyte suspensions were cultured for 72 hours in complete RPMI-1640, with 200U/mL of recombinant rat IL-2 (Sigma Aldrich), at 37°C, 95% humidity and 5% CO2. Before the challenge, YAC-1 cells were stained with 1μM Cell Trace Violet (CTV) in 1XPBS for 20 minutes and washed twice with 1X PBS. Similarly, human PBMCs from the EpiPath cohort (Elwenspoek, Hengesch et al. 2017) were cultured in complete medium with 200U/mL of recombinant human IL-2 (R&D Systems Inc., MN, USA) and left undisturbed overnight. For human NK cells, the cytotoxic response was determined against K562, a human myeloid leukemia cell line.Cells were cultured in suspension in flasks with complete DMEM (DMEM, 10% FBS, 1% Pen/Strep, 1% Glutamine, 1 mM HEPES) and only taken for the assays at the exponential growth phase. Before the assay, K562 were pre-incubated with 1μM CTV for 20 minutes and washed twice with complete RPMI-1640 (RPMI-1640, 10% FBS, 1% Pen/Strep, 1% Glutamine). Effector NK cells (E) and YAC-1 or K562 target cells (T) were plated at E:T ratios ranging from 1:1 to 100:1 for rat splenocytes and 1:1 to 25:1 for PBMCs, for four hours. Fifteen minutes before acquisition, 15μM of TO-PRO3 (Invitrogen, Karlsruhe, Germany) was added, to discriminate viable cells from dead cells (TO-PRO3+).

3.3.8 Natural Killer Cell Degranulation Assay

Human PBMCs were cultured overnight in complete medium with 200U/ml of IL-2 and stimulated with CTV labelled K562 target cells at ratios of (E:T): 1:1, 5:1, 10:1 and 25:1 as described above. At the same time, anti-CD107a antibody was added to each well. After 1h incubation, 0.1μL of GolgiStop (BD

Biosciences) was added per well and the plate was incubated at 37°C, 5% CO2 for a further three hours. Cells were washed with FACS buffer (10 minutes, 300 x g) and stained for NK cell surface markers (Supplementary Table 3) followed by intracellular staining for IFN-γ as described above.

3.3.9 Flow Cytometry

A minimum of 50,000 events were recorded for all the experiments. Immunophenotyping, NK cell maturity and degranulation assays were performed on BD LSR Fortessa (BD BioSciences using FACSDiva software (BD BioSciences, version 8.0). The NK cytotoxicity assays were analyzed on a NovoCyte Quanteon Flow Cytometer (Agilent).

Data Analysis

Flow cytometry data was analysed with FlowJo (Tree Star, Ashland, OR, USA), visNE software (Cytobank, Inc., CA, USA) and Tableau (Seattle, WA, USA). After processing the raw data, 36 flow cytometry .fsc files (12 per experimental group) from the 12-colour initial panel were uploaded onto Cytobank and used to generate viSNE plots according to the following parameters: Events = 50.000; Channels = all 12 antibodies; Compensation = uncompensated; Iterations = 5000; Perplexity = 30. For the illustration menu, the gating of all channels was set for minimum of -2000 and the argument at 200. For further and more detailed analysis, FlowSOM was used with the default settings and all channels and files were selected. Event sampling was set at 50.000; Number of metaclusters at 10; Iterations at 10; and Number of clusters at 49. Results of this analysis were plotted into t-SNEs maps and cell populations were separated according to the presence of each cell marker across the different cell populations.

3.3.10 Statistical analyses and data presentation

Statistical analyses were performed in GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, CA, USA) and FlowSOM (Cytobank, Inc., CA, USA). Tests used to assess statistical differences were One-way ANOVA (Tukey's multiple comparisons test) or Two-way ANOVA (Dunnett's or Sidak's multiple comparisons tests), depending on the number of animals and

parameters in the assay. Figures were subsequently generated using GraphPad Prism and Adobe Illustrator CS6 (version 16.00).

3.4 Results

3.4.1 Maternal deprived animals subjected to stress in adulthood have an increased physiological response to acute stress

At PND49, all animals were subjected to a restraint stress in order to evaluate HPA axis function. Corticosterone and glucose were measured from plasma and whole blood before and upon completion of the acute stressor, respectively. Although the restraint stress did not induce any significant changes in corticosterone and glucose levels in the two MD groups (Fig. 8A and 8B; Tukey's multiple comparisons test, p=0.81 for MD_{15} and p=0.6 for MD_{180} for corticosterone; p= 0.43 for MD_{15} and p=0.14 for MD_{180}, for glucose), the stressor significantly increased the absolute glucose levels in the MD_{180} group (115.9mg/dL ± 1.4 vs 134.4mg/dL ±3.7, Sidak's multiple comparisons test p=0.014) (Fig. 8C). This shows an activation of gluconeogenesis in the liver (Kuo, McQueen et al. 2015) and release into the blood stream, indicative of the fight-or-flight response of a system in need of energy supply.

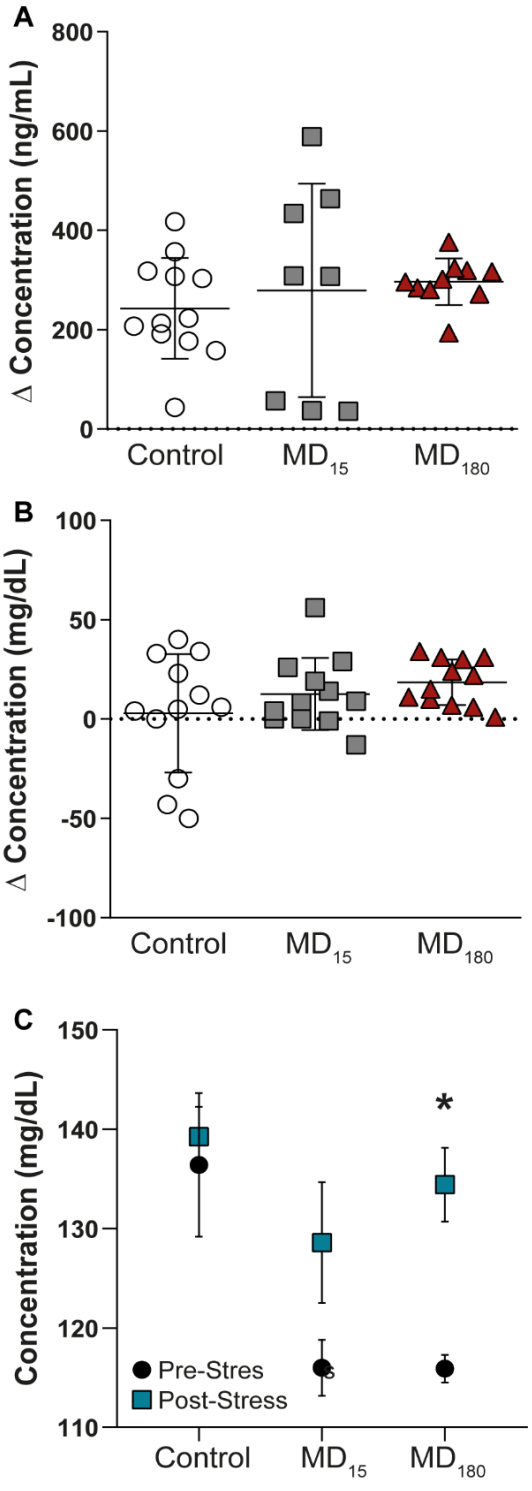
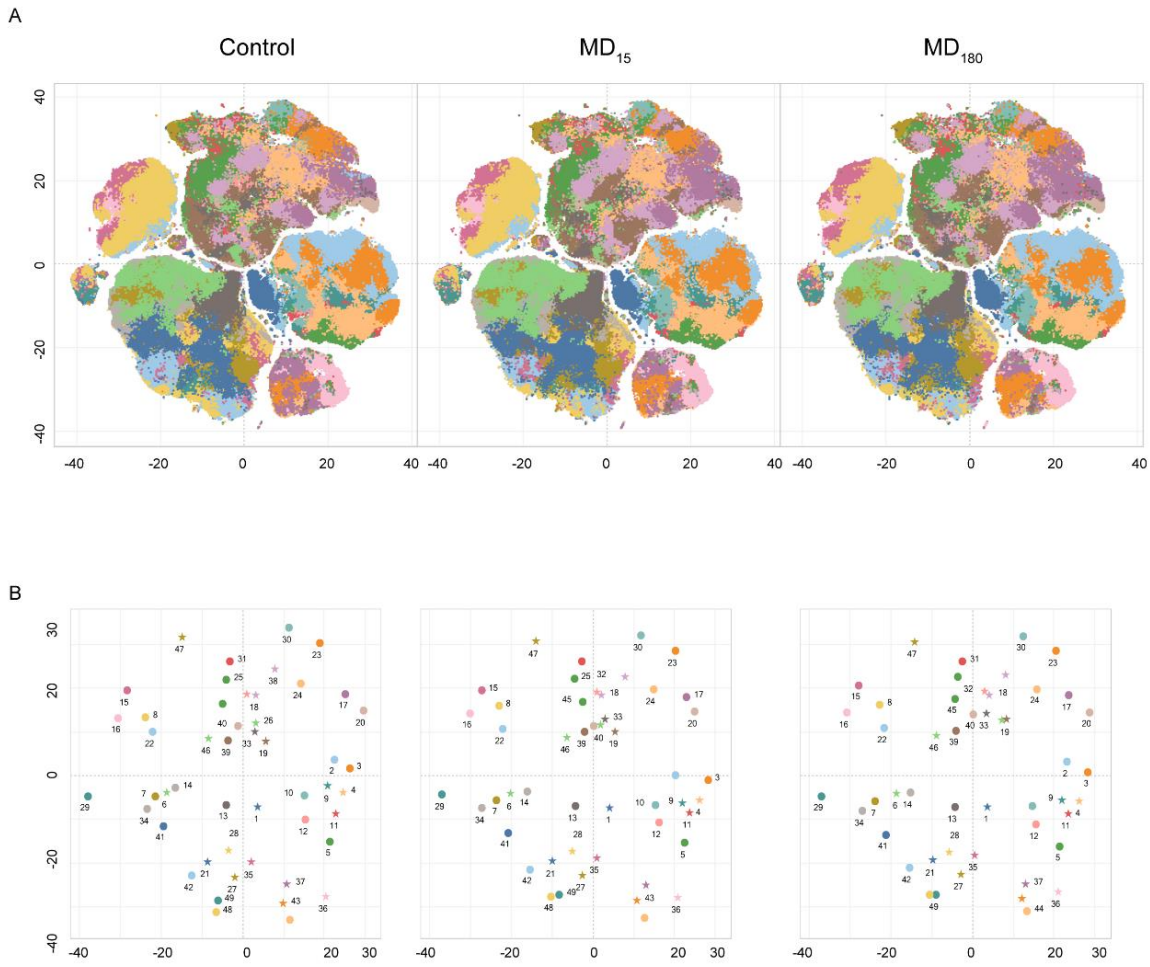


Figure 13 - Acute stress in adulthood has no significant impact on the HPA axis of maternal deprived animals, but changes the glucose absolute levels. (A) Delta corticosterone levels; (B) Delta glucose levels; (C) Absolute glucose levels before and after an acute stress. Data is presented as mean ± SEM of 9 to 12 animals per group. Statistics: One-way and Two-way ANOVA. *p = 0.014

3.4.2 Unbiased immunophenotyping

Flow cytometry was performed for all animals at PND56. After basic data quality checks, t-SNE maps were generated from viSNE and flowSOM analysis. The t-SNE maps show clear differences in the clustering of the data through the abundance and spatial distribution of certain regions (Fig. 9A). The map regions represent the different immune cell subsets from the animals, that were colour defined based on the cell markers used (Supp. Fig. 1). In total, 49 different clusters were identified, twenty of which were found to be statistically different between both maternally separated groups and the control, according to the antibodies that define each cluster (supplementary table 4; Fig. 9B). As seen in previous reports from other experimental paradigms (Sakkestad, Skavland and



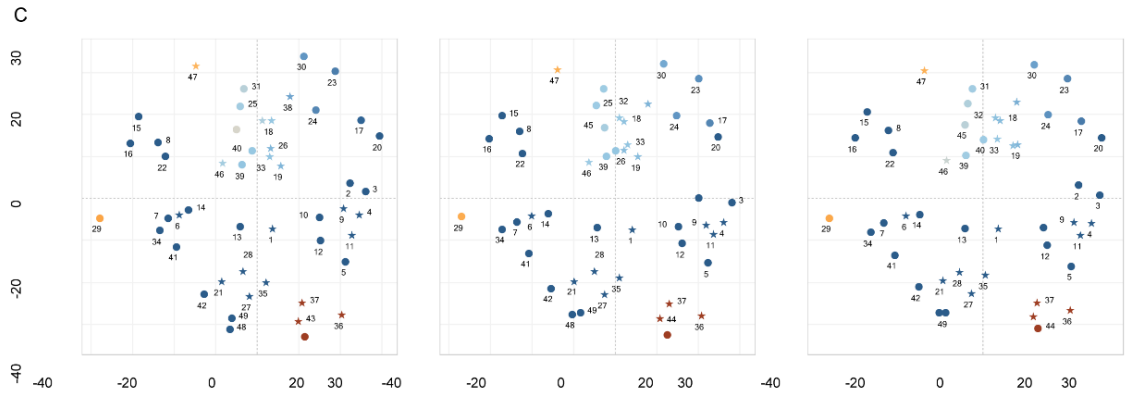


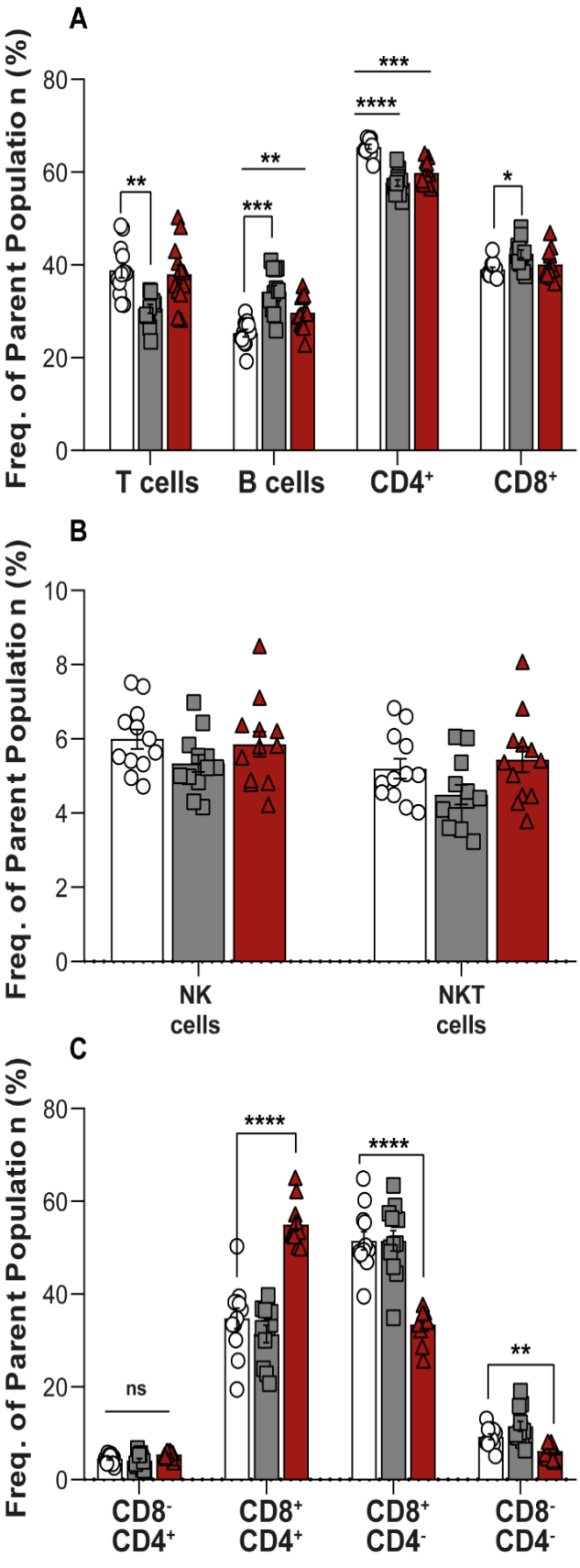
Figure 14 - Unbiased immunophenotyping with viSNE. (A) visNE map obtained through cytobank with the markers a single 13-color antibody combination. Each panel represents the mean of all 12 animals in the control (left), MD15 (middle) and MD30 (right) groups; (B) Identification of different clusters by the principal rat cell surface markers – CD3 CD4, CD8, CD11b/c, CD45RA, FoxP3. Stars represent significantly different clusters, between the different treatment groups at an FDR corrected $p < 0.05$; (C) Clusters colored by CD161a (NK cell marker) intensity. Blue to orange: low to high expression of the marker.

Hanevik 2020; Lohmann et al. 2018; Jang et al. 2020), T ($CD3^+$), T helper ($CD4^+$) and T cytotoxic ($CD8^+$) cells were the most clearly delineated populations within the viSNE plot and where we found the most significant changes after MD (Sup. Fig. 1). B cells ($CD45RA^+$) (Woollett Gr Fau – Barclay et al., 1985; Barclay An Fau – Jackson et al., 1987) together with clusters containing macrophages, dendritic ($CD11b^+$) and T regulatory ($CD25^+$, $FoxP3^+$) cell types were also readily identified (Sup. Fig.2). Surprisingly, some of the most significantly different clusters were associated with the CD161a cell marker, which is one of the primary cell surface markers for NK cells. (Fig. 9C).

3.4.3 Maternal deprivation induces long-term changes in the immune system

The clusters identified in our viSNE analysis were examined in detail with FlowJo. The percentage of $CD3^+$ T cells was significantly decreased in the animals subjected to 15 minutes of MD (30.5 ± 0.95, Dunnett's multiple comparisons test, p=0.001), although no significant changes were found in the group separated for 3 hours, when compared to the control group (37.9 ±1.98 vs 38.8 ± 1.62) (Fig. 10A). B cells, on the other side, were found to be significantly increased in both groups (MD_{15}: 34.2 ± 1.4, $p<0.0008$; MD_{180}: 29.7 ± 1.04, $p<0.0085$), when compared to controls (25.3 ± 0.81) (Fig. 10A). To further investigate how the immune system was impacted, we also looked

at the different types of T cells. $CD4^+$ helper Tcells were found to be significantly decreased in both MD_{15} (57.7 ± 0.71, $p<0.0001$) and MD_{180} (59.8 ± 0.74, $p=0.001$), compared to the control group (65.5 ± 0.51). The cytotoxic $CD8^+$ T cells were significantly increased in the MD_{15} group compared to controls (42.3 ±0.86 vs 39.0 ±0.48, $p=0.022$) but not in the MD_{180} (Fig. 10A). Activated B cells and subsets of $CD4^+$ and $CD8^+$ T cells, which are involved in Th1, Th2 and Th17 types of responses, characterized by the transcription factors T-bet, GATA3 and RORγT, respectively, were also analysed and quantified but did not produce significant changes upon early stress (data not shown).



3.4.4 Levels of NK and NKT-like cells changed after ELA

After our unbiased analysis with viSNE, we quantified the levels of both NK and NKT-like cells using a classical gating strategy (Sup. Fig. 10) in FlowJO. For the two populations, both MD groups did not show significant differences when compared to control (Fig. 10B), but when separating the NKT

Figure 15 - Maternal deprivation induces long-term changes in the immune system. (A) Adaptive immune cell population analysis. T cells gated as CD3+ from total lymphocytes. B cells gated as CD45RA+ gated from total lymphocytes; CD4+ and CD8+ gated from within the T cell population (B) expression levels of NK and NKT-like cells. NK and NKT cells were gated from total lymphocytes as the brighter population expressing CD161a+ and being CD3- or CD3+, respectively.; (C) Sub-gating of the NKT-cell population from panel (B) by CD4+ and CD8+ markers. Data is presented as mean ± SEM of 12 animals per group. Statistics: Two-way ANOVA; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001, NS, Not Significant. Representative Figures for flow data are included in the Supplementary Material.

cells into their functional subgroups defined by CD4 and CD8 expression (Seino and Taniguchi 2005), statistically significant differences appeared (Fig. 10C). Double positive ($CD4^+CD8^+$) NKT-like cells were significantly increased in the MD_{180} (54.9 ± 1.31, Dunnett's multiple comparisons test, $p<0.0001$), whereas 15 minutes of MD had no effect, when compared to the control group (34.77 ± 2.21). On the other hand, double negative ($CD4^-CD8^-$) NKT-like cells were significantly decreased in the MD_{180} group (6.19 ± 0.43, $p<0.0036$) but suffered no changes in the MD_{15} group, compared to controls (9.24 ± 0.62). Furthermore, $CD8^+$ NKT-like cells were also found to be significantly decreased in the group separated for 3 hours in relation to the control group (33.4 ±0.97 vs 51.5 ± 1.92, $p<0.0001$).

3.4.5 Maternal deprivation changes the maturation state of NK cells

To evaluate the effect of MD on NK cell functionality, we quantified the maturation state of these cells using the markers CD27 and CD11b (Chiossone et al. 2009; Inngjerdingen et al. 2011; Hayakawa and Smyth 2006). The process begins with no expression of either receptors (immature NK cells, iNK), followed by gain of CD27 and CD11b receptors, and ends with loss of CD27, representing the most mature NK cells (mNK) (gating strategy: Sup. Fig.4). The double negative cell population ($CD11b^-$, $CD27^-$; Q4 from Sup. Fig.4) does not appear to be influenced by our MD paradigm (Fig. 4A). Following that, the NK cell population that gained CD27 but not CD11b (Q1 from Sup. Fig. 4) was shown to be significantly decreased in the MD_{180} group compared to the control group (67.0 ± 2.79 vs 73.9 ± 3.70, Dunnett's multiple comparisons test, $p=0.0119$). The double positive (DP) population (Q2 from Sup. Fig. 4A) was significantly increased in both maternal deprived groups (MD_{15} 18.28 ± 1.61, $p=0.019$; MD_{180} 16.48 ± 1.29, $p=0.0042$) compared to the control group (9.76 ± 0.87). Finally, $CD27^-CD11b^+$ population (Q3 from sup. Fig. 4), representing the most mature NK cells, was significantly increased in both maternal

separated groups (MD_{15} 5.07 ± 0.66, p=0.0153; MD_{180} 5.03 ± 0.79, p=0.0023), compared to the control group (3.10 ± 0.56) (Fig. 11A).

3.4.6 Long maternal deprivation changes the cytotoxicity of NK cells

The cytotoxic capacity of rat NK cells after MD was measured against the mouse target cell line YAC-1, as previously described in the literature (Poli, Brons et al. 2010). Cells from animals that underwent 3 hours of MD exhibited a significantly decreased cytotoxicity from E:T ratio 12.5:1 (5.94 ± 0.38, Dunnett's multiple comparisons test, p=0.0032) to the highest E:T ratio, 100:1 (16.57 ± 1.77, p=0.0057), when compared to the cells of the group that did not suffer any type of early stress (12.99 ± 1.03; 38.16 ±3.48) (Fig. 11B). Animals that were maternal separated for 15 minutes displayed a similar response to the control group.

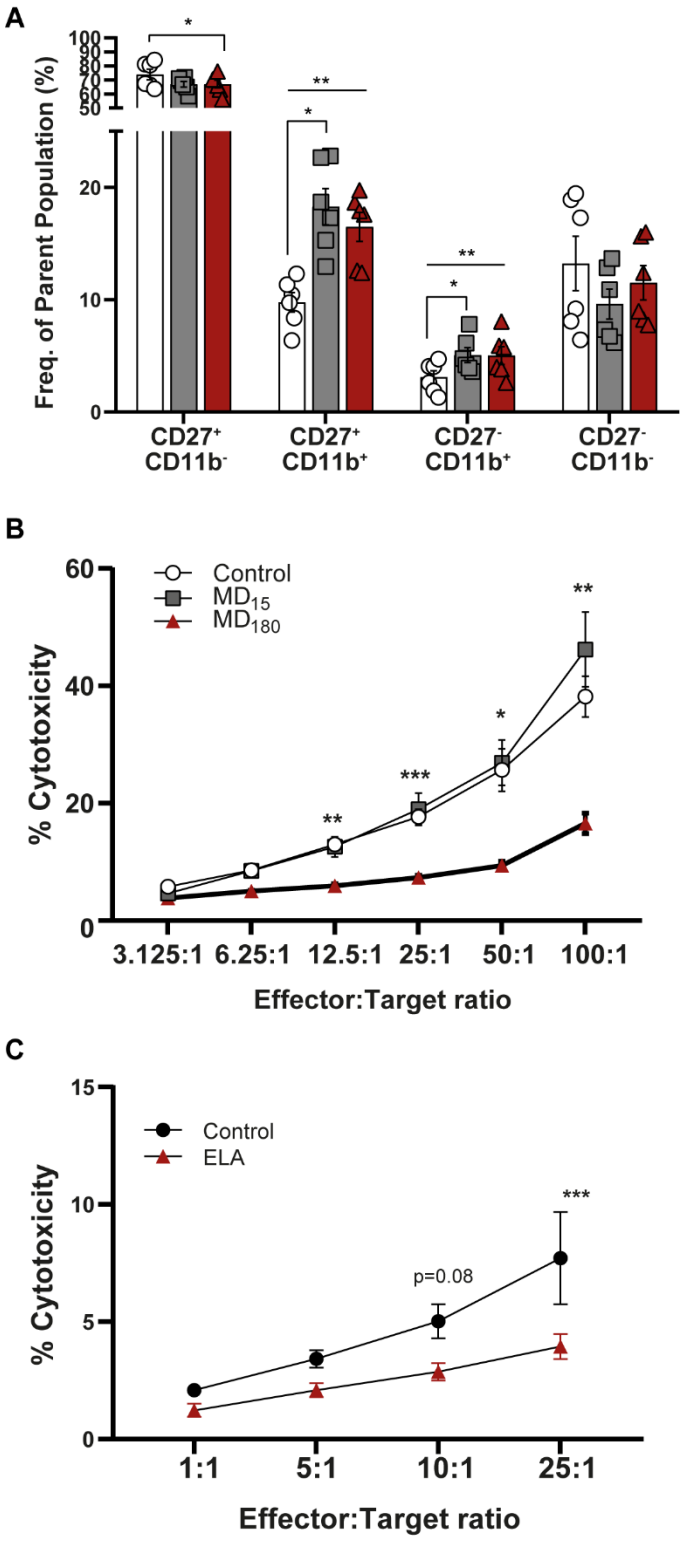


Figure 16 - Early life separation induces functional changes in rat and human NK cells. (A) Maturation state of rat NK cells, defined by CD27 and CD11b markers; (B) Cytotoxic response of rat NK cells to YAC-1 cells; (C) Cytotoxic response of NK cells from institutionalized individuals, to K562 cells. Data is presented as mean ± SEM of 6 animals per group or 14 donors per group. Statistics: Two-way ANOVA; *p<0.05; **p<0.01.

3.4.7 NK cell changes are reproduced in the EpiPath ELA cohort

The cytotoxic response of human NK cells from the EpiPath cohort was measured against K562 cells. Similarly to the rat, NK cells from the individuals that were exposed to ELA had a lower response than the cells from the control group, reaching statistical significance at the highest ratio (E:T, 25:1) (3.93 ± 1.78 vs 7.71 ± 6.52, Sidak's multiple comparison test, p=0.0004) (Fig. 11C). As previously seen in our study (Elwenspoek, Sias et al. 2017), increased titers of CMV could be associated with such a decrease in the cytotoxicity of NK cells. However, there is no statistical correlation in any of the ratios, between CMV titers and NK cytotoxicity (Sup. Fig. 5).

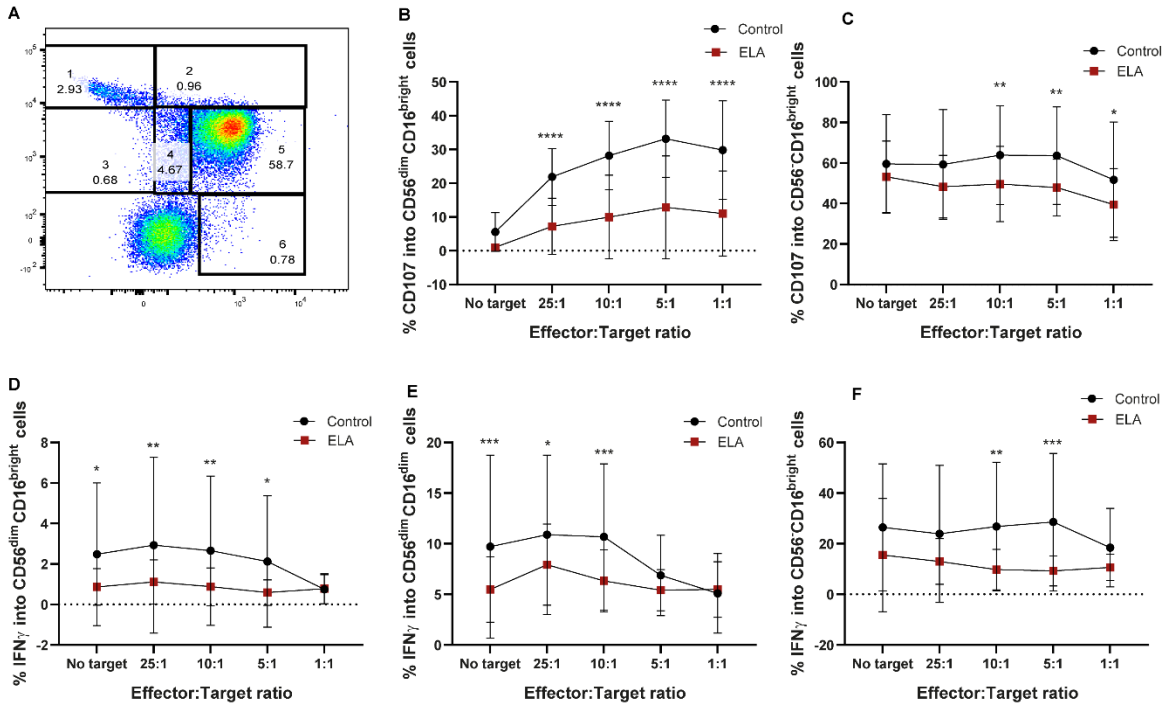


Figure 17 - Maternal deprivation changes the degranulation capacity of the NK cells from ELA individuals. (A) representative image of the NK cell population gating strategy: 1– CD56brightCD16-; 2 – CD56brightCD16+; 3- CD56dimCD16+; 4– CD56dimCD16dim; 5- CD56dimCD16bright; 6 – CD56-CD16bright; (B) and (C) expression of CD107a by populations 5 and 6; (D), (E) and (F) expression of IFN-γ by populations 4, 5 and 6. Data is presented as mean ± SEM of 14 donors per group. Statistics: Two-way ANOVA; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

3.4.8 Early life stress reduces degranulation of NK cells in the EpiPath cohort

Similar to what was previously described (Amand, Iserentant et al. 2017; Poli, Michel et al., 2009), six populations were obtained in the flow cytometry CD16 vs CD56 dot plot: $CD56^{bright}CD16^{-}$, $CD56^{bright}CD16d^{im}$, $CD56dimCD16^{bright}$, $CD56^{-}CD16^{bright}$, $CD56^{dim}CD16^{-}$ and $CD56^{dim}CD16^{dim}$ (Fig. 12A). In all these populations, the expression of CD107a and IFN-γ were measured. Although the majority of NK populations displayed less degranulation capacity in donors that suffered ELA (Sup. Fig. 6), only $CD56^{dim}$, or CD56 negative NK cells ($CD56^{dim}CD16^{dim}$; $CD56^{-}CD16^{bright}$, $CD56^{dim}CD16^{bright}$), the most mature populations, reached statistical significance (Fig. 12). $CD56^{dim}CD16^{bright}$ (population 5) NK cells from the control group displayed significantly higher levels of CD107a expression for all ratios, but did not show any differences when target cells were not presented (**25:1** – 7.2 ± 8.4 vs 21.9 ± 8.4, p<0.0001; **10:1** – 9.9 ± 12.4 vs 28.2 ± 10.1, p<0.0001; **5:1** – 12.9 ± 15.2 vs 33.2 ± 11.4, p<0.0001; **1:1** – 11.04 ± 12.6 vs 29.8 ± 14.6, p<0.0001) (Fig. 12B). The same was observed for the expression of IFN-γ, reaching statistical difference for all ratios except E:T 1:1 (**No target** – 0.87 ± 0.91 vs 2.5 ± 3.5, p=0.0171; **25:1** – 1.12 ± 1.08 vs 2.9 ± 4.3, p=0.0061; **10:1** – 0.88 ± 0.9 vs 2.7 ± 3.7, p=0.0072; **5:1** – 0.6 ± 0.63 vs 2.1 ± 3.2, p=0.0262) (Fig. 12D). Double dim NK cells from ELA donors (population 4: $CD56^{dim}CD16^{dim}$) showed significantly decreased secretion of IFN-γ for all E:T ratios except 5:1 and 1:1, when compared to control donors (Sidak's multiple comparisons test: **No target** - 5.48± 3.2 vs 9.71 ± 9.02, p=0.0009; **25:1** – 7.9 ± 4.01 vs 10.9±7.9, p=0.033; **10:1** –6.3± 3.1vs 10.7±7.2, p=0.0006)) (Fig. 12E). Expression of CD107a was not different between the groups (Sup. Fig. 6). Finally, the $CD56^{-}CD16^{bright}$ (population 6) NK cell population displayed significant differences at the E:T ratios 10:1 and 5:1 with lower expression in the ELA group for both CD107a (**10:1** - 49.6 ± 18.6 vs 63.9 ± 24.4, p=0.0084; **5:1** – 47.9 ± 13.9 vs 63.6 ± 24.1, p=0.003)) and IFN-γ (**10:1** -9.7 ± 7.9 vs 26.7 ± 25.4, p=0.0011; **5:1** – 9.2 ± 5.9 vs 28.5 ± 27.2, p= 0.0002) (Fig, 12C and 12F). CD107a

expression was also significantly decreased in the ELA group at the E:T ratio 1:1 (39.5 ± 17.7 vs 51.7 ± 28.5, p=0.0298) (Fig. 12C).

3.5 Discussion

In this study, we demonstrated how ELA, in the form of maternal separation, has a more widespread influence on the immune system than previously thought. To our knowledge, this is the first unbiased viSNE analysis linking ELA with clear changes in the overall immune profile and, more specifically, the first to provide specific mechanisms of maturation, senescence, and changes in cytotoxicity and degranulation profiles of NK cells after ELA. We initially examined the effect of ELA using the rat MD model. As previously reported by ourselves and others (Koe et al. 2014; Kaidbey et al. 2019; Breivik et al. 2015), the separation and deprivation of maternal care during this period has been associated with increased cognitive impairment,, HPA-axis dysregulation and anxious-like behaviour (Nishi, Horii-Hayashi, and Sasagawa 2014; Roque et al. 2014; Daniels et al. 2004; Aisa et al. 2007; Lundberg et al. 2017).

Stress, in all types of forms, either in early life, adolescence or adulthood activates the HPA axis, leading to the release of cortisol that will bind to GRs and ultimately initiate a cascade of molecular and cellular events (Russell and Lightman ; Finsterwald and Alberini ; Maniam, Antoniadis, and Morris 2014). GRs activation is reported to impact gene transcription (Oakley and Cidlowski 2013) and to inhibit immune responses (Cain and Cidlowski). However, we previously reported that this process is not always so clear-cut (Elwenspoek et al.). We saw clear clinical consequences later in life, specifically in the immune system and in the development of chronic and psychological disorders (Elwenspoek, Hengesch, Leenen, Schritz, Sias, Schaan, Mériaux, et al. 2017; Elwenspoek, Sias, et al. 2017a), however, these changes were not accompanied by alterations in the expression or response of GRs, although the HPA axis was hypo-responsive (Elwenspoek et al. ; Hengesch et al.). Although we did not directly assess the functioning of the GRs, our data confirm our previous report that HPA axis hormones and receptors might not be as intimately involved in the long-term consequences of early life stress as thought. Importantly, we were able to reproduce the immune phenotype previously seen in our EpiPath cohort (Elwenspoek, Hengesch, Leenen, Schritz, Sias, Schaan, Mériaux, et al. 2017), where CD8+ T cells were found to be more activated (Elwenspoek,

Hengesch, Leenen, Schritz, Sias, Schaan, Mériaux, et al. 2017) and more senescent (Elwenspoek, Sias, et al. 2017a) than the cells from the individuals in the control group. In our paradigm, T cells (CD3+) and their subsets (CD4+ and CD8+) were significantly changed, with CD8+ T cells following the same trend as in our ELA study (Elwenspoek, Hengesch, Leenen, Schritz, Sias, Schaan, Meriaux, et al. 2017), confirming the relevance of the MD model for the biological consequences of ELA. Furthermore, we expanded changes in the immune system to B and NK cells. Our unbiased viSNE analysis did not clearly distinguish NK and NKT-like cells, the latter being T cells that share and express NK cell receptors bridging the innate and adaptive immune responses that are implicated in tumour rejection, cardiovascular and neurological diseases (Bendelac, Savage, and Teyton 2007; Cui and Wan 2019; Seino and Taniguchi 2005; van Puijvelde and Kuiper 2017). Little is known about the long-term effects on NK cells after early-life psychosocial stressors, although NK cell numbers have been reported to be impacted (Wyman et al.). However, our previous report (Elwenspoek, Hengesch, Leenen, Schritz, Sias, Schaan, Mériaux, et al. 2017), together with the data reported here, suggest that ELA has a minor impact on circulating NK cell numbers, but is accompanied by a higher activation state and a trend towards increased senescence. Our data suggest that the NK cells have a similar phenotype to the CD8 T cells, previously reported. We see a different secretion of CD107a and IFN-γ from the CD56dim NK cell subsets (CD56dimCD16dim and CD56dimCD16+), as well as from the CD56-CD16+. As discussed by Emily Mace (Mace 2016) and others (Moretta 2010; Poli et al. 2009), these subsets are thought to be the most differentiated ones, as loss of CD56 expression and acquisition of CD16 was proposed to be part of the maturation process. These results follow the increased expression of maturation markers observed in the rats. Altogether, this seems to indicate that, although immature cells in both adoptees and stressed animals are still functional, as they become more mature, they lose their functionality, both in terms of cytotoxicity and degranulation. In a similar way to the increased activation (CD25) and senescence (CD57) of CD8+ T cells, the NK cells appear to lose functionality as they mature, although unlike CD8 T cells, this was independent of CMV exposure and titers (Elwenspoek, Hengesch, Leenen,

Schritz, Sias, Schaan, Mériaux, et al. 2017). It would appear that this mechanism is not applicable in NK cells, as there was no correlation between either CMV titers or seropositivity and NK cell activity. Furthermore, as there was no clear HPA axis phenotype, although there was a trend towards an increase in stress-induced gluconeogenesis, we conclude that the HPA axis is unaffected in our MD paradigm and, as such, cannot be responsible for the NK cell phenotype either.

NK cells are known to be affected by current acute and chronic stress. The early work by Schedlowski et al. showed that acute stress in adulthood, in this case novice parachute jumpers, had significant changes in the circulating lymphocyte subsets as well as functional differences in NK cells immediately post-stress. We expanded on this to demonstrate the kinetics of NK cell redistribution throughout the day, coupled to the circadian HPA axis rhythm, though in the work of Schedlowski it appeared to be associated with noradrenaline levels (Schedlowski, Falk, et al. 1993; Schedlowski, Jacobs R Fau - Stratmann, et al. 1993). Both studies suggested that this rapid mobilisation of NK cells was a natural physiological reaction to an external stressor, in agreement with both their natural role as an immediate initiator of the immune response before adaptation starts, and as an evolutionary mechanism, preparing the body to fight injury or infection after encountering an acute stressor. Sympathetic nervous system control of NK cell action via noradrenaline has been suggested to be an advantage because of the speed with which the immune system can be primed to act after a stressful encounter, as well as the speed in which the priming can be terminated and homeostasis re-established (Schiller, Ben-Shaanan, and Rolls 2021) through the inflammatory reflex (Tracey 2002). There is a similar dearth of literature on the effects of chronic stress on NK cell functioning. In a similar manner to our observation of decreased NK cell functionality, chronic low-dose glucocorticoid administration reduced histone acetylation levels around promoters for two essential NK cell produced effectors: perforin and granzyme B. This was associated with lower mRNA transcript levels, lower protein levels, and NK cells were functionally impaired in a manner similar to what we report in both our rat model and in the EpiPath cohort. The lower perforin and granzyme B levels

decreased their cytolytic activity. Inversely, the same administration regime increased histone acetylation of the IFN-□ and IL-6 promoters, up-regulating transcription and functional protein levels (Eddy et al.). The situation is, however, far from clear-cut. Children with current chronic stress from maternal mental health had higher levels of psychiatric symptoms as well as an increase in the number of illness episodes that was associated with increased NK cell cytotoxicity (Wyman et al.). However, none of the data available so far addresses the long-term effect of early life psychosocial stress and adversity, and the differences in NK cell functionality when the stressor is no longer present. In our previous report from the EpiPath cohort, multiple correction testing during our survey of the complete immune system meant that NK cells only narrowly missed significance (Elwenspoek, Hengesch, Leenen, Schritz, Sias, Schaan, Mériaux, et al. 2017)), and the only other comparable study did not investigate NK cells (Reid et al. 2020). Both of these studies reported that ELA induced a long-term immunosenescence and reduced T-cell functionality that was most probably due to continued re-activation of viruses such as CMV. It would seem logical that the exposure to a period of chronic stress in both models presented here has had a similar effect on NK cells. The two experimental systems show that once the period of ELA has resolved, NK cells are programmed with a long-term hypo-reactivity. As for acute stress preparing the NK cells to deal with an immediate infectious threat or potential wound, we suggest that this long-term hypo-reactivity is a similar evolution. The sensitive early life period would appear to have prepared the NK cells for an environment in which they can expect to be more regularly activated, and as such, to avoid any negative effects associated with NK cell secreted effector molecules or cytokines.

Although NK cells are often associated with a positive regulation of the immune response, they are also associated with the development of immunopathologies. In chronic hepatitis B virus infection, NK cells contribute to both liver inflammation and injury (Chen et al. 2007; Dunn et al. 2007), and aggravate and increase the lethality of bacterial infections in murine models (Kerr et al. 2005; Badgwell et al. 2002). NK cell activity was found to be impaired in patients that suffered from multiple sclerosis (Kastrukoff et al.

2003; Takahashi et al. 2016), type-1 diabetes (Rodacki et al. 2007) and cardiovascular diseases (Hak et al. 2007; Ong, Rose, and Čiháková 2017; Jonasson, Backteman, and Ernerudh 2005), and found to sustain joint inflammation in rheumatoid arthritis patients (Dalbeth and Callan 2002); the risk of the latter three are all increased by exposure to ELA (Huffhines, Noser, and Patton 2016; Dube et al. 2009; Shaw et al. 2017; Baumeister et al. 2016). This raises the possibility of the long-term alteration of the NK cell phenotype underlying the pathophysiological effects of ELA.

The limitations of our pre-clinical study include potential litter effects and the absence of a clear HPA axis phenotype. Similarly, the number of EpiPath participants analysed was limited, however, based on the rat MD data, our power calculation suggested that to see the same phenotype in the cohort only 14 participants were required to have 80% power at alpha 0.05, which we largely exceeded. However, these are clearly outweighed by the reproduction of the functional NK cell phenotype in the EpiPath cohort. Furthermore, the identical phenotype in the MD model and the cohort allowed us to exclude the two most prominent mechanistic hypotheses from the literature – HPA axis control, and continual CMV reactivation. Nevertheless, ELA has a direct impact in the maturation state and later exhaustion of NK cells that may impair their activity and lead to uncontrolled reactions in adulthood.

It is clear that all cells of the immune system are not equally affected by ELA. Here, we have expanded our prior observation of T cell immunosenescence to a novel, unbiased examination of the immune system, identifying NK cells as functionally affected by ELA in both the rat MD model and in our human institutionalization – adoption cohort and NKT-like cells to be differently expressed in the rats. The immature NK cells appear to retain their functionality, however, as they mature towards CD56dim NK cell subsets and CD56- phenotype, their cytotoxic and degranulation potential are reduced. It is now evident that alterations in the HPA axis, either as stress-induced cortisol / corticosterone production or gluconeogenesis, are not responsible for the immune phenotype. The challenge is now to understand how ELA is inducing such changes and the role of both T cell and NK / NKT-like cells functional and expression loss in the long-term ELA-induced disease risk.

3.6 Ethics statement

All animals used in this study were maintained in accordance with all current European Union (Directive 2010/63/EU), national and local ethical guidelines and legal regulations. Rat experiments were performed in accordance with the LIH institutional animal welfare structure requirements as well as the European Union Directive 2010/63/EU as implemented in national legislation. In accordance with the declaration of Helsinki, all participants provided written informed consent and the study protocol was approved by the Ethics Review Panel (ERP, University of Luxembourg, No 13-002) and the National Research Ethics Committee (CNER) of Luxembourg (No 201303/10 v1.4).

3.7 Conflict of Interest

The authors all declare that they have no conflict of interest.

3.8 Authors Contribution

Conceptualization: SBF and JDT; literature review: SBF and JDT; Data collection: SBF, NDP, SM, MMCE, FADL and MT; Data analysis: SBF, NDP, JZ, JDT; Manuscript writing and editing: SBF, NDP, JZ and JDT. All authors read and approved the final version of the manuscript.

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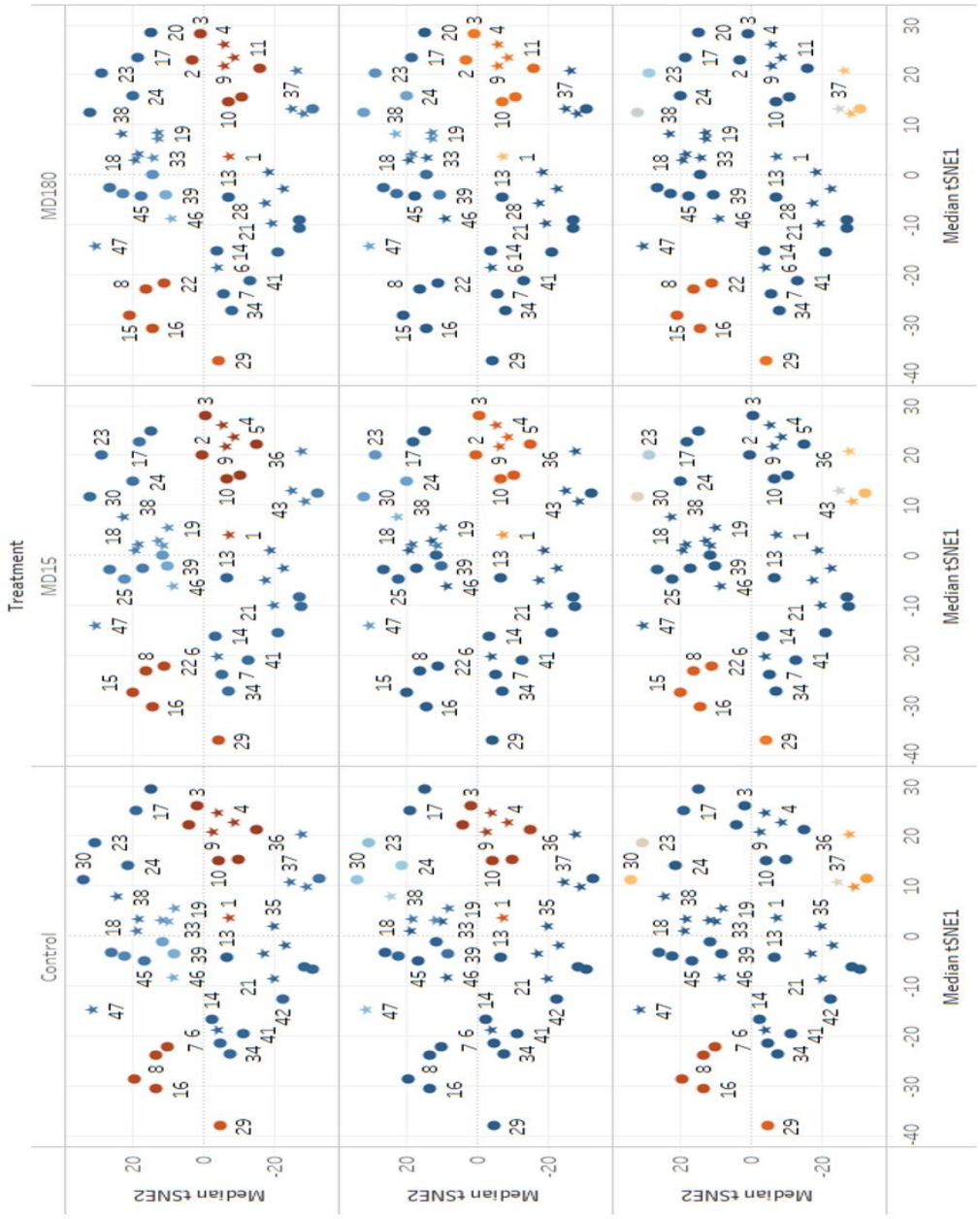
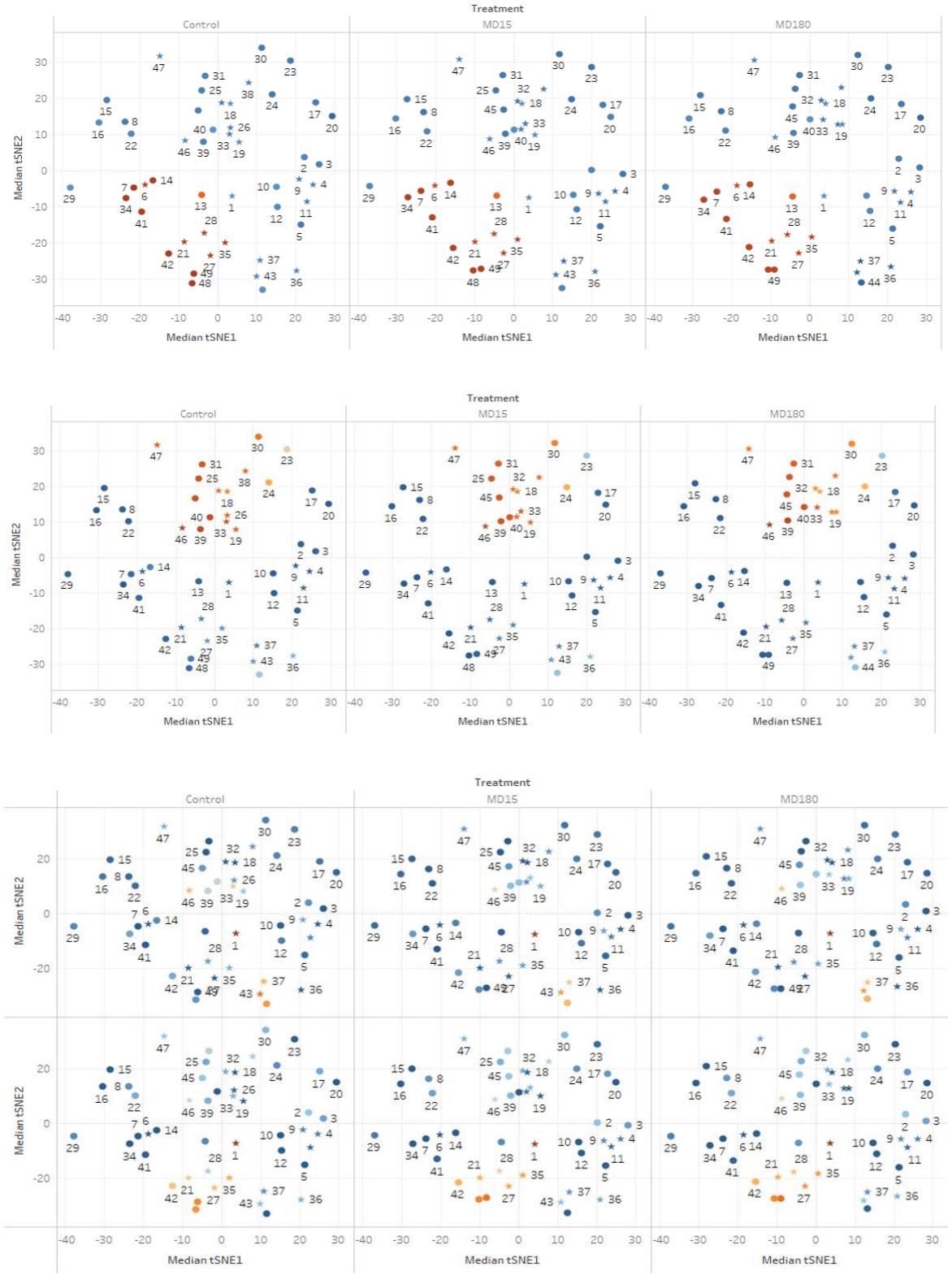
3.10 Abbreviations

MD, Maternal deprivation; NK cell, Natural killer cell; NKT, Natural killer-like T cell; PND, Post-natal day; ACTH, Adrenocorticotropin hormone; CRH, Corticotropin-releasing hormone; GCs, Glucocorticoids;; HPA axis, Hypothalamic–pituitary–adrenal axis; CRP, C-reactive protein; PBMCs, Peripheral blood mononuclear cells; CMV, cytomegalovirus.

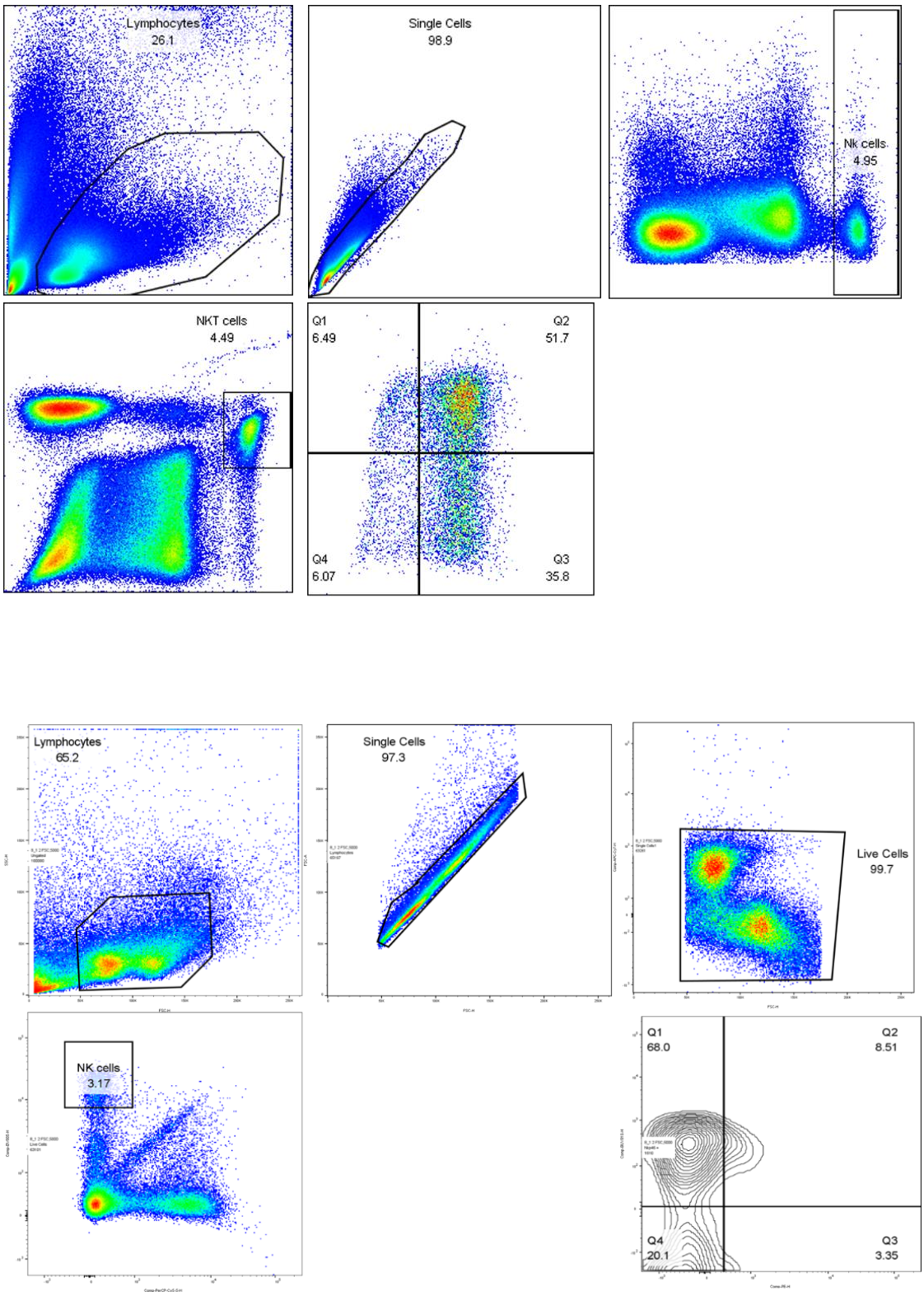
3.11 Supplementary data



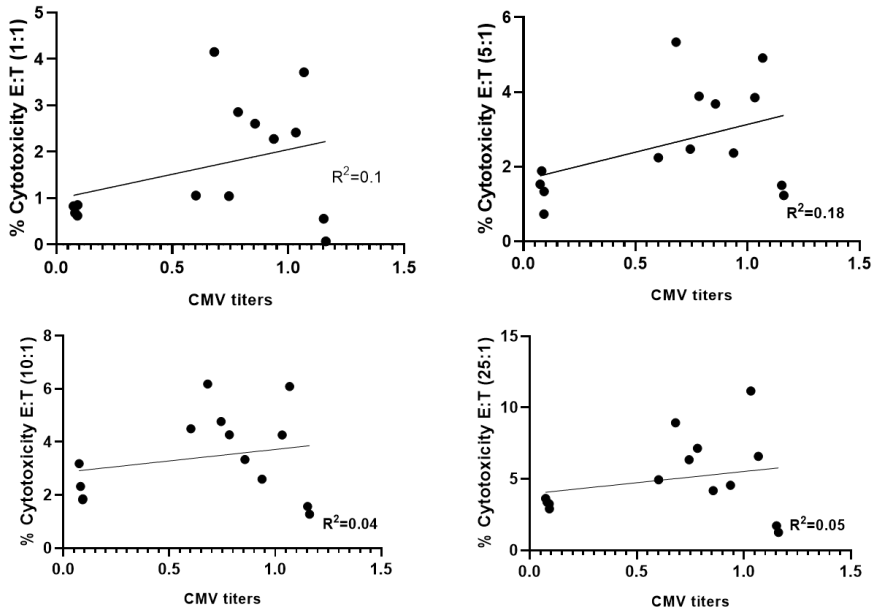
Supplementary Figure 1 - viSNE clusters definition based on the used markers. Clusters colored by CD3, CD4 and CD8 cell markers to identify immune populations associated. Blue to orange: low to high expression of the marker.



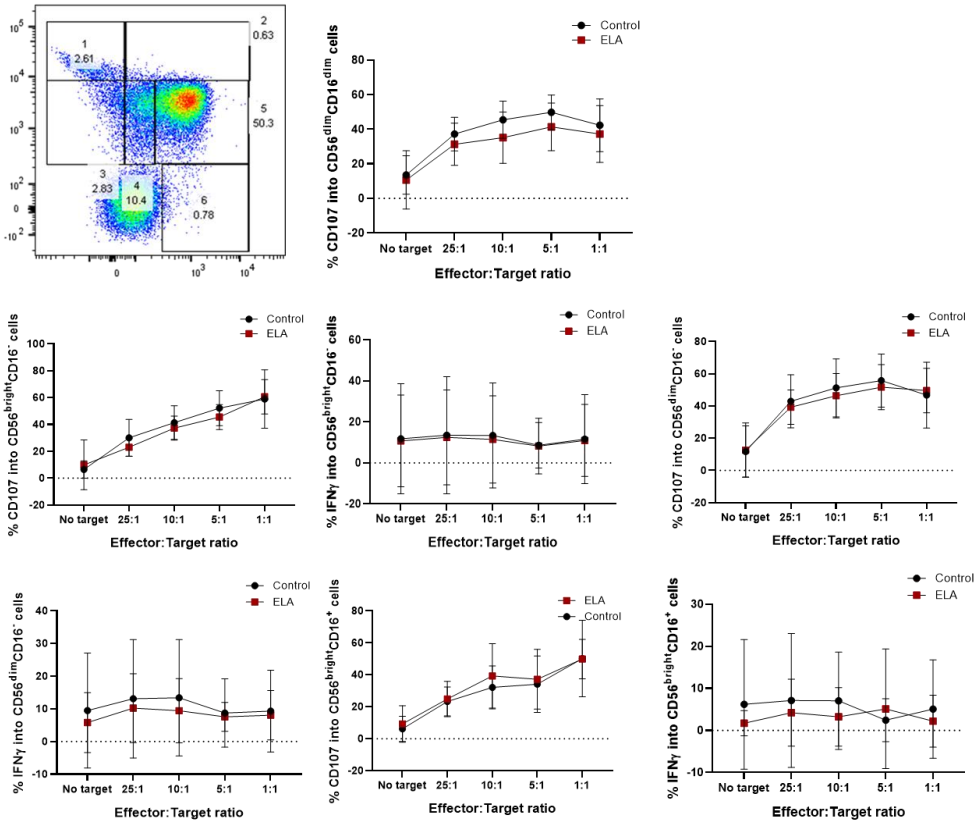
Supplementary Figure 2 - Clusters colored by CD45RA (Top), CD11b/c (Middle), CD25 and FoxP3 (Bottom) cell markers to identify immune populations associated. Blue to orange: low to high expression of the marker.



Supplementary Figure 3 - Flow cytometry gating strategy for quantification of long-term changes in the immune system after maternal deprivation



Supplementary Figure 5 - Correlation analysis between CMV titers and cytotoxic response of NK cells of institutionalized individuals.



Supplementary Figure 4 - (A): representative image of the NK cell population gating strategy: 1– CD56brightCD16-; 2 –CD56brightCD16+; 3- CD56dimCD16+; 4– CD56dimCD16dim; 5- CD56dimCD16bright; 6 – CD56-CD16bright. Rest of the figures show the release of IFN-γ and CD107a by the different NK cell populations. No significant differences were found. Data is presented as mean ± SEM of 14 donor per group.

Chapter IV: Preliminary data on the effects of ELA on the brain

My contribution to this chapter:
Conception of the project. Planning and performing the animal experiments involving rodents. Literature research and current writing of the manuscript.

4. LONG-TERM FUNCTIONAL EFFECTS ON CEREBELLUM AFTER EARLY LIFE PSYCHOSOCIAL STRESS

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4.1 Introduction

Early life adversity, ELA, is known to be a key factor in the development of several diseases later in life (Barker Dj Fau - Osmond and Osmond 1986, Barker 1995), such as depression, anxiety and neurodegenerative disorders (Short and Baram 2019, Stuart, Hinchcliffe et al. 2019). Particularly, during early development, adverse circumstances can severely impact the normal development of the brain (McLaughlin, Sheridan et al. 2014, McLaughlin, Weissman et al. 2019). In addition, such events are also significantly associated to engagement in risky and violent behaviours (Nelson, Bhutta et al. 2020) such as smoking (Felitti, Anda Rf Fau - Nordenberg et al. 1998, Lee, Harari et al. 2020), drug and alcohol abuse (Enoch 2011, Lopez-Patton, Kumar et al. 2016) and violence towards others (Ford, Chapman et al. 2012, Courtney and Maschi 2013, Walsh and Hasin 2015). Both clinical and animal studies strongly suggest that different types of ELA, ranging from separation from the parents, to low social-economic conditions, maltreatment and physical abuse, significantly increase the risk of brain malformation which can lead to the development of a neurodegenerative or neurological disorders (Felitti, Anda Rf Fau - Nordenberg et al. 1998, Weiss, Longhurst Jg Fau - Mazure et al. 1999, Gilman, Kawachi et al. 2002, Fabricius, Wörtwein et al. 2008, Freeman, Tyrovolas et al. 2016, Hui, Feng et al. 2017). Such alterations occur particularly at the pathways level and structural integrity, affecting the good functionality of the brain and leading to disease in the long-term (Smith and Pollak 2020). These changes are similar to the ones observed upon stress in several situations, even outside early life development, and are frequently associated with brain regions that participate in the stress response, such as the amygdala, striatum and hippocampus (Fenoglio, Brunson et al. 2006, Fareri and Tottenham 2016, Youssef, Atsak et al. 2019).

Different brain regions play a role in the stress response, which starts with the activation of the hypothalamic-pituitary-adrenal (HPA) axis. Upon its activation, several hormones are produced inducing different organs to respond to the threat (Smith and Vale 2006). Glucocorticoids are the end product of HPA axis activation and regulate the stress response through glucocorticoid

receptors, which are mainly distributed throughout the brain (Garabedian, Harris et al. 2017). Changes happening in the brain differ on the type of stress suffered, and range from disrupted connectivity between different regions, altered pathways and activation of microglia (Jauregui-Huerta, Ruvalcaba-Delgadillo et al. 2010, Khan, Geiger et al. 2020). These changes will in turn lead to increased inflammation, decreased learning capabilities and memory formation and sleep disturbances which can culminate in the development of diseases such as depression and anxiety (Krishnan and Nestler 2008, Dowlati, Herrmann N Fau - Swardfager et al. 2010, Hodes, Kana et al. 2015). However, it is probable that such changes may be the result of the altered HPA axis (Sarabdjitsingh, Loi et al. 2017) since glucocorticoid levels have been implicated in neurodevelopmental, structural and functional differences in the brain (Cirulli, Berry A Fau - Alleva et al. 2003, Lupien, McEwen et al. 2009).Human data suggests that severe early life adversity results in a smaller grey matter volume (Gorka, Hanson et al. 2014)white matter integrity (Hanson, Adluru et al. 2013) as well as altered connectivity in stress-associated regions, particularly the hippocampus and the pre-frontal cortex (Yan, Rincón-Cortés et al. 2017). Rodent ELA models suggest that the brain retains full structural integrity (Sarabdjitsingh, Loi et al. 2017), but with altered functional connectivity (Yan, Rincón-Cortés et al. 2017). These changes may, in part explain the link between ELA and psychopathology. Many stress-related psychopathologies have been associated with structural anomalies such as reduced hippocampal volume in PTSD patients when compared to control individuals, firstly studied in the mid-90's (Bremner, Randall et al. 1995). MRI based studies that followed found that ELA-associated stress leads to significant decrease of grey matter volume, particularly in the prefrontal cortex, hippocampus and amygdala, in young adults exposed to different types of stress in childhood such as sexual abuse (Sheffield, Williams et al. 2013), physical (Tomoda, Suzuki et al. 2009) and emotional violence (van Harmelen, van Tol et al. 2010) and parental domestic violence (Tomoda, Polcari et al. 2012).

In order to best mimic the changes happening after early life stress in humans, researchers often take advantage of the animal model of maternal

separation (MS) (Vetulani 2013). In this model, the animals are separated from the mother right after birth, from post-natal day (PND) 2 to PND 14, which has been shown to significantly impair the behavior of the animals in adulthood (Cui, Cao et al. 2020), lead to cognitive impairments (Aisa, Tordera R Fau - Lasheras et al. 2007, Janetsian-Fritz, Timme et al. 2018) and altered HPAS axis (Roque, Mesquita et al. 2014). Furthermore, we recently demonstrated that ELA, particularly in form of maternal separation, significantly impacts the immune system, particularly the maturation of NK cells. Moreover, the functionality of such cells was demonstrated to be significantly impaired, with animals subjected to maternal separation having a less cytotoxic and degranulation response from NK cells, to specific target cell line (Fernandes 2021).

This early period is also known to be imperceptibly responsive to stress, as the levels of corticosterone (CORT) significantly decrease around PND2, known as the stress hypo responsive period (SHRP) (Walker Cd Fau - Perrin, Perrin M Fau - Vale et al. 1986, Levine 2001), thus responsiveness to stressors is reduced. However, MS is able to disrupt the SHRP and for that is widely used to model early trauma (Rosenfeld, Gutierrez et al. 1991).

After exposure to the maternal separation paradigm rats develop an anxious phenotype usually characterized in the elevated plus maze (Walf and Frye 2007) and, as expected, MS rats have an altered HPA axis response to a stressor such as a restraint stress (Daniels, Pietersen Cy Fau - Carstens et al. 2004). After restraint stress, the HPA axis activity, notably ACTH and corticosterone levels, of the maternally deprived animals were shown to be significantly lower than in unexposed controls. Furthermore, neurotransmitters levels such as noradrenaline were shown to be significantly lower in MS animals when compared to controls, while serotonin turnover was significantly increased.

In this study we hypothesized that early maternal separation in rats, from postnatal day (PND) 2 to PND 14, would significantly impact the brain and possibly lead to neurological-like diseases. After maternal separation we validated the effect of our model in the EPM as well as in a restraint test in adulthood together with an examination of the brain structural integrity and

connectivity as well as gene expression in four different brain regions (hippocampus, cortex, striatum and cerebellum). Through magnetic resonance imaging (MRI), we were able to measure the volume of four different selected regions, the apparent diffusion coefficient and the fractional anisotropy. Maternal separation significantly changed the anxious-like behavior of the animals but did not induce changes in the volumes of the structures, as previously documented (Sarabdjitsingh, Loi et al. 2017). Nevertheless, it significantly impacted the fractional anisotropy in the cerebellum. All changes were accompanied by differences in gene expression.

4.2 Material and Methods

4.2.1 Animals and Maternal Deprivation protocol

Outcomes from this study result from two independent experiments, as previously described (Fernandes, SB, et al., manuscript under review, Frontiers in Immunology). Briefly, mating process for the Wistar rats was done by Janvier and pregnant females arrived at pregnancy day 2. Litters were naturally delivered between days 21-23 of gestation, litter size adjusted to 12 pups/dam and randomly assigned to one of the following groups (two litters for each group): 15 minutes (MD15) or 3h (MD180) maternal separation from PND2 to PND14 and no separation (Control; CTR). The maternal separation protocol was carried out daily for 3 hours, from 9 a.m to 12a., or 15 minutes, from 9a.m to 9h15a.m. Each litter was placed in a new cage, with no bedding, inside a heated vented animal cabinet (Noroit, France), at 33°C, in order to maintain body temperature. After the separation, litters were returned to their mothers and home cage. Pups from the control litters were only handled to change cages and left undisturbed for the rest of the time until weaning day (PND21). At weaning animals were separated by sex and experimental group, and left undisturbed until further experiments.

The experiments were carried out in accordance with the European Union legislation (2010/63/EU) for the care and use of laboratory animals and were approuved by the local Animal Ethical Committee (DII-2017-18).

4.2.2 Behaviour test: Elevated Plus Maze

At PND42, the anxious-like behavior of the animals was tested with the Elevated Plus Maze. The apparatus consisted of a grey cross-shaped Plexiglas apparatus with two opposed open arms and two opposed closed arms (Stoelting Europe, Dublin, Ireland) placed 65 cm above the floor. The four arms were 50 cm long and 10 cm wide and connected by a central platform (10 x 10 cm). The closed arms were surrounded by grey walls (40 cm high) while the two open arms were completely flat. At the start of the experiment, rats were gently placed in the central square facing one of the closed arms and allowed to

explore freely and undisturbed during a single 10 min session. Sessions were video-recorded with ANY-maze Video Tracking Software (Stoelting Europe, Dublin, Ireland). Distance travelled and time spent in closed (CA) and open arms (OA) were measured, together with open/total arm entry and duration ratios. Percentage of time spent in open arms was calculated as following: (time spent in OA/(time spent in OA +time spent in CA)) x 100. Percentage of distance travelled in each arms was calculated in relation to the total distance travelled.

4.2.3 Brain collection and tissue preparation procedure

At PND56, animals were euthanized by CO2 inhalation and brains immediately dissected and put into a cold solution of 4% PFA with 8mM Gadolinium, for post-mortem MRI analysis. In order to increase the signal-to-noise ratio, brains were left undisturbed in this solution for 21 days, at 4°C.

4.2.4 MRI acquisition

On the analysis day, excised brains were placed in a custom-made 3D holder with fluorinert (3M, MN, USA) and structural MRI was performed using a preclinical MRS*DRYMAG 3.0T (MR solutions, Guildford, UK), with a 17 cm horizontal bore. For the acquisition, the following MRI sequences were used: FSE T1 weighted, FLASH3D, DWI and DTI. Anatomical series (FSE T1w and FLASH3D) were used to calculate the volumes of sub-regions of the brain. Diffusion series (DWI and DTI) were used to calculate the Apparent Diffusion Coefficients (ADC) and Fractional Anisotropy (FA) in the different sub-regions, and used as indicators of neuronal density and connectivity. Acquisition parameters used for the sequences are reported in the Table 1. Images were later analysed using a region-of-interest (ROI)-based aproach. The following brain regions were chosen grounded on literature reports on stress effects on the brain: amygdala, hippocampus, striatum and cerebellum.

Table 2 - MRI Protocols. SR: Spatial Resolution, Sl: Number of Slices, TE: Echo Time, TR: Repetition Time, ET: Echo Train, AVG: Number of Averages, SD: Scan Duration, NOB: number of b-value, NOD: number of diffusion direction

SEQUENCE	PURPOSE	PARAMETERS
FSE T1 weighted	Anatomical, low resolution	**SR:** 0.1 × 0.1 × 1 mm; **Sl:** 13; **TE\TR:** 17\1400 ms; **ET:** 4; **AVG:** 4; **SD:** 5min41sec
DWI	Apparent Diffusion Coefficient (ADC) map	S**R:** 0.2 × 0.2 × 1 mm; **Sl:** 13; **TE\TR:** 22\700 ms; **AVG:** 1; **NOB:** 9; **NOD:** 1; **SD:** 13min26sec
DTI	Principal Diffusion Direction map, Franctional Anisotropy (FA) map	S**R:** 0.2 × 0.2 × 1 mm; **Sl:** 13; **TE\TR:** 22\700 ms; **AVG:** 1; **NOB:** 1; **b-value:** 800; **NOD:** 7; **SD:** 10min27sec
µMRI - FSE	Anatomical, high resolution	**SR:** 0.1 × 0.1 × 0.1 mm ; **Sl:** 128 ; **TE\TR:** 17\600 ms; **ET:** 4; **AVG:** 2; **SD:** 2h46min30sec

Delineation of brain sub-regions: Custom developed scripts written in Matlab (MathWorks, MA, USA) were used to register a brain atlas to MRI. A precise manual delineation of the brain structures was also performed to assess possible morphological changes induced in the brain subregions for the different experimental groups (Supp. Fig. 1). Further statistical and graphical analysis and was performed with GraphPah.

Difiusion parameters: Diffusion images were analyzed in nordicICE (NordicNeuroLab, Bergen, Norway). The ADC maps are scaled in units of 10-5 mm2/s and calculated using equation is S=S_0 e^((-b*ADC)) where S and S0 are the diffusion signals measured with and without diffusion (b=0) respectively; the FA maps provide an index for diffusion asymmetry within a voxel, defined in terms of its eigenvalues (λ) measured in various directions, using equation: FA= √((〖(λ_1-λ_2)〗 ^2+ 〖(λ_2-λ_2)〗 ^2+ 〖(λ_1-λ_3)〗 ^2)/(2(λ_1^2+λ_2^2+λ_3^2))). The value of FA varies between 0 and 1.

Quantification of the diffusion maps per subregion and rat was performed in MATLAB. Median values are reported in the chosen regions of the brains. Further statistical and graphical analysis was performed with GraphPad.

4.2.5 RNA extraction

Total RNA was extracted from samples of four different brain regions (cerebellum, hippocampus, cortex and striatum), from the three experimental groups, through a spin-column procedure, using the RNeasy FFPE Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Quality and quantity were assessed using the RNA 6000 Nano chips with the Bioanalyzer (Agilent, Diegem, Belgium), and RNA integrity number (RIN) considered for further usage. Only samples with RIN > 7 were considered for microarrays analysis. Samples were stored at -80°C until further analysis.

4.2.6 Transcriptome analysis

Affymetrix Rat Clariom S array was used to analyse fourty-seven RNA samples with the GeneChip™ WT Pico Kit (Thermofisher scientific, MA, USA) following the GeneChip 3' IVT Pico Reagent Kit Manual Target Preparation for GeneChip® 3´Expression Expression Arrays User Guide protocol, according to the manufacturer's instructions. All samples were randomized to minimize batch effects. Samples were finally analyzed with TAC 4.0 software (Thermofisher scientific, MA, USA) followed by a non-parametric analysis.

4.2.7 Functional analysis

Analysis of the functionality of the significantly different expressed genes was performed using Gene Ontology (GO) terms and pathways in which the genes were involved analyzed with KEGG pathways and Reactome.

4.2.8 Statistical analysis

Statistical analyses were performed in GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California USA) and R (RStudio, MA, USA). Statistical differences were tested with one-way ANOVA (Tukey's multiple comparisons test), Two-way ANOVA (Dunnett's or Sidak's multiple comparisons tests) or non-parametric analysis depending on the number of animals and parameters in the assay. Figures were subsequently generated using GraphPad Prism and Adobe Illustrator CS6 (version 16.00).

4.3 Results

4.3.1 Early life adversity leads to an increased anxious-like behaviour in adult rats

Since ELA is highly associated with the development of depression and anxiety in adulthood, we evaluated the anxious-like behavior at PND42 with the elevated plus maze. Both groups of maternal deprived animals had a significant decrease in the percentage of distance travelled in the open arms of the maze, when compared to the control group (one-way ANOVA, Tukey's multiple comparison's test: MD15 8.3 ± 2.3 vs 23.9 ± 2.8, $p<0.01$; MD180 14.5 ± 3.2 vs 23.9 ± 2.8, $p<0.05$) (Fig. 13A). Less time spent moving in the open arms is associated with an anxious-like state. To further confirm this, we quantified the time spent in each zone of the maze. Animals that had experienced either 15 minutes or 3 hours of maternal separation spent significantly less time in the open arms zone, when compared to the control group (one-way ANOVA, Dunnett's multiple comparison's test: MD15 10.6±2.3 vs 21.8±1.7, $p<0.01$; MD180 13.4±2.8 vs 21.8±1.7, $p<0.05$) (Fig. 13B).

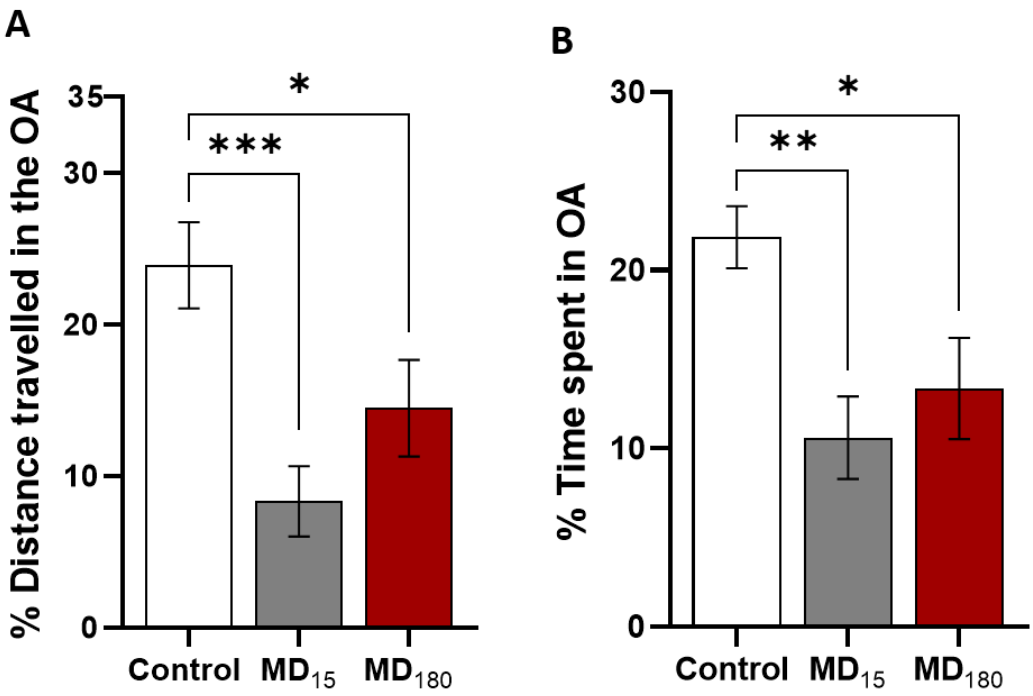

Figure 18 - Maternal separation changes the anxious-like behavior of the animals. (A) Percentage of distance travelled in the open arms of the elevated plus maze; (B) percentage of time spent in the open arms of the elevated plus maze.

4.3.2. Maternal separation does not affect the total volumes of the selected brain regions

To assess whether and how maternal deprivation affects the brain, we measured the volume of different regions-of-interest (ROIs) with images acquired through magnetic resonance imaging (Fig. 14). Analysis of structural MRI showed no differences in the total volume of the selected regions, between both treated groups and the control group (Fig. 14; Table 2). Although we can see a slight decrease in the volume of the cortex after three hours of maternal separation, it did not reach statistical significance (1389776.8 vs 1488192.8; p=0.0939; 2-way ANOVA multiple comparisons; Fig. 14 and Table 2).

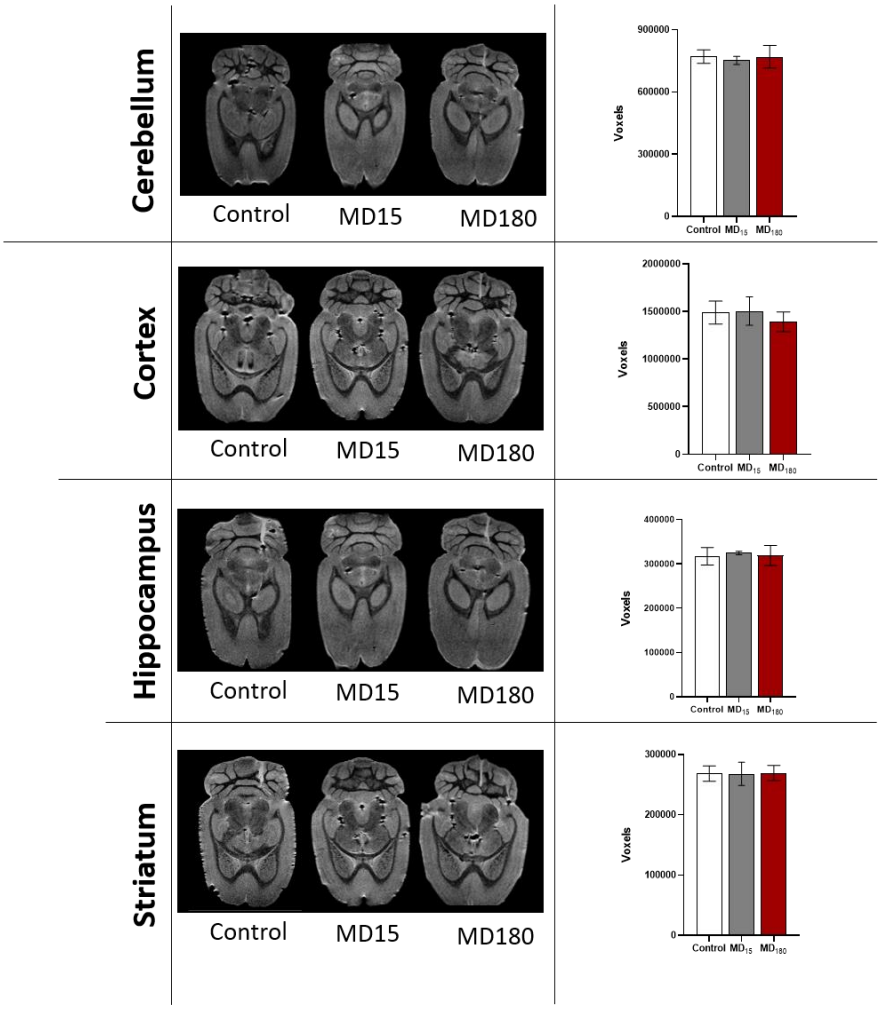

Figure 19 – Maternal separation does not induce changes in the volumes of the different studied regions: Cerebellum, Cortex, Hippocampus and Striatum.

Table 3 - Total volumes of the different selected brain regions. N= 5/6 rats per group. Data is represented as mean ± SD. p values were calculated using 2-way ANOVA, mixed effects multiple comparisons.

TOTAL VOLUME					
		Treatment (Maternal Separation)			
	Control	**15 minutes (MD_{15})**	**p value**	**3 hours (MD_{180})**	**p value**
Cerebellum	770701.8 ± 32739	751886.7 ± 19394.5	0.54	769399.8 ± 54346	0.98
Cortex	1488192.8± 120173.8	1503620.2 ± 149187.4	0.99	1389776.8 ± 104778.8	0.094
Hippocampus	317209.6 ± 19734.6	324470.25 ± 3992.2	0.80	318755.5 ± 22435.9	0.99
Striatum	268489.6 ± 12706.4	267873 ± 19273.1	0.99	269396.7 ± 12369.7	0.99

4.3.3 ELA alters fractional anisotropy uniquely in the cerebellum

After measuring the changes in the volumes of the different ROIs, we evaluated the structural integrity and micro-architecture of the same brain regions through both diffusion weighted imaging (DWI) and tensor imaging. Although the apparent diffusion coefficients (ADC) did not change after acute (MD15) or chronic (MD180) maternal separation (Fig. 15; Table 3) in any of the selected brain regions, fractional anisotropy was significantly impacted in the cerebellum after fifteen minutes (MD15: 0.31 ± 0.028 vs 0.25 ± 0.006, p=0.002) and three hours of maternal separation (MD180: 0.29 ± 0.018 vs 0.25 ± 0.007, p= 0.035) (Fig. 16). No significant differences were found in the cortex, striatum or hippocampus (Fig. 16; Table 4).

Table 4 - Apparent diffusion coefficients of the different selected brain regions. N= 5/6 rats per group. Data is represented as mean ± SD. p values were calculated using 2-way ANOVA, mixed effects multiple comparisons.

APPARENT DIFFUSION COEFICIENT					
		Treatment (Maternal Separation)			
	Control	**15 minutes (MD_{15})**	**p value**	**3 hours (MD_{180})**	**p value**
Cerebellum	90.9 ± 8.5	85.8 ±3.3	0.36	90.5 ± 6.2	0.99
Cortex	89.9 ± 6.3	85.5 ± 4.2	0.27	86.5 ± 3.7	0.41
Hippocampus	81.4 ± 6.2	77.9 ± 3.4	0.43	80.8 ± 4.6	0.96
Striatum	75.2 ± 3.6	72.1 ± 4.2	0.28	75.0 ± 2.4	0.99

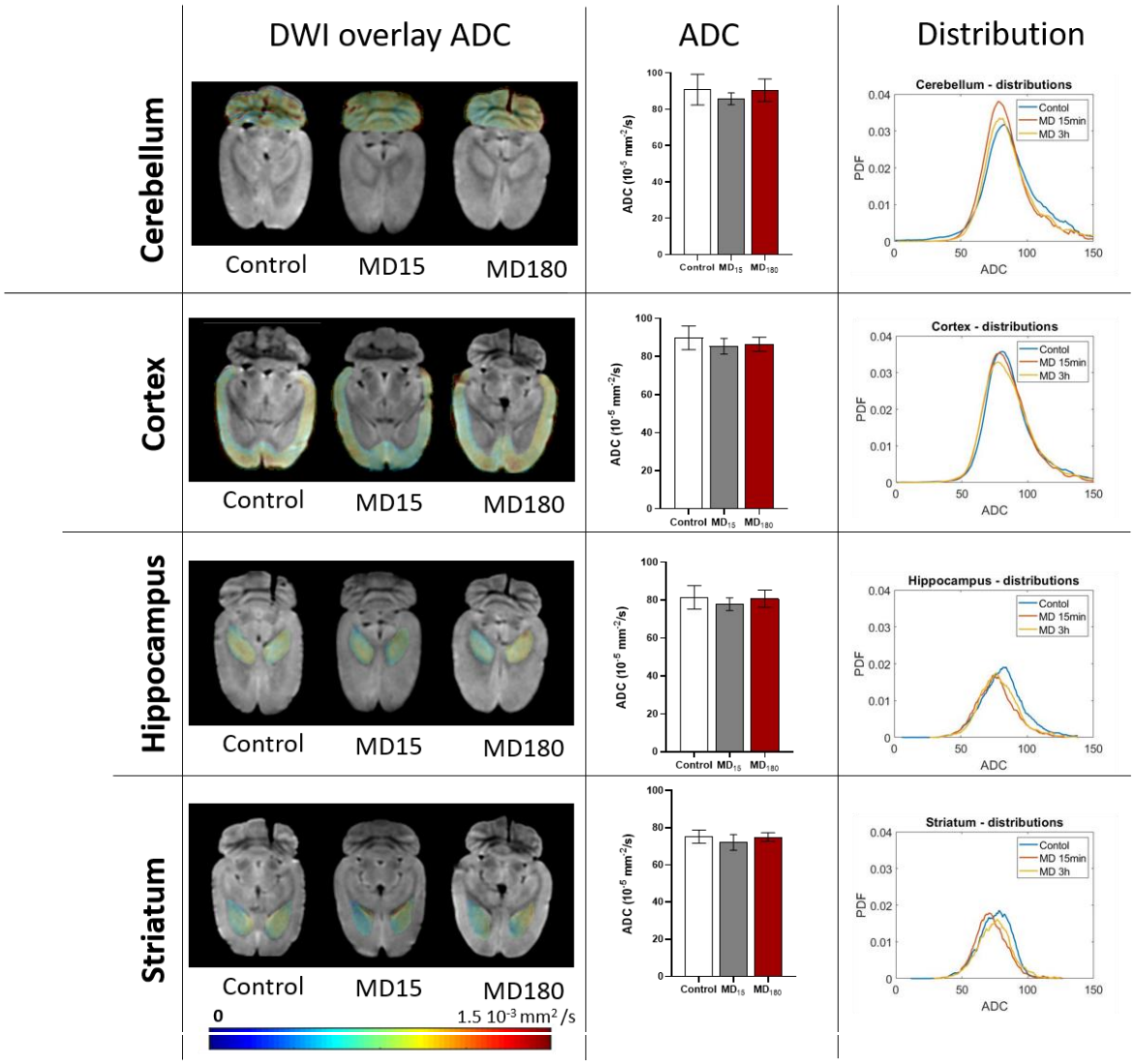

Figure 20 - ADC measured through magnetic resonance imaging (MRI) in the cerebellum, cortex, hippocampus and striatum.

Table 5 - Fractional anisotropy of the different selected brain regions. N= 5/6 rats per group. Data is represented as mean ± SD. p values were calculated using 2-way ANOVA, mixed effects multiple comparisons.

FRACTIONAL ANYSOTROPY					
		Treatment (Maternal Separation)			
	Control	**15 minutes (MD_{15})**	**p value**	**3 hours (MD_{180})**	**p value**
Cerebellum	0.25 ± 0.007	0.31 ± 0.028	0.002	0.29 ± 0.018	0.035
Cortex	0.32 ± 0.02	0.34 ± 0.02	0.11	0.32 ± 0.02	0.97
Hippocampus	0.30 ± 0.03	0.32 ± 0.02	0.54	0.32 ± 0.04	0.44
Striatum	0.31 ± 0.03	0.34 ± 0.05	0.39	0.32 ± 0.04	0.92

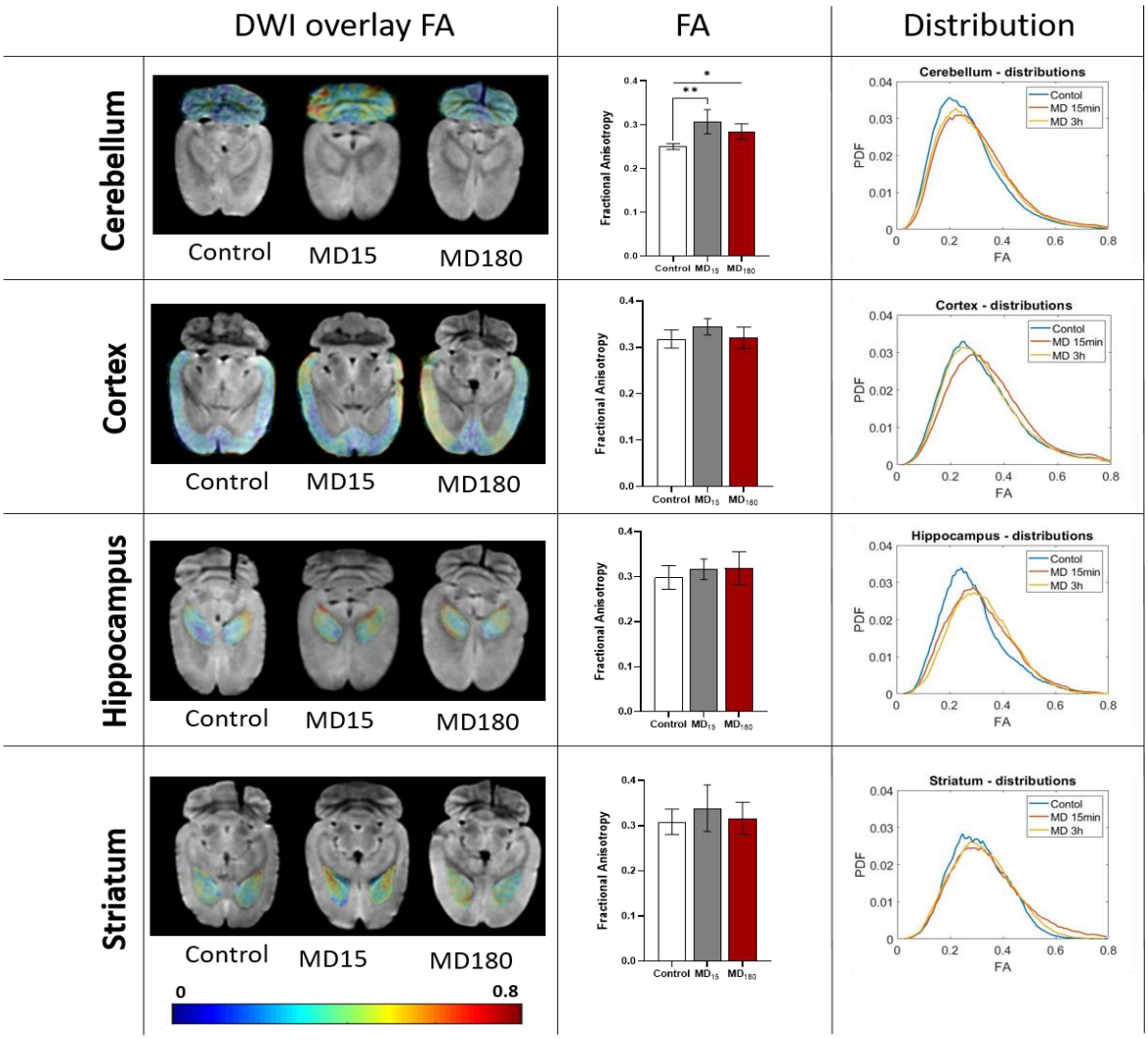

Figure 21 - **Figure 21** - fractional anisotropy measured through magnetic resonance imaging (MRI) in the cerebellum, cortex, hippocampus and striatum.

4.3.4 ELA changes the transcriptome but only in animals maternal deprived for 15 minutes

Upon obtaining changes in the behavior and brain structural integrity after maternal separation, we decided to measure the gene expression in an attempt to justify such drastic changes. Although no differences in gene expression were observed in the animals that were maternal separated for three hours (MD180), we found more than one hundred of differently expressed genes after 15 minutes of maternal separation, when compared to the control group, in the cerebellum (Fig. 17). Furthermore, differences in gene expression were also found between the two maternal separated groups. The same trend was observed for the other three regions (Fig. 17).

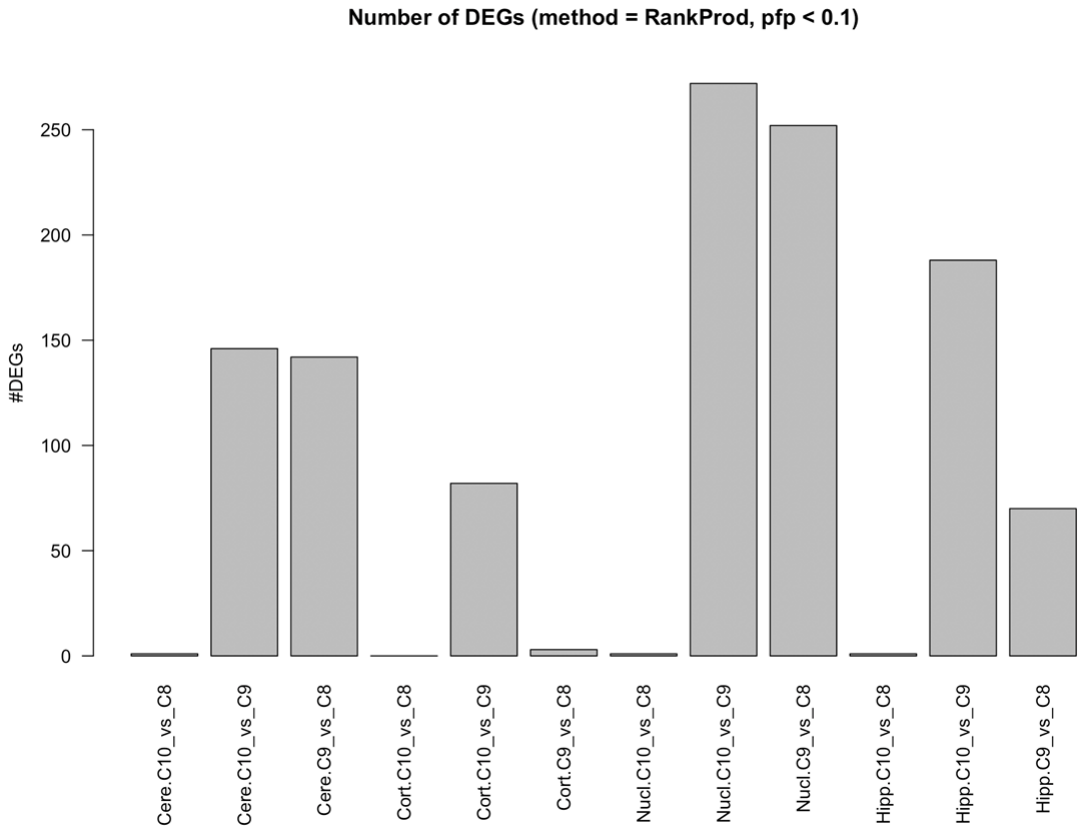

Figure 22 – Number of differentially expressed genes, per brain region, comparing the control group with MD15 (C9 vs C10); control with MD180 (C10 vs C8) and MD15 with MD180 (C10 vs C9).

4.3.5 Differently expressed genes top functions are associated with neurodegenerative disorders

Genes identified as being differently expressed were uploaded into DAVID for gene ontology (GO) and KEGG and Reactome for pathway analysis. GO analysis showed that the DEGs were mainly associated with biological processes such as "metabolic process", "cell adhesion" with the majority being associated with mitochondrial processes such as "electron transport chain", "ATP synthesis", "oxidation-reduction processes" and "mitochondrial respiratory chain complex assembly" (Fig. 18). In terms of pathways, running our samples through KEGG showed that these genes were mostly involved in pathways related with neurodegenerative disorders such as Alzheimer, Parkinson's and Huntington's diseases (Fig. 19).

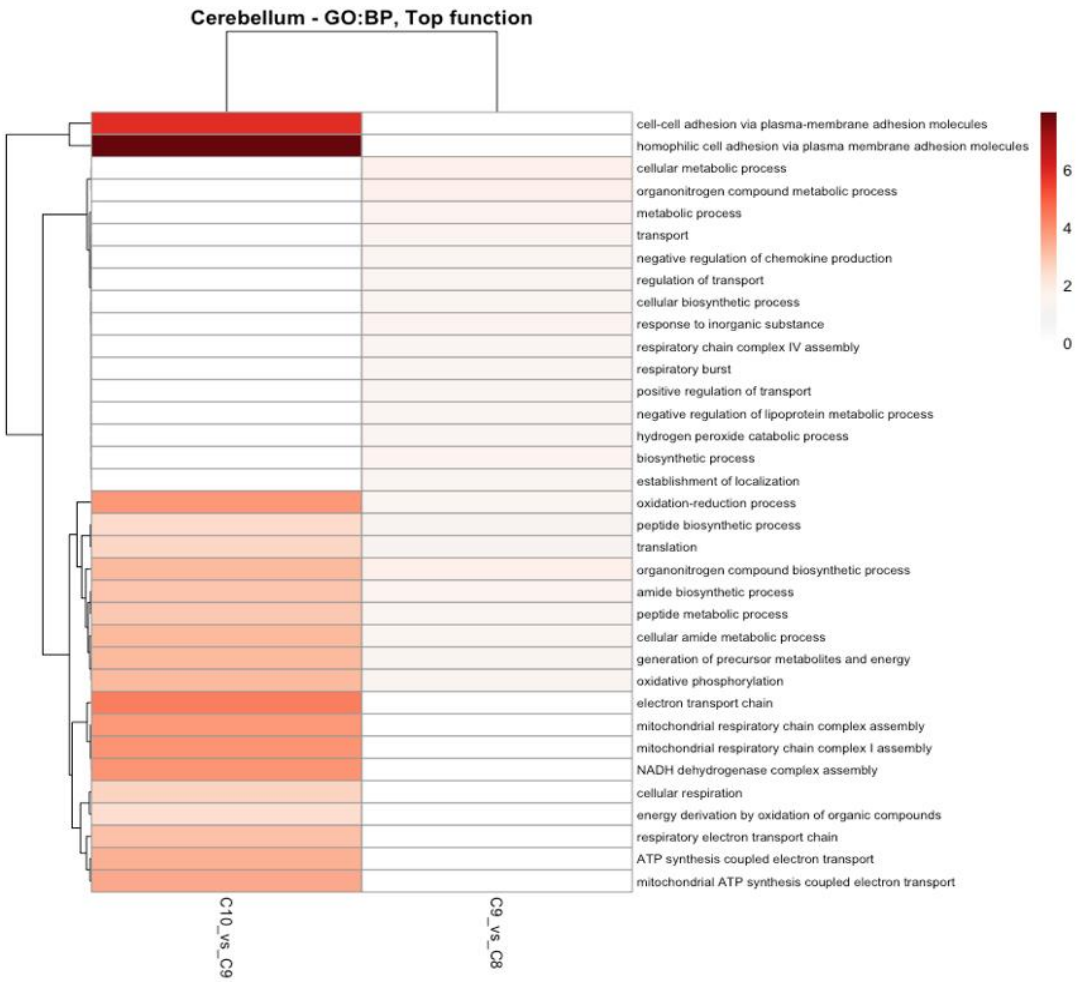

Figure 23 –Gene ontology of differentially expressed genes. Comparison between control group and MD15 (C10 vs C9) and control vs MD180 (C9 vs C8).

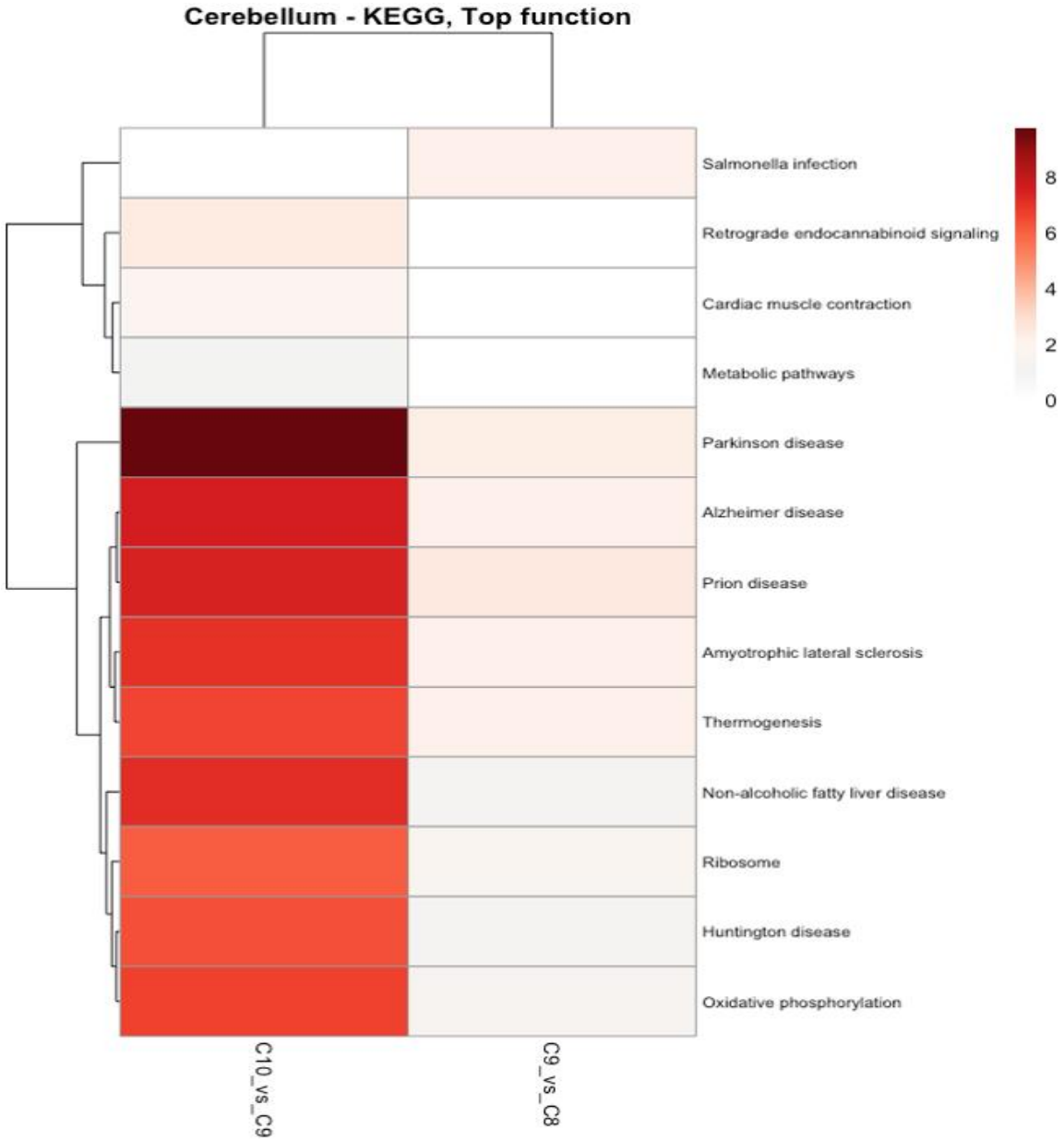

Figure 24 –Gene ontology of differentially expressed genes. Comparison between control group and MD15 (C10 vs C9) and control vs MD180 (C9 vs C8).

Discussion

In this study we show for the first time how early life adversity, in the form of maternal separation, impacts the behavior and structural integrity of the brain of adult animals. Furthermore, we demonstrate that such changes are accompanied by alterations in gene expression, which might be the trigger for the cascade of events described. Interestingly, in the same experiment, we were able to detect a significant increase in the levels of glucose after a stress test in adulthood, that was followed by a strong shift in the immune phenotype (Fernandes et al., 2021). Moreover, similar changes were observed in a cohort study from our group (martha's paper). Effects on the brain after maternal separation have also been reported, with accompanying changes in the anxious-like behavior of the animals (REF), as also reported by us in this manuscript.

Although previous reports (Sarabdjitsingh, Loi et al. 2017) show a significant decrease in the volume of the prefrontal cortex after maternal deprivation, in this study we did not observe such differences in any of the studied regions. However, we were able to detect significant differences in the fractional anisotropy of the cerebellum of maternal separated animals, through diffusion-weighted magnetic resonance imaging, which allows the detection of water motion through the axons of a specific brain region (REF). Several studies mention the relation between changes in FA and the development of brain disorders such as schizophrenia, Alzheimer's and Parkinson's disease but, at the same time, this measure is still very limited as it looks at a macroscopic level, leaving behind important microscopic changes.

Cerebellum is a brain region that has been mostly associated with motor and cognition functions and only recently, its involvement in the stress response and ELA consequences has been studied (Du, Wang et al. 2016, Tomas-Roig, Piscitelli et al. 2016, Roque, Lajud et al. 2019, Tomas-Roig and Havemann-Reinecke 2019, Catale, Gironda et al. 2020). Functional changes observed in

this study refer to increases in fractional anisotropy (FA), which were observed throughout the studied brain regions (hippocampus, striatum, cortex and cerebellum) but only reached significance in the cerebellum. FA allows the measure of brain fibers integrity considering the water diffusion properties (O'Donnell and Westin 2011). Deviations to the normal FA volumes were recently associated with depression (Hermesdorf, Berger et al. 2017), Alzheimer's (AD) (Zhang and Burock 2020) and Parkinson's disease (PD) (Mole, Subramanian et al. 2016). When adding early life stress into the equation, reduction (Meinert, Repple et al. 2019) and increase (Kircanski, Sisk et al. 2019) of the FA values in specific brain regions were observed.

Further findings from our study correlated changes in the FA values in the cerebellum with changes at the gene expression level. Genes significantly different from the control group were associated with neurodegenerative disorders such as Parkinson's, Alzheimer's and Prion's disease but also with biological processes associated with the mitochondria. Impairments at the mitochondrial level have been for years associated to the development of AD, PD and depression (Moreira, Carvalho et al. 2010, Allen, Romay-Tallon et al. 2018, Grünewald, Kumar et al. 2019, Karabatsiakis and Schönfeldt-Lecuona 2020, Wang, Zhao et al. 2020). Together with literature data from our and other clinical studies, we can conclude that prolonged maternal separation, both in humans and rodents, undoubtedly impacts the normal development of different body systems, in particular the neuronal and immune system. Interplay between these and all other systems surely has a saying in the way stress affects the body and proposed mechanisms for the phenotypes following ELA should take all of them into consideration.

Chapter V: DNA methylation as a mechanism behind early life consequences

My contribution to this chapter:
Conception of the project. Planning and performing the animal experiments involving rodents. Setting up and performing the biochemical analysis. Final statistical analysis. Literature research and writing of the manuscript. Manuscript accepted for publication in *Frontiers in Genetics*, in April 2021.

5. N6-METHYLADENINE IN EUKARYOTIC DNA: TISSUE DISTRIBUTION, EARLY EMBRYO DEVELOPMENT AND NEURONAL TOXICITY

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5.1 Abstract

DNA methylation is one of the most important epigenetic modifications and is closely related with several biological processes such as regulation of gene transcription and the development of non-malignant diseases. The prevailing dogma states that DNA methylation in eukaryotes occurs essentially through 5-methylcytosine but recently adenine methylation was also found to be present in eukaryotes. In mouse embryonic stem cells, 6-methyladenine was associated with the repression and silencing of genes, particularly in the X-chromosome, known to play an important role in cell fate determination. Here, we have demonstrated that 6mA is a ubiquitous eukaryotic epigenetic modification that is put in place during epigenetically sensitive periods such as embryogenesis and foetal development. In somatic cells there are clear tissue specificity in 6mA levels, with the highest 6mA levels being observed in the brain. In zebrafish, during the first 120h of embryo development, from a single pluripotent cell to an almost fully formed individual, 6mA levels steadily increase. An identical pattern was observed over embryonic days 7-21 in the mouse. Furthermore, exposure to a neurotoxic environmental pollutant during the same early life period may led to a decrease in the levels of this modification in female rats. The identification of the periods during which 6mA epigenetic marks are put in place increases our understanding of this mammalian epigenetic modification, and raises the possibility that it may be associated with developmental processes.

Keywords:
DNA methylation, 6-methyladnenine, stress, brain, embryo development, developmental neurotoxicity

5.2 Introduction

Although it is known to occur in both cytosine and adenine bases, the prevailing dogma is that DNA methylation essentially occurs on the fifth position of cytosine residues. Five-methyl cytosine (5mC) is an evolutionarily conserved modification, present throughout both eukaryotes to prokaryotes, which is involved in the development and afterward adaptation to the local environment (Tobi et al. 2018; Bonsch et al. 2012; LaSalle 2011). Several studies from famine (Roseboom et al. 2011) to stressful events in life, such as war (Trivedi et al. 2019), suggest alterations in the deposition of this modification and its passage to the second and third generation, thereby inducing part of the heritability of certain phenotypes or disorders. Furthermore, early life adversity, such as psychosocial stress, infections or exposure to pollutants, has shown to play a major role in the development of certain disease phenotypes, and DNA methylation is believed to be the link between these events (Mitchell, Schneper, and Notterman 2016; Vonderwalde 2019; Murphy et al. 2015; Elwenspoek et al. 2019; Duca et al. 2018). Exposures of rodents to Persistent Organic Pollutants (POPs) through the diet of the dams, such as Brominated flame retardants (e.g. hexabromocyclododecane (HBCDD) or Polycyclic aromatic Hydrocarbons (PAHs)), during development was shown to induce significant behavioral changes (increased anxious-like behavior, hyperactivity and altered social behavior) in their offspring (Maurice et al. 2015; Crepeaux et al. 2014). These results underline the existence of a critical window of exposure for brain and behaviour development and suggest that epigenetic modifications could be involved in these behavioural impairments. Furthermore, there is growing evidence that POP exposure might be associated with the occurrence of developmental and neurodegenerative diseases (like Autism, Alzheimer's and Parkinson's), through the regulation of DNA methylation, namely 5-methylcytosine (Grova et al. 2019). Although changes in the levels of 5mC has already been linked to POPs (Duca et al. 2018; Alvarado-Cruz et al. 2018), the mechanism behind exposure and later disease development is still unclear.

On the other hand, adenine methylation (6mA) has been known to be present mainly in prokaryotes since it was first described in E. Coli in 1955 (Dunn and

Smith, 1955) and subsequently in Aerobacter Aerogenes, Mycobacterium tuberculosis and Salmonella (Dunn and Smith, 1958). Since its discovery, 6mA has been associated with important biological processes in bacteria such as DNA replication, regulation of gene expression and cell defense against viruses, through the restriction-modification systems in which DNA adenine methylase (Dam) plays a role (Low and Casadesus 2008; Marinus and Casadesus 2009; Wion and Casadesús 2006; Sanchez-Romero, Cota, and Casadesus 2015). More recently, adenine methylation was reported in eukaryotic organisms such as plants (Zhou et al. 2018), Drosophila melanogaster (Zhang et al. 2015), Danio rerio (Liu et al. 2016) and mammals, such as mouse (Li et al. 2019; Wu et al. 2016; Yao et al. 2017) and human (Xiao et al. 2018; Xie et al. 2018). This is currently controversial as studies now suggest that this is due to bacterial DNA contamination in the original eukaryotic samples or that the currently available antibodies also non-specifically bind to unmethylated adenine (Douvlataniotis et al. 2020; O'Brown et al. 2019; Schiffers et al. 2017). Nevertheless, the enigmatic 6mA is particularly interesting in eukaryotes as the reports available so far have associated it with determining the fate of mouse embryonic stem cells, repression and silencing of genes, particularly in the X-chromosome (Wu et al. 2016) and retrotransposons (Li et al. 2020); adaptation to psychosocial stressors (Yao et al. 2017) and tumorigenesis (Xiao et al. 2018; Xie et al. 2018). One common point would appear to be neuronal tissues. Levels of 6-mA were increased in brain tumor biopsies when compared with normal human astrocytes (Xiao et al. 2018). Furthermore, they showed that knockdown of the demethylase ALKBH1, as knockdown, leads to an increase in the proliferation and tumor formation capacity. Adding to this, sequencing analysis showed that gene regions enriched in 6mA were mainly related to neuronal processes (Wu et al. 2016; Xie et al. 2018; Yao et al. 2017). Other studies also reported an increased 6mA brain levels in specific brain areas like prefrontal cortex and amygdala in mice subjected to stress (Yao et al. 2017; Kigar et al. 2017), suggesting a high sensitivity of adenine methylation to several brain insults.

Many of the studies performed so far have relied on 6mA immunoprecipitation and sequencing (MeDIP-seq) to identify the genomic regions susceptible to methylation. A consensus is starting to emerge, focussing on 6mA in LINE-1

elements. Both Yao and Wu reported strong annotations of 6mA in LINE-1 elements in the pre-frontal cortex after stress and embryonic stem cells respectively (Wu et al. 2016; Yao et al. 2017). Gene ontology analysis showed that in the stress model, 6mA levels negatively correlate with neuronal gene expression and furthermore, the differentially methylated genes overlap with genes present in mental disorders such as depression and autism (Yao et al. 2017) and in the embryonic stem cell model, LINE-1 element methylation impacted their transcription as well as in their neighboring genes (Wu et al. 2016). This is unfortunately contradicted by Xiao who observed significantly higher levels of 6mA in the mitochondria rather than on the genome per se (0.18% vs 0.055%), and Koziol and colleagues who reported 6mA in non-coding regions of the genome (Koziol et al. 2016).

The data on how adenine is modified and at what point during development or cell-type differentiation the modification is introduced are currently limited. Although the measured levels of 6mA are much lower than 5-mC the available data suggests that 6mA levels increase upon fertilization until the 64-cell stage in zebrafish and then gradually decrease as development progresses. Similarly, in the pig embryo, levels peaked at the morula stage (Liu et al. 2016). The methylase responsible for introducing this modification in eukaryotic species such as man has been reported to be N6AMT1 (Xiao et al. 2018) or METTL4 (Ye et al. 2017) (Fig. 1A), while the active demethylase has been independently reported twice as ALKBH1 or Alkbh1 in humans (Xiao et al. 2018) and mice, respectively (Wu et al. 2016). Elegant over-expression and knockout studies of both these enzymes, have demonstrated that a reduction in 6mA levels promotes tumorigenesis (Xiao et al. 2018) whilst increasing 6mA leads to inhibition of glioblastoma formation (Xie et al. 2018).

In this study, we evaluate the levels of 6mA in different eukaryotes species, with a special focus on mammals. Although previous studies affirm that this adenine modification is present in eukaryotes at a low density, we demonstrate that is it widely present in zebrafish, mice, rat and human, specifically in the brain. Furthermore, we report that 6mA steadily increases during embryogenesis in both zebrafish and mice and its global levels show subtle changes in the cerebellum of female rats upon exposure to the brominated

flame retardant HBCDD during early life. Collectively, these results may help shed a light on our understanding of the mammalian epigenetic modifications.

5.3 Material and Methods

5.3.1 Identification of 6mA from existing sequencing results

The MethSMRT database of 6mA signals in single-molecule real-time (SMRT) sequencing was interrogated (Ye et al., 2017). MethSMRT contains single-nucleotide resolution of 6mA throughout many genomes extracted from all publicly available PacBio SMRT sequencing. For the worm (*C. elegans*), brewer's yeast (*S. cerevisiae*), thale cress (*A. thaliana*) and the fruitfly (*D. melanogaster*) the numbers of unmodified adenine residues, 6mA residues and their genomic locations were extracted. Protein sequences from man (*H. Sapiens*), Norway rat (*R. Norvegicus*), house mouse (*M. Musculus*), zebrafish (*D. Rerio*) and the fruitfly (*D. Melanogaster*) were extracted from the NCBI under accession numbers NP_006011.2, NP_001102188.1, NP_001096035.1, NP_001018527.1 and NP_996458.1 respectively, for ALKBH1. For METTL4, accession numbers used for man, Norway rat, house mouse and zebrafish were NP_073751.3, NP_795891.2, NP_001178743.1 and XP_689178.3, respectively. Sequences alignment and phylogenetic trees were obtained with COBALT, a multiple sequence alignment tool from NCBI (Papadopoulos and Agarwala, 2007; Madeira et al., 2019). Genotype-Tissue Expression data for human METLL4 and ALKBH1 enzymes described in this manuscript were downloaded from the GTEX portal on August 19th, (dbGaP Accession phs000424.v8.p2, 19/08/2020).

5.3.2 Zebrafish embryos

Fish were housed in a ZebTec standalone recirculating tank system (Techniplast, Buguggiate, Italy) kept at 28°C on a light:dark (14:10 hour) cycle. Fish were fed twice per day: once with granular food (Special Diet Services, Essex, UK) and then with freshly prepared brine shrimp (Artemia salina). Male and female adult wild-type AB fish were mated and their offspring collected immediately following fertilization. Zebrafish embryos were dechorionated via a 5 min incubation with pronase (2mg/mL diluted in E3 medium; Roche Diagnostics GmbH, Germany) before being washed and raised in E3 medium (0.33 mM CaCl2, 0.17 mM KCl, 5 mM NaCl, 0.33 mM MgSO4,

and 0.1% Methylene Blue, pH 7.4) as previously described (Ernens et al., 2017). To ensure sufficient genetic material for DNA extraction, zebrafish embryos were pooled at different densities across the selected time points as follows; 100 embryos were pooled immediately after fertilization (0h post fertilization), 30 embryos at 24 hpf (hours post fertilization) and 20 embryos at 48, 72 and 120 hpf. Four biological replicates were collected at each time point.

5.3.3 Mice embryos

Female Balb/c mice were group housed (n=5 per cage) for 10-14 days for Lee-Boot cycle synchronization and brought into estrous by exposure to soiled bedding from a male mouse. Two females in estrous and one male were placed in a clean "breeding" cage and left together overnight. Mating was confirmed by the presence of a vaginal plug and all animals were returned to their home cage until sacrifice. Pregnant females were sacrificed at six different time-points, from E7 to E21. The uterus was extracted and placed in 1X PBS (4 °C) in a petri dish. Embryos were removed from the uterus, decapitated and the brain dissected. Hippocampi was dissected and stored at -20°C until further analysis.

5.3.4 Post mortem tissue from human brains

Human material used in this study is from a previously reported study (Cao-lei et al., 2013). Briefly, tissues were collected from five different donors from the region of Chang Mai, in Northern Thailand. The subjects had no underlying diseases and were hospitalized due to either car accidents, blunt chest injury or gunshot. All patients died in the Chiang Mai University Medical Hospital, where the autopsies were carried out 2-10h after the death. A 5mm punch biopsy was collected from the initial one-centimeter sections, for each of the 28 different brain regions.

5.3.5 Perinatal exposure to the brominated flame retardant α-HBCDD (HexaBromoCycloDoDecane) as an environmental early life adversity.

As previously reported (Maurice et al., 2015), pregnant females Wistar dams were exposed daily to the α isomer of HBCDD for 42 days by receiving a volume of HBCDD contaminated eggs at different concentrations (0, 22, 66 ng/kg/day), from gestational day (GD0) onto the weaning of the offspring (PND21), constituting a solid early life adversity model in lab rats (Sengupta P., 2013). Concentrations were calculated according to the human exposure through egg consumption and 22 ng and 66 ng/kg/day were found to correspond, in rats, to the lowest and highest levels of human contamination from eggs.

5.3.6 Behavior analysis

At PND270, sensory and motor impairments were evaluated by using the Locotronic apparatus (Locotronic Intelli-Bio, France), according to the manufacturer's instructions. The later was composed of a starting and arrival box, connected by a horizontal ladder corridor, with 3mm diameter bars (7 mm spacing). Two trials were run and infrared sensors above and below each inter-bar space read the position, number and duration of missteps of the animals. Offspring were sacrificed at PND270.

5.3.7 DNA extraction

DNA was extracted from samples through a spin-column procedure, using the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. DNA quantity and quality were assessed with both nanodrop (ThermoFisher scientific, Belgium) and Qubit (ThermoFisher Scientific).

5.3.8 6mA detection by HPLC MS/MS

Prior to LC-MS/MS analysis, isolated genomic DNA was enzymatically hydrolysed to individual deoxyribonucleosides in a one-step procedure. Sample DNA (1 µg) was supplemented with 2.5 ng [15N3]-2'-deoxycytidine (Cambridge Isotope Laboratories, Inc., France) as internal standard, dried under N2 and then hydrolyzed at 37°C, in a 10µl digestion mix of phospophosphodiesterase I

(300 mU; Sigma-Aldrich, France), alkaline phosphatase (200 U; Sigma-Aldrich), and BenzonaseR Nuclease (250 U, Sigma-Aldrich) in Tris buffer pH 7.9 (20mM Tris; Sigma-Aldrich), for about 8h (Godderis et al., 2015). In each sample, DNA 6-methyl-adenine was determined as previously published (Cardenas, A. et al, 2017; De Nys, S. et al., 2018; Duca et al., 2018) with minor modifications: After hydrolysis, samples were diluted with water (500 µL), filtered using an Amicon Ultra-0.5 Centrifugal filter device (Sigma-Aldrich) and resuspended in a solution of ACN : H2O (70:30, v/v). An aliquot of 10 µL was injected on a hydrophilic interaction liquid chromatography (HILIC) column (Acquity UPCL Beh Amide columns 1.7 µm, 2.1 x 50 mm; Waters Corp), held at a temperature of 40°C. A mixture of 1mM Ammonium Fluoride (A) and acetonitrile (B) was used as the mobile phase for chromatographic separation. A flow rate of 0.4 mL/min was applied. All HPLC solvents and reagents were from Sigma-Aldrich (LC-MS/MS grade). The analyses were carried out using a Waters Xevo TQ-XS triple quadrupole mass spectrometer (Wexford, Ireland) with an electrospray ionization source (ESI) in positive mode. Multiple reaction monitoring (MRM) with an argon collision gas was used to improve quantification, selectivity and sensitivity. MS/MS parameters for the specific detection by MRM are detailed in Supp. Table 1. The peaks were identified as previously described (Grova et al., 2020). Samples have been analyzed in a random manner and without prior identification of the different treatment groups.

Table 6 - MS/MS parameters for MRM detection of modified and unmodified hydrolyzed nucleosides

Compounds Cone	Ionization mode	Transitions (m/z)	Collision energy (eV)	Cone (V)
[15N3]-2'-deoxycytidine (IS)	ESI+	231 → 115	15	12
2'-deoxyadenine	ESI+	268 → 135	18	26
	ESI+	268 → 119	42	26
N^6-methyl-2'-deoxyadenine	ESI+	282 → 149	14	2
	ESI+	282 → 133	40	2

5.3.9 Oligonucleotide synthesis

A spike-in sequence of 201 base pairs was inserted in a pUC57 plasmid backbone of 2710 base pairs, with EcoRV producing a final product of 2911 base pairs (Genecust, Boynes, France). The spike-in sequences were carefully designed to have ten GATC sequences along the 201 base pairs, which is the

prefered motif for dam methyltransferase when modifying the adenine residues (Sup. Fig. 11). The sequences were later amplified with the following primers: forward 5'-GCCTCGTGAAATCCCGTTAG-3' and reverse 5' TGAAGGTGCCAAGAAGTTTCC-3'. The PCR products were then treated with dam methyltransferase for sysnthesis of dot blot positive control.

5.3.10 Enzymatic treatments of DNA for generation of positive controls

Non-methylated Yeast DNA was used as a negative control, and artificially methylated DNA was used as a second positive control. To generate it, 2µg of Yeast DNA were incubated together with nuclease free water, 10X reaction buffer, S-Adenosyl methionine (160µM) and 2µL of EcoGII enzyme (NEB labs, Frankfurt, Germany), for 4h at 37°C.Similarly, 1µg of PCR product of the created oligonucleotide was incubated with nuclease free water, 10X reaction buffer, S-Adenosyl methionine (160µM) and 1µL of dam methyltransferase enzyme (NEB labs), for 4h at 37°C. Both reactions were heat-inactivated at 65°C, for 15 minutes. Signals were detected (Intas ECL Chemocam Imager) after 5 min incubation with ECL Plus Western Blotting Substrate (Pierce; Thermofisher Scientific) following the manufacturers' recommendations. Signal intensity was quantified with ImageJ software (Schneider et al., 2012).

5.3.11 Dot blot

Immunoblotting was performed and normalized as previously described (Wu et al., 2016; Xie et al., 2018). Briefly, DNA was denatured at 95°C for 10 min, flash-cooled on ice and neutralized with 10% (v/v) of 6.6M Ammonium Acetate. Samples (40ng, 4µl per sample) were spotted on a nylon membrane (Whatman Nytran SuperCharge; Sigma), air-dried for 10 min and UV-crosslinked for 90 seconds (Ingenius syngene bio imaging). Membranes were then blocked (5% non-fat milk, 1% BSA in 0.1% PBST) for 1h at room temperature (RT) followed by incubation with the primary anti-N6-methyladenine antibody (Synaptic Systems) diluted 1:1000 in blocking solution overnight, at 4°C. After washing 3 times with 0.1% PBST, membranes were incubated with anti-rabbit IgG

antibody (Thermofisher scientific) diluted 1:5000 in blocking solution for 2h at RT.

5.3.12 Immunohistochemistry

At PND270, brains from the dams of the animals treated with HBCDD were excised, flash frozen, and stored at -80°C until analyzed. For IHC analysis, serial sections (20 µm) were mounted on Super Frost slides (Roth-Sochiel, Lauterbourg, France).

6mA: After temperature equilibration (10 min, RT) slides with cerebellum sections from two female and two male individuals were rinsed with PBS 1x for 5 min, and underwent rehydration and permeabilisation (PBS1X, Triton-X 0.3%), for 10 min. Slides were subjected to antigen retrieval (2M HCl) for 45 min and neutralisation (0.1M Tris-HCl) for 20 min. After blocking for 1h (PBS1x, BSA 1%, Triton-X 03%, RNAse A 50 µg/mL), slides were washed with 1X PBS (3 x 5 min) and incubated with primary antibody (1:500, rabbit-anti-6mA; Synaptic Systems, Gottingen, Germany) for 1h (RT). After washing (3x 5 minutes, 1x PBS) a 1:2000 Alexa Fluor 488-anti-rabit IgG (ab150077; Abcam, Cambridge, UK) was used for visualization. Finally, the slides were washed with 1X PBS (3x 5 minutes) and mounted with anti-fade mounting medium which contained DAPI (ThermoFisher Scientific).

Cytochrome c oxidase: Cerebellum sections were incubated with a cytochrome substrate buffer (100 mg DAB-4HCl (Sigma-Aldrich), 40 mg cytochrome c (Sigma-Aldrich), 36 g catalase (Sigma-Aldrich) in 0.1 M Phosphate buffer, pH 7.4) for 65 minutes at 37°C, in the dark and with agitation, as previously described (Crepeaux et al. 2014). The reaction was stopped by washing the sections for 5 minutes with cold 0.1 M Phosphate buffer containing 10% sucrose. Sections were then fixed in 4% formaldehyde for 30 min, washed three times with 0.1 M Phosphate buffer and dehydrated with ascending ethanol baths (50, 70, 96, 100%) for 3 min. Finally, sections were cleared with toluene twice for 5 min and mounted with Eukitt mounting medium (Sigma-Aldrich). Stained sections were analysed with a BIOCOM system (Les

Ulis, France), using the standard curve to calculate µmol/min/g of tissue from optical density.

5.3.13 Statistical analysis

All results were expressed as mean ± SEM. For each group, a Kolmogorov-Smirnov test for normality of the distributions as well as a Bartlett test for equality of variances were performed. When normality of the distribution and homogeneity of variance were assumed, a two-way ANOVA test was performed to compare different groups, followed by a post hoc Boneferroni test. Locomotor activity in the locotronic apparatus was analysed using non-parametric procedures (Kruskal-Wallis test for comparison among the 3 groups at the 1st and the 2nd trials, Wilcoxon procedure to compare performances between the two trials in each group). Statistical analyses were carried out using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) or GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California USA). Heatmaps were generated in R v3.5.3.

5.4 Results

5.4.1 6-mA as a Genuine Eukaryotic Modification

As the literature casts doubt on the existence of 6mA in eukaryotic DNA, we searched the MethSMRT database for direct 6mA reads from PacBio SMRT sequencing data. Direct reading of 6mA from the SMRT data ensures that the DNA from the target species is genomic DNA and does not represent contamination from bacterial, viral or mitochondrial DNA. 6-mA levels in three different species, *Drosophila melanogaster*, *Caenorhabditis elegans* and *Arabidopsis thaliana* (Fig. 20B) are in accordance with the literature, ranging from 1.25% to 1.75% of the total existent Adenines, representing 200,000 to 290,000 adenines in each of the genomes (Fig. 20C). This modification appears to be more abundant in Exons and Promoter regions in all three species, expanding the observation in human cell lines and in *Drosophila M.* (Xiao et al., 2018; Yao et al., 2018) to *A. thaliana* and *C. elegans*. Furthermore, homologues of three key enzymes in the methylation / demethylation machinery were identified in *H. sapiens*, as well as *D. rerio*, *M. musculus* and *R. norvegicus* (Fig. 20D and 20F). ALKBH1 was also present in D. melanogaster (Fig. 20E). METTL4 is a conserved methyltransferase, as well as N6AMT1, while ALKBH1 is a conserved demethylase. The sequences of these were highly conserved with an average 71% similarity between species (range 42-92%; Fig. 20D, E and F). Available RNA Seq data from the GTEXx and Illumina human body map v2.0 suggests that both ALKBH1 and METTL4 enzymes are expressed throughout the human body and that levels are uniform. We investigated the linear correlation between the tissue expression of both enzymes and discovered that they are similarly expressed in all tissues suggesting that a defined level is required in all tissue of the body, either for housekeeping purposes or as a mechanism to ensure correct functioning of the 6mA machinery (Fig. 21A, 21B and Sup. Fig. 7A). N6AMT1 does not show such a high correlation with ALKBH1 as METLL4 (Sup. Fig. 7B), but shows an equally high tissue distribution and interspecies similarity (Sup. Fig. 8 and 9).

5.4.2 6-methyladenine Detection and Quantification

We established 6mA detection using two independent techniques. Initially we established a LC-MS/MS detection of 6mA with increased selectivity since in addition to compound identification with retention time; we were also able to confirm its presence with MRM (multiple reaction monitoring) transitions. Furthermore, an extra confirmation transition was used to ensure the presence of each target compound. To confirm the later, the ratio "quantification transition to confirmation transition" was determined as the difference from the ratio obtained with standard compounds below 20%. Based on previous literature reports (Wu et al., 2016; Xiao et al., 2018), we also measured 6mA using dot blot with a specific antibody against 6-methyladenine (6mA). This technique allowed us to measure the levels of this modification through quantification of the signal intensity of the DNA dots spotted on a membrane given by the

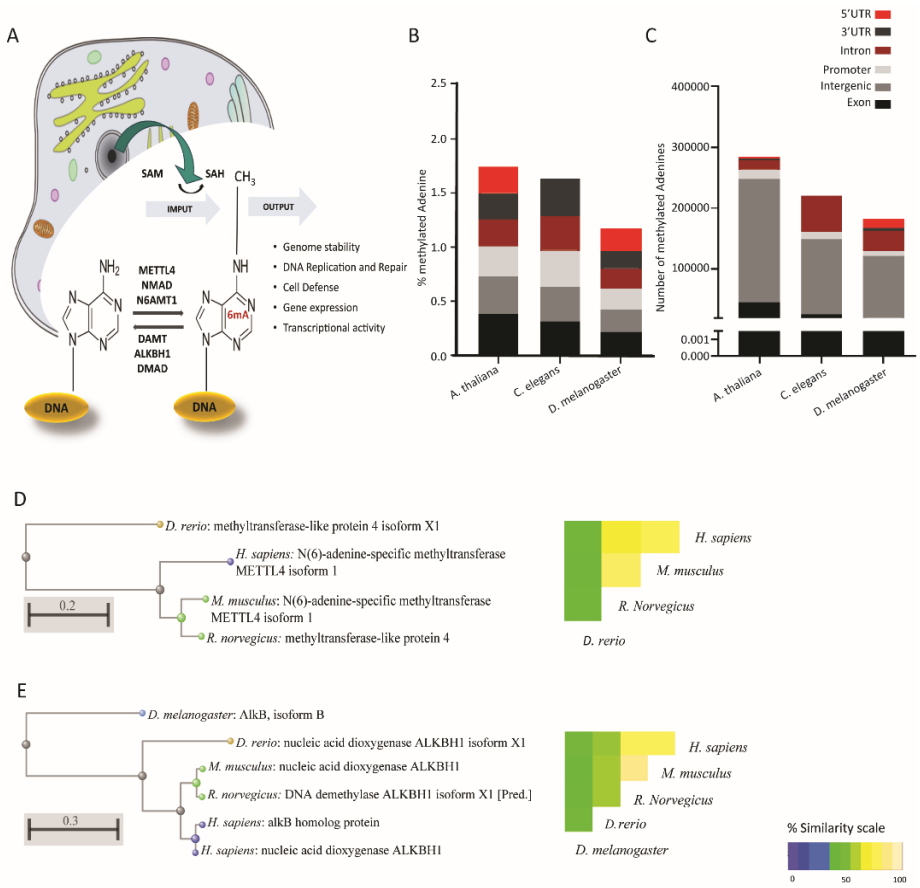

Figure 25 - 6mA is a conserved eukaryotic epigenetic modification with conserved epigenetic machinery. **(A)** Schematic representation of the current knowledge of 6mA. As for 5mC, the methyl group is donated by S-Adenosyl methionine (SAM), leaving S-Adenosyl homocysteine (SAH). Reported methyl transferases include METTL4, NMAD, and N6AMT1. Demethylases include DAMT, ALKBH1 and DMAD. Cellular compartments are not drawn to scale. **(B)** Relative abundance and **(C)** absolute abundance of direct-read 6mA calls from three eukaryotic species extracted from the MethSMART database of direct calls from PacBio sequencing, with identifiable eukaryotic sequence surrounding the methylation call. **(D)** Hierarchical clustering of sequence alignment and percentage sequence similarity for the methyltransferase METTL4. **(E)** Hierarchical clustering of sequence alignment and percentage sequence similarity for the demethylase ALKBH1.

oxidation of the luminol present in the revealing agent. The linearity of detection between the dot blot and LC MS/MS techniques was then evaluated to validate the presence of 6mA in eukaryotic DNA (Fig. 22A). DNA samples, isolated from cerebellum collected from both female and male rats exposed or not to α-HBCDD at 22 and 66 ng/kg/day, was subjected to both types of analysis. Fig. 22A displays a linear correlation between the two sets of data (Spearman analysis, n= 26, r2 = 0.81, p<0.001) confirming the suitability of these two methods to produce comparable results.

6mA antibody cross-reactivity

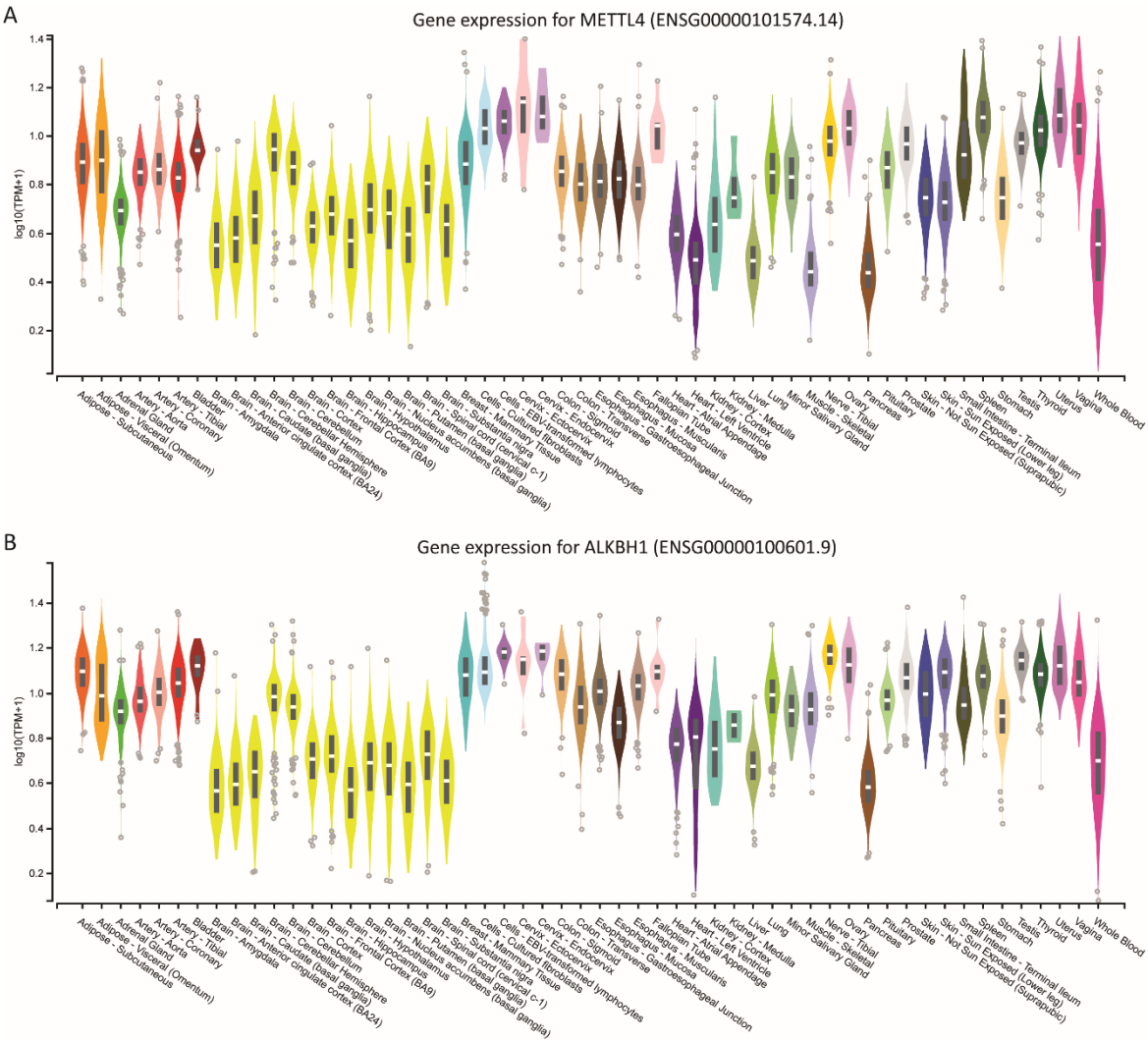

Figure 26 - Genotype-Tissue Expression data for (A) human METLL4 methyltransferase and (B) ALKBH1 demethylase. Data were downloaded from the GTEX portal on August 19th, (dbGaP Accession phs000424.v8.p2, 19/08/2020)

In order to have suitable positive controls, we tested the cross-reactivity of the 6-methyladenine antibody (synaptic systems) with in-lab methylated Yeast DNA and a methylated oligonucleotide. As seen in Figure 22B and supplementary Figure 10, Yeast DNA is devoid of 6mA and when treated with EcoGII, the antibody clearly detects a 40-fold increase of 6mA. For the methylated oligo, when treated with dam methyltransferase, the antibody clearly detects a higher signal (left) when compared to the non-treated PCR product (right). Based on this, all further 6mA results presented in this study are based on dot blot analysis. Finally, by using immunochemistry, we visualized for the first time the presence of 6mA in the cerebellar cells of rats (PND270) exposed or not through the dam to α-HBCDD at 66 ng/kg/day. Methylation of adenine (in red) is shown to be nicely located in the nucleus of cerebellar cells (in blue) (Fig 22C). Figures 22D and 22E are, respectively, representative images of control and treated animals, divided into the different channels, with the same settings, demonstrating that the control group has no 6mA background (Fig. 22E, 1). As only two individuals/sex/group were taken to generate the present images, no quantification of the signal was done.

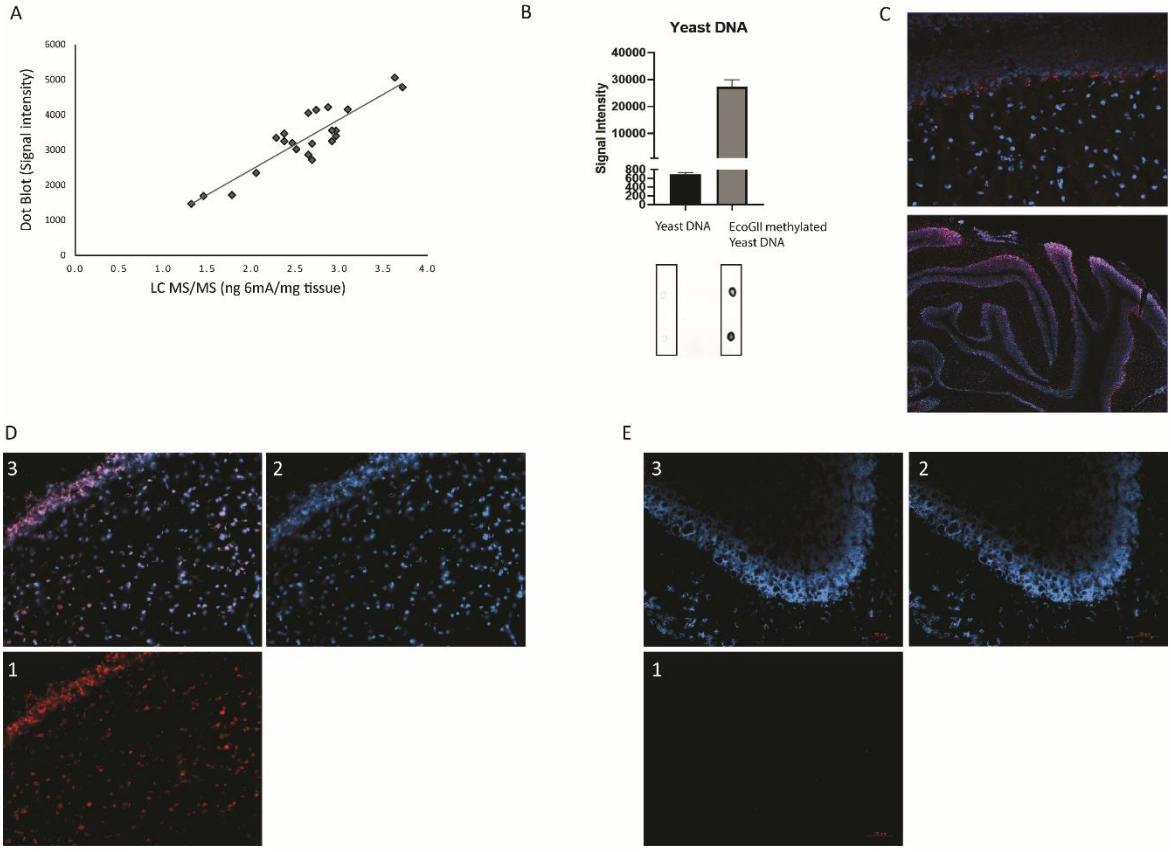

Figure 27 - (A) Representation of the linearity between the dot blot and LC-MS/MS techniques (linear relationship was evaluated by Spearman analysis, n = 26, r 2 = 0.81, p < 0.001); (B) Quantification of positive and negative control dot blots with a representative membrane underneath; (C) Immunofluorescence 6mA detection in cerebellum of female and male rats exposed through the dams to α-HBCDD at PND270. DAPI in blue, primary AB anti-6mA in red; (D) Immunofluorescence 6mA detection separated into (1) red channel – 6mA, blue channel – DAPI (2) and merged channels (3); (E) background immunofluorescence separated into (1) red channel – secondary antibody only, blue channel – DAPI (2) and merged channels (3). All images except C upper: 20x objective. C upper: 60x objective. In all images red scale bar 50 µm

Having convinced ourselves that 6mA was a genuine eukaryotic epigenetic modification that we could detect, we determined its body-wide tissue distribution in a model species, the mouse, and performed a detailed examination in the most relevant human tissue. DNA from nine different adult mice organs, from the cardiovascular to the neuronal and digestive system, was examined by dot blot for 6mA. This modification was detected in all organs and its abundance, unlike the classical 5mC, was not equally distributed throughout the tissues. The highest abundance was in the lung, spleen and brain, represented by prefrontal cortex (PFC), (Fig. 23A) that were 1.8, 1.7 and 1.9 times more abundant than the weakest tissue: the heart. The higher levels in the brain appears to agree with the GTEx RNA-Seq data that suggests lower expression of the demethylase enzymes in the brain. As such, we performed a detailed examination of 6mA presence in twenty-eight different human brain regions from five human donors. Additionally, data from the human brains

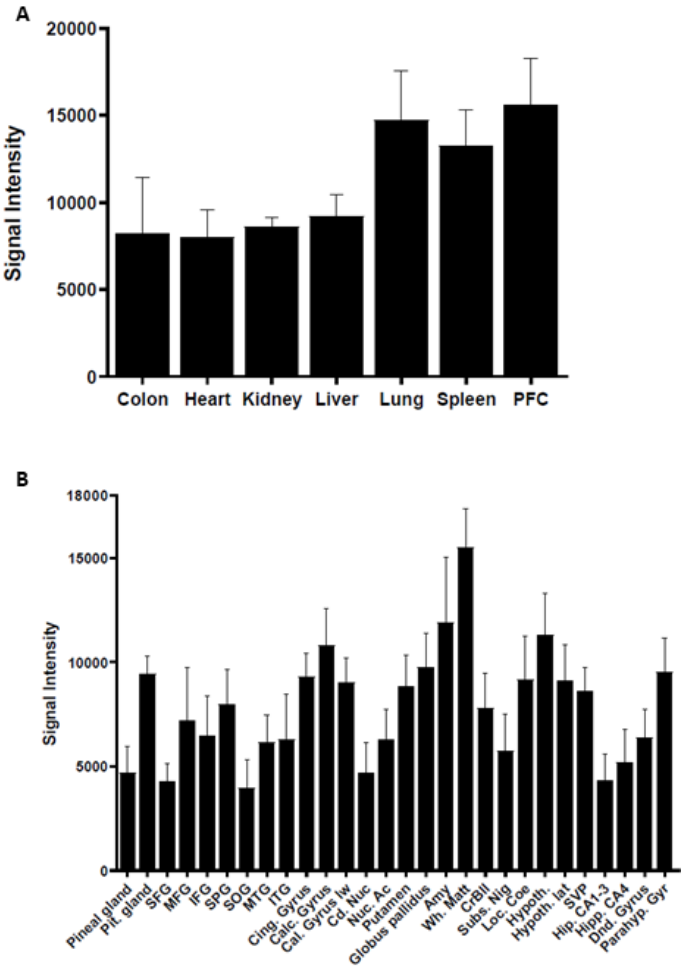

Figure 28 - Quantification of the distribution of 6mA throughout the different tissues in (A) mice and in (B) different human brain regions, via dot blot. Data are from four biological replicates in mice and five in humans, and presented as mean ± SEM

confirm this abundance where tissue specificity also seems to play a role with white matter being the most methylated tissue, having 3.9 times higher levels of 6mA then the weakest tissue: the Superior occipital gyrus (SOG) (Fig. 23B). The amygdala is the second brain region with higher methylation levels, having almost 3 times more methylated adenines than the SOG. This is a region involved in fear conditioning and known to be susceptible to stressful events and, together with our previous findings, these observations consolidate the fact that 6mA may play a role in the response to stress.

5.4.4 Embryonic States of both mice and zebrafish show a linear increase in the levels of 6mA

Mice and zebrafish embryos were collected at different time points, in order to cover the whole gestational period, a critical period in the fetal development. Contrarily to 5mC, dynamic changes throughout early and late development, adenine modifications seem to steadily increase as the embryo develops from a single pluripotent cell to an almost fully formed individual consisting of mainly somatic cells, both in zebrafish and mice (Fig. 24A and 24B). In mice, from day 7 to a fully formed embryo the amount of 6mA significantly increases 3.5 times (***p < 0.001) and the same trend can be observed throughout the pregnancy with significant increases of 1.8 times from day 11 to 16 (*p< 0.05) and 1.6 times from day 14 to 16 (*p=0.01) (Fig. 24A). In zebrafish, the

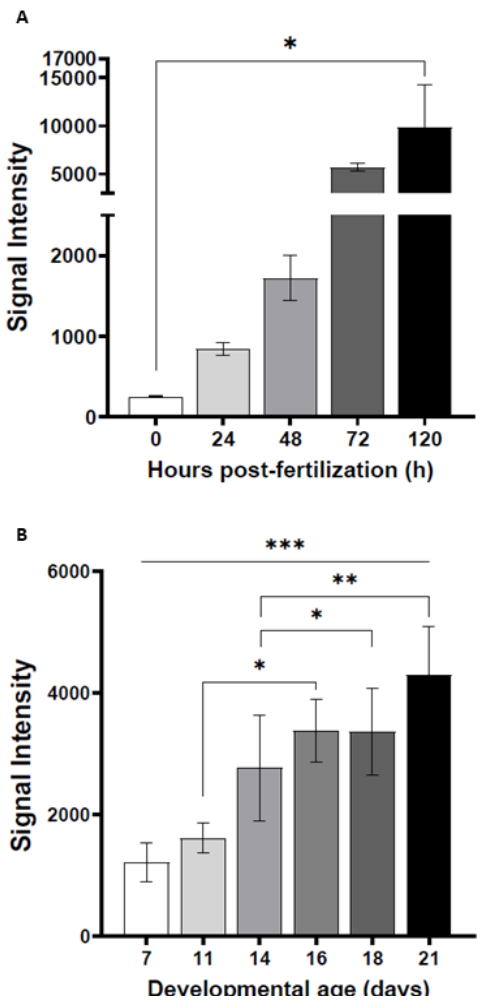

Figure 29 - Quantification of 6mA modification in genomic DNA extracted from (A) zebrafish and (B) mice, at different developmental stages. Later embryonic stages show a higher accumulation of this modification when comparing to the early stages. Data are from 20 to 100 biological replicates for zebrafish and 5 to 8 for mice, and presented as mean ± SEM. *p < 0.05; **p = 0.001; ***p = 0.0003.

same behavior is observed and although is not significantly increasing during the development process, the values of 6mA suffer a 38 fold increase from fertilization to fully formed embryo (*p<0.05) (Fig. 24B). These observations suggest that 6mA may play an important role in the development and can help us understand how a deviation from normal development can trigger the disease phenotypes such as neurodevelopmental disorders later in life.

5.4.5 Early life exposure to α-HBCDD induces change in 6mA levels in F1 generation at PND270

After demonstrating the steadily increase of 6mA throughout development, we evaluated how values of 6mA change in the brain of rats followed by exposure to α-HBCDD through the dams in a known neurodevelopmental toxicity model. There was a trend towards lower 6mA levels 9 months after exposure (ANOVA p<0.1) although there was a sex*treatment interaction (ANOVA interaction effect p=0.024, Supplementary Figure 12A). A similar result was obtained for cytochrome oxidase (sex * treatment = 0.079, Supplementary figure 12B). There were similar trends in locomotor coordination and motor learning abilities of the animals. In the second trial, the time to perform the test was significantly decreased in controls when compared to the first trial, (p<0.05), showing animals learned the task, that became a trend in the 22 ng/kg/day HBCDD-exposed animals (p=0.09) and there was no difference at the highest dose (Supplementary Figure. 12C and D), confirming that HBCDD exposure induced learning deficits, and the same pattern was observed for the number of missteps, confirming that motor deficits were induced.

5.6 Discussion

Epigenetic modifications are known to occur in RNA, DNA and histones and, although they may change the accessibility of the DNA region and/or gene expression, the original DNA sequence remains unaltered (Robin H., 1989). To date, 5mC was the ex-libris of DNA methylation in eukaryotes. Only recently, 6mA, originally described in prokaryotes (Dunn and Smith, 1955; Dunn and Smith, 1958) has been found in eukaryotes and proved to have functional roles (Wu et al., 2016; Yao et al., 2017; Xie et al., 2018; Yao et al., 2018). In this study, we demonstrate not only that in our laboratory 6mA is a genuine eukaryotic DNA modification, but also that it is widely distributed throughout rodents' tissues, with major enrichment in the brain, as recently described (Yao et al., 2017). Furthermore, in a neurodevelopmental toxicity model, the trend in 6mA levels was influenced by perinatal exposure to the pollutant α-HBCDD and associated with the behavioral anomalies seen upto 8 months after exposure.

There is currently some uncertainty in the literature as to whether 6mA is a genuine eukaryotic DNA modification. Concerns have surrounded potential bacterial contamination, RNA presence in DNA readouts and antibody cross-reactivity (Douvlataniotis et al. 2020; O'Brown et al., 2019; Ratel et al., 2006). Initially, we confirmed that the methylation machinery is common to many eukaryotic species and that the available 3rd generation sequencing datasets provide direct-read evidence for the modification being found in regions of identifiable eukaryotic DNA. Furthermore, online available data from GTEX allowed us to explore the body expression levels of the different methylases and methyltransferases involved in the 6mA machinery. Recently, N6AMT1 was identified as a methyltransferase for 6mA and ALKBH1, a demethylase already shown to be present in mice (Wu et al., 2016), were expressed in humans (Xiao et al., 2018). In the same study, Xiao and colleagues also showed that manipulation of these enzymes directly affected the expression of 6mA, particularly in cancer cell lines. Knowledge of tissue-specific expression of these enzymes, as well as the methytransferase METLL4, leads us to hypothesize that, in normal individuals, levels of 6mA are homeostatically maintained.

Expression of these enzymes throughout the human body appear to be similar as we can see in Figure 2 and in the correlation graph from the supplementary data. Differences in 6-methyl adenine levels after stressors might be justified by an imbalance in the expression of such enzymes and their manipulation might help attenuate the outcome.

As potential sources of contamination have previously been reported (O'Brown et al., 2019), we took extra care when handling samples and extracting DNA: we removed the zebrafish chorion prior to DNA extraction, reducing the contamination from bacterial DNA, and as such, the 6mA levels represents that in the uncontaminated zebrafish gDNA. Similarly, mouse embryos were extracted from the sterile in-utero environment and the DNA treated with RNAse, reducing the risk of bacterial and RNA contamination. We also extracted the genomic locations of 6mA from the MethSMRT database. This is an extraction of 6mA calls from direct long reads from PacBio Sequencing, with base-calling that identifies 6mA from the original read. This excludes the hypothesis that 6mA calls are a contaminant as the surrounding eukaryotic sequence is read, confirming the species of origin of 6mA. Furthermore, we demonstrated the concordance of the results of 6mA measurement by two methods (dot blot and LC-MS/MS), which leads us to believe that these two independent techniques are robust enough to allow the detection and quantification of 6mA. Although dot blots might present some limitations in regards to full quantification of this modification, LC-MS/MS completely erases any doubts that may exist. Has previously reported (Liu et al., 2016; Wu et al., 2016; Xiao et al., 2018; O'Brown et al., 2019), levels of 6mA are accurately detected and show no contamination of others adenine modifications (Xiao et al., 2018). Furthermore, levels of 6mA detected with LC MS/MS, dot blot and SMRT-sequencing were shown to be similar in other reports (Wu et al., 2016; Xiao et al., 2018) and in our own data. Positive and negative controls for this modification are yet to be described and for that reason, we have decided to develop two of our own. Both in lab methylated yeast and oligonucleotide provided good quality positive controls as we can clearly see an increase in signal intensity after enzymatic treatment. However, these controls fail to give

an exact measure of the numbers of adenines present in the sequence vs number of adenine methylated.

Moreover, we were able to show, for the first time, that like 5mC, 6mA is present in tissues from all the major eukaryotic organ systems in mice, specifically in the digestive, cardiovascular and immune system. We also provide the first evidence of the presence of 6mA in twenty-eight different human brain regions. Interestingly, the brain region where we measured the higher levels of methylated adenine was the white matter, shown and described to take a great part on the development of the brain tumours (Esmaeili et al., 2018; Louis et al., 2007). These results, together with the RNA sequencing results illustrating the lower levels of the demethylases expression in the human brain, are in line with the recently described high levels of 6mA in Glioblastoma cell lines (Xie et al., 2018) and may help to elucidate the mechanisms behind such events. Furthermore, the role of the conserved methyltransferase and demethylase in the folate cycle and DNA methylation warrants further biochemical investigation. Increasing evidence highlights that chronical stress, particularly in early life, could interfere and regulate the levels of DNA methylation, which, in turn, acts on the expression of certain genes (Uchida et al., 2011; Murgatroyd et al., 2009; Vidrascu et al., 2019). Our results from animals exposed to α-HBCDD during early life are consistent with alterations in DNA methylation, with decreased levels of 6mA in the cerebellum, revealing the importance of this modification in the processes initiated by stress.

Earlier studies detected the accumulation of 6mA during embryogenesis, in genomic DNA from sperm, oocytes and several embryonic stages of pig and zebrafish (Liu et al., 2016). Considering our previous results and the knowledge from embryogenesis, we decided to investigate the behaviour of 6mA during the whole gestation period of zebrafish and mice. Our data from zebrafish embryos confirms the accumulation of methylated adenines during this period and, contrarily to what was previously published by Liu et al., we demonstrate that this modification steadily increases until birth. Recent data confirms this increase during development (Li et al., 2020). The levels of 6mA in the hippocampus of mice embryos provided similar results to the zebrafish, encouraging the hypothesis that 6mA has a role in neurogenesis. Opposite of

what happens with 5mC that changes dynamically throughout embryogenesis (Greenberg and Bourc'his, 2019), 6-methyladenine appears to significantly change its levels from early embryonic days up to the very end of gestation. DNA methylation, namely 5mC or 5hmC, has already been proven to play an important role in neurodevelopment (Szulwach et al., 2011; Ficz et al., 2011; Zhiqin, Beisha and Peng, 2016) and deviations to a normal pregnancy, in various forms, led to neurodevelopmental disorders (Chen, Ozturk and Zhou, 2013; Kundakovic and Jaric, 2017; Palma-Gudiel et al., 2017). The physiological role of 6mA appears to be in the silencing of both genes and transposons such as at long interspersed element 1 (LINE-1) (Li et al., 2020). Although LINE-1 retrotransposons make up around 17% of the human genome, they are highly mobile, and cause somatic mosaicism that has been linked to both neurodevelopmental disorders and psychiatric disorders such as Schizophrenia (Doyle et al., 2017; Bundo et al., 2014).

Finally, changes in 6mA level in cerebellum were measured at PND270 in a rodent model that was shown to concurrently induce neurobehavioral deficiencies (Maurice et al. 2015). Indeed, the neurodevelopmental toxicity of α-HBCDD was recently studied by evaluating neurobehavioural impairments induced by α-HBCDD exposure via the food during the gestation and lactation in dam rats at concentration levels which were representative of human exposure (22 and 66 ng/kg/day) (Sengupta, P., 2013). During the first 3 weeks of life, impairments in motor maturation of pups were observed in a dose-dependent manner depending on the test, whereas no significant differences were reported between male and female pups. At PND26, the anxiety levels of female rats exposed to the lowest dose of α-HBCDD (22 ng/kg/day) was significantly reduced whereas it remained unchanged in males. No significant variations were measured in rats exposed to the higher level of the pollutant (66 ng/kg/day). These observations are in line with the observed changes in DNA methylation (slight decrease in 6mA levels in the cerebellum) and CO activity (slight decrease measured in the interpositus nucleus). However, alterations in locomotor coordination and learning capabilities observed in the same animals later in life, at PND270, appear to be inversely proportional to 6mA levels, suggesting a potential role of 6mA epigenetic change in the brain

toxicity initiated by α-HBCDD exposure. Further investigations need to be carried out to confirm if early life exposure to α-HBCDD may induce changes in 6mA epigenetic hallmarks in the developing brain, which in turn may affect behaviour of the offspring at adulthood.

To conclude, our observation that 6mA levels continuously increase throughout the whole prenatal development suggests that this modification is introduced as the fetus differentiates and fully differentiated somatic cells accumulate. As such, the role of 6mA in the developmental origins of health and disease is obvious. Given the developmental role that is becoming apparent in the literature, we have expanded this. We report that 6mA steadily increases during embryogenesis in both zebrafish and mice and its global levels show subtle changes in the cerebellum of female rats upon exposure to brominated flame retardant during early life. Nevertheless, further studies are required in order to better understand the role and plasticity of 6mA in development and pathophysiological processes. Our findings suggest that the link between 6-methyladenine, neurogenesis, and changes in behavior as well as inflammation in the adult brain is worth exploring in more detail in the future

5.7 Ethics statement

All animals used in this study were maintained in accordance with all current European Union (Directive 2010/63/EU), national and local ethical guidelines and legal regulations. Mouse and zebrafish embryo experiments were performed in accordance with the LIH institutional animal welfare structure requirements as well as the European Union Directive 2010/63/EU as implemented in national legislation. Rat experiments were approved and supervised by the institutional ethics committee of the University of Lorraine (authorization number B54-547-13). The study was approved by the Ethical Committee of the Faculty of Medicine, Chiang Mai University, Thailand as previously published (Cao-Lei et al., 2013).

5.8 Conflict of Interest

The authors all declare that they have no conflict of interest.

5.9 Authors Contribution

Conceptualization: SBF and JDT; literature review: SBF and JDT; Data collection: SBF, SR, NG, SM, RD, LG, HS; Provided samples: AL, IE, YD; Data analysis: SBF, SR, NG, HS, JDT; Manuscript writing and editing: SBF, NG and JDT. All authors read and approved the final version of the manuscript.

5.10 Funding

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5.11 Acknowledgements

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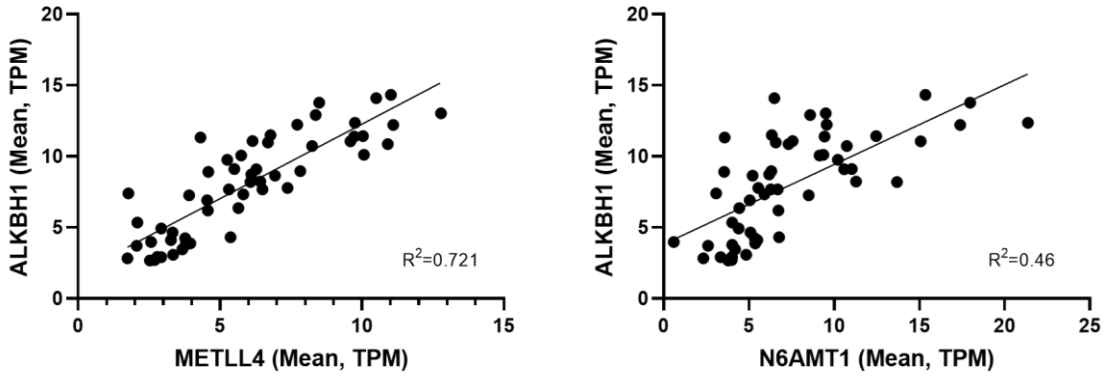
long-term effects of early life adversity over the years as well as Loic Zalko and Jean-Charles Olry (Calbinotox, Nancy) for their expertise in cytochrome c oxidase and behavioural assessment.

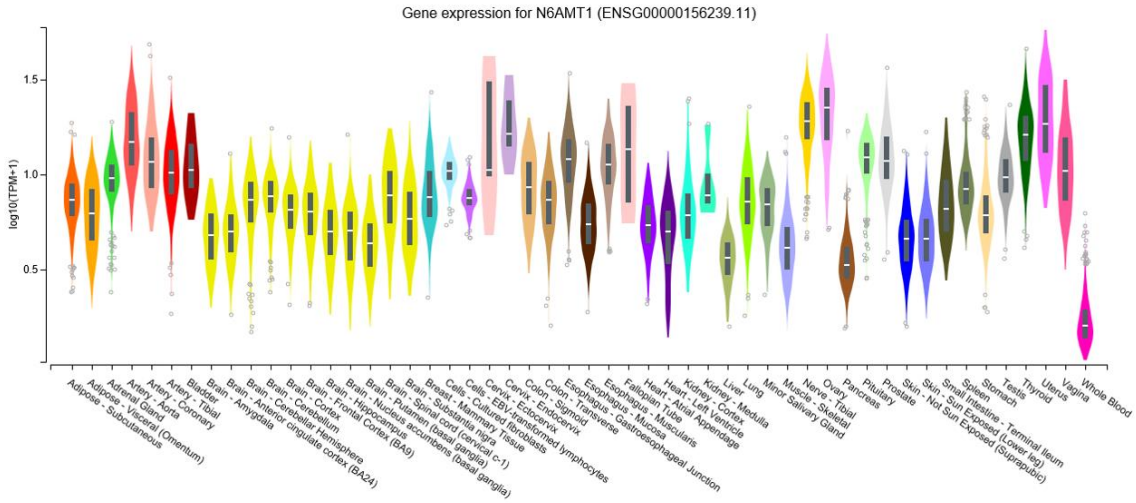
5.12 Abbreviations

6mA, 6-methyladenine; CO, Cytochrome c oxidase; HPF, Hours post fertilization; HBCDD, HexaBromoCycloDoDecane; LC-MS/MS, Liquid chromatography tandem mass spectroscopy; PND, Post-natal day.

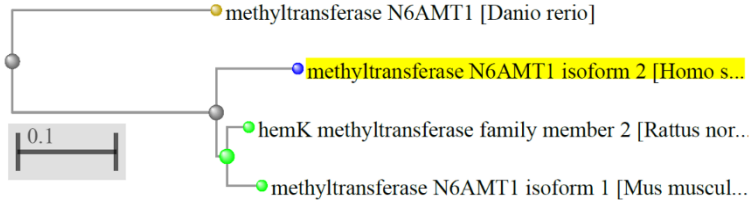
5.13 Supplementary data



Supplementary figure 6 - Representation of the linearity of expression levels for methylase and demethylase enzymes. (A) ALKBH1 vs METLL4; (B) ALKBH1 vs N6AMT1. Data from the GTEX portal on August 19th,2020 (dbGaPAccession phs000424.v8.p2, 19/08/2020) (linear relationship was evaluated by Pearson analysis, R2given in each panel, p< 0.01 in both cases).

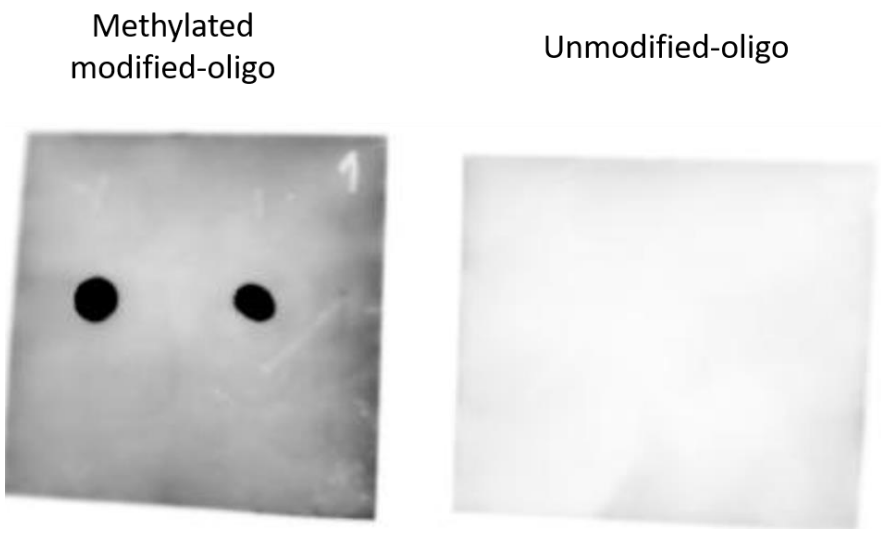


Supplementary figure 7 - Genotype-Tissue Expression data for human N6AMT1. Data were downloaded from the GTEX portal on August 19th, (dbGaPAccession phs000424.v8.p2, 19/08/2020

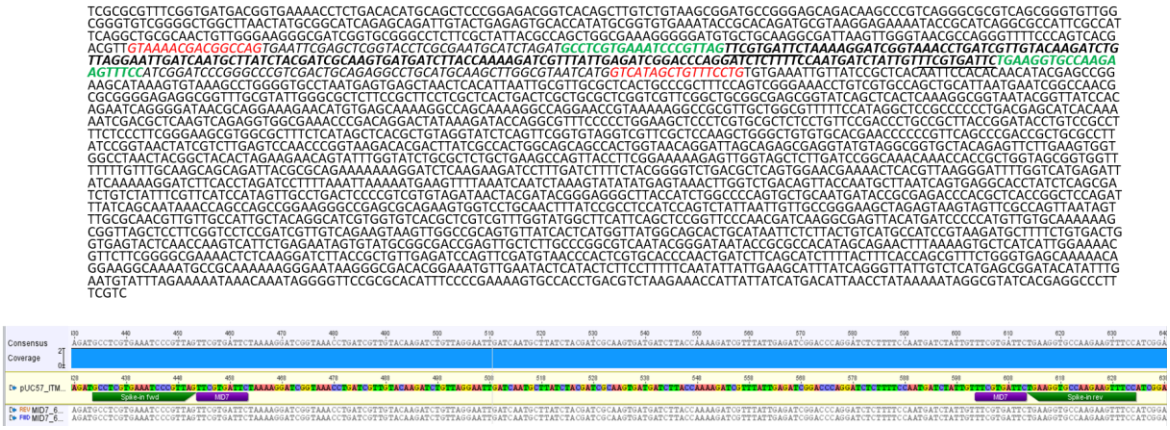


N6AMT1	% similarity
Homo Sapiens	100
Mus musculus	76.1
Rattus Norvegicus	77.5
Danio rerio	53

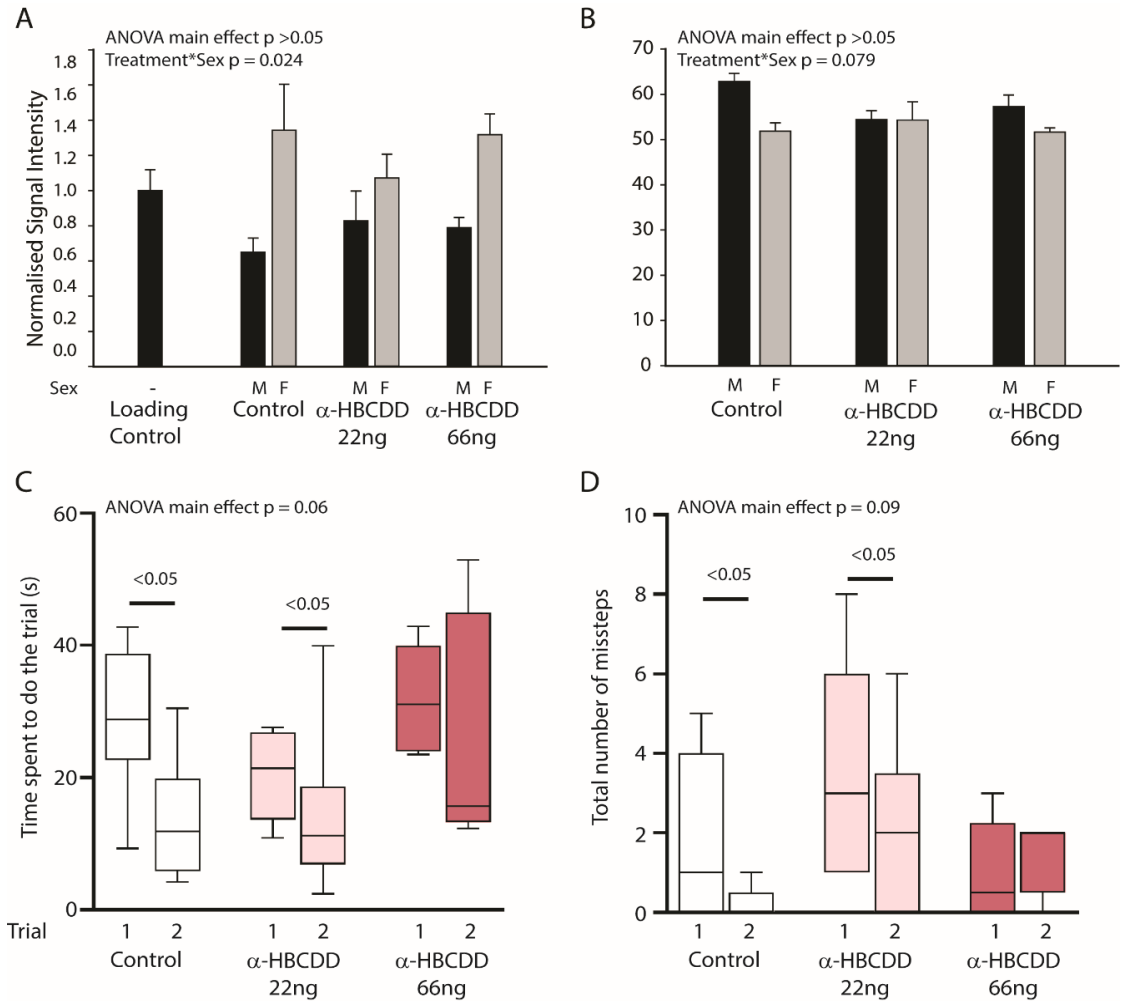
Supplementary figure 8 - Hierarchical clustering of sequence alignment and percentage sequence similarity for the methyl transferase N6AMT1



Supplementary figure 10 - Representative membranes for dot-blot quantification of the artificially methylated DNA control to demonstrate the lack of binding in synthetic DNA without 6mA and the strong signal from the artificially modified DNA.



Supplementary figure 9 - Spike-in oligo sequence and sanger sequence confirmation



Supplementary figure 11 - After demonstrating the steadily increase of 6mA throughout development, we decided to evaluate how values of 6mA change in the brain of rats followed by exposure to α-HBCDD through the dams in a known neurodevelopmental toxicity model. At PND270, the statistical analysis of 6mA levels in cerebellum showed an almost significant difference between the control and the animals treated with α-HBCDD, for both males and females (Supp. Fig 4A; ANOVA main effect p>0.05). Additionally, a significant interaction between sex and treatment (ANOVA interaction effect p=0.024) was observed, which can be explained by the decrease in 6mA observed at 22 ng/kg/day dose, only in females (Supp. Fig 4A). Upon measuring the cytochrome oxidase activity in the interpositus nucleus, the same interaction tendency was observed (sex * treatment = 0.079) (Supp. Fig 4B), which we also justify with the slight decrease at the lowest dose of α-HBCDD in females.

To confirm if the observed changes in cerebellar cells of female rats may result in behavioural impairments, we evaluated the locomotor coordination and motor learning abilities of the animals. The time spent in the apparatus and the number missteps were compared between the two trials. In the second trial, the time spent to perform the test was significantly decreased in controls when compared to the first one. The same tendency was observed between the two trials in the 22 ng/kg/day HBCDD-exposed animals (p=0.09) whereas no difference was observed at the highest dose (Supp. Fig 4C). Similarly, a slight decrease in the number of missteps between the two trials was also observed both in the controls (p=0.1) and the 22 ng/kg/day α-HBCDD group (p=0.06) but not at the highest dose (Supp. Fig 4D). These results suggest that perinatal exposure to α-HBCDD at 66 ng/kg/day may induce a deficit of coordination and motor learning skills in adult female rats, but 22ng/kg/day is not enough to induce such behavioral changes.

Chapter VI: Discussion and conclusion

My contribution to this chapter:
Literature research and writing.

6. GENERAL DISCUSSION

Research focused on stress and its consequences, particularly early life stress, has been studied for several years but the mechanisms leading to the observed outcomes are still poorly understood. This thesis demonstrates that early life adversity in rats (MetCOEPs project), in the form of maternal separation, strongly affects the immune system and the brain functionality. Additionally, it provides evidences for what might be one of the essential mechanisms behind this sequence of events.

After a thoroughly literature search and analysis (Chapter 1), we conclude that adoption in humans and maternal separation in rats clearly identifies with adulthood-impaired immune system, specifically T cells subsets, as previously published by our group (Elwenspoek, Hengesch et al. 2017, Elwenspoek, Kuehn et al. 2017) and others (Roque, Mesquita et al. 2014) but also, as part of being a psycho-sociological stress, interferes with the normal development and functionality of the brain (Bremner 2006, Krugers, Arp et al. 2016, Kraaijenvanger, Pollok et al. 2020). Nevertheless, cell and region specific data, such as from the innate immune system and the brain, was still missing. Therefore, with this thesis we aimed to investigate the particular changes suffered by the innate immune system. After confirming the already existing findings, through an unbiased approach, we moved on to identify clear changes in the natural killer cells, particularly in terms of maturation and cytotoxicity (Chapter 3). Furthermore, we identified functional changes, accompanied by changes in gene expression, in the cerebellum of animals that were maternal deprived (Chapter 4).

Key findings from EpiPath

Increased anxiety and depression disorders diagnosis;

T cell increased activation and senescence

No significant changes in the stress system

Unanswered questions

Which elements or aspects of ELA cause the phenotype?

Does immunosenescence causes inflammation and impairs the immune system?

Is CMV infection a prerequisite for ELA-associated immunosenesnce?

Are DNA modifications underlying these functional immune differences?

This thesis arose from a previous clinical study, EpiPath, which aimed at identifying the long-term effects of adoption in the immune system. Data from the study demonstrates that individuals who suffered from this type of ELA are more prone to engage in risky behaviors, such as smoking and medication use, as well as more sensitive to the development of stress-related disorders, such as depression and anxiety (data not published). Key findings of the study spotlight T cells as the primary immune cell type to be affected by ELA, becoming more active and senescent. Yet, some questions were still left unanswered, which is where the focus of the thesis went (Fig. 25).

There is an ongoing debate on whether ELA affects multiple organs in an independent manner or it firstly affects the stress system, which consequently will induce changes in the neuronal, immune and cardiovascular systems. Although both theories are supported by literature, in EpiPath study we did not find clear changes in the stress system, especially at the cortisol release level and glucocorticoid receptor (*NR3C1*) gene expression (Chapter 2) (Hengesch, Elwenspoek et al. 2018, Elwenspoek, Hengesch et al. 2020). Similarly, in this study, although we did not measure the expression of glucocorticoid receptors, in the maternal separated animals we did not find significant changes in the

corticosterone and glucose release after an acute stress, in adulthood. Moreover, maternal deprived animals similarly displayed an increased anxious-like behavior, when compared to the control group (Chapter 4). Altogether, these results are in line with the previous findings and provide support for the increasing hypothesis that the HPA axis is not the core system in ELA-derived consequences (Fig. 26). Besides confirming and adding new findings to the previous results from our group, we aimed at providing a mechanism that could explain all changes observed in both clinical and experimental studies.

Immune phenotype

Although it existed some literature on the effect of ELA in the immune system, specificity in terms of cell types was still scarce. Similar to data from EpiPath (Elwenspoek, Hengesch et al. 2017, Elwenspoek, Sias et al. 2017) and from maternal separated animals (Roque, Mesquita et al. 2014), we were able to confirm previous findings on changes in the levels of T cells, particularly in the $CD4^+$ and $CD8^+$ T subsets but also expanded the findings to B and NK cells. Clear changes in the innate immune system after ELA are yet to be documented, although changes in the number of NK cells has been previously reported (Wyman, Moynihan J Fau - Eberly et al. 2007). In this study, we report a significant increase in the expression of B cells after maternal separation and a clear increase in the maturation state of NK cells. Same changes in the NK cells' profile were observed on the EpiPath cohort as well as significant decreased in the levels of the cytotoxicity response. This reduction was interestingly only observed in the most mature NK cell populations (Chapter 3). Both B and NK cell populations are naturally the first in line in response to a viral or bacterial infection and increases in the numbers could be justified by an adaptation of the immune system to prolonged stress. Data from EpiPath on the immunosenescence of T cells strongly suggests a mediation by cytomegalovirus infection. In this study however, that is not the case as we performed correlation analysis between CMV titers and NK cell cytotoxicity and laboratory animals are in facilities where there is no presence of CMV (Chapter 3). A possible mechanism that might explain such phenomena is an imbalance

in the number of specific activating and inhibitory receptors at the NK cell surface, such as Ly49, KIRs and NKG2D. These receptors are what makes them able to recognize self and non-self cells, by binding to MHC class I receptors present in cells, thus inhibiting the activation when no infection agent is present (Paul and Lal 2017, Abel, Yang et al. 2018, Poznanski and Ashkar 2019). An imbalance in the number of these receptors would lead to failure in recognize non-self cells and consequently decrease NK response and cytotoxicity. Levels of such receptors were not investigated in this study so conclusions towards this hypothesis can hardly be made. Nevertheless, it would be of the highest interest to focus future ELA research on immune cell markers expression.

Immunosenescence

Most common used markers for immunosenescence is the cell surface marker CD57 or measure of telomere length. Absence of a marker for immunosenescence in rats and the fact that telomere length has proven lack of reliability when measured in cells from blood or when the individuals are young did not allow us to directly quantify this phenomenon in our study. Nevertheless, increased NK cell maturation was observed by the gain of specific cell surface markers. Similarly, in the the human cohort, we were able to identify different types of NK cell population and demonstrate that the most mature populations were indeed the ones further away from the controls, exhibiting impaired responses (Chapter 3). Unlike observed in EpiPath, these findings are not correlated with CMV infection, which raises new questions on the underlying mechanism of ELA-mediated phenotypes.

Brain changes induced by ELA

Considered a psychosocial type of stress, early maternal separation can significantly precipitate the development of several psychiatric diseases and neurodegenerative disorders (Martisova, Aisa B Fau - Guereñu et al. 2013, Hui, Feng et al. 2017, Mravec, Horvathova et al. 2018, He, Zhang et al. 2020,

Tanaka, Hirai et al. 2021). Also demonstrated in clinical studies, ELA in its different forms is highly associated with the development of anxiety and depression, as well as motor and cognitive problems (Felitti, Anda Rf Fau - Nordenberg et al. 1998, Erica L. Weiss, James G. Longhurst et al. 1999, Spence, Najman et al. 2002, Negele, Kaufhold et al. 2015, Rehan, Antfolk et al. 2017), placing the brain at the leading position of organs most affected by stress (McEwen, Bowles et al. 2015). In our study, maternal deprived animals did not only display a clear anxious-like behavior phenotype but also suffered functional changes in the cerebellum. These changes, observed through magnetic resonance imaging (MRI), open a door for new hypotheses regarding ELA effects (chapter 4).

Key Findigns from MetCOEPs

Chapter 2 & 3

No significant changes in GR expression in humans, after ELA

No significant changes in corticosterone levels and glucose in maternal deprived rats, after and cute stress in adulthood

Increased maturation and decreased cytotoxicity capacity of NK cells

Chapter 4

Increased anxious-like behaviour in maternal deprived animals

Altered brain functionality, particularly in the cerebellum

Altered gene expression in brain regions associated with stress

Proposed mechanisms underlying ELA-induced phenotypes

As previously mentioned, both CMV infection and impaired HPA-axis derived mechanisms do not seem to be the explanation for all the observed changes. Although the slight increase observed in absolute glucose levels in our animal study may be awakening the stress response, which in turn would influence the immune system, the non-changing levels of corticosterone do not

allow us to take such conclusion. Furthermore, as described in chapter 2, although we detected some changes in the DNA methylation levels of certain GR promoter regions, in our cohort study, they were not enough to induce differences at the gene expression levels (Elwenspoek, Hengesch et al. 2020). Therefore, development of disease phenotypes after ELA through the HPA axis and dysregulation of GR expression does not appear to be the mechanism.

In chapter 5, we discuss the importance of DNA methylation during early development. 6-methyladenine, a DNA modification recently shown to be present in eukaryotes and participate in important processes, is shown in our study to steadily increase during embryogenesis, both in mice and zebrafish. Furthermore, we were able to associate changes in 6mA levels with motor impairments of rats whose mothers were exposed to pollutants during pregnancy (chapter 5). This allow us to conclude that 6-methyladenine might play a crucial role during early life and might contribute to deviations to normal development. Our study also demonstrates that this modification is widely present in the brain, in both rodents and humans.

Analyzing our data and putting into perspective with the current knowledge on ELA and its consequences, we hypothesize that one of the mechanisms underlying such changes might be DNA methylation. Modifications at the DNA level were already proved to interfere with gene expression, both suppressing and upregulating (Razin and Cedar 1991, Moore, Le et al. 2013). Levels of 6-methyladenine were recently shown to be increased after predator odor exposure in early life, specifically in the amygdala (Kigar, Chang et al. 2017), a brain region responsible for the detection and response to emotional and physical stressors (Ressler 2010). Furthermore, exposure to restraint stress in adulthood also led to the increase of 6mA, which interestingly led to changes in the expression of genes linked to depression, schizophrenia and autism (Yao, Cheng et al. 2017). Although in our study we did not assess the DNA methylation levels, we were able to induce an anxious-like behavior in the animals. Changes observed in the cerebellum together with results from gene expression lead us to believe that changes in DNA, through methylation at the sixth position of adenine, might be triggering the gene expression that later leads to behavioral and brain functional changes.

When being affected by stress, the body responds as a whole and not only through a specific organ. Interplay between the different systems plays an important role and since the 1970s that it is known that the immune system can impact the HPA axis and vice versa. As we already discarded the impairment of the HPA axis, we hypothesize that changes seen in the immune system might be due to the interaction of immune cells with neurotransmitter receptors (Hadden Jw Fau - Hadden, Hadden Em Fau - Middleton et al. 1970). For instance, exposure to pollutants during pregnancy, which on its own already induces a strong immune activation,(Bauer, Diaz-Sanchez et al. 2012, Glencross, Ho et al. 2020) has been linked to an increased risk of developing autism and schizophrenia (Antonsen, Mok et al. 2020, Chun, Leung et al. 2020, McGuinn, Windham et al. 2020). In addition, a recent study identified different immune cells subsets at the boundaries of the brain. Such cells impact the brain by secreting cytokines and modeling brain cells' response. Amongst the identified populations are NK cells, in particular a subset expressing high levels of the CD27 marker. A part from this marker these cells also expressed CD62L, which is strongly associated with a more mature and cytotoxic phenotype, strongly suggesting a high level of involvement of NK cells in autoimmune pathologies (Korin, Ben-Shaanan et al. 2017). Mature NK cells found in both our studies might be interfering with the brain leading to the observed phenotypes.

Changes in the immune system and the brain can also derive from alterations in the gut microbiota. The microbiome research has significantly increased in the past decades and has been the center of attention of different research areas, from neuroscience, to endocrinology and oncology. This "organ" composed of trillions of bacteria has been associated with the development of several diseases as well as immune resistance (Wu and Wu 2012, Hsiao, McBride et al. 2013, Sampson, Debelius et al. 2016, Valles-Colomer, Falony et al. 2019, Zheng, Liwinski et al. 2020). Although specific mechanisms of action are yet to be known, dysbiosis of commensal bacteria has been appointed as a key factor in the development and homeostasis of the immune system, which can consequently induce neuronal disorders. Gut microbiota has been proved to produce metabolites that act as activators of specific immune cell population

(Hooper, Littman Dr Fau - Macpherson et al. 2012, Sharon, Sampson et al. 2016). Furthermore, studies have shown that the microbiota influences the function of microglia with germ-free animals (with no microbiota) having an increased number of immature microglial cells that in turns downregulates the activation of the immune system and might contribute to the development of diseases (Ma, Xing et al. 2019). In addition, studies with GF mice also provided important information on the link between hippocampal neurogenesis and microbiota, as a combination of probiotic bacterial strains given to GF animals noticeably prevented chronic stress induced brain damage and decreased neurogenesis (Ait-Belgnaoui, Colom A Fau - Braniste et al. 2014).

A clear mechanism underlying changes in the immune and neuronal system after early life adversity is yet to be demonstrated but bringing the microbiome as an extra variable into the equation could help developing theories and later on specific targets to help diminish ELA-induced diseases.

Future perspectives

Data from this thesis, together with previous findings from EpiPath, helped providing more insights on the consequences of ELA and possible underlying mechanisms. We were able to answer some of the questions raised from EpiPath but also raise some interesting research questions. One important point raised in the clinical study was the need to dissect the different aspects of ELA in humans by distinguishing the different types of stress. In this study, we focused on maternal separation as an early life stress and provide sufficient data to incite the conception of new clinical trial addressing this stress in particular.

We were able to partially discard the HPA axis as a moderator of changes that followed maternal separation/adoption. Although we provide evidence for the non-action of the stress system in both EpiPath and MetCOEPs study, we can never fully discard the HPA axis and GR activity upon exposure to stress.

Secondly, we were able to expand the previous findings on the ELA-induced immune phenotype. Dysfunction of NK cells, such as increased maturation and decreased cytotoxicity can affect the response of the innate

immune system to a virus of bacteria, and is often observed in individuals with autoimmune diseases (Schleinitz, Vély et al. 2010, Fogel, Yokoyama et al. 2013). Furthermore, in severe diseases provoked by virus, such as the SARS-CoV2 that emerged in china at the end of 2019 and rapidly became a pandemic, an impairment of NK cells has been proved to impede a proper response (Maucourant, Filipovic et al. 2020). In addition, a role for our early life surrounding environment and exposure to stress in the severity of COVID-19 pandemic has been recently addressed (Holuka, Merz et al. 2020).

In our study, CMV infection as a pre-requisite for immunosenescence was also discarded, raising the question on the mechanism behind such biological process. DNA methylation was initially proposed and proved in this study to be present during early development and to dynamically change upon exposure to stress. In addition, we also propose a role for the microbiome even though the sequence of events is yet to be defined: is maternal separation firstly affecting the immune system that in turns induces functional changes in the brain, through the microbiome? Or, is the brain the first system to be affected and the one leading to long-term changes in the immune system?

New research questions

How are NK cells changing over time after exposure to stress?

Is 6mA playing a key role in the development of ELA-induced phenotypes?

What is the role of the microbiome in Early life stress?

What is the sequence of events: brain to immune system or immune system to brain?

Chapter VII: Supplementary data

My contribution to this chapter:
Literature research and partial writing of the manuscript
Manuscript published in the *Int. Journal of Molecular Sciences*, July 2020

7. THE COVID-19 PANDEMIC: DOES OUR EARLY LIFE ENVIRONMENT, LIFE TRAJECTORY AND SOCIOECONOMIC STATUS DETERMINE DISEASE SUSCEPTIBILITY AND SEVERITY?

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7.1 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 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 recognise 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

7.2 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 March 11, 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 foetal 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.

7.3 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 focussed 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 neighbourhoods [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 labourers [23]. The link between the incidence of COVID-19 and lower income neighbourhoods 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 neighbourhood influence the morbidity of SARS-CoV-2 infection and COVID disease rather than the mortality rate.

7.4 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 28). 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.

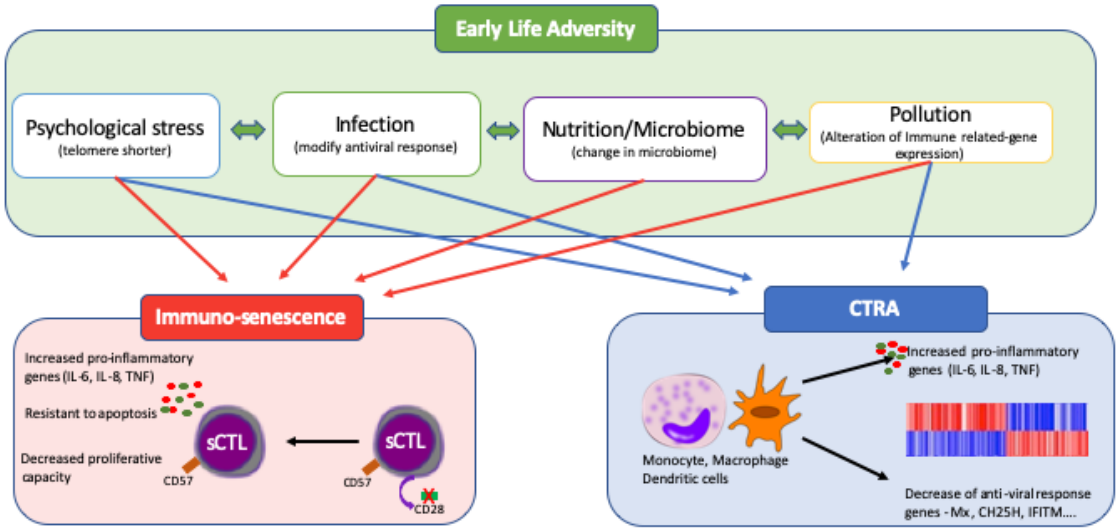

Figure 30 - Immune adaptation mediated by early life adversity. ELA is broken down into its' four key components: psychosocial stress, infectious stress, nutrition and the microbiome; and pollutant exposure. They are linked to increases in the numbers of senescent cytotoxic lymphocyte (sCTL) which, upon 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).

6.4.1. 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 institutionalisation 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 signalling and the peripheral HPA-axis stress system were not epigenetically programmed [40], implying that the immune system was directly impacted.

6.4.2. 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 tumour 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 institutionalisation [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. But the overlap does not end there: sickness, in humans and animals, also changes their social behaviour. Well known behavioural changes include a decrease in activity and expanded sleeping periods [50]. Therefore, social behaviour 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 faeces [58, 59], it will interact, affect, and be affected by the microbiome. Indeed, diarrhoea is now recognised by the Centres 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,

recognise host-specific commensal bacteria derived antigens[65], and results 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 foetal reprogramming 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 ⌈m; PM2.5) and neurodevelopmental (ADHD, Autism) /neurodegenerative (Parkinson, Alzheimer) [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, focussing 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 ⌈g 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 ⌈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.

7.5 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 ionising 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 hyperglycaemia-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 glycaemic control [95]. As such, glycaemic 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 aetiopathological 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 80657 adults that had been exposed to ELA and 161314 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 aetiopathology 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.

7.6 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-©)

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 neutralising antibodies which block viral entry into the host cell by binding to viral surface proteins such as the envelope or capsid protein (Figure 29). When the subsequent cell-mediated immunity enters into force, it is principally CD8+ cytotoxic T lymphocytes (CTLs) that are the effector cells. CTLs recognise 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.

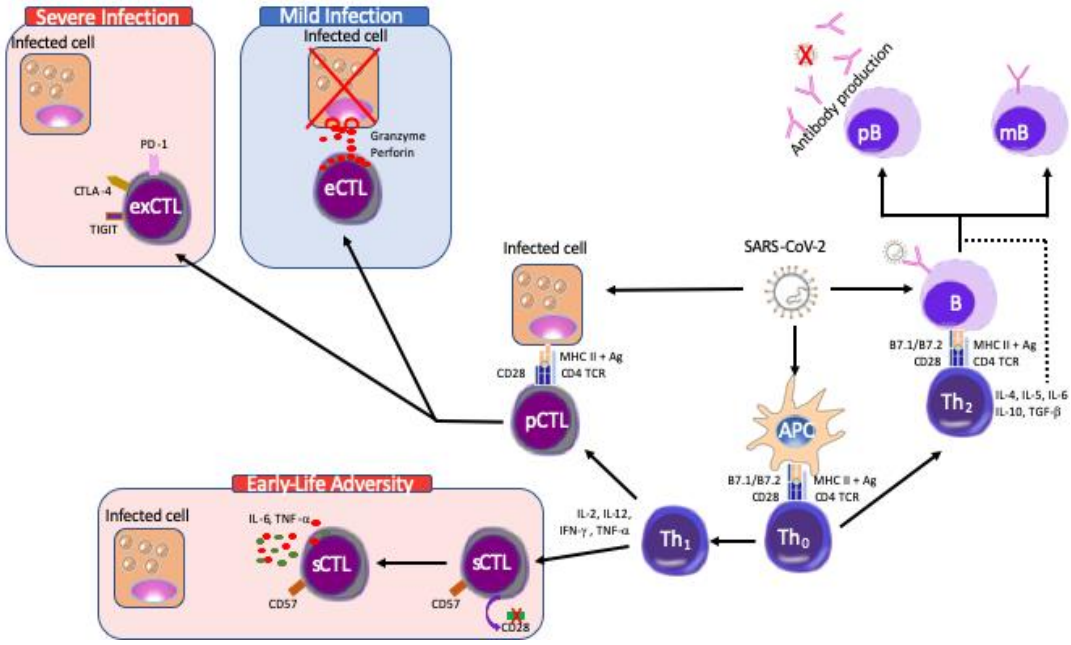

Figure 31 - Imune reaction to coronavirus disease (COVID-19). The adaptive response to SARSCoV-2 is a classical anti-viral response. On the right side, once recognized by antigen presenting cell (APC), Th2 response is activated and induced maturation of B cell. After maturation precursor B cell produces 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 rig

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., analysed 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% (day8) 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 29). 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 29). 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 programmed 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- ® 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 hypothesise 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 remodelling 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 behaviours (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 behaviours 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 hospitalisation 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 favourable 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 lymphopaenia 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 coomorbidities 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 respiratiory tract infections (ARTIs) in low socio-economic characterised 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.

7.7 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 programming 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 foetal 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 programming 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 programming 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 foetus [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 jeopardising 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.

7.8 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 behaviours 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 analysed 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 analysed concurrently with biological material to tease out the effects of the environment in health and disease.

7.9 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 recognise 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.

7.10 Author Contributions

Conceptualisation. JDT, CH. Literature review, all authors; Writing - original draft, all authors; Writing – Review & Editing, all authors.

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7.13 Conflicts of Interest

The authors declare no conflict of interest.

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