Phenotype and Mechanisms of Altered Immune Functions induced by Early Life Adversity

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Abbreviations

ANOVA	Analysis of Variance
ACE	Adverse Childhood Experiences
ACME	Average Causal Mediation Effect
ADE	Average Direct Effect
ASPA	Aspartoacylase
AUC	Area Under the Curve
AUCi	Area Under the Curve increase
BDI	Beck Depression Inventory
BMI	Body Mass Index
CHE	Childhood experience
CI	Confidence Interval
CIEC	Clinical and Epidemiological Investigation Centre
CM	Central memory
CMV	Cytomegalovirus
CNER	National Research Ethics Committee
CNT	Control
CpG	Cytosine nucleotide followed by a guanine nucleotide
CPT	Cold Pressor Test
CRP	C-reactive protein
CTI	Childhood Trauma Index
CTL	Cytotoxic T Lymphocyte
CTQ	Childhood Trauma Questionnaire
Ctrl	Control
DFG	German Research Foundation
EBV	Epstein-Barr Virus
ECG	Electrocardiography
EDTA	Ethylenediaminetetraacetic acid
ELA	Early Life Adversity
EM	Effector Memory
ERP	Ethics Review Panel

FACES	Family Adaptability and Cohesion Evaluation Scales
FNR	Fonds Nationale de Recherche
GC	Glucocorticoid
GR	Glucocorticoid Receptor
GraB	Granzyme B
HLA-DR	Human Leukocyte Antigen - antigen D Related
HPA	Hypothalamic-Pituitary-Adrenal
HR	Heart rate
HSV-1	Herpes Simplex Virus type 1
IFNγ	Interferon gamma
IgE	Immunoglobulin E
IL	Interleukin
IPAQ	International Physical Activity Questionnaire
IQR	Interquartile range
ISI	Inter-Stimulus Interval
ITGA2B	Integrin alpha 2b
LASSO	Least Absolute Shrinkage and Selection Operator
LIH	Luxembourg Institute of Health
M	Mean
MAP	Mean Arterial Pressure
METs	Metabolic Equivalent of Task units
MFI	Median Fluorescent Intensity
NA	Negative Affect
NK cell	Natural Killer cell
PA	Positive Affect
PANAS	Positive Affect and Negative Affect Schedule
PASAT	Paced Auditory Serial Addition Task
PBI	Parental Bonding Instrument
PBMCs	Peripheral Blood Mononuclear Cells
PDE4C	Phosphodiesterase
PTSD	Post-Traumatic Stress Disorder
SCID 1	Structured Clinical Interview for DSM-IV Axis I Disorders
SCID 2	Structured Clinical Interview for DSM-IV Axis II Disorders

SD	Standard Deviation
SECPT	Socially Evaluated Cold-Pressor Test
SEM	Standard error of the mean
SES	Socioeconomic Status
SIgA	Secretory Immunoglobulin A
SLST	Standardized Laboratory Stress Test
T/S	Relative Telomere to Single copy gene ratio
TEMRA	Terminally differentiated Effector Memory
Th cell	T helper cell.
TICS	Trier Inventory for Chronic Stress
TLR	Toll-Like Receptor
TNFα	Tumor Necrosis Factor alpha
TSST	Trier Social Stress Test
VAS	Visual Analogue Scales
WW2	World War 2

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General abstract

Early life adversity (ELA) is associated with a higher risk for diseases in adulthood. Changes in the immune system have been proposed to underlie this association. Although higher levels of inflammation and immunosenescence have been reported, data on cell-specific immune effects are largely absent. In addition, stress systems and health behaviors are altered in ELA, which may contribute to the generation of the 'ELA immune phenotype'. In this thesis, we have investigated the ELA immune phenotype on a cellular level and whether this is an indirect consequence of changes in behavior or stress reactivity.

To address these questions the EpiPath cohort was established, consisting of 115 young adults with or without ELA. ELA participants had experienced separation from their parents in early childhood and were subsequently adopted, which is a standard model for ELA, whereas control participants grew up with their biological parents. At a first visit, blood samples were taken for analysis of epigenetic markers and immune parameters. A selection of the cohort underwent a standardized laboratory stress test (SLST). Endocrine, immune, and cardiovascular parameters were assessed at several time points before and after stress. At a second visit, participants underwent structural clinical interviews and filled out psychological questionnaires.

We observed a higher number of activated T cells in ELA, measured by HLA-DR and CD25 expression. Neither cortisol levels nor health-risk behaviors explained the observed group differences. Besides a trend towards higher numbers of CCR4+CXCR3-CCR6+ CD4 T cells in ELA, relative numbers of immune cell subsets in circulation were similar between groups. No difference was observed in telomere length or in methylation levels of age-related CpGs in whole blood. However, we found a higher expression of senescence markers (CD57) on T cells in ELA. In addition, these cells had an increased cytolytic potential. A mediation analysis demonstrated that cytomegalovirus infection – an important driving force of immunosenescence – largely accounted for elevated CD57 expression. The psychological investigations revealed that after adoption, family conditions appeared to have been similar to the controls. However,

ELA participants scored higher on a depression index, chronic stress, and lower on self-esteem. Psychological, endocrine, and cardiovascular parameters significantly responded to the SLST, but were largely similar between the two groups. Only in a smaller subset of groups matched for gender, BMI, and age, the cortisol response seemed to be blunted in ELA participants. Although we found small differences in the methylation level of the GR promoter, GR sensitivity and mRNA expression levels GR as well as expression of the GR target genes FKBP5 and GILZ were similar between groups.

Taken together, our data suggest an elevated state of immune activation in ELA, in which particularly T cells are affected. Furthermore, we found higher levels of T cells immunosenescence in ELA. Our data suggest that ELA may increase the risk of cytomegalovirus infection in early childhood, thereby mediating the effect of ELA on T cell specific immunosenescence. Importantly, we found no evidence of HPA dysregulation in participants exposed to ELA in the EpiPath cohort. Thus, the observed immune phenotype does not seem to be secondary to alterations in the stress system or health-risk behaviors, but rather a primary effect of early life programming on immune cells. Longitudinal studies will be necessary to further dissect cause from effect in the development of the ELA immune phenotype.

1 General introduction

My contribution to this chapter:

Writing of the manuscript. Literature research and analysis of available studies.

1.1 Introduction

Prevalence of early life adversity (ELA) is astonishingly high. In 2010, the World Health Organization estimated that 39% of the global population were exposed to one or more childhood adversities. This problem is not limited to underdeveloped countries: ELA prevalence is similar in high, middle, and low income countries (Kessler et al., 2010). ELA is an overarching term for a multitude of adverse experiences in early life, ranging from parental separation and childhood maltreatment to low socioeconomic status (SES), each of which have been linked to increased risk for mental and physical diseases. Several large cohort studies have investigated these associations and found increased risks for chronic back pain, cardiovascular diseases, type 2 diabetes, allergies and asthma, autoimmune diseases such as multiple sclerosis and rheumatoid arthritis, migraine, obesity, psychiatric disorders (depression), substance use disorders, and personality disorders (e.g. Anda et al., 2008; Eriksson et al., 2014; Gern et al., 2009; Spitzer et al., 2012; Tomasdottir et al., 2015). These associations appear to follow a dose-response relationship: the greater the adversity, the higher the risk for diseases (Anda et al., 2010). ELA also appears to be linked to a worse outlook after disease onset, as ELA was associated with reduced treatment response in multiple sclerosis patients (Spitzer et al., 2012), increased symptom severity in fibromyalgia (Loevinger et al., 2012), poorer prognosis in breast cancer patients (Witek Janusek et al., 2013), and premature mortality (Brown et al., 2009).

There may be a unifying role for the immune system in the etiology of these multifactorial diseases associated with ELA. The immune system can be divided into the innate and adaptive immune systems. Innate immunity forms the first line of defense against infections, is more evolutionary conserved, and responds to unspecific molecular patterns of pathogens. The adaptive immune system, on the other hand, is composed of highly specialized B and T cells that are vital for building immunological long-term memory against specific pathogens. These two immune systems are intricately intertwined. The innate immune response is critical for the initiation of the adaptive immune response, whereas the adaptive immune system helps the innate immune cells to clear pathogens by labeling them. In addition, there is a delay of a

few days in the response of the adaptive immune system to an infection; the innate immune system plays a crucial role in filling this time gap. It is now becoming clear that there is an ELA-associated immune phenotype affecting specific functions of both innate and adaptive immunity. ELA shapes health at an early age when the foundations are laid for specific diseases such as allergic sensitizations, which develop between birth and age 8 (Lendor et al., 2008; Rowe et al., 2007). However, ELA does not appear to affect all elements of the immune system to the same extent and the molecular mechanisms underlying the development of this phenotype are unknown.

Individuals with a history of ELA have an altered stress response (e.g. Lovallo et al., 2012; Schwaiger et al., 2016) and engage in more risky health behaviors, such as smoking, obesity, alcohol abuse, drug abuse, and sexual risk behavior (Ramiro et al., 2010), which may mediate the relationship between ELA, the immune phenotype, and disease susceptibility. For example, the relationship between ELA and risk for liver disease was reduced by 35-50% when accounting for risky health behaviors (Dong et al., 2003) and life style factors accounted for 50% of the association between ELA and white blood cell counts (Surtees et al., 2003). However, although health behaviors clearly play a role, they do not appear to fully explain the relationship between ELA and disease risk. The stress system, on the other hand, has a direct effect on immune function and may play a fundamental role in the overall ELA phenotype.

In this review, we focus on human studies investigating immune parameters in relation to post-natal adverse experiences. We describe the current understanding of the ELA immune phenotype involving persistent low-grade inflammation, accelerated immunosenescence, and possibly an impairment in cellular immunity. However, it is unclear whether the phenotype we observe is a direct consequence of early life programming of immune cells, or secondary to an altered stress response. Subsequently, we examine two hypotheses as to how the immune phenotype is generated as well as evidence supporting them.

Table 1. Early life adversity and immune cell function.

Immune measure	*Early life adversity type*	*Finding*	*Timing adversity*	*Participants*	*Reference*
NK cell cytotoxicity	Adverse life events	Higher number of lifetime adverse events were associated with low NK cell activity (r=-0.28), independent of diagnosis, SES, and cortisol levels.	Prior to assessment	Adolescent patients with major depression n=20 or conduct disorder n=17, controls n=20 (age 11-18)	Birmaher et al., 1994
	Family stress	NK cell activity was enhanced in children whose parents reported more chronic stress. Family stress also predicted higher rates of illnesses, esp. febrile illnesses, in children.	Current adversity (assessed 4 times over 2 years)	n=169 children (age 5-10)	Wyman et al., 2007
	Childhood trauma (CTQ)	Emotional neglect/abuse correlated with lower NK cell activity	Prior to age of 18	n=40 women with early stage breast cancer (mean age 55)	Witek Janusek et al., 2013
Stimulated IFNγ, T cell activation, NK cell activity	Childhood sexual abuse	Stimulated IFNγ was lower in PTSD group compared to controls. Patients with present PTSD and repeated abuse had lower CD3(+)HLA-DR(+) counts than one-time abused participants. No difference in NK cell activity.	Prior to assessment	Adolescents with present or lifetime PTSD associated with childhood sexual abuse (n=33) and controls (n=10) (age 13-18)	Ayaydin et al., 2016
IgE expression, proliferation and cytokine response	Parental (caregiver) stress	Higher early life chronic caregiver stress was associated with higher total IgE levels, enhanced proliferation response, higher TNFα production, and lower IFNγ production.	During the first 2 years of life (assessed in 2-month intervals)	n=215 children predisposed to atopy (age 18-32 months)	Wright et al., 2004
IgE levels, eosinophil counts and IL-4 production	Low family support	Children and adolescents with lower levels of family support showed higher IgE levels, eosinophil counts, and IL-4 production.	Current adversity	n=78 children and adolescents with asthma (age 9-18)	Chen et al., 2007
Stimulated IL-5 and IFNγ	Low SES	Higher stimulated IL-5 and IFNγ in the low SES group.	Current adversity	Adolescents with persistent asthma with low (n=18) or high (n=12) SES (age 13-18)	Chen et al., 2003
sIgA levels	Childhood physical or sexual abuse	Childhood abuse indirectly predicted lower sIgA levels, mediated by more adult sexual victimization experiences.	Prior to the age of 14	n=89 adults young women (mean age 19.2)	Waldron et al., 2016
Bacterial killing of granulocytes	Parental separation or	20% lower *ex vivo* bacterial killing of *Staphylococcus aureus* in children with separated or divorced parents, independent of	Prior to assessment	Depressed (n=11) and control (n=11) children (mean age 10)	Bartlett et al., 1997

	divorce	depression.			
T cell activation (CD45RA expression)	Sexual abuse	Higher percentage of activated effector T cells, CD8(+)CD45RA(+), in women with ELA and PTSD compared to women without PTSD (with or without ELA), independent of depression and urinary norepinephrine or cortisol levels.	Prior to age of 18	n=24 abused women (of which 11 with PTSD) and n=12 controls (age 18-40)	Lemieux et al., 2008
T cell proliferation	Childhood trauma (CTQ)	Blunted T cell proliferation (*ex vivo* stimulation) associated with depression, however, this association was not modified by ELA.	Prior to age of 18	n=53 women with and without depression or early life adversity (age 22-55)	Lopes et al., 2012
IL-6 production of PBMCs to TLR3 and TLR5 stimulation	Low childhood SES (parental occupation)	Adults raised in low SES produced 35% more IL-6 in response to flagellin and 51% more in response to Poly I:C, independent of current SES, lifestyle practices, and perceived stress.	During first 5 years of life	Healthy adults with low (n=53) and high (n=50) SES in early life (age 25-40)	Miller et al., 2009
IL-6 production of PBMCs to TLR4 stimulation	Harsh family environment	Harsh family environment was a significant predictor for increased IL-6 production and decreased cortisol sensitivity over time, independent of depression symptoms.	Prior to study entry (4 visits over 1.5 years)	n=135 female adolescents with high risk for affective disorders (age 15-19)	Miller and Chen, 2010
IL-6 production of monocytes to TLR4 stimulation	Early life adversity	Increased odds for girls with ELA to develop proinflammatory phenotype: increased IL-6 responses and decreased GC sensitivity, independent of ongoing social stress.	Prior to 15 (6 visits over 2.5 years)	n=147 female adolescents with high risk for affective disorders (age 15-19)	Ehrlich et al., 2016

Abbreviations: NK cell, Natural Killer cell; SES, socioeconomic status; IgE, Immunoglobulin E; sIgA, Secretory Immunoglobulin A Levels; CTQ, Childhood Trauma Questionnaire; IFNγ, Interferon gamma; PTSD, Post-Traumatic Stress Disorder; TNFα, Tumor Necrosis Factor alpha; ELA, Early Life Adversity; IL-6, Interleukin 6; TLR, Toll-like receptor; GC, Glucocorticoid.

1.2 Immune Phenotype of Early Life Adversity

1.2.1 Innate immunity and inflammation

Activation of innate immune cells – e.g. neutrophils, monocytes, macrophages, dendritic cells, and Natural Killer (NK) cells – initiates an inflammatory response, characterized by dilatation of blood vessels, increased blood flow, tissue infiltration of immune cells, and the production of pro-inflammatory markers. Although inflammation is crucial for effective clearance of an infection and tissue repair, an unresolved or overactive inflammatory response leads to chronic low-grade inflammation (Lawrence and Gilroy, 2007).

One of the most robust findings on long-term ELA effects is the association with higher levels of typical markers of inflammation, such as white blood cell count, circulating proinflammatory cytokine levels, and the acute phase molecule C-Reactive Protein (CRP). In a large population-based sample (EPIC-Norfolk) of almost 12,000 people an association was found between increased white blood cell counts and adverse experiences in childhood (Surtees et al., 2003). A meta-analysis of the available studies found an association between trauma exposure and markers of inflammation: Interleukin (IL)-1β, IL-6, Tumor Necrosis Factor alpha (TNFα), and CRP (mean Spearman Rank-order Coefficient = 0.2998, 0.3067, 0.2890, and 0.2455, respectively), but not with IL-2, IL-4, IL-8, IL-10, or fibrinogen (Tursich et al., 2014). However, this meta-analysis examined trauma exposure in general, not at specific ages such as during early life. Baumeister et al. (2015) confirmed these results in a recent meta-analysis focusing on childhood trauma. They observed a similar association of childhood adversity with higher levels of IL-6, TNFα, and CRP. The effect size was largest for TNFα, although all effect sizes were small (Fisher's z [confidence interval]; IL-6: 0.08 [0.03–0.14]; TNFα: 0.23, [0.14–0.32]; CRP: 0.10 [0.05–0.14]). Allostatic load, a measure for general disease risk that combines markers for inflammation, neuroendocrine and metabolic dysregulation, was also found to correlate with ELA in several large population based studies (Carroll et al., 2013; Friedman et al., 2015), although one study only found the association in men (Dich et al., 2015).

Hyper-responsive immune cells may contribute to an increased inflammatory status in individuals with ELA, as many inflammation markers are produced by cells of the immune system. Indeed, three studies found exaggerated IL-6 responses to *ex vivo* stimulation of Toll-Like Receptors 3, 4 and 5 in adolescents raised in harsh family environments (Miller and Chen, 2010), in adults raised in low SES (Miller et al., 2009), and in adolescent girls with ELA (Ehrlich et al., 2016) (Table 1). IL-6 triggers CRP release in hepatocytes, thus, increased IL-6 production may explain elevated CRP levels observed in ELA. Immune cells in ELA may not only be more reactive, they may be in a constant state of low-grade activation. This is supported by a study where spontaneous (non-stimulated) production of pro-inflammatory cytokines in isolated immune cells was higher in women with a history of childhood maltreatment (Boeck et al., 2016). Although these studies attribute the increased production of IL-6 primarily to monocytes, the contribution of other cytokine producing cell types, such as endothelial cells or other immune cells, have not been studied.

Data on innate cell function after ELA are scarce. Granulocyte function, measured by *ex vivo* killing of *Staphylococcus aureus,* was reduced by 20% in children with separated or divorced parents (Bartlett et al., 1997), concordant with an increased susceptibility to Streptococcal infections in children exposed to acute or chronic family stress (MEYER and HAGGERTY, 1962) (Table 1). Low NK cell activity was associated with childhood trauma in both adolescents with depression or conduct disorder (Birmaher et al., 1994) and in adult women in early stage of breast cancer (Witek Janusek et al., 2013). In contrast, Wyman et al. (2007) found *enhanced* NK cell activity in children whose parents reported more chronic stress. Family stress also correlated to higher rates of febrile childhood illnesses, although this was not related to NK cell activity (Wyman et al., 2007) (Table 1). At first sight, the latter findings seem to contradict the other two studies, but these may be explained by important differences in the study populations. Wyman et al. (2007) studied a much younger population where adversity was still present at time of immune assessment, and acute stress has been shown to enhance NK cell activity in young subjects (Naliboff et al., 1991). Finally, Ayaydin

et al. (2016) found no difference in NK cell activity between sexually abused and control adolescents, although their participant numbers were low (Ayaydin et al., 2016).

These data suggest that granulocyte and NK cell functions are inhibited, whereas monocytes may be more reactive in ELA. There is convincing evidence for enhanced levels of inflammation markers in ELA, but the sparse data available on functional differences in specific cell types do not provide a clear overall picture of ELA-associated effects on innate immunity.

1.2.2 Adaptive immunity

Adaptive immunity comprises both humoral and cellular immune functions. Humoral immunity is centered around the production of Immunoglobulins (Ig) including the germinal center reaction, Ig class switching, memory B-cell formation and is associated with activation of T-helper 2 (Th2) cells and production of Th2 cytokines. Ig produced by B cells, mainly protects against extracellular pathogens such as bacteria (IgA, IgG, IgM), however, IgE is involved in atopy, allergy, and asthma and binds allergens. Cellular immunity implies phagocytosis (neutrophils, monocytes, macrophages, mast cells, and dendritic cells) as well as antigen-specific cytotoxic (CD8+) T-lymphocytes, and the release of T-helper 1 (Th1) associated cytokines in response to an antigen principally protects against infected or malignant cells.

1.2.2.1 Humoral immunity.

Data on the effects of ELA on humoral immunity in healthy individuals are scarce. A history of sexual or physical abuse was related to lower salivary IgA levels – which form the first-line of defense against pathogens in mucus – in young women, although this relationship appeared to be fully mediated through adult sexual victimization (Waldron et al., 2016). However, there are abundant epidemiological data showing an association between ELA and diseases where humoral immunity plays an important role, e.g. asthma and allergy (Chen et al., 2003; Schreier et al., 2016). Reports of severe adversity in the form of documented abuse was associated with a 73% greater risk of first hospital treatment of asthma and more frequent asthma-related hospitalizations (Graham-Bermann and Seng, 2005; Lanier et al., 2010), although

the risk was only visible from documented severe abuse rather than self-reported abuse (Scott et al., 2012). A similar doubling of the risk to develop asthma was reported for recent life physical and sexual abuse rather than abuse in the past in 5-13 year olds (Cohen et al., 2008), although Haavet et al. (2014) observed that self-reported exposure to violence increased self-reported asthma risk, but sexual violation did not (Haavet et al., 2004). Recently, Bonfim et al. (2015) reported that emotional abuse, but not physical abuse, was associated with asthma symptom intensity among 6-7 year old non-atopic, but not atopic, children (Bonfim et al., 2015). Overall, we conclude that adversity is linked to an approximate doubling of the risk to develop asthma, although so far, no clear pattern as to the exact type and timing of adversity has emerged.

In allergic and asthmatic patients, the immune system is skewed towards a Th2 response. IgE production is also under the direct control of Th2 cytokines such as IL-4, while IL-4 together with IL-13 induces Ig class switching from IgM to IgE. Additionally, IL-4 and IL-5 are mast cell and eosinophil growth and activation factors, respectively (Weltman and Karim, 2000). IgE triggers effector cells, mostly mast cells and basophils, to release histamine, leading to clinical symptoms such as asthma and anaphylaxis (Galli and Tsai, 2012; Metcalfe et al., 2016; White, 1990). As such, atopy, asthma and allergy are considered to be based upon a Th1/Th2 imbalance, in which the Th2 cytokines are predominant (Dave et al., 2011).

Although data on the mechanistic effects of ELA on the humoral immune system are limited, it would appear that after exposure, atopy, allergy and asthma have a similar molecular basis to that in unexposed individuals. Higher IgE levels in children predisposed to atopy have been associated with the chronic stress levels of their parents (Wright et al., 2004) (Table 1). Similarly, asthmatic children and adolescents reporting low family support showed higher IgE levels and IL-4 production (Chen et al., 2007; Chen et al., 2006), although no difference was found in Th1/Th2 balance in adolescent asthma patients living in low SES neighborhoods (Chen et al., 2003). Low SES, chronic family and household stress was also associated with a pro-inflammatory profile and higher eosinophil counts in asthma (Chen et al., 2007; Chen et al., 2003; Chen et al., 2006), and an increased Th2 cytokine production (Marin et al., 2009). There is also preliminary data

available that suggests intergenerational effects may occur. Although many studies have focused on current family SES, the risk of asthma in children whose parents grew up in low SES conditions, independent of the current family SES, is higher. These children had hyper-reactive PBMCs with significantly higher Th1 and Th2 responses to in-vitro polyclonal stimulation (Chen et al., 2017). Overall, it would appear that atopy, allergy and asthma have a similar phenotype, but an increased incidence and potentially symptom severity after ELA.

We must, however, interpret literature on ELA and SES with some caution. In early life, both ELA and low SES expose individuals to a significantly increased risk of infection. Early life infection is strongly associated with subsequent childhood asthma and allergy (reviewed in Beigelman and Bacharier, 2016). Hospitalization due to respiratory syncytial virus (RSV) bronchiolitis was associated with a four-fold increase asthma at age 6 (21% vs 5% of controls), as well as abnormal pulmonary function (Zomer-Kooijker et al., 2014). Similarly, human rhinovirus (HRV) infection severity associated with an increased risk of developing asthma (Carroll et al., 2009; Escobar et al., 2013) that is maintained until adulthood (Sigurs et al., 2010). In addition, children affected by ELA in low SES environments are more likely to experience high exposure to strong allergenic sensitizers, such as cockroaches, house dust mites and mold, a circumstance which multiplies together with the underlying stress situation in order to promote the genesis of allergic disorders (Gaffin and Phipatanakul, 2009; Krieger et al., 2002).

1.2.2.2 Cellular immunity.

T cells are the major cell type in cellular immunity, which main function is to protect against infected or malignant cells. They develop and mature in the thymus, which is largest at birth and during the first years of life, but shrinks significantly during adolescence. ELA has a detrimental effect on the thymus. Thymus involution was accelerated in severely abused or neglected young children, and the degree of involution correlated with maltreatment duration. In the most severe cases of thymus involution, this was accompanied by atrophy of both the spleen and lymph nodes (Fukunaga et al., 1992). It is therefore not

surprising that ELA has a lifelong impact on cellular immunity, although the exact mechanisms remain obscure.

As cellular immunity is essential for defense against intracellular pathogens and malignancies, an impaired cellular immune response renders an individual more susceptible to viral infections and certain types of cancer. Indeed, women sexually abused in childhood had a higher risk of bacterial vaginosis during pregnancy (Cammack et al., 2011). Similarly, several forms of childhood maltreatment were associated with sexually transmitted infections in a large cohort of almost nine thousand young adults, taking sexual risk behaviors into account. However, this association was only found in women and was less clear for men (Haydon et al., 2011). Finally, basal cell carcinoma patients that suffered from recent life stress in combination with a history of emotional maltreatment had lower immune cell infiltration of the tumor, reflecting a poorer antitumor immune response (Fagundes et al., 2012).

When the cellular immunity fails to keep latent viral infections in check, viral proteins are detected by the humoral immune system, eliciting an antibody response. Consequently, higher antibody levels to common viruses have been used as a proxy for an impaired cellular immunity. Epstein-Barr virus (EBV), Cytomegalovirus (CMV), and Herpes Simplex Virus (HSV) 1 antibody titers have been associated with ELA, including family dysfunction (Fagundes et al., 2013a; Janicki-Deverts et al., 2014), childhood institutionalization, and physical abuse (Shirtcliff et al., 2009). Although often an association was found only in specific subgroups: e.g. EBV titers after traumatic life events in girls only (McDade et al., 2000), CMV titers and early life poverty in an older subgroup only (age 11-16) (Dowd et al., 2012), and EBV titers and physical abuse only when it took place between age 3-5 compared to adolescence (Slopen et al., 2013a). Notwithstanding, these associations hold true for both children where adversity is still present and populations examined many years after ELA exposure. Thus, childhood adversity induces an immediate and long-lasting impairment of latent viral control (Table 2).

While these studies only provide indirect evidence of altered cellular immunity; direct evidence is still largely absent. The effects of ELA on the functionality of CD4(+) and CD8(+) T cells and NK cells have received very little attention. The available data suggests that the number of active T cells correlated with Post-Traumatic Stress Disorder (PTSD) symptoms in women with a history of sexual abuse and current PTSD (Lemieux et al., 2008), although Lopes et al. (2012) found no effect of childhood trauma on T cell proliferation in depressed participants (Lopes et al., 2012) (Table 1). Thus, there is no clear evidence that impaired cellular immunity is a direct effect of altered T cell function.

Table 2. Early life adversity and cellular immunity.

Virus	*Early life adversity type*	*Finding*	*Timing adversity*	*Participants*	*Reference*
EBV	Traumatic life events	Association between EBV levels and traumatic life events in girls: r=0.31 (increased to r=0.45 in girls with high life strain); none in boys: r=0.007.	At any time before assessment	Community-based sample of n=205 EBV(+) children and adolescents (age 9-13)	McDade et al., 2000
EBV	Socioeconomic disadvantage and abuse	Lower parental education and occupational status, but not family income, was associated with EBV levels. Any abuse before 18 was not associated with EBV levels. Association between EBV levels and severe sexual abuse (>10 times) vs no sexual abuse*. Among physical abused: higher EBV levels when abuse took place between 3-5 years of age vs adolescence.	Prior to age 18	Nationally representative sample of n=13,162 EBV(+/-) young adults (age 24-32)	Slopen et al., 2013a
EBV and CMV	Family dysfunction	Positive association between childhood adversities and EBV and CMV levels (correlations r=0.22 and r=0.25).	Prior to age 17	n=104 EBV(+) and n=56 CMV(+) adult breast cancer survivors (mean age 52)	Fagundes et al., 2013a
CMV	Family poverty	Association between family poverty and CMV titers in older subgroup only (age 11-16). Regression coefficient=0.168.	At time of assessment	Nationally representative sample of n=2,226 CMV(+) children and adolescents (age 6-16)	Dowd et al., 2012
CMV	Family dysfunction	All family environment variables, except parental divorce, were associated with CMV titers. Low family warmth was the best predictor for high CMV titers.	Prior to age 18	n=53 CMV(+) healthy adults (age 18-55)	Janicki-Deverts et al., 2014
HSV-1	Industrialization or physical abuse	50% and 20%, resp., higher HSV-1 levels compared to controls. HSV-1(-) participants not excluded from initial analysis, but controlling for serostatus did not affect results.	<age 3, current (< age 11), resp.	HSV-1(+/-) n=41 post-institutionalized, n=34 physically abused, and n=80 control children and adolescents (age 9-14).	Shirtcliff et al., 2009

Abbreviations: EBV, Epstein-Barr virus; CMV, Cytomegalovirus; HSV-1, Herpes simplex virus type 1.

* When the same data set was re-analyzed, excluding seronegative participants from analysis, the association between a history of sexual abuse that occurred >10 times and EBV titers was lost. This means that this association was mainly caused by the difference in serostatus in certain groups, not by difference in titers among seropositive individuals (Slopen et al., 2013b).

Table 3. Early life adversity and telomere length and attrition.

	Early life adversity type	*Finding*	*Timing adversity*	*Participants*	*Reference*
Telomere length in leukocytes	Childhood adverse events	Shorter telomere length was associated with a greater number of reported childhood adverse life events among both anxiety disorder patients and controls (p= 0.005).	Prior to age 16	n=974 adults with or without anxiety disorder (age 30-87)	Kananen et al., 2010
	Childhood maltreatment (CTQ)	Telomeres were shorter in maltreatment group. Analysis of subscales showed an association between shorter telomeres and physical and emotional neglect.	"in childhood"	n=31 adults (age 18-64)	Tyrka et al., 2010
	Childhood trauma (life stressor checklist)	Childhood trauma exposure was linearly associated with shorter telomeres among both PTSD patients and controls (rpartial= −.27, p= 0.005). Childhood trauma seemed to account for the shorter telomeres observed in the PTSD group.	Prior to age 14	n=90 adults with or without PTSD (age 21-49)	O'Donovan et al., 2011
	Childhood difficulties	Childhood difficulties were associated with shorter telomeres in a dose-dependent manner, independent of measures of social adversity or emotional health.	Prior to age 17	n=4,441 women (age 41-80)	Surtees et al., 2011
	Temporary separation from both parents during WW2	No association between parental separation and telomere length. However, the combination of parental separation and traumatic experiences across life span was associated with shorter telomeres.	Median age at separation 4.1	Population based sample of n=1486 older adults (mean age 61.5)	Savolainen et al., 2014
	Early life stress	No association between telomere length and early life stress (nor later life stress, between 16-25).	Prior to age 16	n=677 (age 28-30)	Jodczyk et al., 2014
	Life events or trauma	No association between childhood life events or trauma and telomere length. However, there was a significant association between recent life stress and telomere length (up to 5 years ago).	Prior to age 16	n=2,936 (age 18-65)	Verhoeven et al., 2015
	Physical and sexual child abuse	Moderate physical abuse correlated with shorter telomeres compared to no physical abuse, but no dose-response relationship was observed for increased severity of physical abuse. No associations were found for sexual abuse.	Prior to age 17	n=1,135 women in middle adulthood (mean age 45.5)	Mason et al., 2015
	Stressful events	Number of stressful events in childhood was associated with shorter telomere length (strongest for "being placed away from home"), whereas the number of stressful life events during life was not. The association was mediated by depressive mood (16%) and CRP (9%).	"in childhood"	n=324 men from Danish birth cohort (age 63)	Osler et al., 2016

	Childhood trauma (CTI)	Having experienced any childhood adverse event was weakly and negatively associated with telomere length. However, childhood abuse specifically, recent negative life events and loneliness were unrelated to telomere length.	Prior to age 16	n=496 (age 60–93)	Schaakxs et al., 2016
Telomere length in monocytes and T cells	Childhood trauma (CTQ)	Childhood trauma was associated with shorter telomeres, which could translate into a 7- to 15-year difference in life span.	Prior to age 16	n=132 healthy caregivers and non-caregivers (mean age 70)	Kiecolt-Glaser et al., 2011
Telomere length in CD8(+)CD28(-) cells	Low childhood SES	Fewer years of parental home ownership (low SES) was associated with shorter telomeres in CD8(+)CD28(-) cells, and predicted infection and symptom development when exposed to a cold virus.	Prior to age 18	n=135 healthy adults (age 18-55)	Cohen et al., 2013
Telomere length in saliva (mix of leukocytes, buccal cells, epithelial cells)	Financial and social adversities in childhood	Psychosocial problems – but not financial problems –during childhood predicted telomere length. Adverse lifetime experiences predicted lower telomere length, but this association was most strongly driven by adverse childhood experiences.	Prior to age 18	n=4,598 US retirement study (age >50)	Puterman et al., 2016
Telomere length and mitochondrial DNA copy number in leukocytes	Parental loss and maltreatment	Childhood maltreatment and parental loss (and lifetime psychopathology) was associated with shorter telomeres and higher mitochondrial DNA copy number.	Prior to age 18	n=290 healthy adults (age 18-61) with and without psychopathology	Tyrka et al., 2016
Telomerase activity and telomere length in leukocytes	Parental death/separation, (mental) illness in family	Men with shorter telomeres and high telomerase activity showed greater early life adversity, a blunted neuroendocrine stress recovery and reduced stress reactivity. However, this effect was not evident in women.	Prior to age 16	n=333 healthy older adults (age 54-76)	Zalli et al., 2014
Telomere length and attrition rate in leukocytes	Adverse life events	No association between adverse life events in childhood or during lifetime and telomere length or attrition (after 4 and 6 years). However, recent adverse life events predicted telomere attrition.	Prior to age 12	n=1,094 adults (age 33-79)	Van Ockenburg et al., 2015
	Childhood trauma (CTI)	High childhood trauma index correlated with telomere attrition rate in 6 years, but not telomere length.	Prior to age 16	Community sample of n=2,936 adults (baseline), n=1,860 (6y follow up) (age 18-65)	Revesz et al., 2016

Abbreviations: CTQ, Childhood Trauma Questionnaire; SES, socioeconomic status; PTSD, Post-Traumatic Stress Disorder; WW2, World War 2; CTI, Childhood Trauma Index.

1.2.3 Immunosenescence

Recent findings suggest that ELA accelerates immunosenescence, the aging of the immune system. As we age, immune function declines, similar to what is observed in ELA. The most commonly used marker for immunosenescence is telomere length. Telomeres, tandem TTAGGG repeats at chromosome ends, shorten with every cell division and chronological age. Although 64-70% of the variance in telomere length could be explained by genetic factors, 22% appeared to be determined by environmental factors (Hjelmborg et al., 2015). Since telomere attrition is fastest in early life, adverse environmental factors in childhood are thought to have a greater impact on telomere length. Of the sixteen studies available, eleven demonstrated an association between ELA and shorter telomeres (Table 3) in populations from Finland (Kananen et al., 2010), the UK (Surtees et al., 2011) and the US (Kiecolt-Glaser et al., 2011; Puterman et al., 2016; Tyrka et al., 2016; Tyrka et al., 2010). The link between ELA and shorter telomeres was found in both men and women, and remained significant after controlling for Body Mass Index (BMI), smoking, and current health status. Additionally, it was shown to be independent of recent life events or loneliness (Schaakxs et al., 2016), PTSD (O'Donovan et al., 2011), or anxiety disorder (Kananen et al., 2010). Osler et al. (2016) investigated somatic and mental health factors, life style factors, and low-grade inflammation as possible mediators (Osler et al., 2016). The strongest mediators were depressive mood, which could explain 16% of the relationship between ELA and shorter telomeres, and CRP levels (but not IL-6 or IL-10), which could explain 9%.

Telomere length varies greatly among individuals, which can partly explain inconsistent findings among large scale studies (Table 3; e.g. (Jodczyk et al., 2014; Savolainen et al., 2014). Already at birth, there is a large inter-individual variation, which makes cross-sectional studies hard to interpret. In addition, many studies have investigated telomere length in leukocytes, a heterogeneous mixture of cell types. This may also contribute to the variability of results, as leukocyte composition will differ between individuals and between samplings. However, Cohen et al. (2013) eliminated the problem of cellular heterogeneity by specifically measuring telomere length in CD8(+)CD28(-) leukocytes, and found an association between

shorter telomeres and low childhood SES, in line with the leukocyte length data (Cohen et al., 2013). Ideally, measurements of telomere length should be normalized by cell composition and telomere length at birth. The latter is difficult for obvious reasons. Longitudinal studies, where telomere length is measured at different time points, are a valuable alternative.

As telomere shortening is counteracted by telomerase, telomere attrition accelerates when there is an imbalance between telomerase activity and telomere shortening. The Dutch cohort of 2,936 participants that did not find any association between childhood adversity and telomere length (Verhoeven et al., 2015) is one of the few examples that investigated the attrition rate in relation to ELA. Six years after the first measurement, telomere length was re-measured in a subsample of 1,860 participants. A high score for childhood trauma was one of the predicting factors for a high telomere attrition rate. Other predictors were older age, not having a partner, gastrointestinal disease, long sleep, although the strongest predictor was baseline telomere length (Revesz et al., 2016). However, a second longitudinal study re-measured telomere length after 4 and 6 years, and did not find an association between childhood adverse events nor lifetime adverse events and telomere attrition rate. They did find an association between attrition and recent adverse life events (van Ockenburg et al., 2015).

A growing number of studies have shown that aging is associated with accumulating epigenetic changes (Pal and Tyler, 2016). Horvath (2013) and Hannum et al. (2013) have proposed epigenetic aging metrics using two distinct formulas and 353 and 71 CpG sites, respectively, to predict biological age (Hannum et al., 2013; Horvath, 2013). Telomere length and epigenetic aging are believed to target different pathways, as they were not correlated in large birth cohorts and independently predicted age and mortality (Marioni et al., 2016). Recent research has provided early indications that ELA may also accelerates epigenetic aging. For instance, certain perinatal exposures such as delivery by cesarean section have been found to accelerate epigenetic aging (Simpkin et al., 2016). Using both Hannum's and Horvath's metrics, Miller et al. (2015) found an acceleration of epigenetic aging in adolescents from higher SES compared to lower SES backgrounds, although this difference was only visible among individuals with higher self-control (Miller et

al., 2015). However, others found that mainly cumulative life stress, but not ELA alone, predicted accelerated epigenetic aging (Boks et al., 2015; Zannas et al., 2015).

Overall, the current literature suggests that ELA accelerates telomere attrition, and possibly, also the accumulation of age related epigenetic changes. Finally, the 'ELA immune phenotype' and the 'aged immune system' share the following common features: increased susceptibility to infections and chronic diseases such as cardiovascular disease and type 2 diabetes, low-grade inflammation, thymic involution, and decreased NK cell cytotoxicity (Bauer and Fuente Mde, 2016). Together these data support the hypothesis that immunosenescence is accelerated in ELA.

1.2.4 Revisiting Th1 vs Th2 – ELA timing and regulatory cell subtypes?

The development of the immune system starts in-utero and continues to mature after birth and through early childhood (Marques et al., 2013). Although outside of the scope of this review, prenatal exposure to adversity, such as maternal social of psychological stress during pregnancy, has also been linked to a similar immune phenotype, with a clearer Th2 bias (reviewed in (Entringer et al., 2012, 2015; Marques et al., 2013; Suh et al., 2017). For instance, prenatal stress lead to lower antibody titers after Hepatitis B vaccination (O'Connor et al., 2013), higher risk for infectious diseases (Nielsen et al., 2011), and increased levels of inflammation (Slopen et al., 2015). Much literature is available in the field of asthma research, demonstrating a shift in the Th1/Th2 balance towards a Th2 response (Entringer et al., 2008; O'Connor et al., 2013; Wright et al., 2010) and higher levels of IgE (Scirica et al., 2007; Sternthal et al., 2009) in individuals exposed to prenatal stress, consistent with an increased risk for asthma (Khashan et al., 2012; Lefevre et al., 2011).

The classical immune paradigm of Th1/Th2 imbalance is now being challenged with the discovery of an increasing number of T-cell subsets including regulatory T cells (Treg), Th9, Th17 and Th22 cells that play an important role in shaping and directing the subsequent T-helper response development (reviewed in (Berker et al., 2017; Landgraf-Rauf et al., 2016). Reduction of the immunological mechanisms in atopy, allergy and asthma to Th1/Th2 imbalance does not take into account these newly discovered Th cells, nor

does a shift towards a Th2 response fully explain the clinical phenotype. Regulatory T cells (Treg) expressing FoxP3 prevent allergic inflammation and regulate the Th2 response (Ohnmacht et al., 2015; Vock et al., 2010), particularly by suppressing Th1, Th2, and Th17 cell migration (Akdis and Akdis, 2009). In addition, differences in Treg cells, and their cytokine expression profiles may explain clinical differences observed in allergic and non-allergic asthma (Raedler et al., 2015). Treg cells are counterbalanced by IL-17 producing Th17 cells. Derived from common naïve T cells, Th17 cell differentiation signals inhibit Treg differentiation and vice versa. Increased IL-17 levels have been reported in plasma, lung biopsies, and bronchoalveolar lavage (BAL) from adult asthmatic patients (Chakir et al., 2003; Molet et al., 2001). There is some debate as to the importance of IL-17, as anti-IL-17 treatment has yet to give convincing results (Busse et al., 2013). In contrast, anti-IL-9 treatment (MED528) has showed promising preliminary results in allergic asthma (Holgate, 2012; Kearley et al., 2011; Parker et al., 2011). IL-9 producing Th9 cells are important for the survival of mast cells, T cell proliferation, airway remodeling, and epithelial mucus production. The number of circulating Th9 cells is higher in patients with asthma and the Th9/Th1 ratio is elevated (Hoppenot et al., 2015). Although there are no data available on the regulatory immune cells such as Treg and Th17 cells in ELA, it is interesting to hypothesize that adversity provides a first “hit”, particularly affecting these two cell populations, consequently rendering the individual more susceptible to developing an allergic phenotype.

1.2.5 ELA immune phenotype

Innate and adaptive immune changes as well as immunosenescence are not independent, but form an intertwined self-perpetuating, “vicious” circle (Figure 1). A senescent immune cell is functionally impaired, has dysfunctional mitochondrial activity and increased production of proinflammatory markers (Sahin et al., 2011), thereby producing low-grade inflammation and an impaired cellular immune response. Conversely, inflammation leads to oxidative stress, which is thought to be one of the main drivers of immunosenescence (Bauer and Fuente Mde, 2016). Indeed, Boeck et al. (2016) found an association between childhood trauma and elevated levels of oxidative stress, ROS production, and mitochondrial activity. Mitochondrial activity and ROS production, in turn, correlated with the release of proinflammatory

cytokines by isolated immune cells. On the other hand, an impaired cellular immunity – due to repeated viral infections and reactivation of latent viruses – increases inflammatory markers, such as IL-6 and CRP (Bennett et al., 2012). In addition, frequent infections, chronic inflammation, and certain latent viral infections, such as CMV (Bauer et al., 2015), accelerate immunosenescence. Interestingly, among CMV positive children (5-10 years of age) a higher number of senescent cells was associated with ELA – in this case parental psychiatric symptoms (Caserta et al., 2008). The literature does not currently enable us to distinguish causes from secondary effects, as all of these immune effects seem to be already present in childhood.

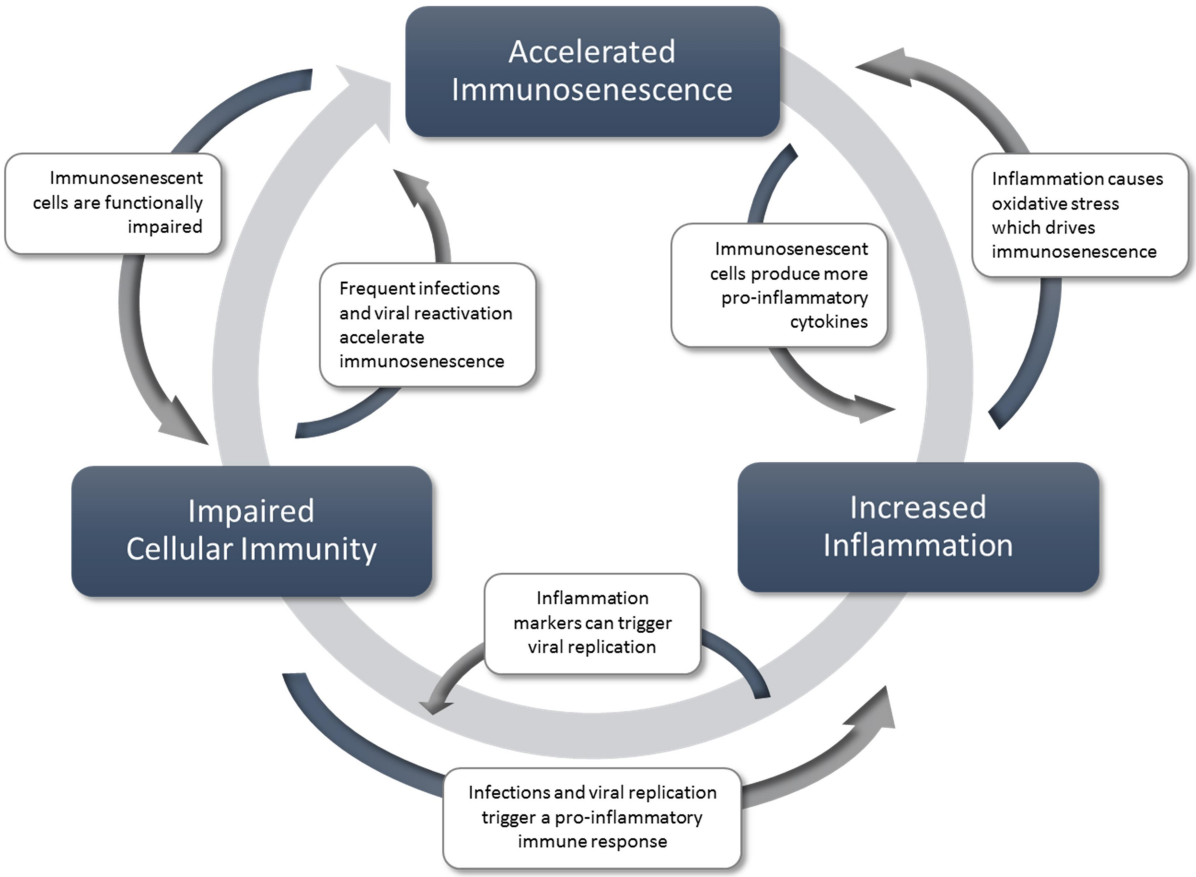

Figure 1. ELA Immune Phenotype. Accelerated immunosenescence, increased circulatory inflammation markers, and impaired cellular immunity are the three principle immune effects associated with ELA and form a self-perpetuating cycle.

1.3 Underlying mechanisms

The molecular mechanisms underlying the development of the ELA-associated immune phenotype are unknown. Here, we propose two refined hypotheses (Figure 2 and Figure 3). Both hypotheses are based on the idea that ELA causes subtle changes in set points, due to epigenetic mechanisms, inducing long-term changes in the transcriptional and proteomic landscapes (Leenen et al., 2016) and resulting in higher disease risk. We focus on DNA methylation, which is one of the most studied and best characterized epigenetic markers. The first hypothesis – based on that previously proposed by Danese et al. (2012) – states that ELA leads to genome-wide systemic epigenetic changes, which independently affects distinct physiological systems (Danese and McEwen, 2012). The second hypothesis is a refinement of that previously proposed by Wright (Wright, 2011). This hypothesis states that the ELA programs the stress response systems, indirectly affecting the immune system and health behaviors.

1.3.1 Hypothesis: Early life adversity independently programs multiple systems

In early childhood, the developing immune system is shaped or programmed by the continuous exposure to a variety of environmental factors, such as microorganisms forming the microbiota or causing infections, maternal immunomodulatory factors via breast milk (e.g. hormones and cytokines), nutrition, as well as psychosocial factors (e.g. stress) (MacGillivray and Kollmann, 2014). This hypothesis (Figure 2) is based on what was originally proposed by Danese et al. (2012), and it suggests concurrent programming of the allostatic nervous, endocrine and immune systems during sensitive periods. This leads to allostatic overload later in life, which in turn increases the risk for age-related diseases. Similarly, Miller et al., (2011) have suggested that ELA causes the immune phenotype by direct epigenetic programming of the immune system. Independently, the brain and stress systems are programmed, together leading to the overall ELA phenotype with an altered stress response, altered (health) behaviors and immune functions. They may then act either alone or in combination to increase the risk for psychiatric, cardiovascular and chronic

inflammatory disorders. There are several lines of evidence supporting this hypothesis, particularly the transcriptional profile and methylation status of immune cells.

Epigenetic mechanisms regulate transcription and it is well established that environmental factors, especially in early life, can influence epigenetic profiles in human immune cells (Leenen et al., 2016). Several studies have found links between whole genome DNA methylation profiles in immune cells and ELA (See (Vinkers et al., 2015) for a comprehensive review on epigenetic changes linked to ELA). Smith et al. (2011) could not find any differences in leukocyte DNA methylation associated to child abuse, although this may have been due to low participant numbers. Other studies have specifically investigated genes involved in the immune system or inflammation and found methylation differences. In a pilot study of 20 women with breast cancer undergoing radiotherapy, childhood trauma was associated with increased expression of genes associated with inflammatory signaling pathways (Han et al., 2015). Adolescents raised in low SES had higher TLR4 mRNA expression, potentially explaining this excessive inflammatory response (Miller and Chen, 2007). Chronic inflammatory diseases, allergies and asthma have been linked to stress and early-life epigenetic modifications (Harb and Renz, 2015). More recently, epigenetic variation, higher methylation status at the promotor of gene ADCYAP1R1 (receptor for pituitary adenylate cyclase-activating peptide) has been associated with asthma, especially in children with history

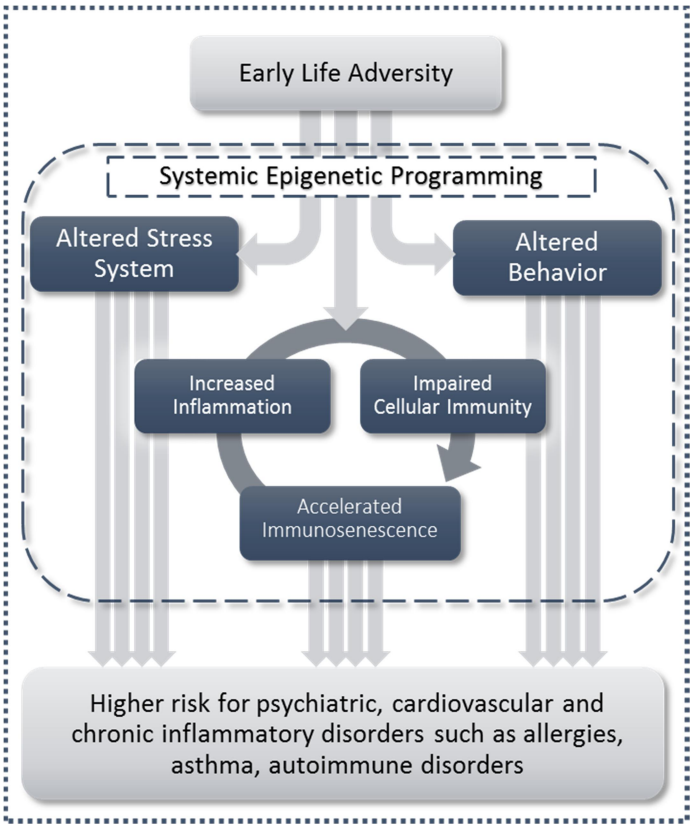

Figure 2. Hypothesis – The immune phenotype is a direct response to epigenetic programming in early life.

of family violence (Chen et al., 2013). Finally, women with PTSD related to childhood maltreatment had increased Nuclear Factor kappa-B activity in leukocytes, which is a transcription factor involved in the inflammatory response, compared to controls (Pace et al., 2012).

Social adversity has been associated with certain changes in gene transcription in immune cells, called the 'conserved transcriptional response to adversity' (CTRA) (Cole, 2014). This has now also been shown specifically for adversity in childhood, which suggests that adversity can cause long-term changes in gene expression. For instance, a similar pattern of gene expression was found in former Nepali child soldiers and this was linked to the severity of trauma (Kohrt et al., 2016). Steven W. Cole characterized this response of more than 200 different genes to adversity as "enhanced expression of proinflammatory genes and decreased expression of genes involved in innate antiviral responses and in antibody synthesis" (Cole, 2014). These are the same systems that are affected by ELA, namely, increased inflammation and impaired cellular immunity, as described in section 2. However, thus far, there is no data demonstrating a link between ELA and an altered antibody synthesis. Although higher antibody titers have been found against common latent viruses, this reflects higher viral reactivation rather than an elevated B cell response (Janicki-Deverts et al., 2014).

The effects of ELA on DNA methylation are not limited to immune genes nor to immune cells, e.g. similar methylation patterns have been found in buccal cells and hippocampal brain tissue (Vinkers et al., 2015). Equally, the effects of ELA on telomere length and attrition are not specific for immune cells. Indeed, studies using buccal cell DNA show similar findings: children (5 years of age) with greater exposure to institutional care also had shorter telomeres in buccal DNA (Drury et al., 2012); greater attrition rate was found in institutionalized children (between 6-15 years of age) (Humphreys et al., 2016) and in children (5-10 years of age) exposed to violence (Shalev et al., 2013b). The percentage of life spent in an institution was correlated with shorter buccal telomeres and unfortunately, high quality foster care could not attenuate this association (Humphreys et al., 2016). However, in a sample of indentured Swiss child laborers with traumatic experiences (with an average age of 76) no relationship was found with telomere

length (Küffer et al., 2016). The fact that changes in DNA methylation patterns and telomere shortening occur in a number of different cell types, suggests that the effects of ELA are not limited to the immune system but are rather systemic.

1.3.2 Hypothesis: Early life adversity programs the stress system, indirectly affecting immune function

This hypothesis (Figure 3), a refinement of the original proposition of Wright et al. (2011) suggests a different hierarchy. Wright et al. (2011) originally proposed a model where prenatal and postnatal exposure to stress leads to altered stress reactivity, in turn increasing the risk for asthma, atopic disorders and reduced lung function. Phenotypic differences in the stress response, particularly immuno-modulatory glucocorticoids, subsequently induce the ELA immune phenotype as well as altered health behaviors (Fagundes et al., 2013b). Indeed, epigenetic changes in the glucocorticoid receptor gene (*NR3C1)* and a co-chaperone of the glucocorticoid receptor (*FKB50*), both of which play a pivotal role in the stress response, have been associated with ELA in several human studies (See Palma-Gudiel et al., 2015 for a comprehensive review). In addition, evidence is accumulating that both stress systems – the hypothalamic-pituitary-adrenal (HPA) axis and the symphatho-adrenomedullary (SAM) system (e.g. Lovallo et al., 2012; Schwaiger et al., 2016) – of adults with a history of ELA are less responsive to acute stress. This relationship

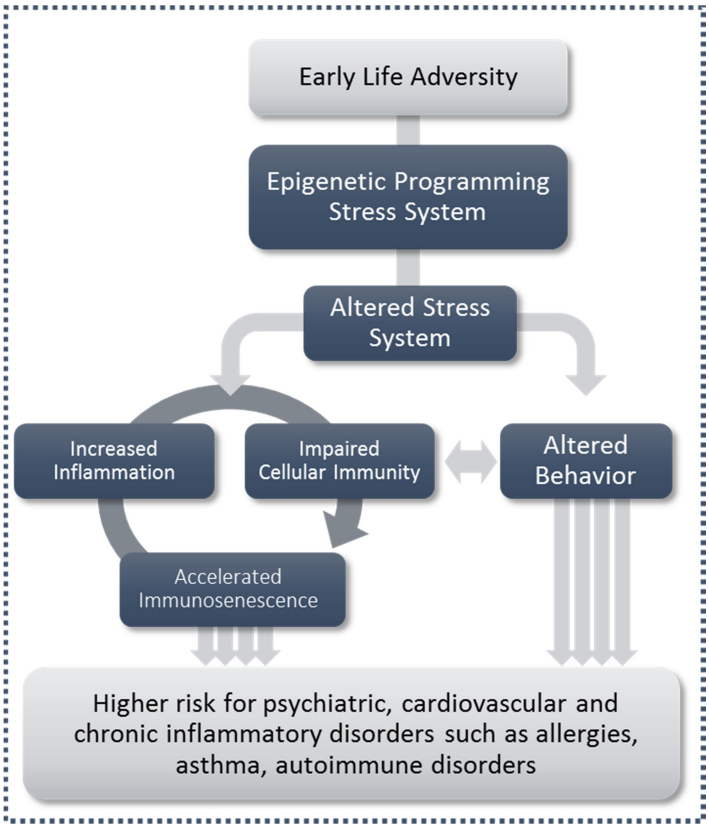

Figure 3. Hypothesis – The ELA immune phenotype is an indirect consequence of an altered stress system.

seems to be dose-dependent: the higher the number of different adverse experiences or the longer the duration of adversity, the lower the stress response (Lovallo et al., 2012). All stress signals can have a direct effect on immune function, since immune cells express receptors to stress hormones and sympathetic nerves innervate lymphoid tissue. Therefore, altered levels of stress hormones in circulation, stress reactivity, or sensitivity of the glucocorticoid receptor will change immune function.

Stress appears to be involved in all three aspects of the ELA immune phenotype. First, acute stress leads to increased inflammatory markers in the circulation, and this effect is augmented in people with a history of ELA (e.g. Carpenter et al., 2010). Lower cortisol stress levels, as observed in ELA, may explain this observation, since cortisol inhibits immune activation. Schwaiger et al. (2016) found differences in stress-induced gene regulation between ELA and controls, with increased activity of proinflammatory upstream signaling in ELA. Thus, these data support that an altered stress response will lead to higher levels of inflammatory markers in ELA.

Second, acute psychosocial stress as well as stress hormones can compromise cellular immunity. A longitudinal study of 19 seropositive women, for example, showed that genital HSV-2 lesion episodes and viral shedding were related to self-reported psychological distress (Horn et al., 2015). A meta-analysis found “a robust positive association between psychosocial stress and symptomatic HSV recurrence” (Chida and Mao, 2009). Patients suffering from Herpes Zoster (n=250), which is caused by a reactivation of Varicella-Zoster Virus, had significantly higher scores of depression and recent negative life events compared to matched controls (n=500) (Lasserre et al., 2012). Rector et al. (2014) found an association between CMV titers with higher anxiety, depression, vital exhaustion, sleep disturbances and lower mental health (Rector et al., 2014). However, parameters of inflammation (CRP) and HPA axis (cortisol awakening response and area under the curve of diurnal cortisol) did not mediate the relationship, although other unmeasured factors of the HPA or SAM axis may be involved. Additionally, glucocorticoids have been shown to directly trigger viral reactivation: *in vitro* glucocorticoid receptor stimulation reactivated latent Epstein-Barr virus (Yang et al., 2010). Consequently, the sensitivity of the glucocorticoid receptor or the

levels of circulating glucocorticoids could affect the susceptibility to viral reactivation. Thus, dysregulation of the stress system may increase the signals that trigger viral reactivation, and potentially permitting replication, even though cellular immunity is intact.

Third, shorter telomeres and reduced telomerase activity have been associated with (chronic) psychological stress, increased levels of stress hormones, and increased stress responses (for an overview see Price et al., 2013). In contrast, Tomiyama et al. (2012) found an association between higher cortisol responses to acute laboratory stressor and shorter telomeres (Tomiyama et al., 2012). Furthermore, acute stress has been shown to increase telomerase activity (Epel et al., 2010). T cells exposed to cortisol showed decreased telomerase activity (Choi et al., 2008). Long-term inhibition of telomerase activity – for instance due to elevated cortisol levels – will hasten telomere shortening. Zalli et al. (2014) investigated the interaction of telomere length, telomerase activity, ELA, and stress response. Participants were divided into three groups according to their telomere length and telomerase activity: short telomeres/high telomerase activity, short telomeres/low telomerase activity, and long telomeres. Men with short telomeres/high telomerase activity had blunted diastolic blood pressure, heart rate, and cortisol stress responses and delayed systolic BP, heart rate variability, and monocyte chemoattractant protein-1 recovery from stress, in comparison with the other groups. Interestingly, the group with short telomeres/high telomerase activity were also associated with greater ELA (Zalli et al., 2014).

Finally, this hypothesis states that an altered stress response will cause risky health behaviors. This is supported by data showing a correlation of a blunted stress response with more social and behavioral problems (Ouellet-Morin et al., 2011) and increased adiposity (Carroll et al., 2008). In addition, low heart rate reactivity to acute psychological stress increased the risk of obesity in a 5 year follow-up (Carroll et al., 2008). In turn, these health behaviors will affect the ELA immune phenotype. Smoking and alcohol abuse have been also linked to the shortening of telomeres (Morla et al., 2006; Pavanello et al., 2011). However, most studies have accounted for health behaviors and still found an association between ELA and shorter telomeres. Schrepf et al. (2014) found that the association between childhood trauma and higher

inflammation levels (CRP) was mediated by emotional eating behaviors and higher BMI. They suggested that people try to cope with stress and trauma by emotional eating, leading to higher adiposity and potentially obesity, as well as inducing low-grade inflammation. Furthermore, BMI has been associated with CMV antibody levels (Dowd et al., 2012), although other similar studies show that the association between ELA and higher antibody titers are independent of BMI (Janicki-Deverts et al., 2014).

In general, stress intervention programs have been successful in reducing inflammation and boosting the immune response. Stress management in children boosted sIgA levels (Hewson-Bower and Drummond, 1996) and reduced the duration of upper respiratory tract infections (Hewson-Bower and Drummond, 2001). In adults, cognitive behavioral stress management, Tai Chi, meditation, and yoga have also been shown to suppress CTRA gene expression profiles (Cole, 2014). Telomerase activity was increased by a 3 month intensive meditation retreat, a mindfulness-based program, and a yoga-based lifestyle intervention (Schutte and Malouff, 2014). Finally, stress reduction training was able to reduce CRP levels in adolescents placed in foster care, which is a model for ELA (Pace et al., 2013). The fact that intervention focusing on relaxation and stress reduction have such a profound effect on immune parameters – and can even partly reverse detrimental effects of ELA on the immune system – suggests that alterations in the immune system are secondary to alterations in the stress system.

1.4 Research objectives and thesis outline

This first chapter has introduced the current understanding of the ELA immune phenotype, characterized by inflammation, impaired cellular immunity, and immunosenescence. In addition, two hypotheses have been discussed of potential underlying mechanisms in which epigenetic modifications, an altered stress response, and health-risk behaviors interact with the ELA immune phenotype resulting in a higher risk for diseases (published in *Psychoneuroendocrinology*). The overall aim of this thesis was to examine the phenotype and mechanisms of altered immune functions induced by ELA in humans. Therefore, the first objective was to recruit a suitable cohort in which these questions could be addressed. The EpiPath cohort was established as part of this thesis and forms the base of the studies reported in the following chapters. Therefore, Chapter 2 is dedicated to the EpiPath cohort, giving a detailed account of the recruitment strategies applied and the EpiPath study protocol. Chapter 2 also describes the demographic characteristics of the complete cohort, as all following chapters have focused on specific subsets of the EpiPath cohort.

The second objective of this thesis was to describe cell-specific differences in the immune system associated with ELA, especially immune cell distribution and activation status using flow cytometry. These data are reported in Chapter 3, which has been submitted for publication in *The Journal of Immunology*. In response to these first results, a nested project was performed on senescence in T cells specifically. These results were submitted to *Frontiers in Immunology* and are reported in Chapter 4. In collaboration with the Institute of Health and Behavior at the University of Luxembourg, EpiPath participants were psychologically evaluated. In Chapter 5, we present these psychological data and their association with immune parameters.

The third objective of the thesis was to address possible underlying mechanisms that may explain the observed changes in the immune system. Alterations in the stress system have been proposed to mediate the effect of ELA on immune function. Therefore, a subset of the EpiPath cohort was subjected to a

standardized laboratory stress test to examine whether individuals with ELA responded differently to an acute stress. The stress test was performed in collaboration of the Department of Clinical Psychophysiology at the University of Trier, who primary focus was cardiovascular reactivity in ELA. Chapter 6 reports the cardiovascular and endocrine stress response in ELA, whereas Chapter 7 presents the response of the immune system to the stress test. Chapter 7 also presents the first epigenetic results of the EpiPath cohort: ELA-specific methylation patterns on the promotor of the glucocorticoid receptor. This manuscript is ready for submission. The final chapter, Chapter 8, is the general discussion, which seeks to place these findings in a broader context.

2 EpiPath cohort

My contribution to this chapter:

Writing of the chapter. Organizing and performing the recruitment of study participants. I was involved in setting up and coordinating visit 1, including scheduling of participants, nurses, and experimental rooms, performing the stress test, and sample collection.

2.1 Overall objectives of EpiPath

'EpiPath' is a multidisciplinary clinical research project investigating the long-term Epigenetic and Pathological effects of early life adversity (ELA). The target population consists of young adults that were adopted from an institution and/or from poor early life living conditions by families from Luxembourg and The Greater Region Saar-Lor-Lux. Using an integrative approach, this project explores the role of epigenetic mechanisms, by collecting large quantities of epigenetic, environmental, behavioral, and psychological data from these individuals, as well as functional data on the status of their immune, cardiovascular, cognitive and stress response systems. Integrating functional, behavioral, environmental, and epigenetic data will provide a broad and unique insight on the influence of ELA on the development of disorders that become manifest in adulthood.

The overarching objective of EpiPath is to understand the impact of ELA on epigenetic methylation patterns and the consequences for cardiovascular, psychological health, stress response, and immune related pathologies, four of the most important public health burdens. This thesis forms the cornerstone of EpiPath, describing the recruitment of the EpiPath cohort and reporting its first scientific output (Chapter 3-7). Future EpiPath publications will combine these first findings with the high-resolution genome-wide epigenetic methylation patterns. It is envisaged that the cohort will be maintained for future studies and for following up on the observations made in this project.

2.2 Recruitment

The EpiPath cohort was recruited using two concurrent recruitment strategies (Figure 4). The general or broad approach aimed at keeping the public informed about our research, increasing awareness and participation among both control as well as ELA individuals. In addition, a targeted strategy was developed to maximize the outreach to ELA individuals specifically. These strategies were approved by the National Research Ethics Committee (CNER) and the Ethics Review Panel (ERP, University of Luxembourg).

2.2.1 Recruitment: general approach

To inform the general public about the relevance of EpiPath, several articles were published during the time of recruitment: "Early damages, late consequences" (*Frühe Schädigungen, späte Folgen*, Luxemburger Wort, 2014), "EpiPath: From epigenetics to Pathology" (*Epipath: de l'épigénétique à la pathologie*, Semper, 2014) "Epigenetics: How childhood experiences influence our health" (*L'épigénétique: Comment les experiences de l'enfance influencent notre santé*, Semper, 2014), and "Is stress during childhood bad for your adult health?" (Science.lu, 2016). Flyers and summaries of the study were published on all collaborators' websites, including Luxembourg Institute of Health (LIH), Trier University, University of Luxembourg, and LuxClin (Luxembourg Clinical Investigations Network, platform dedicated to clinical research in Luxembourg). EpiPath staff promoted EpiPath at events such as the *Open Doors event of House of BioHealth* (LIH), *Medical Research Day Luxembourg*, and other science events for the general public. Flyers were distributed in more than one hundred waiting rooms of hospitals and general practitioners.

Additional recruitment efforts focused specifically on young adults, as EpiPath's target age group was 18-35 years of age. For example, over 200 letters were send to recent high school graduates from *L' Athénée de Luxembourg*. EpiPath flyers were regularly posted and shared on social media and on Universities' websites in Luxembourg, Saarland and Trier. Lastly, each EpiPath participant received a small number of flyers to hand out to friends and colleagues. With these broader strategies, we recruited 25 adoptees and 73 control participants (Figure 4).

2.2.2 Recruitment: targeted approach

Adoptions in Luxembourg are under the supervision of the "Central Authority for adoptions" (Autorité Centrale des Adoptions) of the Ministry of Family and Integration (Ministère de la Famille et de l' Intégration). This Central Authority accredits non-Governmental Organizations (NGOs) that are involved in adoptions. Most of the NGOs are specialized according to countries or geographic regions. With the help of these organizations, letters were send to parents that adopted children during the 1980s or 1990s, in which we explained the objective and the implications of the study. The parents were contacted in monthly batches to reduce the time delay and potential dropout between the initial contact, recruitment, and the on-site visits. The parents were asked to talk to the adoptees to encourage their participation. If they agreed, the EpiPath recruiter contacted the adoptees.

The following organizations offered their help in the EpiPath recruitment:

- *Roumänesch Kanner an Noud*, active during the early nineties assisting in adoptions from Romania, provided contact details of 95 families.
- *Amicale Internationale d'Aide a l'Enfence asbl* (AIEAE) was active during the required time period, with ministerial accreditation from 1993 onwards, assisting in adoptions from South Korea and India. AIEAE could not provide us with contact details directly, but forwarded our letter to 200 families.
- *Association Luxembourg-Pérou* was created in 1991, still active in assisting in adoptions from Peru, provided contact details of 100 families.
- *Hand an Hand asbl* provided contact details of 70 families.

Four-hundred-sixty-five letters were send out; however, the response was limited. Subsequently, all adoptive parents were contacted by telephone of whom telephone numbers were publicly available (n=130), using a script that was drafted in collaboration with experienced psychologists of the University of Luxembourg. In total, 52% of parents contacted by telephone were interested in the study and happy to cooperate, of which 39% provided contact details of their children at the initial phone call, whereas others

asked for time to discuss with spouse and/or children. Despite the overall awareness and understanding of the relevance of EpiPath, 48% of parents contacted by telephone were unable to help. The predominant reasons being (1) loss of contact with adoptive children, (2) the topic of adoption was too emotionally charged between parents and children to address, or (3) the children were not interested in participating. Nonetheless, with this targeted approach we successfully recruited 17 additional adoptees adding up to a total of 42 adoptees (Figure 4).

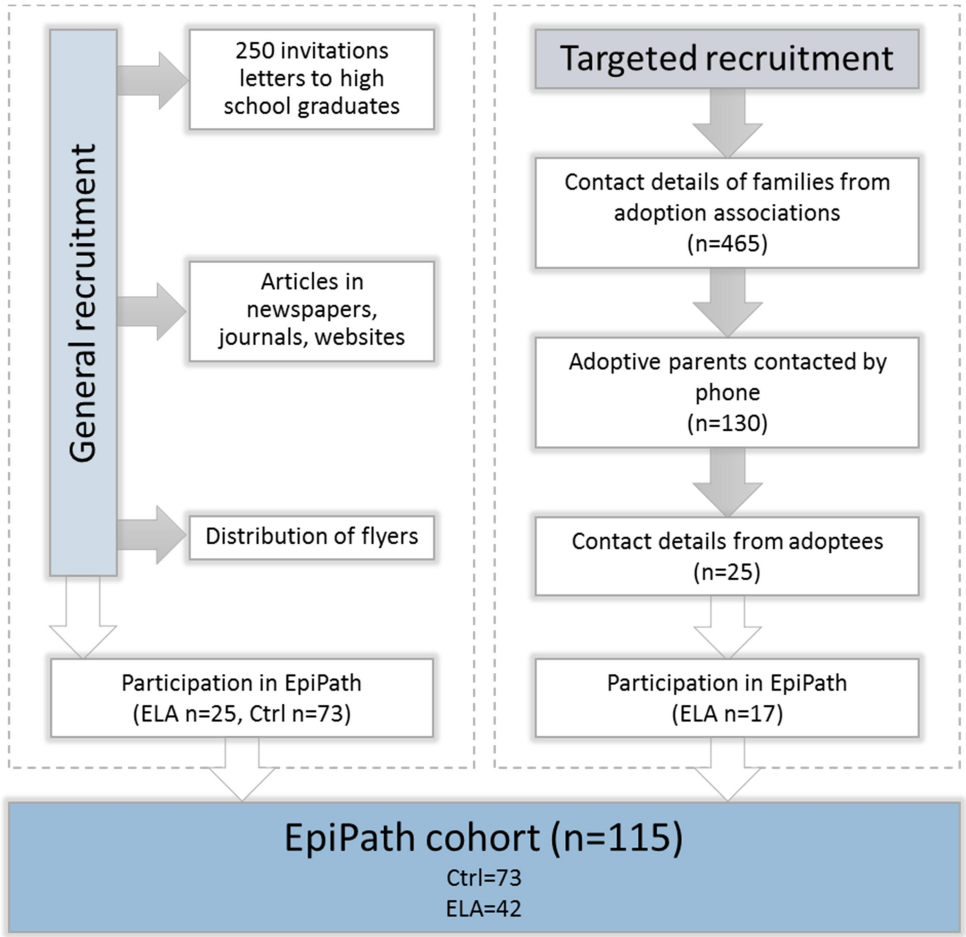

Figure 4: Recruitment strategies. The EpiPath cohort was recruited through concurrent general and targeted strategies.

2.2.3 Inclusion and exclusion criteria

Individuals expressing interest in participating in EpiPath received an elaborate description of the study by email and were subsequently screened on inclusion and exclusion criteria per telephone. To have an

optimal response to the SLST participants were recruited between 18 and 35 years old. Current psychotic mental health problems that would put participants at risk from suffering adverse consequences of taking part in the study (e.g. psychotic disorders) were formal exclusion criteria, in addition to health conditions that were not compatible with the examinations. After appointments were scheduled, each participant received a confirmation email and was reminded by email and telephone to minimize dropout. All 115 interested and eligible individuals were successfully included in the EpiPath cohort.

2.2.4 Demographics EpiPath cohort

The demographics of the complete EpiPath cohort are represented in Figure 5. ELA and controls were similar in their average physical activity (international physical activity questionnaire, IPAQ; Figure 5D) and hours of sleep per night (estimated by participant; Figure 5E). Sex was equally distributed between groups (Figure 5F). ELA participants were slightly, yet significantly, older than the controls (Figure 5B). As expected, the number of ELA participants with a chronic disease was higher than in controls (Friedman et al., 2015; Gilbert et al., 2015) (Figure 5G), as was the use of respective medication (Figure 5I). There was a non-significant trend towards higher prevalence of allergies (Figure 5H), higher BMI (Figure 5C), and smoking in ELA (Figure 5J). As all studies in this thesis investigated a subset of this cohort, each chapter reports the corresponding demographics.

A

	Controls (n=73) median (IQR)	ELA (n=42) median (IQR)	p-value
Age (years)	22 (21-23)	24 (20-25.5)	**0.021**
Sex (women)	57.5%	65.9%	0.501
Chronic diseases	6.3%	39.5%	**<0.001**
Allergies	24.1%	45.9%	0.051
Medication use	9.4%	36.8%	**0.002**
Smoking	14.3%	31.7%	0.052
BMI (kg/m2)	22.5 (20.6-24.3)	23.0 (21.1-26.2)	0.051
Physical activity (IPAQ, METs)	3779 (1744-5826)	3201 (1452-8633)	0.864
Average sleep per night (hours)	7.5 (7.0-8.0)	7.0 (6.3-8.0)	0.251

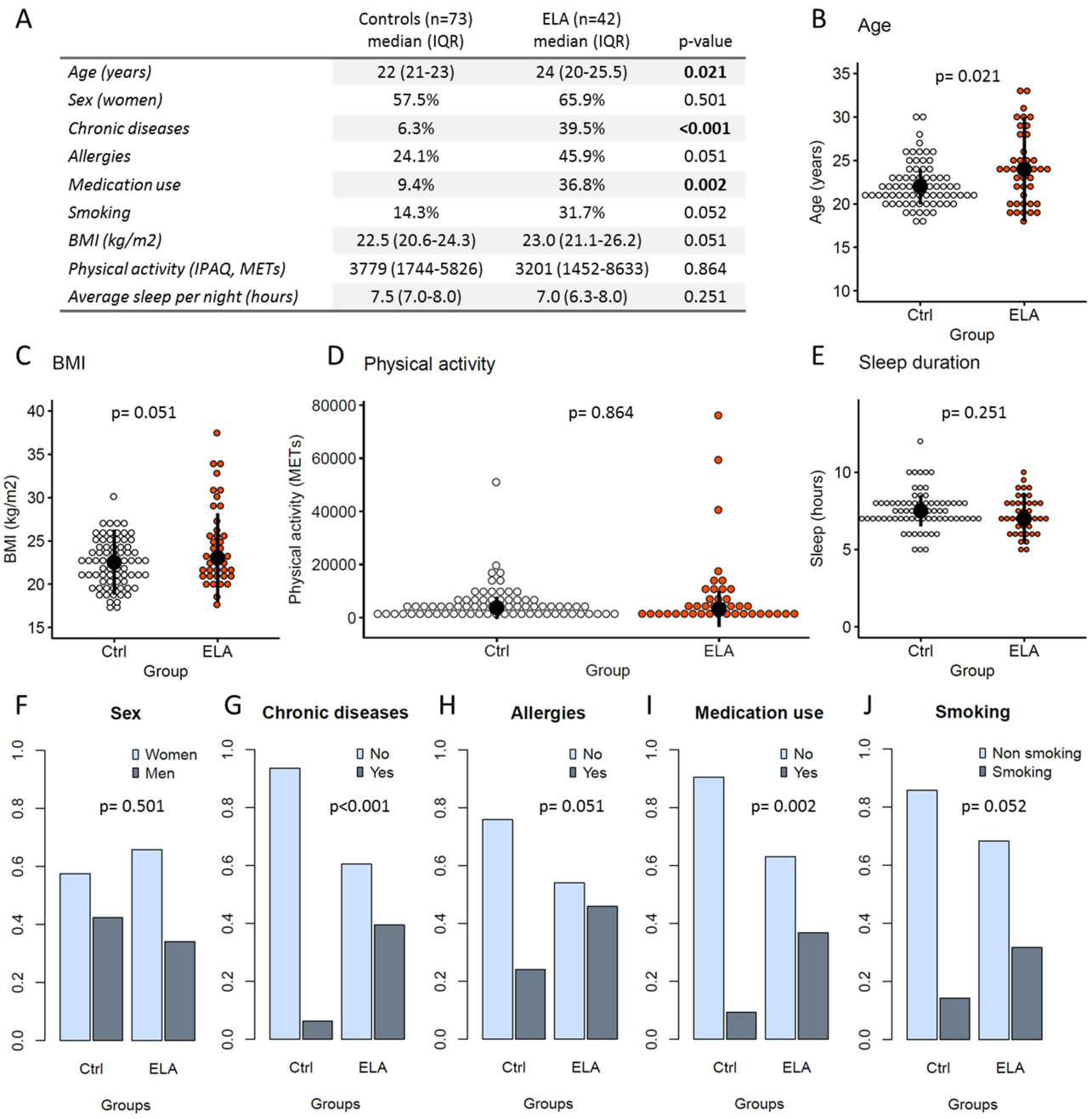

Figure 5: Demographics EpiPath cohort. A. Table of demographics. Statistics: Wilcoxon rank-sum test for numerical variables; Chi-square test for categorical variables; compared to control group; significant p-values are shown in bold. B-J. Graphical representation of EpiPath cohort demographics. B-E. Black circle represents median and error bars represent interquartile range (IQR). F-J. Relative proportions of number of participants in respective categories.

2.3 EpiPath study protocol

The recruited EpiPath cohort was invited to two clinical visits and a follow-up (Figure 7). The first visit consisted of physiological assessments, including the response to a standardized laboratory stress test (SLST). On a different day, a physiological assessment was performed during the second visit. Finally, during one year, the cohort received monthly surveys about their health. In accordance with the declaration of Helsinki, all participants provided written informed consent and the study protocol was approved by the ethics committees in Luxembourg (CNER and ERP). This thesis presents data collected at these clinical visits, but each chapter focusses on a different aspect of EpiPath using the respective data set (Figure 7 and Figure 6), e.g. ELA-associated consequences for immune function (Chapter 3 and 4), psychological health (Chapter 5), and stress response (Chapter 6 and 7). By applying distinct exclusion criteria, each study investigated a subset of the EpiPath cohort. Figure 6 visualizes the overlap between the participants that gave a blood sample for immune function and epigenetic investigations (Chapter 3, 4, 5 and 7), that underwent the psychological assessment in Visit 2 (Chapter 5), and that participated in the SLST (Chapter 6 and 7).

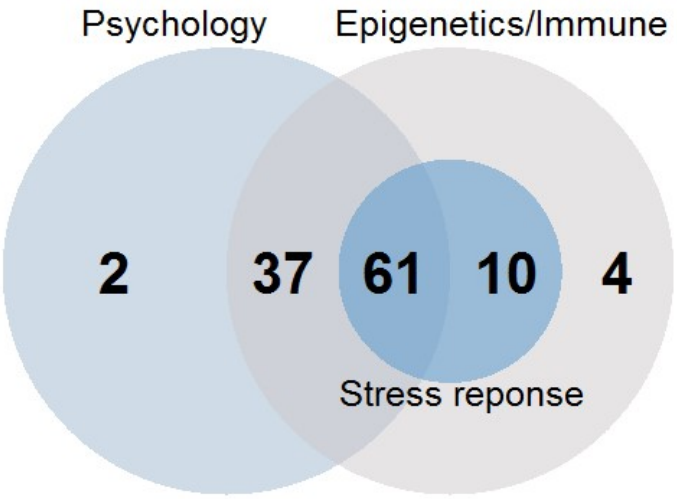

Figure 6: Overlap between subsets of the EpiPath cohort. The circles represent the participants that underwent the psychological assessment in Visit 2 (Chapter 5), that gave a blood sample for immune function and epigenetic investigations (Chapter 3, 4 and 7), and that participated in the SLST (Chapter 6 and 7), respectively.

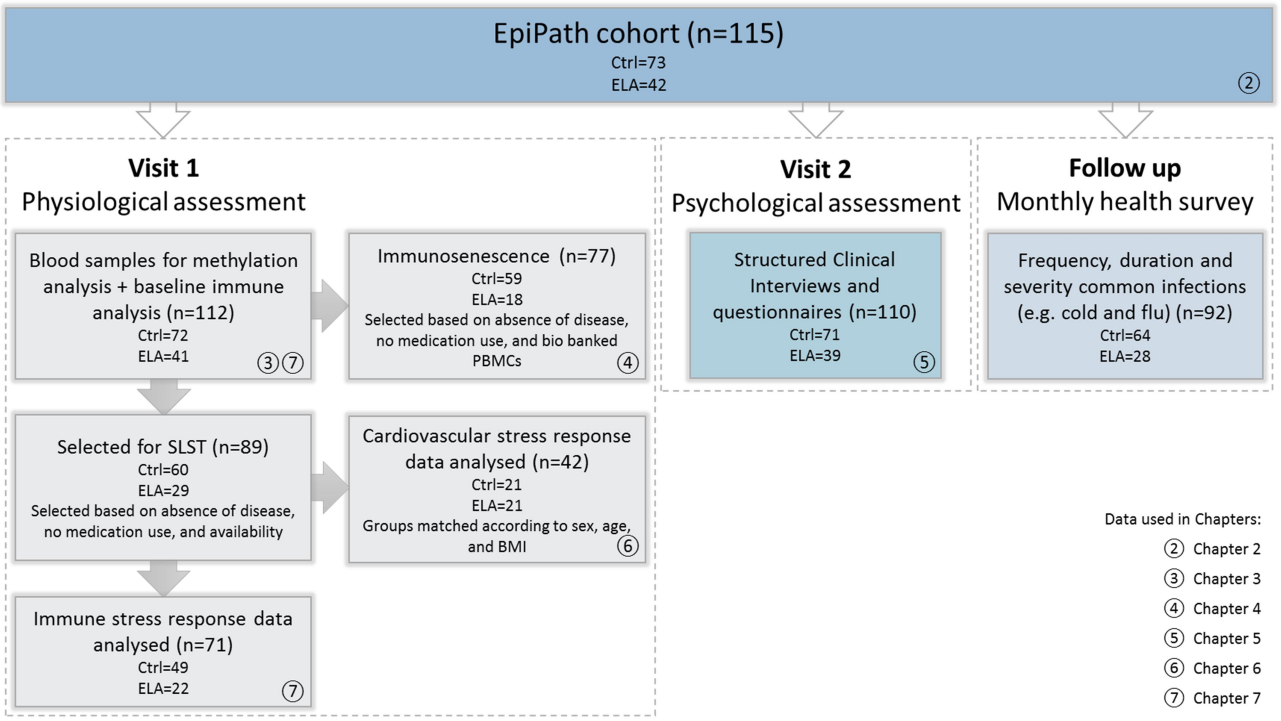

Figure 7: Overview of data generated in the different parts of the EpiPath study. The EpiPath protocol consisted of two clinical visits (visit 1: physiological assessment and visit 2: psychological assessment) and a one-year follow-up with monthly questionnaires about health. Each box represents the number of participants that were included in the respective analysis. The numbers in the right hand corners refer to the chapters in which the data is presented.

2.3.1 Visit 1

During Visit 1, a 3-minute SLST was performed, consisting of the "Paced Auditory Serial Addition Task" (PASAT) (Schachinger et al., 2003) combined with the "Socially Evaluated Cold-Pressor Test" (SECPT) (Schwabe et al., 2008) (Figure 8). The PASAT was originally developed to investigate cognitive impairment after traumatic brain injury, but more recently has been successfully used to induce psychological stress (Philippsen et al., 2007; Schwabe et al., 2007). In the PASAT performed in EpiPath, participants were presented with an audio recording of 60 one-digit numbers with a stimulus interval of 2.5 seconds. They were instructed to calculate the sum of the two last numbers heard and say the answers aloud during each interval. The responses were recorded on audiotape for post hoc analysis. At the same time, the SECPT was performed, which combines physical and psychological stress components. The cold-pressor test – the physical component of the test – is one of the most commonly used laboratory stress tests and has been

known for many decades to induce stress (Hines and Brown, 1932a). This test can be applied on any limb or even the forehead, which is immersed in ice-cold water for a few minutes. Here, participants were asked to place both feet in a cold water bath (ca. 4°C) for exactly 3 minutes. Participants were encouraged to keep their feet in the cold water for the entire test period, although they could withdraw their feet at any time if the pain was unbearable. Although the cold-pressor test reliably induces SAM responses, such as increased heart rate, blood pressure, norepinephrine, and epinephrine (al'Absi et al., 2002; Bolli et al., 1981; Robertson et al., 1979), HPA axis activation is only low to moderate (al'Absi et al., 2002; Gluck et al., 2004) or absent (Duncko et al., 2007; McRae et al., 2006). To improve the HPA axis response, Schwabe et al, (2008) added a social-evaluative element to the cold-pressor test, consisting of continuous observation by the investigator and videotaping. This SECPT reliably activated both SAM and HPA systems. In EpiPath, the social-evaluative elements were continuous observation by a researcher in a white coat instructed not to respond to the participant and audiotaping of PASAT responses through a microphone.

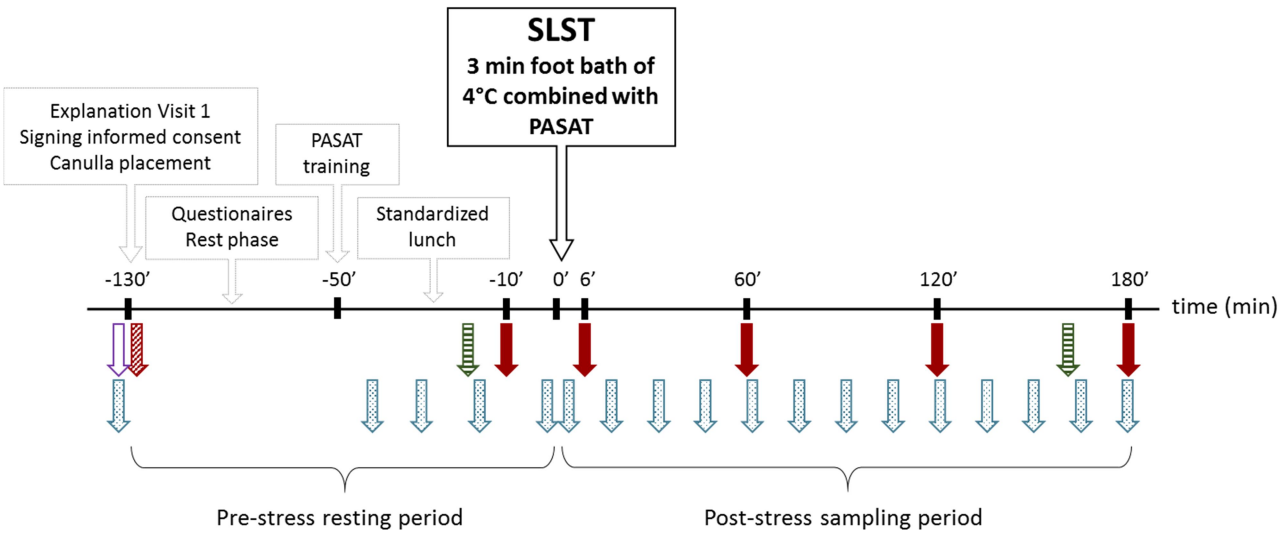

Figure 8: Study protocol Visit 1. ⇨ *Buccal swab.* ➡ *Baseline blood sample for Ex Vivo stimulation experiments, and isolation of PBMCs, RNA and DNA.* ⇨ *Urine sample.* ➡ *Blood samples for stress response of immune cells, cytokine levels, and cortisol.* ⇨ *Saliva sample for cortisol measurement. Abbreviations: PASAT, Paced Auditory Serial Addition Task; SLST, standardized laboratory stress test.*

Visit 1 took place at the Clinical and Epidemiological Investigation Center (CIEC, Luxembourg Institute of Health), which offers an infrastructure to prepare and perform clinical research projects in accordance with Good Clinical Practice (ICH-GCP) and Quality Assurance. In collaboration with the University of Trier, a detailed minute-to-minute protocol was developed. Staff performing Visit 1 consisted of one researcher from the University of Trier, one researcher from LIH, and one nurse from the CIEC. The nurse was responsible for placing the cannula and all biological samples. The researcher from Trier was the main contact person to the participant during the visit and was responsible for all cardiovascular measurements (heart rate, blood pressure, and ECG) throughout the visit. The researcher from LIH was responsible for the overall coordination of the visit (booking nurses, researchers from Trier and lab technicians, reserving two experimental rooms in CIEC, making appointments with and reminding participants), and processing of biological samples on site. A fourth EpiPath staff member, a lab technician, was responsible for sample transport from the CIEC to the laboratories of LIH, and the immediate processing of baseline samples. Mock Visit 1s were used to test and optimize the Visit 1 protocol as well as train all members of the EpiPath staff.

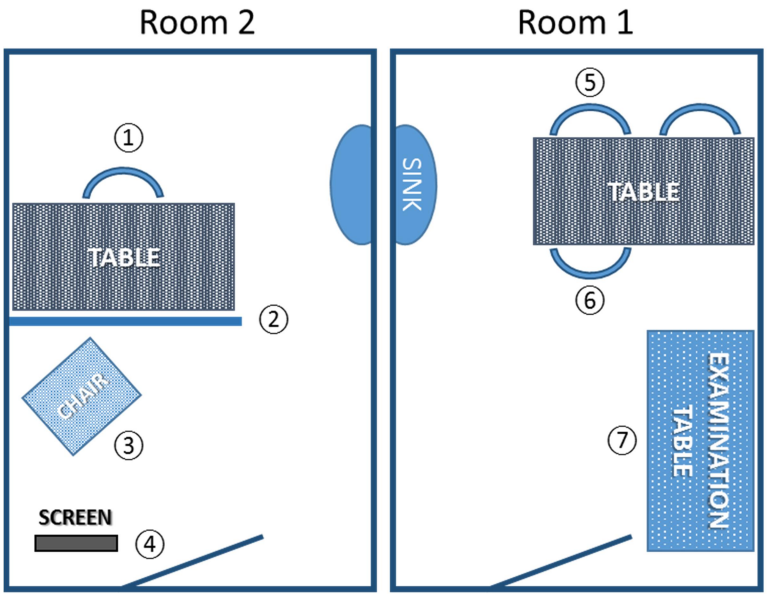

Figure 9: Experimental rooms. Two experimental rooms were used during visit 1. Room 2: ① Researcher from Trier with equipment for cardiovascular activity markers; ②Separation screen, making ① invisible for participant; ③ Participant, during PASAT training and SLST; and ④ Computer screen with PASAT instructions. Room 1: ⑤ Researcher for initial explanations. ⑥ Participant, when in Room 1. ⑦ Examination table for blood drawing.

Participants arrived at 11:00 am and were welcomed by and introduced to the EpiPath staff. In experimental Room 1 (Figure 9) the researcher from Trier explained the day and answered all the participant's questions before written informed consent was obtained. The nurse inserted an indwelling intravenous cannula and baseline blood was drawn within the first 30 min of the study. These samples, which were transported to LIH laboratories and processed immediately, were used for baseline immune measurements and epigenetic profiling (Chapter 3, 4 and 7). The participant was given a folder with questionnaires and ample time to fill them out. Participants that were offered a short visit (due to time constrains or failing to fulfil the specific inclusion criteria for the SLST) left after completion of the questionnaires and did not perform the SLST (n=18). For all other participants, electrodes were attached in a standard electrode II configuration and in thenar and hypothenar electrode positions to measure ECG and electrodermal activity, respectively, with a Biopac MP150 system (Bertsch et al., 2012; Buchholz et al., 2003; Schachinger et al., 2004; Schachinger et al., 2001). The participant was brought to Experimental Room 2 for the "PASAT training", in which the PASAT was explained and the participant could practice, to make sure the task was well understood. Back in Room 1, the participant received a standardized lunch (cheese sandwich) during a 30 min lunch break. At circa 01:00 pm the participant was brought back to Room 2, where they underwent the 3-minute SLST. Before and after the test was a 10 min rest phase, where the participant was instructed to relax without moving or talking. The remaining 3 hours of Visit 1 were spent in Room 1. At several time points before, during, and after the stress test participants' autonomic and cardiovascular activity markers, subjective ratings, and blood samples were assessed by established procedures. Cardiovascular and immune stress response data are presented in Chapter 6 and 7, respectively. Figure 8 gives an overview of all biological samples taken during Visit 1 and Table 4 shows the total of collected samples in the EpiPath study.

Table 4: Collected samples during visit 1. Saliva, plasma, RNA, blood pellets, and urine samples were collected at several time points before and after the SLST, whereas PBMCs, buccal swabs, and DNA were only collected during the first time point (baseline). Abbreviations: SLST, standardized laboratory stress test; PBMCs, peripheral blood mononuclear cells; RT, room temperature.

Samples	**Time points**	**Number of samples**	**Storage**
Saliva	18	2,000	-20°C
Plasma	7	700	-80°C
RNA	6	600	-20°C (RNA later)
PBMCs	Baseline	600	Nitrogen tank
Buccal swabs	Baseline	112	RT (stabilized DNA)
Isolated DNA (fresh blood)	Baseline	112	-20°C
Blood pellets	7	700	-20°C
Urine	2	200	-20°C

2.3.2 Visit 2

During Visit 2, a clinical psychological evaluation was performed to assess mental disorders on Axis I and II of the DSM IV-TR using a semi-structured interview (SCID-I and SCID-II). High levels of reliability and validity of both SCIDs have been reported by several studies over the last 2 decades (e.g. Lobbestael et al., 2011). The SCID yields lifetime-, period-, and point-prevalence rates for the following mental disorders: all affective disorders, all anxiety disorders, hypochondrias, somatisation disorder, substance abuse and dependence, conversion disorder and pain disorder, bulimia nervosa and anorexia nervosa (SCID-I), and personality disorders (SCID-II). The interviews were carried out by a specially trained psychologist (V. Schaan M.Sc.) under the supervision of a chartered Clinical Psychologist/Psychotherapist (Prof. Dr. Claus Vögele). These data are presented in Chapter 5.

In addition, participants filled out a range of questionnaires designed to assess ELA and related personality constructs (e.g. attachment security, rejection sensitivity, depression, self-esteem). All psychological assessments (i.e. interview, questionnaires) are routinely used in research and clinical practice and only validated German translations were used. In addition to the psychological evaluation, data was collected on the pre- and immediate post-adoption period from the participants.

2.3.3 Follow-up

After completion of Visit 1, participants were followed up for a 1-year period. Using an online self-reporting system participants reported monthly on health in general and upper respiratory tract infections in particular (symptoms, severity, duration number of working/school days lost). The LimeSurvey system (www.limesurvey.org) was used, providing secure participant restricted access to the questionnaire. To ensure compliance with the monthly reporting, participants were invited per email to complete the questionnaire at the end of each month. These data are foreseen to be published together with the whole genome methylation analysis.

3 Pro-Inflammatory T Cell Status Associated With Early Life Adversity

My contribution to this chapter:

Writing of the manuscript. Recruitment of study participants. Performing all immunological measurements, including the flow cytometry experiments. Final statistical analysis. I was involved in setting up and coordinating visit 1, including scheduling of participants, nurses, and experimental rooms, performing the stress test, and sample collection.

3.1 Abstract

Early life adversity (ELA) has been associated with an increased risk for diseases in which the immune system plays a critical role. The 'ELA immune phenotype' is characterized by inflammation, impaired cellular immunity, and immunosenescence. However, data on cell-specific immune effects are largely absent. In addition, stress systems and health behaviors are altered in ELA, which may contribute to the generation of the ELA immune phenotype. The present investigation tested cell-specific immune differences in relation to the ELA immune phenotype, altered stress parameters, and health behaviors in individuals with (ELA, n=42) and those without a history of ELA (Ctrl, n=73).

Relative number and activation status (CD25, CD69, HLA-DR, CD11a, CD11b) of monocytes, NK cells, B cells, T cells and their main subsets were assessed by flow cytometry. ELA was associated with significantly reduced numbers of CD69+CD8+ T cells ($p=0.022$), increased numbers of HLA-DR+CD4+ and HLA-DR+CD8+ T cells ($p<0.001$), as well as increased numbers of CD25+CD8+ T cells ($p=0.036$). ELA also showed a trend towards higher numbers of CCR4+CXCR3-CCR6+ CD4 T cells.

Taken together, our data suggest an elevated state of immune activation in ELA, in which particularly T cells are affected. Although several aspects of the ELA immune phenotype were related to increased activation markers, neither stress nor health-risk behaviors explained the observed group differences. Thus, the state of immune activation in ELA does not seem to be secondary to alterations in the stress system or health-risk behaviors, but rather a primary effect of early life programming on immune cells.

Keywords: Early life adversity, immune programming, HLA-DR, Th17 cells, health behaviors, stress.

3.2 Introduction

Adverse circumstances or experiences during early childhood (Early Life Adversity, ELA) have profound and lasting effects on both mental and physical health. ELA has been associated with an increased risk for diseases, such as cardiovascular diseases (Friedman et al., 2015; Korkeila et al., 2010), obstructive pulmonary disease (Anda et al., 2008), asthma (Scott et al., 2008), autoimmune diseases (Dube et al., 2009), diabetes (Eriksson et al., 2014), cancer (Brown et al., 2010; Felitti et al., 1998), arthritis (Dube et al., 2009), and mental disorders, especially depression and post-traumatic stress disorder (PTSD) (Ehlert, 2013; Kessler et al., 2010). Although these conditions differ widely in symptoms, clinical presentation, and therapy; the immune system – inflammation in particular – appears to be a common denominator (Gibney and Drexhage, 2013; Shurin and Smolkin, 2008).

Although data on the effects of ELA on specific immune cell types are scarce, there is evidence supporting an ELA-associated immune phenotype, i.e. persistent low-grade inflammation, impairment of cellular immunity, and accelerated immunosenescence (Baumeister et al., 2015; Osler et al., 2016; Revesz et al., 2016; Shirtcliff et al., 2009) reviewed in (Elwenspoek et al., 2017). The three aspects of the ELA immune phenotype form a vicious circle, and with currently available data it is not yet possible to distinguish cause from effect. Senescent immune cells produce more pro-inflammatory markers (Sahin et al., 2011), contributing to low-grade inflammation, which, in turn, accelerates immunosenescence through oxidative stress (Bauer and Fuente Mde, 2016). Likewise, impaired cellular immunity – through frequent reactivation of latent viruses and viral infections – accelerates immunosenescence (Bauer et al., 2015) and increases inflammation (Bennett et al., 2012). Finally, the immune function of aging cells deteriorates (Sahin et al., 2011), thereby contributing to an impaired cellular immune system. However, these findings on the ELA immune phenotype are not conclusive, as reports from similar ELA cohorts did not find clear associations with inflammation, impaired cellular immunity, or immunosenescence (Carpenter et al., 2012; Slopen et al., 2013a; Verhoeven et al., 2015).

The development of the ELA immune phenotype is not necessarily a direct consequence of early life programming of the immune cells. In addition to alterations in the immune system, ELA has been associated with changes in the stress system (Carpenter et al., 2007; Carpenter et al., 2011; Elzinga et al., 2008; Leitzke et al., 2015; Lovallo et al., 2012; McLaughlin et al., 2014a; McLaughlin et al., 2015; Schwaiger et al., 2016; Voellmin et al., 2015), especially the hypothalamic-pituitary-adrenal axis, and with increased health-risk behaviors (Ramiro et al., 2010). The immune and stress systems closely interact, as sympathetic nerves innervate lymphoid tissues during acute stress, and immune cells express receptors for stress hormones. Health-risk behaviors such as smoking (Shiels et al., 2014), and low physical activity (Jurdana et al., 2015), and its consequences (e.g. high BMI; Park et al., 2005) are related to low-grade inflammation. Thus, changes in both the stress system and health behaviors affect immune function and could, therefore, promote the generation of the ELA immune phenotype.

Stress hormones, but also other non-immune stimuli, may modulate immune activation markers that are typically upregulated on the surface of immune cells during infection, such as HLA-DR, CD38, CD25 and CD69. Infusion with stress hormones (epinephrine, cortisol, and glucagon) has been shown to reduce $CD25^+$ T cells by 50% (Januszkiewicz et al., 2001). Young adults with high perceived stress during an academic examination period showed increased T cell numbers expressing HLA-DR (Maes et al., 1999). Smokers had higher levels of $CD38^+HLA\text{-}DR^+$ cells compared to non-smokers (Valiathan et al., 2014). Higher numbers of $HLA\text{-}DR^+$ and $CD69^+$ cytotoxic T lymphocytes (CTLs) were found in depressed hip fracture patients compared to healthy controls (Duggal et al., 2014). To date, there is only one report of activation markers on specific cell types in relation to ELA: higher HLA-DR expression was found on T cells in children with a history of maltreatment (Bielas et al., 2012).

In the present study, our model of ELA were young adults that had experienced parental loss in early life and were subsequently adopted. Separation from parents in childhood is a strong stressor that negatively impacts lifelong health and a well-known model for ELA in animals and human (Gunnar and Quevedo, 2007; Sanchez et al., 2001). In addition, the majority of participants were institutionalized before adoption, which

is considered to be a form of social deprivation or structural neglect (van IJzendoorn et al., 2011) due to the lack of social and emotional interactions with a stable caregiver. Unfortunately, this is characteristic for even the best institutions (Gunnar et al., 2000; Gunnar et al., 2009; Gunnar et al., 2007).

Although ELA has been associated with altered immune responses later in life, little is known about the role of specific immune cell types and the activation markers that are involved in this process. As the immune system plays a critical role in ELA-associated diseases, we hypothesized that there are cell-specific differences associated with ELA in circulating immune cells as well as their activation status. We expected these differences to be related to other aspects of the ELA immune phenotype. Finally, because of the close interplay between behavior, stress and immune systems, we expected that health-risk behaviors and an altered stress system would explain at least part of our findings of cell-specific differences related to ELA.

3.3 Materials and methods

3.3.1 Participant enrollment and study protocol

Participants between 18-35 years of age with either with a history of ELA (separation from parents and subsequent adoption) or raised by their biological parents (Ctrl) were recruited between July 2014 and March 2016 from Luxembourg and The Greater Region Saar-Lor-Lux. In accordance with the declaration of Helsinki, the study protocol was approved by the National Research Ethics Committee (CNER) of Luxembourg (No 201303/10 v1.4) and the Ethics Review Panel (ERP, University of Luxembourg, No 13-002). All participants provided written informed consent. The study design consisted of two separate visits (Figure 1), one for physiological measurements and a standardized laboratory stress test, and a subsequent clinical psychological assessment. To compensate for time, effort and inconvenience participants were reimbursed up to €150.

3.3.2 Laboratory visits

First visit. To minimize variability, participants were asked to refrain from drinking caffeinated drinks (>1h) or alcohol (>24h), smoking and physical exercise on the day of the first visit. Women were either in the luteal phase of the menstrual cycle or using hormonal contraceptives. Prior to blood collection, participants completed a visual analog scale assessing perceived stress, and saliva was collected for 2 min under the tongue with a SalivaBio Oral Swab (Salimetrics, Newmarket, UK). At approximately 11:30 am an indwelling cannula was inserted and blood was drawn as a baseline measurement. Sodium Heparin anti-coagulated blood samples were transported at room temperature in the dark and were manipulated within 2 h of blood collection. For plasma collection, EDTA blood samples were centrifuged within 15 min of blood collection at 4°C, transported on ice and stored at -80°C within 6 h until further analysis. Age, sex, smoking status (yes/no), height, and weight were recorded and the International Physical Activity Questionnaire (IPAQ; Booth, 2000) administered. While the present paper reports on baseline data, a selection of

participants underwent a standardized laboratory stress test, which are reported elsewhere (Chapter 6 and 7).

Second visit. Participants filled out the Childhood Trauma Questionnaire (CTQ) (Klinitzke et al., 2012) to exclude any trauma in the control group, and answered questions about the age at adoption, if applicable. All participants were administered the German version of the Structured Clinical Interview for DSM-IV Axis I and Axis II Disorders (SCID-I and –II; Fydrich, 1996; Wittchen, 1997), as reported elsewhere (Chapter 5).

3.3.3 Ex vivo stimulation, saliva and plasma measurements

Undiluted whole blood was incubated for 19 h (37°C, 5% CO2, 95% humidity level) with phytohemagglutinin-M, pokeweed mitogen, or lipopolysaccharide from E coli 0111:B4 (2µg/mL, 0.5µg/mL, 5ng/mL, respectively; Sigma-Aldrich, Overijse, Belgium). After centrifugation, supernatants were harvested and immediately stored at -80°C until further analysis. IL-6 concentrations were determined with the Human IL-6 ELISA Set in a 1:50 dilution (BD OptEIA, Erembodegem, Belgium). Salivary cortisol levels were determined with the Salimetrics Salivary Cortisol ELISA Kit (CV: 7% intra-assay, 11% inter-assay; Salimetrics). Plasma samples were assayed undiluted to determine IL-6 levels (Human IL-6 High Sensitivity ELISA, SB Molecular Biology Reagents: Isogen Life Science, Utrecht, Netherlands), in 1:3,000 dilution for CRP levels (USB Molecular Biology Reagents: Isogen Life Science), and in 1:21 dilution for HSV-1 IgG, EBV-VCA IgG, and CMV IgG antibody indexes (Calbiotech, El Cajon, CA, USA). All ELISAs were performed in duplicate, following manufacturer's instructions, and read on a SpectraMax Plus 384 Microplate Reader (Molecular Devices, Berkshire, UK).

3.3.4 Flow cytometry

Immediately after blood collection, whole blood was stained using pre-mixed antibody panels (Table 5 and Table 6) for 15 min at 4°C in the dark. Cells were washed in 1x FACS buffer (1x Dulbecco's PBS, Lonza BioWhittaker, Verviers, Belgium; 0.1% Bovine Serum Albumin Cohn Fraction V, Sigma-Aldrich), fixed overnight in BD Lysing Buffer (BD Biosciences, Erembodegem, Belgium), and washed in 1x FACS buffer the

next morning. Leukocytes were identified by dual scatter and 50,000 leukocyte events were acquired on the FACSCanto (BD Biosciences). Cytometer Setup and Tracking beads (BD Biosciences) were used to standardize instrument settings between experiments. Doublets were excluded using a pulse geometry gate (FSC-H x FSC-A). Each sample was checked for run stability to eliminate artifacts caused by poor flow. Dead cells were excluded with Fixable Near-IR Dead Cell Stain (Lifetech). Granulocytes and lymphocytes were gated on the basis of forward and side scatter. Eight 6-color immunophenotyping panels were used to measure the relative number and activation status (CD25, CD69, HLA-DR, CD11a, CD11b) of the following cell types: monocytes (CD14+CD16+/-), NK cells (CD3-CD19-CD56+CD16+/-), B cells (CD19+), T cells (CD3+), CTLs (CD8+), Th cells (CD4+), Th1 (CCR4-CXCR3+CCR6-), Th2 (CCR4+CXCR3-CCR6-), Th17 (CCR4+CXCR3-CCR6+) (Table 5 and Table 6). At least two technical replicates were measured for the main cell subtypes, except for the three Th subsets and CD16+ monocyte subset, which were measured in singlets. The subsequent analysis, using FlowJo (version 10.0.7, Tree Star, Ashland, OR) and R (version 3.3.2; R Core Team, 2016), were performed on the mean percentage of the parent population.

Table 5. Flow cytometry antibodies.(Supplementary material)

Fluorochrome	Ab	Clone	Company	Order #
FITC	CD4	EDU-2	Immunotools	21278043S
FITC	CD3	MEM-57	ImmunoTools	21270033
FITC	CD11a	TB-133	ImmunoTools	21330113
FITC	CD11b	MEM-174	ImmunoTools	21279113
FITC	CD14	MEM-18	ImmunoTools	21270143X2
FITC	CD19	LT19	ImmunoTools	21270193S
PE	CD19	LT19	ImmunoTools	21270194X2
PE	CD69	FN50	Biolegend	310906
PerCP-Cy5.5	CD3	HIT3a	Biolegend	300328
PerCP-Cy5.5	CD196	TG7/CCR6	Biolegend	335505
PerCP-Cy5.5	CD16	3G8	Biolegend	302028
PerCP-Cy5.5	CD8a	RPA-T8	eBioscience	45008841
PerCP-Cy5.5	CD19	HIB19	Biolegend	302230
PE-Cy7	CD15	SSEA-1	Biolegend	323030
PE-Cy7	CD183	G025H7	Biolegend	353720
PE-Cy7	CD56	CMSSB	eBioscience	25056742
PE-Cy7	CD3	UCHT1	Biolegend	300420
APC	CD14	18D11	ImmunoTools	21620146X2
APC	CD194	L291H4	Biolegend	359408
APC	CD25	BC96	eBioscience	17025942
Alexa Fluor 647	HLA-DR	L243	Biolegend	307622
APC-Cy7	Life Dead		Lifetech	L10119

Table 6. Flow cytometry panels.(Supplementary material)

Panel	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-Cy7
1	CD4	CD69	CD196	CD183	CD194	L/D
2	CD11b	CD69	CD16	CD15	CD14	L/D
3	CD3/CD19/CD14	CD69	CD16	CD56		L/D
4	CD4	CD69	CD8a	CD3		L/D
5	CD11a	CD69	CD19	CD3	CD14	L/D
6	CD4	CD14	CD8a	CD3	HLA-DR	L/D
7	CD4	CD19	CD8a	CD3	CD25	L/D
8	CD3	CD19/CD14	CD16	CD56	CD25	L/D

3.3.5 Telomere length

Telomere length was measured by quantitative polymerase chain reaction (qPCR), as previously described (Cawthon, 2002), with minor modifications. Briefly, relative telomere length was determined from the ratio of telomere and single copy gene (*36B4*) Ct values (T/S ratio). Amplifications were performed in 25µL reactions containing 1x PCR Buffer, 1mM MgCl2, 200nM dNTP, 0.2µM primers, 0.02U/µL Platinium Taq DNA Polymerase, Sybr Green I (1.0× *36B4*; 0.2x telomere) (All reagents, Invitrogen, Aalst, Belgium), and 1% DMSO (New England Biolabs, Hitchin, Hertfordshire, UK). Thermal cycling (CFX96, BioRad, Temse, Belgium) conditions were 95°C, 5 min; 45 cycles of 95°C, 15 sec; Ta of either 58°C (*36B4*) or 54°C (telomere), 30 sec; 72°C, 30 sec (only for 36B4).

3.3.6 Data reduction and statistical analysis

IL-6 secretion data was reduced to a composite score: data were log10-tranformed to fulfill the requirements for normal distribution and converted to z-scores of which the sum was taken. Manufacturer's recommended cut-offs were used to distinguish seropositive from seronegative participants for the different herpes viruses tested. In the final statistical models antibody levels of both seronegative and seropositive participants were included. Self-reported physical activity was expressed in minutes per week and weighed by its energy requirements as previously described (Ainsworth et al., 2000). Salivary cortisol, plasma IL-6, and plasma CRP values were used untransformed. Obvious outliers caused by

documented technical errors were eliminated from analysis (Telomere length, n=2 participants; flow cytometry, n=0-13 participants).

Differences in immune variables between groups (Ctrl vs. ELA) were tested using the Wilcoxon rank-sum test with false discovery rate corrected p-values using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). Immune cell types that differed significantly between groups were selected for further investigation. To identify variables that affected these cell types, Least Absolute Shrinkage and Selection Operator (LASSO) linear regression was used for 1000 bootstrap samples after a 10-fold imputation of missing values. Variables with non-zero estimates were counted and an exploratory cut-off of 50% was applied to select important variables, which were subsequently used to build multivariable linear regression models. P-values of 5% were considered significant. All statistical analyses were performed in R (version 3.3.2; (R Core Team, 2016) using Hmisc version 4.0-2 (Harrell Jr and others, 2016) and glmnet version 2.0-5 (Friedman et al., 2010).

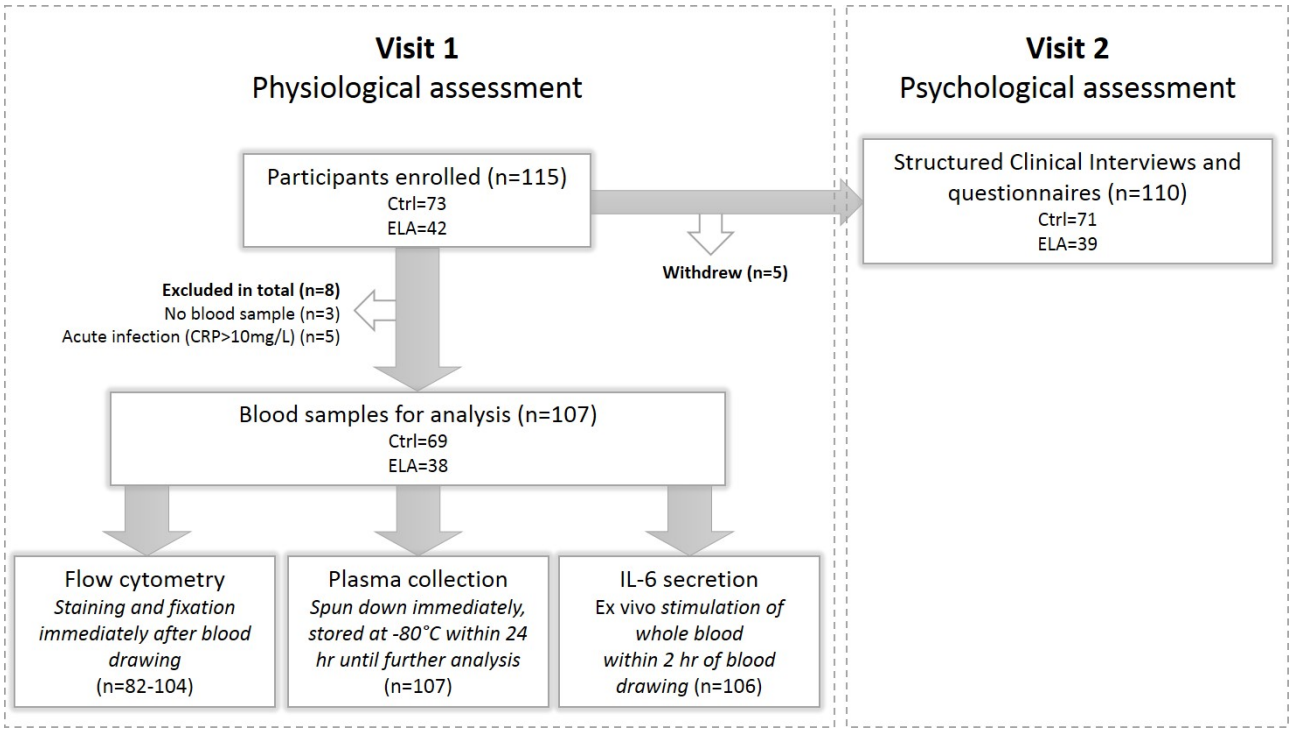

Figure 10. Recruitment summary of all participants enrolled and study protocol. Abbreviations: Ctrl, control; ELA, early life adversity; IL-6, interleukin 6.

3.4 Results

3.4.1 Cohort description

We enrolled a total of 115 young adults, of which 42 had a history of ELA and 73 controls that were raised by their biological parents (Ctrl) (Figure 10). Three ELA participants were adopted directly from the birth family, one participant was adopted from a foster family, but the majority had experiences the additional stress of institutionalization prior to adoption. The final analysis was performed on 40 individuals with ELA and 69 controls (Table 8), after excluding three participants without a blood sample. The control group was successfully matched for sex (p=0.354, Chi-square test); however, there was a small but statistically significant difference in age (Ctrl: median 22 years [IQR 21-23], ELA: median 24 years [IQR 20-25.5]; p=0.036, Wilcoxon rank-sum test). Both groups had low CTQ scores, suggesting that they had no memory of childhood trauma (p=0.659, Wilcoxon rank-sum test). As expected (Friedman et al., 2015; Gilbert et al., 2015), a higher percentage of ELA participants reported a doctor-diagnosed chronic disease (Ctrl: 9%, ELA: 43%; p<0.001, Chi-square test), such as asthma or migraine (Table 7).

Table 7. Self-reported doctor-diagnosed chronic diseases. (Supplementary data)

Group	Disease
Ctrl	Migraine
Ctrl	Hypothyroidism
Ctrl	Hypothyroidism
Ctrl	Hypothyroidism
Ctrl	Sinusitis (possibly chronic)
ELA	Migraine
ELA	Chronic Hepatitis B infection
ELA	Depression
ELA	Chronic borreliose (Since 2 years)
ELA	Asthma, increased blood prolactine
ELA	Depression
ELA	Migraine
ELA	Migraine
ELA	Autoimmune disease
ELA	Hypothyroidism
ELA	Asthma
ELA	Borderline personality disorder
ELA	Acne
ELA	Attention-deficit hyperactivity disorder
ELA	Asthma
ELA	Arthrosis in knee

3.4.2 ELA immune phenotype, stress system and health behaviors

A number of variables for each arm of the ELA immune phenotype – inflammation, cellular immunity, and immunosenescence – were tested in both groups (Table 8). Telomere length and levels of

the circulating inflammation markers CRP and IL-6 were similar between groups (p>0.05, Wilcoxon rank-sum test). Nevertheless, IL-6 secretion after *ex vivo* stimulation with phytohemagglutinin-M, pokeweed mitogen, and lipopolysaccharide was lower in ELA (p=0.027, Wilcoxon rank-sum test on sum scores). To test cellular immune function, antibody titers of several common herpes viruses were measured as a proxy for viral reactivation, but no significant differences were found between the groups in titers nor seropositivity (p>0.05, Wilcoxon rank-sum test, Chi-square test, respectively). However, a trend towards EBV seropositivity was found in ELA (p=0.053, Chi-square test).

*Table 8. Cohort demographics and health data. Statistics: Wilcoxon rank-sum test for numerical variables; Chi-square test for categorical variables; compared to control group: # p<0.10, * p<0.05, ** p<0.01, *** p<0.001. Abbreviations: IQR, interquartile range; CRP, C-reactive protein; IL-6, interleukin 6; HSV-1, Herpes simplex 1; EBV, Epstein-Barr virus; CMV, Cytomegalovirus; BMI, body mass index; IPAQ, international physical activity questionnaire; METs, Metabolic Equivalent of Task units.*

		Variable	Controls (n=69) (median, IQR)	ELA (n=40) (median, IQR)
Demographics		Age (years)	22 (21-23)	**24.0 (20.0-25.5)***
		Sex (women)	57%	68%
Health		Chronic disease	9%	**43%*****
ELA immune phenotype	*Immune activation*	Plasma CRP (mg/L)	2.0 (1.3-3.3)	2.0 (1.1-3.9)
		Plasma IL-6 (pg/mL)	0.6 (0.3-1.1)	0.7 (0.3-1.1)
		IL-6 secretion (sum z-score)	0.7 (-0.7- 1.7)	**-0.4 (-2.6-1.2)***
	Cellular immunity	HSV-1 seropositive/titers[1]	37% / 1.9 (1.7-2.0)	45% / 1.8 (1.7-2.0)
		EBV seropositive/titers[1]	46% / 1.9 (1.4-2.3)	**68%#** / 2.1 (1.5-2.7)
		CMV seropositive/titers[1]	12% / 1.3 (1.1-1.9)	19% / 1.2 (1.1-1.2)
	Immunosenescence	Telomere length (relative T/S)	1.2 (0.8-1.9)	1.1 (0.7-1.7)
Stress		Salivary cortisol (µg/dL)	0.26 (0.17-0.34)	0.23 (0.19-0.34)
		Stress rating (Visit 1)	1.2 (0.6-2.6)	1.5 (0.2-3.1)
Health behaviors		Smoking	12%	**30%***
		BMI (kg/m^2)	22.6 (20.2-24.3)	**23.2 (21.3-26.8)***
		Physical activity (IPAQ, METs)	3772 (1706-5784)	3051 (1452-8633)

[1] Among seropositive participants.

Both groups were similar in salivary cortisol levels and perceived stress before the blood sample was taken (p>0.05, Wilcoxon rank-sum test). In terms of health behaviors, a higher number of individuals in the ELA group were smokers (Ctrl: 12%, ELA: 30%; p=0.043, Chi-square test) and had a higher BMI (Ctrl: median 22.6 kg/m^2 [IQR 20.2-24.3 kg/m^2], ELA: median 23.2 kg/m^2 [IQR 21.3-26.8 kg/m^2]; p=0.046, Wilcoxon rank-sum test) than in the control group, although physical activity levels were similar (p>0.05, Wilcoxon rank-sum test) (Table 8).

3.4.3 Differences in cell subsets and activation status

Individuals in the ELA group showed a trend towards lower numbers of monocytes (Ctrl: median 6.7% [IQR 5.5-7.9%], ELA: median 5.7% [IQR 4.7-7.3%]; p= 0.067, Wilcoxon rank-sum test) and higher numbers of $CCR4^+CXCR3^-CCR6^+$ T helper (Th) cells (Ctrl: median 9.2% [IQR 7.6-10.4%], ELA: median 10.1% [IQR 8.7-11.9%]; p=0.060, Wilcoxon rank-sum test) (Table 9a). Nevertheless, Natural Killer (NK) cells, B cells, total $CD3^+$ T cells, as well as T cell subsets such as CTLs and total Th cells, and Th cell subsets Th1 or Th2 cells were comparable between groups (p>0.05).

There were a number of significant differences in expression of activation markers (Table 9b). A reduced number of CTLs ($CD8^+$) expressing the early activation marker CD69 were found in the ELA group (p=0.022). This was true for almost every cell type tested, albeit as a trend (monocytes ($CD14^+$), p=0.060; total T cells ($CD3^+$), p=0.083, and T cell subsets: total Th cells ($CD4^+$), p=0.067, and Th cell subsets: $CCR4^+CXCR3^-CCR6^-$ (Th2), p=0.060; $CCR4^+CXCR3^-CCR6^+$ (Th17), p=0.060; total NK cells ($CD56^+$), p=0.060, and NK cell subset: $CD56^{dim}CD16^+$, p=0.060; Wilcoxon rank-sum test). There was also a trend towards lower numbers of monocytes expressing integrin CD11b (p=0.060, Wilcoxon rank-sum test) in ELA participants. In contrast, HLA-DR^+ on total T cells, but also on the two main subsets – CTLs and Th cells – were significantly higher in the ELA group (p<0.001, Wilcoxon rank-sum test). Also CD25 on CTLs was higher in ELA (p=0.036), and we could observe a similar trend in $CD25^+$ total T cells and Th cells (p=0.065, 0.060, respectively, Wilcoxon rank-sum test). CD25 expression on B cells and NK cells, and integrin CD11a expression on monocytes, T cells, and B cells did not differ between groups (p>0.05) (Table 9b).

3.4.4 Current stress levels, health and immune parameters

We investigated the associations between current stress levels, health behaviors, and the ELA immune phenotype. Potential confounders were identified by multivariable linear regression models with LASSO penalization in combination with bootstrap sampling, using an exploratory cut-off of 50% for the percentage of non-zero estimates (Table 10). Monocyte numbers were associated with sex and IL-6 secretion (non-zero estimates: 84.6%, 68.3%, respectively). After including these variables in a linear regression model, the association between monocyte numbers and ELA disappeared (Table 11). Age (51.3%) was identified as important variable in predicting CD69+ Th cells. The model including age suggested that the group difference in CD69+ Th cells reflected the group difference in age (Table 8), as the association with ELA disappeared. Similarly, the group difference in CD69+CTLs reflected the difference in BMI, which was selected as important variable (54.3%). Sex was an important variable for CD69+ NK cells (56.2%), but after including sex in the model there was still a significant association between ELA and CD69+ NK cell numbers. After including sex (61.5%) and BMI (50.6%) the association between ELA and CD69+CD56dim NK cells numbers disappeared. CMV titers (66.2%, not excluding seronegative participants) and telomere length (54.7%) were identified as potential confounders in HLA-DR+ CTLs. Because CMV seropositivity and HLA-DR+ CTLs have been associated with immunosenescence (Rea et al., 1999; van de Berg et al., 2010), we also tested for interactions. Although there was no interaction between telomere length and CMV titers, we found a trend towards an interaction between ELA and

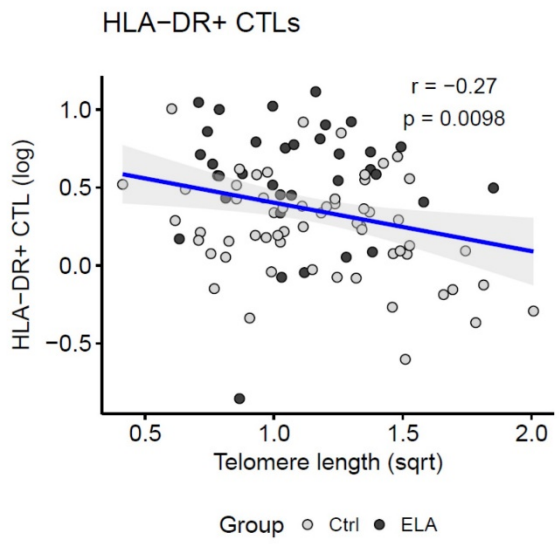

Figure 11. Negative correlation between activated CTL numbers (HLA-DR+ CTLs) and telomere length (T/S). Statistics: Pearson's product-moment correlation (log or square root transformed variables). Abbreviations: CTL, Cytotoxic T Lymphocyte; Ctrl, control; ELA, early life adversity.

telomere length (β=0.176, SE=0.098, p=0.075). Shorter telomere length was associated with higher HLA-DR+ CTLs numbers (r=-0.27, p=0.010, Pearson's product-moment correlation; Figure 11). After taking this interaction into account, the association between ELA and HLA-DR+ CTLs numbers disappeared. However, this may also be explained by the number of combinations and sample size in the model, leading to an underpowered analysis. CRP levels were related to HLA-DR+ Th cell levels, but did not explain the ELA association. Also, when excluding participants with CRP plasma levels >10 mg/L, indicative of a current or recent infection, this relationship disappeared.

3.4.5 Dose-response relationship with ELA severity

Finally, we investigated if there was a dose-response relationship between ELA severity and cell-specific immune alterations. Age at adoption was used as a proxy for ELA severity, as it is proportional to the time spent in an institution and/or otherwise adverse environment that led to the adoption, which is related to worse long-term ELA outcomes (Drury et al., 2012; Hodel et al., 2015). In the initial analysis, we observed no correlation between age at adoption and the expression of activation markers. However, after excluding individuals with CRP plasma levels >10 mg/L, indicative of a current or recent infection, we observed a trend (Figure 12), in that those that were adopted at a later age tended to have more T cells expressing HLA-DR and more CTLs expressing CD25 (r=0.33, p=0.079; r=0.35, p=0.067; respectively; Pearson's product-moment correlation without p-value adjustment).

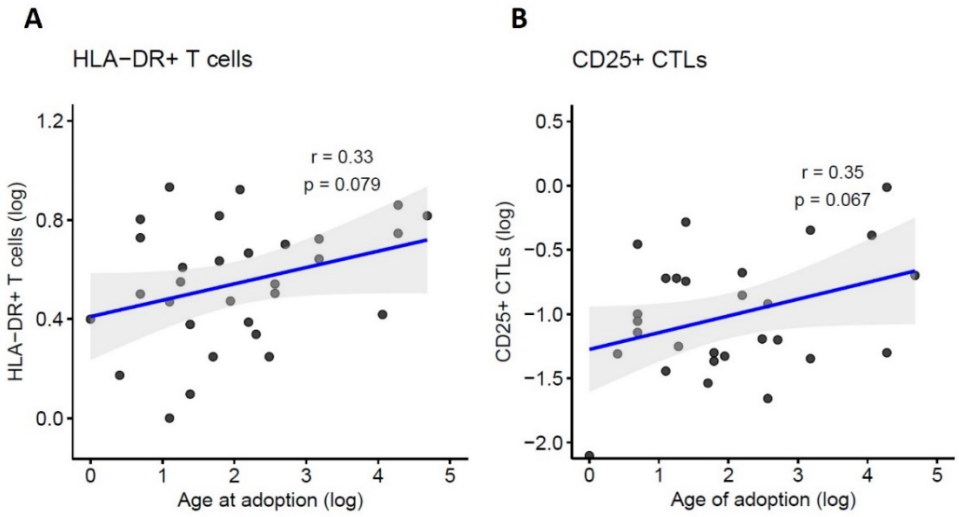

Figure 12. Dose-response relationship between ELA severity (age at adoption) and HLA-DR+ T cells (3a), CD25+ CTLs (3b), among ELA participants only. Participants with CRP>10mg/L, indicative of a current or recent infection, were excluded from this analysis. Statistics: Pearson's product-moment correlation (log transformed variables).

*Table 9. First screening of immune differences between controls and ELA in primary immune cell phenotypes (2a) and activation markers (2b). Numbers represent percentage of parent population (median, IQR) and p-values after Wilcoxon rank-sum test and corrected p-values represent p-values after false discovery rate correction for multiplicity. # p<0.10, * p<0.05, ** p<0.01, *** p<0.001. Abbreviations: IQR, interquartile range; Th cell, T helper cell; CTL, Cytotoxic T Lymphocyte; NK cell, Natural Killer cell.*

2a Cell types	Participants	Ctrl		ELA		Uncorrected	Corrected
	n	Median	IQR	Median	IQR	p-values	p-values
Monocytes ($CD14^+$)	Ctrl=69, ELA=40	6.7	5.5-7.9	5.7	4.7-7.3	0.028	0.067#
Classical ($CD14^+CD16^-$)	Ctrl=63, ELA=38	6.3	5.5-8.7	5.9	4.4-8.6	0.483	0.698
T cells ($CD3^+$)	Ctrl=69, ELA=40	70.0	64.2-72.7	68.2	64.4-73.2	0.828	0.901
Th cells ($CD4^+$)	Ctrl=69, ELA=40	56.1	49.6-62.8	56.0	52.3-61.8	0.816	0.901
$CCR4^-CXCR3^+CCR6^-$ (Th1)	Ctrl=58, ELA=38	8.4	7.1-12.0	8.0	6.9-9.9	0.510	0.698
$CCR4^+CXCR3^-CCR6^-$ (Th2)	Ctrl=58, ELA=38	8.3	6.9-9.7	8.4	6.8-10.3	0.603	0.743
$CCR4^+CXCR3^-CCR6^+$ (Th17)	Ctrl=58, ELA=38	9.2	7.6-10.4	10.1	8.7-11.9	0.021	0.060#
CTLs ($CD8^+$)	Ctrl=68, ELA=40	32.2	28.3-37.8	33.1	30.3-36.0	0.353	0.594
B cells ($CD19^+$)	Ctrl=69, ELA=40	11.3	8.9-15.2	12.8	10.7-15.3	0.164	0.288
NK cells ($CD56^+$)	Ctrl=67, ELA=40	8.9	6.7-14.0	9.4	6.6-13.4	0.905	0.926
$CD56^{bright}CD16^-$	Ctrl=66, ELA=40	4.9	3.6-7.4	5.0	2.8-8.4	0.894	0.926
$CD56^{dim}CD16^+$	Ctrl=66, ELA=40	95.1	90.9-98.5	94.1	90.3-98.5	0.718	0.830
2b Activation markers							
CD69+ *Monocytes ($CD14^+$)*	Ctrl=68, ELA=39	4.94	3.6-6.7	3.80	2.1-5.3	0.020	0.060#
T cells ($CD3^+$)	Ctrl=69, ELA=39	4.57	3.6-6.1	3.72	2.6-5.0	0.038	0.083#
Th cells ($CD4^+$)	Ctrl=68, ELA=39	3.61	2.6-4.6	2.73	1.7-4.2	0.029	0.067#
$CCR4^-CXCR3^+CCR6^-$ (Th1)	Ctrl=52, ELA=34	1.31	0.8-2.0	1.09	0.8-1.4	0.065	0.133
$CCR4^+CXCR3^-CCR6^-$ (Th2)	Ctrl=52, ELA=35	7.67	4.2-11.4	3.96	2.9-8.8	0.016	0.060#
$CCR4^+CXCR3^-CCR6^+$ (Th17)	Ctrl=53, ELA=34	2.51	1.5-3.8	1.69	1.0-2.4	0.014	0.060#
CTLs ($CD8^+$)	Ctrl=68, ELA=39	5.07	4.2-7	4.04	3.0-5.2	0.002	**0.022***
B cells ($CD19^+$)	Ctrl=68, ELA=40	0.30	0.2-0.4	0.24	0.1-0.3	0.082	0.159
NK cells ($CD56^+$)	Ctrl=67, ELA=38	2.21	1.7-3.1	1.69	1.2-2.5	0.014	0.060#
$CD56^{bright}CD16^-$	Ctrl=62, ELA=33	1.98	1.2-2.9	1.65	1.0-2.7	0.571	0.743
$CD56^{dim}CD16^+$	Ctrl=67, ELA=39	2.16	1.5-3.2	1.67	1.1-2.3	0.018	0.060#
HLA-DR+ *T cells ($CD3^+$)*	Ctrl=64, ELA=37	1.86	1.4-2.8	4.06	2.5-6.1	<0.001	**<0.001****
Th cells ($CD4^+$)	Ctrl=62, ELA=35	1.77	1.2-2.7	3.86	2.6-6.1	<0.001	**<0.001****

2a Cell types		Participants	Ctrl		ELA		Uncorrected	Corrected
		n	Median	IQR	Median	IQR	p-values	p-values
	CTLs ($CD8^+$)	Ctrl=59, ELA=36	1.65	1.2-2.1	2.91	2.0-3.8	<0.001	**<0.001*** **
CD25+	*T cells ($CD3^+$)*	Ctrl=65, ELA=39	1.95	1.6-2.9	2.75	1.7-4.3	0.025	0.065#
	Th cells ($CD4^+$)	Ctrl=65, ELA=39	2.67	2.1-3.9	3.89	2.3-6.4	0.017	0.060#
	CTLs ($CD8^+$)	Ctrl=54, ELA=36	0.05	0.03-0.12	0.13	0.05-0.34	0.005	**0.036***
	B cells ($CD19^+$)	Ctrl=65, ELA=39	0.42	0.24-0.66	0.50	0.34-0.9	0.161	0.288
	NK cells ($CD56^+$)	Ctrl=56, ELA=32	0.13	0.06-0.19	0.13	0.0795-0.255	0.496	0.698
CD11a+	*Monocytes ($CD14^+$)*	Ctrl=64, ELA=38	88.45	80.8-96.2	88.00	65.9-95.6	0.491	0.698
	T cells ($CD3^+$)	Ctrl=65, ELA=38	53.90	43.9-60.3	53.65	37.9-64.3	0.926	0.926
	B cells ($CD19^+$)	Ctrl=65, ELA=38	2.18	1.46-3.85	2.57	1.52-4.03	0.674	0.805
CD11b+	*Monocytes ($CD14^+$)*	Ctrl=64, ELA=37	26.30	10.95-46.35	10.80	6.59-28.8	0.021	0.060#

*Table 10. Selection of possible confounding variables. The columns represent the dependent variables and the rows the independent variables. The percentage is shown for how often a parameter estimate of an independent variable was unequal to zero in LASSO regression that was applied to 1000 bootstrap sample datasets after a 10-fold imputation of missing values. A cut-off of 50% was applied (shown in bold and with *). Abbreviations: BMI, body mass index; CMV, Cytomegalovirus; CRP, C-reactive protein; CTL, Cytotoxic T Lymphocyte; EBV, Epstein-Barr virus; HLA-DR, Human Leukocyte Antigen - antigen D Related; HSV-1, Herpes simplex 1; IL-6, interleukin 6; IPAQ, international physical activity questionnaire; LASSO, Least Absolute Shrinkage and Selection Operator; METs, Metabolic Equivalent of Task units; NK cell, Natural Killer cell; Th cell, T helper cell.(Supplementary data)*

Potential confounding variables	Monocytes	Th17	$CD69^+$ Monocytes	$CD69^+$ T cells	$CD69^+$ Th cells	$CD69^+$ Th2	$CD69^+$ Th17	$CD69^+$ CTL	$CD69^+$ NK cells	$CD69^+$ $CD56^{dim}$ NK	$HLA\text{-}DR^+$ T cells	$HLA\text{-}DR^+$ CTL	$HLA\text{-}DR^+$ Th	$CD25^+$ T cells	$CD25^+$ CTL	$CD25^+$ Th	$CD11b^+$ Monocytes
Age (years)	12.4	43.6	12.7	49.8	**51.3**	22.8	16.4	44.9	4.2	4.4	10.8	13.4	17.7	14.7	32.4	16.4	11.4
Gender (Male)	**84.6**	9.2	6	5.7	4.4	5	3	11.7	**56.2**	**61.5**	5.2	13.3	12.2	4.3	9.1	4.2	9.6
Smoking	6.3	16.9	2.9	1.9	6.8	4.9	4.7	4.2	4.9	6.9	5.4	12.7	10.8	10.3	3.2	7.3	4.8
BMI	36.9	6.7	16.4	45.5	34.4	2.5	2.3	**53.8**	39.3	**50.6**	5	36.2	7.8	30	8.9	34.1	6.8
Physical activity (IPAQ, METs)	18.6	20.4	29.8	5	11.7	2.3	0.9	13.9	2.7	3.3	15.6	20.2	35.9	2	8.4	4	2.8
Chronic disease	9.8	3.3	41.3	29.6	36.8	3.3	1.3	29.3	12.2	11.1	5.1	17.1	28.3	3.6	11.8	6.2	3.6
Plasma CRP (mg/L)	11.2	15.3	4.7	5.8	22.3	45	0.2	22.5	13.4	17.6	39.5	26.3	**68.5**	19.3	30.8	27.2	5.7
Plasma IL-6 (pg/mL)	35.8	13.8	1.5	9.2	12.6	7.3	4.3	11.5	6.5	9	2.5	9	5.7	1.4	2.3	2.9	2.5
IL-6 secretion (sum z-score)	**68.3**	6.1	8.9	6.1	4.2	7.8	2	21.2	8.2	8.6	12.1	18.8	15.2	3.9	4	9.5	8.1
HSV-1 titers	44.6	7.2	4.7	4.8	4.1	4.1	1.9	27.5	5	3.9	3.9	7	7.3	2.1	6.8	3	3.2
EBV titers	13	42.2	21	3.1	3.1	4.2	2.6	18.5	24.3	20	13.3	17.5	31.6	4.2	2	8.4	7.3
CMV titers	9	4.9	5.5	1.8	5.3	2.2	0.8	15.6	19.9	31.8	22.1	**66.2**	15.7	3.2	11.2	4.6	0.8
Salivary cortisol (µg/dL)	12.3	22.6	4.3	0.5	2.1	1.7	2.8	4.1	2.4	2	12.8	21.8	24.1	5.8	1.3	5.4	3.8
Stress rating (Visit 1)	17.3	14.5	23.3	1.7	2.5	2.9	0.9	6.7	6	7.9	10.6	16.4	14.6	8.9	8.4	16.4	5.8
Telomere length (relative T/S)	34.4	21.8	5	1.7	10.6	5.2	1.7	3.5	7.4	14.9	12.3	**54.7**	14.8	2.5	4.1	3.6	1.5

*Table 11. Multivariable linear regression model predicting influence of confounders selected with LASSO (>50%) on group differences in immune cell types. Abbreviations: IQR, interquartile range; SE, standard error; Th cell, T helper cell; CTL, Cytotoxic T Lymphocyte; NK cell, Natural Killer cell. Statistics: Linear regression, # p<0.10, * p<0.05, ** p<0.01, *** p<0.001.*

	Sample size	**Estimates**		
	n	b	SE	p-values
Monocytes	Ctrl: 68 ELA: 40			
Group ELA		-0.48	0.35	0.174
Male sex		1.45	0.34	**<0.001*** **
IL-6 secretion		0.17	0.06	**0.009** **
CD69^{+}Th cells	Ctrl: 68 ELA: 39			
Group ELA		-0.07	0.05	0.154
Age		-0.02	0.01	**0.003** **
CD69^{+}CTLs	Ctrl: 68 ELA: 39			
Group ELA		-0.061	0.035	0.087$^{\#}$
BMI		-0.014	0.005	**0.002** **
CD69^{+}NK cells	Ctrl: 67 ELA: 39			
Group ELA		-0.11	0.05	**0.024***
Male sex		0.14	0.05	**0.002** **
CD69^{+}CD56^{dim}NK cells	Ctrl: 67 ELA: 39			
Group ELA		-0.088	0.050	0.081$^{\#}$
Male sex		0.148	0.048	**0.002** **
BMI		-0.014	0.007	**0.032***
HLA-DR^{+}CTLs	Ctrl: 58 ELA: 35			
Group ELA		0.006	0.150	0.968
CMV titer		-0.152	0.051	**0.003** **
Telomere length		0.210	0.074	**0.006** **
Group ELA: Telomere length		0.176	0.098	0.075$^{\#}$
HLA-DR^{+}Th cells	Ctrl: 60 ELA: 35			
Group ELA		0.246	0.055	**<0.001*** **
CRP levels		0.032	0.006	**<0.001*** **

3.5 Discussion

This study aimed at examining cell-specific immune differences of individuals with a history of ELA. Our data suggest that those who had experienced ELA had an elevated state of immune activation, characterized by higher expression of the activation markers HLA-DR and CD25, implicating CTLs in particular. We observed a trend between ELA and higher numbers of $CCR4^{+}CXCR3^{-}CCR6^{+}$ Th cells, which have been defined as Th17 cells (Acosta-Rodriguez et al., 2007; Annunziato et al., 2007). In the final statistical model numerous potentially confounding factors were taken into account. Although several aspects of the ELA immune phenotype were related to increased activation markers, neither stress nor health-risk behaviors explained the observed group differences. Thus, the state of immune activation in ELA is not secondary to alterations in the stress system or health behaviors.

Although HLA-DR is constitutively expressed on antigen presenting cells such as B cells and monocytes, on T cells its expression is induced during cell activation. Similarly, CD25, the alpha chain of the IL-2 receptor, is upregulated on T cells after T cell receptor stimulation. Thus, our data suggest a higher level of activation of both Th cells and CTLs in individuals that experienced ELA. Elevated numbers of $HLA\text{-}DR^{+}$ T cells have been found in patients with autoimmune diseases and chronic viral infections. Indeed, in our data $HLA\text{-}DR^{+}$ CTLs numbers were significantly related to chronic infection with CMV, whereas $HLA\text{-}DR^{+}$ Th cell numbers were related to CRP plasma levels. However, in our final statistical model, neither current inflammation nor infection proved to be the underlying cause of ELA-associated T cell activation.

Expansion of T cells expressing HLA-DR has also been related to normal aging (Rea et al., 1999). Telomeres – shortening with every cell cycle – are a commonly used marker of immunosenescence (Bauer and Fuente Mde, 2016). A growing body of research indicates an association between ELA and telomere shortening, suggesting that ELA accelerates the normal process of aging (Kananen et al., 2010; Puterman et al., 2016; Surtees et al., 2011). Although differences in telomere length are only detectable in very large

cohorts due to strong inter-individual variation, we observed a clear association between shorter telomeres and higher numbers of HLA-DR$^+$ CTLs, which explained the effect of ELA on HLA-DR$^+$ CTLs. Since these cells are at very low percentages in circulation, it is unlikely that they are solely responsible for the observed difference in telomere length. However, the process of immunosenescence is most probably underlying both shorter telomeres and higher numbers of HLA-DR$^+$ CTLs. Thus, these data provide additional evidence for the hypothesis that ELA accelerates immunosenescence.

A cut-off of CRP levels >10 mg/L is commonly used in studies of low grade inflammation because it may indicate a recent or current infection. However, recent reports recommend to use a higher cut-off such as 30 mg/L, as CRP levels >10 mg/L have also been observed in chronic inflammation related to obesity (Ishii et al., 2012). As none of our participants had CRP levels above 30 mg/L, we included all of them in the initial analysis. However, we also examined the effects of excluding those with higher CRP levels (i.e. >10 mg/L). Indeed, applying the cut-off eliminated the association between HLA-DR$^+$ Th cells and CRP levels, suggesting that HLA-DR expression was not related to low grade inflammation. Also, the duration of ELA (i.e. age at adoption) tended to increase T cell activation status only when excluding those with CRP levels >10 mg/L. This suggests that higher CRP levels, which may have been caused by a subclinical infection, masked this dose-response relationship.

This is the first study to suggest a trend of higher Th17 cell numbers in ELA. There are a number of observations that provide circumstantial evidence to support this observation. Th17 cells are an effector CD4$^+$ T cell subset that play a critical role in infection, autoimmunity, and inflammation and promote central nervous system inflammation by disruption of the blood brain barrier (Kebir et al., 2007). A higher prevalence of ELA has been found among multiple sclerosis (MS) and rheumatoid arthritis (RA) patients (Spitzer et al., 2012). These are diseases in which Th17 cells play an important role (Wilke et al., 2011). Similarly, both ELA and Th17 cell numbers are putative risk factors for schizophrenia: a meta-analysis showed a medium to large effect of increased rates of ELA among schizophrenia patients (Matheson et al.,

2013). In addition, higher percentages of Th17 cells and IL-17 have been reported in schizophrenia patients (Drexhage et al., 2011), and a correlation was found between Th17 cell numbers and schizophrenia symptom severity (Ding et al., 2014). Thus, elevated percentages of circulating Th17 cells offer a potential explanation for the ELA-associated risk for autoimmune diseases and schizophrenia.

Other immune alterations in ELA may underlie the shift in Th cell differentiation in favor of Th17 cells. For instance, Th17 cell differentiation is promoted by IL-6 (Bettelli et al., 2006), a cytokine that is elevated in individuals with ELA (Baumeister et al., 2015). Interestingly, we found lower percentages of $CD69^+$ on most immune cells in ELA participants. Since CD69 is a well-known activation marker, this finding may appear to contradict the rest of the data. However, reduced CD69 expression may actually be related to the increased numbers of Th17 cells. CD69 is believed to inhibit Th17 differentiation through activation of the Jak3−Stat5 signaling pathway (Martin and Sanchez-Madrid, 2011). Therefore, lower expression of CD69 may promote Th17 differentiation leading to higher Th17 numbers. Indeed, antibody-induced CD69 blockade in a mouse model for arthritis led to disease exacerbation (Sancho et al., 2006). However, it is still unclear which factors down-regulate CD69 expression in immune cells. Taken together, these data suggest that ELA favors Th17 differentiation, perhaps via inhibited expression of CD69 leading to increased Th17 numbers, promoting inflammation and Th17-associated diseases.

The immune differences presented here could not be statistically accounted for by health-risk behaviors or stress parameters, even though these are known to strongly affect the immune system. Thus, the observed immune differences do not appear to be secondary to differences in stress response systems or health behaviors, but are rather primary differences possibly resulting from early life programming of the immune system.

One of the major strengths of this study is that we combined all aspects of the immune phenotype in one cohort and controlled for a wide range of confounding factors. Secondly, blood samples were taken at the same time of day, minimizing the effects of circadian rhythm on immune cell redistribution. Although

our inclusion of individuals reporting chronic diseases may appear like a weakness in this study, it is well known that individuals with ELA have a higher risk for chronic diseases (Friedman et al., 2015) and have 1.4 to 3.5 higher odds of poor general health (Gilbert et al., 2015). This means that our sample gave an accurate reflection of disease prevalence in individuals that experienced ELA, as the overall prevalence of chronic diseases in our samples was comparable to the reported prevalence in this age group (Center for Disease Control and Prevention, 2009). Importantly, including health as a binary variable did not affect the associations between ELA and altered immune parameters.

We did not observe differences in circulating inflammatory markers, antibody titers against common herpes viruses, or telomere length in the EpiPath cohort, which have been reported previously in large ELA cohorts (e.g. Lin et al., 2015; Puterman et al., 2016; Slopen et al., 2013a). Although our group sizes were not sufficient to see differences in measures with high inter-individual variation, they were more than sufficient to see significant differences in altered expression of activation markers. Moreover, most of our ELA participants were adopted at a very young age, with no memory of the time before adoption (Chapter 5). Thus, adversity in this model may have been less severe as compared to other models of ELA, such as childhood abuse. However, with the choice of our model we eliminated the common problem of recall and reporting bias in ELA studies.

ELA has been associated with differences in the immune system; however, this is the first study to investigate a broad range of specific immune cell subsets in relation to ELA. ELA was associated with higher numbers of T cells expressing HLA-DR and CD25 and there was a trend towards elevated numbers of Th17 cells. This was independent of current health, parameters of current stress, and health-risk behaviors. Future studies are needed to further dissect the role of different aspects of an adverse early life environment, such as early life infection, nutrition, and psychological stress. Overall, our data suggests that ELA programs immune cells, leading to a long-term shift in T cell differentiation and activation status towards a more pro-inflammatory phenotype.

3.6 Acknowledgments

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Jonathan D Turner assisted in writing of the manuscript, which was revised into its final format by all co-authors. Xenia Hengesch, and Hartmut Schächinger were responsible for the first visit for physiological assessment. Violetta Schaan and Claus Vögele were responsible for the second visit for psychological assessment. The study was conceived by Claude P Muller and Jonathan D Turner with the support and contribution of Hartmut Schächinger and Claus Vögele. All authors read and approved the final manuscript.

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4 T cell immunosenescence after early life adversity: mediation by CMV infection

My contribution to this chapter:

Writing of the manuscript. Recruitment of study participants. Performing the immunological measurements, including CMV titers. Setting up and assisting in the flow cytometry measurements. Final statistical analysis. I was involved in setting up and coordinating visit 1, including scheduling of participants, nurses, and experimental rooms, performing the stress test, and sample collection.

4.1 Abstract

Early life adversity (ELA) increases the risk for multiple age-related diseases, such as diabetes type 2 and cardiovascular disease. As prevalence is high, ELA poses a major and global public health problem. Immunosenescence, or aging of the immune system, has been proposed to underlie the association between ELA and long-term health consequences. However, it is unclear what drives ELA-associated immunosenescence and which cells are primarily affected.

We investigated different biomarkers of immunosenescence in healthy participants, who were either parent-reared (Ctrl, n=59) or had experienced separation from their parents in early childhood and were subsequently adopted (ELA, n=18). No difference was observed in telomere length or in methylation levels of age-related CpGs in whole blood, containing a heterogeneous mixture of immune cells. However, when specifically investigating T cells, we found a higher expression of senescence markers (CD57) in ELA. In addition, senescent T cells ($CD57^+$) in ELA had an increased cytolytic potential compared to senescent cells in controls. With a mediation analysis we demonstrated that cytomegalovirus infection, which is an important driving force of immunosenescence, largely accounted for elevated CD57 expression observed in ELA.

Leukocyte telomere length may obscure cell specific immunosenescence; here, we demonstrated that the use of cell surface markers of senescence can be more informative. Our data suggest that ELA may increase the risk of cytomegalovirus infection in early childhood, thereby mediating the effect of ELA on T cell specific immunosenescence. Thus, future studies should include cytomegalovirus as a confounder or selectively investigate cytomegalovirus seronegative cohorts.

Keywords: Early life adversity, immunosenescence, CD57, cytomegalovirus, telomere length, T cells.

4.2 Introduction

Adverse and stressful events in childhood, such as parental loss, low childhood socioeconomic status, or institutionalization, have been associated with elevated levels of inflammation (Baumeister et al., 2015) and an increased risk for multiple age-related diseases, such as cardiovascular disease (Friedman et al., 2015; Korkeila et al., 2010) and type 2 diabetes (Eriksson et al., 2014). As many as 39% of children worldwide are estimated to experience one or more forms of early life adversity (ELA) (Kessler et al., 2010), placing a high economic burden on health care systems – and society in general – through medical costs and lost productivity (Fang et al., 2012). Although ELA is a major and global public health problem, it is currently unknown how its detrimental consequences can be prevented or reversed.

Many efforts have been made to understand the mechanisms underlying long-term effects of ELA. One of the mechanisms proposed is accelerated aging of the immune system, also known as immunosenescence (Elwenspoek et al., 2017; Shalev et al., 2013a). Immunosenescence refers to the process of progressive deterioration of immune functions that go hand in hand with normal aging (Bauer and Fuente Mde, 2016). Although senescence is characterized by an irreversible arrest in cell proliferation, senescent cells are not inactive, but show elevated levels of cytotoxicity and produce more pro-inflammatory cytokines (Tu and Rao, 2016). Accelerated immunosenescence negatively impacts health, leading to increased lifetime morbidity and mortality. Thus, if ELA affects the rate of immunosenescence, this may explain an increased risk and earlier onset of age-related disorders.

Indeed, evidence is accumulating that ELA accelerates immunosenescence. One of the most used proxies for immunosenescence is telomere length. Telomeres are tandem repeats at the ends of chromosomes that shorten with age and with every cell division (Blackburn, 1991). For instance, naïve T cells have longer telomeres than terminally differentiated T cells that went through more replication cycles (Weng et al., 1995). A considerable number of studies have investigated telomere length in individuals with

a history of ELA, but results vary in size and significance. Ridout et al. (2017) included 41 studies (n= 30,773) in a meta-analyses and could demonstrate a significant association between ELA and shorter telomeres, although with a small to medium effect size (Ridout et al., 2017). A number of tissues were included, including buccal cells, but the majority of studies focused specifically on leukocytes.

However, leukocytes are a heterogeneous mixture of immune cells; ELA may affect some cell types more than others. Few studies have investigated immunosenescence in specific immune subtypes. To our knowledge, only Cohen et al. (2013) measured telomere length in a specific immune subset. They investigated a terminally differentiated and senescent subset of T cells, $CD8^{+}CD28^{-}$ cells, and found shorter telomeres associated with low childhood socioeconomic status (Cohen et al., 2013). These data suggest that ELA specifically affects the aging of T cells, although other cell types are probably affected as well.

T cell senescence is characterized by a loss of naïve T cell populations, which are essential to combat novel antigens from infection or vaccination. At the same time, memory cell types such as effector memory and terminally differentiated T cells gradually increase during aging (Koch et al., 2008). It is possible to differentiate between naïve, central memory, effector memory, and terminally differentiated T cell populations, using lineage markers such as CD45RA and CCR7 (Sallusto et al., 1999). Moreover, several cell surface markers have been identified that are either up- or down-regulated as T cell senescence progresses, such as CD57, which allow for cell type specific analysis of immunosenescence (Strioga et al., 2011).

Apart from telomere length and surface molecules as biomarkers for senescence, there is emerging literature on various epigenetic indicators of cellular aging, based on an accumulation of age-related changes in DNA methylation profiles (Pal and Tyler, 2016). Epigenetic indices have been shown to predict mortality and biological age independently from telomere length, suggesting that epigenetic aging targets an alternative pathway to telomere length (Marioni et al., 2016). Furthermore, epigenetic aging signatures have been shown to predict age more precisely than telomere length (Weidner et al., 2014). The

association between ELA, telomere shortening, and age-related diseases, suggests that also the 'epigenetic clock' ticks faster in ELA. Early results imply that psychological factors and early environment can predict epigenetic aging (Boks et al., 2015; Simpkin et al., 2016; Zannas et al., 2015). However, to date, few studies have addressed this in ELA specifically, and results are ambiguous (Elwenspoek et al., 2017).

It remains an open question as to what drives ELA-associated immunosenescence. Besides ELA, several other environmental factors have been found to modulate the rate of immunosenescence, such as persistent viral infections (Bauer et al., 2015). Herpes simplex virus (HSV), Epstein-Barr virus (EBV), and cytomegalovirus (CMV) are among the most prevalent viral infections that establish latency after primary infection and reactivate when the immune system is compromised. Latent infections with CMV in particular are believed to play an important role in immunosenescence and are associated with age-related alterations of T cell immunity (Tu and Rao, 2016). Moreover, ELA increases the risk of herpes infections and has been implicated in increased reactivation in children and adults (Janicki-Deverts et al., 2014; Shirtcliff et al., 2009; Slopen et al., 2013a).

In the present study, we investigated T cell specific immunosenescence (T cell differentiation and CD57 expression) in healthy participants with and without a history of ELA, in addition to epigenetic aging at age-related CpGs. Participants in the ELA group had experienced separation from their parents in early childhood and were subsequently adopted, which is a standard model of ELA. With a mediation analysis we examined whether CMV titers may account for immunosenescence observed in ELA.

4.3 Material and methods

4.3.1 Participants

Participants were selected from the EpiPath cohort, based on health (absence of chronic or acute diseases and medication use) and adequate number of bio-banked peripheral blood mononuclear cells (PBMCs) for investigation. The EpiPath cohort was recruited between 2014 and 2016 from Luxembourg and The Greater Region Saar-Lor-Lux and consisted of young adults, aged 18-35, that were either parent-reared (Ctrl) or experienced separation from their parents in early childhood followed by adoption (ELA). 59 Ctrl and 18 ELA participants were included in this study (Figure 13). One adoptee was directly adopted from the birth family, all others experienced the additional stress of institutionalization, which is considered to be a form of social deprivation and structural neglect (van IJzendoorn et al., 2011). All participants gave their written informed consent. The study design was approved by the Ethics Review Panel of University of Luxembourg (ERP, No 13-002) and the National Research Ethics Committee (CNER, No201303/10) in compliance with the declaration of Helsinki.

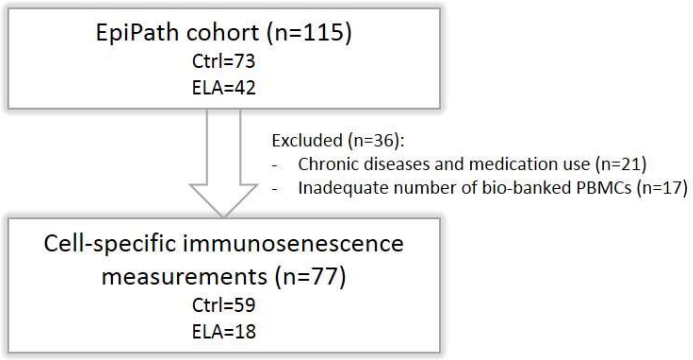

Figure 13. Selection of participants from within the complete EpiPath cohort.

4.3.2 Blood samples

Blood samples were collected in sodium heparin coated tubes for PBMC isolation and in EDTA coated tubes for DNA isolation. To minimize inter-individual variation all samples were collected at the end of the morning (ca. 11:30 am ±30 min); participants were asked to refrain from smoking, strenuous physical exercise, and drinking caffeinated or alcoholic beverages on the day of the clinical visit; women were either using hormonal contraceptives or were in the luteal phase of their menstrual cycle. Furthermore,

participants' age and sex were recorded. At a second visit, information about the age at adoption was obtained and the Childhood Trauma Questionnaire (CTQ) was administered.

4.3.3 Telomere length and age-related CpGs

DNA isolation and telomere length measurements in the EpiPath were reported previously in Chapter 3. Methylation levels were measured at age-related CpGs in ASPA (cg02228185), ITGA2B (cg25809905), and PDE4CA (cg17861230) according to Weidner et al. (2014). In brief, unmethylated cytosine residues in each DNA sample were converted to uracil with a bisulfite treatment (EpiTect Bisulfite Kit, Qiagen, Venlo, Netherlands) and regions of interest were amplified with PCR (PyroMark PCR Kit, Qiagen) in the bisulfite-modified DNA according to manufacturer's protocols. PCR products were pyrosequenced on a Pyromark ID with Pyrogold reagents (Biotage, Uppsala, Sweden) and methylation levels were analyzed with Pyro Q-CpG SW (Biotage). A sample of pooled DNA was run in each batch as internal control, which was used to calculate relative methylation levels. These relative methylation levels were used for all further analyses.

4.3.4 PBMCs Isolation and flow cytometry

All cell culture products were from Lonza BioWhittaker (Versviers, Belgium), unless otherwise stated. PBMCs were isolated within 3 h of sample collection using Ficoll-Paque density gradient centrifugation. Briefly, EDTA blood was diluted in sterile 1x PBS, layered over Ficoll-Paque™ PLUS (Fisher Scientific, Erembodegem-Aalst, Belgium) in Leucosep tubes (Greiner Bio-One, Vilvoorde, Belgium), and centrifuged for 5 min at 300g. PBMCs were washed twice with PBS and stored at 4.10^6 cells/1mL/aliquot in 80% Heat Inactivated Fetal Bovine Serum (Gibco, Paisley, United Kingdom) and 20% DMSO (Sigma-Aldrich, Saint-Louis, USA) in liquid nitrogen until analyzed.

PBMCs were thawed quickly and rested overnight at 37°C, 5% CO_2 in RPMI 1640 medium, with 10% Heat Inactivated Fetal Bovine Serum, 1% Penicillin/Streptomycin, 1% Sodium Pyruvate (Gibco), 1% non-essential amino acids (Gibco), and 1% ultra-glutamine. PBMCs (1.10^7 cells/mL) were incubated with GolgiPlug and GolgiStop (final concentrations of 2µL/mL and 1µL/mL, respectively, BD BioSciences) for 5 h at 37°C, 5%

CO_2. All subsequent steps were performed at 4°C and protected from ambient light. PBMCs were washed twice with 1x FACS Buffer (1x PBS, 1% Bovine Serum Albumin Cohn Fraction V [Sigma-Aldrich, Saint-Louis, USA], 0.1% NaN3, 2mM EDTA [Sigma-Aldrich], pH 8.0) and stained with a LIVE/DEAD dye and antibodies against CD4, CD3, CD8, CD45RA, HLA-DR, CCR7, and CD57 (Table 12) for 30 min. Then, PBMCs were permeabilized and fixed with BD Cytofix/Cytoperm™ (BD BioSciences, San Diego, USA) for 20 min, followed by a 30 min intracellular staining of granzyme B and perforin (Table 12).

Thirty thousand lymphocyte events were acquired on the BD LSRFortessa (BD BioSciences) using FACSDiva (BD BioSciences, version 8.0). Data analysis was performed with FlowJo (version 10.2, Tree Star, Ashland, OR) using the gating strategy presented in Figure 14. T cell differentiation was determined by CCR7 and CD45RA expression: naïve ($CCR7^+CD45RA^+$), central memory (CM, $CCR7^+CD45RA^-$), effector memory (EM, $CCR7^-CD45RA^-$), and terminally differentiated cells (TEMRA, $CCR7^-CD45RA^+$). Relative numbers of cells (e.g. $CD57^+$ cells) and median fluorescent intensity (MFI) were analyzed.

Table 12. Flow cytometry panel.(Supplementary material)

Fluorochrome	Ab	Clone	Company	Cat. N°
BUV395	CD4	SK3	BDBioSciences	563550
BUV496	CD3	UCHT1	BDBioSciences	564809
BUV805	CD8	RPA-T8	BDBioSciences	564912
PacBlue	CD45RA	HI100	BioLegend	304123
Bv711	HLA-DR	G46-6	BDBioSciences	563083
PE	GranzymeB	GB11	BDBioSciences	561142
PE-Dazzle	CD197	150503	BDBioSciences	562381
PE-Cy7	Perforin	B-D48	BioLegend	353316
APC	CD57	HCD57	BioLegend	322314
APC-Cy7	L/D		LifeTech	L10119

4.3.5 Statistical analysis

Group differences in telomere length, relative methylation levels of age-related CpGs, CMV titers, CTQ sum scores, and age at adoption were investigated with a Wilcoxon rank sum test with continuity correction. In the initial analysis, we constructed linear regression models to investigate group differences in cell types (flow cytometry data), in which 'cell type' was included as outcome variable and both groups and experimental day were included as fixed effects; the latter to account for variation between experiments. Cell percentages were transformed with the arcsine-transformation (asin(sign(x) *

sqrt(abs(x)))) to stabilize variance and MFI values with a log-transformation to approximate normality. ANOVAs were performed on each model to test the significance of the group effect. The Benjamini-Hochberg procedure was performed on all p-values generated by the ANOVAs to correct for multiplicity (Benjamini and Hochberg, 1995). To investigate the relationship between CMV levels and CD57 expression, Spearman's rank correlation rho were determined. To investigate the mediating effect of CMV on the association between ELA and senescent cells, a mediation analysis was performed in R (version 3.3.3 (R Core Team, 2016) using *mediation* version 4-4.5 (Tingley et al., 2014)). Because age at adoption can be considered to be proportional to the duration of adversity and can thus be used as proxy for ELA severity (Julian, 2013), in the mediation analysis, age at adoption (months) was used as continuous variable for ELA, in which controls were set to 0. P-values below 5% were considered significant.

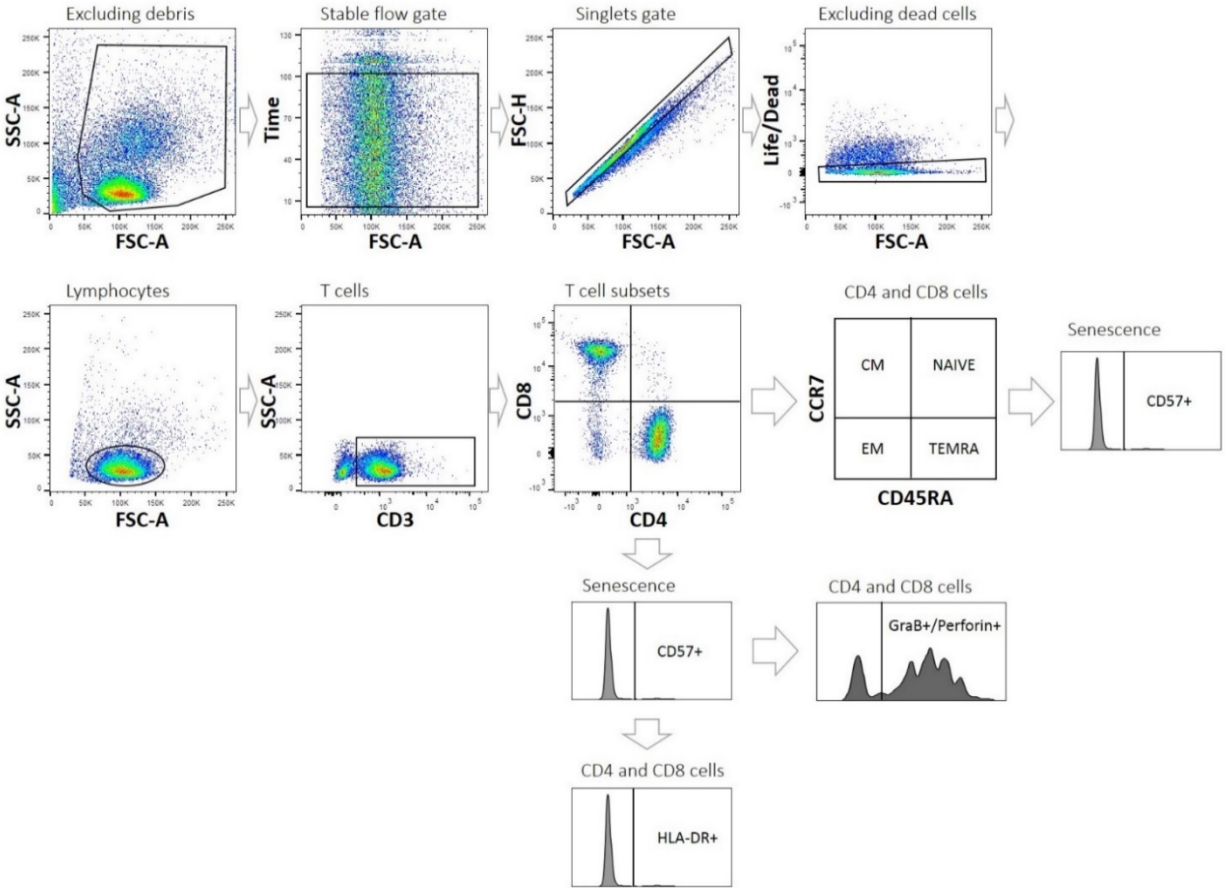

Figure 14. Gating strategy. (Supplementary material)

4.4 Results

4.4.1 Participant characteristics

The Ctrl and ELA groups did not differ in age or sex. The median age at adoption was 3.4 months. Adoptions took place at an early age, so participants had no memory of the time before adoption. Consequently, the CTQ scores, based on the participant's memory of trauma experiences before age 16, reflects experiences after adoption, which was similar between groups. Thus, apart from the adoption the experimental groups were comparable in age, sex, and subsequent childhood trauma exposure (Table 13).

Table 13. Participant characteristics. Statistics: For continuous variables the Wilcoxon rank sum test with continuity correction was applied and for categorical variables the Pearson's Chi-squared test with Yates' continuity correction. Abbreviations: Ctrl, control; CTQ, childhood trauma questionnaire; ELA, early life adversity; IQR, interquartile range.

	All (n=77)	**Ctrl (n=59)**	**ELA (n=18)**	**p-value**
Age (median years [IQR])	22 [20-24]	21 [20-23]	23 [20-25]	0.702
Sex (% female)	61.0%	59.3%	66.7%	0.777
Age at adoption* (median months [IQR])	0 [0-0]	0 [0-0]	4.3 [0-15]	<0.001
Childhood trauma (median CTQ scores [IQR])	1.2 [1.1-1.4]	1.2 [1.1-1.4]	1.2 [1.1-1.4]	0.934

* 'Age at adoption' was used as a proxy of ELA severity (Julian, 2013); therefore, controls were set to 0.

4.4.2 Telomere length and epigenetic aging

Immunosenescence in leukocytes was measured with two distinct techniques. First, as previously reported, we did not observe a difference in telomere length between the two groups in the complete EpiPath cohort (Chapter 3). Also in the subset of participants used in the present investigation, which only included healthy participants, there was no effect of ELA on telomere length (median [IQR]; Ctrl: 1.2 [0.8-1.8], ELA: 1.1 [0.8-1.7], p=0.714; Figure 15a). Second, methylation levels at three age-related CpGs that have been linked to chronological and biological age were measured (Weidner et al., 2014). We found similar methylation levels in ELA and Ctrl (ASPA Ctrl: 1.01 [0.97-1.03], ELA: 1.01 [0.98-1.04], p=0.452;

ITGA2B Ctrl: 1.05 [1.00-1.12], ELA: 1.08 [1.02-1.12], p=0.438; PDE4C Ctrl: 0.99 [0.93-1.10], ELA: 1.05 [0.97-1.13], p=0.258; Figure 15b-d).

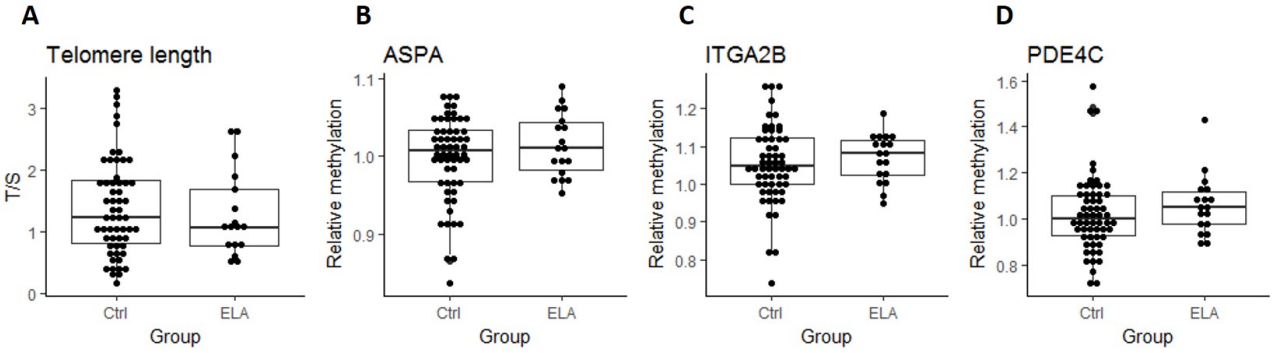

Figure 15. Markers for senescence. Relative telomere length in T/S (A), methylation levels at the age-related CpGs ASPA, ITGA2B, and PDE4C (B-D), respectively. Statistics: Wilcoxon rank sum test with continuity correction. Abbreviations: Ctrl, control; ASPA, aspartoacylase; ELA, early life adversityITGA2B, integrin alpha 2b; PDE4C, phosphodiesterase; T/S, relative telomere to single copy gene ratio.

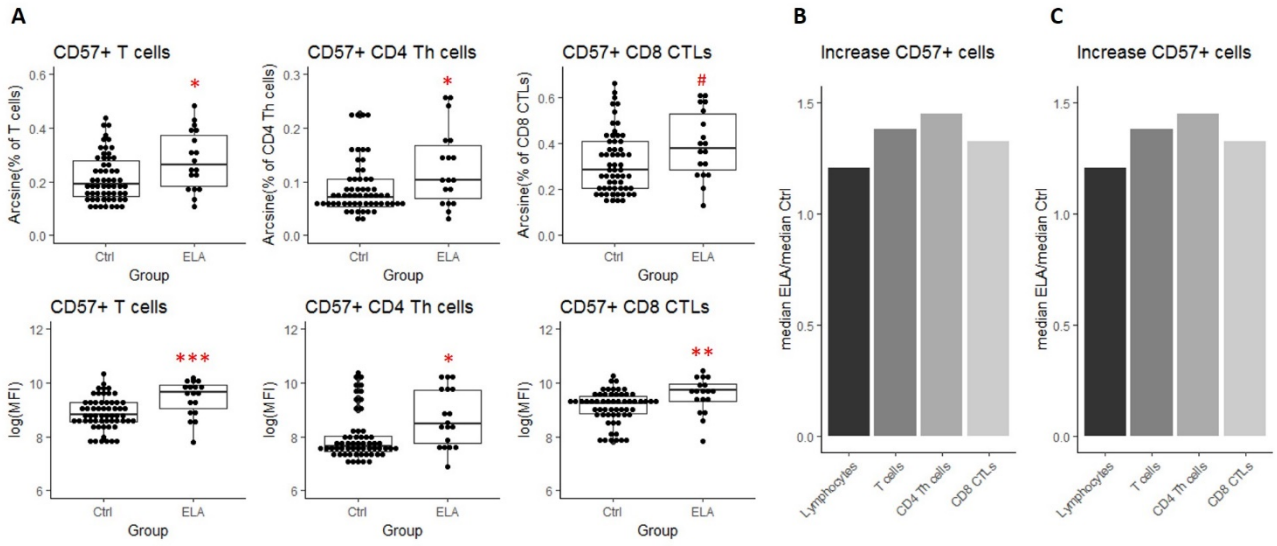

*Figure 16. Senescence marker CD57 on T cells. ELA participants have more CD57+ T cells (total and subsets Th and CTLs) and the expression of CD57 per cell is higher (MFI) (A). Y axes show percentage of parent after an arcsine transformation, or log-transformed MFI values. Factor increase in CD57+ cells between Ctrl and ELA (B). Factor increase in CD57 expression on CD57+ cells (MFI) between Ctrl and ELA (C). Statistics: linear model with group and experimental day as independent variables, and arcsine-transformed percentages or log-transformed MFI of CD57+ cells as dependent variable. P-values are corrected for multiplicity with false discovery rate. # p<0.10, * p<0.05, ** p<0.01, *** p<0.001. Abbreviations: Ctrl, control; ELA, early life adversity; MFI, median fluorescent intensity; CTL, cytotoxic T lymphocyte; Th cell, T helper cell.*

4.4.3 T cell specific senescence

A 10-color flow cytometry panel was used to investigate cell specific immunosenescence. Although immunosenescence is related to changes in the ratio of naïve and memory T cells, the ratios and numbers of naïve, central memory (CM), effector memory (EM), and terminally differentiated T cells (TEMRA) were similar between Ctrl and ELA (data not shown). However, we found a higher number of T cells expressing the senescence marker CD57. ELA was associated with a significant increase in both the total number of T cells (linear regression, adjusted p=0.017) and T helper (Th) cell subset (adjusted p=0.038), expressing CD57 (Figure 16a). The cytotoxic T lymphocytes (CTLs) showed a similar trend towards higher CD57, albeit this did not reach statistical significance (adjusted p=0.061). The increase in $CD57^+$ cells between Ctrl and ELA appeared to be highest in Th cells, showing almost a 1.5-fold increase (Figure 16b), and lowest in overall lymphocytes, consisting of a mixture of B cells, T cells, and NK cells.

There was also a significant group effect on the intensity of CD57 fluorescence (MFI, T cells: adjusted p<0.001, Th cells: p=0.024, CTLs p=0.001; Figure 16a), suggesting that ELA does not only have more senescent cells, the senescent

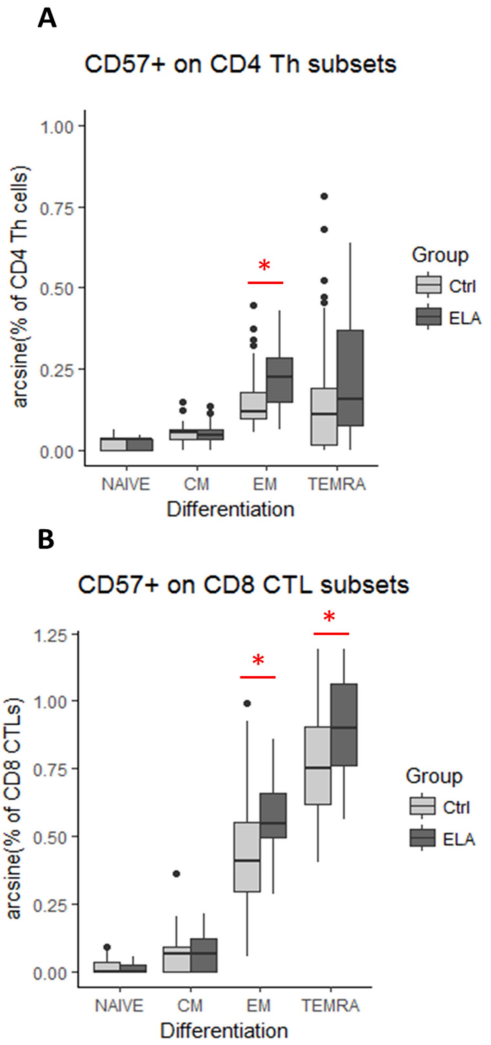

*Figure 17. CD57 expression in different stages of T cell differentiation in CD4 Th cells (A) and CD8 CTLs (B). Statistics: linear model with group and experimental day as independent variables, and arcsine-transformed percentages of CD57+ cells as dependent variable. P-values are corrected for multiplicity with false discovery rate. * p<0.05, ** p<0.01, *** p<0.001. Abbreviations: Ctrl, control; CM, central memory; ELA, early life adversity; EM, effector memory; CTL, cytotoxic T lymphocyte; TEMRA, terminally differentiated effector memory;*

cells also have a higher level of senescence. The mean fluorescent intensity (MFI) of CD57 was 5-10% higher in ELA than Ctrl on lymphocytes, total T cells, Th and CTLs. CTLs showed the lowest increase in CD57 expression (Figure 16c). As expected (Sallusto et al., 2004), $CD57^+$ cells were not equally distributed among the different stages of T cell differentiation (Figure 17). In both Th and CTL subsets, EM and TEMRA cells had the highest number of $CD57^+$ cells. The increase in $CD57^+$ cells in ELA was mainly happening in EM Th cells (Figure 17a) and in both TEMRA and EM CTLs (Figure 17b).

4.4.4 Cytolytic potential and activation

Cytolytic potential was measured by granzyme B and perforin staining. As expected (Chattopadhyay et al., 2009), $CD57^+$ cells had higher granzyme B, perforin, and HLA-DR expression (Figure 18A-B), suggesting higher cytolytic potential and a higher activation status in senescent Th and CTLs. When comparing the senescent cells ($CD57^+$) between groups, granzyme B and perforin expression was elevated in ELA, although the expression of the activation marker HLA-DR was similar (Figure 18C-D). The level of fluorescent intensity of neither

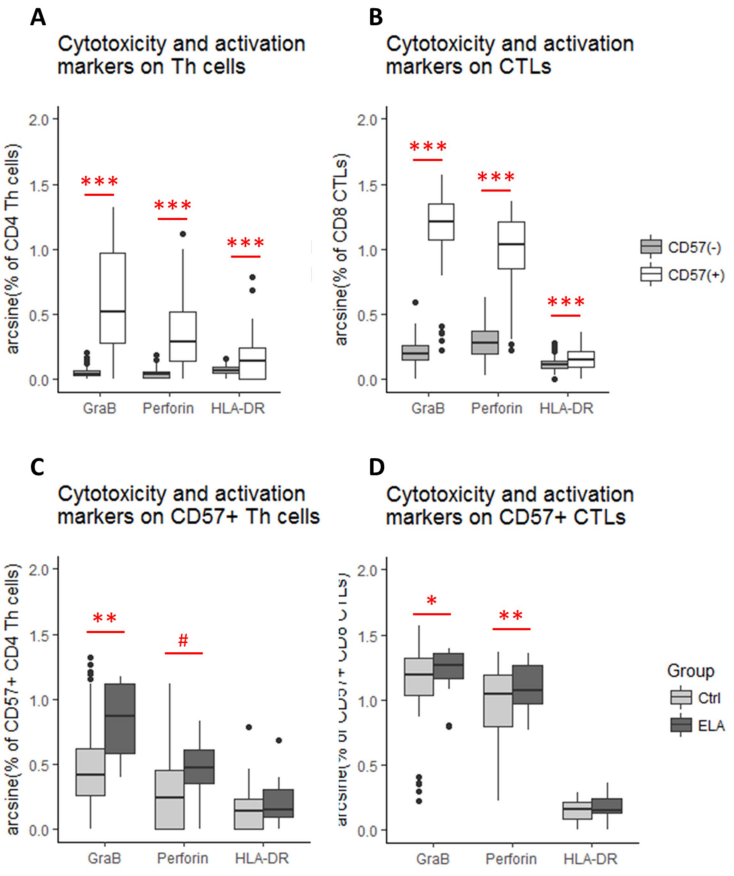

*Figure 18. Cytolytic potential and activation status: CD57 positive versus CD57 negative Th cells (A) and CTLs (B). ELA versus controls among CD57 positive Th cells (C) and CTLs (D). Statistics: Paired Wilcoxon rank sum test with continuity correction (A-B), linear regression with group and experimental day as fixed effects, and percentage of cells as outcome variable (C-D). P-values are corrected for multiplicity with false discovery rate. # p<0.10, * p<0.05, ** p<0.01, *** p<0.001. Abbreviations: Ctrl, control; ELA, early life adversity; GraB, granzyme B; HLA-DR, Human Leukocyte Antigen - antigen D Related; CTL, cytotoxic T lymphocyte; Th cell,*

granzyme B nor perforin differed between the groups (data not shown).

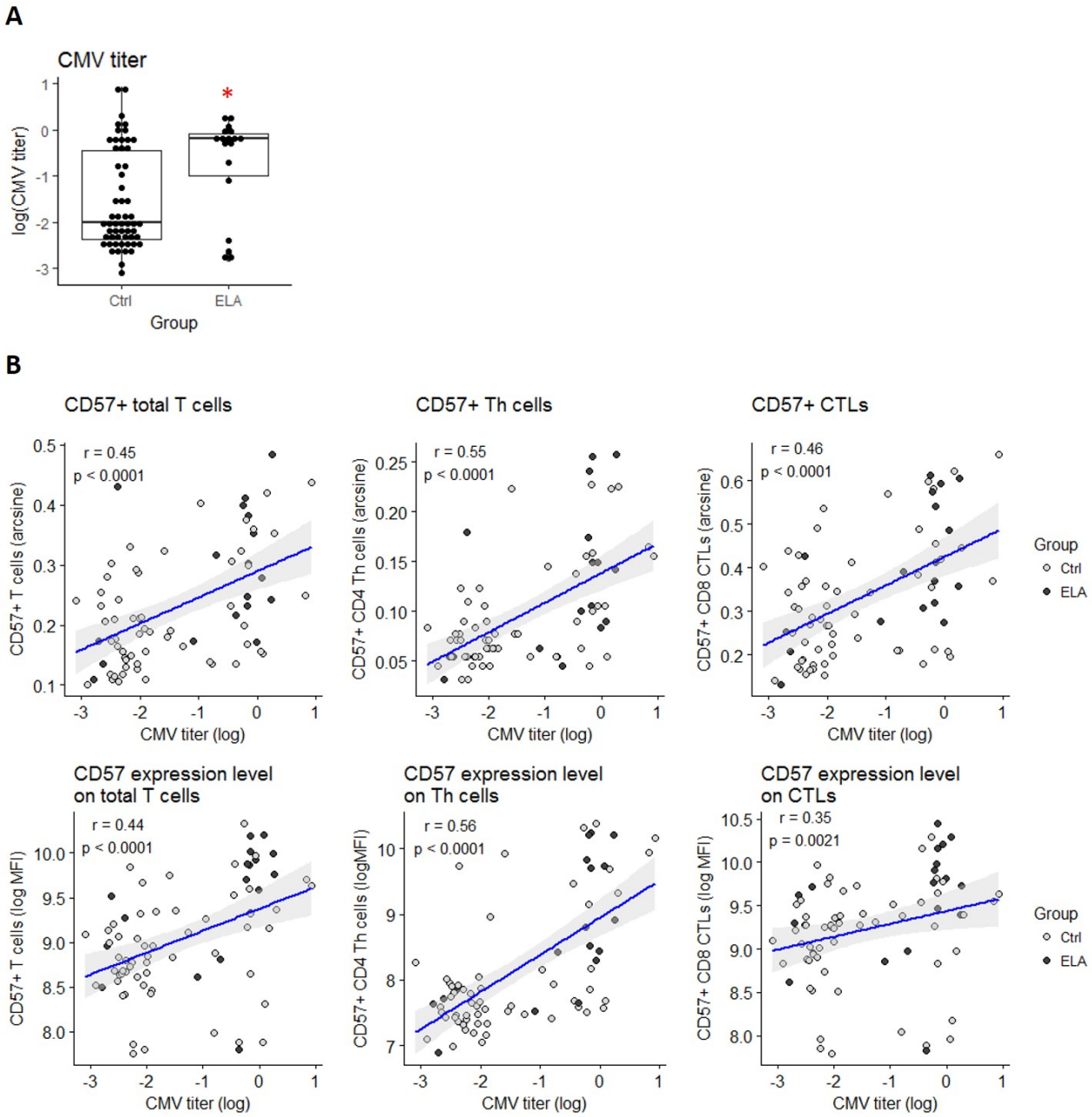

*Figure 19. Influence of CMV infection. CMV titers are significantly higher in ELA (A). Statistics: Wilcoxon rank sun test with continuity correction. Positive correlations between CMV titers and CD57+ numbers and expression levels on T cells (total, and subsets Th and CTLs) (B). Statistics: Spearman's rank correlation rho. # p<0.10, * p<0.05, ** p<0.01, *** p<0.001. Abbreviations: CMV, Cytomegalovirus; Ctrl, control; ELA, early life adversity; MFI, median fluorescent intensity; CTL, cytotoxic T lymphocyte; Th cell, T helper cell.*

4.4.5 Influence of CMV infection

In the final step of the analysis, we investigated whether CMV infection had a mediating effect on the relationship between ELA and immunosenescence. Initially, we tested the difference in CMV titers between the two groups. Indeed, titers were higher in the ELA group (medium [IQR], Ctrl: 0.13 [0.09-0.64], ELA: 0.82 [0.33-0.94]; p=0.023; Figure 19a), caused by an increased number of seropositive participants. Sample size did not allow for further stratification into seropositive and seronegative groups. Subsequently, we tested the correlation between the number of senescent cells ($CD57^+$) or the level of CD57 expression on these cells and CMV titers. Indeed, we found highly significant and strong correlations in T cells, and its subsets Th and CTLs (Figure 19b). In contrast, titers of EBV and HSV, herpes viruses that cause similar lifelong latent infections, were not elevated in ELA (EBV: Ctrl: 0.91 [0.53-1.78], ELA: 1.43 [0.72-2.10], p=0.213; HSV: Ctrl: 0.22 [0.12-1.78], ELA: 0.29 [0.13-1.82], p=0.906), nor where they correlated to the number of senescent T cells (EBV: rho=0.10, p=0.370; HSV: rho=-0.01, p=0.900). A causal mediation analysis demonstrated a large mediating effect of CMV titers (Figure 20), which could explain most of the total effect of ELA (age at adoption) on T cell senescence (Table 14).

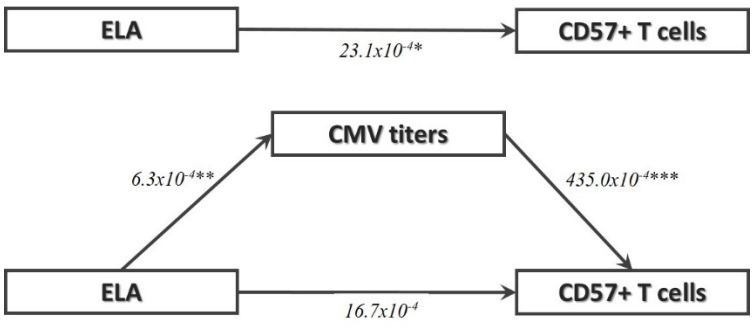

Figure 20. Mediation model, with ELA as independent variable, T cell immunosenescence as dependent variable, and CMV titers as mediator variable. In this model, ELA was treated as continuous variable (age at adoption) and all Ctrl were set to 0. Abbreviations: CMV, Cytomegalovirus; ELA, early life adversity.

Table 14. Causal Mediation Analysis. Nonparametric Bootstrap Confidence Intervals with the Percentile Method. Abbreviations: ACME, Average Causal Mediation Effect; ADE, Average Direct Effect; CI, confidence interval; prop., probability.

	Estimate	**95% CI Lower**	**95% CI Upper**	**p-value**
ACME	0.000633	0.000315	0.002202	0.00**
ADE	0.001674	-0.001614	0.002890	0.07
Total effect	0.002307	0.000586	0.003754	0.03*
Prop. mediated	0.274381	0.099298	0.661469	0.03*

4.5 Discussion

4.5.1 Discussion

In the present study, we have shown that ELA is associated with higher levels of T cell senescence in healthy participants (selection of EpiPath cohort). Even though there was no difference in telomere length or methylation levels at age-related CpGs in leukocytes, we observed a significant difference when specifically investigating T cells. Not only did we find a higher number of senescent cells ($CD57^+$), these cells also expressed higher levels of CD57, a cell surface marker for senescence, and were more cytotoxic in ELA compared to controls. The difference was highest in cells in later stages of differentiation, such as EM and TEMRA cells, while differentiation *per se* was not altered in ELA (according to lineage markers CD45RA and CCR7). Importantly, we found that the effect of ELA on immunosenescence was mediated by CMV infection specifically, rather than being the consequence of continued reactivation of latent viruses in general.

Our findings have important implications for the present literature on senescence in ELA. Most evidence for accelerated immunosenescence in ELA comes from telomere length, but none of these studies have accounted for CMV infections. Our results suggest that the association between ELA and shorter telomeres – or immunosenescence in general – may have been largely mediated by CMV infection. Although we did not observe shorter telomeres in the present study, nor in the complete EpiPath cohort (Chapter 3), we did find higher numbers of $CD57^+$ T cells in ELA. It has been demonstrated previously that this cell type has shorter telomeres (Brenchley et al., 2003). The large inter-individual variation in telomere length and the heterogeneity of leukocytes, most probably masked this effect.

CMV is a likely mediator between ELA and immunosenescence. First of all, because there is a clear link between CMV infection and immunosenescence. CMV infection is related to expanding populations of specific memory T cells (Klenerman and Oxenius, 2016; Weltevrede et al., 2016), and a shrinking population of naïve T cells (Wertheimer et al., 2014), similar to what is observed in aging (Koch et al., 2008). In

agreement with the results presented here, CMV specific T cell have been found to express senescence markers, such as CD57 and KLRG1 (Vieira Braga et al., 2015), but are still highly cytotoxic (Klenerman and Oxenius, 2016). Interestingly, CMV infection affects reactivation of other latent viruses. Reactivation of HSV increased with age, but only in CMV seropositive individuals (Stowe et al., 2012). Similarly, only in CMV seropositive individuals, EBV reactivation was associated with inflammatory markers in circulation (Bennett et al., 2012). Inflammation and chronic antigen exposure as a results of viral reactivation further enhances immunosenescence (Tu and Rao, 2016). Finally, CMV infection has been related to reduced lymphocyte telomere length (van de Berg et al., 2010) and is associated with decreased telomerase activity (Dowd et al., 2013), an enzyme that can partially counteract telomere loss.

Second, there is reason to believe that children in adverse circumstances are at higher risk for CMV infection. For instance, the likelihood of CMV infection is higher in children raised in poverty and low socioeconomic status (Dowd et al., 2012; Voigt et al., 2016). Poverty is reliable predictor of more severe forms of ELA such as childhood abuse and neglect (Cancian et al., 2013; Lefebvre et al., 2017; Slack et al., 2004). There is no clear epidemiological data on the prevalence of infection in international adoptees, as were included in the present study. However, most adopted children have been institutionalized prior to adoption, which arguably increases the risk for CMV infection, as is the case for day-care center attendance (Voigt et al., 2016). This is supported by a US study that found a 45% seroprevalence of CMV in a group of 247 internationally adopted children between 1-2 years of age (Hostetter et al., 1991) – much higher compared to the 21-22% reported in a German population samples of 1-2 year olds (Voigt et al., 2016). Indeed, we found higher CMV titers in the adoptees compared to controls.

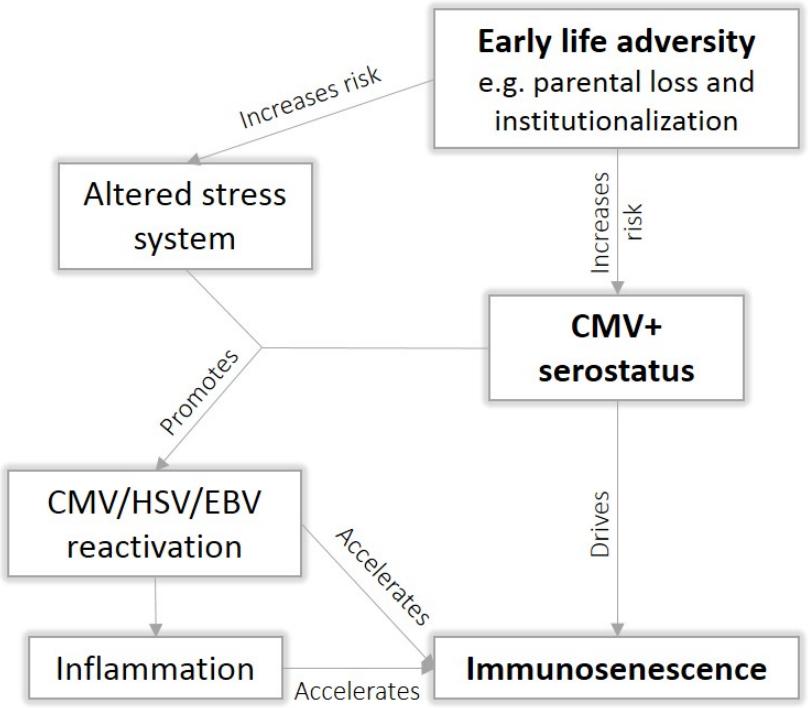

Figure 21. Proposed interaction between ELA and CMV to cause immunosenescence.

Although our statistical analysis suggest complete mediation by CMV, is it unlikely that CMV infection alone can explain the negative health consequences related to ELA. Especially because among seropositive individuals, ELA is associated with impaired viral control and increased CMV reactivation (Dowd et al., 2012; Fagundes et al., 2013b; Janicki-Deverts et al., 2014). Furthermore, among CMV positive children ELA was associated with an increased percentages of senescent CTLs ($CD8^+CD28^-CD57^+$ cells) (Caserta et al., 2008). Unfortunately, our sample size did not allow for further stratification according to CMV serostatus. Thus, even though CMV infection alone has been related to immunosenescence, ELA appears to amplify this effect.

Psychological stress plays an important role in viral reactivation (Chida and Mao, 2009), possibly due to immunosuppressive effect of the stress hormone cortisol that could compromise an effective response of the immune system. Interestingly, ELA has been association with an altered stress response (Lovallo et al., 2012). When we incorporate our CMV mediation model into the existing literature, it becomes clear that CMV and ELA interact on several levels, resulting in accelerated immunosenescence (Figure 21). We speculate that ELA increases the risk for CMV infection, leading to an immune response that drives T cell differentiation and thereby further accelerating immunosenescence. The effect of ELA on the stress system

may subsequently compromise viral control, leading to more frequent viral reactivation, which further promotes immunosenescence. Viral reactivation results in elevated levels of inflammation, which in turn may accelerate immunosenescence via oxidative stress. Immune functions decline, ultimately resulting in an earlier onset of age-related diseases. However, to date, there is insufficient data to validate this hypothesis.

4.5.2 Strengths and limitations

Differences in ethnic background between the Ctrl and ELA group and small sample size were the main limitations in this study. Nevertheless, we have taken several measures to reduce variability. ELA participants came from various regions in the world, and there was not one particular region overrepresented, so we believe that a possible effect of genetic background would have been limited. Furthermore, all blood samples were collected at the same time of day, which minimized the impact of circadian rhythms of hormones and immune cells on the results. It is important to note that in the original sample of the EpiPath cohort, ELA participants were almost 4-times more likely to have a chronic disease than controls. Participants with any chronic disease had higher CMV titers than healthy participants, which may have been secondary to medication use or the disease itself. To avoid effects of present disease and medication on the immune variables, we specifically selected a healthy subset of the cohort. Finally, we accounted for possible variation between experimental days by including this as a factor in the final statistical model.

4.5.3 Conclusion

By using specific cell surface markers of senescence, we were able to detect higher levels of T cell senescence associated with ELA in a relatively heterogeneous sample of individuals. Moreover, these differences were present many years after ELA had occurred. Leukocyte telomere length may obscure cell specific immunosenescence, therefore, the use of cell surface markers of senescence or measuring telomere length on isolated cell subsets will be more informative. Although CMV appears to play an important role, it is unclear whether CMV infection is a prerequisite for ELA-related immunosenescence. To our knowledge, there is no data showing an association between accelerated immunosenescence and ELA in CMV seronegative individuals. Future studies should include CMV as a confounder or selectively investigate CMV seronegative cohorts.

4.6 Acknowledgments

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Jonathan D Turner and Krystel Sias assisted in writing the manuscript, which was revised into its final format by all co-authors. The study was conceived by Claude P Muller and Jonathan D Turner with the support and contribution of Claus Vögele and Hartmut Schächinger. All authors read and approved the final manuscript.

5 Effects of Early Life Adversity on Mental and Physical Health in Early Adulthood

This chapter was created in collaboration with the Institute for Health and Behaviour of the University of Luxembourg and written by Violetta Schaan.

My contribution to this chapter:

Recruitment of study participants. I was involved in setting up and coordinating visit 1, including collection, transportation, and storage of saliva, DNA, and plasma samples. I was involved in the telomere length and salivary cortisol measurements, and performed the plasma IL-6 measurements, as well as the analysis of these data.

5.1 Introduction

Early parental separation can cause intense and even traumatic stress to the child concerned with adoption representing one of the most final and life changing events. Experience of significant early life stress (ELS) and adversity (ELA) prior to adoption and during the adoption process are frequently observed in adopted children (e.g. Gunnar et al., 2009). These include physical and psychological maltreatment as well as transitory placements in orphanages. The detrimental effects of ELA on mental health and social adjustment of these children are well documented and efforts have been made to elucidate its long-term pathophysiological impact by focusing on immune function (e.g. Elwenspoek et al., 2017).

5.1.1 Adoption and mental health

Adoptees have an elevated risk for developing behavioral and emotional problems during childhood (e.g. externalizing behavior, overall distress, depression; Cubito and Brandon, 2000; Wierzbicki, 1993) that are likely to become manifest as mental disorders in later life (Shonkoff et al., 2012). Furthermore, adoptees are more likely to face problems at school with respect to both academic achievement and peer-relationships (Brodzinsky et al., 1984; Verhulst et al., 1992; Wierzbicki, 1993). In addition, adoptees report lower self-esteem and higher emotional distress than non-adoptees (Lindblad et al., 2003; Miller et al., 2000), more social maladjustment, social loneliness and problems with intimacy (Feeney et al., 2007; Hjern et al., 2002). These problems might translate into difficulties in partner relationships and problems in finding employment during adulthood (Feeney et al., 2007). Furthermore, adoptees have been found to be less frequently married, to be less likely to have children and to have lower social competencies (Brodzinsky et al., 1984; Lindblad et al., 2003; Tieman et al., 2006).

The reasons for adoption are divers but ELA including maltreatment, such as neglect or abuse are common. In addition, the separation from one's main caregivers is difficult for children and may cause extreme insecurity. The healing trajectory from early maltreatment is complex. If maltreated, flight or fight

reactions are rarely possible: when being hit by a parent, for instance, it is difficult for the child to counter-attack or to run away. Expressions of anger are maladaptive and better suppressed than expressed. Freezing as a dissociative process helping to numb emotional and physiological impressions might help to survive, and to escape from these overwhelming situations (Chu and Dill, 1990). The main attachment person is associated with positive and with negative feelings making the relationship unpredictable, ambiguous and confusing for the child. Internal working models of attachment are assumed to be construed and subsequently transferred onto other social interactions and relationships (Steele et al., 2003).

5.1.2 Adoption and attachment styles

When adopted, children might be left with a feeling of being unwanted, rejected and abandoned (Feeney et al., 2007). Attachment styles have been observed to importantly mediate the effect of negative family experiences during childhood (e.g. before the adoption) and problematic social adjustment in later life (Jaeger et al., 2000; Riggs and Jacobvitz, 2002; Zimmerman et al., 2002). Secure attachment that has been associated with better relationship quality and the open expression of thoughts and feelings has been shown to buffer these negative effects (Feeney, 1999; Jaffari-Bimmel et al., 2006). Preoccupied, insecure and disorganized attachment styles that are more prevalent in adoptees seem to be problematic for the child's social development, for instance regarding peer rejection and acceptance, social esteem or social problems (Borders et al., 2000; van Ijzendoorn and Juffer, 2006). Time spent in a harmful family system or an institute might be critical, since the formation of a secure attachment bond with a main caregiver is impossible, leading the child to form undifferentiated relationships (van den Dries et al., 2009). Irhammer and Bengtsson (2004) observed that the development of insecure attachment organization was associated with late adoption (Irhammar and Bengtsson, 2004). Children that experience rejection at home (e.g. maltreatment or neglect) might develop negative working models of attachment, characterized by ideas that the self is unworthy of consideration and love and that others are untrustworthy and rejecting

(Bartholomew and Horowitz, 1991), and cope with these experiences by becoming increasingly sensitive to rejection. Coherently, anxious and insecure (i.e. avoidant and ambivalent) attached individuals show higher rejection sensitivity implying lower sense of belonging and lower perceived control over social interactions (Feldman and Downey, 1994; Khoshkam et al., 2012). Rejection sensitivity has been shown to be associated with negative behavioral tendencies such as anger and aggression, and interpersonal difficulties (Leary et al., 2006; Liu et al., 2014). Due to their social uncertainty (i.e. rejection sensitivity), adoptees might be more motivated to acquire relationship-threatening information, i.e. a tendency to be more suspicious, to make more sinister attributions in ambiguous situations and to entertain more paranoid cognitions about others, which have been shown to be maladaptive and to promote social rejection (Marr et al., 2012). While strong rejection sensitivity may not only play a role in the biased identification of ambiguous situations as threatening, it might also modify the intensity of experienced emotion thereby contributing to mental ill health. Attachment, interpersonal behavior and academic problems might importantly affect family interactions at home and vice versa. In a sample of adopted adolescents Beijersbergen and co-workers (2008) found that those with a dismissing attachment classification were more stressed during a mother-child conflict task than those classified as securely attached (Beijersbergen et al., 2008). As adoptees have been shown to be at risk to develop insecure attachment styles, these results reflect an increased vulnerability of adoptees to experience familial and interpersonal interactions as more stressful than non-adoptees. To the best of our knowledge, however, there is no research comparing interpersonal interactions in families with adopted children versus non-adopted children. Furthermore, studies on perceived chronic stress comparing adoptees to non-adoptees seem to be still missing.

5.1.3 Adoption and personality disorders

Taking into account the difficulties adoptees are likely to experience during a sensitive period of personality development, it is not surprising that they continue to have more interpersonal difficulties characterized by delinquent, oppositional, aggressive and antisocial behavior (Austad and Simmons, 1978;

Offord et al., 1969; Sharma et al., 1998), as well as other behavioral problems (Bimmel et al., 2003; Juffer and van Ijzendoorn, 2005). Those childhood difficulties can translate into personality disorders in later life. Previous studies investigating personality disorders in adoptees have mostly focused on specific disorders. They report increased odds for antisocial, schizotypal, schizoid, paranoid, avoidant, dependent, and obsessive-compulsive personality disorders, as well as externalizing disorders including attention-deficit/hyperactivity disorder, conduct disorder and oppositional defiant disorders in adoptees as compared to non-adoptees (Cadoret et al., 1995; Crowe, 1974; Keyes et al., 2008; Reichborn-Kjennerud et al., 2007; Tienari et al., 2003). There are only few reports investigating the whole spectrum of personality disorders in adult adoptees. Simon and Senturia (1966), for example, found a higher incidence of personality disorders in a sample of 35 adopted patients as compared to the entire clinical sample (Simon and Senturia, 1966). Additionally, a recent epidemiological study analyzed data from 378 adoptees and 42.503 non-adoptees and found adoptees to be at an increased risk for histrionic, antisocial, avoidant, paranoid, schizoid or obsessive-compulsive personality disorders. Unfortunately, they did not include a measure for borderline personality disorder as they assessed participants using the Alcohol Use Disorder and Associated Disabilities Interview Schedule (AUDADIS; Westermeyer et al., 2015). The current study aimed at closing this gap in the literature, expecting an increased risk for borderline personality disorders in adoptees, based on their increased vulnerability for social and emotional difficulties.

5.1.4 Adoption and physiological, endocrine and immunological abnormalities

In addition to a higher risk for mental ill health, there is also evidence that ELA can negatively impact physical health (Elwenspoek et al., 2017). Individuals with a history of ELA, for example, have been found to show altered stress responses (e.g, Lovallo et al., 2012; Schwaiger et al., 2016) and abnormalities in immune function and hormone levels. Hypothalamic–pituitary–adrenal (HPA) axis responses to stress, for example, are reduced in participants reporting childhood maltreatment, as reflected in a blunted cortisol response to stress (Carpenter et al., 2007). This is in line with previous findings of hypocortisolism in

children raised in Romanian orphanages (Carlson and Earls, 1997), foster care (Bruce et al., 2009a; Gunnar and Vazquez, 2001) or those surviving extreme environmental conditions (e.g. earthquake; Goenjian et al., 1996). Cortisol dysregulation has been associated with depression, anxiety disorders and post-traumatic stress disorder (PTSD; Bierer et al., 2006; Burke et al., 2005; Griffin et al., 2005; Hansen-Grant et al., 1998; Holsboer et al., 1995; Wessa et al., 2006; Yehuda, 2005, 2006). While some disorders seem to be associated with hypercortisolism (e.g. depression), others (e.g. PTSD) have been linked with hypocortisolism. Nevertheless, there are also contradictory reports (Inslicht et al., 2006; Keller et al., 2006; Peeters et al., 2004; Young et al., 2004), so that replication of these findings is wanting. These inconsistencies in the literature could be related to HPA axis programming during early developmental stages as for instance changes in cortisol release (chronic or stress induced) might be differently related with psychological functioning depending on whether or not ELA was present during childhood. It might for instance be that cortisol release is associated with mental health in the general population, but that early changes in HPA axis functioning shadow those associations in persons who experience ELA. The current study, therefore, aimed at investigating the link between cortisol levels and mental disorders depending on ELA status.

Immune function not only depends on dispositional (i.e. genetic) factors but also on environmental factors such as stress. Cytokines, such as interleukins (e.g. IL-6), serve an important immune-modulatory function. IL-6 has been shown to be strongly implicated in aging (Daynes et al., 1993; Ershler, 1993) and immune regulation (Daynes et al., 1993; Van Snick, 1990), in that increased levels in IL-6 reflect inflammation or trauma (Lutgendorf et al., 1999). However, IL-6 has not only been related to physical but also to mental ill-health and other psychological factors, e.g. depression (Dentino et al., 1999), poor sleep efficiency and poor social relationships (Friedman et al., 2005). In this study, we aimed at further investigating the relationship between IL-6, mental well-being and the impact of ELA on those associations.

Another biomarker of immune function, or more precisely immunosenescence, is telomere length. Telomeres are protective caps that are situated at the end of chromosomes. With time, telomeres progressively shorten, reflecting a normal process of aging. This process of telomere attrition, however, can be enhanced by environmental factors such as stress. Recent studies not only found correlations between telomere length and perceived stress and mental health, but also with mood disorders and personality factors such as pessimism (Epel et al., 2004; Epel et al., 2006; Huzen et al., 2010; Simon et al., 2006). There is also evidence that ELA might be associated with telomere erosion (Kananen et al., 2010). By including leukocyte telomere length in the current study, we aimed at further investigating the relationship between telomere length and mental well-being, and the impact of ELA (i.e. adoption) on those associations.

In summary, the current study was designed to further investigate the processes that may be involved in conferring the increased risk for mental and physical ill health in those experiencing ELA. In contrast to previous research, we used face-to-face structural clinical interviews in addition to self-report questionnaires, focusing not only on psychological vulnerability but also on family interactions at home. Specifically, we included the family context and investigated if adoptees experience more social tension during family interactions than non-adoptees. Additionally, we broadened the focus by investigating differences in perceived chronic stress. We hypothesized that adoptees experience more chronic stress during social interactions than participants raised by their biological parents. Previous research failed to provide data on the whole range of personality disorders (e.g. by excluding borderline personality disorder). As we specifically expect an increased vulnerability for enhanced social and emotional difficulties in adoptees, it seems crucial to consider borderline personality disorder when presenting data on personality disorders. With regard to immune changes, we aimed at investigating the link between cortisol, IL-6, telomere length and mental ill health, depending on ELA status.

5.2 Material and methods

5.2.1 Procedure

All participants were recruited between July 2014 and March 2016 in Luxembourg and the Greater Region Saar-Lor-Lux. The study design was approved by the National Research Ethics Committee (CNER) and the Ethics Review Panel (University of Luxembourg). All participants provided written informed consent. The study (Figure 22) consisted of two separate visits: one visit for physiological measurements including a stress test, and a second visit for clinical psychological assessments. A detailed description of the procedure for the first visit is reported elsewhere (Chapter 2, 3, and 6). In short, participants underwent a 3 min standardized laboratory stress test that consisted of a combination of a cold pressor test and a mental arithmetic task. At several time points before and after the test, blood and saliva samples were collected and cardiovascular parameters were recorded. All participants were reimbursed with €150 for their full participation. Prior to participation, all volunteers were screened for exclusion criteria such as age between 18-35 years old. To reduce interindividual variation, baseline blood samples of each participant were collected between 11 am and 12 pm, participants were asked to not consume alcohol or caffeine, smoke, or exercise on the day of the first visit, and women either used hormonal contraceptives or were in the luteal phase of the menstrual cycle. While the present paper only includes relevant baseline and mental health data, findings from the stress testing and the full analysis of baseline immune differences are reported elsewhere (Chapter 3 and 6).

Second visit. Participants were invited to the University of Luxembourg to attend a three-hour screening session involving several questionnaires and the German version of the Structured Clinical Interview for DSM-IV Axis I and Axis II Disorders (SCID-I and –II;(Fydrich, 1996; Wittchen, 1997). All interviews were conducted by the first author – a psychologist and clinical psychology trainee, supervised by the last author, a chartered clinical psychologist. Feedback to the participant and contact information on clinical services

was provided, if requested by the participant. In case of suicidal ideation, an emergency procedure was

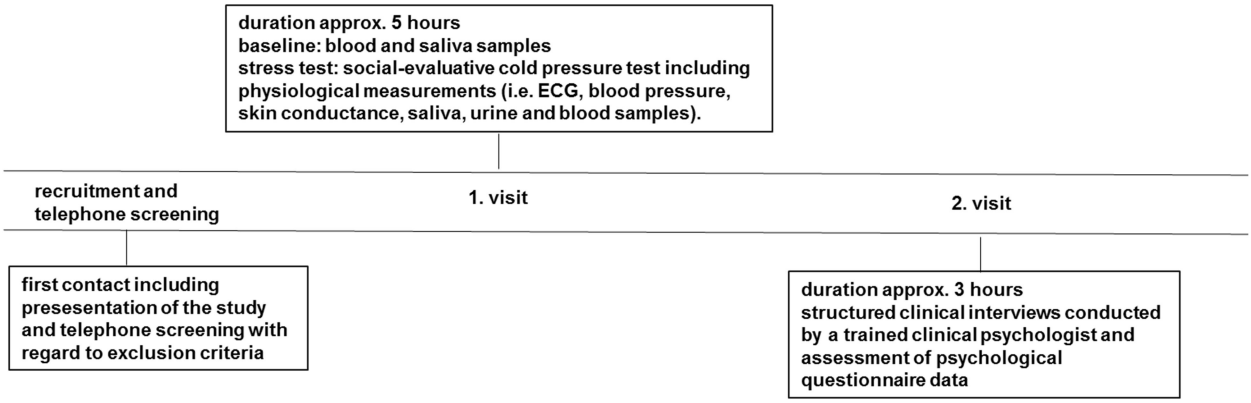

Figure 22. Illustration of the general project procedure.

followed as approved by the Ethics Review Panel (University of Luxembourg). In the present sample, there were no cases of emergency hospitalization.

5.2.2 Sample

Thirty-five adoptees (11 men) of 42 who attended the first visit and 70 control participants (24 men) of 73 attending the first visit took part in the interview session and provided data regarding their history of adoption: 5 adoptees were adopted from Brasil, 2 from China, 2 originated from Columbia, 1 from Nepal, 10 were born in Peru, 7 in Rumania, 1 in Seoul, 1 in Sri Lanka, 1 in Thailand and 4 in South Korea (Figure 23). One adopted participant was unsure about her origin. Five of the interviewed adoptees lived in foster families prior to adoption. Mean age at adoption of the interviewed sample was 14.9 month (SD=24.16 month, range: 1-108 month). Adoptees reported lower educational

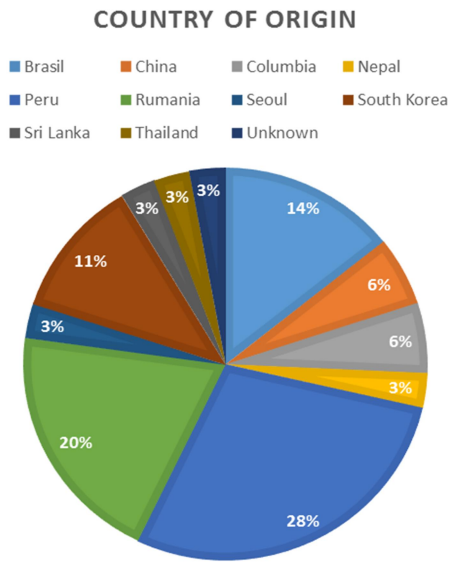

Figure 23. Country of origin

background than non-adoptees (adoptees: M=3.37; SD= 1.33; non-adoptees: M= 3.85 SE= 0.912; for details please refer to Table 15; Chi^2=16.07; p=.003) and approximately the same number of siblings (adoptees: M=2.29, SD=0.975; non-adoptees: M=2.36, SD=1.029). Mean age of interviewed adoptees was 24.63 years (SD=3.85), and 22.28 years (SD=2.54, t(49,09)=-3.325, p=.002) in the control group.

Table 15. Educational background by group

Educational background	**Non-adoptees**	**Adoptees**
	N (%)	N (%)
Certificate of secondary education	4 (5.8%)	3 (8.6%)
General certificate of secondary education	2 (2.9%)	9 (25.7%)
Advanced technical college entrance qualification	4 (5.8%)	3 (8.6%)
University-entrance diploma	49 (71%)	12 (34.3%)
University degree	10 (14.5%)	8 (22.9%)

5.2.3 Psychological data

Childhood trauma was measured using the Childhood Trauma Questionnaire (CTQ, Klinitzke et al., 2012). This 28-item questionnaire assesses childhood trauma on five subscales: Emotional Abuse (α=.78), Physical Abuse (α=.85), Sexual Abuse (α=.52), Emotional Neglect (α=.87) and Physical Neglect (α=.41). The CTQ has been shown to have good psychometric properties in previous research (Klinitzke et al., 2012) and also in this study, psychometric properties are convincing (α=.79 for the global scale).

The Beck Depression Inventory (BDI-II, Kuhner et al., 2007) was used to assess depressive symptoms. The inventory consists of 21 items and has been shown to have good psychometric properties (α>.84 in Kuhner et al., 2007), in this study: α .86).

Chronic stress was assessed using the Trier Inventory for Chronic Stress (TICS; Schulz, 2004). The 57-item inventory assesses chronic stress on the following nine dimensions: Work Overload, Social Overload,

Pressure to Perform, Work Discontent, Excessive Demands from Work, Lack of Social Recognition, Social Tensions, Social Isolation, and Chronic Worrying. All items are rated on a five-point Likert scale ranging from 0 =never to 4 very often and reflecting the frequency of specific experiences (e.g. "Although I try, I do not fulfill my duties as I should."). The psychometrics properties are excellent in this study (α=.95).

Participants were interviewed with the two parts of the structured clinical interview for, DSM-IV disorders (SCID-I: DSM-IV axis 1 disorders, i.e. major mental disorders; SCID-II: DSM-IV axis 2 disorders, i.e. personality disorders). A questionnaire preceded the SCID-II interview to shorten the interview time, as negated items could be skipped. If previously agreed by the participant, interviews were audiotaped for validation purposes. The SCID-I and II are currently used as the gold standard in determining clinical diagnoses. In the present study 20% of the interviews were rated by a second trained person to assess interrater-reliability.

The Circumplex Model of Marital and Family Systems and the Family Adaptability and Cohesion Evaluation Scales (FACES; Olson, 2011) was used to assess family flexibility (e.g. flexibility with regard to leadership and relationship rules), cohesion (i.e. emotional bond between family members) and communication (i.e. ability to alter levels of flexibility and cohesion). The questionnaire consist of 62 items assessing the two dimensions: cohesion and flexibility each measured with three different subscales that are rated on a 5-point Liker-scale ranging from 1=strongly disagree to 5=strongly agree. The subscales 1) disengaged, 2) balanced cohesion and 3) enmeshed measure the dimension of family cohesion while the subscales 4) rigid, 5) balanced flexibility and 6) chaotic measure family flexibility. Ratio scores are calculated for Cohesion (= Balanced Cohesion/(Disenganged + Enmeshed)) and Flexibility (Balanced Flexibility/(Rigid + Chaotic)) with higher scores reflecting a healthier family system. The instrument also allows calculating a score for global family satisfaction and communication within the family. In addition, participants were asked to indicate how often they had experienced conflicts with their parents and siblings, how intense they were and how long they usually lasted on a scale ranging from 1 (not often/not intense/very short) to

6 (very often/very intense/very long). Furthermore, participants were asked to fill out the Parental Bonding Instrument (PBI; Parker, 1979, 1983) for both fathers and mothers, with each version consisting of 21 items that are answered on a 4-point Likert scale ranging from 0=very unlikely to 3= very likely.

5.2.4 Immune parameter

The methods of the following measurements that were performed in this cohort have been reported elsewhere (Chapter 3): salivary cortisol concentrations, plasma levels of IL-6 and leukocytes telomere length (T/S).

5.2.5 Statistical analysis

All data were analyzed using *SPSS 18*. Randomization effectiveness with regard to demographic variables and personality traits were controlled using preliminary Chi-square tests, *t*-tests or analyses of variances (ANOVA). Kolmogorow-Smirnow and Mauchly's tests were performed to test for the normal distribution and sphericity assumptions, respectively. All scores were z-transformed and outlier identification was realized for all variables. Extreme values (>3 *SD*s above the mean) were set to missing.

5.3 Results

5.3.1 Psychological vulnerability

To test the hypothesis that adoptees report more psychological symptoms, an ANOVA was calculated using the depression scores of the BDI as dependent variable. Adoptees (M=.486, SD=.86) reported significantly higher BDI scores than control participants (M=-.335, SD=70; t(102)=-5.165, p<.001). Chi^2-tests were carried out to test for group differences in the frequency of SCID-I and SCID-II diagnoses. Adoptees were diagnosed with a higher number of SCID-I (24 out of 35) and SCID-II (9 out of 35) diagnosis compared to non-adoptees (SCID-I: 11 out of 71, Chi^2=22.692, p<.001, Table 16; SCID-II: 1 out of 71, Chi^2=16.210, p<.001, Table 17). We further categorized the diagnoses following the DSM-IV into anxiety disorders, mood disorders, substance (abuse) disorders, eating disorders, acute stress disorder and PTSD. There was high comorbidity between these disorders, explaining the case numbers in the tables below.

Table 16. Number of SCID-I diagnoses shown separately for adoptees and non-adoptees (percentages in parentheses).

SCID-I	**Adoptees N=35**	**Non-adoptees N=71**	**Chi^2**
Anxiety Disorder	13 (31%)	9 (12.3%)	8.533, p=.003
Mood Disorder	14 (33.3%)	3 (4.1%)	22.281, p<.001
Substance	9 (21.4%)	4 (5.5%)	8.785, p=.003
Abuse	7 (16.7%)	3 (4.1%)	6.828, p=.009
Dependency	3 (7.1%)	1 (1.4%)	3.313, p=.069
Eating Disorders	1 (2.4%)	2 (2.7%)	1.580, p=.209
Acute Stress Disorder	1 (2.4%)	0	2.048, p=.152
PTSD	1 (2.4%)	0	2.048, p=.152

Table 17. Number of SCID-II diagnoses shown separately for adoptees and non-adoptees (percentages in parentheses). The table shows in total 10 diagnosed personality disorders for adoptees, as one out of the 9 adoptees being diagnosed with a personality disorder fulfilled the criteria for a comorbid personality disorder consisting of a borderline and an antisocial personality disorders diagnosis. NOS= personality disorder not otherwise specified.

SCID-II	Adoptees N=35	Non-adoptees N=71	Chi^2
Narcissistic	1 (2.4%)	0	2.048, p=.152
Histrionic	0	0	
Dependent	2 (4.8%)	0	4.135, p=.042
Obsessive compulsive	1 (2.4%)	0	2.048, p=.152
Borderline	3 (7.1%)	0	6.263, p=.003
Antisocial	1 (2.4%)	0	2.048, p=.152
NOS	1 (2.4%)	1 (1.4%)	0.266, p=.606
insecure	1 (2.4%)	0	2.048, p=.152

5.3.2 Childhood trauma

Pairwise comparisons between groups were carried out using the CTQ sum score as dependent variable. Adoptees (M=-.089, SD=0.75) did not report more traumatic experiences than control participants (M=-.087, SD=0.80, t(102)=-0.12, p=.990). We also calculated a MANOVA entering all subscales as dependent variables. The MANOVA failed to reach significance with F(5,93)=0,611, p=.692, η^2=.032. No group differences on the subscales reached statistical significance (p>.25).

5.3.3 Self-esteem

An ANOVA using adoption status as independent variable and self-esteem as dependent variable showed significantly lower self-esteem scores for adoptees (M=-.263, SD=.88) than for non-adoptees (M=.186, SD=.96; t(101)= 2.432, p=.017).

5.3.4 Chronic stress

Using a MANOVA we tested for group differences in chronic stress, with the different subscales of the TICS as dependent variable and group (adoption vs. control) as between subject factor, yielding a significant

effect (F(10,95)=2.315, p=.017, η^2=.196). The between subject effects revealed significant differences for the subscales: Social Overload (F(1,104)=8,568, p=.004, η^2=.075), Social Tension (F(1,104)=11,015, p=.001, η^2=.096), Chronic Worrying (F(1,104)=4.619, p=.034, η^2=.043), Screening scale for chronic stress (F(1,104)=4.072, p=.045, η^2=.038), Social Isolation (F(1,104)=3.3966, p=.049, η^2=.037), Lack of Social Recognition (F(1,104)=4.153, p=.044, η2=.038) and Work Discontent (F(1,104)=5.909, p=.017, η^2=.054). None of the other subscales reached significance: Excessive Demands from Work (F(1,104)=3.859, p=.052, η^2=.036), Pressure to Perform (F(1,104)=.257, p=.613, η^2=.002) and Work Overload (F(1,104)=.363, p=.548, η^2=.003; for mean values please refer to Figure 24). We also calculated sum scores reflecting high demands and lack of satisfaction. The multivariate analysis entering both scales as dependent variables was significant (F(2, 107)=4,692, p=.011, η^2=.081), indicating than adoptees scored significantly higher on the scale lack of satisfaction than non-adoptees (*F*(1,109)=9,160, *p*=.003, η^2=.078; adoptees: *M*=.376, *SD*=.95; non-adoptees: *M*=-.206, *SD*=.97). There was no difference regarding the factor high demands (F(1,109)=1.112, p=.294, η^2=.010; adoptees: *M*=.136, *SD*=1.06; non-adoptees: *M*=-.074, *SD*=.96).

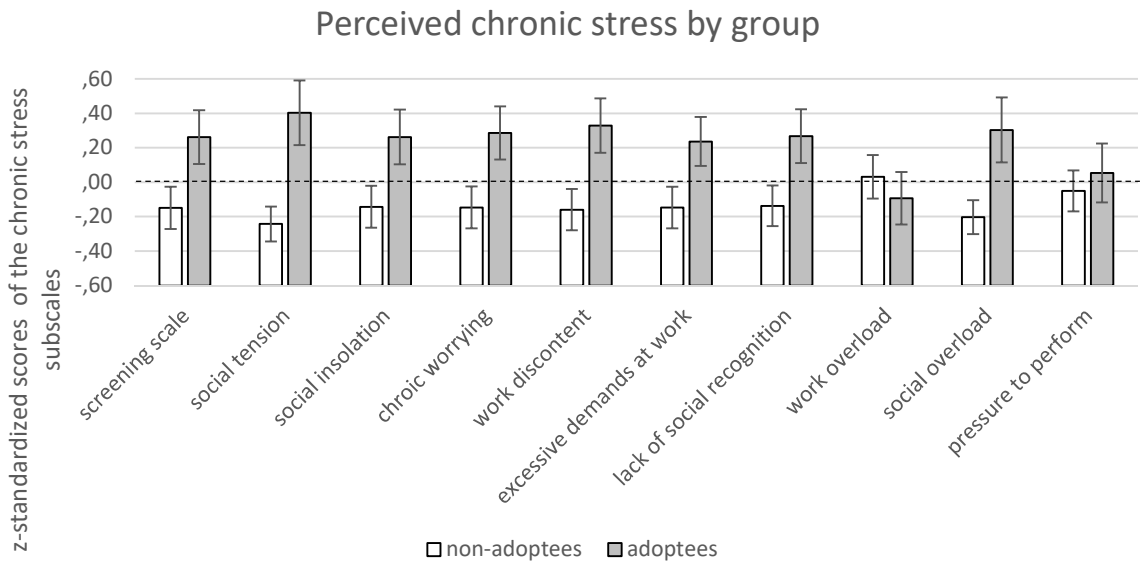

Figure 24. Illustration of the chronic stress subscale scores per group (non-adoptees vs. adoptees). Error bars indicate one standard error.

5.3.5 Family dynamics

We also tested if adoptees report more conflict in family relationships than control participants do by calculating two separate MANOVAs entering frequency of conflicts, intensity and duration for both parental conflicts and conflicts with siblings, respectively. Neither analysis reached significance (p>.40). We calculated another MANOVA entering the subscales of the FACES (cohesion, flexibility, communication, satisfaction) as dependent variables. There were no difference between adoptees and non-adoptees regarding these family dynamics (p>.40). Additionally, we analyzed parental care and overprotection as measured with the parental bonding inventory. Parental overprotection and care both from fathers and mothers were introduced as dependent variables and adoption status was used as independent variable. As was the case for the previous analyses there were no significant effects (p=.26).

5.3.6 In-depth analysis

Regression analyses revealed significant predictions for depressive symptoms: when adoption (*b*=.357, *p*>.001), rejection sensitivity (*b*=.179, *p*>.034) and chronic stress (*b*=.352, *p*>.001) were introduced as predictors for depression, the model predicted up to 38% of the total variance in the BDI sum score (*F*(3, 98)= 19.617, *p*<.001).

To investigate how the different scales measuring mental well-being (i.e. chronic stress, self-esteem, depression and rejection sensitivity) related to one-another in both groups, we calculated correlations separately for both groups (control vs. adoptees). We used Spearman rank correlations for dichotomous variables (i.e. SCID-I, SCID-II), and Pearson's correlations for continuous variables. SCID-II data are not reported for the control group, as there were not enough diagnoses to calculate meaningful results. To assure that the observed differences in correlations coefficients between both groups were significant a Fisher r-to-z transformation (website: http://vassarstats.net/rdiff.html) was used. Chronic stress correlated significantly in the control group with self-esteem (r=-.748, p<.001), and depression (r=.376, p=.001) but also in the adoption group (self-esteem: r=-.406, p=.010; depression: r=.574, p<.001). Participants who

scored high on self-esteem reported lower rejection sensitivity scores (control group: r=-.252, p=.034; adoption group: r=-.418, p=.017). Rejection sensitivity scores were not significantly correlated in the control condition with depression scores as measured with the BDI (r=.203, p=.09) whereas this correlation was highly significant in the adoption group (r=.500, p=.004, z=.155, p=.121). The post-hoc Fisher r-to-z transformation however suggests that the observed differences in correlation coefficients were not significant. While there was no correlation between chronic stress and SCID diagnoses in the control group (SCID-I: rho=.078, p=.526), there was a strong correlation in the adoption group (SCID-I: rho=.407, p=.019, z=1.61, p=.107; SCID-II: rho.365, p=.037). Overall, chronic stress was strongly associated in both groups with self-esteem (control group: r=-.681 p<.001; adoption group: r=-.370, p=.02) and depression (control group: r=.314, p=.009; adoption group: r=.488, p=.005). Childhood trauma was neither associated with self-esteem (r=-.141, p=.449) and rejection sensitivity (r=.277, p=.124) in the adoption group nor in the control group (self-esteem: r=-.197, p=.099; rejection sensitivity: r=.206, p=.085). However, depression scores correlated significantly with childhood trauma in the control group (r=.343, p=.003) but not in the adoption group (r=.075, p=.688). However, this difference in correlation coefficients was not significant (z=1.26, p=.207).

5.3.7 Immune data

Outliers caused by documented technical errors were eliminated from analysis and data z-standardized. In a first step, we conducted three separate analyses of variance to investigate group differences at baseline with regard to cortisol, IL-6 and telomere length. In a second step, we calculated correlations for the entire sample between cortisol, IL-6 and telomere date and mental health symptoms. To investigate those correlations in more detail, we analyzed the data separately for each group.

5.3.8 Cortisol

We conducted an ANOVA using salivary cortisol levels as dependent variables and adoption as between subject factor. There were no significant differences between adoptees (*M*=-.077, *SD*=.88) and non-adoptees (*M*=-.116, *SD*=.74; *t*(1, 106)=-.241, *p*=.810).

5.3.9 Interleukine 6

The ANOVA using plasma IL-6 as dependent variable and group (adoption vs. control) as between-subject factor did not yield any significant results (adoptees: *M*=-.0815, *SD*=.87; non-adoptees: *M*=-.163, *SD*=.66; t(1,101)=-.535, *p*=.594).

5.3.10 Telomere length

We conducted an ANOVA to investigate differences in telomere length between the adoption (*M*=-.079, *SD*=.88) and the control group (*M*=-.047, *SD*=.93). The main effect of group was not significant (*t*(1,105)=.177, *p*=.860).

5.3.11 Correlational analysis

To investigate differences in immune parameters such as cortisol response, telomere length and IL-6 levels between the groups and in relation with psychological indicators of mental disorders and chronic stress, we split the data according to group (adoption vs. control group).

In the control group, SCID-I data significantly correlated with plasma IL-6 (rho=.269, p=.034) and telomere length (rho=-.248, p=.043). There was no significant correlation between these parameters in the adoption group (plasma IL-6: rho=-.182, p=.310, z=2.05, p=.04; telomere length: rho=.036, p=.846, z=2.82, p=.005). Additionally, cortisol as measured in saliva correlated significantly with the depression score in the control group (r=.401, p=.001) but not in the adoption group (r=-.256, p=.158, z=3.06, p=.002). Chronic stress correlated significantly in the adoption group with plasma IL-6 (r=.381, p=.02), whereas no correlation could be observed in the control group (r=-.036, p=.782, z=2.03, p=.042).Regression analysis involving cortisol, IL-6 and telomere length revealed no significant predictions for depressive symptoms.

5.4 Discussion

ELA as experienced by adoptees during their pre-adoption period has been shown to lead to elevated risks of developing behavioral and emotional problems (e.g. Cubito and Brandon, 2000; Wierzbicki, 1993), insecure attachment styles (Borders et al., 2000; van Ijzendoorn and Juffer, 2006) and more social problems (Feeney et al., 2007; Hjern et al., 2002). Those childhood difficulties might increase the risk for mental disorders as well as personality disorders in later life as that has been reported for adoptees (Grant and Higgins, 2003; Simon and Senturia, 1966) but never been investigated using a structured clinical interview for the whole spectrum of personality disorders.

With the current study, we aimed to further understand the pathway of ELA to mental ill health. In contrast to previous research, we included face-to face structural clinical interviews as well as self-reported questionnaire data to assess psychological well-being and family interactions at home. By extending previous research with our scope on chronic stress, the quality of family relationships and the whole spectrum of personality disorders, we strove to provide new insight into the processes conferring the increased vulnerability of adoptees to suffer from mental ill health. Adding indicators of immune changes, we intended to provide a deeper understanding of the ELA – well-being relationship.

The results illustrate an increased vulnerability of adoptees to suffer from more depressive symptoms than non-adoptees, which is in agreement with previous reports (Wierzbicki, 1993). The SCID-I and SCID-II data, reflecting more diagnosis of mental and personality disorders in adoptees as compared to non-adoptees, support these findings and is in line with the research conducted by for instance Grant and colleagues (2003) or Simon and Senturia (1966). The inclusion of the borderline personality disorder revealed to be highly meaningful, as it reflected 30% of all personality disorders diagnosed in the adoption sample, highlighting their difficulties to regulate their emotions especially in social situations. People suffering from borderline personality disorder often have instable social relationships and reduced

occupational functioning (Salz, 1983). Indeed, adoptees scored higher on the subscale “lack of satisfaction” as compared to non-adoptees with increased odds to experience social overload, social tension, social isolation, lack of social recognition, chronic worrying and work discontent in their daily life. Additionally, the finding that adoptees scored lower on self-esteem than non-adoptees is coherent with their increased vulnerability to a) fulfill the diagnosis of a mental disorder and/or a personality disorder, b) of experiencing chronic stress and c) to suffer from more depressive symptoms. However, interpersonal problems do not seem to reflect family interactions at home, as no difference between adoptees and non-adoptees could be observed. It might be that either family interactions are not perceived as threatening and therefore do not trigger dysfunctional emotional and behavioral reactions or that adoptive parents found effective ways to adapt to the special needs of their children. On one hand, it might be that they found strategies to help their children regulate their emotions at home and within family interactions. However, the translation of those skills seem to remain difficult outside. On the other hand, it might be that parents adapted themselves to meet their children’s need and to avoid conflicts at home. An investigation of family interactions in adoptees using interaction tasks have been established by previous research with children of younger ages (Allen et al., 1994; Allen et al., 2003) but we do not know of any comparable studies in adults. To our knowledge these family interaction tasks unfortunately only focused on autonomy and family relatedness but not on functional or dysfunctional parent-child interactions (Allen et al., 1994; Allen et al., 2003) – therefore not helping us to shed light on the above discussed interpretations. It would be very valuable to assess adolescents and their parents with structural clinical interviews and subsequent interaction tasks with a focus on functional and dysfunctional communication to understand how the family achieves to live together in harmony.

Results of chronic stress being significantly associated with reduced self-esteem and higher depression scores support previous observations (Lo, 2002; Tafet and Bernardini, 2003). Participants who reported more childhood trauma or rejection sensitivity, suffered from more depressive symptoms as has been

reported previously (Schaan and Vogele, 2016). Additionally, participants with higher self-esteem scored lower on rejection sensitivity, which is in line with a study from Ford and Collins (2010), who found that individuals with low self-esteem reacted stronger to social rejection as compared to participants with high self-esteem (Ford and Collins, 2010). The finding that chronic stress correlated with SCID-I and SCID-II diagnosed mental disorder in the adoptive sample probably reflects the increased difficulty to cope with daily activities while being burdened with a mental or personality disorder. It seems likely, that the correlation did not reach significance in the control group due to the low number of diagnosed participants (11 out of 71). Overall, results of the regression analyses demonstrate that individuals with a history of adoption, high rejection sensitivity and more chronic stress were more likely to suffer from depressive symptoms.

Interestingly, we did not find differences between adoptees and non-adoptees regarding baseline measurements of saliva cortisol, plasma IL-6 data and telomere length. The saliva cortisol measurement was only based on one time point and variations between groups might not have been visible due to small variations per participants. Recent literature suggests a blunted saliva cortisol response over the day with differences between family-reared adoptees and a post-institutionalized control group in the morning but not in the afternoon (Tarullo and Gunnar, 2006). Additionally, changes in the HPA-axis were observed to rebound after post-institutionalization to a normal diurnal cortisol pattern (Gunnar et al., 2001; Kertes et al., 2008). Therefore, it might be that we missed the time-spot for significant differences by measuring cortisol years after adoption or at a non-meaningful time point during the day (at noon and not in the morning). Furthermore, it might be that in our sample of adoptees the HPA axis was not (yet) affected by ELA (Chapter 7). It would be very valuable if future studies would have a closer longitudinal look at HPA axis functioning depending on ELA to understand the underlying moderators of change.

The non-significant finding regarding IL-6 levels between both groups is in line with a study from Carpenter and colleagues (2010), who also did not find differences in IL-6 levels between maltreated and

control participants at baseline. The ability of proinflammatory cytokines to inhibit hippocampal neurogenesis was identified as one candidate mechanism for their detrimental effects on mood. It might be that our participants were too young to display dysregulations of plasma IL-6, as dysregulations of immune parameters might accumulate over time (Koss and Gunnar, 2017). Additionally, IL-6 is difficult to measure, because in absence of disease and inflammation these levels are often below the detection limit for most methods. Although we used the most sensitive method, there were still some values below the detection limit, which makes the data less sensitive to detect small differences between both groups.

Previous studies have found associations between ELA and shortened telomeres (e.g. Kananen et al., 2010; Surtees et al., 2003; Tyrka et al., 2016), however, there were also some studies not being able to replicate those findings (Mason et al., 2015; Verhoeven et al., 2015); for a review refer to Elwenspoek et al., 2017). As other studies mostly included clinical populations that by definition are more likely to show elevated IL-6 levels (Elwenspoek et al., 2017), differences in findings might be related to the choice of study populations. The results are in line with previous studies that found no differences between both groups. It might be that not all immune cells are equally affected and that a measure of telomere length in the whole blood masks cell specific effects (Chapter 4). Telomere length displays a very high inter-individual variation, already at birth. This is why expected differences are small and large populations necessary to find significant results. Additionally a reference or baseline measurement measured some years before the study or directly at birth would have increased the power to find variations between both groups. Other studies looking at telomere length often use more than 1000 participants (e.g. Surtees et al., 2003; Verhoeven et al., 2015).

However, within the two groups, psychological health was differently related to these above-mentioned immune parameters: in the control group, mental health (either measured by a SCID-I diagnosis or the BDI depression score) correlated with plasma IL-6, telomere length and saliva cortisol, whereas no such correlation could be found in the adoption group. IL-6 concentration was found to be increased in people

suffering from major depression compared with control participants (Dowlati et al., 2010), supporting the correlations found in our non-adoptive sample. Additionally, telomere length was recently observed to be inversely associated with lifetime depression exposure and shown to be shortened in people diagnosed with a mood disorders (Hartmann et al., 2010; Simon et al., 2006; Wolkowitz et al., 2011). Saliva cortisol was found to be associated with depressive symptomatology (Pruessner et al., 2003b), again supporting the correlations found for non-adoptees. Interestingly, those associations were not found in the adoptive sample. As the variances within both groups did not differ significantly from each other, these results do not seem to be related to different distributions in both groups. This finding might indicate a relative undocking of emotional disturbances and immune system functioning in adoptees. However, future studies are needed to validate and to provide a deeper understanding of the underlying mechanism of these findings.

5.5 Acknowledgments

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6 Blunted Endocrine Response to a Combined Physical and Cognitive Stress Test in Participants who Experienced Adverse Childhood Events

This chapter was created in collaboration with the Department of Clinical Psychophysiology at University of Trier and written by Xenia Hengesch.

My contribution to this chapter:

Recruitment of study participants; I was involved in setting up and coordinating visit 1, including scheduling of participants, nurses, and experimental rooms, performing the stress test, sample collection, salivary cortisol measurements and analysis.

6.1 Abstract

Early life adversity (ELA) impair health in adulthood. Altered stress physiology has been proposed as a mediator of this long-term effect. However, large inhomogeneity of stress tests used, as well as deficits in control of physical, cognitive and social stressor components, hampered previous studies to conclude on whether ELA enhance or diminish stress reactivity in adult participants.

In a first, randomized, controlled validation study (n=56) the combination of a bilateral feet cold pressor test (CPT) and a standardized mental arithmetic task (Paced Auditory Serial Addition Task; PASAT) was shown to evoke strongest stress responsiveness.

In a second study the above described stress procedure was used in 21 participants who had experienced severe ELA (separation from parents, institutionalization, and adoption in early childhood), and in 21 age-matched control volunteers. Blunted stress reactivity of the hypothalamus-pituitary-adrenal (HPA) axis was found in the ELA group, while cardiovascular reactivity was similar, and self-report ratings were even higher in the ELA group as compared to the control group.

These results demonstrate the validity and feasibility of a new CPT and PASAT stress procedure, and suggest a selectively inhibited HPA axis, but unchanged cardiovascular responsiveness in ELA participants during exposure to a physical and cognitive stressor. This pattern of response separation may best be explained by ELA selectively enhancing central feedback-sensitivity to glucocorticoids.

6.2 Introduction

It is well established that early life adversity (ELA), e.g. parental separation, and/or childhood maltreatment may impair physical and mental health in adulthood (Felitti et al., 1998; Wegman and Stetler, 2009) (Kendler et al., 1992) (Chrousos, 2009). It is surprising, however, that ELA-associated disorders manifest many years after ELA-exposure (Green et al., 2010). This raises questions about the long-term mechanisms underlying the etiology of ELA-associated disorders. One hint towards an answer to this question is the finding that ELA particularly increase the incidence of stress-related mental and cardiovascular disorders (Batten et al., 2004; McCauley et al., 1997), suggesting that stress physiology, and Autonomic Nervous System (ANS) and Hypothalamus-Pituitary-Adrenal (HPA) axis function may contribute to ELA pathophysiology. Indeed, ELA were found to be associated with increased sympathetic tone and disturbances of the HPA axis in late childhood, e.g. at age of 10-12 years (Gunnar et al., 2009).

There is corroborating evidence from rodent models. Mother-pup separation in the neonatal period, as well as reduced maternal care behavior in early life decrease offsprings' physiological adaptive reactivity to stress episodes throughout later life (Francis et al., 1999; Francis and Meaney, 1999; Liu et al., 1997; Plotsky and Meaney, 1993). Evidence from research involving humans, however, is inconsistent. While some studies have found ELA to be associated with hyper-responsiveness of the stress response systems, especially the HPA axis (Heim et al., 2000; Pesonen et al., 2010), others report hypo-responsiveness and a suppression of the stress response to a psychosocial stressor in individuals maltreated in childhood (Carpenter et al., 2007; Carpenter et al., 2011).

Such differences may be explained by a large degree in heterogeneity in severity, onset and duration of ELA (Heim et al., 2008), but also in the inhomogeneity of stress tests employed across these studies. Furthermore, many laboratory stress paradigms are hampered by standardization problems relating to

protocol, timing, intensity, cognitive load, social components, and other implementation issues (Steptoe and Vogele, 1991).

Laboratory-based physical stress paradigms such as the Cold Pressor Test (CPT) generally offer a greater possibility of standardization, as they are shorter and less complex than psychosocial stressors, e.g. the Trier Social Stress Test (TSST). The CPT was first described 80 years ago by Hines and Brown (Hines and Brown, 1932b). Previously, it has often been used in autonomic nervous system and cardiovascular research and clinical assessment. Today, however, the CPT represents an established and widely used tool in psychobiological research, too. In the standard version of the CPT, participants are requested to immerse one hand into ice water for several (usually 2–3) minutes. This procedure reliably triggers activation of the sympathetic nervous system, as indexed by elevated blood pressure, heart rate and increased skin conductance (Lovallo, 1975). It also leads to a rise in cortisol (al'Absi et al., 2002; Bullinger et al., 1984), the end product of the HPA axis. Other CPT protocols have been established, such as foot (Previnaire et al., 2012), elbow (Sanger et al., 2014) and forehead (Saab et al., 1993) exposure to cold. Bilateral hand immersion CPT (Suter et al., 2007) has been used to take into consideration potential effects of unilateral stimulation, such as unwanted asymmetric brain activation. And recently, a bilateral feet version of the CPT version has been developed (Larra et al., 2015) to overcome practical limitations of the standard version, and also to induce stronger autonomic and endocrine responses.

Threatening the physical or the social ego is an important feature of stress (Dickerson and Kemeny, 2004). Accordingly, adding a social-evaluative component increases neuroendocrine reactivity to the CPT (Schwabe et al., 2008). However, a small paradox emerges regarding safety recommendation obligatory in CPT protocols. The CPT, although generally unproblematic when specific exclusion criteria are respected, still possesses a small risk of inducing a pain-induced pre-syncopal state and – in case appropriate (para)medical support is missing – full fainting event (Wirch et al., 2006). Therefore, close supervision and monitoring of participant's behavior and vital signs is mandatory during CPT application, and ethical

reglementations also encourage this information to be explicitly disclosed to the participants. Therefore, monitoring may be perceived by the participant as a cue of safety and support. This, however, would represent an implication in fundamental opposition to stress features like danger, insecureness, and unpredictability. We therefore aimed to extend the CPT by a mental challenging component Performing on a mental task during CPT application would therefore not only increase the absolute load of the stress procedure, but also direct the social evaluation to actual cognitive task performance.

Indeed, combining different elements is frequently done in stress protocols. The psychosocial stress paradigm (e.g.) TSST included a cognitive challenge element. A mental arithmetic task (e.g. “Count backwards from 2000 by 17!”) is standard part of the TSST and usually applied some minutes following the TSST mock job interview. The test involves having to start all over again (from the beginning) when a calculation mistake occurs (which may happen quite often). This is frustrating for most of the participants, and was invented to enhance the social exposure threat component of the TSST. However, this procedure makes task standardization and the assessment of task performance more difficult. This is further aggravated by the fact that the counting pace is not strictly controlled (e.g. by electronic timing).

There are versions of mental arithmetic tasks, which overcome these drawbacks. The Paced Auditory Serial Addition Task (PASAT) (Gronwall, 1977) was developed as a neuropsychological tool to test sustained divided attention, concentration, working memory and speed of information processing (Diehr et al., 1998). This test offers an alternative to standard mental arithmetic tasks, since it is feasible, short, challenging, reproducible, with different item-versions, and the task may be continued without interruption or intervention in case of calculation or omission errors. The PASAT has been used previously as a means to induce stress (Mathias et al., 2004; Philippsen et al., 2007), but not in combination with the CPT.

We hypothesized that the combination of the physical stressor CPT, the mandatory monitoring of participants’ actual state, and the challenging math-test PASAT will increase overall stress reactivity, and

that such a new stress protocol will be suitable to study the effect of ELA on stress reactivity in adulthood. The first aim (Study 1: Stress Test Validation) of the current study was to test the assumption that the combination of the PASAT and the CPT would increase cardiovascular, endocrine (saliva cortisol), and self-perceived emotional stress reactivity over and beyond reactivity to single presentations of the PASAT and CPT. The second aim (Study 2: Stress Testing ELA Participants) was to apply this new stress test to volunteers with and without ELA experience (separation from biological parents, institutionalization, adoption), and to explore any differences in psychological, cardiovascular and/or HPA axis stress reactivity.

6.3 Methods

Study 1: Stress Test Validation

6.3.1 Participants

56 healthy volunteers (28 females; age: 23 yrs., ranging from 19 to 32 yrs.) were recruited using announcements posted on the University of Trier Intranet. Exclusion criteria were acute or persistent medical and mental disorders, severe cold intolerance (e.g. Raynaud's disease), current medication except for the occasional use of pain killers (paracetamol, aspirin, or NSAR), heavy smoking (>10 cigarettes per day), regular consumption of alcohol (>30 g/day), and illicit drug intake within the last 6 months. All criteria were checked beforehand by an interview. Participants gave their written informed consent prior to study participation and received financially compensation. All procedures of the study were approved by the ethical committee of the medical association of Rhineland-Palatinate.

6.3.2 Procedures

Participants visited the psychophysiological lab at the University of Trier on two separate days, first for a screening visit, and then one day later for the experimental session. For the screening visit, exclusion criteria were checked, and blood pressure and heart rate were measured using a standard oscillometric blood pressure measurement device to ensure arterial normotension. Participants were then familiarized with the general laboratory settings, and the stress test procedures. Thereafter, participants underwent a PASAT teaching session to make sure that they would understand the arithmetic instruction given with the PASAT (see below). The screening visit lasted for approx. 40 minutes.

The experimental session started in the afternoon and lasted for approx. 120 minutes. Upon arrival, participants were seated in a comfortable chair and asked to relax. Individuals were randomly assigned to one of four conditions (fully crossed treatments): (i) bilateral feet version of the CPT with simultaneous

PASAT; (ii) CPT without PASAT; (iii) control (warm water) test version with PASAT; (iv) control without PASAT. Participants were asked to take off their shoes and socks. Headphones, microphone, and physiological recording devices (see below) were attached to deliver instructions, record speech, and monitor ECG continuously and blood pressure intermittently (at -13, -8, and -3 minutes before the intervention). At minutes -30, -20, and -10 prior to the intervention saliva samples were taken for cortisol assessment. At minute -2 participants were asked to provide self-report ratings (see below). Then, the CPT/control intervention (see below) with or without PASAT started. One minute after the end of the CPT/control intervention, participants were asked to rate their current stress, arousal and anxiety, and to also rate their perceived pain retrospectively. Furthermore, they provided post-intervention saliva samples at minutes +3, +15, +30, and +60 after starting the CPT, and blood pressure and heart rate measurements were taken at minutes +4, +12, and +20 after starting the CPT.

6.3.3 Cold Pressor Test / control intervention

One minute before the intervention, the experimenter entered the testing room, set a towel-covered (to avoid specific anticipation effects) water basin on the ground in front of the test person and said that the test procedure would now start. Participants were instructed to put both feet including the ankles into the water, and to only take them out when asked to do so. 15 seconds after feet immersion the PASAT (see below) started and lasted for 160 seconds. The water temperature of the CPT was 2-3°C, and 36 °C in the control version. Blood pressure and heart rate measurements were started at minute 1 and 2 after feet immersion. CPT/control duration was 3 minutes. They were then asked to lift both feet out of the water basin, which was removed immediately, and to remain quietly seated. Participants' feet were covered with fresh and dry towels. During the stress/control procedure there was no interaction between experimenter and the participants, and they were not informed about the testing time left.

6.3.4 Paced Auditory Serial Addition Task (PASAT)

We used a computerized version of the PASAT in which participants were asked to listen to a series of single – digit numbers delivered via stereo headphones from a computer. Their task was to add each new number to the previous as quickly and accurately as possible and to report the sum aloud. Verbal responses were digitized and recorded in standard wave files for offline analysis. Stimuli were presented with an inter-stimulus interval (ISI) of 2.5 s. The total task consisted of 64 stimuli and lasted for 160 s. Percentage of correct responses was used as the main PASAT performance indicator. Omission errors and false responses (Schachinger et al., 2003) were not separated.

6.3.5 Blood Pressure and Heart Rate Measurement

Heart rate was derived from ECG. Standard Ag/AgCl electrodes (ECG Tyco Healthcare H34SG Ag/AgCl electrodes of 45 mm diameter) were used for ECG (standard lead II configuration) recording with a Biopac MP150 system and ECG100C amplifier modules. ECG data were stored on a disk with a sampling rate of 1 kHz at 16 bit resolution. Beat detection was performed offline by WinCPRS (Absolute Aliens Oy, Turku, Finland), as was artifact control. Heart rate was averaged over 1-minute intervals. Systolic, diastolic, and mean arterial blood pressure (MAP) was measured with a standard cuff oscillometric Dinamap blood pressure monitor (Dinamap SX 1846, Critikon, US), with only MAP data being reported in the present paper.

6.3.6 Cortisol

Saliva was collected using standard absorbent swabs (Salivettes, Saarstedt; Nümbrecht, Germany). Samples were kept at +6°C until the end of the experiment and were then stored at -20°C, until analyzed. Cortisol concentration was determined by time-resolved immunoassay with fluorescence detection (Dressendorfer et al., 1992).

6.3.7 Stress and Pain Ratings

Self-reported levels of stress, arousal, anxiety and pain were assessed using visual analogue scales (VAS), displayed on a VGA screen placed about 60 cm in front of the participant, and ranging from 0 to 100 arbitrary Units (aU).

6.3.8 Data Reduction and Statistics

Blood pressure and heart rate assessed one minute prior to the CPT/control were defined as baseline scores. Stress scores were defined as maximum (peak) values during intervention. Reactivity (Δ) scores were defined as change scores (Δ = stress - baseline). Saliva cortisol values at 20 and 10 minutes before intervention and the value immediately after the intervention were averaged individually and defined as baseline. Baseline corrected cortisol was calculated by subtracting baseline from post-intervention data. The area under the curve with respect to increase (AUC_i) was calculated for statistical analyses (Pruessner et al., 2003a).

Two-factorial ANOVAs were conducted to compare the main effects of ***CPT*** (2-level between subjects factor: cpt vs. control) and ***PASAT*** (2-level between subjects factor: pasat vs. control) and the interaction between ***CPT*** X ***PASAT*** on baseline and stress reactivity scores.

A two-factorial ANOVA was conducted to compare the main effects of ***CPT*** (2-level between subjects factor: cpt vs. control) and ***PASAT-TIME*** (2-level within subjects factor: 1st half vs. 2nd half) and the interaction between ***CPT*** X ***PASAT-TIME*** on PASAT performance (% correct responses).

Two-factorial ANOVAs were conducted to compare the main effects of ***CPT*** (2-level between subjects factor: cpt vs. control) and ***PASAT*** (2-level between subjects factor: pasat vs. control) and the interaction between ***CPT*** X ***PASAT*** on pre-post differences in mood ratings. Pre- and post-intervention ratings of stress, arousal, and anxiety are illustrated in Figure 2. However, pre-post differences of these ratings were used for

statistical testing, as were retrospective ratings of pain aversion, pain intensity, and tense arousal during the 3-minute intervention period.

Furthermore, unpaired t-tests (or Wilcoxon test; if explicitly stated) were used to directly assess the effects of PASAT, wherever appropriate. Exact p-values are reported unless otherwise stated. Testing was two-tailed, unless explicitly indicated to be one-tailed. Data in the text and tables represent means ± SD, and in figures mean ± SEM.

Study 2: Stress Testing ELA Participants

6.3.9 Participants

In total, 42 young adults participated in this study, of whom 21 (ELA group; 14 females; age: 22.3 yrs., ranging from 19 to 30 yrs.) were adopted by Luxembourgish families after institutionalization in early childhood during the 1990s, from international and national orphanages, or families. 21 age-matched participants (14 females; age: 21.3 yrs., ranging from 19 to 28 yrs.) without adoption history were recruited as control group. The cohorts are part of the EpiPath project, an ongoing study conducted in Luxembourg and Trier, Germany. Participants gave written informed consent prior to the study and received financial compensation for participation. There were no special exclusion criteria, other than arterial hypertension and cold-sensitivity (see Study 1). If individuals reported acute illness (e.g. seasonal cold), study participation was postponed until fully recovered. Structural clinical interviews for DSM-IV mental disorders (SCID 1;(Spitzer et al., 1992)) revealed a higher percentage of mental disorders in ELA subjects (52,4%) compared to controls (4,8%) (details of these results are reported elsewhere). All procedures of the study were approved by the responsible ethics committees.

6.3.10 Procedures

The study was conducted in the early afternoon at the Clinical Research Unit of the Luxembourg Institute of Health. Upon arrival, participants were seated in a comfortable chair and asked to relax, and a small venous catheter was attached for blood collection, the results of which are reported elsewhere. They were then familiarized with the general laboratory settings, as well as the stress test procedures, and underwent a complete PASAT teaching session to make sure that they would understand the arithmetic instruction given with the PASAT (see Study 1). After 60 minutes of rest the stress experiment started. All participants received the bilateral feet version of the CPT with simultaneous PASAT. Before, headphones, a microphone, and physiological recording devices (see Study 1) were attached to deliver instructions, record speech, and monitor ECG continuously and blood pressure intermittently (at -13, -8, and -3 minutes before intervention). At minutes -30, -20, and -5 prior to the intervention saliva samples were taken for cortisol assessment. At minute -2 participants were asked to provide mood and pain ratings (see Study 1). Then, the CPT started. One minute after the end of the CPT intervention, individuals rated their current stress, arousal and anxiety, and pain retrospectively. Furthermore, they provided post-intervention saliva samples at minutes +3, +15, +25, +40, and +55 after starting the CPT, and blood pressure and heart rate measurements were taken at minutes +12, +14, and +16 after starting the CPT. Current mood was also assessed using the Positive Affect and Negative Affect Schedule(PANAS, (Watson et al., 1988)) before and after the stress procedure. Responses of the PANAS questionnaire with the instruction “how do you feel at the moment” are coded on a fivepoint Likert scale and have been found to be reliable with Cronbach´s alpha for PA = .89 and for NA = .85.

Procedures for the CPT, PASAT, ratings, ECG, blood pressure and heart rate measurements were as described for Study 1. Salivary cortisol levels were determined by a different lab by using commercially available immunoassays with the Salimetrics Salivary Cortisol ELISA Kit (CV: 7% intra-assay, 11% inter-assay;

Salimetrics). Samples were also kept at +6°C until the end of the experiment and were then stored at -20°C, until analyzed.

6.3.11 Data Reduction and Statistics

Blood pressure and heart rate values assessed at -13, -8, and -3 before CPT were individually averaged, and defined as baseline scores. Salivary cortisol values at -20 and -5 minutes before intervention were also averaged individually and defined as baseline. All other data reduction steps were as described for see Study 1.

Group differences between the ELA and control groups (childhood experience; CHE) were carried out using t-tests (independent class variable ***CHE-GROUP***: ELA vs. control) for BMI, baseline and reactivity scores of cardiovascular data and cortisol, as well as pre-post changes of ratings. AUC_I data was skewed and thus non-parametrically analyzed for group differences by Wilcoxon test. Within groups, stress reactivity data was tested for significance by t-tests (results are reported in Table 18).

A two-factorial ANOVA was conducted to compare the main effects of ***CHE-GROUP*** (2-level between subjects factor: *ELA* vs. control) and ***PASAT-TIME*** (2-level within subjects factor: 1^{st} half vs. 2^{nd} half) and the interaction between ***CHE-GROUP*** X ***PASAT-TIME*** on PASAT performance (% correct responses).

Two-factorial ANOVA were carried out to compare the main effects of ***CHE-GROUP*** and ***TIME*** (2-level within subjects factor: before vs. after CPT) and the interaction between ***CHE-GROUP*** X ***TIME*** on Positive and Negative Affect (PANAS scales).

Using T-tests we compared pre-post differences in self perceived emotion, as well as retrospective ratings of pain aversion, pain intensity, and tense arousal during the 3-minute stress period between groups. Pre- and post-stress ratings of stress, arousal, and anxiety are illustrated in Figure 4. However, pre-post differences of stress, arousal, and anxiety were used for statistical testing (t-tests).

6.4 Results

Study 1: Stress test validation

6.4.1 Cardiovascular and cortisol baseline

As expected, there were neither significant main effects for ***CPT*** (F[1:52]=0.0; p=.91), and ***PASAT*** (F[1:52]=0.3; p=.61), or an interaction effect between ***CPT*** X ***PASAT*** (F[1:52]=0.7; P=.42) on MAP baseline, nor were there significant main effects for ***CPT*** (F[1:52]=0.3; p=.62), and ***PASAT*** (F[1:52]=1.7; p=.19), or an interaction between ***CPT*** X ***PASAT*** (F[1:52]=0.0; P=.93) on HR baseline. Furthermore, there was no main effect for ***CPT*** (F[1:52]=0.2; p=.63). Nevertheless, there was a marginally significant effect for ***PASAT*** (F[1:52]=3.2; p=.08), but no interaction between ***CPT*** X ***PASAT*** (F[1:52]=1.5; P=.22) on baseline cortisol.

6.4.2 Mean arterial blood pressure reactivity

MAP reactivity data are illustrated in Figure 25 (upper left graph). There were significant main effects for ***CPT*** (F[1:52]=16.9; p<.0001) and ***PASAT*** (F[1:52]=19.7; p<.0001), but no interaction between ***CPT*** X ***PASAT*** (F[1:52]=0.7; p=.41) on MAP reactivity. Subgroup comparisons using t-tests revealed that PASAT-specific MAP increase was 14.4 ± 9.1 mmHg (t=4.2; p<.0003) when PASAT was added to the control procedure, and 9.9 ± 11.3 mmHg (t=2.3; p<.03) when PASAT was added to the CPT procedure.

6.4.3 Heart rate reactivity

HR reactivity data are illustrated in Figure 25 (lower left graph). There were significant main effects for ***CPT*** (F[1:52]=4.0; p<.05) and ***PASAT*** (F[1:52]=12.3; p<.001), and a significant interaction between ***CPT*** X ***PASAT*** (F[1:52]=5.3; p<.03) on HR reactivity. Subgroup comparisons using t-tests showed that the PASAT-specific HR increase (vs. control/control) was 21.5 ± 13.4 bpm (t=4.2; p<.0003). However, there was no significant PASAT-specific HR increase when PASAT was added to the CPT procedure (4.5 ± 14.3 mmHg, t=0.8; p=.41).

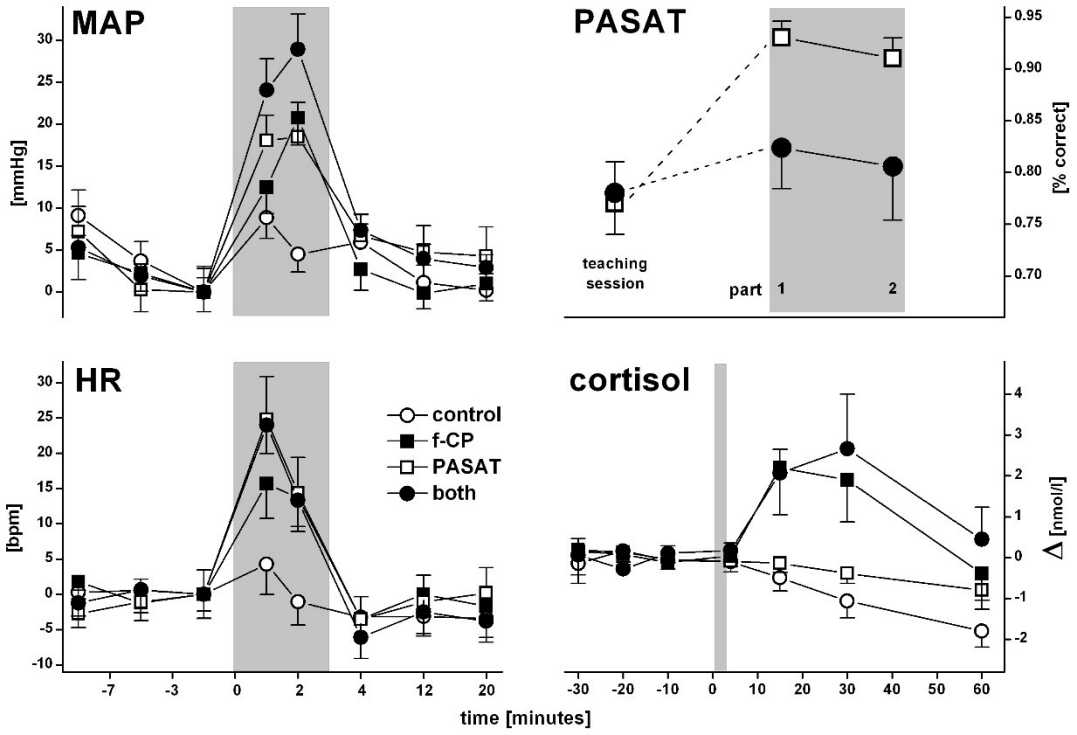

Figure 25. Mean arterial blood pressure (MAP), heart rate (HR) and saliva cortisol reactionsto a both-feet version of the CPT, with (both: closed circles) and without (f-CP: closed squares) simultaneous PASAT, as compared to a control (warm water) condition with (PASAT: open squares) or without (control: open circles) simultaneous PASAT. Upper right graph illustrates PASAT performance (percent correct responses) during f-CP and warm water control. Performance data during the teaching session is added for descriptive purpose. Grey areas indicate time of intervention. Data represent mean ± SEM

6.4.4 Saliva cortisol reactivity

Salivary cortisol reactivity (AUC_i) is illustrated in Figure 25 (lower right graph). There was a significant main effect for ***CPT*** (F[1:52]=11.8; p<.002), but there was neither a main effect for ***PASAT*** (F[1:52]=0.9; p=.34), nor a significant interaction between ***CPT*** X ***PASAT*** (F[1:52]=0.0; p=.95) on cortisol reactivity. Subgroup comparisons using non-parametric Wilcoxon tests revealed a PASAT-specific AUC_i increase of 35.4 ± 57.8 nmol*min/l (p<.035, one-tailed) when PASAT was added to the control procedure. However, there was no significant PASAT-specific AUC_i increase when PASAT was added to the CPT procedure (27.7 ± 182.8 nmol*min/l; p=.40, one-tailed).

6.4.5 PASAT performance

Data are illustrated in Figure 25 (upper right graph). Performance data during the teaching session is given for descriptive purpose, only. There was a significant main effect for ***CPT*** (F[1:25]=4.7; p<.04), but no significant main effect for ***PASAT-TIME*** (F[1:25]=2.1; p=.16), or an interaction between ***CPT*** X ***PASAT-TIME*** (F[1:25]=0.0; p=.94) on PASAT performance.

6.4.6 Pre-post differences in perceived stress

There were significant main effects for ***CPT*** (F[1:52]=37.1; p<.0001) and ***PASAT*** (F[1:52]=4.5; p<.04), but no interaction between ***CPT*** X ***PASAT*** (F[1:52]=1.4; p=.25) on pre-post changes in perceived stress. Subgroup comparisons by t-tests showed that PASAT-specific increases in stress ratings were present (11.9 ± 12.0 aU, t=2.6; p<.015) when PASAT was added to the control procedure, but not (3.4 ± 15.1 aU, t=0.6; p=.55) when PASAT was added to the CPT procedure (Figure 26).

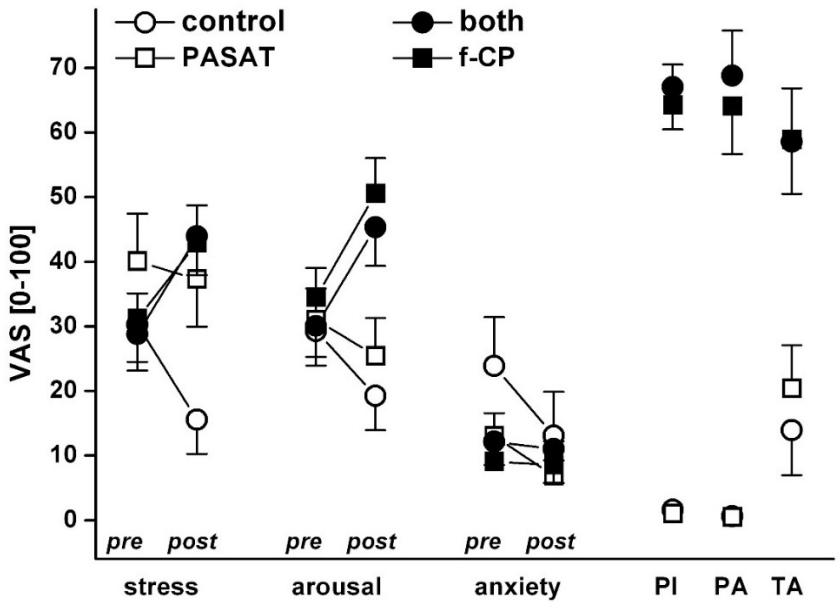

Figure 26. Subjective ratings (visual analogue scale: 1 to 100 aU) of stress, arousal, and anxiety before (pre) and after (post) intervention, as well as retrospective ratings of pain intensity (PI), pain adversity (PA), and tense arousal (TA) during intervention. Data represent mean ± SEM.

6.4.7 Pre-post differences in perceived arousal

There was a significant main effect for ***CPT*** (F[1:52]=19.5; p<.0001), but no significant main effect for ***PASAT*** (F[1:52]=0.1; p=.73), or an interaction between ***CPT*** X ***PASAT*** (F[1:52]=0.3; p=.62) on pre-post changes in perceived arousal. Subgroup comparisons by t-tests revealed no PASAT-specific increases in arousal ratings (4.5 ± 15.8 aU, t=0.8; p=.46) when PASAT was added to the control procedure, nor (0.8 ± 23.3 aU, t=0.1; p=.92) when PASAT was added to the CPT procedure (Figure 26).

6.4.8 Pre-post differences in anxiety ratings

There was a significant main effect for ***CPT*** (F[1:52]=6.0; p<.02), but no significant main effect for ***PASAT*** (F[1:52]=0.5; p=.47), and no interaction between ***CPT*** X ***PASAT*** (F[1:52]=0.8; p=.38) on pre-post changes in anxiety ratings. Subgroup comparisons by t-tests showed no PASAT-specific increases in anxiety ratings (5.4 ± 14.4 aU, t=1.0; p=.36) when PASAT was added to the control procedure, nor (0.6 ± 9.3 aU, t=0.1; p=.87) when PASAT was added to the CPT procedure (Figure 26).

6.4.9 Retrospective pain intensity ratings

There was a significant main effect for ***CPT*** (F[1:52]=192.2; p<.0001), but no main effect for ***PASAT*** (F[1:52]=0.1; p=.81), and no interaction between ***CPT*** X ***PASAT*** (F[1:52]=0.1; p=.71) on retrospective pain intensity ratings. Subgroup comparisons by t-tests revealed no PASAT-specific increases in anxiety ratings (0.6 ± 3.0 aU, t=0.5; p=.60) when PASAT was added to the control procedure, nor (2.8 ± 23.9 aU, t=0.3; p=.76) when PASAT was added to the CPT procedure (Figure 26).

6.4.10 Retrospective pain aversion ratings

There was a significant main effect for ***CPT*** (F[1:52]=161.8; p<.0001), but no significant main effect of ***PASAT*** (F[1:52]=0.2; p=.65), and no interaction between ***CPT*** X ***PASAT*** (F[1:52]=0.2; p=.64) on retrospective pain adversity ratings. Subgroup comparisons by t-tests revealed no PASAT-specific increases in adversity

ratings (0.1 ± 1.4 aU, t=0.2; p=.82) when PASAT was added to the control procedure, nor (4.7 ± 26.8 aU, t=0.5; p=.64) when PASAT was added to the CPT procedure (Figure 26).

6.4.11 Retrospective tense arousal ratings

There was a significant main effect for ***CPT*** (F[1:52]=30.8; p<.0001), but no significant main effect for ***PASAT*** (F[1:52]=0.1; p=.68), and no interaction between ***CPT*** X ***PASAT*** (F[1:52]=0.2; p=.65) on retrospective tense arousal ratings. Subgroup comparisons by t-tests revealed no PASAT-specific increases in tense arousal ratings (6.4 ± 25.1 aU, t=0.7; p=.51) when PASAT was added to the control procedure, nor (0.3 ± 30.0 aU, t=0.0; p=.98) when PASAT was added to the CPT procedure (Figure 26).

Study 2: Stress Testing ELA Participants

There were 21 subjects in each group, with 14 women in in the ELA group, and 14 women in in the control group. Chi-square tests on the sex distribution between ***CHE-GROUPs*** showed no statistical difference (χ^2=0.83; p=.36).

6.4.12 BMI and baseline data

Neither did BMI differ between ***CHE-GROUPs***, nor did baseline HR and baseline saliva cortisol measures (Table 18). Nevertheless, baseline MAP was significantly (p<.05) lower in ELA compared to control participants (Table 18).

6.4.13 Stress reactivity

All reactivity measures were different from zero in both ***CHE-GROUPs*** (Table 18). There was no difference in MAP and HR reactivity between ***CHE-GROUPs*** (Table 18 and Figure 27). However, peak salivary cortisol responses were significantly (p<.04) lower in the ELA group, and the AUC_i cortisol response

showed a similar difference (with a tendency towards statistical significance) between ***CHE-GROUPs*** (p=.08, two-tailed) (see Table 18 and Figure 27).

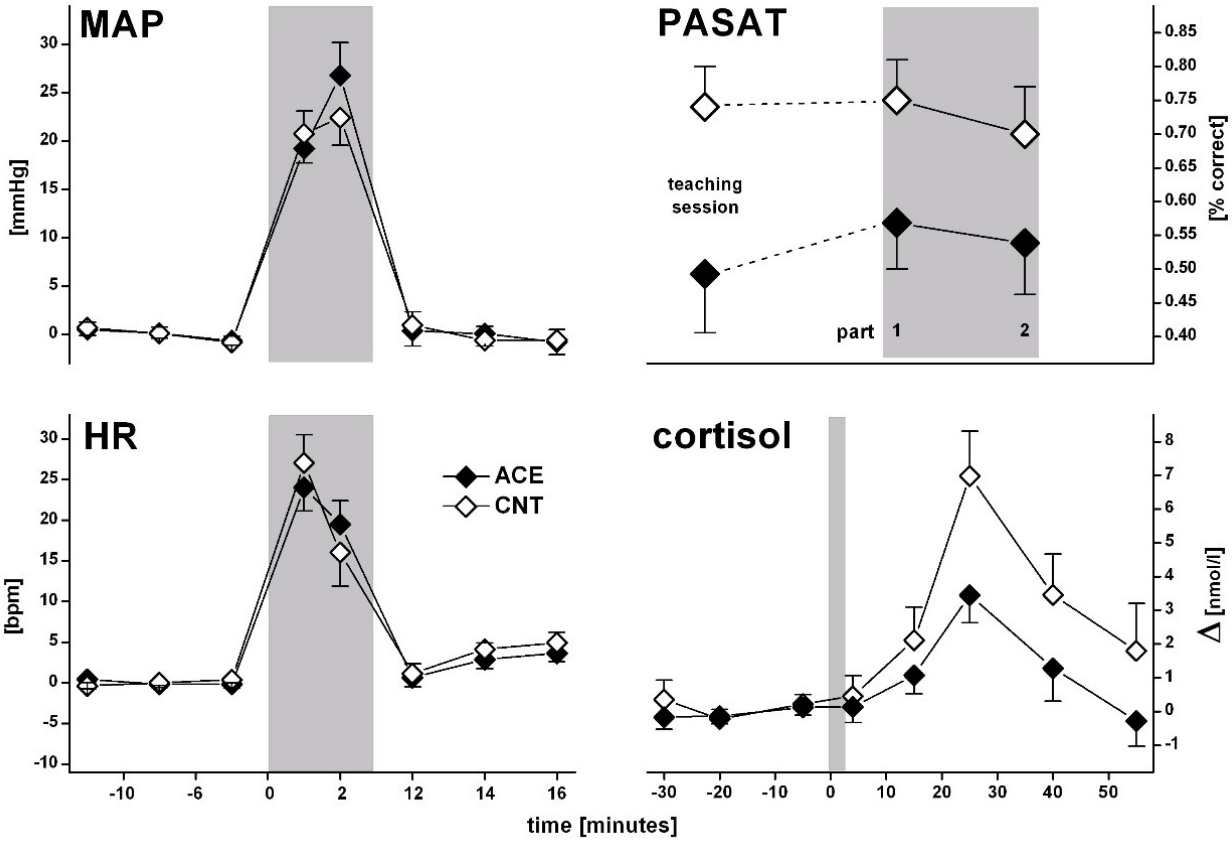

Figure 27. Mean arterial blood pressure (MAP), heart rate (HR) and saliva cortisol reactions to a both-feet version of the CPT with simultaneous PASAT in subjects experiencing adverse childhood events (ELA: closed diamonds), as compared to age-matched control subjects (CNT: open diamonds). Upper right graph illustrates PASAT performance (percent correct responses) per group. Performance data during the teaching session is added to indicate that group differences were present before stress testing. Grey areas indicate time of intervention. Data represent mean ± SEM.

6.4.14 PASAT performance data

Due to technical problems PASAT recordings were flawed for 3 participants. ANOVA testing of PASAT correct responses during the first and second half of the testing phase revealed a significant main effect for ***CHE-GROUP*** (F[1:39]=4.3; p<.05), a marginally significant main effect for ***PASAT-TIME*** (F[1:39]=3.8; p=.06), but no interaction between ***CHE-GROUP*** X ***PASAT-TIME*** (F[1:39]=0.3; p=.62). However, the difference in PASAT performance was already present when comparing group data assessed during the learning session,

which revealed significantly (t=2.6; p<.01) lower scores of correct responses in ELA than in control participants (Figure 27, upper right graph).

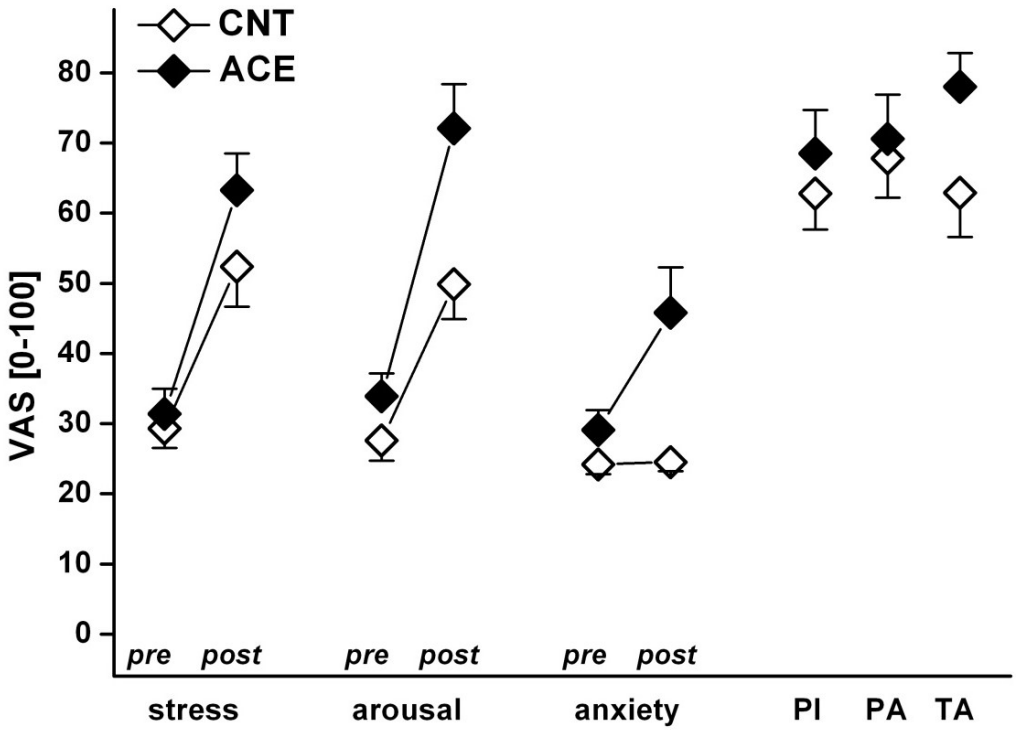

Figure 28. Subjective ratings (visual analogue scale: 1 to 100 aU) of stress, arousal, and anxiety before (pre) and after (post) intervention, as well as retrospective ratings of pain intensity (PI), pain adversity (PA), and tense arousal (TA) during intervention, in subjects experiencing adverse childhood events (ELA: closed diamonds), as compared to age-matched control subjects (CNT: open diamonds). Data represent mean ± SEM.

6.4.15 Pre-post differences in ratings of perceived stress, arousal, and anxiety

Pre-post changes in perceived stress did not differ reliably between groups (ELA: +31.9 ± 27.8, CNT: +23.1 ± 26.5 aU; t=1.1; p=.29). Pre-post changes in perceived arousal, however, were significantly different between groups (ELA: +38.3 ± 30.6, CNT: +22.3 ± 20.8 aU; t=2.0; p<.05), as were pre-post changes in anxiety ratings (ELA: +16.6 ± 25.0, CNT: +0.4 ± 4.6 aU; t=2.9; p<.005) (Figure 28).

6.4.16 Pain intensity and aversion ratings, and tense arousal ratings

Pain intensity (t=0.7; p=.48), and pain aversion (t=0.3; p=.74) ratings did not differ significantly between groups. However, the analysis of tense arousal ratings revealed an almost significant statistical group

difference (t=1.9; p=.06) with tense arousal ratings in the ELA group (t=1.9; p=.06) as compared to the control group (Figure 28).

6.4.17 Positive and Negative Affect (PANAS)

Positive Affect ratings increased (5.1 ± 6.3 aU) from 24.8 ± 8.6 aU before to 29.9 ± 8.6 aU after stress testing in the ELA subjects, and increased (5.0 ± 6.4 aU) from 23.9 ± 6.6 aU before to 28.9 ± 6.4 aU after stress testing in control subjects. ANOVA testing of Positive Affect ratings revealed a significant main effect for ***TIME*** (F[1:42]=28.1; p<.0001), but no significant main effect for ***CHE-GROUP*** (F[1:42]=0.2; p=.66), and no interaction between ***CHE-GROUP*** X ***TIME*** (F[1:42]=0.0; p=.94).

Negative Affect ratings increased (4.1 ± 4.8 aU) from 13.5 ± 3.8 aU before to 17.6 ± 6.5 aU after stress testing in the ELA subjects, but increased only slightly (1.5 ± 2.4 aU) from 11.6 ± 2.9 aU before to 13.2 ± 3.2 aU after stress testing in control subjects. ANOVA testing of Negative Affect ratings revealed a significant main effect of ***TIME*** (F[1:42]=24.4; p<.0001), a significant main effect of ***CHE-GROUP*** (F[1:42]=7.3; p<.01), and a significant interaction between ***CHE-GROUP*** X ***TIME*** (F[1:42]=5.1; p<.03). Subgroup comparisons by t-tests revealed a trend (T=1.8; p=0.07) towards a significant difference between groups in Negative Affect ratings before stress testing, and significant group differences in post stress ratings (T=2.9; p<.01), as well as stress-induced pre-post changes (T=2.3; p<.05).

Table 18. Cardiovascular (MAP and HR) and saliva cortisol baseline and stress reactivity in subjects who experienced adverse childhood events (ELA) and age-matched control subjects (CNT). Data represent mean ± SD; stars in brackets indicate significant difference from zero: (*) – p<.05, (**) – p<.005, (***) – p<.0005.

	ELA	CNT	T	p
BMI (kg/m^2)	23.9 ± 3.6	22.9 ± 3.6	1.1	0.30
Baseline physiology				
MAP (mmHg)	81.2 ± 5.6	85.2 ± 6.9	2.1	0.05
HR (bpm)	70.8 ± 10.8	72.9 ± 10.7	0.7	0.52
Saliva Cortisol (nmol/l)	7.3 ± 2.7	7.6 ± 3.9	0.3	0.80
Physiological stress reactivity				
ΔMAP (mmHg)	23.0 ± 8.8 (***)	21.6 ± 9.6 (***)	0.5	0.61
ΔHR (bpm)	21.8 ± 10.4 (***)	21.5 ± 16.1 (**)	0.1	0.96

6.5 Discussion

We used a fully-crossed between-group design to test the hypothesis that stress reactivity is enhanced when combining CPT and PASAT, over and beyond the stress reactivity induced by either stress paradigm alone. The most important finding of this validation study (Study 1) was an additional increase in blood pressure reactivity when the PASAT was combined with the CPT. Such effects were not observed for HPA axis reactivity or changes in anxiety, stress and tension. Furthermore, we also found that the CPT impairs cognitive performance (PASAT percent correct responses).

We used the combined stress paradigm version (CPT + PASAT) in a second study (Study 2) to investigate stress reactivity differences between volunteers with and without ELA. In ELA participants, cardiovascular reactivity was similar, stress-ratings were higher, but HPA axis stress reactivity was blunted compared to the control group.

The CPT is a powerful procedure which has successfully been used to study the impact of stress on learning (Duncko et al., 2007), memory processes (Duncko et al., 2009; Smeets et al., 2008), emotional behavior (Suter et al., 2009), attention (Sanger et al., 2014), startle (Deuter et al., 2012; Schulz et al., 2011), and pain (Edwards and Fillingim, 2005). Adding a social evaluative component to the CPT increases stress reactivity, especially responsiveness of the HPA axis (Schwabe et al., 2008). However, the CPT is a passive test and does not require active performance and cooperation, so that remains unknown (and uncontrolled) on which particular aspect the social evaluation is directed to. In order to provide focus for social monitoring, we added a cognitively challenging component to the CPT. The PASAT was chosen as an additional cognitive task (mental arithmetic), as it combines several advantages: it is practical, well validated, it provides a cognitive performance read-out, and it has been shown to induce a stress response (Philippsen et al., 2007; Schwabe et al., 2007). Indeed, data from Study 1 corroborate the stress eliciting effect of PASAT.

Adding PASAT to the CPT considerably increases mean arterial blood pressure (MAP) reactivity (by approx. 10 mmHg). The final MAP stress reactivity was approaching 30 mmHg, suggesting that the test has a very strong cardiovascular stress effect. Other published stress protocols, validated in big cohorts, e.g. (Becker et al., 1996), induce lower MAP reactivity.

Furthermore, the results suggest that the CPT impairs PASAT performance. This effect is likely to be due to distraction and/or pain induced by the CPT. Comparing the first and the second half of the total test period showed no effects for time, suggesting that this effect is independent of habituation and/or sensitization. Combining CPT and PASAT offers the potential to also address questions on whether cognitive performance is differently impacted by stress in different groups and/or under different conditions.

Study 2 used the combination of CPT and PASAT to induce stress in participants who experienced ELA (separation from biological parents, institutionalization, adoption) and age-matched controls. The procedure clearly resulted in stress in all participants. MAP and heart rate reactivity were comparable in ELA and control participants. Emotional distress ratings were even higher in the ELA group, especially negative emotion and mood. Nevertheless, HPA axis responses to stress were significantly lower in the ELA than in the control group. This pattern suggests a physiologically based response separation (i.e. response fractionation) – potentially initiated by increased glucocorticoid feedback sensitivity in ELA individuals (Lupien et al., 2009; Mirescu et al., 2004).

Cognitive performance (PASAT correct responses) was lower in ELA participants. It is unlikely that CPT stress is responsible for this effect, because the difference in performance was visible already during the PASAT training sessions, and thus, prior to stress induction. Rather, cognitive performance levels may be lower in ELA individuals compared to healthy controls, and this may be related to early childhood history and separation from biological parents, as suggested by previous research (Loman and Gunnar, 2010).

Some limitations of the present studies need to be addressed. Due to administrative reasons salivary cortisol was analyzed in different laboratories, which may have impacted negatively on the comparability of the results between the 2 studies. In a similar vein, stress tests were conducted in different laboratories and settings: Study 1 was carried out in a University Psychophysiology Lab, and Study 2 in a clinical research unit in a hospital setting. Thus, contextual differences and different cortisol determination techniques may be responsible for the different HPA axis responses seen in both studies. Nevertheless, both studies included an appropriate control condition/group, so it could be argued that each study can provide valid conclusions in a stand-alone fashion.

6.5.1 Conclusion

The present results demonstrate the validity and feasibility of a new stress protocol, consisting of a bilateral CPT, combined with the PASAT. Using this new stress paradigm, we found clear evidence for selectively blunted HPA axis stress responsiveness in participants who experienced ELA, which may be best explained by ELA selectively enhancing central feedback sensitivity to glucocorticoids.

6.6 Acknowledgments

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7 Glucocorticoid receptor signaling in leukocytes after early life adversity

My contribution to this chapter:

Writing of the manuscript. Recruitment of study participants. Performing the flow cytometry experiments. Final statistical analysis. I was involved in setting up and coordinating visit 1, including scheduling of participants, nurses, and experimental rooms, performing the stress test, and sample collection. I assisted in GR pyrosequencing and mRNA expression experiments.

7.1 Abstract

Early life adversity (ELA) has been associated with a clear immune phenotype of higher levels of inflammation and accelerated immunosenescence. However, it is still unclear whether this immune phenotype is a direct result of ELA, or an indirect consequence of dysregulated hypothalamic-pituitary-adrenal (HPA) axis activation.

We, therefore, examined GR signaling in immune cells and the *in vivo* immune response to acute stress in the EpiPath cohort. Participants had either been exposed to 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, nor in the immune, endocrine, or psychological response to a standardized laboratory stress test.

In sum, we found no evidence of HPA dysregulation in participants exposed to ELA in the EpiPath cohort. These findings suggest that HPA axis programming does not underlie our observed ELA immune phenotype. Longitudinal studies will be necessary to investigate whether immune perturbations may precede GR dysregulation.

Keywords: Early life adversity, methylation, *NR3C1*, glucocorticoid receptor, standardized laboratory stress test.

7.2 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 (Chapter 1, 3, and 4). However, it is still unclear whether this immune phenotype is a direct result of ELA, or an indirect consequence of dysregulated neuroendocrine systems, in particular the hypothalamic-pituitary-adrenal (HPA) axis.

The HPA axis is intertwined and interacts closely with the immune system. Physiological and psychological 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 the synthesis and release of glucocorticoids (GCs) from the adrenal gland. GC effects are mediated by the constitutively expressed glucocorticoid receptor (GR). While the unbound receptor resides in the cytosol bound to a chaperone protein complex, upon GC binding, the GR translocates to the nucleus, where it acts as a transcription factor or repressor on target genes (Oakley and Cidlowski, 2013). HPA axis activation modulates immune function by down-regulating pro-inflammatory cytokines, upregulating anti-inflammatory cytokines, 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 et al., 2016) as well as HPA axis dysregulation, such as altered epigenetic regulation of the GR gene, GR signaling affecting GR sensitivity, and a dampened response to acute stress (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 et al., 2015). One of the most consistent findings is hypermethylation of the 1F promoter and/or exon in association with ELA (Martin-Blanco et al., 2014; Perroud et al., 2011; Romens et al., 2015; Tyrka et al., 2012; van der Knaap et al., 2014). The human 1F promoter contains the NGFI-A binding region originally identified by Weaver et al. (2004) in the rat, who reported an association between methylation levels and maternal care (Weaver et al., 2004).

The first exons are transcribed to mRNA in a tissue specific manner, but are not translated into protein (Palma-Gudiel et al., 2015; Turner and Muller, 2005). 1B and 1C transcripts exhibit the highest expression ratios and are most widely expressed over different tissues. 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 et al., 2009), peripheral blood (Labonte et al., 2014; Perroud et al., 2014), and saliva (Vukojevic et al., 2014). However, few studies have investigated the relationship between methylation levels at specific first exons and the corresponding transcripts (Cao-Lei et al., 2013).

GCs also act as negative feedback of the HPA axis by inhibiting CRH and ACTH release, which is essential for homeostasis. The sensitivity of this negative feedback loop can be tested *in vivo* with the dexamethasone (DEX)/CRH test, in which the HPA axis is inhibited by the administration of a GR agonist (DEX), which is followed by the administration of CRH. A higher-than-normal cortisol response suggests decreased sensitivity of the feedback mechanism. However, findings from this test are contradictory, as both increased (Heim et al., 2008; Tyrka et al., 2008) and decreased (Carpenter et al., 2009) GC responses have been found in ELA.

GR sensitivity is 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 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 et al., 2016). Indeed, GR resistance has been associated with higher risk to develop a cold and a greater pro-inflammatory response to a cold (Cohen et al., 2012).

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 et al., 2015). Induced NFkB activity has been found in ELA (Pace et al., 2012), although it is still unclear if this can be explained by upstream differences in GILZ expression levels.

Evidence is accumulating from cross sectional studies that adults with a history of ELA have an altered stress response. ELA has been associated with blunted reactivity in heart rate, blood pressure, and GC peak in response to acute stress (Carpenter et al., 2007; Carpenter et al., 2011; Elzinga et al., 2008; Lovallo et al., 2012; Schwaiger et al., 2016; Voellmin et al., 2015). This relationship seems to be dose-dependent: the larger the number or the duration of adversity, the lower the stress response (Lovallo et al., 2012; Voellmin et al., 2015). An inverse correlation has been found between the GC and immune response to acute stress (McInnis et al., 2015). In line with this, two studies have observed an increased IL-6 stress response to a stress test in ELA (Carpenter et al., 2010; Pace et al., 2006). GCs are also known to affect immune cell trafficking (Cain and Cidlowski, 2017).

We have previously shown higher immune activation and senescence in the EpiPath cohort, which had been exposed to separation from parents and/or institutionalization followed by adoption in early childhood (Elwenspoek et al., 2017a; 2017b; manuscripts under review). To date, there is no data specifically addressing immune cell trafficking, FKBP5 and GILZ expression, or GR sensitivity of immune cells

in the context of ELA. Therefore, we examined GR signaling in immune cells and the *in vivo* immune response to acute stress in the same cohort. We measured methylation levels at the 1F and 1H alternative first exons of the GR, mRNA expression levels of first exon-specific GR transcripts as well as expression of the GR target genes FKBP5 and GILZ. To examine GR sensitivity in immune cells, we determined the susceptibility to DEX inhibition on immune function. A subset of the cohort underwent a standardized laboratory stress test (SLST), to examine their stress response and in particular their immune response to stress. Together, these data allowed us to investigate the impact of ELA on HPA axis functioning at multiple levels.

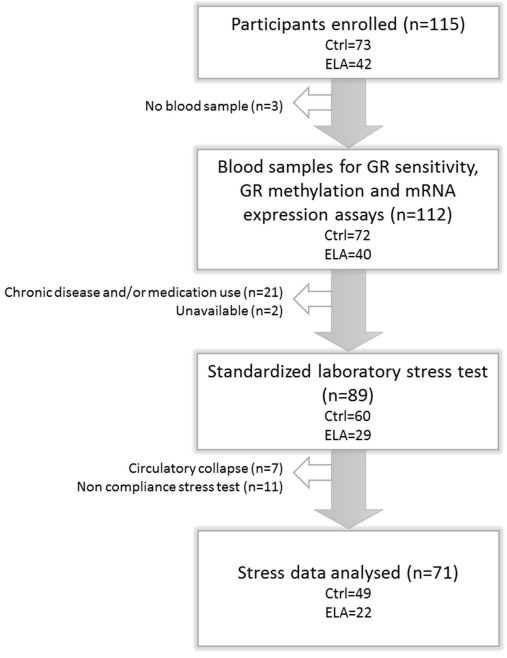

Table 1. Demographics EpiPath subset used for GR experiments.

	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

Table 2. Demographics EpiPath subset used in stress test.

	All (n=71) median [IQR]	Ctrl (n=49) median [IQR]	ELA (n=22) median [IQR]	Corrected p-values
Age (years)	22 [20-24]	21 [20-23]	23 [20-24]	0.567
Sex (% female)	56%	53%	64%	0.567
Smoking (% yes)	20%	12%	36%	0.205
Body mass index	22.9 [21.0-25.2]	22.9 [20.8-24.4]	23.1 [21.7-25.4]	0.567
Childhood trauma (CTQ scores)	1.2 [1.1-1.4]	1.2 [1.1-1.4]	1.2 [1.1-1.3]	0.567

Figure 29. Recruitment summary of all participants enrolled, showing the selection of participants used for each analysis. Table 1 and 2 show the demographics of the two subsets of the EpiPath cohort used in this study.

7.3 Material and methods

7.3.1 Subject enrolment

The EpiPath cohort, as previously described (Chapter 3), 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. Exclusion criteria for participating in the SLST were chronic diseases, medication use, and severe cold intolerance (Figure 29). 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.

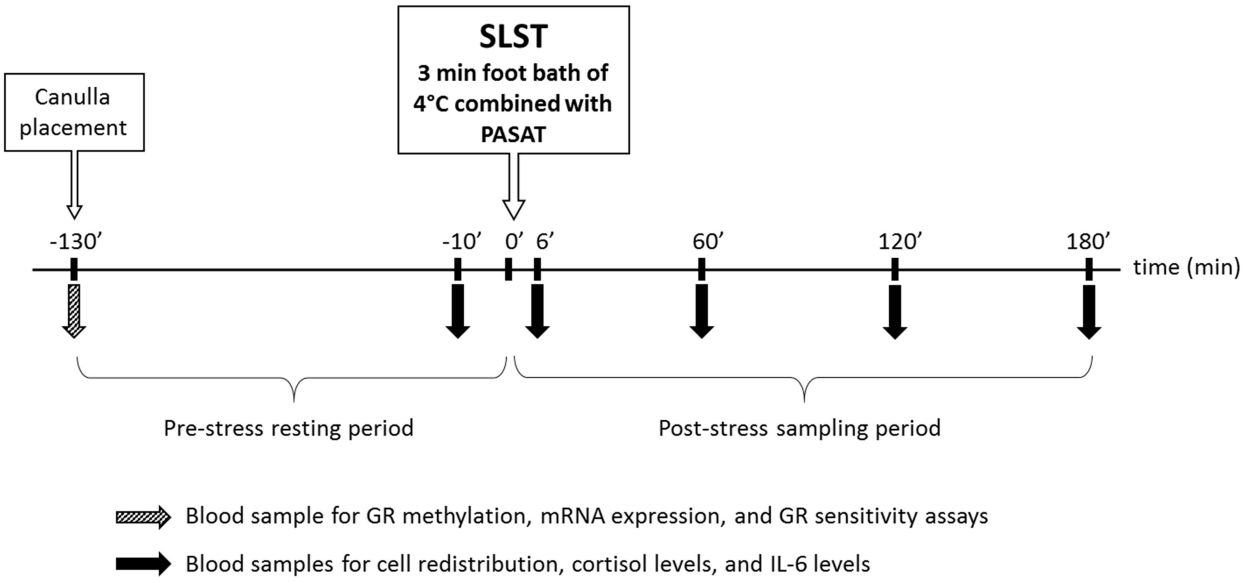

Figure 30. Study protocol. Abbreviations: SLST, standardized laboratory stress test; GR, glucocorticoid receptor; PASAT, Paced Auditory Serial Addition Test.

7.3.2 Study protocol

Participants were invited to two laboratory visits: at visit 1, participants underwent a SLST, and at visit 2, participants were psychologically evaluated. 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. The cannula placement was followed by a 2 h resting period, which allowed the participant to acclimatize to the lab and recover from needle/anticipatory stress (Figure 30). During this resting phase the participant received an explanation and training for the Paced Auditory Serial Addition Test (PASAT), a standardized lunch; they completed non-arousing questionnaires and answered questions about height, weight, age, sex, and smoking status (yes/no). Around 13:30 am participants underwent the SLST, consisting of a foot bath of 4°C combined with the PASAT during 3 min (Chapter 6). Immediately before and at several time points after the SLST, EDTA blood samples were collected, cardiovascular parameters (heart rate, mean arterial pressure) recorded, and participants completed visual analog scales assessing perceived stress, arousal and anxiety. At visit 2, participants filled out the childhood trauma questionnaire (CTQ) and were asked about their age at adoption if applicable.

7.3.3 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 (Table 19). 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 (Lei Cao-Lei., et al, 2013). All PCR and pyrosequencing primers are given in Table 19.

7.3.4 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 Table 20). 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 (Table 20) 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 et al., 2007).

Table 19. 1F and 1H GR PCR primers and conditions and sequencing primers for pyrosequencing. (Supplementary material)

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'	**gTYGagggggaggaa**
	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**

Table 20. Sequences of forward and reverse primers and conditions used in real time qPCR analyses. All primers were purchased at Eurogentec, Liege, Belgium. (Supplementary material)

			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

7.3.5 GR sensitivity assay

Whole blood was stimulated with 0.5µg/mL pokeweed mitogen (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).

7.3.6 Endocrine and immune response to SLST

ELISA. Plasma was collected from blood samples before and after the SLST, which were centrifuged at 4°C within 15 min of blood drawing, transported on ice and frozen at -80°C within 6 h. Plasma cortisol levels

were determined in 1:10 dilution with the Cortisol ELISA kit (Enzo, Brussels, Belgium). IL-6 levels in undiluted plasma samples were determined with the Human IL-6 High Sensitivity ELISA (SB Molecular Biology Reagents: Isogen Life Science, Utrecht, Netherlands). Both ELISAs were performed following the manufacturers' instructions, in duplicate, and read on a SpectraMax Plus 384 Microplate Reader (Molecular Devices, Berkshire, UK).

Flow cytometry. Immediately after each blood collection, whole blood was stained for 15 min with antibody mastermixes of six 6-color immunophenotyping panels to measure the relative number and activation status (CD69, CD11a, CD11b) of monocytes ($CD14^{+}CD16^{+/-}$), NK cells ($CD3^{-}CD19^{-}CD56^{+}CD16^{+/-}$), B cells ($CD19^{+}$), T cells ($CD3^{+}$), CTLs ($CD8^{+}$), Th cells ($CD4^{+}$), Th1 ($CCR4^{-}CXCR3^{+}CCR6^{-}$), Th2 ($CCR4^{+}CXCR3^{-}CCR6^{-}$), Th17 ($CCR4^{+}CXCR3^{-}CCR6^{+}$) (Table 21 and Table 22). All stainings were performed at 4°C in the dark. Samples were washed once (FACS buffer containing 0.1% Bovine Serum Albumin Cohn Fraction V, Sigma-Aldrich and 1x Dulbecco's PBS, Lonza BioWhittaker, Verviers, Belgium), incubated overnight at 4°C in BD Lysing Buffer (BD Biosciences, Erembodegem, Belgium), and washed again before acquiring 50,000 leukocytes on the FACSCanto (BD Biosciences). To standardize instrument settings between measurements, Cytometer Setup and Tracking beads (BD Biosciences) were used. Dead cells were excluded with Fixable Near-IR Dead Cell Stain (Lifetech). Relative cell numbers were extracted from the data using FlowJo (version 10.0.7, Tree Star, Ashland, OR).

7.3.7 Statistical analysis

Stress responses in immune variables from different time points were expressing in terms of change scores between baseline and peak values. Visual analogue scale scores for perceived stress and pain were reduced to sum scores. 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).

Table 21. Flow cytometry antibodies. (Supplementary material)

Fluorochrome	Ab	Clone	Company
FITC	CD4	EDU-2	Immunotools
FITC	CD3	MEM-57	ImmunoTools
FITC	CD14	MEM-18	ImmunoTools
FITC	CD19	LT19	ImmunoTools
PE	CD19	LT19	ImmunoTools
PE	CD69	FN50	Biolegend
PerCP-Cy5.5	CD3	HIT3a	Biolegend
PerCP-Cy5.5	CD196	TG7/CCR6	Biolegend
PerCP-Cy5.5	CD16	3G8	Biolegend
PerCP-Cy5.5	CD8a	RPA-T8	eBioscience
PerCP-Cy5.5	CD19	HIB19	Biolegend
PE-Cy7	CD15	SSEA-1	Biolegend
PE-Cy7	CD183	G025H7	Biolegend
PE-Cy7	CD56	CMSSB	eBioscience
PE-Cy7	CD3	UCHT1	Biolegend
APC	CD14	18D11	ImmunoTools
APC	CD194	L291H4	Biolegend
APC-Cy7	Life Dead	-	Lifetech

Table 22. Flow cytometry panels.. (Supplementary material)

Panel	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-Cy7
1	CD4	CD69	CD196	CD183	CD194	L/D
2		CD69	CD16	CD15	CD14	L/D
3	CD3/CD19/CD14	CD69	CD16	CD56		L/D
4	CD4	CD69	CD8a	CD3		L/D
5		CD69	CD19	CD3	CD14	L/D

7.4 Results

7.4.1 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 2 represents the smaller subset of participants that were selected to undergo the SLST (n=71). Comparable to the whole EpiPath cohort, also in this subset groups were similar in age, sex, smoking status, body mass index, and CTQ scores (p>0.05).

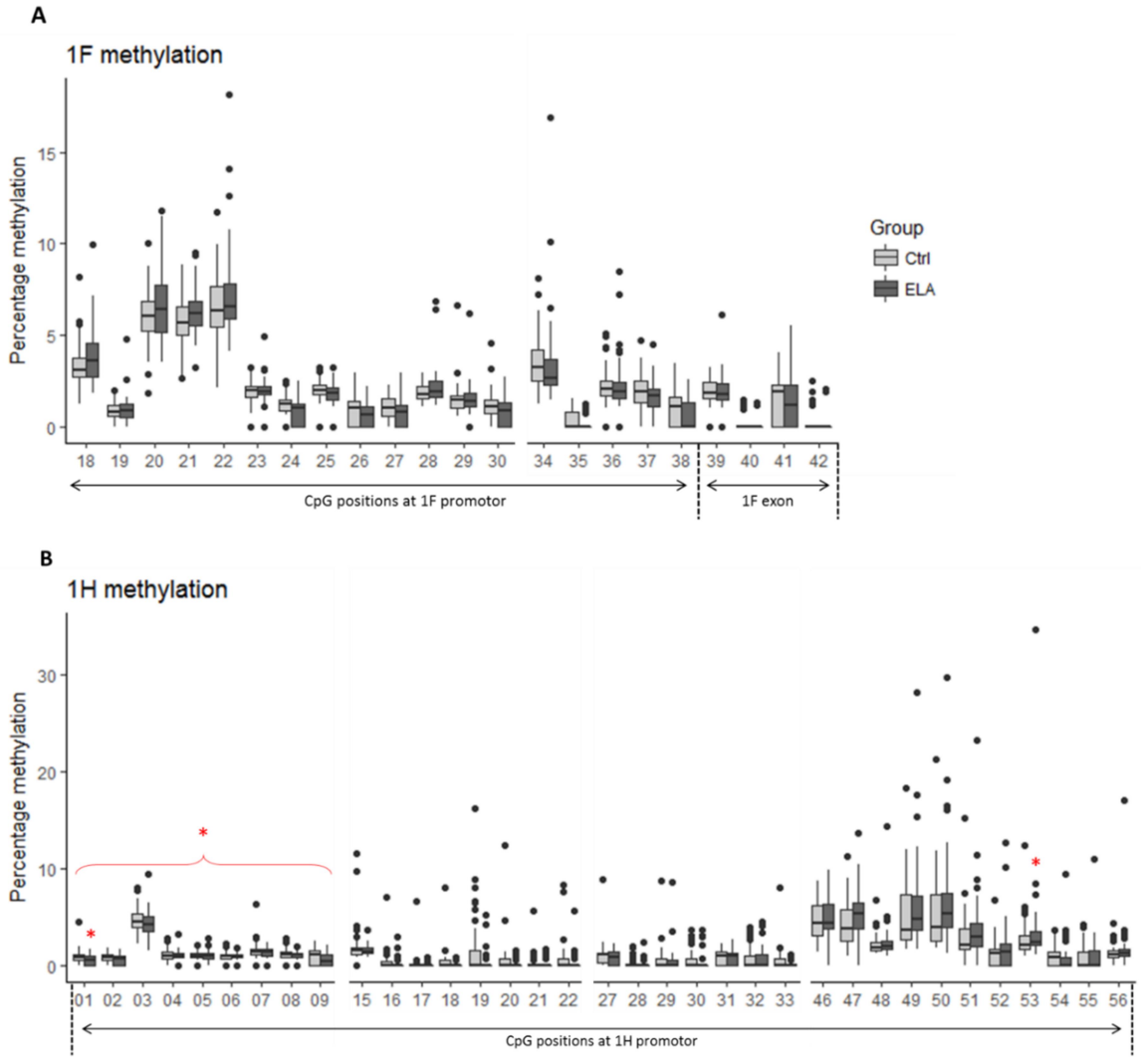

Figure 31. GR DNA methylation levels in Ctrl vs ELA in two first exon promoter regions: 1F (A) and 1H (B).

7.4.2 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 31). 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).

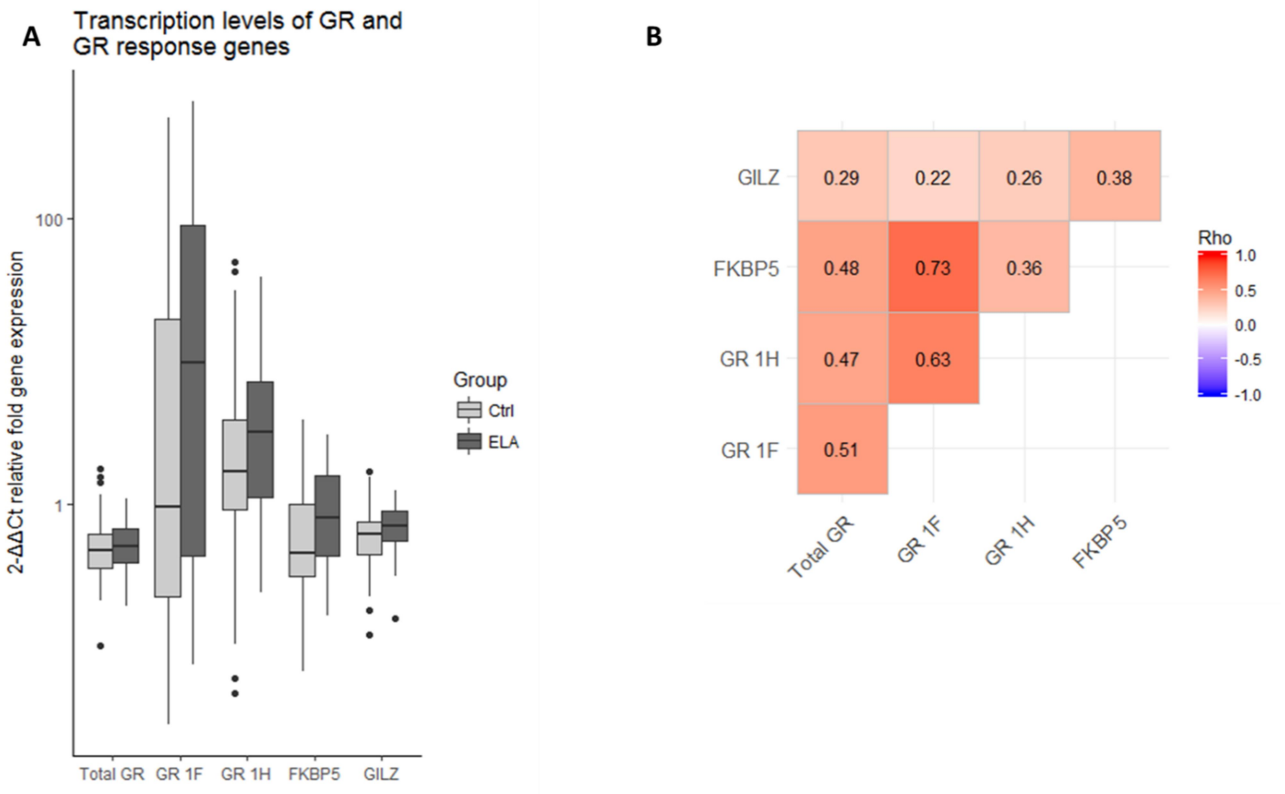

Figure 32. Transcription levels of GR and GR target genes FKBP5 and GILZ (A) and correlation matrix between all four mRNA transcripts (B). All expression levels are relative to a housekeeping gene expression (2-ΔΔCt relative fold gene expression). Statistics: Spearman correlations. Values in correlation matrix show rho values, only shown when significant (p<0.05, after false discovery rate correction).

7.4.3 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. Our data showed high inter-correlation between 1F and 1H transcripts (Figure 32), suggesting that first exon expression levels are co-regulated (Coa-Lei 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 32). 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 32). GILZ was strongest related to 1H transcripts (rho=0.26, p=0.010; Figure 32). However, mRNA levels of GR expression as well as the expression of the two target genes were similar between ELA and controls ($p>0.05$).

*Table 23. Group differences in stress response to SLST. Statistics: Wilcoxon rank-sum test or Student's T test for numerical variables; Chi-square test for categorical variables. # p<0.10, * p<0.05, ** p<0.01, *** p<0.001.*

Difference in stress response between Ctrl and ELA		**Ctrl (n=49)**	**ELA (n=22)**	**p-value**
Psychological	Retrospective account pain experience (sum of intensity, unpleasantness, tension) median [IQR]	21.9 [17.6-23.6]	24.5 [18.1-26.9]	0.091#
	Subjective stress response (sum of anxiety, arousal, and stress deltas) mean [SD]	6.0 [4.5]	8.6 [6.7]	0.102
HPA axis	Plasma cortisol (delta ng/mL) median [IQR]	5.7 [0.7-12.4]	3.1 [0.4-10.3]	0.374
Cardiovascular	MAP (delta) mean [SD]	21.8 [8.3]	23.0 [8.8]	0.595
	HR (delta) median [IQR]	21.5 [12.3-26.2]	20.2 [14.7-26.8]	0.980
Immune system	Plasma IL-6 (delta) mean [SD]	3.4 [2.8]	2.9 [2.2]	0.522
	Monocytes (delta) median % [IQR]	1.7 [0.8-5.0]	2.2 [1.1-4.7]	0.456
	CD16+ monocytes (delta) median % [IQR]	2.5 [1.5-3.4]	1.3 [0.5-2.7]	0.126
	NK cells (delta) median % [IQR]	3.2 [1.1-4.4]	2.6 [0.7-5.7]	0.999

7.4.4 GR sensitivity

To determine GR sensitivity, whole blood from participants was stimulated with pokeweed mitogen. The concentration of dexamethasone that was needed to achieve a 50% inhibition of IL-6 production was used as a measure of GR resistance. Higher methylation levels at GR 1H CpG 5 predicted higher GR resistance (rho=0.40, p<0.001), whereas higher methylation at GR 1F CpG 28 and 29 predicted lower GR resistance (rho=-0.25, p=0.011; rho=-0.24, p=0.012). As expected, higher levels of GR 1F mRNA transcripts were related to lower GR resistance (rho=-0.42, p<0.001). GR resistance showed a similar relationship with GR 1H transcripts, albeit as a trend and to a lesser extent (rho=-0.20, p=0.053). However, there was no group difference in GR resistance between ELA and controls (Wilcoxon rank sum test, p=0.194).

7.4.5 Psychological, endocrine, cardiovascular, and immune stress response to the SLST

As expected, psychological, endocrine, and cardiovascular parameters (see Table 23) significantly responded to the SLST (data not shown). ELA participants had a stronger negative experience of the pain during the SLST, although this did not reach statistical significance (Wilcoxon rank-sum test, p=0.091, Table 3). The relative increase in heart rate, MAP, and plasma cortisol were comparable between groups (Wilcoxon rank-sum test and Student's T test, resp., p>0.05, Table 23). Immune cell redistribution and plasma IL-6 was measured in response to the SLST. Four immune parameters significantly increased after stress (comparing baseline with peak values): plasma IL-6, total monocytes, CD16+ monocytes, and NK cells. Monocytes that express both the LPS co-receptor CD14 and FCγIII receptor CD16, have been classified as non-classical monocytes and are characterized by inflammatory properties (Mukherjee et al., 2015). As expected, NK cells increased in circulation and returned to baseline levels within minutes in response to an acute stress (Breen et al., 2016). Total monocytes, CD16+ monocytes, and IL-6 plasma levels increased gradually over the 3 hours post-SLST. However, there was no difference between groups (Wilcoxon rank-sum tests on baseline-peak deltas, p>0.05).

Figure 33 presents the correlations between the different stress response parameters. The retrospective experience of pain during the SLST correlated with the increase (delta peak) in self-reported stress (rho=0.40, p=0.002), heart rate (rho=0.29, p=0.041), plasma cortisol (rho=0.38, p=0.041), MAP (rho=0.28, p=0.042), and monocytes in circulation (rho=0.43, p=0.027). Self-reported stress correlated with heart rate response (rho=0.48, p<0.001). In turn, heart rate responses were associated with monocyte responses (rho=0.44, p=0.027). NK cell increase in circulation correlated with MAP (rho=0.37, p=0.042) and plasma cortisol (rho=0.43, p=0.027) stress response.

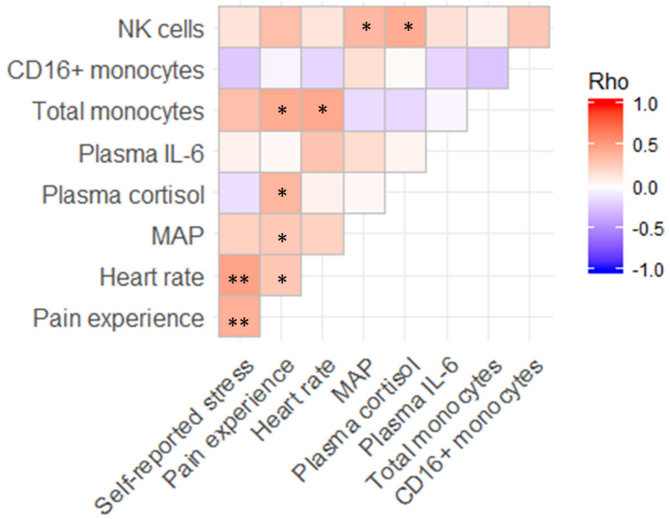

*Figure 33. Correlation matrix between stress response parameters. Statistics: Spearman correlations. # p<0.10, * p<0.05, ** p<0.01, *** p<0.001 after false discovery rate correction.*

7.4.6 Associations between mRNA expression and methylation levels at single CpGs

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 34a). The first 5 CpGs of the 1F region are positively correlated with transcription 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 34b 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).

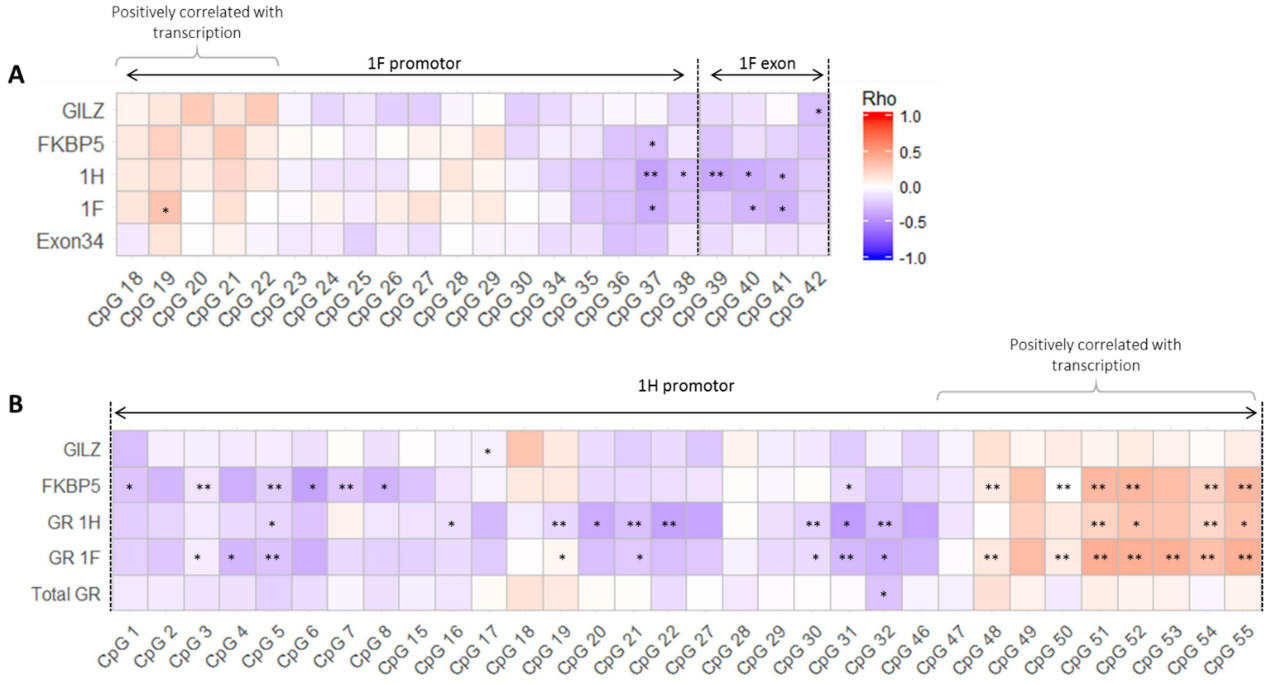

*Figure 34. Correlation matrix between relative mRNA expression of GR and GR target genes and methylation levels at single CpGs in the GR 1F (A) and GR 1H (B) promoter regions. Statistics: Spearman correlations. # p<0.10, * p<0.05, ** p<0.01, *** p<0.001 after false discovery rate correction.*

7.4.7 Associations between stress response parameters and methylation levels at single CpGs

Overall methylation levels of 1F promoter and exon region (sum) predicted a reduced pain experience (rho=-0.32, p<0.001) and lower self-reported stress (rho=-0.46, p=0.016) after the SLST. Figure 35a shows the analysis on single CpG level. Lower methylation levels at position 18 and 21 predicted higher IL-6 plasma levels in response to stress (rho=-0.47, p=0.009; rho=-0.59, p<0.001; resp.). Lower levels of methylation at position 18 were also associated with a higher heart rate increase after stress (rho=-0.35, p=0.020). Lower methylation at position 27 correlated with higher MAP stress response (rho=-0.37, p=0.011). Lower methylation at positions 24, 27, 34, and 38 predicted a stronger experience of the pain induced by the cold water bath (rho=-0.39, p=0.009; rho=-0.45, p<0.001; rho=-0.33, p=0.034; rho=-0.36,

p=0.017, resp.). Finally, lower methylation levels at positions 34, 35, 36, 37, 39, and 42 predicted higher psychological stress response (rho=-0.56, p<0.001; rho=-0.32, p=0.045; rho=-0.38, p=0.009; rho=-0.31, p=0.047, rho=-0.32, p=0.046; rho=-0.38, p=0.009; resp.).

Overall methylation levels at the 1H promoter, however, predicted a higher response in heart rate (rho=0.46, p<0.001) and monocytes (rho=0.41, p=0.032). Although when looking at single CpG, there were no significant correlations between methylation levels at the GR 1H promoter region and the level of the stress response (Figure 35b).

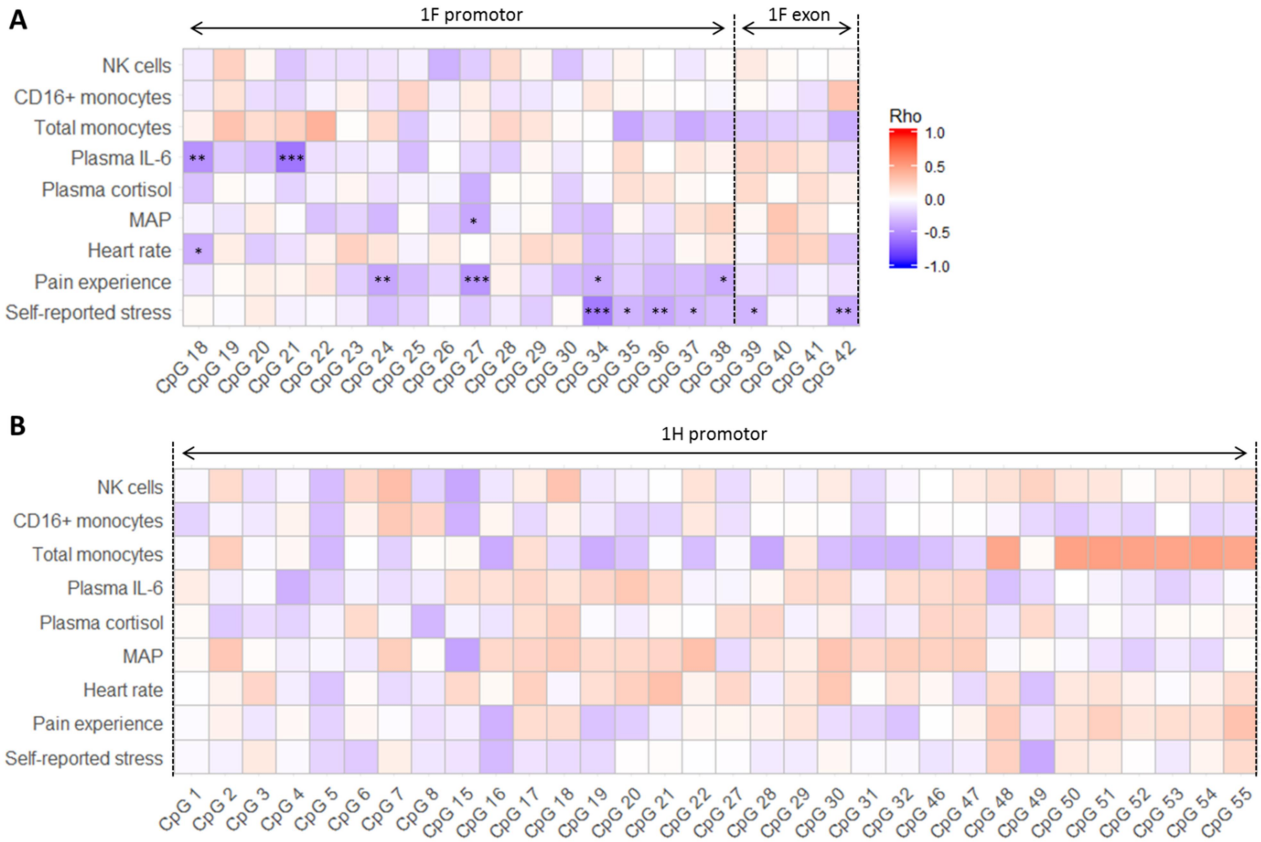

*Figure 35. Correlation matrix between psychological and physiological stress response and methylation levels at single CpGs in the GR 1F (A) and GR 1H (B) promoter regions. Statistics: Spearman correlations. # p<0.10, * p<0.05, ** p<0.01, *** p<0.001 after false discovery rate correction.*

7.5 Discussion

In the present study, we investigated HPA axis 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, as well as the subjective and immune response to an acute stressor. Moreover, we showed that expression of GR transcripts correlated with GR target gene expression. We applied the SLST on a selection of the EpiPath cohort, which induced a robust stress response in immune parameters, such as monocytes, NK cells, and plasma IL-6. To our knowledge, this is the first study investigating the effect of the SLST on immune cell redistribution.

Interestingly, in this cohort, ELA was associated with a higher prevalence of chronic diseases and psychological disorders, and we could demonstrate a clear immune phenotype (Chapter 3, 4, and 5). Here, we investigated whether this phenotype was dependent on disturbances in HPA axis signaling. First, we demonstrated 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. Second, we demonstrated that the endocrine, cardiovascular, and immune responses to acute stress were similar between ELA and controls. These data suggest that the HPA axis is unperturbed in our cohort and that the observed phenotype was not secondary to HPA axis dysfunction.

Here, ELA was characterized by the early separation from parents and institutionalization prior to adoption, which are known and well-established adverse childhood experiences (Julian, 2013; Phillips and Carver, 2015; van IJzendoorn et al., 2011). 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 et al., 2009) and circulating immune cells (Martin-Blanco et al., 2014; Perroud et al., 2011; Romens et al., 2015; Tyrka et al., 2012), although others have not found such an association (Steiger et al., 2013). 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 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 (Martin-Blanco et al., 2014; Perroud et al., 2011). In addition, we included CpGs within the NGFI-A binding region originally 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.

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 et al., 2017a; manuscript under review), 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 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 et al., 2014).

It has become increasingly clear that there is no one-to-one relationship between methylation and transcription (Leenen et al., 2016). Although studies have reported correlations between higher GR methylation and lower GR expression, other studies found no association (Hogg et al., 2013) or in the opposite direction (Labonte 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 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.

Only a few studies have investigated associations between *GR* methylation levels and stress response and the results are inconclusive. For instance, Rooij et al. (2012) found that higher methylation of the 1C promoter predicted a higher stress response to a psychological stress paradigm (de Rooij et al., 2012). Tyrka et al. (2012) reported that higher methylation at the 1F exon predicted decreased cortisol response to the dex/CRH test (Tyrka et al., 2012). Edelman et al. (2012) showed that higher methylation at the 1F promoter predicted lower stress reactivity to the Trier Social Stress Test, although only in women (Edelman et al., 2012). Li-Temple et al. (2016) demonstrated that *GR* genotype and 1F and 1H promoter methylation predicted the response in blood pressure to the 'socially evaluated cold pressor test' (Li-Tempel et al.,

2016). Although there was no correlation between cortisol stress response and methylation levels at 1F or 1H in the present study, we did see that higher methylation at certain CpGs and/or over the whole 1F and 1H regions predicted lower perceived stress, pain experience, and IL-6 response, and higher heart rate and total monocyte response.

Possibly, the effects of ELA on the HPA axis were not detectable yet. With a median age of 22, the participants in our cohort were relatively young, as compared to e.g. the cohort used by Melas et al. (2013) (mean age of 57) that had experienced early parental death. HPA axis dysregulation may develop over time and may not have been present or visible yet. This notion is supported by the fact that blunted stress responses are not reliably shown in children and adolescents (Gunnar et al., 2009), whereas methylation changes – potentially upstream of GR dysregulation – are reported in young cohorts with ELA (Romens et al., 2015). This raises the question whether HPA axis dysregulation may be secondary to the immune changes. Indeed, pro-inflammatory cytokines directly influence the HPA axis. In animal studies, IL-6 has been shown to activate the HPA axis, independent from CRH (Bethin et al., 2000) and IL-1a has been shown to play a role in GR resistance through the p38 mitogen-activated protein kinase signal transduction pathway (Wang et al., 2004).

Finally, not all immune cell types were equally affected by ELA and differences were only detectable when examining specific immune subsets (Elwenspoek et al., 2017a; 2017b; manuscripts under review). Therefore, a limitation of the present study is that we used DNA and RNA isolated from whole blood, which contains multiple different cell types and may have obscured cell specific changes in GR signaling. Nevertheless, cell type specific response to the SLST showed no differences either, so this explanation seems rather unlikely.

In this study, we found no evidence of HPA dysregulation in participants exposed to ELA, suggesting that HPA axis programming does not underlie our observed ELA immune phenotype (Figure 36). 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. 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.

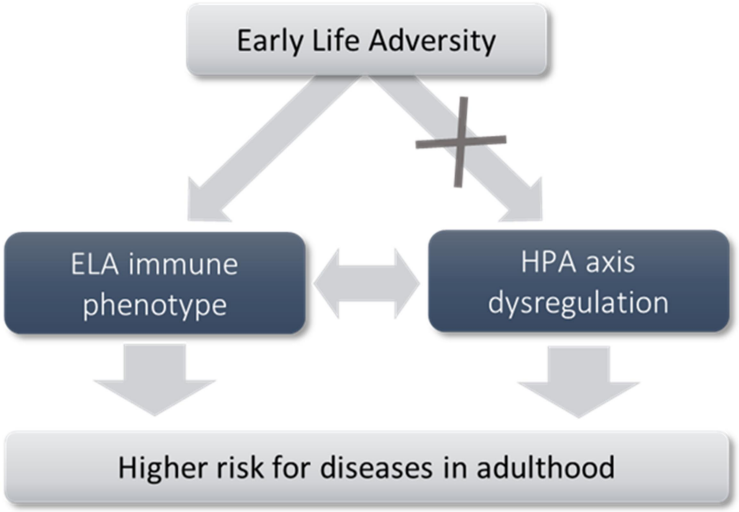

Figure 36. Proposed model. ELA results in an ELA immune phenotype, possibly through epigenetic programming, which leads to an increased risk for diseases in adulthood. Although the HPA axis was not dysregulated in our ELA cohort, the ELA immune phenotype may promote HPA axis dysfunction and once HPA dysregulation is established it may further undermine immune functions in ELA.

7.6 Acknowledgments

We would like to thank Sophie B Mériaux, Fanny Bonnenberger, Stephanie Schmitz, Krystel Sias, Martyna Marynowska, Fleur AD Leenen, 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), Xenia Hengesch and Johannes Finke (University of Trier) for assisting in sample collection during the visit 1. Krystel Sias and Jonathan D Turner assisted in writing of the manuscript. Xenia Hengesch, Fleur AD Leenen, and Hartmut Schächinger were involved in performing the SLST at visit 1. Violetta K Schaan and Claus Vögele were involved in performing visit 2. The study was

conceived by Claude P Muller and Jonathan D Turner with the support and contribution of Hartmut Schächinger and Claus Vögele.

8 General discussion

8.1 Lessons learned from EpiPath

This thesis demonstrates that early life adversity (ELA), in the form of separation from parents in early life followed by institutionalization, has long-term effects on immune function. In Chapter 1, we identified an ELA immune phenotype from the existing literature defined by three characteristics: inflammation, impairment of the cellular immune response, and immunosenescence (Elwenspoek et al., 2017). However, even though this immune phenotype was relatively clear, cell specific data was largely absent. Hence, we investigated all aspects of the ELA immune phenotype from a cell specific perspective and were able to identify T cells as the immune cell type that was particularly affected by ELA. We found a higher level of T cell activation (Chapter 3) and senescence (Chapter 4) associated with ELA.

This thesis was part of EpiPath, an international and multidisciplinary cohort study, aiming to investigate – besides the immunological effects – the long-term psychological and cardiovascular effects of ELA (Figure 37). As expected, also in our cohort, individuals with a history of ELA had a higher risk for SCID-I or SCID-II diagnoses as well as psychological difficulties, such as lower self-esteem and higher chronic stress (Chapter 5). However, the effects of ELA on cardiovascular reactivity were less clear. Although cortisol response was blunted and stress-ratings were higher in ELA compared to controls (in subgroups matched for BMI, age, and sex), there were no differences in cardiovascular reactivity to a laboratory stress test (Chapter 6).

Chapter 1 compared two hypotheses: (1) “ELA independently programs multiple systems” and (2) “ELA programs the stress system, indirectly affecting immune functions”. Although the current literature provides compelling evidence for both of them, no definite mechanism has been established that can explain the development of the ELA immune phenotype. In the EpiPath cohort, we did not find convincing evidence that the HPA axis was affected by ELA. First of all, variations in cortisol levels could not explain the higher activation status of T cells (Chapter 3). Secondly, although we found minor modifications in methylation levels at certain CpGs in the GR gene (*NR3C1*), this did not lead to differences in GR mRNA expression (Chapter 7). Finally, immune parameters, such as circulating inflammation markers and immune cells, showed a similar response to acute stress in ELA compared to controls. Although we observed a

blunted cortisol stress response in the matched groups used in Chapter 6, this was not found when all participants that successfully completed the stress procedure were included (Chapter 7). Taken together, we can conclude that the immune phenotype observed in EpiPath is not secondary to an altered stress system.

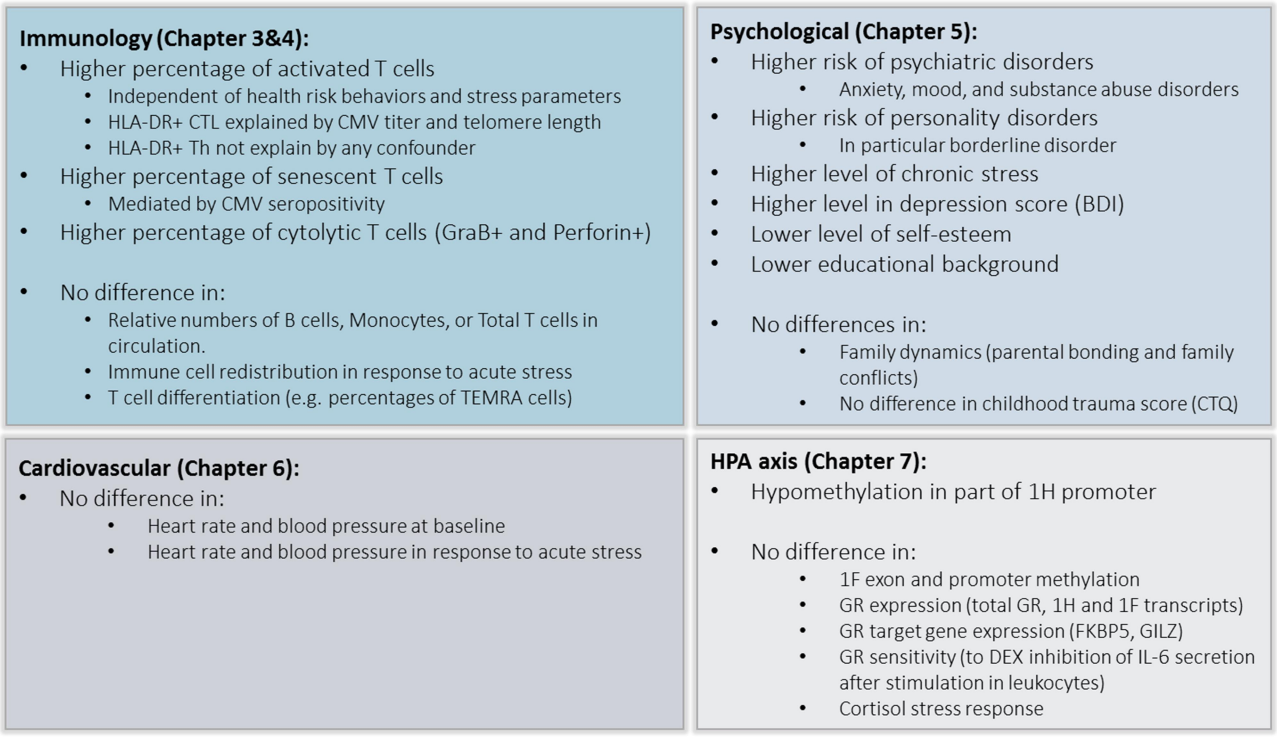

Figure 37. Summary of EpiPath findings

8.2 Key advances made with EpiPath

8.2.1 Inflammation

Our data mainly implicate T cells, but additional cell types may contribute to the ELA-associated low-grade inflammation. In contrast to other studies (Baumeister et al., 2015), we found lower levels of stimulated IL-6 production and no difference in IL-6 plasma levels. The latter may be explained by the fact that IL-6 is difficult to detect in plasma, especially in young and healthy participants. IL-6 plasma levels are often below the detection limit of even the most sensitive methods. Both T cells and monocytes are principal producers of IL-6. While other studies attributed ELA-associated inflammation largely to monocytes (Miller and Chen, 2010; Miller et al., 2009), *ex vivo* production of IL-6 was measured in PBMCs,

so the contribution of T cells cannot be excluded. Increased secretion of IL-6 may be due to hyperreactive T cells and/or monocytes, or may be caused by a relative increase of (activated) T cells or monocytes in the sample. Lower levels of *ex vivo* IL-6 production in our study could be largely explained by a lower proportion of monocytes found in ELA (Chapter 3, Table 11). Although we did not observe high levels of classical inflammation markers, our results imply a relative increase in pro-inflammatory T cells, as we found higher numbers of T cells in an activated and senescent state in ELA (Chapter 3 and 4). These changes may occur prior to a detectable increase in inflammation markers in circulation.

8.2.2 Cellular immune function

Several studies have suggested that the cellular immune system is impaired in ELA, based on a decreased control of latent herpes virus infections. However, antibody titers against specific herpes viruses are at best an indirect proxy of cellular immune capacity, and give no information of altered immune functions at a cellular level. It is important to keep in mind that even when cellular immunity is intact, external and internal factors, such as health risk behaviors or high levels of stress hormones, may result in elevated antibody titers. Importantly, impaired cellular immunity suggests increased susceptibility to infectious diseases and malignancies, but there is no epidemiological data confirming this link in ELA. Nevertheless, our data suggest that the function of immune cells are indeed affected by ELA. Interestingly, this effect may be largely mediated by cytomegalovirus (CMV) infection and immunosenescence (Figure 38).

Studies on the effects of ELA on the humoral immune function are scare, although several lines of indirect evidence suggest that antibody production may be impaired. For instance, individuals exposed to (childhood) trauma show transcriptional differences in genes involved in antibody production. In addition, immunosenescence is characterized with a reduced antibody response to new antigens, which in turn is believed to be accelerated in ELA. In asthmatic children, ELA has been associated with higher IgE levels (Wright et al., 2004). However, since it has not been shown that elevated IgE levels persist into adulthood, it is unclear whether these are really long-term consequences or an immediate and transient effect of

adversity. Our data did not imply any changes in humoral immunity. We did not find any differences in the incidence of allergies in our cohort (Chapter 2, Table 8), nor did we observe any differences in B cell activation, measured in expression of activation markers CD25, CD69, and CD11a (Chapter 3, Table 9). Nevertheless, it would be very informative to investigate antibody responses to a vaccination in individuals with a history of ELA.

8.2.3 Immunosenescence

In addition to an impaired cellular immune response, immunosenescence is related to an altered CD4:CD8 T cell ratio or an increase in regulatory T cells, which have not yet been investigated in an ELA. Many of these effects may only become apparent over time as cohorts age. The most used marker of immunosenescence in telomere length and evidence is accumulating that ELA is associated with shorter telomeres. However, this marker is not very sensitive when used in a cross sectional design, particularly when telomere length is measured in a mixture of cell types such as the blood and when the participants are young. Our data have demonstrated that expression of senescence surface markers can reveal cell specific immunosenescence in ELA, even in a cross sectional sample of young adults. Furthermore, our data imply that CMV infection plays an important role and may mediate the association between immunosenescence and ELA. This has important implication for the telomere length and attrition literature that have not controlled for CMV infection.

8.2.4 Underlying mechanisms

HPA-axis function and mediators were studied extensively in the EpiPath cohort, but our data does not support the notion that an altered stress system underlies the ELA-immune phenotype. Also health risk behaviors – smoking, BMI, and physical activity – did not account for the observed immune differences. However, other confounders may have been included such a poor eating habits, alcohol and other drug use, which may also affect immune function.

Previous studies have found altered epigenetic and transcription profiles in ELA. DNA methylation is one of the most studied epigenetic markers, which was originally believed to suppress gene expression in an all-

or-nothing fashion. It is becoming increasingly clear that the gene regulation by DNA methylation is much more complex than previously assumed. Clear associations between expression and methylation levels in the respective gene promoters are limited (Lam et al., 2012). ELA and other environmental influences are mainly associated with subtle changes in DNA methylation, which are believed to "redistribute the transcriptional landscape, affect translational isoform production, and orchestrate the final proteomic landscape" (Leenen et al., 2016).

Here, we did not observe convincing changes in GR methylation or transcription related to ELA (Chapter 7). However, ELA may have caused small changes in inflammation related genes, such as NFkB, which were not studied in this thesis. However, the whole methylome of the EpiPath cohort is currently under investigation and clear epigenome-wide differences have been observed (Figure 38). Finally, future studies should not limit their investigation to DNA methylation, and include other epigenetic mechanisms, such as histone modification and microRNAs, which play an important role in transcriptional regulation and are understudied in ELA.

Box 1: Key advances made

1. Establishment of a cohort that may be followed up for longitudinal data collection
2. We have shown that ELA has cell type specific long-term effects on the immune system
3. We identified T cells as the immune cell type primarily affected by ELA
4. In ELA, a higher number of T cells showed an active and senescent phenotype
5. Our findings suggest that CMV infection may underlie ELA-associated T cell immunosenescence and activation
6. We could exclude HPA axis dysregulation as mediator of the immune phenotype

8.3 Parental loss, followed by institutionalization, and adoption as model for ELA

8.3.1 Adoption is inevitably associated with ELA

Parental separation in rodents and primates (maternal separation in particular) leads to altered behaviors, functional changes in the biological stress systems, and negatively impacts prefrontal cortex development, which persist into adulthood (Arnsten, 2009; Cirulli et al., 2003; Gunnar and Quevedo, 2007; Sanchez et al., 2001).

Children that are given up for adoption have all experienced separation from their birth parents and provide the human equivalent of these animal models. Indeed, parental loss predicts poorer adult health (Phillips and Carver, 2015), and increased stress sensitization (Slavich et al., 2011). Typically, adopted individuals have experienced institutional case, such as an orphanage. There is accumulating evidence from human studies that early institutionalization negatively impacts: physical development (Johnson et al., 2010), cognitive development (Nelson et al., 2007), attention, emotion and behavior (Stevens et al., 2008), gray matter volume and white matter organization (Govindan et al., 2010; Hanson et al., 2013; Hodel et al., 2015; McLaughlin et al., 2014b; Sheridan et al., 2012), HPA axis activity (Gunnar et al., 2001; Gunnar and Vazquez, 2001; Johnson et al., 2011; Koss et al., 2014), and autonomic nervous system functioning (Esposito et al., 2016).

8.3.2 Institutional care causes ELA by definition

Ijzendoorn et al. (2010) has stated that “The institutional setting itself is in most cases pathogenic and should be classified as a type of child maltreatment, particularly in the form of structural neglect” (van IJzendoorn et al., 2011). Several research teams have described the caregiving in institutions around the world. Although often basic needs in medical care, nutrition, and safety are met, typically social and emotional interactions with caregivers are extremely limited ((Groark and McCall, 2011), Central America (Groark et al., 2011), Romania (Smyke et al., 2007), Russia (St. Petersburg, 2008; Tirella et al., 2008), Greece

(Vorria et al., 2003)). The work of Gunnar et al. (Gunnar et al., 2000; Gunnar et al., 2009; Gunnar et al., 2007) has shown that even in the best institutions, paid employee caregivers who work in rotating shifts, are universally in charge of a relatively large number of children, with ratios of children:caregiver regularly exceeding 20:1. This, by definition, results in infants receiving continuously unstable care, uniformly lacking in both quality and quantity (Smyke et al., 2007). This lack of a stable caregiver is the most potent stressor currently known for human (and non-human primate) infants (Johnson et al., 2002), and it has also been shown that the lack of a stable carer in what would otherwise be considered an enriched environment significantly alters both social and emotional behavior for many years post adoption.

8.3.3 Duration of institutional care as measure for ELA severity

Time spend in an institution has been found to be proportional to the detrimental consequences on biological, social, emotional, neurological, and cognitive systems. For instance, longer length of institutionalization has been associated with shorter telomeres measured in buccal cells (and high quality foster care could not attenuate this association) (Drury et al., 2012; Humphreys et al., 2016), more atypical sensory discrimination, praxis, and sensory modulation scores (Lin et al., 2005), worse growth and cognitive scores (Miller et al., 2005), smaller hippocampal volumes (Hodel et al., 2015), increased disinhibited social behavior (Bruce et al., 2009b), and impaired emotional understanding (Camras et al., 2006). Overall, “institutionalization beyond a certain age is associated with a step-like increase in risk for lasting social and behavioral problems, with the step occurring at an earlier age for children who experienced more severe levels of deprivation” (Julian, 2013). Thus, duration of institutional care or age at adoption are valid proxies for ELA severity.

8.3.4 Strength and limitations of our ELA model

The findings from the psychological assessment (Chapter 5) are in line with the current belief that exposure to ELA is a risk factor for psychological problems and the development of psychiatric disorders later in life. These results confirm that the participants who experienced separation from their parents in

early childhood followed by institutionalization and adoption indeed experienced ELA and that ELA has left a clear psychological mark.

ELA is an overarching term for adverse and stressful circumstances or events in early childhood, which can vary from low socio-economic status to parental divorce to childhood maltreatment. Despite the variation in models used for ELA, findings are similar and all suggest negative long-term consequences for mental and physical health. This suggests that also our findings may be generalized and hold true for other forms of adversity in early childhood. Parental loss followed by institutionalization and adoption was chosen as model for ELA in this thesis, because the detrimental long-term consequences are clear and well established (as discussed above). Furthermore, a major strength of this model compared to other models of ELA is that it can be measured in an objective way and is not affected by recall bias.

The use of this model also has some limitations. In European countries, most adoptions are international, which inevitably leads to a heterogeneous sample of ELA participants. Also in our cohort, participants came from a range of different countries, whereas the control sample was predominantly Luxembourgish. The control group mainly controlled for socioeconomic factors during childhood and adolescence. However, we believe that the ethnic differences had a limited effect on the results, since there was not a particular country of origin overrepresented in the sample. In the present cohort, also ELA was heterogeneous, as some were adopted directly from their birth families without spending time in an institution. Finally, there might have been third factors that were not measured but explain the found associations, for instance nutrition in the homes, infections (hygiene), or environmental pollution. We were not able to control for such factors, as information about the specific circumstances of each institution was not available. Nevertheless, without dissecting all early life environmental factors, EpiPath shows the long-term effects of a combination of adversities that children typically experience who are given up for adoption.

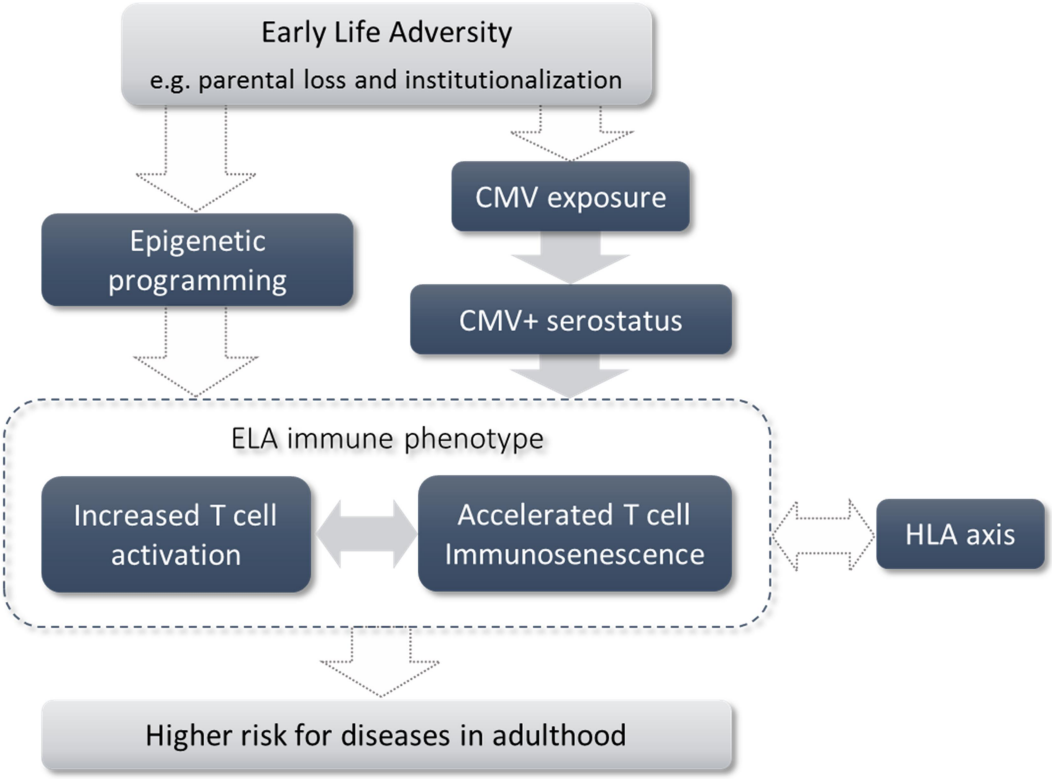

Figure 38. Graphical representation conclusions EpiPath study. Associations suggested by EpiPath are represented by closed arrows. Open arrows represent associations suggested by other work. ELA may increase the risk of early CMV infection, which appears to play an important role in the development of the ELA immune phenotype. Especially T cells are affected by ELA, leading to higher levels of activation and senescence. Although we found no evidence of an altered HPA axis function, immune activation may promote HPA axis dysregulation, which in turn may further promote immune perturbations. First results from whole genome methylation profiling of the EpiPath cohort suggest ELA affects epigenetic programming, which may also influence the ELA immune phenotype (data not presented in this thesis).

8.4 Future perspectives

This thesis has shed more light on the ELA-immune phenotype (see box 1), but it has also generated new interesting and relevant research questions (see box 2). First of all, despite the relative short live spans of circulating immune cells – 6 month to 1 year for T cells, a few hours for monocytes (Parihar et al., 2010) – the ELA immune phenotype appears to be maintained for years. To date, it is unclear how this is possible and which mechanisms are responsible for maintaining the immune differences.

The broad screening of immune cells in circulation revealed a trend towards higher Th17 cells, a cell type that has not been implicated in ELA before. Higher levels of Th17 cells are risk factors for schizophrenia (Drexhage et al., 2011; Matheson et al., 2013) and autoimmune diseases (Wilke et al., 2011).

ELA has also been associated with a higher risk for these diseases, but also to many others, including cardiovascular diseases. Therefore, if these results can be confirmed in a larger cohort, Th17 levels may be used as a biomarker to identify which individuals that are at risk for developing schizophrenia and autoimmune diseases.

ELA is often a combination of several adversities; future research should try to dissect the various aspects of ELA, e.g. distinguishing between physiological stressors, infections, nutrition, and pollution. These questions will need to be addressed in pre-clinical models.

The results presented in this thesis are from a cross sectional design, which can only give us a snapshot of the immune function of an individual. Therefore, it is difficult to draw conclusions about causality. Nevertheless, our results imply that immunosenescence may drive the development of the ELA immune phenotype, leading to higher levels of inflammation and an impaired cellular immunity. Furthermore, now that the cohort is established, the basis is laid for a longitudinal study. Longitudinal data on immune function, and the progression of immunosenescence in particular, would be a valuable addition to the current descriptive phenotype.

Although an increasing body of literature demonstrates immunosenescence in ELA, none of these studies have controlled for CMV infection. CMV is known to drive immunosenescence (Tu and Rao, 2016), and studies have shown that ELA amplifies this effect. However, it is unclear if ELA is associated with immunosenescence in CMV negative individuals. In other words, is CMV infection a prerequisite for ELA-associated immunosenescence?

Work on the EpiPath cohort is still ongoing. Although we did not find changes in DNA methylation of the GR promoter specifically, the methylation profile of other regions may explain the immune findings. To identify these regions, an unbiased approach is used to measure whole genome methylation.

Now that it is clear that ELA represents a public health problem, possible interventions and buffering factors should receive more attention. The use of body-mind therapies, such as yoga, mindfulness, Tai Chi, and meditation, in clinical populations has grown over the last decades. Data from clinical trials suggest

that these therapies can reduce symptoms and improve quality of life (Bower and Irwin, 2016) and they have been effectively used against stress (Chiesa et al., 2009), depression (Piet J et al., 2011), and anxiety (Bohlmeijer et al., 2010). Only in recent years, evidence is accumulating that these therapies can positively affect the immune system. Although the results on circulating inflammation markers, such as CRP, IL-6, TNFa, are inconsistent, studies could show quite convincingly that these therapies affect transcription profiles from inflammation pathways, in particular reducing the activity of NFkB and NFkB target genes. These therapies have not yet been applied in populations with ELA. Understanding long-term effects of *positive* and *nurturing* environments may help define effective prevention strategies.

We have studied a severe form of ELA, where children lost their parents and were exposed to unstable care. Animal models have demonstrated that repeated maternal separation for 6 hours a day has profound behavioral and biological impacts on the offspring. In modern western society, many children spend up to 10 hours a day and 5 days a week in day care. Also in day care centers, care is relatively unstable with high children:caretaker ratios. However, day care has been related to both positive as negative outcomes and interacts with experiences at home as well as the characteristics of the child itself (Bradley and Vandell, 2007). Finally, to put this thesis in a broader societal context, we would like to end with the question what the effect of minder forms of early life separation may be. Can day care have similar consequences as severe forms of ELA in sensitive or predisposed children?

Box 2: New research questions that emerged from EpiPath

1. How is the ELA immune phenotype maintained over time?
2. Does ELA promote the differentiation of Th17 cells?
3. Which elements or aspects of ELA cause the immune phenotype?
4. Does immunosenescence cause inflammation and an impaired cellular immune system?
5. Is CMV infection a prerequisite for ELA-associated immunosenescence?
6. Are modifications of DNA methylation in immune genes underlying these functional immune differences?
7. What are the effects of less severe forms of ELA?

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Presentations and meeting participations

2013:

- Life Sciences PhD days 2013, Luxembourg (L) *participant*
- Doctoral symposium 'Genetics meets Psychology', October 31, 2013, Trier (DE) *participant*
- 13th Euroconference on clinical cell analysis ESCCA, November 12-14, 2013, Luxembourg (L) *participant*

2014:

- UniGR-Workshop Systems Biology, Epigenetics & Systems Analysis, January 22, 2014, Saarbrücken (DE) *participant*
- European Behavioral Pharmacology Society (EBPS) Workshop: Immune Influences on Brain & Behaviour, June 28-30, 2014, Brighton (GB) *poster*
    - *Martha MC Elwenspoek, Johannes Finke, Mauro Larra y Ramirez, Jonathan D Turner, Claude P Muller. Acute stress affects CD69+ expression in lymphocyte subsets in young healthy volunteers.*
- Life Sciences PhD days 2014, September 15-16, Luxembourg (L) *poster*
    - Martha MC Elwenspoek, Johannes Finke, Mauro Larra y Ramirez, Jonathan D Turner, Claude P Muller. The effect of an acute stressor on immune parameters in healthy volunteers.
- 44th Annual Meeting of the German Society for Immunology (DGfI), September 17-20, 2014 Bonn (DE) *talk*
    - Talk: The effect of acute stress on the immune sytem.
- Trierer Stressforschung Quo Vadis, December 8, 2014, Trier (DE) *poster*
    - Poster: Martha MC Elwenspoek, Xenia Hengesch, Hartmut Schächinger, Jonathan D Turner, Claude P Muller. Early Life Adversity changes Immune Parameters and the Immune Response to Stress.

2015:

- Educational short course GEBIN, April 22-23, 2015, Munich (DE) *talk*
    - Talk: The long-term effects of early life adversity on immune function
- 11th Scientific Meeting of the German Endocrine-Brain-Immune-Network (GEBIN), April 23-25, 2015, Munich (DE) *talk*
    - Talk: Early life adversity alters the activation status of T cells
- Treffen des Forschungsschwerpunkts "Psychobiologie des Stresses", June 8, 2015, Trier (DE) *participant*
- The International Society of Psychoneuroendocrinology (ISPNE) Annual Meeting: Stress and the Brain: from fertility to senility, September 8-11, 2015, Edinburgh (GB) *poster*
    - Poster: MMC Elwenspoek, X Hengesch, FAD Leenen, H Schächinger, CP Muller, JD Turner. Increased methylation of the GR 1F and 1H promoters in post-institutionalized adults.
- Life Sciences PhD days 2015, September 21-22, Luxembourg (L) *talk*
    - Talk: Epipath: Long-term effects of early life adversity.

2016:

- 23rd Annual Scientific Meeting of the PsychoNeuroImmunology Research Society (PNIRS), June 8-11, 2016, Brighton (GB) *poster*
    - Poster: MMC Elwenspoek, V Schaan, X Hengesch, C Voegele, H Schaechinger, CP Muller, JD Turner. Early life adversity associates with increased depressive symptoms and few active T cells in adulthood.
- Life Sciences PhD Days 2016, November 24-25, Luxembourg (L) *participant*

Publications

1. Chapter 1 is published in part (section 1-3) as: **Elwenspoek MMC**, Kuehn A, Muller CP, Turner JD. The effects of early life adversity on the immune system. Psychoneuroendocrinology (2017) 82:140-54. doi: 10.1016/j.psyneuen.2017.05.012. PubMed PMID: 28549270

2. Chapter 3 has been submitted to the *Journal of Immunology* and is under revision as: **Elwenspoek** MMC, Hengesch X, Leenen FAD, Schritz A, Schaan VK, Meriaux SB, Schmitz S, Bonnemberger F, Schächinger H, Vögele C, Turner JD, Muller CP. Pro-Inflammatory T Cell Status Associated With Early Life Adversity.

3. Chapter 4 has been submitted to *Frontiers in Immunology* and is under revision as: **Elwenspoek MMC**, Sias K, Hengesch X, Schaan VK, Leenen FAD, Adams P, Meriaux SB, Schmitz S, Bonnemberger F, Ewen A, Schächinger H, Vögele C, Muller CP, Turner JD. T cell immunosenescence after early life adversity: mediation by CMV infection.

4. Chapter 5 was created in collaboration with the Institute for Health and Behaviour of the University of Luxembourg and written by the first author: Schaan VK, **Elwenspoek MMC**, Schulz A, Hengesch X, Turner JD, Muller CP, Schächinger H, Vögele C. Effects of Early Life Adversity on Mental and Physical Health in Early Adulthood. *Manuscript in preparation*

5. Chapter 6 was created in collaboration with the Department of Clinical Psychophysiology at University of Trier and written by the first author: Hengesch X, **Elwenspoek MMC**, Schaan VK, Turner JD, Larra MF, Finke JB, Zhang X, Vögele C, Muller CP, Schächinger H. Blunted Endocrine Response to a Combined Physical and Cognitive Stress Test in Participants who Experienced Adverse Childhood Events. *Manuscript in preparation*

6. Chapter 7 is ready to be submitted as: **Elwenspoek MMC**, Hengesch X, Leenen FAD, Sias K, Beatriz Fernandes S, Meriaux SB, Schmitz S, Bonnemberger F, Schächinger H, Vögele C, Muller CP, Turner JD. Glucocorticoid receptor signalling in leukocytes in early life adversity.

Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertationsschrift selbständig verfasst und keine anderen als die angegebenen Hilfsquellen verwendet habe. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Luxembourg, August 2017

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