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Impact of adverse early life events on physiological stress responses

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General Abstract

Early life adversity (ELA) poses a high risk for developing major health problems in adulthood including cardiovascular and infectious diseases and mental illness. However, the fact that ELA-associated disorders first become manifest many years after exposure raises questions about the mechanisms underlying their etiology. This thesis focuses on the impact of ELA on affective processing (Paper I), immunology (Paper II) and psychobiological stress reactivity (Paper III) in adulthood.

The first study (Paper I) investigated the impact of parental divorce on affective processing of visual emotional information in young adults. A special block design of the “affective startle modulation paradigm” was developed and revealed blunted startle responsiveness during presentation of aversive stimuli in participants who experienced parental divorce during childhood. Nurture context potentiated startle in these participants suggesting that visual cues of childhood-related content activates protective behavioral responses. The findings provide evidence for the view that parental divorce leads to altered processing of affective context information in early adulthood.

A second investigation (Paper II) was conducted to examine the link between aging of the immune system and long-term consequences of ELA. In a cohort of healthy young adults who were institutionalized early in life and subsequently adopted, higher levels of T cell senescence were observed compared to parent-reared controls. Furthermore, the results suggest that ELA increases the risk of cytomegalovirus infection in early childhood, thereby mediating the effect of ELA on T cell-specific immunosenescence.

The third experiment (Paper III) addresses the effect of ELA on cardiovascular, neuroendocrine and subjective stress reactivity. An extended version of the Cold Pressor Test (combined with a cognitive challenging task) revealed blunted salivary cortisol responses in adults with a history of adoption, while cardiovascular stress reactivity was similar to control participants. This pattern of response separation may best be explained by selective enhancement of central feedback-sensitivity to glucocorticoids resulting from ELA, in spite of preserved cardiovascular/autonomic stress reactivity.

The work presented in this thesis emphasizes the presumably negative impact of early life stress (two different forms of parental separation) on emotional processing, stress responsiveness and immune system function. The evidence provided in these studies may help to explain the emergence of long term negative effects in ELA subjects' health status in adulthood. Altered stress psychobiology may represent the link between ELA and the development of stress-related disorders. Prospective human studies should further concentrate on the psychobiological stress mechanisms responsible for ELA-induced pathophysiology and psychopathology.

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Index of Publications

This doctoral thesis consists of four chapters including chapter I as general background. Chapter II to IV have been published as *original research articles* in international peer-reviewed journals. All articles are presented in their originally published form except for changes in formatting (i.e. figure and table labeling, heading and references).

Content	***has been published as***
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Index of Abbreviations

AA	approach - avoidance
ACE	adverse childhood experience
Ag/AgCl	silver/ silver chlorid
ANOVA	analysis of variance
ASPA	aspartoacylase
aU	arbitrary unit
AUC_i	area under the curve with respect to increase
BMI	body mass index
bpm	beats per minute
cm	centimeter
CM	central memory
CMV	Cytomegalovirus
CNT	control
CPT	cold pressor test
CTL	cytotoxic T lymphocyte
CTQ	childhood trauma questionnaire
Ctrl	control
daU	differential arbitrary unit
dB	decibel
EBV	Epstein-Barr virus
ECG	Electrocardiogram
ELA	early life adversity
EM	effector memory
EMG	Electromyogram
eVAS	electronic visual analog scale
FACES	Family adaptability and cohesion evaluation scale
GraB	granzyme B

HLA-DR	Human Leukocyte Antigen - antigen D Related
HPA	hypothalamic-pituitary-adrenal
HR	heart rate
HSV-1	Herpes simplex 1
Hz	hertz
IAPS	international affective picture system
ICC	inter class correlation
IQR	interquartile range
ISI	inter stimulus interval
ITGA2B	integrin alpha 2b
kg/m^2	kilogram per square meter
kHz	kilohertz
MAP	mean arterial blood pressure
MFI	median fluorescent intensity
mmHg	millimeter of mercury
ms	millisecond
NA	negative affect
NEG	negative
NSAR	non steroidal antiinflammatory drugs
NUR	nurture
PA	positive affect
PANAS	positive affect and negative affect schedule
PASAT	paced auditory serial addition task
PBMCs	peripheral blood mononuclear cells
PDE4C	phosphodiesterase
POS	positive
PTSD	post traumatic stress disorder
SAS	statistical analysis system
SEM	standard error of the mean
SOA	stimulus onset asynchrony
TEMRA	terminally differentiated effector memory

Th cell	T helper cell
T/S	relative telomere to single copy gene ratio
TSST	Trier Social Stress Test
yrs	year

Chapter I: General Background

1.1 Introduction

Exposure to early life adversity is a well-known health determinant that increases the risk for the development of major health problems such as mental illness (McCauley, Kern et al. 1997; Felitti, Anda et al. 1998; Chapman, Whitfield et al. 2004), cardiovascular disorders (Felitti, Anda et al. 1998; Batten, Aslan et al. 2004; Dong, Giles et al. 2004) and infectious/inflammatory diseases (Danese, Pariante et al. 2007; Shirtcliff, Coe et al. 2009). An estimate of nearly 40% of children worldwide are subject to some form of ELA, e.g. maltreatment, household dysfunctions, parental separation or loss (Kessler, McLaughlin et al. 2010). ELA-associated disorders become manifest many years after exposure (Greenfield and Marks 2010; McLaughlin, Green et al. 2010) and, in contrast to other diseases, represent a substantial socioeconomic burden (Fang, Brown et al. 2012). As ELA has been hypothesized to lower the threshold for the development of physical and mental disorders duration, onset and severity of the exposure determinates the impact on health in later life (Heim, Mletzko et al. 2008), also depending on gender differences (McCormick, Smythe et al. 1995; Heim, Bradley et al. 2009). Findings of reduced hippocampal volume in depressed abused individuals as compared to depressed non-abused ones (Vythilingam, Heim et al. 2002) also raise questions about the causal role of early adversity. Given that especially stress-related disorders are shown to be more prevalent in adults who experienced ELA (McCauley, Kern et al. 1997; Batten, Aslan et al. 2004), limbic brain structures such as the hippocampus and the amygdala might be involved in such long-lasting effects. Recent studies found sufficient evidence for a context dependent effect of ELA on brain functioning (Elsey, Coates et al. 2015) that supports the concept of an experience dependent plasticity (Heim, Mayberg et al. 2013). The environmental impact of ELA may also be present in the main stress response system, the hypothalamus-pituitary-adrenal (HPA) axis which is conditioned particularly during early life. Moreover, HPA axis functioning (see section 1.2.3) has been suggested as a potential pathway by

which ELA continues to impair health into adulthood (Barton, Zakreski et al. 2016). Another mechanism proposed is accelerated aging of the immune system, known as immunosenescence (Shalev, Entringer et al. 2013; Elwenspoek, Kuehn et al. 2017) (see section 1.2.2); also, ELA has been associated with elevated levels of inflammation (Baumeister, Akhtar et al. 2016).

This thesis work examines a specific type of ELA in order to extend our knowledge of the mechanisms underlying the etiology of ELA-related disorders. In favor of controlling for potential confounds due to comorbidity, e.g. diseases associated with ELA, the experimental investigations reported in this thesis focus on separation experiences: first, parental divorce representing one of the most common type of ELA (Green, McLaughlin et al. 2010; Greenfield and Marks 2010), and second, individuals who were institutionalized and subsequently adopted.

The thesis consists of four chapters. Starting with this introduction and an overview of the state of research, I will introduce the background needed to understand the aim of the investigations, including a short description of the experiments. Chapter II to IV contain the original published reports on the three experimental studies.

1.2 Methodological background

1.2.1 Startle reflexivity

Research on emotional processing using startle responses gained importance in the last decades (Lang, Bradley et al. 1990), i.a. due to its methodological advantages regarding triggering, recording and quantification. Startle responses protect the organism against harm and prepare for action, e.g. fight or flight (Lang and Davis 2006), orchestrated by the amygdala (Ulrich-Lai and Herman 2009). A sudden and unexpected stimulus activates the polysynaptic startle reflex (Davis 1984; Koch 1999) that consists of neuroendocrine, autonomic, and behavioral changes – such as activation of the hypothalamus-pituitary-adrenal (HPA) axis, increased respiration, hypervigilance, startle eyeblink potentiation and other responses. Somatic muscle contractions represent a major output component of this reflex and appear fast, e.g.

within few hundred milliseconds and less. The eye blink is the most sensitive and consistent startle response across human individuals. Eye blinks are relatively easy to assess and show little habituation during repeated startle stimulation, for example, in experiments of 30 min duration with 40-50 startle-eliciting probes, which are considered feasible (Lang, Bradley et al. 1990). Facial electromyography detects the electrical activity of the orbicularis oculi muscle (Blumenthal, Cuthbert et al. 2005), closest to the neural path of innervation comprising three synapses such as cochlear root nucleus, nucleus reticularis pontis caudalis and facial motor nucleus. The EMG waveform is rectified and integrated and scored for (i.a.) peak latency and amplitude. The latter is supposed to be highly sensitive to emotional, motivational and attentional states of the individual. Given that these variations are under experimental control, eye blink responses may serve as a probe of the individuals' internal condition (Grillon and Baas 2003). Consequently, the emotional context in which startle eye blink is elicited influences the direction of responses. Positive emotional states attenuate and negative ones enhance the startle response. This phenomenon has been named "affective startle modulation" and is supposed to be mediated by the central nucleus of the amygdala that projects to the nucleus reticularis pontis caudalis, concerning startle potentiation. The nucleus accumbens is supposed to play a role in startle attenuation by pleasant cues (Grillon and Baas 2003). According to the emotional priming model (Lang, Bradley et al. 1998) that is related to aversive emotional context the defensive motivational system is primed and allows for facilitated elicitation of (aversive) defensive reflexes, such as the startle reflex. Given an appetitive emotional context, the reflex is inhibited (Bradley, Lang et al. 1993). Numerous studies have shown that an individuals' emotional state may lead to either startle potentiation or attenuation (Kuehl, Lass-Hennemann et al. 2010; Ferreira de Sá, Plein et al. 2014). It may be suggested that an emotional state causing "aversive" potentiation may also favor adverse cardiovascular activation and, eventually, stress related disorders. In psychobiological experiments emotional context was manipulated by paradigms and stimuli with positive or negative hedonic valence, such as pictures (Bradley, Cuthbert et al. 1990), films (Kaviani, Gray et al. 1999), music (Roy, Mailhot et al. 2009), odors (Pause, Adolph et al. 2009), anticipation (Sabatinelli, Bradley et al. 2001) and imagination (McTeague, Lang et al. 2009).

Applying the paradigm of affective startle modulation by visual foreground stimuli of variable emotional and nurture related content to a group of young adults who experienced parental divorce might broaden our knowledge of the impact of early adverse events in adulthood. Furthermore, analysis on potential modification of startle responses in these individuals might offer valuable clues to the pathophysiological embedment of early adversity. In matters of divorce experiences it is hypothesized that nurture cues with basically positive emotional valence are perceived as aversive state in ELA individuals causing startle potentiation which might favor stress related disorders.

1.2.1.1 Affective startle modulation and ELA

Based on the recently reported experience-dependent plasticity (Heim, Mayberg et al. 2013) there is accumulating evidence of ELA induced changes in cortical and limbic brain structures. Observations of increased amygdala volume in humans with exposure to early adverse events (Buss, Pruessner et al. 2012; Pechtel, Lyons-Ruth et al. 2014) support this view. Moreover, findings of hippocampal impairment in adult victims of childhood abuse, related to e.g. memory deficits (Bremner, Randall et al. 1995), lead to the assumption that ELA might pose an important factor of cognitive and behavioral variations. Thus, startle methodology serves as a feasible method to investigate altered information processing reflecting cognitive changes. Enhanced startle has been found in women with self-reported physical or sexual abuse in early childhood, suggesting a long-lasting sensitization of the startle eye blink reflex due to the experience of ELA (Jovanovic, Blanding et al. 2009). Furthermore, another study reported an amygdala over-activity associated with emotional maltreatment that leads to changes in startle responsiveness (van Harmelen, van Tol et al. 2013).

In the matter of child maltreatment, parental divorce is regarded as one of the most common adverse events in childhood (Green, McLaughlin et al. 2010). Indeed, there is accumulating evidence that divorce may represent a serious form of emotional maltreatment, e.g. emotional neglect. Children raised by a single parent reported significantly higher rates of emotional neglect compared to children living with two parents (Sedlak, Mettenburg et al. 2010). Given the fundamental role of the limbic system in emotion, startle responsivity may serve as a biomarker of varied affective processing in adults following parental divorce. However, it remains unclear whether

startle enhancement in ELA participants is the result of a per se increased startle reflex, or whether participants' actual emotional processing leads to a context-dependent startle potentiation.

Study 1 (41 healthy volunteers) provides evidence for altered affective context information processing in young adults due to the experience of parental divorce in early childhood. The effect of positive, negative and nurture-related cues on startle responsiveness in healthy adults with and without parental divorce in early childhood was investigated by means of a novel block design based on the affective startle modulation paradigm. Both the need to prevent potential carry-over effects between different stimulus categories and the possibility to clearly distinguish between general and context-driven effects of divorce justified the use of the special block paradigm. Nurture-related stimuli revealed startle potentiation in ELA participants pointing to a negative emotional conflict that activates protective behavioral responses. Furthermore, blunted startle modulation by aversive cues in adults with parental divorce was observed that may result from a tolerance-like effect induced by repeated negative experiences. Additionally, higher values of emotional abuse and emotional neglect were observed in ELA individuals. Together with the context-dependent effect of ELA on startle responsiveness, the results provide novel evidence regarding the impact of the exposure to parental divorce on limbic system functioning and emotional processing.

1.2.2 Immune system and immunosenescence

The immune system, acting as a defender of the human body against pathogens, consists of unspecific (innate) and specific (adaptive) immune responses. By recognizing danger such as infections innate immune cells are able to destroy infected cells and release proteins which recruit further cells to fight the infection. This immune response is known as inflammation (Lawrence and Gilroy 2007). Upon the recruitment of such specific effector cells the innate immune response can develop to an adaptive immune response in which T lymphocytes play a vital role, resulting in cellular immunity and humoral immunity orchestrated by B lymphocytes. The ability of executing a robust and adequate immune response is attenuated by aging (Bauer and Fuente Mde 2016) which implicates a loss of immune function with increasing age: a process referred to as immunosenescence (Shalev, Entringer et al. 2013), to which T cells are supposed

to be very susceptible (Tu and Rao 2016). However, senescent cells are not inactive but show an increased cytotoxicity and produce more pro-inflammatory cytokines. A cell-type specific analysis of immunosenescence can be done by using cell surface markers (e.g. CD57) which are either up- or down-regulated as T-cell senescence progresses (Strioga, Pasukoniene et al. 2011). Furthermore, environmental factors – among others – such as persistent viral infections have been found to modulate the rate of immunosenescence (Bauer, Wieck et al. 2015). For instance, latent infection with Cytomegalovirus (CMV) is believed to represent a mediator of immunosenescence and has been associated with age-related alterations of T-cell immunity (Moss and Khan 2004).

1.2.2.1 ELA-associated immunosenescence

Dysregulation of the immune system might play an important role in increasing the risk for long-term pathologies in individuals exposed to ELA (Danese and McEwen 2012; Fagundes, Glaser et al. 2013). Parental loss, institutionalization, and maltreatment has been associated with increased levels of inflammation (Danese, Caspi et al. 2011; Baumeister, Akhtar et al. 2016). Childhood sexual abuse, indeed, has been linked to an elevation as well as a decrease in several immune system parameters (Ayaydin, Abali et al. 2016) which might also link ELA to chronic diseases and a proinflammatory state in adulthood (Baumeister, Akhtar et al. 2016; Boeck, Koenig et al. 2016). The current literature describes an *ELA immune phenotype* characterized by impairment of the cellular immune system, chronic inflammation state and accelerated immunosenescence (Ehrlich, Ross et al. 2016). An increased level of inflammation can result in T-cell proliferation shortening telomeres (Aviv 2004) which are tandem repeats at the ends of chromosomes and which become shorter with age and with every cell division (Blackburn 1991). Many studies have reported an association between ELA and reduced telomere length, probably also related to accelerated cell aging and mortality (Epel, Blackburn et al. 2004; Kiecolt-Glaser, Gouin et al. 2011). Moreover, experience of institutionalization in childhood has been linked to diminished telomere length (Drury, Theall et al. 2012). However, the underlying mechanisms behind the development of the *ELA immune phenotype* remain relatively unknown, raising the question whether this is a direct effect of immune programming or indirect effect caused by behavioral changes and/or variation in stress reactivity. Nevertheless, there

is accumulating evidence for the view that ELA accelerates immunosenescence and, thereby, leads to an increased risk and earlier onset of age-related disorders, elevated lifetime morbidity and mortality (Chou and Effros 2013; Childs, Durik et al. 2015).

The second experiment (77 volunteers) focused on investigating accelerated immunosenescence related to ELA. Blood samples of 18 healthy adults with experience of institutionalization and subsequent adoption and 59 controls were collected. Blood plasma, extracted from EDTA tubes, was used to determine CMV titers. Upon analysis, results showed that the effect of ELA was associated with CMV infection specifically, rather than being the consequence of continued reactivation of latent viruses in general. Specific cell surface markers were used indexing high numbers of senescent cells with increased levels of cytotoxicity in adults exposed to ELA. Despite missing direct evidence regarding reduced telomere length in ELA individuals, results of a mediation analysis indicate that CMV infections represent a crucial mediator of the association between accelerated immunosenescence and ELA.

1.2.3 Autonomic nervous system and HPA axis reactivity to stress

Stress has been defined as an actual or anticipated disruption of homeostasis in which neural and neuroendocrine systems are activated subsequently (Tsigos and Chrousos 1994; Tsigos and Chrousos 2002). The autonomic nervous system (ANS) and the hypothalamus-pituitary-adrenal (HPA) axis are the two primary systems of this response to stress with the aim to maintain homeostasis (Cannon 1939). The ANS contains two branches, the sympathetic and parasympathetic nervous system which effect rapid alterations in physiological states and thereby mediate the initial stress response (Ulrich-Lai and Herman 2009).

The brain, with central control relays located in the hypothalamus and brain stem, is the coordinator triggering the response to any stress-related stimulus. In case of a homeostatic perturbation, the brainstem activates the sympatho-adrenomedullary (SAM) system, a shortlived response which in turn is modulated and counteracted by the parasympathetic branch (Ulrich-Lai and Herman 2009) that, therewith, controls the duration of the autonomic response. SAM activation represents the classical fight-or-flight reaction resulting in elevated levels of catecholamines, increasing blood pressure, heart rate and force of contraction, peripheral vasoconstriction and energy

mobilization. Exposure to stressors also activates neurons in the paraventricular nucleus (PVN) of the hypothalamus resulting in a secretion of releasing hormones, such as corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) which in turn promote the adrenocorticotropic hormone (ACTH) secretion in the anterior pituitary. Subsequently, this secretion initiates the synthesis and release of glucocorticoids (GC) in the adrenal cortex resulting in energy mobilization. GCs represent the final effectors of the HPA axis and play a fundamental role in the termination of acute stress responses via a negative feedback mechanism. GCs, mainly cortisol in humans, operate via a binary receptor system, mineralocorticoid receptors (MR) and glucocorticoid receptors (GR), functioning in a different manner (Gunnar and Quevedo 2007; Ulrich-Lai and Herman 2009). The maintenance of homeostasis and prevention of disturbances is mediated by MR. GR facilitates recovery from disturbances and prepares for future events. Moreover, it is hypothesized that an imbalance of GR-MR mediated effects leads to a loss of the ability to maintain homeostasis if challenged by an adverse event, followed by neuroendocrine dysregulation and impaired behavioral adaptation which again might lead to stress-related disorders, i.a. depression (De Kloet, Vreugdenhil et al. 1998).

1.2.3.1 The stress response system and ELA

Maternal separation is known to cause long-term consequences for HPA axis functioning later in life (Francis, Diorio et al. 2002; Sanchez, Noble et al. 2005) and to increase the risk of developing physical and mental disorders (Felitti, Anda et al. 1998; Wegman and Stetler 2009). Despite previous findings of both higher risk of early onset and higher rates of major depression in women compared to men (Weissman and Klerman 1977; Wittchen, Essau et al. 1992; Kessler, McGonagle et al. 1993), reports of sex-differences in ELA-related mental disorders are rare and inconsistent. While studies of rodent models observed increased HPA-axis responsivity after prenatal stress exposure in male offspring (Mueller and Bale 2008), human research revealed a higher risk of stress-induced HPA axis dysfunction in female victims, presumably contributing to the fact that onset of depression is associated with ELA in both male and female victims (Weiss, Longhurst et al. 1999). Furthermore, varying results of hyper-responsiveness of the HPA axis and ANS (Heim, Newport et al. 2000) and otherwise a suppression of endocrine responsivity to laboratory stressors (Carpenter,

Carvalho et al. 2007) demonstrate the diverse impact of ELA on the stress response system in adulthood. However, this variation in the direction of effects on stress responsivity may also reflect differences in duration, onset and type of ELA (Heim, Mletzko et al. 2008), as well as being a function of stressor categorization (Dayas, Buller et al. 2001), i.e. physical and psychological stress. Thus, different stressors require diverging adaptations (Goldstein 2010; Pacak and Palkovits 2001) that result from afferent inputs which mediate stressor-specific information to the HPA axis and ANS (Dickerson and Kemeny 2004; Ulrich-Lai and Herman 2009). A combination of the two main types of stressor, e.g. recently reported within the Maastricht Acute Stress Test (MAST) protocol (Smeets, Cornelisse et al. 2012), might help to elucidate basic effects of ELA on stress responsivity (avoiding contingent variation), also concerning sex-specific differences.

The Cold Pressor Test (CPT) (Hines and Brown 1932), well validated in psychobiological research, is known as a physical stressor triggering the activation of the sympathetic nervous system and HPA axis. Despite various versions, e.g. exposure to cold of both hands (Suter, Huggenberger et al. 2007), elbow (Sanger, Bechtold et al. 2014) or forehead (Saab, Llabre et al. 1993), the bilateral feet CPT was shown to induce the most robust endocrine and autonomic responses (Larra, Schilling et al. 2015). There is reliable evidence that the combination with a social evaluative task, e.g. the mental arithmetic task of the Trier Social Stress Test (TSST) within the MAST protocol, ensures strongest stress responses.

In a similar vein, the third study (44 volunteers) of the present thesis aimed at ensuring pronounced endocrine and autonomic responses by means of an extended stress protocol, in order to investigate the impact of ELA in terms of parental separation (institutionalization/adoption) on stress reactivity. After preparation for physiological measurements, 22 control and 22 ELA participants conducted a PASAT teaching session to ensure understanding of the arithmetic instructions. The following application of a bilateral feet CPT in conjunction with the feasible and short Paced Auditory Serial Addition Task (PASAT, (Gronwall 1977)), simultaneously, induced very strong cardiovascular activation (e.g. increase of mean arterial pressure [MAP] ~30 mmHg) and endocrine responses within the cohort. However, stress reactivity of salivary cortisol was blunted in ELA participants, as compared to the control group. Indeed, autonomic responses to the extended stress protocol were preserved and

similar to non-ELA participants. Group differences were analyzed by two factorial ANOVA. The results justify the assumption of a response separation due to ELA exposure potentially initiated by increased glucocorticoid feedback sensitivity (Mirescu, Peters et al. 2004). Additionally, the study supports prior research reporting higher levels of negative mood after stress in ELA individuals (Zakreski, Barton et al. 2016). However, the experiment was not able to clarify sex differences.

1.3 General conclusion

The here presented work aims at broadening the knowledge of the impact of adverse early life events on physiological responses in adulthood. As a summarizing result, the thesis is capable of highlighting a multilevel effect of early life adversity that emerges years after exposure. On the one hand, there is sufficient evidence of alterations in emotional processing (Paper I). Otherwise, results corroborate the impact of ELA on an immunological level (Paper II) and modifications in stress physiology (Paper III) represent the third level.

Nevertheless, future research should try to substantiate the findings to better address the underlying cause of ELA-related disorders. Continuative, this might support the identification of children and adults who experienced some form of ELA. Prevention programs and selective therapies based on the causality of ELA-related disorders may help to avoid such diseases in adulthood being a considerable burden for the social, medical, educational and psychological public services.

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Chapter II: Enhanced Startle Reflexivity During Presentation of Visual Nurture Cues in Young Adults Who Experienced Parental Divorce in Early Childhood

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2.1 Abstract

Adverse childhood experiences (ACE) may influence stress and affective processing in adulthood. Animal and human studies show enhanced startle reflexivity in adult participants with ACE. This study examined the impact of one of the most common ACE, parental divorce, on startle reflexivity in adulthood.

Affective modulation of acoustically-elicited startle eye blink was assessed in a group of 23 young adults with self-reported history of parental divorce, compared to an age- and sex-matched control group (n=18). Foreground pictures were either aversive (e.g. mutilation and injury), standard appetitive (e.g. erotic, recreational sport), or nurture pictures (e.g. related to early life, parental care), intermixed with neutral pictures (e.g. household objects), and organized in three valence blocks delivered in a balanced, pseudo-randomized sequence. During picture viewing startle eye blinks were elicited by binaural white noise bursts (50ms, 105 dB) via headphones and recorded at the left orbicularis oculi muscle via EMG.

A significant interaction of group X picture valence (p = .01) was observed. Contrast with controls revealed blunted startle responsiveness of the ACE group during presentation of aversive pictures, but enhanced startle during presentation of nurture-related pictures. No group differences were found during presentation of standard appetitive pictures. ACE participants rated nurture pictures as more arousing (p = .02) than did control participants.

Results suggest that divorce in childhood led to altered affective context information processing in early adulthood. When exposed to unpleasant (vs. neutral) pictures participants with ACE showed less startle potentiation than controls. Nurture context, however, potentiated startle in ACE participants, suggesting visual cuing to activate protective behavioral responses.

Keywords: adverse childhood experiences, startle eyeblink, emotion

2.2 Introduction

Adverse childhood events (ACE) are believed to induce negative long-term consequences, which may become evident decades after exposure. Physical and mental health problems tend to increase (Felitti, Anda et al. 1998; Wegman and Stetler 2009), and especially stress-related psychological and cardiovascular disorders are shown to be more prevalent in adult participants who experienced ACE (McCauley, Kern et al. 1997; Batten, Aslan et al. 2004).

Rodent models suggest that long-lasting changes in stress responsivity may link ACE to stress-related disease. Indeed, mother-pup separation in the neonatal period, as well as reduced maternal care behavior in early life, decreases the offsprings' physiological adaptive reactivity to stress episodes throughout later life (Plotsky and Meaney 1993; Liu, Diorio et al. 1997; Francis, Diorio et al. 1999; McCormick, Kehoe et al. 2002). However, human research has revealed conflicting results. ACEs have been found to induce hyper-responsiveness of the stress response systems, especially of the hypothalamus-pituitary-adrenal (HPA) axis (Heim, Newport et al. 2000), but other studies observed hypo-responsiveness and a suppression of the stress response to a

psychosocial stressor in individuals maltreated in childhood (Carpenter, Carvalho et al. 2007; Carpenter, Shattuck et al. 2011). Such differences may be partially explained by variance in severity, onset, and duration of ACE (Heim, Mletzko et al. 2008). Different types of ACE have been characterized in humans, varying in nature and incidence. In an attempt to achieve a mostly homogenous sample we restricted recruitment to a single criterion, the experience of parental divorce in early childhood; this is the most common ACE (Rothman, Edwards et al. 2008; Green, McLaughlin et al. 2010; McLaughlin, Green et al. 2010). Indeed, separation of parents and child has been found to enhance the risk of developing a psychological disorder (Kendler, Neale et al. 1992; Neher and Short 1998; Kristjansson, Sigfusdottir et al. 2009) even when separation was only temporary (Raikkonen, Lahti et al. 2011). Separation due to parental divorce has a similar effect on mental health (Hallstrom 1987; Chase-Lansdale, Cherlin et al. 1995), especially when the divorce occurs before the child's age of 9 years. A recent study reports high negative long-term impact of divorce on adult health with the exposure aged between 0-7 years (Thomas and Hognas 2015). Although the evidence suggests mild alterations in HPA axis activity of young adults from divorced parents (Bloch, Peleg et al. 2007), neuroendocrine reactivity to a social stressor was found to be significantly lower in participants from divorced families compared to those with married parents (Kraft and Luecken 2009).

Limbic brain structures such as the hippocampus and the amygdala may be involved in the long-lasting influence of ACE on stress responsiveness. These structures play an important role in the initiation and processing of stress responses. The amygdala appears to orchestrate the stress response (Lang and Davis 2006; Ulrich-Lai and Herman 2009), while stress hormone feedback on the hippocampus inhibits and terminates the neuroendocrine stress response (Tsigos and Chrousos 2002). Indeed, a reduced hippocampal volume has been observed in depressed participants with childhood abuse compared to depressed non–abused individuals (Vythilingam, Heim et al. 2002) and non-depressed control participants (Saleh, Potter et al. 2017), while other studies observed an increased amygdala volume in human participants who experienced ACE (Buss, Pruessner et al. 2012; Pechtel, Lyons-Ruth et al. 2014). Given the fundamental role of the limbic system in emotional and memory processing, it is not surprising that ACE influence a variety of cognitive functions and behaviors. Indeed, memory deficits have been found in adult human survivors of childhood abuse

(Bremner, Randall et al. 1995), as well as in rodents experiencing early maternal deprivation (Oomen, Soeters et al. 2011), effects presumably mediated by hippocampal impairment. Amygdala over-activity (van Harmelen, van Tol et al. 2013) and information-processing biases for facial displays of emotion (Gibb, Schofield et al. 2009) have been found in human adults reporting ACE.

ACE-induced over-activity of the amygdala may also lead to changes in startle responsiveness. However, only a few studies have addressed this research topic. The startle reflex is a physiological response to a sudden and unexpected stimulus (Davis 1984; Koch 1999). It consists of several components, such as somatic and facial muscle responses (e.g. eye blink), autonomic nervous system activation (e.g. heart rate and electrodermal responses), endocrine responses (e.g. HPA axis activation), and behavioral changes (e.g. acceleration of response times). Responses are orchestrated by the amygdala and prepare the organism for rapid action, e.g. fight and flight (Lang and Davis 2006). The startle eye blink is the most sensitive and consistent startle response across human individuals and can be easily and reliably elicited in laboratory settings by the presentations of abrupt and intense acoustic stimuli (Koch 1999).

There is evidence that previous stress episodes may influence the startle reflex. Several studies have shown startle hyper-responsiveness in patients suffering from posttraumatic stress disorder (PTSD) (Ornitz and Pynoos 1989; Morgan, Grillon et al. 1997; Metzger, Orr et al. 1999; Lipschitz, Mayes et al. 2005). Similarly, enhanced startle has also been found in women with self-reported physical or sexual abuse in early childhood, suggesting a long-lasting sensitization of the startle eye blink reflex due to the experience of ACE (Jovanovic, Blanding et al. 2009). However, it remains unclear whether startle enhancement in ACE participants is the result of a per se increased startle reflex, or whether participants' actual emotional processing is leading to a contextually-driven startle potentiation.

An ideal experimental model to clarify this question is offered by the "affective startle modulation paradigm" (Lang, Bradley et al. 1990). The cognitive mechanism underlying affective startle modulation is best explained by motivational priming, with the emotional context in which startle is elicited influencing the magnitude of the startle response. Given an aversive emotional context the defensive motivational system is

primed to facilitate the processing of defensive reflexes, such as the startle reflex. Given an appetitive emotional context the reflex is inhibited. Many studies, including our own (Lass-Hennemann, Deuter et al. 2010; Ferreira de Sá, Plein et al. 2014), have shown that an individual's emotional state may lead to either startle potentiation or attenuation, irrespective of the sensory modality by which the emotional state was induced: pictures (Bradley, Cuthbert et al. 1990), films (Kaviani, Gray et al. 1999) and music of positive or negative hedonic valence (Roy, Mailhot et al. 2009). However, most often visual stimuli, such as pictures included in the International Affective Picture System (Lang, Bradley et al. 2008), are used. We used this type of foreground stimuli as well; however, since we were interested in an early childhood/nurture context, we additionally included high quality pictures of laughing babies, mother/child interactions, and nurture-related objects (e.g. pacifiers). Most often, affective startle modulation is examined by randomized presentation of affective foreground stimuli intermixed with neutral pictures. We were concerned about carry-over effects between adjacent pictures – especially in the sequence of either erotic or violence presentations followed by nurture/baby cues or vice versa – which may create a socially unacceptable association that might disturb participants' willingness to participate and/or tending to induce unfavorable response biases. To avoid such carry-over effects a special block design was created. Affective pictures of three different qualities (negative valence, positive valence, and nurture-related) were presented in three different blocks of a single valence intermixed with non-emotional, neutral stimuli (e.g. household objects) serving as a neutral reference within each block. Separating valence by block but with neutral fillers (Bradley, Cuthbert et al. 1996) removes carry-over effects. Similar designs have been successfully used before and have proved to be feasible (Schlam, Japuntich et al. 2011). Thus, the design used in the current study allowed us to examine the impact of nurture cues vs. neutral cues, and to compare the effects with the results found after presentation of general affective stimuli.

2.3 Methods

2.3.1 Participants

Forty-nine undergraduate students with or without history of parental divorce were recruited by announcements published at the University of Trier and the Trier University of Applied Sciences. Exclusion criteria were acute or persistent medical and psychiatric diseases, current medication except the occasional use of pain killers (paracetamol, aspirin, or NSAR), heavy smoking (>10 cigarettes per day), regular drinking of alcohol beverages (>30 g/day), illicit drug intake within the last 6 months, current or past hearing problems (e.g. tinnitus), or presence of any ACE other than parental divorce. Presence of childhood abuse was checked beforehand by an interview and the Childhood Trauma Questionnaire (CTQ; see section 2.3.7) and participants were excluded in case of sexual and/ or physical abuse.

All study procedures were approved by the local ethical committee and participants gave written informed consent prior to study participation. Financial compensation was 20 €. Eight participants (3 with parental divorce, 5 without parental divorce) were later excluded from statistical analysis because of sexual and physical abuse (1 participant), physical abuse (1 participant) and complete habituation of startle eye blink (referred to as "startle non-responders": participants that fail to respond to the startle noise probe, with no detectable startle eye blink response in 10 consecutive trails, 6 participants). The final sample consisted of 23 women and 18 men, of which 23 participants belonged to the ACE group (ACE; 13 women). Volunteers who were themselves parents were not included. Participants of the control group (CNT; 11 women) had non-divorced parents through the time of testing, and no other childhood trauma.

2.3.2 Procedures

Experimental sessions started in the afternoon between 1 and 5 pm with an interview screening session. Participants were then asked to fill out several questionnaires (see

section 2.3.7), and were then made familiar with the laboratory settings and procedures. They were then seated in the lab chair and devices, electrodes, and headphones were attached. The startle session lasted about 30 minutes. It was followed by subjective rating of all pictures, which lasted about 20 minutes. At the end, participants were debriefed, thanked and received the expense allowance.

2.3.3 Visual Stimuli

In total, 90 pictures served as visual foreground stimuli. They were selected from the International Affective Picture System (IAPS) (Lang, Bradley et al. 2008), as well as from other public internet sources. Half of them were affective pictures, the other half were emotionally neutral. Affective pictures were positive (POS: opposite sex nudes, sport and fun activities), negative (NEG: injury, mutilation, pollution), or nurture-related (NUR: scenes of parental care, baby items, pacifiers). They were assigned to three corresponding valence blocks (3-level within participant factor *VALENCE*). Hence, each valence block consisted of 15 unique pictures of similar affective valence, supplemented with 15 unique pictures of neutral content (e.g. household objects). This was done to avoid re-exposure to identical pictures. Over and within blocks the sequence of picture presentation was fully randomized.

2.3.4 Stimulus Presentation

E-prime software (E-prime 2.0, PST Software, Inc.) was used to control the timing of stimulus presentation during the startle modulation session. Acoustic startle stimuli were white noise bursts of 105 dB, 50ms duration, and instantaneous rise time, presented binaurally via audiometric headphones (Holmco PD-81, Holmberg GmbH & Co. KG, Germany). Visual stimuli were presented with 1280 x 800 pixel resolution on a 15'' TFT-monitor (participant to screen distance was 70 cm).

2.3.5 Startle Experiment

Participants were asked to keep their eyes open and directed to the centre of the screen, to not speak unless obviously necessary, and to avoid body movements. The first six startle probes were presented during a blank screen and served as habituation trials. They were not included in further statistical analysis. In total, 30 pictures were

presented per *VALENCE* block, 15 per condition “affective picture”, 15 per condition “neutral picture”. Their sequence was randomized. Each picture was displayed for 6 seconds, followed by a dark inter-stimulus-interval (ISI) of 4 seconds. In 80 percent of the trials a startle stimulus was presented at a stimulus-onset-asynchrony (SOA) of 3.5 to 5.5 seconds. No startle stimuli were presented during ISI. One *VALENCE* block lasted 5 minutes, and was followed by a 5 minute break during which participants were asked to relax. The order of *VALENCE* blocks was pseudo-randomized so that across all participants per *GROUP*, each *VALENCE* level was equally often the first, second, or third block in the sequence.

2.3.6 Physiological Recordings

Activity of the orbicularis oculi muscle was continuously measured via Electromyography (EMG) using standard Ag/AgCl electrodes (Tyco Healthcare H124SG electrodes) placed below the left eye with an inter-electrode distance of 1.5 cm. The reference electrode was placed on the forehead (Blumenthal, Cuthbert et al. 2005). Data were recorded using a Biopac MP150 recording system (Biopac Systems, Inc.) with 16 bit resolution and a sampling rate of 1 kHz (Blumenthal, Cuthbert et al. 2005). Hardware band-pass filter settings were 10 to 500 Hz, followed by a 28 Hz software high-pass filter (van Boxtel, Boelhouwer et al. 1998). The raw signal was rectified and integrated online with a time constant of 10 ms (Blumenthal 1994).

2.3.7 Questionnaires

Childhood Trauma Questionnaire (CTQ)

The 28–item short version of the CTQ (Bernstein, Stein et al. 2003) was used in German translation (Klinitzke, Romppel et al. 2012). The CTQ consists of three “abuse” subscales (emotional, physical, and sexual abuse), and two “neglect” subscales (emotional and physical neglect). Each subscale contains five items/ questions. Responses on Likert scales range from 1 (“never true”) to 5 (“very often true”). CTQ reliability is high, with Cronbach´s alpha for subscales ranging from .81 to .95 in previous publications (Bernstein, Stein et al. 2003), and from .70 to .81 in the current study. Subscale interclass correlation (ICC) range from .30 (‘emotional neglect’ – ‘sexual abuse’) to .88 (‘physical neglect’ – ‘emotional neglect’) in previous reports

(Bernstein, Stein et al. 2003), and from .31 ('physical abuse' – 'sexual abuse') to .77 ('physical abuse' – 'emotional abuse') in the current study. The subscales of sexual abuse and physical abuse were used to assess exclusion criterion (scores ≥6 for subscale sexual abuse, ≥8 for subscale physical abuse; per CTQ manual). Aside from sexual and physical abuse, other childhood trauma (e.g. death of parent or family member) were excluded by an interview. Thus, the remaining 3 subscales (emotional neglect, emotional abuse, physical neglect) were used to describe the emotional impact of divorce.

Family Adaptability and Cohesion Evaluation Scale (FACES) IV

Family Adaptability and Cohesion Evaluation Scale (FACES) IV is the latest version of a family functioning self–report assessment test (Olson, Sprenkle et al. 1979). The FACES IV questionnaire assesses family functioning in terms of "cohesion" (3 subscales: cohesion, disengagement, enmeshment) and "flexibility" (3 subscales: flexibility, rigidity, chaos) on a five-point Likert scale ranging from 1 "not at all" to 5 "very much". A ratio score of "cohesion" and "flexibility" and a total ratio score is created. In addition, a family communication and a satisfaction scale is included. In the literature Cronbach´s alpha ranges from .75-.87 (Franklin, Streeter et al. 2001) (.79 - .96 for the current study). ICC scored between .006 ('chaotic' – 'enmeshed') and .77 ('chaotic' – 'flexibility') in previous reports (Franklin, Streeter et al. 2001) and between .005 ('chaotic' – 'enmeshed') and .75 (disengaged – cohesion) in the current study.

Positive Affect and Negative Affect Schedule (PANAS)

Participants' current mood was assessed by means of PANAS (Watson, Clark et al. 1988). The PANAS questionnaire consists of two subscales (positive affect, PA; negative affect, NA) containing ten items each. Likert scale responses range from 1 "not at all" to 5 "very much". Cronbach´s alpha scored .89 for PA and .85 for NA in previous reports (Watson, Clark et al. 1988) and .70 for PA and .55 for NA in the current study. As both scales are designed to measure affectivity on two largely independent dimensions, interclass correlation of the two subscales is -.15 and .11 for the current study.

2.3.8 Subjective Ratings

After the startle experiment all pictures were presented again. Participants were asked to rate “subjective valence” anchored 'very unpleasant' on the left extreme, and 'very pleasant' on the right extreme of an electronic visual analogue scale (eVAS: -100 to 100 arbitrary units [aU]). In a similar way “subjective approach-avoidance (AA) tendency” was rated ('want very much to avoid' to 'want very much to approach'), as well as the “subjective arousal level” ('not arousing at all' to 'very much arousing').

2.3.9 Startle Data Analysis

Startle eye blink responses were analysed offline with a C++ based, semi-automated customised program (CLIP). Baseline was defined as the average signal level in the 50ms interval preceding startle stimulus onset (Lass-Hennemann, Deuter et al. 2010). An algorithm identified signal peaks in the time interval of 20 to 150ms after startle stimulus onset. Startle response amplitude was defined as the difference between peak and baseline signal. Each eye blink response was manually confirmed. The resulting time series were corrected for non-responses (failure to respond to the probe) and artefacts. Non-responses were scored as zero and included in the analysis (1.5 % of all responses). Artefact trials (due to sampling errors, spontaneous eye blinks coinciding with startle stimulus presentation, trials with excessive background noise or atypical multiple peaks) were set to missing and were excluded from analysis (3.4% of all responses). Non-response and artefact (noisy) trial rates of the current study are within the expected range (zero-responses: 1.1% to 3.4%; noisy trials 2.8% to 14.0%; (Deuter, Kuehl et al. 2012; Deuter, Best et al. 2014)). Differences between groups (t-tests) are neither found for noisy (ACE: 3.2 +/- 0.4; CNT: 3.6 +/- 0.3; p=.55), nor zero response (ACE: 1.7 +/- 0.1; CNT: 1.5 +/- 0.1; p=.16) trials. Eye blink response amplitudes were log-transformed by $\log_e(3+X)$ [3µV was defined as the minimum reliably detectable response amplitude], and averaged per participant, block, and condition (affective picture, neutral picture) by including zero-responses. Thus, log-transformed eye blink response magnitude served as the principal startle measure. Affectively induced startle modulation was calculated as the startle response found

during a particular valence picture presentation minus the response found during neutral picture presentation (within the same block).

2.3.10 Statistical Analysis

Eye blink response magnitude and subjective ratings were analysed in a two-factorial mixed-design repeated-measures ANOVA, with the between-groups factor GROUP (2 levels: ACE vs. control) and the within-participant factor VALENCE (3 levels: POS, NEG, NUR). Main effects and the interaction (GROUP X VALENCE) term are reported for each analysis. The Greenhouse-Geisser correction was applied whenever sphericity adjustment was required (adjusted *p* values are reported with uncorrected degrees of freedom). Significant interactions were followed by simple independent t-test based contrasts comparing means of both GROUPs at each VALENCE level.

Habituation of eye blink response magnitude was analysed in a two-factorial mixed-design repeated-measures ANOVA, with the between-groups factor GROUP and the within-participant factor BLOCK-NUMBER (levels 1 to 3). Main effects and interaction are reported. The analysis of habituation was performed on startle responses to neutral stimuli.

Valence, arousal, and approach/avoidance ratings of neutral picture were analysed for carry-over effects of emotionally-valenced pictures on neutral pictures by ANOVA, with between-subject factor GROUP, within-participant factor VALENCE, and GROUP X VALENCE interaction.

Mean differences for questionnaire data were tested by group t-tests. All statistical analyses were conducted with SAS 9.1 running on Win 7 platform. The critical alpha-level set to α=.05 (two-tailed). Mean (+/- SD) is reported in text and tables. Mean and 95% confidence limits are illustrated in Figure 1 and Figure 2.

2.4 Results

Thirteen women and ten men (n=23; age: 23.6 +/- 3.7 yrs.) participated in the final ACE group. Their age at parental divorce was 4.8 (+/- 3.4) years. Ten women and eight men (n=18) participated in the control group (age: 24.3 +/- 4.2 yrs.). Groups did not differ in age (t-test; p=.62) or sex distribution (chi-square; p=.95). Given the relatively small numbers of participants participating in the present study, we abstained from including gender as a factor.

2.4.1 Questionnaires

Results of the questionnaires are presented in Table 1. The three reported subscales of the CTQ show higher values for the ACE group compared to the controls, with significant group differences for the subscales 'emotional abuse' and 'emotional neglect'. There was a marginal tendency towards a difference between the groups for physical neglect as well.

Significant differences between the groups were found for FACES IV 'balanced cohesion' and 'balanced flexibility' levels, pointing towards more normal family functioning in control participants compared to ACE participants. A significant difference was also shown for the subscale 'disengagement', with higher values for ACE participants compared to controls. A total ratio score of cohesion and flexibility regarding balanced versus unbalanced family systems was calculated. The greater the value above 1 the more balanced the family system. A one sample t-test showed a significant difference from 1 for both the control group (t (17)=8.3, p=.000) and the ACE group (t (22)=4.0, p=.001). This suggests a healthy family system within both groups. However, the cohesion ratio and the total ratio revealed a significant difference between the groups, indicating a higher level of balanced family system in the control participants compared to the ACE participants. Supporting the hypothesis of this family assessment model of better communication and satisfaction within balanced family

systems, we found a significant difference of the ‘communication’ scale between the groups, with higher values for the controls.

There was no significant difference in current mood of the young adults between the groups as assessed by PANAS.

Analysis of questionnaires

	Control Group	ACE Group	*t*	*p*
CTQ				
emotional abuse	6.71 (1.26)	10.13 (5.01)	3.15	.004*
emotional neglect	10.47 (3.45)	15.09 (5.83)	3.13	.003*
physical neglect	3.35 (0.79)	4.26 (2.20)	1.83	.078
FACES IV				
flexibility levels				
balanced flexibility	24.22 (2.96)	21.04 (6.12)	-2.18	.036*
rigidity	16.78 (2.76)	15.04 (4.54)	-1.51	.139
chaos	16.00 (3.94)	18.09 (6.43)	1.28	.209
cohesion levels				
balanced cohesion	28.44 (3.22)	23.48 (6.73)	-3.11	.004*
disengaged	16.67 (3.13)	21.30 (4.99)	3.64	.001*
enmeshed	13.17 (3.52)	11.78 (3.03)	-1.33	.193
ratio scores				
cohesion ratio	2.93 (1.05)	2.02 (1.28)	-2.52	.016*
flexibility ratio	2.02 (0.63)	1.68 (0.82)	-1.52	.136
total ratio	2.48 (0.75)	1.85 (1.00)	-2.31	.027*
family scales				
communication	38.72 (5.26)	33.22 (10.96)	-2.12	.042*
satisfaction	36.50 (5.07)	33.57 (8.88)	-1.33	.191
PANAS				
positive affect scale	28.44 (3.60)	30.21 (4.45)	1.36	.180
negative affect scale	11.94 (1.51)	11.87 (2.11)	-0.18	.900

Table 1: Analysis with ACE and control group on questionnaires: CTQ, FACES IV, and PANAS. CTQ: Childhood Trauma Questionnaire; FACES IV: Family Adaptability and Cohesion Evaluation Scale IV; PANAS: Positive Affect and Negative Affect Schedule; *: significant at $p<.05$; independent samples t-test. Data represent mean +/-SD. ACE: adverse childhood experiences

2.4.2 Startle Eye Blink Habituation

The average log-transformed startle eye blink magnitude for responses elicited during presentation of neutral pictures decreased with BLOCK-NUMBER (experiment time): (1st) 3.96 +/- 0.6; (2nd) 3.73 +/- 0.7; (3rd) 3.60 +/- 0.7 [log µV], main effect of BLOCK-NUMBER (F [2,76]=25.6, p=.0001), but no main effect of GROUP (F [1,38]=0.0, p=.96) and no interaction of GROUP X VALENCE (F [2,76]=0.8, p=.45). Polynomial contrast revealed a linear habituation trend (F [1,38]=38.6, p=.0001) but no quadratic trend (F [1,38]=2.2, p=.14).

2.4.3 Startle Eye Blink Modulation

Log-transformed startle eye blink magnitude data per condition – separately for affective and control pictures – is illustrated in Figure 1. However, affective startle modulation scores (the difference between startle elicited during affective and control picture presentation) were used for statistical testing. ANOVA revealed a significant main effect of VALENCE (F [2,76]=11.4, p=.0001), but no main effect of GROUP (F [1,38]=0.2, p=.63). A significant interaction of GROUP X VALENCE emerged (F [2,76]=5.3, p=.01). Contrasting t-tests between GROUPs at each VALENCE level revealed lower affective startle eye blink modulation in the ACE group (vs. the control group) during foreground presentation of negative pictures (t (22)=2.9, p=.007), and higher startle eye blink during presentation of nurture pictures (t (22)=2.1, p=.04). No significant difference between GROUPs was present during foreground presentation of standard positive pictures. A dependent t-test clarifying whether there are differences in affective startle modulation across conditions within each group revealed significant differences within the control group between the negative and the nurture condition (t (17)=2.5, p=.025), as well as the negative and the positive condition (t (17)=2.9, p=.01). Within the ACE group, a significant difference was observed between the negative and the positive condition (t (22)=4.1, p=.0001), and a trend towards statistical significance was detectable between the nurture and the positive condition (t (22)=1.7, p=.09). Note, that the above comparisons refer to affective startle modulation scores, calculated by subtracting neutral from affective startle data.

Although graphical inspection of the "negative" block suggested enhanced startle during presentation of neutral pictures in ACE participants, statistical testing of control startle did not reveal any GROUP (F [1,38]=0.01, p=.94) or VALENCE (F [2,76]=1.0, p=.37) main effects, nor a VALENCE x GROUP (F [2,76]=1.0, p=.38) interaction. Furthermore, a dependent t-test of startle during presentation of neutral pictures in the "negative" block against startle during presentation of neutral pictures averaged over the other two blocks in ACE participants revealed only a marginal trend towards a significant difference (0.15 +/- 0.09 [log µV]; t (22) =1.6, p=.12).

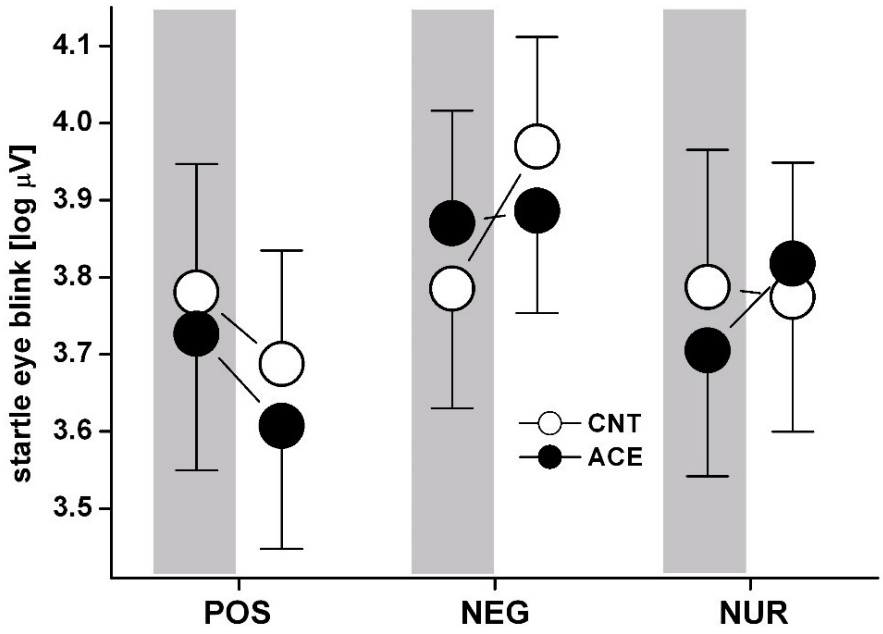

Figure 1: Startle eye blink responses to positive, negative and nurture stimuli. Data represent control group (CNT; open circle) and ACE group (ACE; closed circle) means (+/- SEM) of startle eye blink response during positive (POS), negative (NEG), and nurture (NUR) blocks, separately for valence picture presentation (white area) and neutral picture presentation (grey area). Contrasting t-tests between the groups revealed lower startle eye blink modulation ("affective – control") in the ACE group during presentation of negative stimuli and higher startle modulation during presentation of nurture pictures.

2.4.4 Subjective Ratings

Results of valence ratings are illustrated in Figure 2 (first graph: Valence). Note, that all illustrated and all statistically tested data represent the difference between affective and neutral pictures within a corresponding block. ANOVA revealed a significant main effect of VALENCE (F [2,74]=327, p=.0001), but no main effect of GROUP (F [1,37]=0.4, p=.54) and no GROUP X VALENCE interaction (F [2,74]=1.5, p=.23). Post-hoc comparisons revealed significant differences between all valence levels (POS vs. NEG: t (40)=20.3, p<.0001; NEG vs. NUR: t (40)=22.0, p=.0001; POS vs. NUR: t (40)=2.0, p=.04). Results of arousal ratings are illustrated in Figure 2 (second graph: Arousal.). ANOVA revealed a significant main effect of VALENCE (F [2,74]=82, p=.0001), but no main effect of GROUP (F [1,37]=0.1, p=.74) and no GROUP X VALENCE interaction (F [2,74]=2.1, p=.14). Post-hoc comparisons revealed significant differences between all arousal levels (POS vs. NEG: t (40)=2.8, p<.01; NEG vs. NUR: t (40)=10.6, p=.0001; POS vs. NUR: t (40)=11.5, p=.0001). For arousal ratings, exploratory testing for mean differences at the single VALENCE level “NUR” revealed a significant GROUP contrast (t (40)=2.4, p=.02). Results of approach/avoidance (AA) ratings are illustrated in Figure 2 (last graph: AA). ANOVA revealed a significant main effect of VALENCE (F [2,74]=86, p=.0001), but no main effect of GROUP (F [1,37]=0.3, p=.59) and no GROUP X VALENCE interaction (F [2,74]=1.9, p=.17). Post-hoc comparisons revealed significant differences between the “NEG” valence level and the other levels (POS vs. NEG: t (40)=11.7, p<.0001; NEG vs. NUR: t (40)=12.4, p=.0001), but no difference between “POS” and “NUR” levels (t (40)=0.9, p=.35).

There was no significant difference in valence, arousal, or approach/avoidance ratings in neutral pictures depending on which VALENCE block they were displayed, nor were there GROUP main effects, nor interactions (all p>.1).

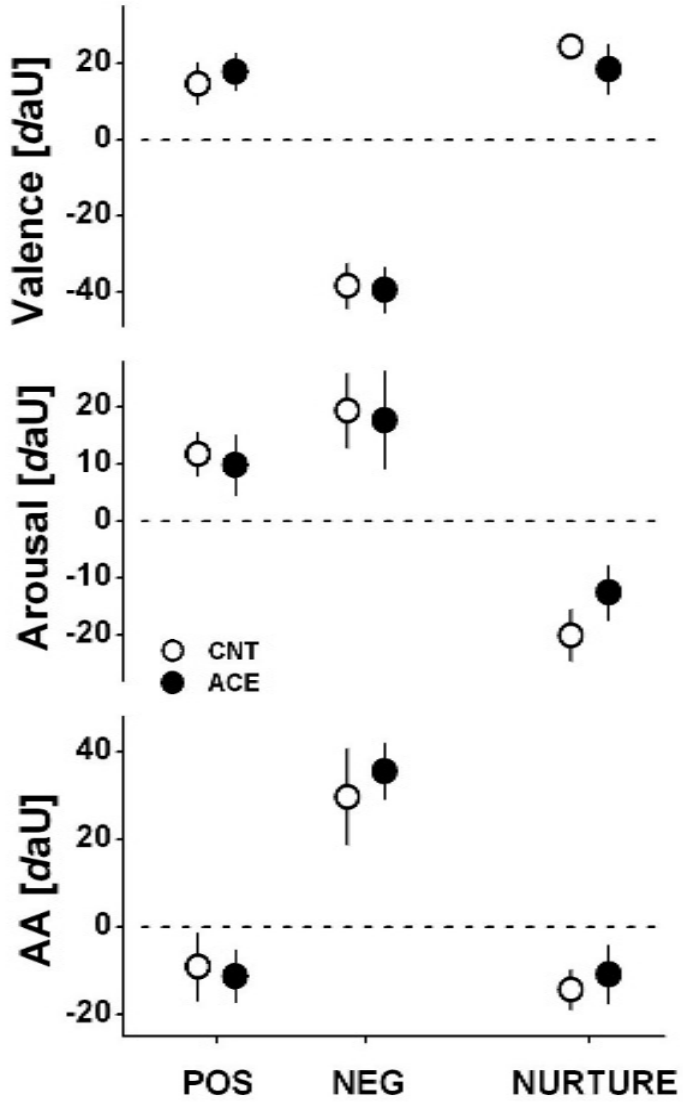

Figure 2: Subjective valence, arousal, and approach-avoidance rating scores of positive, negative and nurture pictures. Data represent mean +/- 95% confidence limits in differential arbitrary units (daU: difference between affective and control pictures) of the control group (CNT; open circles) and the ACE group (ACE; closed circles) for the three different blocks: positive (POS), negative (NEG), and nurture (NURTURE). A significant main effect of VALENCE was observed for valence scores, arousal scores, and approach-avoidance scores. A significant GROUP differences was shown for the VALENCE level "NURTURE" of arousal scores. AA: Approach-avoidance tendency

2.5 Discussion

The aim of the current study was to clarify the question of whether divorce as a potential ACE alters startle responsiveness in a generalized or contextually-driven way. A special block design of the affective startle modulation paradigm was created, on the one hand, to avoid carry over effects of nurture and general affective stimuli and, on the other hand, to clearly distinguish a generalized from a contextually-driven effect. There was no evidence of an overall enhanced startle reactivity in participants reporting parental divorce (ACE participants). However, ACE participants (as compared to controls) showed significantly blunted startle responsiveness during presentation of aversive pictures and enhanced startle responsiveness during presentation of nurture-related pictures. No group differences were found during presentation of standard appetitive pictures, nor neutral control pictures.

Parental divorce is a frequent event. However, there is still a debate concerning the severity of its long-term consequences for the affected children. In our study the Childhood Trauma Questionnaire revealed significant differences between ACE and control groups, indicating higher levels of emotional neglect and higher values of emotional abuse in participants experiencing parental divorce in early childhood. This finding is supported by recent studies indicating that children raised by a single parent reported significantly higher rates of emotional neglect compared to children living with two parents (Sedlak, Mettenburg et al. 2010). Neglect is defined as an act of omission, and the present level of emotional neglect in ACE participants might at least in part be induced by the mere absence of one parent. In addition, we found differences in the FACES IV test which demonstrate that control participants were confronted with more stable family structures. Even though the FACES IV questionnaire revealed values representing healthy family systems within both groups, the two groups differed in the scales of cohesion and flexibility, indicating that a more balanced family system had existed in the families of control participants. Variations in cohesion also reflect a more balanced and, therewith, a healthier family structure. Overall (and not surprisingly), our

data indicate that adults experiencing parental divorce in childhood also experienced a more unfortunate family functioning, presumably interfering with an adequate emotional family support. Other studies have also found parental divorce representing an ACE (Green, McLaughlin et al. 2010), apparently strong enough to increase the risk of developing psychopathologies later in life (Hallstrom 1987; Thomas and Hognas 2015). Major depression appears to be exclusively related to the experience of emotional neglect and abuse (Gibb, Chelminski et al. 2007), but this association is further aggravated when an unbalanced family structure is present (Reinherz, Paradis et al. 2003). The latter finding is also of relevance for our study, since this supports what we found in our ACE participants.

Although the reported results justify the assumption that parental divorce might be related to the development of psychopathologies, especially major depression, we abstained from detailed clinical testing of psychological functioning in our participants. However, past psychological problems qualifying for pharmacological or psychotherapeutic interventions were exclusion factors, and thus not present in our study population. Furthermore, we assessed current mood and emotionality on the experimental day by PANAS questionnaire, which did not reveal any evidence of a disturbed emotional state.

We did not find a general startle hyper-responsiveness as has been found for posttraumatic stress disorder (Lipschitz, Mayes et al. 2005) and physical abuse experienced during childhood (Jovanovic, Blanding et al. 2009). Moreover, startle habituation across experimental blocks was similar in ACE and control participants. However, we observed context-dependent changes of startle responsiveness during presentation of negative as well as nurture cues. Startle modulation in ACE participants during the “negative” (aversive) block was blunted, it was enhanced (potentiated) during the “nurture” block, and similar to controls in the “positive” (standard appetitive) block. This study was not designed to explain why and when in individual development these changes occurred. However, blunted startle modulation in the “negative” block might be the result of two different processes: (1st) relatively reduced startle responsiveness during presentation of standard negative pictures (e.g. as a theoretical tolerance-like effect induced by repeated negative experiences, and subsequent desensitization against aversive cueing), or (2nd) relatively increased startle responsiveness during presentation of neutral pictures in the “negative” block (e.g.

carry-over/conditioning effect from aversive to neutral pictures, and subsequent emotional generalization). The latter possibility would predict differences in startle responsiveness during neutral control picture presentation between the blocks. These were not found in the ACE group (only a marginal statistical significance in exploratory analysis was present), nor in the control group. However, this study may have been underpowered to detect such a difference. Furthermore, this effect was found in startle, but not in ratings. Therefore, future studies will need to replicate this finding by using extended (and maybe more specific) stimulus material.

Since startle represents a protective reflex repeatedly shown to be potentiated under conditions of danger and adversity, and, given that the family environment of ACE participants during childhood was disturbed by instability, adversity, and presumably uncertainty, it may be speculated that ACE participants adapted to childhood-related context by potentiating protective reflexivity. This is exactly the finding of this study, accompanied by the result of increased arousal ratings of nurture cues in ACE participants. However, nurture cues were rated positively by ACE participants, so that increased arousal ratings would be expected to enhance pleasantness and diminish startle responsiveness. This is not the first study to identify such a discrepant effect. Several studies have found enhanced startle during the presentation of increasingly arousing appetitive pictures, such as food pictures during food deprivation (Drobes, Miller et al. 2001) (Ferreira de Sá, Plein et al. 2014). Such an effect may best be described by the model of frustrative non-reward (Amsel 1958; Wagner 1963), suggesting that the frustration of missing the possibility to approach and acquire the visually displayed nurture-object may evoke a negative emotional conflict. Furthermore, ratings of nurture cues are likely influenced by a social expectancy bias resulting in overestimation of item pleasantness, and it might be that ACE participants are more vulnerable to such a biasing effect. A higher social expectancy bias would mask lower pleasantness of nurture cues in ACE participants, corresponding to higher startle responses. However, this is just a speculation which cannot be empirically addressed by the current study.

To our knowledge, this is the first study demonstrating long-term contextually-driven differences in psychophysiological responding in participants who experienced parental divorce. The novel block design employed in this startle modulation paradigm may have helped to identify these differences by avoiding carryover effects between

adjacent stimuli of different affective quality. The observed effect may not be capable of characterizing the impact of general adverse childhood events on physiological reactivity in adulthood, rather it supports the evidence of alterations in physiological response due to the experience of parental divorce in early childhood. In conclusion, divorce constitutes a special form of ACE that does not generalize the impact of childhood trauma on overall startle responsiveness in adulthood. In summary, the block design represents a valid model capable of defining a clear effect of the presented stimuli on the acoustic startle response. The observed alterations of the eye blink reflex suggest that divorce is capable of inducing context-dependent consequences in protective responses. Together with an increased level of emotional neglect and the more unbalanced family structure in the ACE group, our results provide evidence that parental divorce may play a relevant role in behavioural and physiological differences in adaptation to emotional cues in adulthood. Future studies will need to clarify whether the described contextually-driven startle differences may be feasible to identify ACE participants at risk for the development of emotional disorders later in life.

2.6 Acknowledgements

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Chapter III: T cell immunosenescence after early life adversity: an association with cytomegalovirus infection

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3.1 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 a healthy subset of the EpiPath cohort. Participants 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.

3.2 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, Akhtar et al. 2015) and an increased risk for multiple age-related diseases, such as cardiovascular disease (Korkeila, Vahtera et al. 2010; Friedman, Karlamangla et al. 2015) and type 2 diabetes (Eriksson, Raikkonen et al. 2014). As many as 39% of children worldwide are estimated to experience one or more forms of early life adversity (ELA) (Kessler, McLaughlin et al. 2010), placing a high economic burden on health care systems – and society in general – through medical costs and lost productivity (Fang, Brown 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 (Shalev, Entringer et al. 2013;

Elwenspoek, Kuehn et al. 2017). 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 (Chou and Effros 2013; Childs, Durik et al. 2015). 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, naive T cells have longer telomeres than terminally differentiated T cells that went through more replication cycles (Weng, Levine 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, Levandowski 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, Janicki-Deverts 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, Larbi 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, Lenig 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, Pasukoniene 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, Harris 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, van Mierlo et al. 2015; Zannas, Arloth et al. 2015; Simpkin, Hemani et al. 2016). However, to date, few studies have addressed this in ELA specifically, and results are ambiguous (Elwenspoek, Kuehn 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, Wieck 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 (Moss and Khan 2004; Moss 2010). Moreover, ELA increases the risk of herpes infections and has been implicated in increased reactivation in children and adults (Shirtcliff, Coe et al. 2009; Slopen, McLaughlin et al. 2013; Janicki-Deverts, Cohen et al. 2014).

In the present study, we investigated T cell specific immunosenescence (T cell differentiation and CD57 expression) in 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. This study cohort is a healthy subset of the EpiPath cohort, excluding all participants with acute or chronic diseases. With a mediation analysis we examined whether CMV titers may account for immunosenescence observed in ELA.

3.3 Materials and Methods

3.3.1 Participants

Healthy participants were selected from the EpiPath cohort (Elwenspoek et al., 2017; manuscript under review), based on 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 3). 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, Palacios 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.

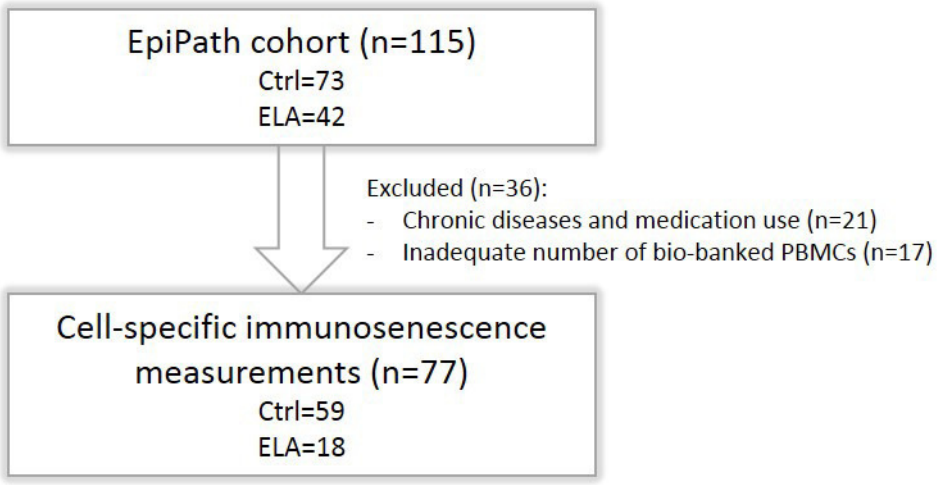

Figure 3: Selection of participants from within the complete EpiPath cohort.

3.3.2 Blood Samples

Blood samples were collected in sodium heparin coated tubes for PBMC isolation and in EDTA coated tubes for DNA and plasma 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.

3.3.3 CMV titers

EDTA blood samples were centrifuged at 4°C within 15 min of blood collection and the plasma phase was collected. Plasma samples were transported on ice and stored at -80°C within 6 h until further analysis. Plasma was used in a 1:21 dilution to determine

CMV IgG antibody indexes by ELISA (Calbiotech, El Cajon, CA, USA). As a control, HSV-1 and EBV IgG antibody indexes were also determined with ELISAs from the same company. The ELISAs were performed following manufacturer's instructions, in duplicate, and read on a SpectraMax Plus 384 Microplate Reader (Molecular Devices, Berkshire, UK).

3.3.4 Telomere length and age-related CpGs

DNA isolation and telomere length measurements in the EpiPath were reported previously in Elwenspoek et al. (2017) (manuscript under review). 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.

3.3.5 PBMCs Isolation and flow cytometry

All cell culture products were from Lonza BioWhittaker (Versviers, Belgium), unless otherwise stated. PBMCs were isolated within 3h 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 5min at 300g. PBMCs were washed twice with PBS and stored at 4.106 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% CO2 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 (1.107 cells/mL) were incubated with GolgiPlug and GolgiStop (final concentrations of 2µL/mL and 1µL/mL, respectively, BD BioSciences) for 5h at 37°C, 5% CO2. 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 2) for 30min. Then, PBMCs were permeabilized and fixed with BD Cytofix/Cytoperm™ (BD BioSciences, San Diego, USA) for 20min, followed by a 30min intracellular staining of granzyme B and perforin (Table 2).

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

Table 2: Flow cytometry panel

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 4. 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.

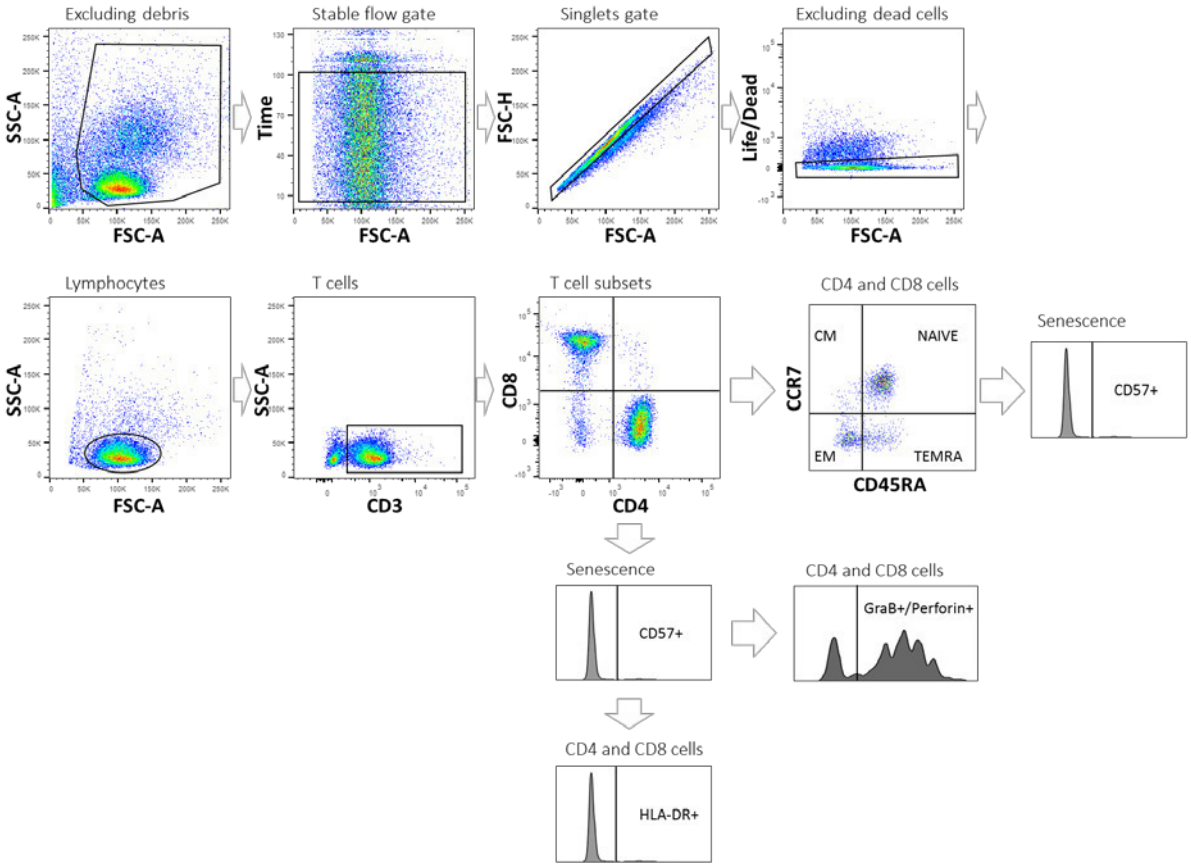

Figure 4: Gating strategy

3.3.6 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). When plotting CMV titers, a 'high' and 'low' antibody group emerged. Log(CMV titers) = -1.2 was chosen as cut-off. 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, Yamamoto 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.

3.3.7 Results

3.3.8 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, subsequent childhood trauma exposure, and all participants were in good health (Table 3).

	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

Table 3: Participant characteristics. * 'Age at adoption' was used as a proxy of ELA severity (Julian 2013); therefore, controls were set to 0. 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.

3.3.9 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 (Elwenspoek et al., 2017; manuscript under review). 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 5a). 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 5b-d).

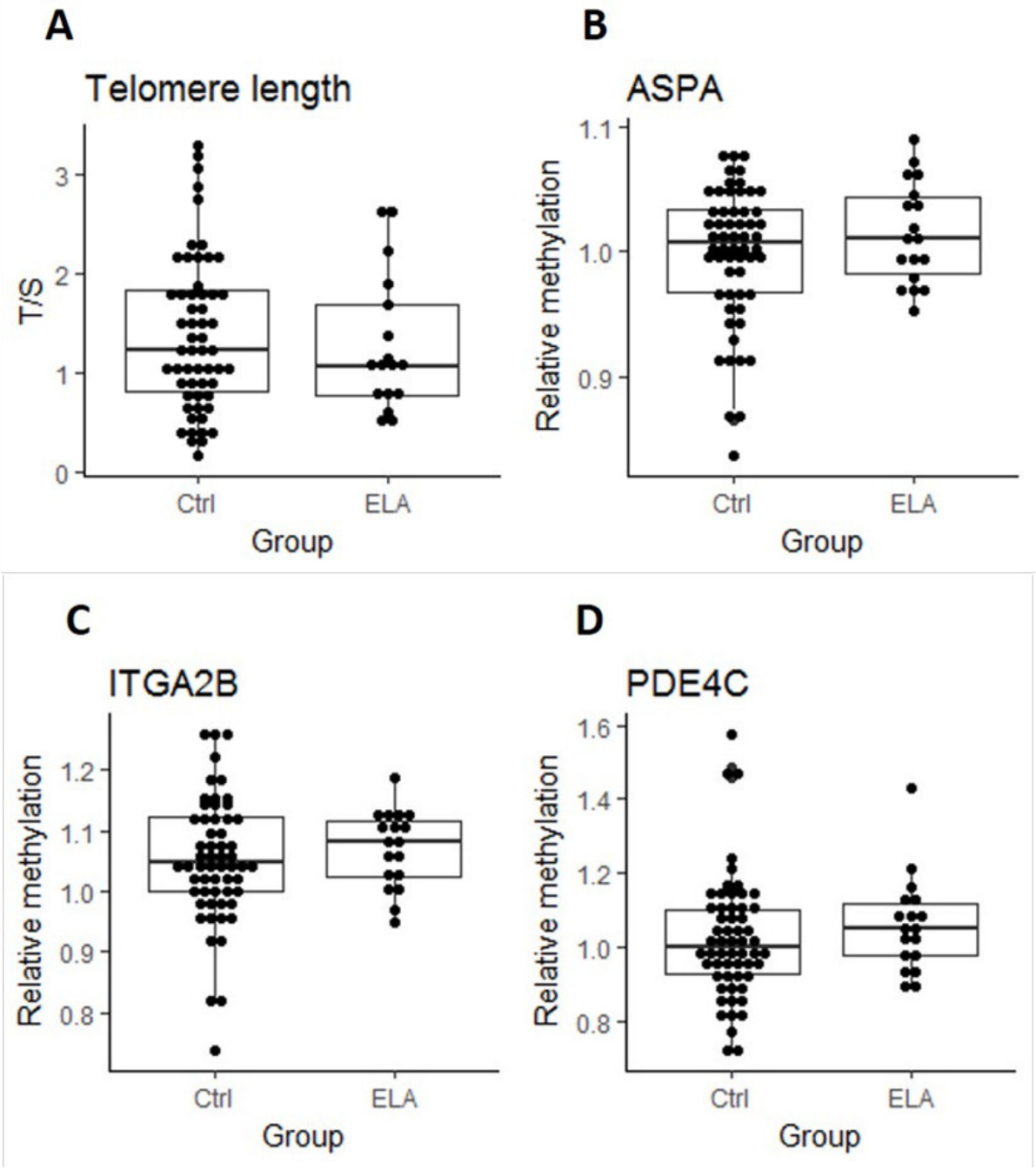

Figure 5: Markers for senescence. Relative telomere length in T/S (A), methylation levels at the age-related CpGs ASPA, ITGA2B, and PDE4C (B-D), respectively. Statistics: Wilcoxon rank sum test with continuity correction. Ctrl: control; ASPA: aspartoacylase; ELA: early life adversity; ITGA2B: integrin alpha 2b; PDE4C: phosphodiesterase; T/S: relative telomere to single copy gene ratio.

3.3.10 T cell specific senescence

A 10-colour flow cytometry panel was used to investigate cell specific immunosenescence. Although immunosenescence is related to changes in the ratio of naive and memory T cells, the ratios and numbers of naive, 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 6a). 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, 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 6b), suggesting that CD57 expression was higher in ELA. 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. As expected (Sallusto, Geginat et al. 2004), $CD57^+$ cells were not equally distributed among the different stages of T cell differentiation (Figure 7). 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 7a) and in both TEMRA and EM CTLs (Figure 7b).

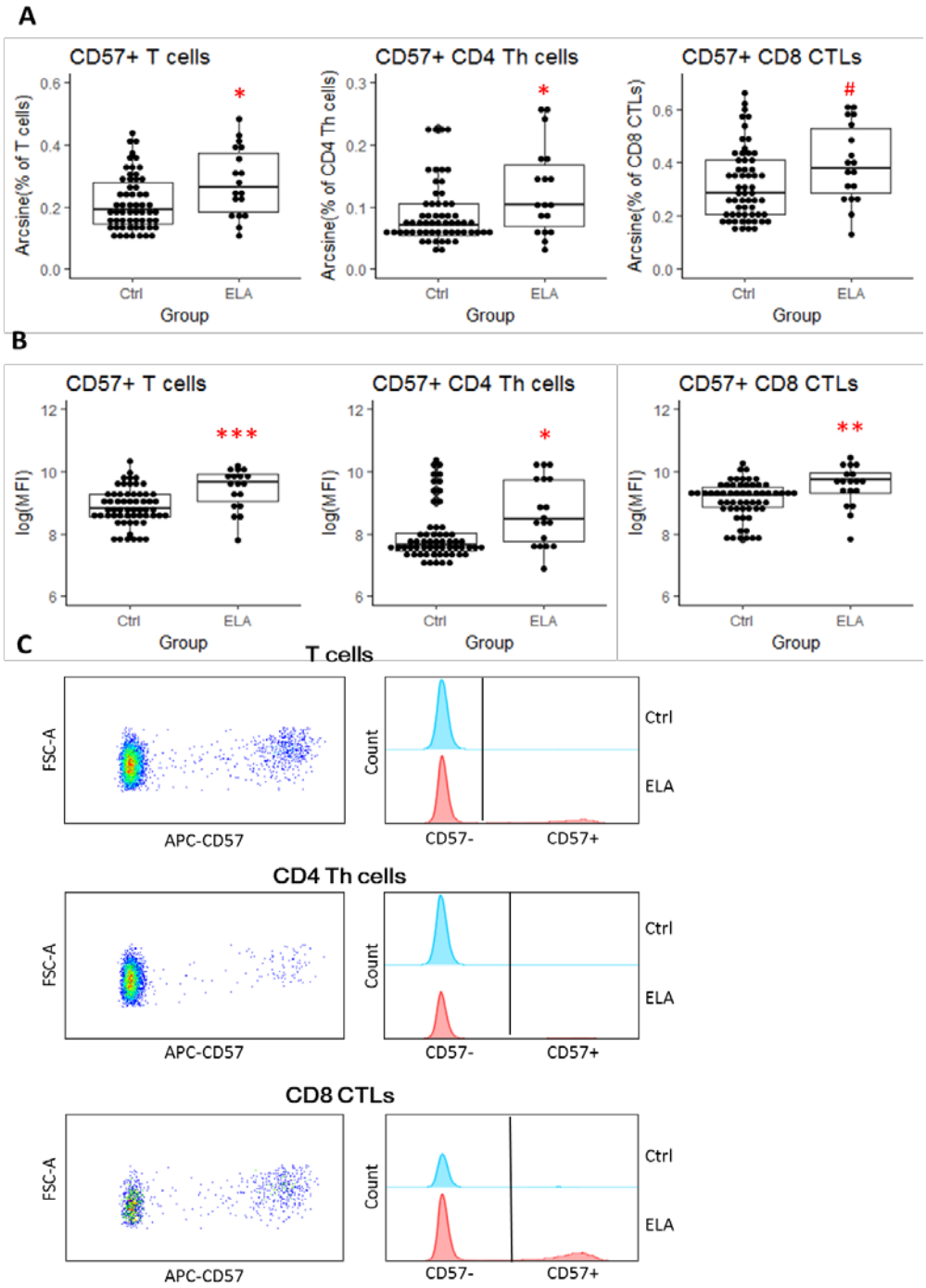

Figure 6: Senescence marker CD57 on T cells. ELA participants have more CD57⁺ T cells (total and subsets Th and CTLs) (A) and the expression of CD57 per cell is higher (MFI) (B). Y axes show percentages of parent populations after an arcsine transformation, or log-transformed MFI values. Representative dot plots and histograms of CD57 expression on T cells and its subsets (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. # $p<0.10$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Ctrl: control; ELA: early life adversity; MFI: median fluorescent intensity; CTL: cytotoxic T lymphocyte; Th cell: T helper cell.

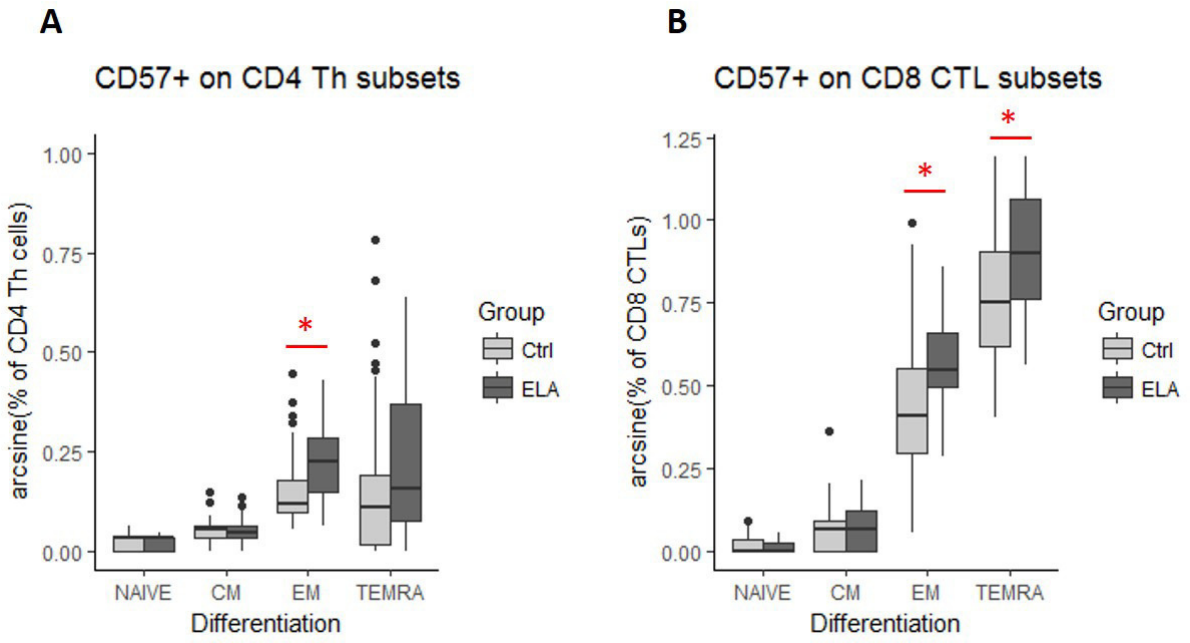

Figure 7: CD57 expression in different stages of T cell differentiation in CD4 Th cells (A) and CD8 Tc cells (B). Statistics: linear model with group and experimental day as independent variables, and arcsine-transformed percentages of $CD57^+$ cells as dependent variable. P-values are corrected for multiplicity with false discovery rate. * $p<0.05$. Ctrl: control; CM: central memory; ELA: early life adversity; EM: effector memory; CTL: cytotoxic T lymphocyte; TEMRA: terminally differentiated effector memory; Th cell: T helper cell.

3.3.11 Cytolytic potential and activation

Cytolytic potential was measured by granzyme B and perforin staining. As expected (Chattopadhyay, Betts et al. 2009), $CD57^+$ cells had higher granzyme B, perforin, and HLA-DR expression (Figure 8 a,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 8 c,d). The level of fluorescent intensity of neither granzyme B nor perforin differed between the groups (data not shown).

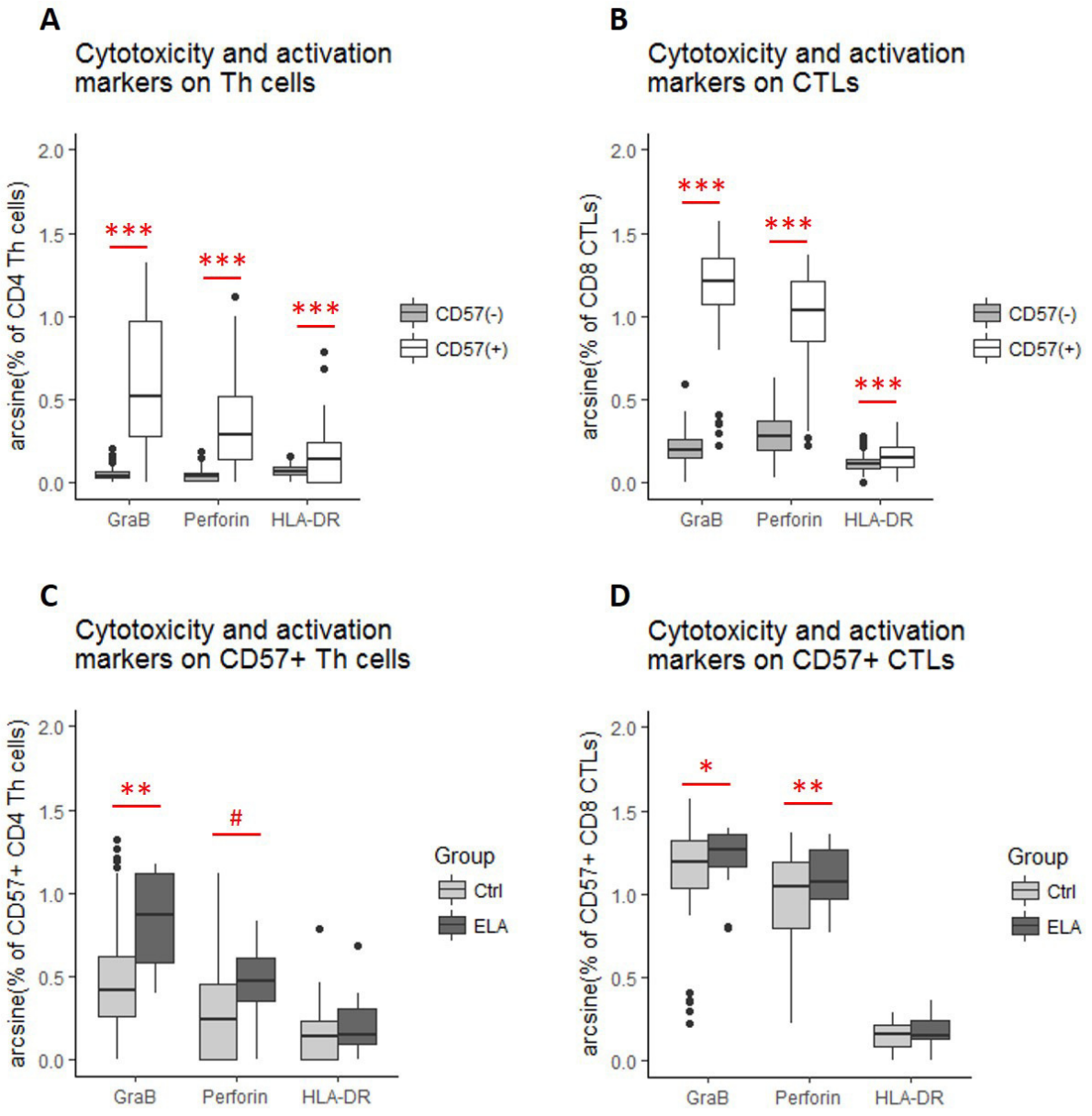

Figure 8: Cytolytic potential and activation status: CD57 positive ($CD57^+$) versus CD57 negative ($CD57^-$) Th cells (A) and CTLs (B). ELA versus controls among CD57 positive ($CD57^+$) Th cells (C) and CTLs (D). Statistics: Paired Wilcoxon rank sum test with continuity correction (A-B), linear regression 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. # $p<0.10$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Ctrl: control; ELA: early life adversity; GraB: granzyme B; HLA-DR: Human Leukocyte Antigen - antigen D Related; CTL: cytotoxic T lymphocyte; Th cell: T helper cell.

3.3.12 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 9a), caused by an increased number of seropositive participants. To further investigate the relationship between CMV titers and CD57 expression, we divided the control participants into two groups with either 'high' or 'low' CMV titers. Participants with high CMV titers had significantly higher levels of $CD57^+$ CD4 EM (p<0.001) and TEMRA (p<0.001) cells, as well as higher $CD57^+$ in all subsets of CD8 (Naïve, p=0.007; CM, p<0.001; EM, p=0.001; TEMRA, p=0.020; Figure 9b). 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 Tc cells (CTLs) (Figure 5c). 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 10a), which could explain 27.4% of the total effect of ELA (age at adoption) on T cell senescence (Table 4). Interestingly, when investigating ELA as mediator of the effect of CMV on senescent T cells, we only found a trend. This suggests that ELA is not just a marker for CMV exposure, but that there are other-ELA related factors involved (Table 5 and Figure 10b).

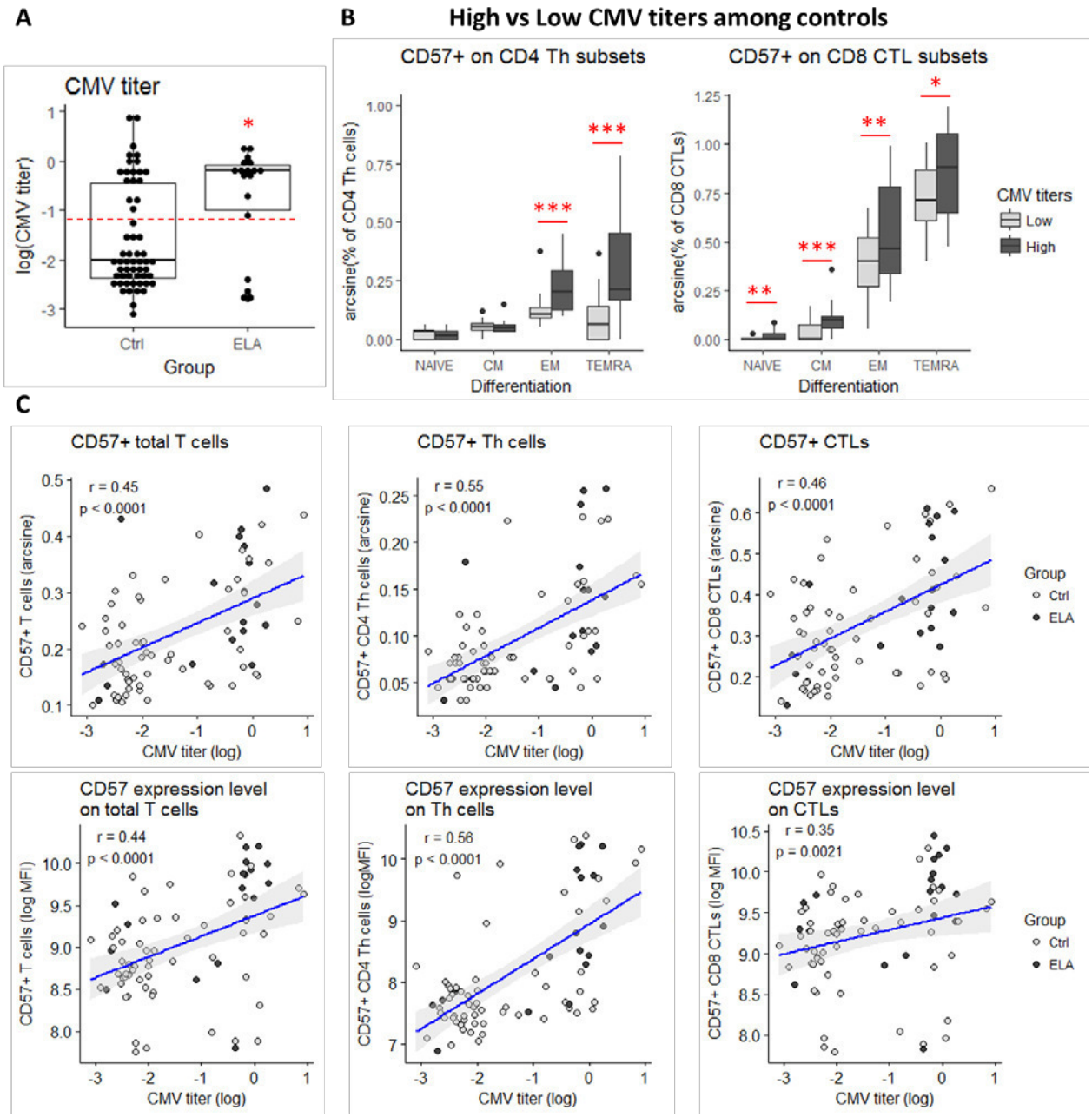

Figure 9: Influence of CMV infection. CMV titers in control and ELA participants (A). Dotted line in (A) represents the cut-off between 'high' and 'low' titers used in (B). CD57 expression in subsets of CD4 Th cells and CD8 CTLs within the control group, separated on 'high' (log(CMV) > -1.2) versus 'low' (log(CMV) < -1.2) CMV titers. Correlations between CMV titers and CD57⁺ numbers and expression levels on T cells (total, and subsets Th and CTLs) (C). Statistics: CMV titers (A), Wilcoxon rank sum test with continuity correction; linear model with CMV titers (high/low) and experimental day as independent variables, and arcsine-transformed percentages of CD57⁺ cells as dependent variable (B); Spearman's rank correlation rho (C). * p<0.05, ** p<0.01, *** p<0.001. CMV: Cytomegalovirus; Ctrl: control; ELA: early life adversity; MFI: median fluorescent intensity; CTL: cytotoxic T lymphocyte; Th cell: T helper cell.

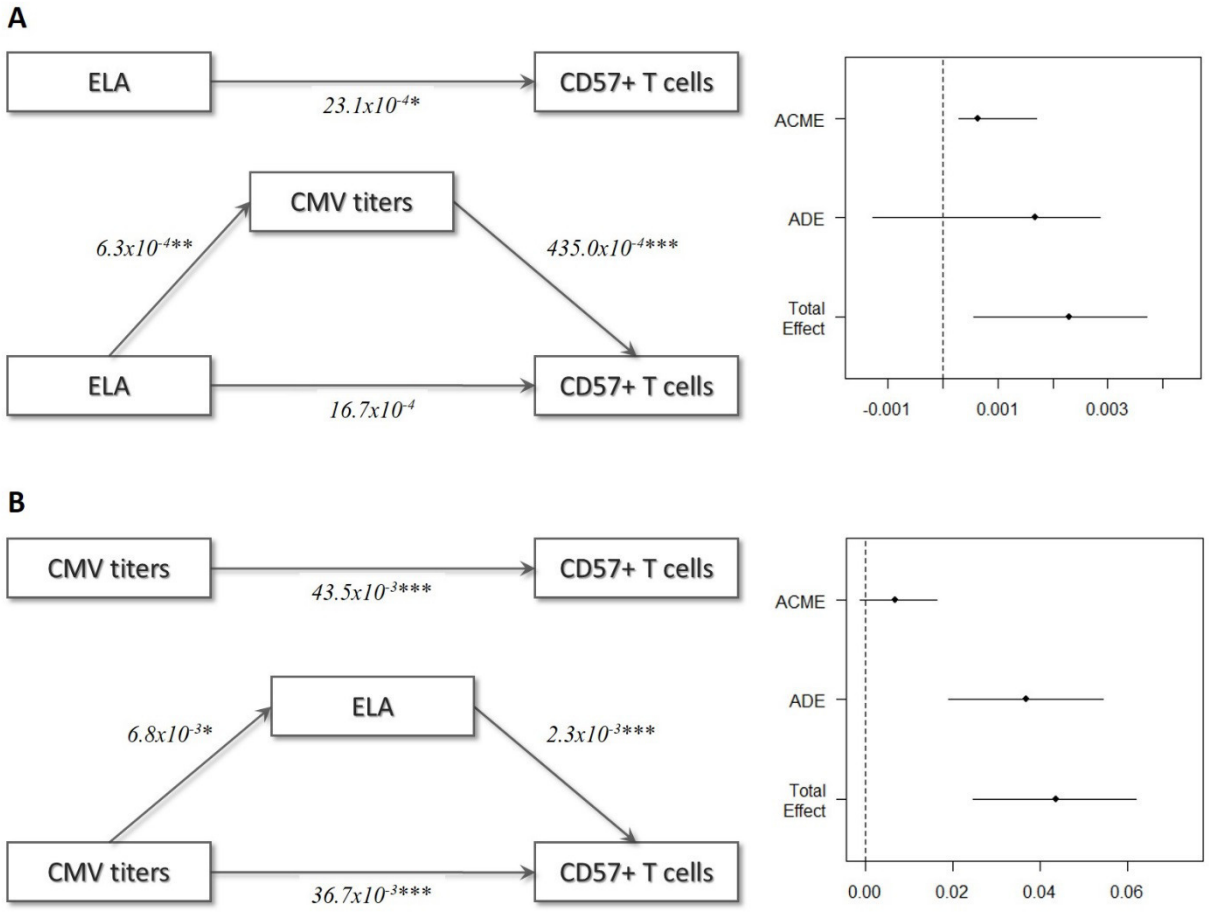

Figure 10: Mediation models. (A) ELA as independent variable, T cell immunosenescence as dependent variable, and CMV titers as mediator variable. (B) CMV titers as independent variable, T cell immunosenescence as dependent variable, and ELA as mediator variable. In these models, ELA was treated as continuous variable (age at adoption) and all Ctrl were set to 0. ACME: Average Causal Mediation Effect; ADE: Average Direct Effect, CMV: Cytomegalovirus; ELA: early life adversity.

	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*

Table 4: Causal Mediation Analysis: CMV as mediator. Nonparametric Bootstrap Confidence Intervals with the Percentile Method. ACME: Average Causal Mediation Effect; ADE: Average Direct Effect; CI: confidence interval; prop.: probability.

	Estimate	**95% CI Lower**	**95% CI Upper**	***p*-value**
ACME	0.00681	-0.00105	0.01637	0.08
ADE	0.03673	0.01905	0.05431	0.00***
Total effect	0.04354	0.02472	0.06183	0.00***
Prop. mediated	0.15635	-0.02606	0.38365	0.08

Table 5: Causal Mediation Analysis: ELA as mediator. Nonparametric Bootstrap Confidence Intervals with the Percentile Method. ACME: Average Causal Mediation Effect; ADE: Average Direct Effect; CI: confidence interval; prop.: probability.

3.4 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). Control participants with 'high' CMV titers showed a higher number of senescent cells, compared to controls with 'low' titers, which was particularly apparent in late differentiated effector Th cells (EM and TEMRA). Importantly, we found that the effect of ELA on immunosenescence was associated with 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 (Elwenspoek et al., 2017; manuscript under review), we did find higher numbers of CD57+ T cells in ELA. It has been demonstrated previously that this cell type has shorter telomeres (Brenchley, Karandikar 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, Eilers et al. 2016), and a shrinking population of naïve T cells (Wertheimer, Bennett et al. 2014), similar to what is observed in aging (Koch, Larbi et al. 2008). CMV seropositivity has been shown to reduce life expectancy by almost 4 years in an elderly population, especially due to an increase in cardiovascular deaths (Savva, Pachnio et al. 2013). 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, Hertoghs 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, Peek et al. 2012). Similarly, only in CMV seropositive individuals, EBV reactivation was associated with inflammatory markers in circulation (Bennett, Glaser 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, Griffiths et al. 2010) and is associated with decreased telomerase activity (Dowd, Bosch et al. 2013), an enzyme that can partially counteract telomere loss. However, these studies looked at total T cells and leukocytes, respectively, so it is unclear whether this effect is limited to CMV-specific T cells or also affects telomere biology in other immune cells.

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, Palermo et al. 2012; Voigt, Schaffrath Rosario et al. 2016). Poverty is reliable predictor of more severe forms of ELA such as childhood abuse and neglect (Slack, Holl et al. 2004; Cancian, Slack et al. 2013; Lefebvre, Fallon et al. 2017). 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, Schaffrath Rosario 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, Iverson et al. 1991) – much higher compared to the 21-22% reported in a German population samples of 1-2 year olds (Voigt, Schaffrath Rosario et al. 2016). Indeed, we found higher CMV titers in the adoptees compared to controls.

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, Palermo et al. 2012; Fagundes, Glaser et al. 2013; Janicki-Deverts, Cohen et al. 2014). Furthermore, among CMV positive children ELA was associated with an increased percentages of senescent CTLs (CD8+CD28-CD57+ cells) (Caserta, O'Connor 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, Farag 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 11). 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.

3.4.1 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 (Table 4). 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.

It is important to note that freezing and thawing as well as resting overnight of PBMCs may have introduced artifacts in the analysis. For instance, resting overnight before staining has been shown to decrease viability and can change T cell phenotype (Kutscher, Dembek et al. 2013). Nevertheless, we could repeat our previous findings on fresh samples of the same cohort concerning HLA-DR expression on T cells (Elwenspoek et al., 2017; manuscript under review).

3.4.2 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.

3.5 Funding

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3.7 Additional figures

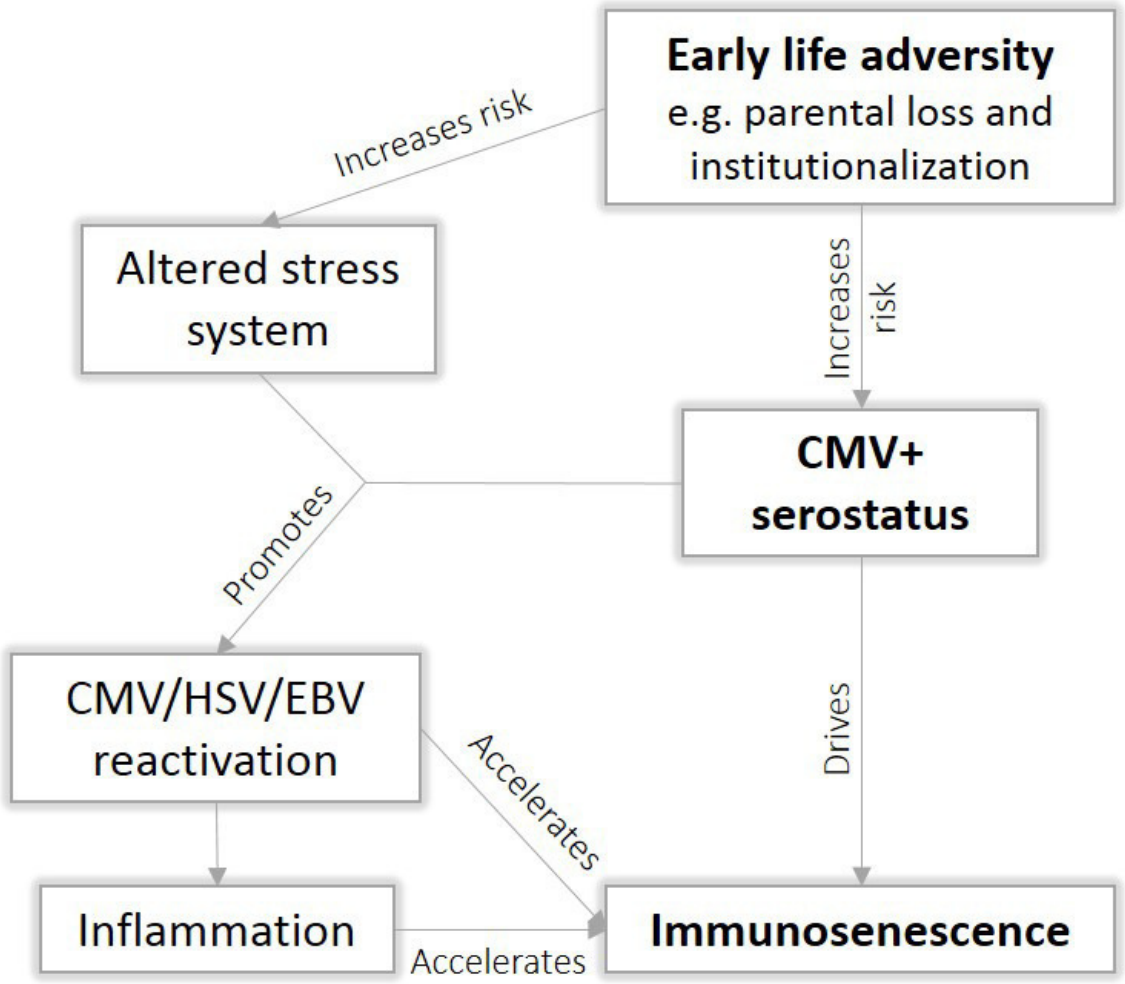

Figure 11: Proposed interaction between ELA and CMV to cause immunosenescence. CMV: Cytomegalovirus; HSV: Herpes simplex virus; EBV: Epstein-Barr virus; ELA: early life adversity

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Chapter III: T cell immunosenescence after early life adversity: an association with cytomegalovirus infection

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Chapter IV: Blunted Endocrine Response to a Combined Physical-Cognitive Stressor in Adults with Early Life Adversity

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4.1 Abstract

The negative health effects of early life adversity (ELA) continue long into adulthood. Changes in the physiological response to psychosocial stressors have been proposed to mediate this effect. However, many previous studies have come to contradicting conclusions as to whether ELA induces a long-term increase or decrease in stress reactivity. Therefore, we tested the association of ELA exposure and adult stress reactivity in a sample of early life adoptees and controls.

Two previously validated stressful elements (bilateral feet CPT and the Paced Auditory Serial Addition Task (PASAT)) were combined in an extended Cold Pressor Test (CPT). This test was performed on 22 participants who had experienced severe ELA (separation from biological parents, institutionalization, and adoption in early childhood), and in 22 age-matched control participants.

A prior history of ELA was associated with blunted reactivity of the hypothalamic-pituitary-adrenal (HPA) axis (Cohen´s d = 0.680). Cardiovascular reactivity remained

unchanged, and affective reactivity (self-report ratings) were increased in participants exposed to ELA compared to the control group (range Cohen´s d: 0.642 – 0.879).

Our results suggest that the activity of the HPA axis reactivity was inhibited in ELA participants. Importantly, cardiovascular stress responsiveness was not affected by ELA. This separation of the HPA axis and cardiovascular stress responses may best be explained by ELA selectively enhancing central feedback-sensitivity to glucocorticoids, but preserving cardiovascular/ autonomic stress reactivity.

Keywords: Adoption; Early Life Adversity; Cold Pressor Test; HPA Axis; Stress Reactivity; Paced Auditory Serial Addition Task

4.2 Introduction

It is well established that early life adversity (ELA), such as parental separation or maltreatment, may impair physical and mental health in adulthood (Chrousos, 2009; Felitti et al., 1998; Wegman & Stetler, 2009). Many, ELA-associated disorders manifest many years after the initial exposure (Greenfield & Marks, 2010; Matheson, Shepherd, Pinchbeck, Laurens, & Carr, 2013), raising questions about disease etiology and the mechanisms underlying such long-term effects. In particular, ELA is known to increase the incidence of stress-related mental and cardiovascular disorders (Batten, Aslan, Maciejewski, & Mazure, 2004; McCauley et al., 1997), suggesting that stress physiology, such as autonomic nervous system (ANS) and hypothalamic-pituitary-adrenal (HPA) axis function, may contribute to ELA pathophysiology. Altered HPA axis functioning has been proposed as the path by which ELA continues to impair health into adulthood (Barton, Zakreski, & Pruessner, 2016). This is supported by the observation that exposure to ELA induces a measurable increase in sympathetic tone and induces perturbation the HPA axis during early adolescence, e.g. at ages 10-12 (Gunnar, Frenn, Wewerka, & Van Ryzin, 2009). However, conflicting results have been reported. Some studies have associated ELA with hyper-responsiveness of stress response systems, especially the HPA axis (Heim et al., 2000; Pesonen et al., 2010),

others report hypo-responsiveness and a suppression of responses to psychosocial stressors in adults maltreated as children (Carpenter et al., 2007; Voellmin et al., 2015).

Gender may play a role in ELA-induced dysregulation of the HPA axis and subsequent risk for mental disorders (Heim et al., 2009), and there is evidence on a higher risk of stress-induced dysfunction of the HPA axis in female victims contributing to a higher risk for developing trauma-induced depression more frequently than males (Weiss, Longhurst, & Mazure, 1999). Rodent models, however, showed an increased HPA axis reactivity in male offspring after prenatal stress exposure (Mueller & Bale, 2008), suggesting that potential gender effects should receive more attention in ELA research.

Different stress tests have been used to study stress physiology in ELA participants, varying with respect to timing, intensity, cognitive load, social components, and others factors (Steptoe & Vogele, 1991). We used an extended Cold Pressor Test (CPT) protocol, which combines the cognitive challenging component of a mental arithmetic task (Paced Auditory Serial Addition Task; PASAT) (Gronwall, 1977) and a bilateral feet CPT version (Larra, Schilling, Rohrig, & Schachinger, 2015). This was done to (i) ensure strong stress responses, (ii) avoid asymmetric brain activation and other lateralization effects potentially induced by unilateral cold stimulation, and (iii) to provide a reliable cognitive performance measure under stress. Furthermore, and in contrast to comparatively more complex psychosocial stressors such as the Trier Social Stress Test (TSST), CPT variants offer better opportunity for standardization of stress onset and offset. The same is true for the PASAT, which was originally developed as a neuropsychological tool for assessing sustained divided attention, concentration, working memory and speed of information processing (Diehr, Heaton, Miller, & Grant, 1998). However, the PASAT has also previously been demonstrated to induce stress by itself (Philippsen et al., 2007). The PASAT may overcome the drawbacks of other mental arithmetic tasks, since it is challenging, stable (e.g. in contrast to some counting tasks the PASAT does not become more or less difficult over time) and replicable (Schachinger, Cox, Linder, Brody, & Keller, 2003).

The aim of the current study was to compare neuroendocrine, cardiovascular/autonomous, and subjective stress reactivity of ELA vs. control participants, and in addition, to address potential gender differences.

4.3 Methods

4.3.1 Participants

The ELA group consisted of 22 adults (14 females; age ranging from 19 to 30 yrs.) who were adopted by Luxembourgish families after being institutionalized in the 1990s in international orphanages during their early childhood. The mean age at adoption was 6.4 months. Twenty-two healthy participants (11 females; age ranging from 19 to 28 yrs.) without institutionalization/ adoption history served as control group. Gender distribution ($\chi 2 = 0.83$; n.s.), body mass index (BMI) (ELA: 23.9 ± 3.6 kg/m2; control: 22.9 ± 3.6 kg/m2) and age (ELA: 22.5 ± 3.04 yrs.; control: 21.8 ± 2.8 yrs.) did not differ between the groups. Cohorts are part of the [edited out for blind review] project, conducted in Luxembourg. Participants gave written informed consent prior to the study and received financial compensation. Exclusion criteria were cold intolerance (e.g. Raynaud's disease), current medication (except occasional pain killers and oral contraceptive pills), heavy smoking (> 15 Cig/day), alcohol consumption (>30 g/day), illicit drug intake within the last 3 months (participant's report), and body mass index (BMI) below 19 or above 30 kg/m2. All procedures of the study were approved by the Luxembourg National Research Ethic Committee (Commission Nationale d'Ethique de Recherche) and the National Committee for Data Protection (Commission Nationale pour la Protection des Données).

4.3.2 Procedures

The study was conducted in the noon at the Clinical and Epidemiological Investigation Center (CIEC) of the Luxembourg Institute of Health (LIH). Upon arrival, participants were seated in a comfortable chair and asked to relax. A small indwelling intravenous cannula was inserted into arm the for blood collection. Blood chemistry and genetic data contributed to a different and bigger cohort, and will be reported elsewhere. In this study ECG was recorded by Tyco Healthcare H34SG Ag/AgCl electrodes (lead II

configuration) with a Biopac MP150 system and ECG100C amplifier modules (1 kHz, 16 bit resolution). Heart rate (HR) was determined offline using WinCPRS (Absolute Aliens Oy, Turku, Finland), and averaged over 1-minute intervals. A standard cuff oscillometric Dinamap blood pressure monitor (Dinamap SX 1846, Critikon, US) was used and mean arterial blood pressure (MAP) data are reported here. All participants completed a PASAT teaching session to ensure that they understood the test (see 2.3). After a 60 minute rest period all participants performed the CPT (see 2.4) with simultaneous PASAT. Blood pressure (BP) was recorded at minutes -13, -8, -3, +1, +2, +12, +14, and +16. Saliva samples (see 2.5) were taken at minutes -30, -20, -5, +3, +15, +25, +40, and +55. Subjective ratings (see 2.6) were provided at minutes -2 and +4, when current mood (how do you feel at the moment) was also assessed using the Positive Affect and Negative Affect Schedule (PANAS) (Watson, Clark, & Tellegen, 1988).

4.3.3 Paced Auditory Serial Addition Task (PASAT)

Participants listened to series of 64 single – digit numbers delivered at 2.5 s inter-stimulus interval via headphones from a computer. Their task was to add each new number to the previous as quickly and accurately as possible and reporting the sum aloud. Verbal responses were digitized and recorded in standard wave files for offline analysis. Percentage of correct responses was used as the main PASAT performance indicator. Omission errors and false responses (Schachinger, Cox et al. 2003) were not separated.

4.3.4 Cold Pressor Test (CPT)

The investigator entered the room with a bin of ice-cold water (temperature between 2-3°C). Volunteers placed both feet including the ankles into the water. 15 seconds later PASAT started and lasted for 160 seconds. The CPT lasted 180 seconds. An investigator was always present in the experimental room and monitored - but did not communicate with - the participant. There was no information or indication about the time left.

4.3.5 Salivary 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, Kirschbaum et al. 1992) (Salimetrics Salivary Cortisol ELISA Kit; CV: 7% intra-assay, 11% inter-assay).

4.3.6 Ratings

Self-reported levels of stress, arousal, anxiety and pain were assessed using visual analogue scales (VAS) ranging from 0 to 100 arbitrary Units (aU).

Participants' current mood was assessed by means of Positive Affect and Negative Affect Schedule (PANAS) (Watson, Clark et al. 1988). This questionnaire consists of two subscales (positive affect, PA; negative affect, NA) containing ten items each. Likert scale responses range from 1 "not at all" to 5 "very much" measuring affectivity on two largely independent dimensions.

4.3.7 Data Reduction and Statistics

BP and HR at minutes -13, -8, and -3 were individually averaged as baseline, as were salivary cortisol at minutes -30, -20 and -5. Reactivity (∆) scores were calculated as peak change scores (∆ = max. stress value – baseline value). For cortisol, the area under the curve with respect to increase (AUCi) was calculated (Pruessner, Kirschbaum et al. 2003), as were peak (maximum) responses.

Group differences were explored by two factorial ANOVA examining the influence of two independent variables (ELA and GENDER) on one dependent variable (MAP; HR; cortisol responses; subjective ratings; performance measures). The main effects of ELA (two-level between subject factor: ELA vs. control) and GENDER (two level between subject factor: female vs. male), as well as their interaction (ELA x GENDER)

was assessed for baseline and reactivity scores of the variables MAP and HR, cortisol responses as well as subjective and performance measures.

All testing was two-tailed. Data in the text and tables represent means ± SD, and in figures mean ± SEM. Exact test statistics and p-values are reported.

4.4 Results

4.4.1 Differences in baseline and stress reactivity

A main effect of ELA was found for baseline MAP, with lower values in the *ELA* group. Furthermore, there was a main effect of GENDER for baseline HR, indicating higher HR in females. No other main or interaction effects emerged for baseline values. Reactivity (∆) scores for MAP and HR did not differ between ELA nor GENDER groups, and ANOVA did not reveal an interaction of ELA X GENDER (see Figure 12 and Table 7).

Peak salivary cortisol responses were different between ELA groups (p=.03), with lower cortisol reactivity in *ELA* participants compared to *controls*, but no main effect of GENDER, and no significant interaction effect of ELA x GENDER (see Figure 12 and Table 7). Overall, statistical testing of AUCi cortisol data revealed similar effects, albeit just a tendency towards statistical significance (p=.078) of the ELA group effect (see Table 7).

4.4.2 Differences in PASAT performance

Due to technical problems PASAT recordings were missing in three participants. ANOVA testing of PASAT correct responses revealed a significant main effect for ELA, indicating lower performance in *ELA* participants compared to *controls* (see Table 7). There was no significant main effect of GENDER, and no significant interaction effects of ELA x GENDER.

4.4.3 Differences in subjective rating of stress, arousal, and anxiety

Baseline stress and arousal ratings did not significantly differ between ELA, nor GENDER groups. However, baseline stress ratings showed a trend (p=0.093) towards statistically significant lower stress ratings in *females*. Baseline anxiety significantly differed between GENDER groups, showing higher values in *male* participants (see Table 7). Pre-post difference scores of anxiety and arousal, but not stress ratings, were higher in *ELA* as compared to *control* participants. There were no significant interaction effects of ELA x GENDER.

4.4.4 Differences in positive and negative affect (PANAS)

No significant main effects of ELA and GENDER were found for baseline positive affect (PA) and negative affect (NA), although *ELA* participants tended (p=0.076) to express higher negative affect as compared to *controls*. Pre-post differences of PA showed a significant main effect of GENDER (p=.058), suggesting a higher increase of positive mood induced by the stress procedure in *male* participants compared to *females*. A significant interaction effect of ELA x GENDER emerged, indicating the highest PA increase in *male controls*, and the lowest PA increase in *female controls*, while there was apparently no GENDER difference within the *ELA* group. Negative affect (NA) increased higher in the *ELA* than the *control* group (main effect of ELA). There was no main effect of GENDER, and no significant interaction effect of ELA x GENDER on NA (see Table 7).

4.5 Discussion

Neuroendocrine, cardiovascular, and subjective stress responses to an extended CPT were assessed in 22 ELA (with history of adoption) and 22 control participants (without history of adoption). Although ELA participants showed higher stress-induced changes in arousal and anxiety ratings, as well as similar cardiovascular stress reactivity, their

HPA stress axis responsiveness was blunted as compared to controls. Consistent with prior findings (Zakreski, Barton et al. 2016), negative affect at baseline and stress-induced increase in negative affect were higher in ELA participants, linking early adversity to negative emotion and mood after stress. Selectively blunted HPA axis responsiveness to stress in ELA participants suggests a physiologically based response separation (Mirescu, Peters et al. 2004; Lupien, McEwen et al. 2009).

This study adds to the existing literature that ELA is associated with blunted HPA axis responses to stress in the presence of increased subjective stress responsiveness. Consistent with prior findings (Sapolsky and Meaney 1986; Mirescu, Peters et al. 2004), there is substantial evidence of a glucocorticoid feedback hypersensitivity in ELA participants driving the reported changes.

Despite a gender difference in baseline HR with higher values in women than man, gender differences and interactions of gender and early life history for cardiovascular and endocrine reactivity to stress were not detected.

Stress results were achieved by a new CPT stress protocol. The CPT is a powerful procedure which has successfully been used to study the impact of stress on learning (Duncko, Cornwell et al. 2007), memory processes (Duncko, Johnson et al. 2009), emotional behavior (Suter, Huggenberger et al. 2009), attention (Sanger, Bechtold et al. 2014), startle (Schulz, Plein et al. 2011; Deuter, Kuehl et al. 2012), and pain (Edwards and Fillingim 2005). Adding a social-evaluative component to the CPT increases stress reactivity, especially responsiveness of the HPA axis (Schwabe, Haddad et al. 2008). However, the CPT is a passive test not requiring active performance and only little cooperation. Therefore, the particular aspect on which social evaluation is focused may remain vague to the participants. Adding a cognitively challenging task to the CPT resolves this problem. The PASAT was chosen, since it is more practical, well-validated, provides a cognitive performance read-out, and has been shown to induce stress responses (Philippsen, Hahn et al. 2007; Schwabe, Szinnai et al. 2007). Indeed, strong cardiovascular stress responses were found (MAP increase by approx. 30mmHg), whereas other published stress protocols induce much lower MAP response (e.g. (Becker, Pepine et al. 1996)). Strong stress reactivity was also present in HPA axis and heart rate responses, as well as in positive and negative affect changes, suggesting high validity of the stress protocol used.

Between-study variation in stressor strength may also be an important reason for divergent findings on ELA-induced changes in stress responsiveness, potentially explaining why several previous studies using less potent laboratory stressors have not detected differences in neuroendocrine reactivity between participants exposed to early-life stress and healthy controls (see also (Del Giudice, Ellis et al. 2011)).

PASAT performance scores (percentage of correct answer) were lower in ELA participants compared to controls. This difference was already pre-existing during the teaching session, but may have aggravated by concurrent CPT stress. Supported by previous research (Loman and Gunnar 2010), this strengthens the assumption of a link between impaired ability to cognitive performance and the exposure to ELA.

Further potentially trauma-induced effects were represented in the results of subjective ratings indicating higher stress-induced arousal and anxiety in ELA participants. Such differences, as well as the higher level of negative mood within the ELA group, justify the assumption of early adversity associated with a lower ability to subjectively cope with stressful situations in adulthood.

Recent evidence also suggests that the ELA effects on HPA stress axis responsiveness might critically depend on contextual factors experienced during ELA (Heim, Mletzko et al. 2008). Thus, it has to be mentioned as a limitation that the current study was unable to retrospectively collect validated data about the closer circumstances leading to institutionalization of ELA participants. This aspect will need to be further investigated by prospective studies. Future research should try to substantiate the finding of blunted stress responsiveness to better address the underlying cause of ELA-related diseases in adulthood. Prevention programs for adults might establish selective therapies based on the causality of such disorders (Hewson-Bower & Drummond, 2001). Results of increased subjective stress responses may help prevention programs for adopted children to improve therapeutic strategies. Thereby, children with early adversities might learn to better cope with negative emotion and increased anxiety after stress at an early stage.

4.5.1 Conclusion

The present study found evidence for a selectively blunted HPA stress axis in ELA participants. Cortisol responsiveness to stress were lower in participants who

experienced ELA, while cardiovascular (autonomous) stress reactivity was preserved, and subjective stress responses (negative affect) were even enhanced.

4.6 Figures and Tables

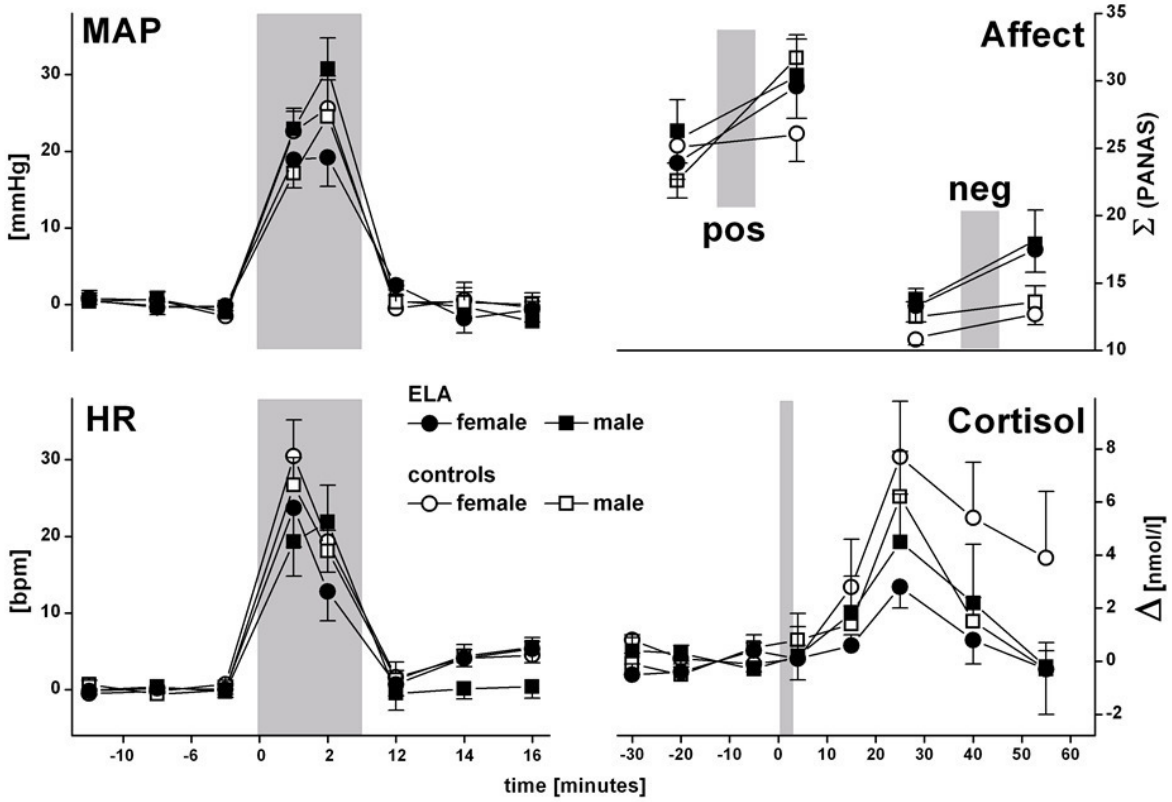

Figure 12. Cardiovascular, cortisol and affect data before, during, and after CPT: a) mean arterial blood pressure (MAP), b) heart rate (HR), c) Positive (pos) and Negative (neg) Affect sum scores and d) saliva cortisol in response to stress in volunteers experiencing early life adversity (ELA: female, closed circles; male, closed squares), as compared to age-matched control participants (CNT: female, open circles; male, open squares). Grey areas indicate time of stress intervention. Data represent mean ± SEM; beats per minute (bpm); millimeter of mercury (mmHg).

			Hemodynamics			
	group		***BL-MAP*** [mmHg]	**∆*-MAP*** [mmHg]	***BL-HR*** [bpm]	**∆*-HR*** [bpm]
ELA	female	mean	81.38	26.97	74.71	28.35
		SD	5.16	15.75	9.58	11.61
	male	mean	80.95	32.16	64.00	28.12
		SD	6.74	10.07	9.75	14.29
CNT	female	mean	82.72	29.81	76.21	33.51
		SD	5.95	12.49	8.79	21.59
	male	mean	87.57	24.96	69.60	24.12
		SD	7.12	11.60	11.74	16.65

			Salivary Cortisol		
			BL [nmol/l]	***PEAK*** [nmol/l]	***AUCi*** [nmol*min/l]
ELA	female	mean	6.92	2.85	57
		SD	2.13	3.17	120
	male	mean	8.02	4.50	113
		SD	3.48	4.97	227
CNT	female	mean	6.82	7.71	254
		SD	2.51	7.08	301
	male	mean	8.33	6.22	124
		SD	4.97	5.64	152

			PASAT performance [%correct]
ELA	female	mean	51.1
		SD	35.8
	male	mean	55.1
		SD	31.1
CNT	female	mean	66.3
		SD	30.0
	male	mean	79.4
		SD	26.8

			VAS Ratings [aU]					
			BL-Stress	***DIF-Stress***	***BL-Arousal***	***DIF-Arousal***	***BL-Anxiety***	***DIF-Anxiety***
ELA	female	mean	9.42	32.07	14.14	46.28	5.42	15.64
		SD	17.87	26.30	16.81	27.72	13.21	19.77
	male	mean	14.87	31.50	13.37	24.25	15.62	18.37
		SD	15.31	32.16	14.06	31.94	11.45	35.48
CNT	female	mean	3.90	27.00	5.00	19.18	2.09	0.27
		SD	4.86	30.35	5.19	20.70	2.25	1.55
	male	mean	14.72	19.18	10.18	25.36	6.27	1.00
		SD	16.84	22.53	18.74	21.41	9.01	6.48

			PANAS			
			BL-POS	***DIF-POS***	***BL-NEG***	***DIF-NEG***
ELA	female	mean	23.92	5.71	13.35	4.14
		SD	9.78	6.52	4.53	4.27
	male	mean	26.25	4.12	13.75	4.12
		SD	6.51	6.24	2.25	6.03
CNT	female	mean	25.18	0.90	10.81	1.90
		SD	8.23	4.08	1.25	1.97
	male	mean	22.63	9.09	12.45	1.18
		SD	4.43	5.61	3.75	2.78

Table 6: statistical characteristics of ELA and control group: for baseline (BL) and stress reactivity (∆), for hemodynamics: mean arterial pressure (MAP), heart rate (HR), for salivary cortisol, for Paced Auditory Serial Addition Task (PASAT), for visual analog scale (VAS) ratings: subjective ratings of stress, arousal, anxiety in baseline (BL) and pre-post difference after stress, and for Positive Affect and Negative Affect Scale (PANAS) in baseline (BL) and pre-post difference after stress in subjects who experienced early life adversity (ELA) and age-matched control subjects (CNT). Arbitrary Units (aU); beats per minute (bpm); millimeter of mercury (mmHg).

ANOVA results

Hemodynamics

		BL-MAP [mmHg]	**∆*-MAP*** [mmHg]	***BL-HR*** [bpm]	**∆*-HR*** [bpm]
ELA	F	4.43	0.14	0.48	0.01
	p	**.042**	.711	.493	.912
GENDER	F	2.24	0.00	7.11	0.94
	p	.142	.955	**.011**	.337
ELA * GENDER	F	1.17	1.55	1.11	0.88
	p	.287	.220	.299	.353

Salivary Cortisol

		BL [nmol/l]	***PEAK*** [nmol/l]	***AUCi*** [nmol*min/l]
ELA	F	0.06	4.85	3.25
	p	.803	**.033**	.078
GENDER	F	1.66	0.10	0.15
	p	.204	.754	.699
ELA * GENDER	F	0.00	0.83	2.43
	p	1.000	.369	.127

PASAT performance [%correct]

ELA	F	4.16
	p	**.049**
GENDER	F	1.29
	p	.264
ELA * GENDER	F	0.00
	p	1.000

		VAS Ratings [aU]					
		BL-Stress	***DIF-Stress***	***BL-Arousal***	***DIF-Arousal***	***BL-Anxiety***	***DIF-Anxiety***
ELA	F	0.22	1.11	1.96	4.33	2.67	8.12
	p	.644	.299	.160	**.044**	.110	**.007**
GENDER	F	2.96	0.42	0.10	1.49	4.16	0.00
	p	.093	.520	.758	.230	**.048**	.955
ELA * GENDER	F	0.63	0.02	0.58	2.64	2.01	0.13
	p	.430	.891	.449	.112	.164	.723

		PANAS			
		BL-POS	***DIF-POS***	***BL-NEG***	***DIF-NEG***
ELA	F	0.14	0.01	3.33	4.85
	p	.714	.937	.076	**.033**
GENDER	F	0.02	3.82	0.54	0.39
	p	.892	.058	.465	.537
ELA * GENDER	F	1.03	7.84	0.81	0.00
	p	.317	**.008**	.374	1.000

Table 7: Results of two factorial ANOVA on baseline (BL) and stress reactivity (∆), for hemodynamics: mean arterial pressure (MAP), heart rate (HR), for salivary cortisol, for Paced Auditory Serial Addition Task (PASAT), for visual analog scale (VAS) ratings: subjective ratings of stress, arousal, anxiety in baseline (BL) and pre-post difference after stress, and for Positive Affect and Negative Affect Scale (PANAS) in baseline (BL) and pre-post difference after stress for factor early life adversity (ELA) and gender and their interaction (ELA*GENDER). Data represent mean ± SD. F-values and exact p-values are reported as ANOVA Test results. Arbitrary Units (aU); beats per minute (bpm); millimeter of mercury (mmHg).

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Erklärung

nach §9 Abs.1 der Promotionsordnung des Fachbereichs I der Universität Trier vom 13.11.2008, geändert am 25.05.2016.

Hiermit versichere ich, dass ich die vorliegende Arbeit selbst verfasst und keine außer den angegebenen Hilfsmitteln und Referenzen benutzt habe. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

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