Psychosocial factors and Cortisol sampled from Hair and Saliva

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2017

Thesis submitted for the degree of Doctor of Philosophy

DECLARATION

I, Bianca Serwinski, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed:

Date:

ABSTRACT

The conventional sources to assess cortisol levels are saliva, plasma or urine specimens, which are subject to a variety of factors and provide only momentary cortisol exposure. A rather new technique is the analysis of hair cortisol which might overcome some of the methodological issues associated with the other specimens. The aim of the PhD was two-fold.

Firstly, I investigated the associations between hair cortisol concentration and different socioeconomic factors, work-related stress and psychosocial factors. Study I employed a design with two time points four years apart and therefore was able to evaluate psychosocial and socioeconomic factors cross-sectionally and as a dynamic entity. Lower income and worsening income change was associated with elevated hair cortisol. Moreover, an effect of status incongruity, a mismatch between education and income, on hair cortisol was found. Study III looked at the impact of examination stress on cortisol in hair and saliva in medical and law students. The student groups differed slightly in their hair cortisol levels at baseline and also in levels of anxiety, impeding proper conclusive findings. Perceived stress and anxiety were not related to hair cortisol but to salivary cortisol. The use of avoidant coping mechanisms was associated with elevated hair cortisol levels.

My second aim was to evaluate the long-term consistency of cortisol in saliva and hair and also saliva-hair correlations over corresponding time-intervals. Using two distinct longitudinal studies (Study I and II), the findings indicate that a flatter rate of decline in salivary cortisol over the day was associated with elevated hair cortisol concentrations several years later, 4 (female sample) and 8 years ago

(in men only), while no relationship could be found between hair cortisol in relation to the AUC and the CAR. Another dataset (Study III) assessing salivary and hair cortisol over corresponding intervals revealed positive associations between the AUC and hair cortisol.

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LIST OF PUBLICATIONS

Some of the research (of Study I; Chapter 3) described in this thesis has been published. Other are being prepared for publication (Study I + II; Chapter 4 and Study III; Chapter 5). Furthermore, some of the research has been presented at international conferences.

Publications:

Serwinski, B., Salavecz, G., Kirschbaum, C. & Steptoe, A. (2016). Associations between hair cortisol concentration, income, income dynamics and status incongruity in healthy middle-aged women. *Psychoneuroendocrinology, 67,* 182–188.

Book chapter:

Steptoe A. & Serwinski B. (2016). Cortisol awakening response. In Fink, G. *Stress Concepts and Cognition, Emotion, and Behavior.* Handbook of Stress Series Volume 1, Elsevier Inc.

Awards:

Serwinski B. (2015). Coping as a stress-buffer during academic stress - findings from hair and salivary cortisol. *Young Investigator Colloquium Award, Scientific Meeting of the American psychosomatic Society*, Savannah.

Conferences:

Serwinski B. (2015). Stress, negative mood, and salivary cortisol output. *Annual Scientific Meeting of the American Psychosomatic Society,* Savannah.

Serwinski B. (2015). Associations between hair and salivary cortisol – a study of temporal relationships. *Annual Scientific Meeting of the American Psychosomatic Society,* Savannah.

Serwinski B. (2014). Hair cortisol as a biomarker of stress: strong associations between cortisol in hair and salivary cortisol*. Iberoamerican Congress of Health Psychology,* Spain.

Serwinski B. (2014). Lower heart rate variability over the working day is associated with elevated hair cortisol levels. *International Congress of Behavioural Medicine,* Netherlands.

ACKNOWLEDGEMENTS

This period of doctoral studies in an environment with excellent scientific research facilities has provided an exceptional opportunity for me to grow as a person and I am utterly thankful to all who enabled and facilitated this journey.

First of all, I would like to express my deep appreciation and gratitude to Professor Andrew Steptoe for his substantial professional and organisational support. I attribute the success of my PhD to his invaluable advice, patience and understanding. Not only his level of expertise and capability to inspire, but also his approachability, responsiveness and his demeanour in general reflect his ability as an excellent supervisor - I could not have wished for more. I am very grateful to my second supervisor Dr Lydia Poole for sharing her ideas on the manuscript, for the insightful and inspiring comments and discussions and her unwavering support towards the end of my PhD. I also thank Dr Marta Jackowska for her indispensable support in the early phases.

I would like to thank all researchers involved in the Daytracker Study, especially Dr Gyöngyvér Salavecz, who facilitated my data collection in Budapest, which was a wonderful experience. I am grateful for all participants who kindly dedicated their time, saliva and hair, and made this research possible. I would like to thank the TU Dresden and Professor Clemens Kirschbaum for their support in analysing the cortisol samples and the Medical Research Council (MRC) for funding my PhD.

I have no words to express my gratitude to my parents (my whole family and also in-laws) for their continuous, unconditional support. Danke für Eure Unterstützung, die Ihr mir zuteilwerden lasst und für all die Zeit, Energie und Geduld, die Ihr in mich investiert habt. Babcia - dziękuję za życzliwość i inspiracji. Gracias por darme tanto cariño y apoyo.

The love, support, optimism and patience of my husband, Isra, has been instrumental to my success; thank you for bringing the best out of me and for teaching me that there is no problem without a solution. Gracias, mi vida, que siempre estás ahí, apoyándome y dándome las alas para continuar mi viaje hacia mis objetivos.

Last but not least, I would like to acknowledge and thank all my friends (especially Kata, Steph, Tanja, Karin, Ella, Catarina, Filipa, Silvia and Los Familigos) for their emotional support and for being there and my current and former office mates and friends at UCL (especially Milagros, Carol, Ruth, Sara, Wendy, Ula and Madiha) who contributed to a wonderful, friendly atmosphere and experience.

LIST OF ABBREVIATIONS

ACTH:	Adreno-Corticotrophic Hormone
AESI:	Academic Expectations Stress Inventory
ANCOVA:	Analysis of Covariance
ANOVA:	Analysis of Variance
ASS:	Academic Stress Scale
AUC:	Area-Under-the-Curve
BMI:	Body Mass Index
CAR:	Cortisol Awakening Response
CATS:	Cognitive Activation Theory of Stress
CHD:	Coronary Heart Disease
CI:	Confidence Interval
COPE:	Coping Inventory
COPEAU:	Coping with Pre-Exam Anxiety and Uncertainty
CRH:	Corticotrophin-Releasing Hormone
CRP:	C-Reactive Protein
CVD:	Cardiovascular Disease
DAI:	Differential Performance Anxiety Inventory
DASS:	Depression, Anxiety and Stress Scale
DCM:	Demand-Control Model
DHEA:	Dehydroepiandrosterone
DV:	Dependent Variable
ELISA:	Enzyme-Linked Immunosorbent Assay
ELSA:	English Longitudinal Study of Ageing
ERI:	Effort-Reward Imbalance
GAD:	Generalised Anxiety Disorder
HCC:	Hair Cortisol Concentration
HESI:	Higher Education Stress Inventory
HPA:	Hypothalamic-Pituitary-Adrenal
IL:	Interleukin
IV:	Independent Variable
LC-MS:	Liquid Chromatograph–Mass Spectrometry
M:	Mean
PCC:	Plasma Cortisol Concentration
PMSSS:	Perceived Medical Student Stress Scale
PNS:	Parasympathetic Nervous System
PSS:	Perceived Stress Scale
PTSD:	Posttraumatic stress disorder
SACS:	Strategic Approach to Coping Scale
SCC:	Salivary Cortisol Concentration
SD:	Standard Deviation
SEM:	Standard Error of the Mean
SES:	Socioeconomic Status
SNS:	Sympathetic Nervous System

SPSS:	Statistical Packages for the Social Sciences
STAI:	State-Trait Anxiety Inventory
TAI:	Test Anxiety Inventory
TICS:	Trier Inventory for the Assessment of Chronic Stress
TNF-α:	Tumor Necrosis Factor-Alpha
UCC:	Urinary Cortisol Concentration
UCL:	University College London
UK:	United Kingdom

OVERVIEW

This PhD investigates the associations between stress, psychosocial factors and cortisol and compares cortisol in two distinct body tissues, i.e. saliva and hair. The thesis commences with a literature review about stress, including the development of stress theory and stress research methods, and outlines evidence of the link between psychosocial, stress-related factors and health. It is followed by a literature review on cortisol, discussing its role in determining health, including the impact of stress and psychosocial factors (focusing on stress exposure and appraisal, coping and social support). The subsequent chapter outlines methodological aspects in cortisol sampling and introduces a novel assessment method (i.e. hair cortisol analyses) that attempts to somewhat overcome these difficulties. Then, four chapters present findings from three studies to investigate the associations between hair cortisol concentration and different psychosocial and stress-related factors, to evaluate the long-term consistency of cortisol in saliva and hair and to evaluate saliva-hair correlations over corresponding time-intervals.

The studies that are included in this thesis are:

1) The Daytracker Study - a prospective study investigating the associations between psychosocial factors and biological functioning in the work environment in women in two contrasting cultures. This study initiated in 2007/2008 and I conducted the follow-up assessment in 2012/2013, including planning, recruitment, data input and analyses. Two chapters resulted from this study: Chapter 3, in which I analyse the relationship between hair cortisol and socioeconomic factors, work

stress and social support (both longitudinally and cross-sectionally) and Chapter 4, in which I assess the long-term consistency of cortisol in saliva and hair.

2) The English Longitudinal Study of Ageing (ELSA) - a longitudinal panel study of a representative cohort of men and women aged 50 years and older (based on a subset of the Health Survey for England). I performed the statistical analyses for this dataset using cortisol measures of two time points (8 years apart) to evaluate the long-term consistency of cortisol in saliva and hair and results are presented in Chapter 4.

3) The Academic Stress Study – a cross-sectional study consisting of two phases (during average academic stress and during high academic stress) in which medical and law students provide salivary and hair cortisol and measures on psychosocial factors. I conducted the whole study including planning, recruitment, data input and analyses. Two chapters resulted from this study, Chapter 5, in which I explore saliva-hair correlations over corresponding time-intervals in the two phases, and Chapter 6, in which I evaluate whether the impact of academic stress is visible in hair specimen and whether coping strategies act as moderators of stress-related cortisol output.

INTRODUCTION

Integrative models of disease aetiology have offered the possibility to determine the potential key elements that significantly contribute to illness. Since the development of the biopsychosocial model in 1977, research has advanced over subsequent decades in considering the mediating role that psychological and social structural factors play in determining individual health outcomes (Borrell-Carrio, Suchman, & Epstein, 2004; Engel, 1977). Further, the literature has predominantly provided evidence that chronic stress fosters disease (Chrousos, 1998; Cohen, Janicki-Deverts, & Miller, 2007). From a life course epidemiology perspective, the pathways involving psychological factors (including managing stressors) in relation to various health outcomes is best described with the chain of risk model (additive model). This model posits different exposures, including behaviours and biological mechanisms, increase disease risk via pathways that explain (probabilistically rather than deterministically) how one factor might add or contribute to the potentially damaging effect of another (Kuh, Ben-Shlomo, Lynch, Hallqvist, & Power, 2003; Steptoe & Freedland, 2010). Figure 0.1 depicts such a model of the relationship between psychosocial factors and the preceding states of morbidity and mortality, namely health behaviours, psychobiological processes and symptoms. Epidemiological research has identified psychological states as both aetiologic and prognostic factors contributing to morbidity and disease-specific and overall survival, as in the case of depression and cardiovascular disease (CVD) mortality (Dickens, 2015). As can be observed in this diagram, the arrow between psychological factors and morbidity and mortality indicates a unidirectional

pathway in which psychological factors have a direct effect on incident rates. The other relationships in this model are bidirectional in nature, as indicated by the arrows. Psychological factors have been identified in the initiation, promotion and progression of certain diseases; they affect the underlying psychobiological processes and also influence the engagement in detrimental health behaviours that are in turn linked with psychobiological mechanisms as well as having direct effects on the disease outcome. Contrariwise, psychological factors can also be a consequence of poor health behaviours, psychobiological processes and an existing condition. Noteworthy, psychological as well as psychobiological processes are influenced and shaped by individual's biological vulnerability factors (genetic, biochemical, and physiologic and constitutional aspects) and environmental factors that are not depicted in this diagram.

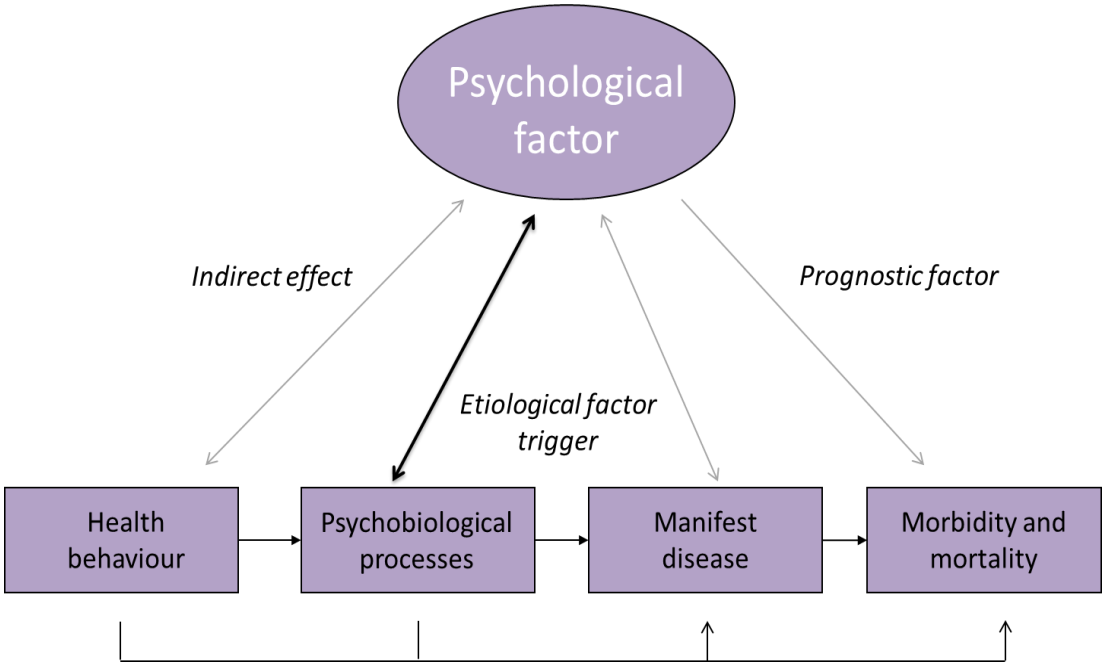

Figure 0.1 Potential mechanisms through which psychosocial factors contribute to ill-health (adapted from Hamer, 2012).

This PhD focuses on the relationship highlighted in the diagram: the association between psychological factors and psychobiological processes. The term psychosocial factor have been regarded as two sets of factors: psychological trait or state characteristics, including depression, anxiety, social isolation and secondly, exposures to external adversities such as work stress, financial strain, early life stress or grief. Psychobiological processes broadly embrace all related bodily functions such as genetic factors, immune system responses, endocrine functioning, nervous system processes and glucose control. The Hypothalamic-Pituitary-Adrenal (HPA) axis, and more specifically its main driving hormone, cortisol, and its interaction with psychological and psychosocial factors are at core of this PhD. Yet, implications and broader explanations for the different pathways (indicated in grey) will be included at relevant sections. Discussing their independent and possibly synergistic effects provides a thorough understanding of the mechanisms involved in disease aetiology.

CHAPTER 1. LITERATURE REVIEW: STRESS, CORTISOL AND HEALTH

1.1 Chapter overview

This chapter introduces the reader to the concept of stress (including psychosocial factors), describing the development of stress theory and the approaches used to investigate stress. This is followed by a literature review on cortisol in relation to health. Particularly, cortisol will be defined and its function and its role in determining health will be discussed. Literature on cortisol in relation to different physical and mental health outcomes will be outlined, with a focus on stress and psychosocial factors as modifiers or direct causes of disease.

1.1.1 The concept of stress

In 1914-1930, Cannon developed the concept of fight-or-flight and the affiliation to the term homeostasis and its consequential biological breakdown (Johnson, Kamilaris, Chrousos, & Gold, 1992). The concept of fight-or-flight outlines a physiological reaction in response to both physical and emotional stimuli that prompts the individual to react by fighting or fleeing. The two main divisions of the autonomic nervous system, the part of the nervous system that controls non-conscious bodily functions, are the parasympathetic nervous system (PNS) and the sympathetic nervous system (SNS). The SNS is responsible for the 'fight or flight' response by increasing for example heart rate and force, muscle contraction, glucose production, dilation of the eyes and by reducing all bodily processes not critical for survival (Chrousos & Gold, 1992). The PNS, on the other hand,

counterbalances the actions of the SNS and reinstates a resting state and controlling homeostasis by conserving energy (slower heart rate, increased intestinal activity). Following this in 1950, the term 'stress' was coined by Selye and conceived as the General Adaptation Syndrome, a framework comprising three stages: 1) the alarm stage (similar to Cannon's fight-or-flight concept) involving the essential and necessary hormonal adjustments to the threat with a consequential normalising decline, 2) the stage of resistance involving sustained activation of the physiological response when facing prolonged stressors and 3) the stage of exhaustion whereby the second stage persists even longer, resulting in an inability to respond adequately when confronting a stressor (Selye, 1973a). He placed the HPA-axis at the centre of the stress process. In addition, an important notion that Selye pointed out was two distinctive categories of stress. Eustress is a beneficial response to an external stressor and distress is the typical negative attribution the term 'stress' is typically understood to mean in today's society (Selye, 1976).

Selye defined stress as the bodily response to any stimulus. This conceptualisation overlooks the fact that changes in bodily function are required in response to all demands. An effective biological regulatory system is one that accommodates change and flexibility in adapting to the environment. This concept led to the terms allostasis and allostatic load that have since gained recognition and are commonly used in the field of contemporary biobehavioural and stress research (Danese & McEwen, 2012; Logan & Barksdale, 2008; McEwen, 1998). Allostasis is defined as the process of maintaining stability (homeostasis) through physiological change and adjustment. Allostasis is beneficial and ensures survival through

temporary bodily adaptation processes. This is important and suitable for acute stressors. Yet, in the long run with sustained or repeated challenge, allostatic load occurs, which is a dysfunctional consequence of allostasis. It is characterised as the inability to restitute or habituate to repeated or chronic stressors and hence contributes to the so-called 'wear and tear on the body' (McEwen, 2000a). Repeated, or chronic, stress creates these compensatory physiological mechanisms that over the long term can accelerate disease processes. While these biological accounts formed the foundations for the concept of stress, they labelled stress as a purely biological-behavioural reaction and based the link between stress and health on a biomedical orientation. Since then, various models have been offered in an attempt to describe an integrative concept of stress that includes the psychological dimension.

For example, in the Transactional Theory, Lazarus and Folkman (Lazarus, 1993; Lazarus & Folkman, 1987) introduced a cognitive-relational element by focusing on the individual cognitive appraisal and coping strategies used in situations involving stress. This offered an important advancement to the concept of stress, as cortex-related processes were added to the basic pituitary-related processes (Le Moal, 2007), and thus the notion emerged that stress was a psycho-biological phenomenon. Stress or so-called 'strain' is thought to arise when facing an imbalance of the expected needs and resources available to deal with the perceived demands of a situation. The Transactional Theory posits that two cognitive elements mediate the stressor and the response to the stressor. Primary appraisal focuses on the nature of the stressor as having either a potentially

damaging or benign effect and secondary appraisal entails the evaluations of available coping resources suitable to handle the situation. Stressors have been determined from two different perspectives. The first is the environmental perspective, a pure stimulus-based definition of stress, where the objective account of stressful life events, measurements of chronic stressors and within-day events (daily hassles, interpersonal discussions) take centre-stage (Holmes, 1982). Temporal aspects and the magnitude of the stressors are thought to play a moderating role. Secondly, the psychological perspective considers perceptions and subjective evaluations of the stimuli, including the emotional reaction to it; thus the focus is on the transaction or relationship between the individual and the environment (Cohen, 1986). Lazarus and Folkman (1987) introduced this psychological (i.e. cognitive, affective, coping) dimension into stress models. A fundamental feature of the appraisal processes in their model is that individual differences in perceptions determine the cognitive elements and hence adaptive coping or maladjustment to the stressor, rather than the event itself. Coping, as a volitional management strategy to deal with the stressor, and the psychosocial factors influencing the coping process will be elaborated later on, as it forms a main part of one of the studies of this PhD (Study III; Chapter 6).

Ursin and Eriksen developed the Cognitive Activation Theory of Stress (CATS) (Ursin & Eriksen, 2004). Underpinning this model of stress is the cognitive aspect of expectation regarding the stressor and the subsequent response and outcome. According to this model stress results from a discrepancy between a set value (expectation) and the actual value (reality). Expectancies of the stressor relate

to defence mechanisms such as having a distorted view about the nature of the stimulus, whilst the expectation of the response outcomes relate to actual coping mechanisms. Coping can be subdivided into either successful coping with the external factor, hopelessness or helplessness. Hopelessness, the belief that actions lead to failures, can be regarded as a negative coping style resulting in negative coping behaviours (which will be elaborated later on; see section 1.1.8). Helpless individuals avoid any kind of action as they fail to acknowledge subjective control or a connection between their actions and the outcome. The CATS attempts to integrate time elements, previous experiences and outcome expectancies in the cognitive aspect of stress appraisal. It has been particularly useful in studies on objective work settings and also employee's individual stress management capacity (Reme, Eriksen, & Ursin, 2008).

From these cognitive-based theoretical explanations it is apparent that an individual's perception of the stimulus and the subsequent subjective experiences and resulting emotions are crucial elements in defining stress. A body of research has indicated that the nature of the stimulus, its strength, dimensions and duration, together with the individual's personal (also genetic) constitution, evaluations, coping capacities and resources, which are themselves shaped by former/ early-life experiences and expectancies, impact on successful stress adaptation and responses (Le Moal, 2007; McVicar, Ravalier, & Greenwood, 2014). Most contemporary views of stress and stress reactions define it as an interaction between the different bodily systems, such as the neurological or endocrinological systems, that ultimately generate cognitive, behavioural, biochemical and physical

pathways. Hence, the evolution of the concept stress has been shaped by discoveries from a multidisciplinary body of research ranging from brain biochemistry, psychoendocrinological and psychoimmunological origin to cognitive and behavioural analyses, encompassing also the modern viewpoints of epigenetic processes. Subjective health complaints and psychological somatisation are concepts that are easier to understand and explain owing to the findings of psychological stress processes being linked to somatic mechanisms (Eriksen & Ursin, 2002). In the following section, the different methodologies for stress research will be briefly described before outlining the key psychosocial factors of interest in the studies conducted as part of the present PhD, whilst providing evidence on the pathophysiological consequences of maladaptive psychosocial factors and stress processes.

1.1.2 Stress research methods

Adaptive stress resilience prevents continual or chronic allostatic responses and thus the plethora of adverse physiological consequences. Stress resilience is the ability to physiologically respond to the stressor within the adaptive range and the ability to terminate this response once the stressor has ended.

1.1.2.1 Stress induction

Stress physiology has been extensively studied with laboratory mental stress testing using standardised controlled stimuli as stressors and markers for autonomic nervous system and neuroendocrine functioning as outcomes. There are

several tools used in psychobiological research to experimentally induce stress, such as the 'Trier Social Stress Test' and cognitive challenge tasks, e.g. colour-word interference or mirror tracing (Kirschbaum, Pirke, & Hellhammer, 1993). These methods employ artificially induced stressful situations which occur in a controlled environment without real-life implications. Ecological validity of acute laboratory stress testing has been widely criticised for these reasons. However, such methods are valuable for establishing causality – they allow us to better understand the biological changes during stress. By manipulating conditions and controlling for confounding factors, effects of physiological responses can be attributed to the manipulated exposure stimulus. The acknowledgment that an individuals' means of coping with stress affects neuroendocrine responses and ultimately health outcomes, led researchers to study individual physiological reactions to laboratory-induced stressors in more detail, taking psychosocial factors, general life stress and demographic factors into account (Chida & Hamer, 2008).

1.1.2.2 Stress responsiveness and reactivity

Stress reactions and stress resilience have often been measured with assessments of the stress hormone, cortisol. Cortisol represents one type of physiological functioning and a suitable marker for stress, since upon experience of a psychological or physiological stressor, cortisol is secreted. Being the end product of the hypothalamic-pituitary-adrenal axis (HPA-axis), different signal pathways are involved in prompting the adrenal glands to secrete cortisol within minutes (the body's stress reaction and the production of cortisol will be explained in detail in

section 1.2). Stressors activate brainstem and forebrain limbic structures, which are directly and indirectly affecting the HPA-axis, creating a cascade of different precursor hormones that ultimately generate cortisol secretion. Stress physiology and perception of stressor significance are is linked to cognititive factors, such as prior experiences and to anticipation and therefore involve regions of the memory and brain reward circuit (Ulrich-Lai & Herman, 2009). Although cortisol is a stress responsive hormone, it follows a typical diurnal pattern with fluctuations during both day and night. Peak cortisol levels develop within the first 30 minutes after awakening and its lowest point is reached around midnight (Pruessner et al., 1997). These transitory fluctuating cortisol concentration levels can be assessed via saliva, blood or urine sampling. Stress reactivity studies have predominantly used saliva sampling, due to its feasibility and easy of collection. Rather than considering diurnal profile of cortisol release, stress reactivity studies are interested in fluctuations over a short time period. The procedure includes taking saliva samples prior to, during and after the stress task at varying interval levels and cortisol concentrations over this period can be compared. This way, baseline levels, stress response and recovery can be evaluated and compared within and between individuals. Laboratory-based acute challenge paradigms typically increase cortisol levels in most individuals; however an over-reactive stress response (higher cortisol levels during and immediately after the stress task) or a diminished recovery (post-stress cortisol levels) have been taken to be indicative of low stress resilience (Kudielka, Hellhammer, & Wust, 2009).

Disturbed responses in acute stress settings might mimic the experience of recurring physiological activation in response to chronic stress in everyday life. An exaggerated response or diminished recovery may indicate constant activation of the stress response upon minor daily hassles and therefore be linked to adverse health outcomes. Using evidence of associations with future health outcomes and also results from observational studies, laboratory-based stress research has been very effective in furthering our understanding of psychoneuroendocrinological and psychoneuroimmunological mechanisms involved in disease development, onset and progression. Specifically, acute laboratory-induced stress reactions have been shown to predict risk factors, future development or incidence of certain diseases, e.g. progression of coronary artery calcification, hypertension or proinflammatory cytokines (Hamer, Endrighi, Venuraju, Lahiri, & Steptoe, 2012; Hamer & Steptoe, 2012; Kunz-Ebrecht, Mohamed-Ali, Feldman, Kirschbaum, & Steptoe, 2003). Studies have also found stress-induced cortisol responses to positively correlate with biological functioning in everyday life, such as total cortisol output over the day in middle-aged adults (Kidd, Carvalho, & Steptoe, 2014). Likewise, the cortisol awakening response (CAR), the rise in cortisol within the first 30 minutes after awakening, was also shown to provide an indication for stress reactivity to laboratory-induced stress (Chida & Steptoe, 2009).

Therefore, cortisol reactivity to laboratory-induced mental stress by itself appears to be an important index for health-related outcomes. More importantly, this type of research methodology allows us to further our understanding of which psychosocial factors play a role in the magnitude of the stress response in a

controlled environment (Miller, Chen, & Zhou, 2007). A meta-analysis evaluating psychophysiological stress research from over 30 years of study reported positive psychological state and trait characteristics (such as positive affect and optimism) to be associated with reduced cortisol responsiveness to acute stress paradigms (Chida & Hamer, 2008). Further, the trait characteristic hostility, a character trait of unfriendliness and anger towards others, was found to mediate post-stress cortisol recovery levels, and also other immunological and cardiovascular markers during stress in patients with advanced coronary artery disease (Brydon et al., 2010) and also in patients with diabetes (Hackett, Lazzarino, Carvalho, Hamer, & Steptoe, 2015).

The allostatic load model and also the basic principles of Selye's General Adaptation Syndrome imply that the HPA-axis is an exhaustible entity and that individuals with chronic allostatic load eventually respond less well to stressors. Indeed, studies have identified that an impaired or reduced habituation to stressors and the stress response is more prevalent in people with disorders related to adrenal exhaustion (Grissom & Bhatnagar, 2009; Kudielka, von Kanel, et al., 2006). Impaired physiological adaptation of the HPA-axis to chronic stress has been suggested as a potential mechanism in which exhaustion is associated with increased disease vulnerability (Kudielka, von Kanel, et al., 2006). Most cortisol literature, as will be outlined in the next section (Section 1.2), reports the health detrimental associations with elevated cortisol levels. Nevertheless, lower cortisol excretion in saliva, urine, and blood were observed in patients with posttraumatic stress disorder (PTSD) and also in burn-out and exhaustion or fatigue related

disorders (e.g. chronic fatigue syndrome) (Rimes, Papadopoulos, Cleare, & Chalder, 2014; Yehuda et al., 1990). The dexamethasone suppression is a method to assess cortisol responsivity and adrenal gland function (Ceulemans, Westenberg, & Vanpraag, 1985). It involves administration of the synthetic glucocorticoid dexamethasone that suppresses naturally occurring ACTH release from the pituitary gland. The amount of cortisol released is an indication of HPA function. Studies using this test have shown cortisol hypersuppression following administration in PTSD patients, indicating an alteration in HPA-axis negative feedback inhibition, or what has been described as an exaggerated sensitisation of the HPA-axis (Yehuda, Halligan, Golier, Grossman, & Bierer, 2004).

Experimentally induced stress reactivity testing has been criticised for varying impacts of stressor types used. Indeed, a meta-analysis reviewing 208 laboratory-based studies reported that psychological stressors effectively induced HPA-axis reactivity but that their effects and their magnitude varied depending on the task (Dickerson & Kemeny, 2004). Specifically, tasks that included uncontrollable and social-evaluative components were the most effective and recommended. Certainly, individual differences, emotional reaction and consequential sympathetic activation mediate the relationship between social stress tasks and cortisol increase. Social psychology and personality psychology have identified social-evaluative threat in stress tasks to evoke ego threat. Ego threat is a construct that involves negative judgement or feedback about all kinds of personal-related aspects challenging the person's self-image or favourable self-views, ranging from general and interpersonal skills to personality (Leary, Terry,

Allen, & Tate, 2009). The fact that social-evaluative threat, including for instance social evaluation, most strongly elicits cortisol as well as other cardiovascular and proinflammatory responses helps to explain the strong associations between social stress in everyday life and varying health outcomes (Bosch et al., 2009).

1.1.2.3 Stress evaluation in naturalistic studies

Other research methods to assess the impact of stress on health are through epidemiological and observational/ naturalistic studies. Both epidemiological and naturalistic research do not to intervene in participants' daily lives (Steptoe, 2005). Prospective longitudinal epidemiological studies are methodologically robust and can identify the aetiologic and prognostic factors contributing to ill health and can specify the mediating psychobiological factors. The advantage of these large population-based studies is that disease-specific incidents and/ or survival estimates can establish determinants of disease while controlling for several covariates. They also enable the analysis of dose-response associations between psychological factors, biomarkers and disease. However, they are not always feasible, especially if monitoring involves repeated and detailed assessment of everyday life. On the other hand, naturalistic trials provide practical means to assess physiological functioning in the individual's natural settings. For instance, this type of design enables assessment of the impact of affective states in everyday life on cortisol (Adam, 2006). Further advantages include that naturalistic designs make possible the study of the covariation between psychological experience and biology over time in real life environments. Similarly, these assessments are

possible in situations of real relevance to people, such as during periods of work stress. A major drawback of naturalistic studies is that they often involve small sample sizes and therefore lack power to control for a wide range of possible confounding factors or covariates.

1.1.2.4 Intervention studies

The purpose of randomised-control intervention studies is to establish causal associations. This type of design is more effective at reducing confounding. The exposure (e.g. treatment or behaviour) under study will be assigned to one or more experimental group(s). Comparing the experimental groups to the control group can identify dose-response associations between the investigated factors and establish whether an effect is causal. For instance, stress research has employed intervention studies (e.g. mindfulness programs or cognitive behavioural therapy) to assess changes in cortisol levels and psychological symptoms (Hoge et al., 2013). When this type of research has high internal and external validity, they provide a good opportunity for establishing the cause-effect relationship between the intervention and the outcome (Kendall, 2003). However, they can be costly, complex and are limited to certain research questions and populations.

1.1.2.5 Overview of stress research methods

Overall, stress research has employed varying methods to investigate HPA-axis functioning: acute laboratory stress responses, sensitivity to negative feedback inhibition (e.g. dexamethasone suppression), observational or naturalistic

monitoring studies and also intervention studies. Reviews by Foley and Kirschbaum (2010) and Kudielka, Hellhammer and Wust (2009), analysed factors moderating HPA-axis responses to acute mental stress tasks over a period of 25 years. They both report on high intra- and inter-individual variability: variability between individual respondents, between patients and healthy controls and between pathologies. Some of this variation is due to confounding factors such as socio-demographic characteristics and lifestyle behaviour, but some is of biological origin, in particular genetic factors (e.g. glucocorticoid-receptor variants). Further factors that are relevant include general life stress exposure, situational factors prior to the experiment, subjective-psychological reactions, other interpersonal factors, social environment and psychopathology.

All these variables play a role in contributing to inconsistent findings and might lead us to question the internal validity of stress research experiments. Scientific developments, especially the incorporation of multidisciplinary research measurements and approaches, have led to an improvement in methodological designs and thus a better understanding of the modulating role psychosocial factors play in the stress response (Steptoe, 2005). Inferences made from laboratory-based research that are corroborated by field research in naturalistic settings have strong ecological validity and more efficiently generalise to the population and real-life situations. The fact that subgroups with certain psychosocial traits do not respond to superimposed acute stress in the same way as other groups do endorses the role that these individual psychosocial factors play in the relationship. The next section

examines the evidence on the associations between cortisol and varying psychosocial factors in more detail.

1.1.3 Psychosocial factors

Nowadays, there is general support for the notion that psychological factors and psychosocial stress impacts physical and mental health. However, only in the last two to three decades have psychological parameters been accepted to play a crucial role in determining physical health outcomes, as for instance in the development and progression of CVD (Steptoe, 1981). Previously, the focus was exclusively on biological risk factors (e.g. blood pressure, cholesterol), together with certain health behaviours, such as smoking. This shift from a reductionist to a holistic, multi-factorial perspective has been prompted by the introduction of the biopsychosocial model by George Engel in 1977 (Engel, 1977). He objected to the biomedical model which did not incorporate psychological (including the behavioural) and social aspects and instead proposed an integrated model combining all these factors. By assessing the contributory properties of these three aspects, the biopsychosocial model implies that health and disease processes are multidimensional and that these bio-psycho-social elements are intertwined and not independent entities. As such, it allows consideration for the impact of cognitive and emotional aspects and social circumstances on health maintenance and disease development and simultaneously the psychological and social consequences of psychobiological processes and illness. It regards the individual's state within a continuum between health and disease. This model not only

identifies and understands the roots rather than symptoms for different diseases, accepting hence also psychosomatic symptoms, but also creates treatment efforts tailored from different angles. These incorporate psychological treatments where medical agents seem not to be effective or appropriate. Opposed to the biomedical model, this approach also turns the passive patient into an active one highlighting the individual's influence and control of psychological and social factors.

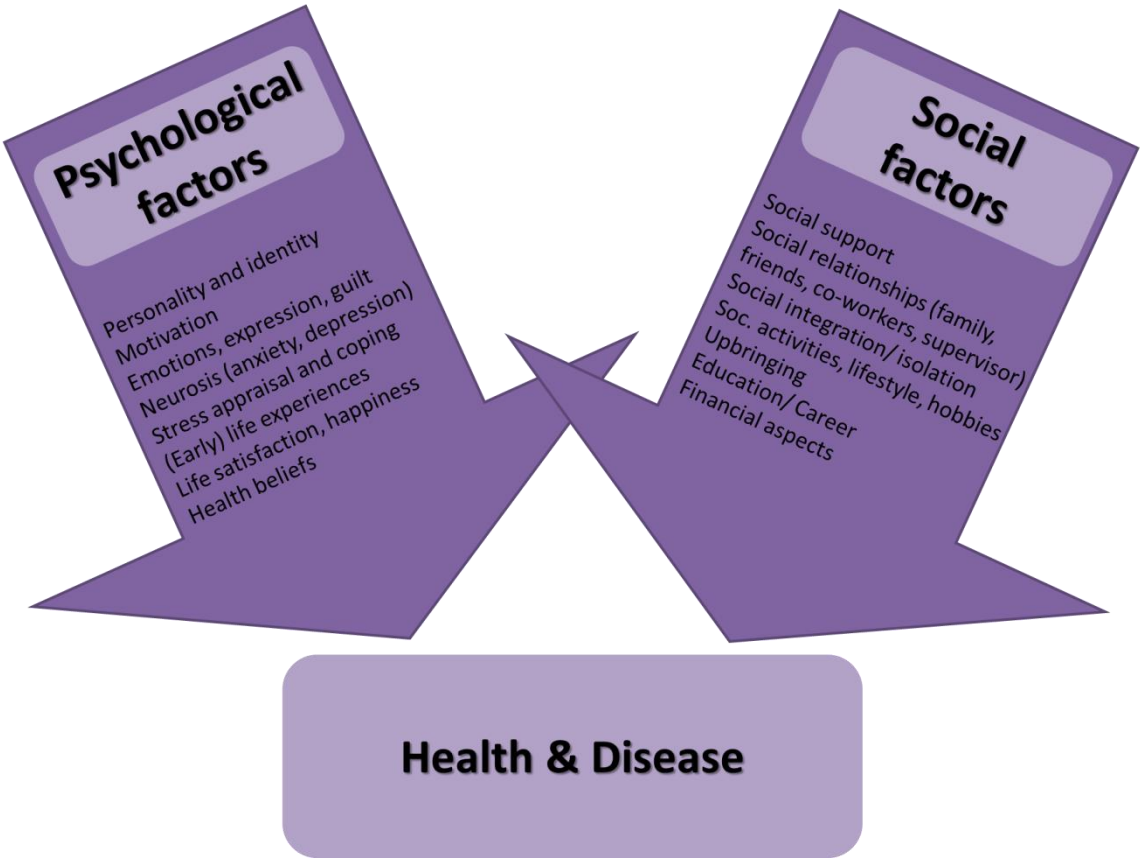

Figure 1.1 The psychological and social elements that drive mental and physical health.

Figure 1.1 depicts different psychological and social factors that have been related to health and disease. Psychological factors include, for example, personality and trait characteristics (such as extraversion, self-esteem, rumination,

locus of control, humour and optimism), emotion regulation and expression (i.e. behavioural reactivity), stress appraisal and coping, life experiences, anxiety and depression. Social factors, on the other hand, involve interactive social elements and present social support, social relationships with family and friends, with the partner (marital happiness), with co-workers and supervisors, work-related stress, social stressors (e.g. caregiving burden), social integration or isolation, lifestyle activities, upbringing, education, career and financial aspects. Although in the figure (Figure 1.1) the two areas are represented as distinct entities, psychological factors and social factors are impossible to disentangle and are indeed bidirectional in nature. Psychological factors are often shaped by upbringing and social experiences and social relationships and interpretation of social encounters are influenced by individual psychological trait characteristics. The term 'psychosocial' factor has often been used in the literature as a more suitable construct. It conveys the reciprocal association between social factors and an individual's psychological processes, i.e. cognitions and behaviours. In the present thesis, however, psychological and (psycho)social factors are presented throughout as distinct concepts.

Stress research up until the emergence of positive psychology in the very late 1990's focused solely on the link between negative psychological attributes in relation to health risk factors. The new era of positive psychology research opened up new possibilities beyond psychopathology (Seligman & Csikszentmihalyi, 2000). Evidence on negative health outcomes associated with negative psychological attributes raised the question as to what extent these associations are indeed

attributable to lack of stressors or the presence of positive emotions during the day. Positive psychology has gained more and more empirical attention over the last two decades and established independent protective health effects of positive psychological characteristics as well as a potential buffering action against illness (Steptoe, Dockray, & Wardle, 2009). Likewise, the absence of positive psychological well-being has been found to be linked to detrimental health outcomes, regardless of level of negative attributes.

Since then, there has increasingly emerged more evidence supporting the role that positive psychological attributes play in positively affecting mental and physiological well-being and functioning independent of the presence of negative emotions. For instance, a meta-analysis of prospective studies on well-being demonstrated how positive attributes (e.g. happiness, optimism) independently contributed to longevity (Chida & Steptoe, 2008). These effects persisted even when controlling for protective health behaviours, such as physical activity, no habitual alcohol use and non-smoking. There is a body of evidence suggesting that psychological and psychosocial factors contribute to the development and progression of certain diseases. In the following section, the psychological and psychosocial factors that are of interest for this thesis, including some evidence linking them to physical and mental health, will be presented in the arrangement as depicted in Table 1.1.

Table 1.1 The psychological and psychosocial factors of interest for this thesis.

Exposure to stressors	Psychological measures	Social factors
Perceived stress Work stress Financial strain Experience of life events	Anxiety Coping	Social support

1.1.4 Perceived stress

In psychosocial sciences, there are different ways of assessing stressors. Based on the concepts of coping, stress appraisal has emphasised the distinction between objective and subjective accounts of stressors (Cohen, 1986). The experience of objective stressors can be amplified or dampened by the degree to which stressors are appraised as stressful or not and by subjective appraisal and coping mechanisms. As such in the stress literature, life experiences or the experience of chronic stressful circumstances are generally regarded as objective stressors (which would include the experience of stressful life events) and the individual perception of the stressor and coping mechanisms as stress responses. Nevertheless, this distinction is not clear cut and the literature uses the different concepts interchangeably to refer to the term stress (Cohen, 1986). The construct stress has been used in the context of the external stressor, the physiological and mental stress response itself and also the exposure to chronic strain (Steptoe & Ayers, 2005). Adding to this, stressors can be acute or chronic, which is not always

clearly differentiated. Acute stressors might include daily hassles and short-term social adversities (e.g. discussions with partner, family, friends or colleagues). Chronic stress refers to living under long-standing stressful circumstances such as work, parenting and family stress, relationship problems, chronic disease, care-giver burden and abuse.

The Perceived Stress Scale (PSS) is one of the most extensively used psychometric questionnaires for measuring stress (Cohen, Kamarck, & Mermelstein, 1983). The PSS assesses the extent to which an individual perceives that given life circumstances or situations are stressful and exceed abilities to cope with them. One of the first studies that showed the impact of increased stress levels on biological processes related to increased susceptibility to the common cold, infections and delayed wound healing (Cohen, Tyrrell, & Smith, 1991; Kiecolt-Glaser, Marucha, Malarkey, Mercado, & Glaser, 1995). Further evidence supports the link between high levels of perceived stress and fatigue (Nater, Maloney, Heim, & Reeves, 2011), pain (Ginis et al., 2003), sleep disturbances (Morin, Rodrigue, & Ivers, 2003), health symptoms and quality of life (Wilkins-Shurmer et al., 2003), depression (Dahlin, Joneborg, & Runeson, 2005), cellular ageing (Epel et al., 2004), several pathophysiological changes relating to CVD (Dimsdale, 2008). A study with 73,424 men and women aged 40 to 79 years reported of a 2-fold increased age-adjusted risk of stroke and CVD mortality in those reporting high levels of perceived stress, adjusting for crucial cardiovascular risk factors (Iso et al., 2002). Strong evidence exists that mental (affective, cognitive), behavioural and psychophysiological (autonomic, endocrine, immunological) processes act as

underlying mechanisms (Krantz, Whittaker, & Sheps, 2011; Steptoe, 1991; Steptoe & Ayers, 2005). In line with the differentiating concepts of acute versus chronic stress and the beneficial aspects of the fight-or-flight response, a meta-analysis of 30 years of inquiry of the impact of stress on the immune system concluded short-term stressors are associated with an adaptive up-regulation of immune processes and long-term stressors with a suppression of immune function (Segerstrom & Miller, 2004).

Psychosocial factors are key to linking stress and health. While timing of the stressor, duration and severity are critical elements in determining the impact on both physical and mental health, it is the individual differences in perceptions and evaluations of certain events and in psychological characteristics that moderate the harmful effects of stress. For instance, individuals who perceive that stress affects their health negatively, report a larger amount of stress and show an increased risk of premature death (Keller et al., 2012). Therefore, it seems that not all stress has a negative impact on health, highlighting the aforementioned difference between eustress and distress.

Certain psychological and also social factors have been shown to act as stress moderators or even stress buffers and might therefore explain to some extent the relationships with better health outcomes. Optimism, locus of control, positive affect, adaptive and active coping strategies, humour and social support have been found to have a moderating effect on the magnitude of perceived stress on psychological well-being, life satisfaction and also physical illness (Abel, 2002; Crockett et al., 2007; Makikangas & Kinnunen, 2003; Polman, Borkoles, & Nicholls,

2010). The sections below provide more detailed evidence on the link between specific psychological and psychosocial factors and mental and physical health. The first study of this PhD (Study I; Chapter 3) focuses on one particular life domain, which is socioeconomic factors, work stress and financial strain. Study III (Chapter 6) makes use of examination stress in university as a naturalistic stressor.

1.1.5 Work stress and financial strain

Work- and finance-related issues have been reported to be the main source of stress for many people, and more than one in three employee in Europe is thought to suffer from inordinate work stress (European Commission, 2011). Work stress can result from low support from supervisors, co-workers, time pressures and workload to accomplish tasks, low changeability of skills, experiencing both excessive but also low demand and having low control over tasks and decisions (Karasek, 1979). Also an imbalance between the efforts (e.g. workload, commitments) spent at work and work-related gratification (e.g. perceived rewards in social, emotional, intellectual or financial aspects) has been shown to generate work strain (Siegrist, Peter, Junge, Cremer, & Seidel, 1990). Occupational or job burnout is a common and often-discussed consequence of job stress, characterised by mental, emotional and physical exhaustion, often observed in excessive irritability, cynicism and reduced personal performance and accomplishment (Maslach, Schaufeli, & Leiter, 2001; Schaufeli & Peeters, 2000).

Likewise, financial hardship has been reported to be a significant source of stress. Financial stability is a critically important life domain as many essential daily

activities and opportunities for education, self-realisation and achievement are dependent on financial resources. Income has often been used as a proxy for socioeconomic status (SES) and studies evaluating the effects of income vary in their methods of income assessment. Collapsing different income levels to certain categories for comparison has been widely used (Martikainen, Adda, Ferrie, Smith, & Marmot, 2003).

Over the last two to three decades, research in occupational health and public health has increasingly reported an association between adverse psychosocial work environments and worse mental and physical health (Ganster & Rosen, 2013; Melchior et al., 2007). Epidemiological cohort studies evaluating the socioeconomic determinants of health demonstrate a social gradient of health, i.e. lower income/ employment grade/ socioeconomic position has been related to a three-fold increase in mortality rate from CHD (Marmot, Rose, Shipley, & Hamilton, 1978). Further, personal debt has been associated with a three-fold increased risk for mental disorders in a population cohort study of 7,461 respondents (Meltzer, Bebbington, Brugha, Farrell, & Jenkins, 2013). Work stress has been established as a risk factor for a variety of health disorders, predominantly CVD, the metabolic syndrome and diabetes (Chandola, Brunner, & Marmot, 2006; Heraclides, Chandola, Witte, & Brunner, 2012; Kivimaki et al., 2012). The impact of work stress on health gradients, however, might vary according to sex; men are more vulnerable to work stress than women for which non-work domestic stress factors are more predictive (Matthews, Power, & Stansfeld, 2001). Studies have identified blood pressure, neuroendocrine pathways, lipoproteins and various

immunomarkers as the underlying mechanisms linking work stress, low income and health (Catalina-Romero et al., 2013; Liao, Brunner, & Kumari, 2013; Nakata, 2012; Steptoe, Siegrist, Kirschbaum, & Marmot, 2004).

There seems to be a connection between financial strain and work-related stress. Employees under financial stress have been found to be more likely to experience work stress and behave with work-related stress responses and behaviours such as worse emotional health and absenteeism (Jinhee & Garman, 2003). A population-based study of 3,374 working participants reported that work-related risk factors (such as job demands, lack of job control and social support) made a large contribution to the relationship between low income and worse mental health outcomes (Virtanen et al., 2008). However, higher degrees of financial stress do not necessarily mean higher degrees of work stress. In fact, type of work stress might differ according to income group or occupational position. High income jobs involve work stress relating to higher demand and pressures. Indeed, work environment has been identified as a crucial mediator in the association between income levels and health (Hemstrom, 2005). Furthermore, financial hardship, instability and debts can be a major source of additional stress, such as marital/ relationship and family problems, lower educational prospects, feelings of worthlessness, feelings of insecurity because of living conditions/ neighbourhood, as often seen in low-income families (Dakin & Wampler, 2008). This highlights the complexity in studying the relationship of income and health outcomes and the difficulty in attributing financial stress as the lone culprit for

worse physical and mental health outcomes. Chapter 3 makes use of a study (Study I) that assesses work stress and financial strain in a sample of working women.

1.1.6 Life events

A life event is any occurrence that might change an individual's circumstances and might include illness diagnosis or management, divorce, unemployment, retirement, illness or death of a loved one, criminal victimisation and abuse. Life events can also include positive events such as marriage and having children, however, in the context of stress research, life events have been treated as threatening and stressful and attributed to negativity in the first instance (Park, 2010). Trauma and stressful life events (including early life experiences) have been researched extensively and a body of evidence supports a strong link between life events and health (Danese & McEwen, 2012; Tosevski & Milovancevic, 2006). Associations between stressful life events and mental and psychiatric illnesses seem to be more robust than associations between life events and physical illness (Salleh, 2008). However, the magnitude, duration, predictability and controllability of a life event have all been shown to be of importance in moderating the impact that these can have on mental and physical health. Further, Schwarzer and Schulz (2003) indicate that life events are often considered as an independent variable, rather than in the context of other psychosocial factors. The same objective stressor might generate different degrees of subjective perception in two individuals, which might be attributable to individual differences in genetic vulnerability but also protective factors, such as better coping styles and higher levels of social support.

A systematic literature evaluating the evidence of the impact of life events on depression using prospective studies reported a strong positive relationship (Tennant, 2002). However, the authors found that depression type, severity and duration of depressive episode, likelihood of relapse or recurrence depended on the nature and magnitude of the stressor. Further, personality factors such as autonomy, negative cognition and anger expression, were found to moderate these relationships. The experience of serious life events have been shown to predict onset or symptom exacerbation of various illnesses such as CVD, arthritis and gastrointestinal disorders (Salleh, 2008). In a 7 year follow-up study, the experience of life events has been associated with a two-fold increased CVD mortality rate in middle-aged men (Rosengren, Orthgomer, Wedel, & Wilhelmsen, 1993). A case-control study with over 20,000 participants from 52 countries corroborated the impact of prior stressful life events on acute myocardial infarction incidence (Rosengren et al., 2004). A review supported an association between severe life events and breast cancer development (Butow et al., 2000) and this was confirmed in another study with over 10,000 women that analysed the impact of separate life events 5 years preceding diagnosis of breast cancer (Lillberg et al., 2003). Divorce/separation, death of spouse and death of a close relative or friend were associated with a two-fold increased risk in breast cancer incidence compared to individuals without the experience of such major life events. Negative/ stressful life events will be assessed as part of a study on examination stress (Study III; Chapter 6).

1.1.7 Anxiety

Anxiety is a feeling of exaggerated worry, fear and agitation and can be a cause of generalised anxiety disorder (GAD), post-traumatic stress disorder (PTSD), panic disorder and phobias (Spielberger, 1972). Anxiety is often triggered by irrational thoughts but can also lead to maladaptive responses to anticipations and uncertainty (Newman, Llera, Erickson, Przeworski, & Castonguay, 2013). These states of chronic, unsubstantiated worry can create distress and restrict daily work and social life. Anxiety as a state characteristic can be a normal reaction to stress, however, it can be exaggerated in individuals who do not suffer from an anxiety disorder. Anxiety symptoms do not necessarily differ qualitatively from symptoms in clinical anxiety disorder, but rather the frequency, severity and duration of symptoms differentiate subclinical from clinical anxiety (Newman et al., 2013). Anxiety is associated with worse mental health overall. For instance, there is a high rate of comorbidity between GAD and substance use, mood disorders (e.g depression), and personality disorders (Grant et al., 2005). Risk factors for incidence of anxiety have been shown to range from personality characteristics such as neuroticism and behavioural inhibition to the experience of stressful life events and history of mental health (Moreno-Peral et al., 2014).

A body of evidence supports the relationship between anxiety and physical health outcomes, e.g. CVD (Tully, Cosh, & Baune, 2013), gastrointestinal health and musculoskeletal pain (Pacella, Hruska, & Delahanty, 2013), diabetes, arthritis and stroke (Tomaka, Thompson, & Palacios, 2006), measured cross-sectionally and also longitudinally (Smith, Fernengel, Holcroft, Gerald, et al., 1994). A systematic review

and meta-analysis in cancer patients report anxiety to be an even stronger risk factor for mortality than depression (Mitchell, Ferguson, Gill, Paul, & Symonds, 2013; Sokoreli, Vries, Pauws, & Steyerberg, 2016). Uncertainty about the disease prognosis might trigger more anxiety. Also, the fact that hypochondriasis, a phenomenon of often unsubstantiated exaggerated health anxiety without organic cause, has been linked with worse health, endorses the role that anxiety plays in the development of disease (Alberts, Hadjistavropoulos, Jones, & Sharpe, 2013).

1.1.8 Coping

As mentioned in section 1.1.1, the physiological stress responses do not simply depend on exposure to threatening or challenging conditions, but on psychological appraisals and coping. Successful dealing of stressors is through cognitive, emotional, behavioural and physiological mastering and thus restoring the initial homeostatic state. Psychological coping can be defined as volitional management of thoughts and behaviours assessing the internal and external demands of situations that are appraised as stressful. As already outlined, the transactional model of stress implies that responses arise when exposure to challenges exceed the psychological and social coping resources that the individual can bring to bear on the situation (Lazarus, 1966). Lazarus and Folkman's conceptual framework on stress categorised coping into problem-focused and emotion-focused coping (Lazarus & Folkman, 1987). A problem-focused approach is regarded as any form of taking direct action to change something in the situation and thus reduce the stress exposing stimulus, whilst an emotion-focused approach

is regarded as behaviours to deal with the emotional state itself, specifically, decreasing emotional distress by blaming, distraction, seeking emotional social support or wishful thinking.

Problem-focused, task- or action-oriented coping has been regarded as an adaptive coping strategy and emotion-oriented and avoidant-oriented coping as maladaptive coping styles. But these may not be generalisable or suitable for handling all types of stressful situations, as unchangeable situations are difficult to deal with using problem-focused coping strategies. Thus action-oriented approaches are considered adaptive when intervening with action is applied to situations that are indeed controllable and changeable; they are valuable by creating alternatives, acquiring new skills, actively seek support or alter external or internal aspects that one can control. Individual differences of perceptions, termed person antecedents of perceptions (such as personal beliefs, values, goals, self-esteem, motivation, available resources and social support) determine the cognitive elements and hence adaptive coping or maladjustment to the stressor rather than the event itself (Carver, 1997). For instance, individuals with higher self-esteem might be more likely to believe in the availability of resources, affecting the belief in their ability to cope successfully, and might hence appraise the stimulus as a challenge rather than threat. Yet, situational factors, for instance time duration, intensity, complexity and degree of controllability of the threat (termed environmental antecedents) correspondingly influence its nature and possible appraisal and coping judgements (Lazarus & Folkman, 1987).

Coping is an important factor influencing the enduring detrimental effects of stress (Baum & Posluszny, 1999). Meta-analytic reviews report avoidant coping styles such as denial, distancing or self-control and also emotion-focused coping being related to negative overall health (Aldwin & Park, 2004; Cheng, Lau, & Chan, 2014; Penley, Tomaka, & Wiebe, 2002). Survival in elderly and also in chronic conditions such as cancer, CVD and HIV have also been shown to be moderated by coping aspects and illness perceptions, controlling for important covariates such as initial health status, age and socioeconomic background (McIntosh & Rosselli, 2012; Struthers, Chipperfield, & Perry, 1993; Svensson et al., 2016; Watson, Homewood, & Haviland, 2012). Adaptive coping mechanisms are stress-reducing strategies with long-term health in mind rather than short-term well-being, gratification and pleasure (Everly & Lating, 2013). These are for example behavioural aspects of food consumption, relaxation, exercise or meditation rather than smoking, denial or interpersonal withdrawal. Interestingly, coping has been linked to health behaviours, with avoidant coping styles being associated with increased maladaptive health behaviours (Doron, Trouillet, Maneveau, Neveu, & Ninot, 2015). Coping has been shown to play a key role in moderating the impact stress can have on the individual. The concept of the stress-buffering effect of coping will be explored hand-in-hand with social support in the section below on social support as the stress-buffering hypothesis has been primarily developed from social support research (Cohen & Wills, 1985). Coping will be assessed in Study III (Chapter 6) in relation to examination stress.

1.1.9 Social support

Social support can be defined as the presence and provision of support by other individuals (Kaplan, 1987). Specifically, on the one side there is the structure or existence of support and on the other side the function of social support (the actuality of appropriate support as a resource). Based on conceptual analyses, social support can be fractioned into three main dimensions of support: emotional, instrumental and informational support (Langford, Bowsher, Maloney, & Lillis, 1997). Emotional support is the empathic psychological support by providing care, love and trust through communication, gestures and actions. Instrumental support is providing concrete tangible material or financial assistance. Informational support is providing support by listening, discussing and advising the problem at hand. Overall, these provide a sense of social involvement, affiliation and integration, belongingness and usefulness.

Studying the interrelationships between health and social environments have identified social support as a vital protective factor for health. In fact, social connection has been identified as a critical element of human need and a body of research indicates that social support is crucial for survival (Seppala, Rossomando, & Doty, 2013). Both biological and behavioural mechanisms have been suggested as the relevant pathways linking social support to physical and mental health (Reblin & Uchino, 2008). Perceived social support has often been found to independently minimise the impact of stressors on the underlying biological mechanisms in the cardiovascular, neuroendocrine, and immune system (Roy, Steptoe, & Kirschbaum, 1998; Uchino, 2006). Repair and physiological functioning, such as wound healing

have been shown to be affected by social support (Cacioppo & Hawkley, 2003). Social support is also a central predictor of clinical characteristics and outcomes of acutely hospitalized patients. For example, not only did higher levels of social support and integration in acute myocardial infarction patients predict better health status, less depressive symptoms and better quality of life one year after admission, but also a positive dose-response relationship for not being re-hospitalised (Bucholz et al., 2014; Rodriguez-Artalejo et al., 2006). Prosocial behaviours through enhanced compassion and adaptive health behaviours are possible behavioural pathways over which social support is often suggested to be linked to both mental and physical well-being (Seppala et al., 2013). Using 182 studies, a meta-analysis evaluated the effect of social support on 15 health outcome variables and found that social support predicted all of them including physical and mental symptomatology, coping, stress and also self-actualisation (Wang, Wu, & Liu, 2003).

There exist two different pathways through which social support is thought to result in better health outcomes (Cohen & Wills, 1985). The direct-effect model of social support suggests that social support directly and constantly positively affects mental and physical health, regardless of exposure to stress. Therefore, long-term low levels of social support can have cumulative adverse health effects over time. Conversely, the stress buffering model (or also called the stress-buffering hypothesis) has been proposed in which social support acts as a buffer for the adverse effects of stress and stressful events (Cohen & Wills, 1985; Uchino, Cacioppo, & Kiecolt-Glaser, 1996). People with high levels of social support do not

show the same associations between perceived stress and health than individuals with low levels of social support (Barrera, 1986). Evidence exists for both models (Hashimoto, Kurita, Haratani, Fujii, & Ishibashi, 1999; Hughes, 2007). For instance, Mezuk et al. (2010) reported results from a population sample of over 6,000 men and women that social support showed an independent effect on inflammation, but that there was also an interaction between stress exposure and social support in relation to inflammatory processes. Lower levels of social support was associated with higher levels of inflammation (i.e. C-reactive protein levels; CRP); however, social support also buffered the association between stress and higher CRP concentration. Similarly, in cancer patients, social support has been shown to be directly associated with better cancer-related physical functioning but also to buffer the adverse psychological outcomes such as depression and anxiety after diagnosis (Carpenter, Fowler, Maxwell, & Andersen, 2010). In this way, social support moderates the impact of stress on health through psychophysiological, psychological and also behavioural pathways.

In over 2,000 community-based sample of adults, cluster analyses revealed that low social support and maladaptive coping styles (avoidance coping and passive reactions, characterised by preoccupation about unchangeable problems) could be identified as risk clusters for increased levels of stress, anxiety and depression (Wijndaele et al., 2007). These interactions emphasise the protective role that social support and adaptive coping can have in psychopathology in adolescence. A different study showed the stress-buffering effects that high parental support, peer support and active coping style had on anxiety and

depressive symptoms in foreign Mexican students reporting high levels of acculturative stress (Crockett et al., 2007). Family, peer and teacher support and active coping has also been shown to moderate the stress-impact bullying and victimisation had in adolescents while distraction coping intensified these effects (Konishi & Hymel, 2009). Figure 1.2 depicts the proposed pathways through which social support and coping can modulate psychological and behavioural factors on the one hand and biological processes on the other hand, creating a stress-buffering effect. The biological pathways include endocrinological responses, which will be outlined in detail in the next section. Perceived social support will be assessed in Study I (Chapter 3) in relation to socioeconomic factors and work stress in a sample of working women.

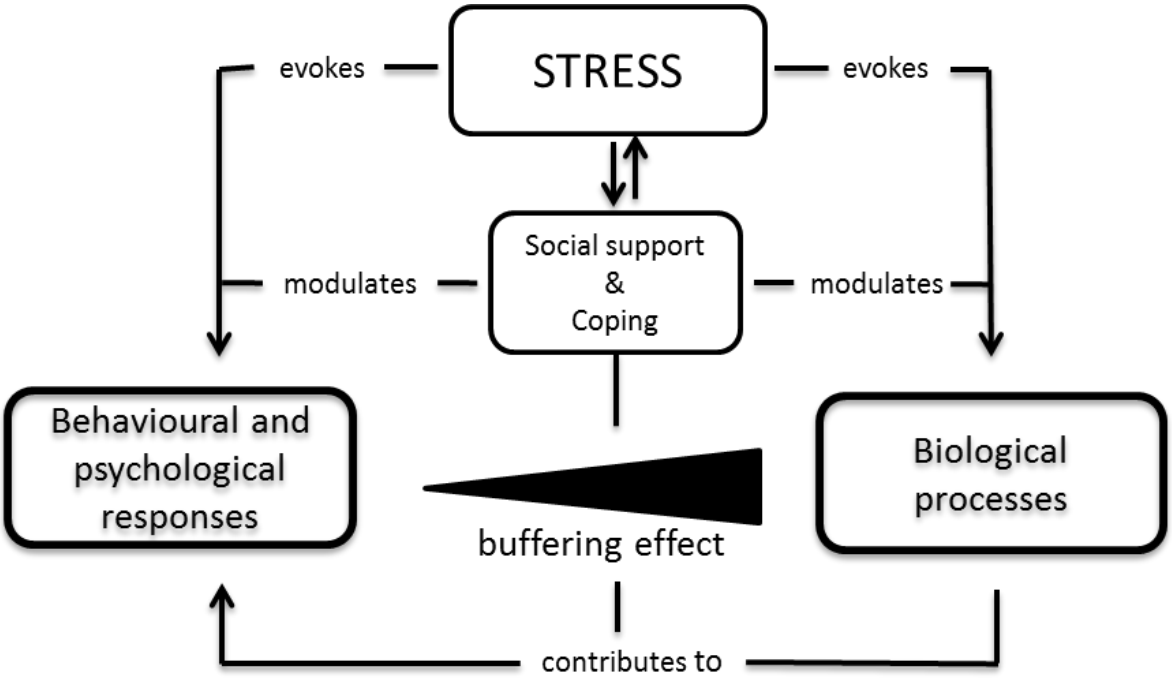

Figure 1.2 The stress-buffering hypotheses for social support and coping.

1.2 Cortisol

1.2.1 Cortisol metabolism

Cortisol is the predominant glucocorticoid hormone, ubiquitous and essential for life. It is produced by the zona fasciculata of the adrenal cortex and is released as a function of the HPA-axis. Figure 1.3 depicts the HPA-axis and the secretion of cortisol. The hypothalamus secretes corticotrophin-releasing hormone (CRH) into the hypophyseal portal system, blood vessels that connect the hypothalamus with the anterior pituitary, which in turn prompts the pituitary gland to release adreno-corticotrophic hormone (ACTH). Being carried to the adrenal cortex, ACTH subsequently stimulates the synthesis of cortisol and other glucocorticoids. Cortisol secretion is regulated by feedback inhibition of CRH and ACTH both at the hypothalamus and at the pituitary: when adequate levels are present, cortisol binds to receptors in the hypothalamus and anterior pituitary which consequently inhibit secretion of CRH and ACTH, resulting into reduced cortisol secretion.

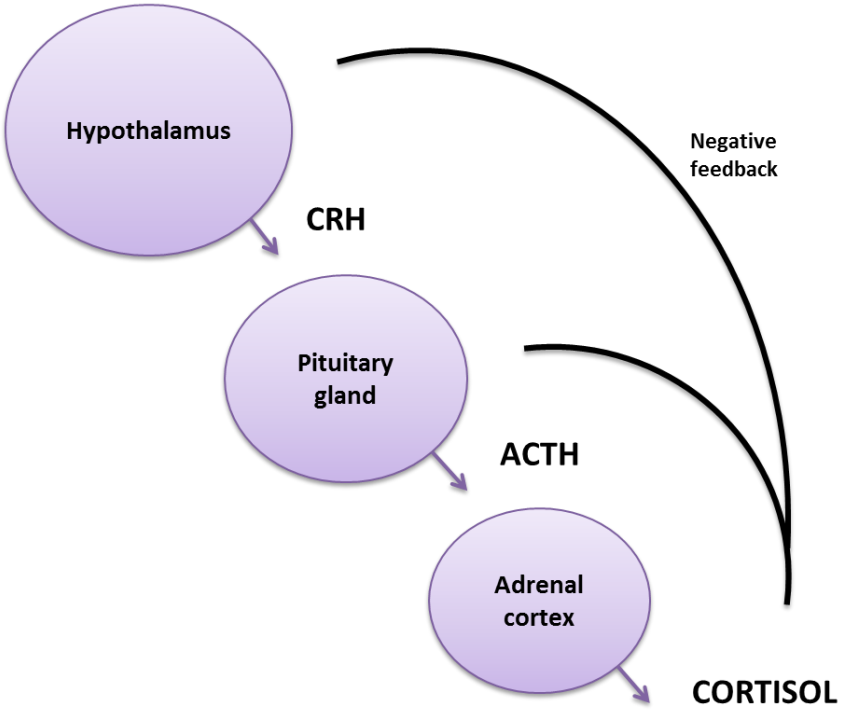

Figure 1.3 The HPA-axis, depicting the sequence of production of the precursor hormones of cortisol and the negative feedback loop.

1.2.2 Cortisol function

Cortisol is a stress responsive hormone, yet it follows a typical diurnal pattern with vital regulatory bodily functions. This pronounced circadian rhythm shows fluctuations during both day and night. Cortisol peaks within the first 30 minutes after awakening and its nadir is around midnight (Pruessner et al., 1997). The abundant presence of corticoid receptors within nearly every cell of our body explains its multifaceted actions. Being the end product of the HPA-axis, it presents a vital connecting actor between the central nervous system and the endocrine system. Cortisol affects multiple bodily systems; its main functions include the maintenance of homeostasis, the mobilisation of micronutrients for energy production and regulation of glucose levels, immune and inflammatory responses,

activation of the central nervous system, vascular reactivity by modifying catecholamine sensitivity as well as modulation of the growth and reproductive axes (Chrousos, 1996; Clow, Hucklebridge, Stalder, Evans, & Thorn, 2010; Fardet, Kassar, Cabane, & Flahault, 2007; Sapolsky, Romero, & Munck, 2000; Yang & Zhang, 2004). Findings also implicate glucocorticoid action in the maintenance of many brain functions including working memory and also emotion-cognition processes, such as emotional appraisal of an event (Herbert et al., 2006; Mizoguchi, Ishige, Takeda, Aburada, & Tabira, 2004).

The multidimensional characteristics of cortisol become apparent in conditions with dysregulations of the HPA-axis (hypo- or hypercortisolism) and after extended use of exogenous steroids. Excessive cortisol levels produce a plethora of detrimental 'side-effects' involving the various above mentioned bodily systems and functions; findings that will be presented throughout the next sections. Cortisol, as well as all other glucocorticoids, can be categorised as having either a permissive or a regulatory effect, either supporting (permissive) or limiting (regulatory) defence mechanisms for action (Sapolsky et al., 2000). Specifically, the permissive action of cortisol is to enable other hormones to exert their full functions, a process vital for homeostasis maintenance (Johnson et al., 1992). Its contradictory action, as a regulator, arises in response to stress, and is essential in preventing an overreaction of the stress-related functional systems. An example of this would be cortisol's immunosuppressive effect preventing the immune system from being overactive.

Alongside the rhythmic fluctuation of cortisol biosynthesis, cortisol secretion is influenced by external and psychological factors. Specifically, following a psychological or physiological stressor, cortisol secretion increases above its basal levels to facilitate the fight-or-flight response (Cannon, 1914). This stimulated activity is vital for survival for several reasons. At the expense of other important, mainly anabolic, processes that are not necessary for immediate survival, cortisol induces certain bodily, mainly catabolic, processes, along with the other more rapidly acting stress hormones, adrenaline and noradrenaline. Cortisol induces a temporal rise in glucose production for muscle energy, by inhibiting insulin, whose normal function is to regulate the storage of glucose in the liver. Cortisol also narrows the arteries to increase heart rate and blood pressure, and output of the heart. These adjustments to the cardiovascular system bring about the immediate bodily stress reactions an individual feels when facing a physical or psychological stressor. Negative feedback regulation ensures these stress-induced biochemical and hormonal imbalances return to levels within the suitable physiological range.

However, there are factors and circumstances under which this reduction or recovery of imbalance does not effectively occur, resulting in excessive or prolonged circulating cortisol levels, which can be detrimental to several bodily functions. The acknowledgement of the paradox of the stress-induced physiological activation system, with its protective and restoring function, but with its simultaneously damaging effect if prolonged, originated from Selye (Selye, 1973b) with the General Adaptation Syndrome model (outlined in section 1.1.1). Hence, both the resting diurnal cortisol profile with its distinct parameters and the

magnitude and recovery rate of the stress response have implications for health, and dysregulations of any of these systems can lead to illness and disease (McEwen, 2000b).

1.2.3 Circadian activity and the different salivary cortisol parameters

The sampling methods for cortisol, their development and advantages and disadvantages will be outlined and analysed in detail in Chapter 2. Briefly, blood and also urine have been used as the first specimens to assess cortisol in the early 1950s (Ayres, Garrod, Simpson, & Tait, 1957; Braunsberg & James, 1961). Subsequent detection of cortisol from saliva specimens in the 1980s suggested this method of sampling to have several methodological and practical advantages over cortisol assessment from blood and urine (Kirschbaum & Hellhammer, 1989; Vining, Mcginley, Maksvytis, & Ho, 1983). However, all of these hormonal measures can be influenced by the analytical method, experimental, situational and random variables and by many other factors, such as gender, age, body mass index (BMI), pregnancy, lifestyle and psychosocial factors (Aardal & Holm, 1995).

A circadian rhythm is an approximate 24-hour cycle of physiological changes within an endogenous network of gene clocks, controlled and synchronized by the suprachiasmatic nucleus with its sensitivity to light (Spiga, Walker, Terry, & Lightman, 2014). Research has been mixed regarding the first appearance of a cortisol circadian rhythm in humans, with findings ranging from 2 weeks to 9 months of age. Interestingly, there is evidence to suggest that the infants' underlying physiological and behavioural characteristics determine onset and

stability of the cortisol circadian rhythm (de Weerth, Zijl, & Buitelaar, 2003). The circadian rhythm of the HPA-axis has been identified using intensive blood samplings over a 24-hour period; Figure 1.4 shows a representative cortisol diurnal profile obtained from plasma.

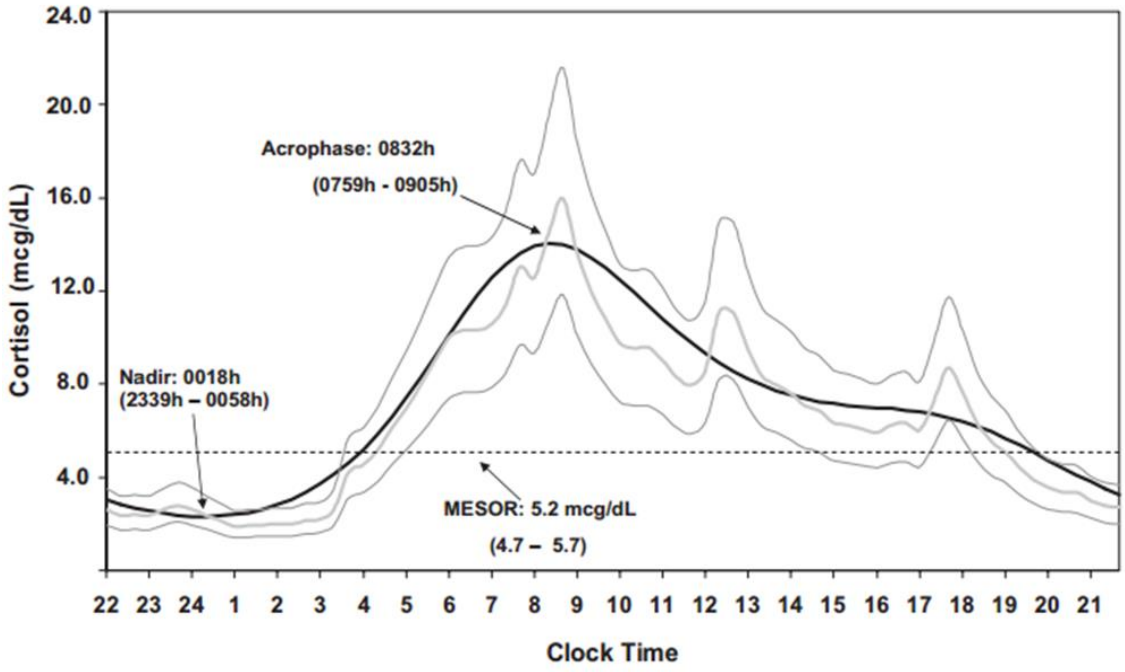

Figure 1.4 Typical cortisol diurnal profile obtained from 20 min blood sampling over a 24-hour period showing the averaged profile (indicated by the black thick line; grey lines are raw mean values ± 2 SD; from Debono et al., 2009).

The reference range of timed cortisol samples has been determined in an attempt to identify hypo- and hypercortisolism. For example, for salivary cortisol the 8am value ranges between 4.0 – 28.0 nmol/l and the 11pm value is <5.0 nmol/l. The cortisol increase after awakening is typically 9 nmol/l with a range of 4–15 nmol/l in healthy individuals, which reflects an increase of 50-160% in the first 30-45 minutes post-awakening (Clow, Thorn, Evans, & Hucklebridge, 2004). Sleep-laboratory studies using polysomnographical recordings, have suggested that this

morning cortisol rise acts as a distinct feature of the HPA-axis, supporting the notion that it is a response to the sleep-wake transition itself rather than part of the rhythmic circadian cortisol secretion (Wilhelm, Born, Kudielka, Schlotz, & Wust, 2007). Its physiological role has also been suggested to be involved in the activation of the metabolic and immunological systems in the sleep-wake transition (Petrovsky & Harrison, 1997). It is believed that heritability of the cortisol awakening response is around 40%, distinctive of the heritability of diurnal cortisol levels (Wust, Federenko, Hellhammer, & Kirschbaum, 2000). After this initial increase, as the day progresses, cortisol levels decline steadily until reaching its lowest point in the late evening/night hours. Studies assessing pituitary-adrenal dynamics have identified pulsatile release of ACTH of 15 to 18 pulses over a 24-hour cycle, with each secretary burst inducing a rise of approximately 2.5 nmol/l in cortisol. It is advocated (and will be elaborated on in section 1.3) that the magnitude of cortisol secretory episodes rather than pulse frequency play a bigger role in determining total cortisol output and its effects on psychological and physiological health (Faghih, Dahleh, Adler, Klerman, & Brown, 2015; Kalsbeek et al., 2012; Linkowski et al., 1985).

This dynamic nature of cortisol secretion requires several samplings per day to obtain a reliable indicator of the profile. From the typical profile three important features are of importance as measures of the diurnal circadian cortisol rhythm. Figure 1.5 depicts the derivation of the main parameters graphically (for illustration purpose four sampling points are shown: at awakening, 30 minutes post-waking, an afternoon sample and a bedtime sample). As there is variation among researchers

about how these parameters are calculated, only the basic concepts are displayed. Firstly, is the increase shortly after awakening, as indicated by the acrophase. Secondly, following this peak there is a decline in cortisol throughout the rest of the day which may vary in rate. Together these two features form the third: the area underneath, which indicates cumulative cortisol output. Studies using cortisol analyses have predominantly used these three parameters to indicate markers of the profile, and hence physiologic indicators of HPA responsiveness, namely the cortisol awakening response (CAR; red), the area-under-the-curve (AUC; purple) and the cortisol diurnal slope (blue). An important methodological focus of this PhD utilises these three parameters (Study I and II as in Chapter 4; Study III as in Chapter 5 and 6). For an understanding on how these are differently and/ or similarly associated with particular psychological and physiological health outcomes, their meaning and derivation will be briefly explained.

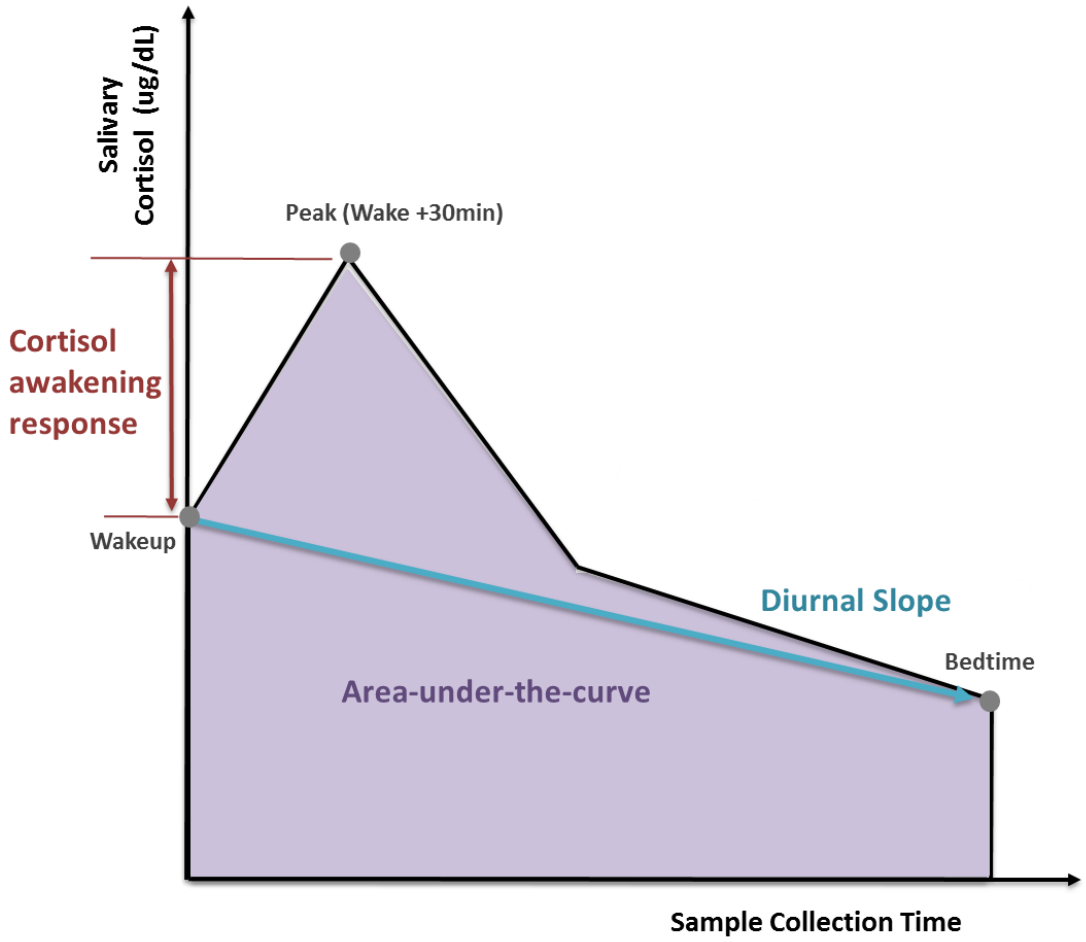

Figure 1.5 The derivation of the different salivary cortisol parameters.

i) CAR. Different calculations for the CAR exist. The simplest is the difference between the awakening and the 30 minutes post-waking value, a calculation that has been mostly used in CAR research. A different calculation is the assessment of the area-under-the-curve of the CAR, although rarely used by researchers. Some studies have assessed the CAR in more detail by several measurements in smaller intervals, i.e. 0, 15, 30 and 45 minutes post-waking (as outlined in Edwards, Evans, Hucklebridge, & Clow, 2001). The awakening value and the 30 minutes post-waking value have also been used in isolation as potential indicators of psychological and physiological outcomes.

ii) AUC. The AUC assesses the amount of cortisol that is secreted throughout the day and can be calculated in several ways. For example, several adjusted AUC measures exist, each with a different reference point (e.g. with or without the awakening value and / or the CAR), resulting in AUC of awakening to bedtime, AUC of 30 min post-waking to bedtime, an early decline AUC of 30 min post-waking point value to a certain set point and a late decline AUC of certain set point to bedtime. Preussner and colleagues (2003) proposed a formula for computing the AUC by breaking down the total area into smaller trapezoids and adding those respective areas to obtain the whole AUC value.

iii) The slope. The slope is the general decline of cortisol throughout the day. The cortisol slope is defined by cortisol values either from waking until a certain afternoon or bedtime sampling time. Generally a less positive value indicates a slower rate of decline and is termed a flatter cortisol slope.

There are several factors that might affect circadian activity. Sex differences in the incidence of certain stress-related disorders and sex-disease dimorphisms imply underlying HPA-axis functioning differs among men and women (Karlamangla, Friedman, Seeman, Stawksi, & Almeida, 2013; Kudielka & Kirschbaum, 2005). Another factor thought to be relevant to cortisol secretion is age. Although with increasing age, the circadian rhythmicity of cortisol is maintained, amplitude in cortisol secretion changes.

1.3 Evidence linking cortisol with physical and mental health outcomes

There are several mechanisms by which elevated cortisol might affect health. Cortisol has widespread regulatory functions in varying bodily systems, majorly affecting the immune system, the digestive and the cardiovascular system (Chrousos, 1998). In the following sections, the literature on the link between cortisol and various health outcomes will be presented with a particular focus on the role of stress and psychosocial factors in these cortisol-driven health risks.

1.3.1 Cortisol and immune functioning

As already outlined above, cortisol contains bidirectional regulatory capacities - it can have a permissive effect but can also suppress immunological processes. Studies using gene profiling reveal that cortisol up-regulates and down-regulates the gene expression of cells involved in the innate immune response depending on its necessity of action (Galon et al., 2002). Hence, cortisol is one of the major immune-modulatory agents. Adequate levels stimulate the body's immune response and accelerate the healing process; cortisol also switches off immune action when this repair stage is reached. However, aberrant cortisol levels have been associated with several immune-related diseases and a disturbance in the interplay between the endocrine and the immune system. The interaction between these two systems is coordinated by cortisol and other glucocorticoids and cytokines, a group of proteins important for cell signalling. The innate immune response, which is the early stage of the immune process, involves proinflammatory cytokines, such as tumor necrosis factor–alpha (TNF-α), interleukin (IL)-1 and 6 and

interferon alpha. The subsequent adaptive immune response entails T cell cytokines such as IL-2 and interferon gamma. These cytokines activate the HPA-axis and cortisol release, leading to a negative feedback mechanism and cortisol's immunosuppressive effect as cytokine synthesis is subsequently reduced by the release of cortisol (Silverman, Pearce, Biron, & Miller, 2005). Cortisol is therefore especially important for the shift from inflammatory to anti-inflammatory immune responses (Chrousos, 2000).

The detrimental effects of excessive cortisol upon the immune process were first observed by administration of excessive, pharmacologic dosages of glucocorticoids (Jefferies, 1991). Glucocorticoid treatment such as prednisone, dexamethasone, and hydrocortisone are used to treat autoimmune diseases, allergies and asthma. Whilst one might argue that the effects of synthetic cortisol might differ in strength and effect from the endogenously produced cortisol, correlational analyses reveal a strong relationship between excessive cortisol in glucocorticoid-treated patients and increased levels of various immunomarkers. A disease called Cushing's syndrome is a condition characterised by several symptoms, such as weight gain, facial plethora, high blood pressure, glucose intolerance and weakness, and inflammatory disturbances (Hellman, Weitzman, Roffwarg, Fukushima, & Yoshida, 1970). This condition is most commonly caused by adenoma, a tumour or overgrowth of the pituitary gland, or by supraphysiological amounts of exogenous glucocorticoid treatment (Newell-Price, Bertagna, Grossman, & Nieman, 2006). The sustained excessive exposure to glucocorticoids that occurs in patients with Cushing's syndrome leads to inflammatory disturbances

that might explain some of the comorbidities associated with the disease (Anagnostis, Athyros, Tziomalos, Karagiannis, & Mikhailidis, 2009; Etxabe & Vazquez, 1994). Conversely, Addison's disease, a state of chronic adrenal insufficiency, leads to extremely low levels of circulating cortisol. It is characterised by extreme weakness, weight loss, pain, lymphoid tissue hypertrophy and hypoglycaemia (Vegiopoulos & Herzig, 2007). Most research links elevated cortisol secretion with worse health outcomes but both elevated and reduced cortisol levels are reported to pose a threat to health (McEwen, 2007). Although there is divergent evidence in this respect, hypocortisolism appear to be a consequence of prolonged elevated cortisol levels and an exhausted overactive HPA-axis, resulting in a down-regulation of HPA-axis activity (Hek et al., 2013). Research has suggested that chronic stress and the consequential prolonged exposure to elevated cortisol levels is related to glucocorticoid receptor resistance (insufficient glucocorticoid signalling), a result of decreased hormone bioavailability or reduced hormone sensitivity. This resistance interferes with down-regulation of inflammatory response, ultimately playing a vital role in onset and progression of chronic diseases (Cohen et al., 2012).

A body of research demonstrates a link between basal cortisol activity and various inflammatory markers. In a large population based sample, a lower CAR, a flatter diurnal decline and higher AUC were associated with higher levels of IL-6, and a flatter slope was associated with TNF-α and IL-10 (DeSantis et al., 2012; Rook, 1999). Studies also show that controlled laboratory-based stress tasks can induce transient inflammatory responses, endorsing the role played by

psychoneuroendocrinological processes in the immune response and its responsiveness to stress. A review by Steptoe and colleagues (2007) presents robust findings of increased levels of circulating IL-6 and IL-1β following acute mental stress tasks, which are moderated by individual differences in vulnerability to stress responsiveness. Not only acute, but also chronic stress, such as that faced by spousal caregivers, has been associated with sustained overproduction of inflammatory markers, even after controlling for the effects of health behaviours, health status and medications (Kiecolt-Glaser et al., 2003). These studies demonstrate that stress-induced HPA-axis activity contributes to alterations in the immune system. These endocrine-immune interactions in turn might accelerate disease processes and result in pathophysiological consequences. Whilst a body of research points towards dysregulated cortisol secretion as the roots for an impaired regulation of inflammatory activity, the relationship between cortisol and inflammation is complex and not fully understood (Cohen et al., 2012).

1.3.2 Cortisol and cardiovascular activity

CVD is a collective term for types of diseases that affect the heart or the blood vessels. It includes coronary heart disease, angina, heart attacks/ myocardial infarctions and stroke. The strongest modifiable risk factors for CVD range from high blood pressure and high levels of cholesterol, diabetes and obesity to smoking and sedentary behaviour (Yusuf, Reddy, Ounpuu, & Anand, 2001). Cortisol parameters have been independently linked to severity of CVD and an increased risk of CVD mortality in a prospective cohort study with over 4,000 civil servants

followed up for a period of 8 years (Kumari, Shipley, Stafford, & Kivimaki, 2011). Other cardiovascular risk factors influenced by cortisol such as decreased heart rate variability, unfavourable blood profiles and obesity, have all been suggested as the mediating pathways of cortisol's association with CVD (Fraser et al., 1999; Thayer & Fischer, 2009; Walker, Soderberg, Lindahl, & Olsson, 2000). Specifically, hypertension and elevations of cholesterol and triglycerides have been associated with higher AUC (Whitworth, Williamson, Mangos, & Kelly, 2005). Also, increased cortisol reactivity to laboratory-based stress tasks were linked to incident hypertension over a 3 year follow-up period in 479 initially healthy middle-aged adults (Hamer & Steptoe, 2012).

Atherosclerosis, a precursor to coronary heart disease, refers to the hardening and narrowing of the coronary arteries caused by a build-up of plaque. Atherosclerosis constitutes a common cause of heart attacks and strokes. A body of research has supported associations between the different cortisol parameters and clinical indicators of atherosclerosis. For example, a greater AUC was positively associated with the number of plaques in the carotid arteries using diagnostic ultrasonography scans (Dekker et al., 2008). Assessing the carotid artery intima-media thickness (the innermost layers of the artery walls) with carotid artery ultrasound or radiographic scans is another imaging method to detect the presence, progression and regression of atherosclerosis (Stein et al., 2008). A greater thickness (superior to 1.0 mm) serves as a surrogate pre-clinical marker of atherosclerosis and risk for future cardiovascular events. A higher intima-media thickness of the artery was found to be associated with several aberrant indices of

cortisol, for example a higher CAR in middle-aged women (Eller, Netterstrom, & Hansen, 2001) and a flatter cortisol slope in overweight children (Toledo-Corral et al., 2013). Carotid artery intima-medial thickness was also found to be positively associated with workplace demands (Everson et al., 1997) and also in another study with work stress and the CAR, the AUC and the slope, suggesting that psychosocial and stress factors might drive the associations the relationship between cortisol and intima-media thickness (Thole, 2014).

Another study found morning serum cortisol to be independently associated with proinflammatory and prothrombotic activity (referring to the promotion of thrombosis or clotting of the blood) in 285 middle-aged female coronary artery disease patients, suggesting that abnormality of blood coagulation might be driven in part by elevated cortisol secretion (von Kanel, Mausbach, Kudielka, & Orth-Gomer, 2008). Endothelial function is a critical determinant of atherosclerosis, a disease characterised by a thickening of the artery-walls with fatty substances/ plaques. Many risk factors for atherosclerosis, such as diabetes, hypercholesterolemia and hypertension, are linked to endothelial dysfunction (the pathological state of the endothelium); risk factors that in turn are partly induced by a dysregulated HPA-axis (Tabrizchi, 2005). Quantitation of calcium deposits in the coronary arteries is another indicator of CVD risk. Coronary calcium sores can be obtained from electron-beam, helical or spiral computerised tomography scans and increased levels of calcium point towards a higher degree of diseased arteries. A flatter diurnal cortisol decline was related to higher levels of coronary calcification in over 700 middle-aged adults, even after adjusting for many

covariates including gender, age, ethnicity, socioeconomic status, smoking, diabetic treatment, blood pressure and triglycerides (Matthews, Schwartz, Cohen, & Seeman, 2006). This study underlines the pivotal role cortisol plays in the atherosclerotic disease process. Furthermore, excessive cortisol reactivity to laboratory-induced stress was found to be related to progression of coronary calcification, implicating mental stress as a contributing risk factor for heart disease by stimulating direct damage of the arteries (Hamer et al., 2012).

Additional evidence for a bidirectional relationship between CVD and psychological factors (such as [comorbid] depression) that are linked to cortisol themselves, strongly endorses the role that psychoneuroendocrinological processes play in the development and progression of CVD. CVD patients with post-diagnostic depressive symptoms are associated with aggravated adverse cardiac outcomes via both physiological (e.g. inflammatory processes, neurohormonal dysfunction) and behavioural pathways (health-behaviours and lack of social interaction), leading to recurrent cardiac events and poor survival. In fact, comorbid depression is highly prevalent in patients with CVD (up to 40%), contributing to the development and progression of the disease (Celano & Huffman, 2011). Depression has also been identified as an independent risk factor for cardiovascular pathology and its complications (Huffman, Celano, Beach, Motiwala, & Januzzi, 2013). Research documents that depressed CVD patients, who respond to laboratory stressors with HPA-axis hypoactivity, also show greater inflammatory processes, i.e. higher CRP, IL-6 levels (Nikkheslat et al., 2015). Moreover, a flatter cortisol slope has been linked to depression in CVD patients, supporting the role of stress or mood and the

consequential impairment in diurnal patterns of cortisol in the pathogenesis and progression of CVD (Bhattacharyya, Molloy, & Steptoe, 2008).

A meta-analysis reported work-related stress to be associated with a 50% excess risk of CHD (Kivimaki & Kawachi, 2015). Occupational burn-out, a condition marked by prolonged exposure to work-related emotional and interpersonal stressors resulting in exhaustive fatigue, work- and people-related cynicism and feelings of inefficacy and worthlessness, has been linked with increased CVD risk (Melamed, Shirom, Toker, Berliner, & Shapira, 2006). Numerous evidence suggests HPA-axis and SNS-PNS dysregulations, systemic inflammation and weakened immune function, the metabolic syndrome, and poor health behaviours, to be potential mechanisms of this association (Melamed et al., 2006). More specifically, in a study with 6,576 healthy middle-aged adults designed to evaluate the effect of behavioural and pathophysiological factors in the relationship between stress and CVD, Hamer and colleagues (2008) demonstrated the contribution that lifestyle behaviours have in the relationship between stress and CVD risk. Compared to the less stressed people, people who were more stressed were at a higher relative risk of CVD. Health behaviours might to some extent explain this association, suggesting that stress encourages unhealthy coping strategies and habits, such as smoking, unhealthy eating and physical inactivity.

Most evidence on the association between cortisol and CVD risk is based on observational studies and therefore does not prove a causal connection. While longitudinal studies might be particularly informative for exploring risk factors in

relation to development of CVD, the inherent limitations impede valid assessment of cause-and-effect relationships.

1.3.3 Cortisol and metabolic dysfunction

Gastrointestinal disturbances are a further result of chronic hypercortisolism. Evidence linking the HPA-axis system with energy homeostasis comes from positive correlations between cortisol and leptin, insulin and glucose levels (Huybrechts et al., 2014). In a blind randomised controlled trial with 20 males, exogenous administration of cortisol compared to a placebo resulted in increased food consumption, energy expenditure and weight gain, endorsing the role that cortisol has on directly and indirectly acting on the regulatory mechanisms of appetite (Tataranni et al., 1996). This direct action can be partly explained by the fact that cortisol secretion positively correlates with secretion of the appetite hormone, ghrelin (Garin, Burns, Kaul, & Cappola, 2013). Cortisol has been shown to contribute to the development of the metabolic syndrome, a combination of high blood pressure, diabetes and central obesity (Anagnostis et al., 2009; Steptoe, Kunz-Ebrecht, Brydon, & Wardle, 2004). Specifically, it has been proposed that cortisol might play a key part in redistributing stored energy into abdominal fat depots, which might explain its strong association with central adiposity (Dallman et al., 2004).

Observational studies suggest that cortisol patterns play an important role in the aetiology of type 2 diabetes. A study with 3270 participants followed up for a period of 10 years indicated that evening cortisol levels predicted new onset of

diabetes and that a flatter diurnal cortisol pattern was associated with future impaired fasting glucose metabolism (Hackett, Kivimaki, Kumari, & Steptoe, 2016). Diabetes itself is considered as an independent risk factor for several diseases such as cardio- and cerebrovascular diseases, and enhanced HPA-axis activity has been shown to predict the presence and number of chronic diabetes complications (such as oral hypoglycaemic agent use, neuropathy, macroangiopathy, nephropathy). Such evidence highlights cortisol's role not only in the progression of diabetes but also in crucial pathophysiological consequences of the disease (Chiodini, Adda, et al., 2007).

1.3.4 Cortisol and brain and cognitive function

Cortisol's function on the brain's structure and connectivity can be observed in studies examining the effects of excessive cortisol levels and the subsequent brain atrophy and loss of functionality. For example, hippocampal damage is a consequence of raised cortisol levels which eventually leads to memory loss and declining cognitive functioning (de Kloet, Joels, & Holsboer, 2005; Lupien et al., 1998). Interestingly, there seems to be a dose-response relationship between cortisol levels and the degree of hippocampal atrophy. Several studies support the notion that cortisol is the key contributing factor to hippocampal atrophy in several neuropsychiatric disorders, for example traumatically stressed war veterans (Gurvits et al., 1996; Sapolsky et al., 2000). Studies evaluating the impact of traumatic experiences which lead to excessive cortisol secretion show that all the brain areas (hippocampus, amygdala and the prefrontal cortex) that are implicated

in the stress response become affected (Wu & Zhao, 2004). A systematic review concluded that cortisol-mediated neurotoxicity and the resulting different cognitive disorders depends on stress type, timing and duration of exposure and on the period of lifespan of the individual (Lupien, McEwen, Gunnar, & Heim, 2009). Specifically, as brain formation is occurring in distinct phases across the lifespan, stress and traumatic experiences impact cortisol-induced impairments in cognitive function differently depending on the level of development in the lifespan period, i.e. prenatal, postnatal, childhood, puberty, adolescence, early adulthood, adulthood, aging (Glover, 2014; Lupien et al., 2009). Ultimately, cognitive decline is a typical ageing process, however, higher cortisol levels have been found to be related to faster ageing-related cognitive function and decline in the healthy elderly (MacLullich et al., 2005). In a follow-up study with over 500 healthy high-functioning elderly persons, high levels of urinary cortisol predicted incidence of cognitive disorders, i.e. dementia, and level of impairment over a period of 7-years (Karlamangla, Singer, Chodosh, McEwen, & Seeman, 2005).

Yet, the causal link between elevated cortisol levels, cognitive deficits and hippocampal atrophy is not fully established. Genetically sensitive studies suggest that a pre-existing condition (as indicated by a smaller hippocampal volume) might provide a risk factor for the development of pathological stress responses rather than presenting merely a consequence of stress exposure (Gilbertson et al., 2002). While a body of evidence suggest that early life experiences (such as childhood violence victimization) are linked to later brain functioning impairment, more recent findings do not support the causal nature of this relationship (Danese et al.,

2017). Using two large cohort studies and controlling for genetic and environmental factors, the authors found that the cognitive deficits observed in victimized individuals were largely explained by cognitive deficits that were present prior to childhood trauma, suggesting a noncausal association between childhood trauma and later cognititive impairment.

1.3.5 Cortisol and age-related processes

The effect of age on the circadian rhythm of cortisol has long been an interesting field of research and studies have repeatedly found age-related impairments to tissue responsiveness and regulation of cortisol secretion (Ferrari et al., 2001). Whilst age is often considered a moderating variable in the relation between cortisol and health, age-related differences in these associations highlight the importance of understanding the role that cortisol plays in premature ageing. The ageing process is complex but research has demonstrated tangible dynamic changes that occur in the interplay between the immune, endocrine and nervous systems. While the earliest findings focused mostly at the intracellular level, such as cellular oxidation and mutations, extracellular functioning has received much more attention in recent years due to greater recognition of the dynamic interplay between the different systems. The different secretory mechanisms at cellular and receptor level (such as the precursor hormone of ACTH, pro-opiomelanocortin, and the action of mineralocorticoid and glucocorticoid receptors) contribute to an adjustment in the whole somatic network of an individual, thus affecting the different immunological and neuroendocrinological pathways (Gupta & Morley,

2014; Straub, Cutolo, Zietz, & Scholmerich, 2001). Mixed findings have been reported regarding the changes in HPA-axis activity across the adult lifespan; some studies have reported an elevated CAR, an increased AUC and a flattened diurnal amplitude and cortisol decline with older age (Deuschle et al., 1997; Nater, Hoppmann, & Scott, 2013), but not others (Wust, Wolf, et al., 2000). It has been argued that differences in cortisol levels during the lifespan relate to an age-associated decline in neuroendocrine function that contributes to pathologic conditions that typically accompany the ageing process (Sapolsky, 1999). These seem to result from impaired HPA feedback and reduced glucocorticoid receptor sensitivity.

1.3.6 Cortisol and other illnesses

Circadian disruption has been repeatedly observed in cancer patients. Disrupted feedback inhibition resulting in flatter diurnal slopes has been associated with accelerated cancer progression (Spiegel, Giese-Davis, Taylor, & Kraemer, 2006) and with disease severity, symptoms and complications in cancer (Abercrombie et al., 2004; Bower et al., 2005). Further evidence indicates the possible modulatory effect of psychosocial factors and stress on the link between circadian disruption and cancer prognosis (Sephton, Sapolsky, Kraemer, & Spiegel, 2000). In female newly diagnosed breast cancer patients, increased levels of depressive symptoms and marital dissatisfaction was associated with an increased AUC, and higher levels of neuroticism with an increased CAR (Vedhara, Tuinstra, Miles, Sanderman, & Ranchor, 2006). Higher levels of social support have also been associated with

lower AUCs in over 100 metastatic breast cancer patients, supporting the protective or buffering role as outlined previously (Turner-Cobb, Sephton, Koopman, Blake-Mortimer, & Spiegel, 2000). A meta-analysis of 20 randomised-control trials evaluated for methodological quality using the Cochrane's risk of bias supports the beneficial effect that psychosocial interventions tailored to reduce emotional distress in cancer patients have on reducing cortisol concentration and also immune parameters such as lymphocyte counts (Oh & Jang, 2014).

Rheumatic conditions, such as fibromyalgia and rheumatoid arthritis, are characterised by muscular or musculoskeletal pain involving excessive inflammation. The HPA-axis with its effect on immunological processes was shown to affect rheumatic disease outcomes, endorsing the psychoneuroendocrinological pathway's involvement in its aetiology and progression (Huyser & Parker, 1998). The cortisol profile is often aberrant in rheumatoid patients, with some studies showing elevated CARs (Straub, Paimela, Peltomaa, Scholmerich, & Leirisalo-Repo, 2002) and others normal CARs but elevated diurnal levels (Catley, Kaell, Kirschbaum, & Stone, 2000). Apart from methodological challenges, such as varying sampling methods, adherence and confounding lifestyle factors, these inconsistencies have been suggested to stem from variations in disease status and duration, treatment, medical history and also general stress (Jessop & Harbuz, 2005). Findings suggest that psychological factors precipitate or exacerbate rheumatic diseases, for example some studies have demonstrated an association between stress and the onset of fibromyalgia (Kivimaki et al., 2004). Furthermore, chronic stress attributable to the condition itself might also account for cortisol

elevation, as muscular or musculoskeletal pain prevent daily activities and are often accompanied by increased levels of stress (Herrmann, Scholmerich, & Straub, 2000). Hence, inconsistencies in findings might result from studies that were in fact masked by stress as a confounding factor.

Cortisol is also known to affect bone decalcification which in turn results in an increased risk for osteoporosis (Shaker & Lukert, 2005). Elevated cortisol levels have been linked to a decreased synthesis of important skeletal growth factors, which might partially explain the inhibitory influence cortisol has on bone formation (Mccarthy, Centrella, & Canalis, 1990). Unfavourable cortisol profiles have been found to contribute to frailty and reduced physical capacities in older adults (Johar et al., 2014). Osteoporosis is a common complication of Cushing's disease (often termed secondary osteoporosis), further supporting cortisol as a major contributing factor in this disease process (Khanine et al., 2000). Osteoporotic fractures have even been proposed to be manifestations of otherwise asymptomatic elevated cortisol levels. Studies report hypercortisolism in asymptomatic patients referred for osteoporosis; treatment aimed at normalisation of cortisol levels have shown to permit bone regrowth and remineralisation (Chiodini, Mascia, et al., 2007; Nieman, 2007).

Atopic disorders are allergic hypersensitivity reactions towards certain allergens and irritants and include asthma, hay fever and inflammatory skin disorders such as atopic dermatitis/ eczema, psoriasis and urticaria. Allergic disorders have been strongly associated with adrenal suppression, i.e. attenuated cortisol responses to laboratory-based stressor and lower basal cortisol

concentration (Priftis, Papadimitriou, Nicolaidou, & Chrousos, 2008). Further, research suggests that restoration of endogenous cortisol levels improve disease activity and severity and symptomatology in atopic dermatitis patients (Haeck et al., 2007). Inflammation (and activation of cytokines, chemokines and neuropeptides) has been suggested as the principal mediating pathway between stress and exacerbations of atopy. A hyporesponsive HPA-axis leads to more inflammation and stronger asthmatic symptoms, endorsing the role that stress and cortisol plays in development and exacerbations of atopic disorders. In fact, in a study of over 500 children, exposure to maternal distress (such as depressive or anxiety disorder as per physician diagnosis) predicted lower cortisol levels in asthmatic children, controlling for age and birth weight (Dreger, Kozyrskyj, HayGlass, Becker, & MacNeil, 2010). Literature reviews suggest that stress and acute negative life events are associated with a two-fold increased risk of asthma onset and attacks (Chen & Miller, 2007; Wright, Cohen, & Cohen, 2005). Allergic sensitisation and skin disorders influence the patient's emotional and psychosocial well-being (Langley, Krueger, & Griffiths, 2005) and hence psychological stress can impair quality of life on the one hand but can also exacerbate the condition on the other hand. This stress-related aspects of atopic disorders complicate the relationship these disorders have or manifest in relation to cortisol.

1.3.7 Cortisol and pregnancy-related complications

Elevation in steroid hormone production during pregnancy is well known and this function favours lipogenesis, fat storage and protection for the developing

foetus, for example. With increasing trimesters, cortisol increases steadily, providing essential supportive functions for the mother and the foetus. Nonetheless, pregnancy-related cortisol increases above typical levels can have detrimental prenatal and postnatal consequences on the offspring, which highlight the multifunctional effects that cortisol exerts on the human body. In particular, stress-induced increased cortisol levels are associated with an increased risk of perinatal complications and an enduring impact on the infant's physiological and mental development, including shaping its HPA-axis and responsiveness to stress up until adulthood (Field & Diego, 2008; Weinstock, 2005). In fact, timing of prenatal exposure to elevated maternal cortisol levels (stress-related and above typical levels) was shown to moderate the infant's mental and motor development as measured repeatedly within the first postnatal year. Stress-induced elevated cortisol levels early in gestation inhibited development whilst this elevation later in gestation favoured development, controlling for several covariates such as prenatal medical history, demographics, infants' ethnicity, gender and birth order (Davis & Sandman, 2010).

1.3.8 Cortisol and mental health

1.3.8.1 Cortisol and depression

Cortisol is thought to play a crucial role in the aetiology of several mental disorders including depression, anxiety and posttraumatic stress disorder (Brown, Varghese, & McEwen, 2004; O'Donovan et al., 2010; Yehuda, Golier, & Kaufman, 2005). Disturbed cortisol rhythms are common in a substantial proportion of

depressed individuals (Herbert, 2013). While most research indicates higher cortisol levels in the depressed (Brown et al., 2004; Goodyer, Herbert, Tamplin, & Altham, 2000), evidence is somewhat mixed (Chida & Steptoe, 2009; Knorr, Vinberg, Kessing, & Wetterslev, 2010). A meta-analysis using 20 case–control studies concluded that saliva cortisol concentration could not distinguish between depression versus non-depression (Knorr et al., 2010). However, the studies differed vastly in sampling times of saliva collection; in addition, heterogeneity in morning cortisol values was large which might present a potential bias. This meta-analysis included studies with a wide range of individuals, i.e. inpatients, outpatients and from the general population. While this might be a potential advantage, it might have introduced often overlooked potential flaws such as severity of depression, comorbidity and diversity of samples. In fact, the link between depression and cortisol (especially the CAR) has been shown to differ depending on the intensity, onset and episode frequency of the disease (Cowen, 2002; Hardeveld et al., 2014; Mangold, Marino, & Javors, 2011); therefore, depression aspects seem to play a moderating role and sub-group analyses are warranted.

The CAR especially, has attracted a lot of attention for research in mental disorders. Prospective and long-term cohorts have provided insights into the contributory and causal mechanisms between dysfunctional HPA-axis and depression. Prospective studies with mood disorder patients suggest that the CAR seems to be a risk factor (despite having a time-limited predictive stability) for future depression rather than a resultant factor from depression. In a longitudinal

study of 270 young adults, a higher CAR predicted depression significantly (up to a 3.5-fold increased risk) over a period of 2.5 years (Vrshek-Schallhorn et al., 2013), supporting previous evidence over a 1 year period in 230 adolescents (Adam et al., 2010). However, evidence on cortisol levels predicting onset or recurrence of depression might also potentially present a marker of susceptibility, i.e. the biological effect of the developing mental disorder in terms of subclinical symptoms in the premonitory phase. Hence, it remains unclear to what extent elevated awakening cortisol levels represent the causal aetiological factor, and potentially an exacerbating element for symptoms, or a consequence of the underlying mental disorder.

A meta-analysis assessing psychoendocrinological stress responses (cortisol levels before stressor onset, during stress tasks and during recovery period/ after stressor offset) in depressed compared to non-depressed controls revealed a typical blunted reactivity and weakened recovery pattern in the recovery period in depressed individuals (Burke, Davis, Otte, & Mohr, 2005). The pattern was characterised by lower cortisol levels during stress tasks and higher cortisol levels during recovery; remarkably, this association increased with severity of depression. This endorses the role that stress and impaired stress reactivity play in depression. In fact, a systematic review suggests that twin studies strongly support stressful life events as a strong risk factor (equally valued as genetic risk factors) for depression onset and recurrence of episodes (Tennant, 2002). Both recent and distant events significantly predict onset of depression; nevertheless, stressor chronicity or duration (long-term stressors) were linked to higher severity and duration of

depression. Studies assessing gene-by-environment interactions have identified stressful life events and stress as a fundamental aetiological feature of depression and indeed cortisol as the key biological mediator between stress and depression (Caspi et al., 2003; Risch et al., 2009).

It is widely accepted that the neurobiological basis of depression in particular is based on a reduction in serotonin receptor function (Nemeroff, 2002). However, research has also suggested a corticosteroid-serotonin interaction, in which cortisol seems to drive low serotonin levels via a complex inter-related system, such as lowered tryptophan levels (Cowen, 2002; Nemeroff, 2002). Interestingly, in a small scale study of 8 depressed, 12 anxiety patients and 8 healthy controls, exogeneously administered cortisol produced a stimulatory effect of increased serotonin uptake (as examined in lymphocytes) in healthy participants, while no such inducement was exhibited in the two mental disorders (Tafet et al., 2001). Nevertheless, identification of causal mechanisms are complicated by the interactive action with the immune system (i.e. high levels of inflammation in depression), the nervous system and the endocrine system, and warrant further research (Tiemeier, 2003).

1.3.8.2 Cortisol and anxiety

Also anxiety disorder has been linked to HPA-axis dysregulation. Anxiety has been repeatedly linked to lower hourly-assessed night-time and morning plasma cortisol levels in pre-pubertal children (Feder et al., 2004), to lower morning salivary cortisol levels in middle-aged adults (O'Donovan et al., 2010) and to a lower CAR in

adults and the elderly (Hek et al., 2013; Walker, O'Connor, Schaefer, Talbot, & Hendrickx, S. Walker, O'Connor, Schaefer, Talbot, & Hendrickx, 2011). Also stress reactivity patterns towards laboratory-induced stress tasks in anxiety patients have been reported to be blunted and longer in the recovery period (Takahashi et al., 2005; Yoon & Joormann, 2012). However, the direction of associations have been somewhat inconsistent, with some studies suggesting lower and others suggesting higher cortisol levels (Chida & Steptoe, 2009; Hek et al., 2013; Vreeburg et al., 2010). For instance, late-life generalised anxiety disorder in 70 older adults compared to non-anxious age-matched controls was associated with higher CARs and higher AUCs which also positively corresponded with symptom severity, controlling for anxiolytic medication (Mantella et al., 2008). In a study on 91 breast cancer patients, anxiety has been associated with a flatter diurnal slope (Giese-Davis, Sephton, Abercrombie, Duran, & Spiegel, 2004).

Differences in cortisol rhythmicity in anxiety may underlie variations in type and severity of the mental disease (Kallen et al., 2008). Also distinguishing between state and trait anxiety might be an important factor, as state anxiety might be considered as the first psychological basis of the stress response whereas trait anxiety might present a persistent typical state of uneasiness and worry in daily life. In fact, a study with over 1,700 children reported persistent rather than current anxiety to be related to a higher CAR (Greaves-Lord et al., 2007). Not controlling for differences in aspects of the mental disorder can therefore generate equivocal results.

A methodological difficulty in investigating mental disorders is the putative focus on “pure” diseases, such as distinct depression or anxiety diagnoses. With the attempt to optimise classification of diagnosis and treatment approaches and also to understand the underlying mechanisms, researchers have been interested in the overlap in anxiety and depression. Although the two can be differentiated (Clark & Watson, 1991), it has been argued that they might both underlie general affective distress and negative affectivity based on e.g. information processing biases (Levine, Zagoory-Sharon, Feldman, Lewis, & Weller, 2007). Interestingly, a literature review suggested that half of studied patients with depression and/or anxiety actually suffer from a comorbid second mental disorder (i.e. anxiety disorder or depression, respectively), impacting recovery and recurrence rates and level of psychosocial disability (Hirschfeld, 2001). A study with 1427 depressed, anxious patients and healthy controls reported a higher CAR in patients with comorbid depressive and anxiety (Vreeburg et al., 2010). In another study assessing cortisol reactivity in individual depression and anxiety disorder and a combination of both against healthy matched controls, only the depressed patients with comorbid anxiety disorders had an exaggerated cortisol reactivity in response to laboratory-induced stressors (Young, Abelson, & Cameron, 2004), suggesting an additive effect on psychoneuroendocrinological processes and potential overlooked aspects that might lead to heterogeneity in findings.

1.3.8.3 Cortisol and PTSD

Evidence of the impact of stress, psychological and psychosocial factors and cortisol on the initiation, promotion and progression of PTSD elucidates the psychoendocrinological mechanisms involved in this disease. In a substantial proportion of individuals suffering from posttraumatic stress disorder lower basal cortisol levels and a diminished CAR has been recorded (Meewisse, Reitsma, De Vries, Gersons, & Olff, 2007; Yehuda et al., 2005). However, there are also inconsistencies in the literature on PTSD and cortisol (Bonne et al., 2003). Higher levels of cortisol and catecholamines have been reported right after the traumatic event in 82 adolescents, which even predicted subsequent development of PTSD at 6 weeks following the incident (Delahanty, Nugent, Christopher, & Walsh, 2005), although the opposite has also been reported (Sherin & Nemeroff, 2011). A review reported exaggerated cortisol responses to laboratory-induced stress in patients with PTSD (de Kloet et al., 2006). In line with this, cortisol responsivity together with sympathetic responses are increased in women with PTSD when prompted with traumatic personalised reminders about their childhood abuse (Elzinga, Schmahl, Vermetten, van Dyck, & Bremner, 2003). PTSD has been linked to reduced hippocampal volume (Bremner, 2006) and prolonged exposure to elevated cortisol in stressful situations might explain the high degree of hippocampal degeneration in this mental disorder.

While methodological factors (such as varying cortisol sampling methods and inclusion/ exclusion criteria) might play a key role in yielding mixed findings, PTSD is a perplexing mental disorder with symptom complexity. Repeated exposure

to traumatic events can cause cumulative effects and generate even a different type of PTSD called complex PTSD (Cloitre et al., 2009). Further, parental PTSD and trauma exposure have been found to predict lower 24-hour urinary cortisol levels in adult offspring with PTSD (Yehuda, Halligan, & Grossman, 2001). Even without own lifetime PTSD, urinary cortisol levels were lower in adult offspring of Holocaust survivors compared to traumatised and non-traumatised control offspring (Yehuda et al., 2000). These findings suggest that parent-child trauma transmission might be relevant for adult offspring HPA-axis functioning, regardless of the level of trauma. A systematic review and meta-analysis concluded that analyses required subgroup division to reveal reduced cortisol levels in PTSD which emerged only under certain conditions such as type of abuse (physical or sexual, as opposed to emotional abuse) and in females (Meewisse et al., 2007). Furthermore, variation in measurement methods induced heterogeneity among population samples and studies. PTSD is a disease that has attracted much interest with its conflicting results. Chapter 2 introduces a novel method for cortisol analysis, which has aided in the understanding of the mixed results in PTSD; findings which will be explained in section 2.8.3.

1.3.9 Cortisol and health overview

To recapitulate, cortisol has been related to a range of physical and mental health outcomes and the pathological mechanisms involve inflammatory processes, gluconeogenesis, hypertension and hypercholesterolemia, and altered neurotransmitter concentrations. The multiple pathways highlight the

multidimensional features of cortisol's function. Table 1.2 summarises the main health outcomes identified by aberrant HPA-axis functioning and the proposed cortisol-induced mechanisms. Although stress and psychological factors are a common denominator for several mental disorders, a bidirectional relationship between these variables is thought to exist. The findings suggest that changes in HPA-axis functioning belong to the biological pathways through which stress contributes to physical and mental health outcomes and increased risk for psychopathology. Studies are only beginning to disentangle how stress may affect different health outcomes by psychoneuroendocrinological mechanisms, but a body of research indicates that stress drives susceptible people to ill-health, as there are individual characteristics that determine the presence and magnitude of the supposed stressor.

Table 1.2 Mechanisms through which aberrant cortisol levels affect physical health.

Cortisol-induced effect	Health outcome
Suppressed immune function, e.g. reduced count of leukocytes, including killer T cells (Cohen et al., 2012; Janicki-Deverts, Cohen, Turner, & Doyle, 2016)	Susceptibility to infections
Increased energy and oxygen, which stimulates blood glucose levels and lipolysis (Djurhuus et al., 2002; Macfarlane, Forbes, & Walker, 2008), glucocorticoid receptor dysfunction (Bjorntorp & Rosmond, 1999), increased gluconeogenesis (Levitt et al., 2000)	Metabolic dysfunction and metabolic syndrome
Blocked insulin action in liver glycogenesis and glucose intolerance (Holmang & Bjorntorp, 1992; Levitt et al., 2000), enhanced activity of the enzyme 11β HSD1 (Stomby, Andrew, Walker, & Olsson, 2014)	Diabetes
Increased fat storage (Bjorntorp & Rosmond, 2000), lipolysis (Djurhuus et al., 2002), activity of the enzyme 11β HSD1 (Stomby et al., 2014), increased appetite (Epel, Lapidus, McEwen, & Brownell, 2001)	Obesity, increase in abdominal adipose tissue
Hypercholesterolemia (Nayak, Carter, & Feldman, 1962; Whitworth et al., 2005), hypertension (Pirpiris, Yeung, Dewar, Jennings, & Whitworth, 1993), coronary atherosclerosis (Dekker et al., 2008)	Cardiovascular disease
Hippocampal Atrophy (Sapolsky, 2000), reduced hippocampal glucose metabolism, disruption of synaptic plasticity (Sapolsky, 1999), altered neurotransmitter concentrations (Stalder & Kirschbaum, 2013)	Cognitive dysfunctions, e.g. memory impairments
Suppressed immune function, e.g. suppressed activity of natural killer cells (Sephton et al., 2000)	Cancer (e.g. breast cancer
Low ratio of cortisol to proinflammatory cytokines, e.g. IL-6 and TNF-α (Straub et al., 2002)	Rheumatoid arthritis
Reduced bone apposition and density, increased bone mineral resorption, calcium malabsorption (Chiodini & Scillitani, 2008)	Osteoporosis
Heightened inflammatory responses (Leung, 2000)	Atopic disorders
Altered neurotransmitter concentrations (Stalder & Kirschbaum, 2013)	Mental disorders

1.4 Chapter summary

This chapter provided the reader with an understanding about stress and its relationship to physical and mental health, outlining the development of stress theory and stress research. Cortisol has been largely used in studies that investigate stress. Cortisol release follows a circadian rhythm that maintains homeostasis and allows for proper functioning of the main systems of the body, but is also secreted in response to stress to facilitate physiological, mental and behavioural reactions towards the stressor. There is evidence that aberrant cortisol levels (both hyper- and hypo-cortisolism) are detrimental to health, such as CVD, diabetes, obesity and cancer, atopic disorders and to accelerated age-related processes. Cortisol also plays a role in the aetiology and progression of mental disorders. The literature suggests that chronic stress can trigger and/ or moderate diseases. People under chronic stress excrete cortisol that overrides the circadian pattern and over a sustained time period, this can have significant impact on the different bodily systems. Psychosocial factors such as stress appraisal, coping, social support and dispositional characteristics were shown to be relevant to the relationship between stress and disease. This PhD investigates the associations between stress exposure, perceived stress and psychosocial factors and cortisol (Chapter 3: socioeconomic factors, work stress and social support; Chapter 6: academic stress exposure and perception, and coping mechanisms). There are inconsistencies in the literature and the failure to control for confounding variables in observational studies is a major problem in traditional cortisol assessment. The next chapter will outline

methodological aspects in cortisol sampling and introduce a novel assessment method that attempts to somewhat overcome these difficulties.

CHAPTER 2. METHODOLOGICAL ASPECTS IN CORTISOL SAMPLING

2.1 Chapter Overview

The complexity of the stress system has led to inconsistent findings and until recently, psychoneuroendocrinological studies in daily life were seriously hampered by methodological obstacles. This chapter introduces the different traditional cortisol specimens and describes the methodological drawbacks associated with them, especially with regards to salivary cortisol assessments. The subsequent sections will draw upon a relatively novel method to assess cortisol, which is the analysis of hair. An overview of hair, its structure and life cycles will aid the understanding of the mechanisms through which cortisol is thought to be incorporated into the hair shaft. This is followed by a literature review on hair cortisol studies in relation to physical and mental disease, stress-exposure in both clinical and non-clinical populations and an outline of the methodological aspects involved in hair cortisol analyses.

2.2 Traditional methods of assessing cortisol

The traditional sources for analysing cortisol in psychoneuroendocrinological stress research are saliva, blood and urine specimens. The first studies were based on blood and urine samples. While urinary measurements cover cortisol secretion for a period of up to 12-24 hours, blood analyses can detect secretion over the preceding minutes. Urine sampling has the advantage that it measures the daily total cortisol excretion and can thus easily detect chronic hypo- or hypercortisolism.

In blood, there are two forms of cortisol. While most is bound to carrier proteins (cortisol binding protein and albumin), a minor portion is in its biologically active free form (Levine et al., 2007). Analyses to measure the active form are complex and costly, especially as several measurements need to be taken due to the dynamic diurnal rhythm. In the late nineties the detection of cortisol in saliva facilitated ambulatory cortisol measurements. The validity of salivary cortisol assessment was based on remarkably high correlations of r = 0.9 with plasma cortisol, whilst absolute levels in salivary cortisol are approximately 50% lower compared to plasma cortisol and thus not comparable per se (Kirschbaum & Hellhammer, 1989). The same marked circadian rhythm profile was also observed in salivary cortisol. These early studies also supported strong temporal relationships based on evidence of rapid blood-saliva transfer that occurred within 1 minute after cortisol injection (Walker, Riadfahmy, & Read, 1978).

Owing to the development of salivary cortisol assessment two to three decades ago, blood- and urine-based findings could be corroborated and important findings could be clarified such as the finding that there is an approximately 15 minute delay in reactivity after stress-induction (Kirschbaum & Hellhammer, 1989). The rise in research studying the effects of cortisol on prenatal development and on the infant's HPA-axis and stress response functioning reflected salivary cortisol's popularity (Clements, 2013). This led to further research questions to be developed and hence an advancement of our understanding of psychoneuroendocrinological processes (Kirschbaum & Hellhammer, 1994; Lehnert et al., 1989).

Saliva sampling is non-invasive and thus more feasible than blood sampling, as it is not influenced by blood draw pain- or stress-induced reactivity rises in cortisol secretion; further it is far more practical as it can be sampled in domestic settings without interrupting the individual's daily routine. Chewing on the cotton roll stimulates saliva production of 0.5-1 ml volume from which cortisol can be extracted, independently of flow rate (Kirschbaum & Hellhammer, 1989). Salivary cortisol has a low cortisol deterioration rate, and has been shown to be relatively stable for more than a week at room temperatures and for several months when refrigerated, and it is not affected by repeated freezing and/ or thawing of the samples (Garde & Hansen, 2005). Together, its high correlation with the free unbound plasma cortisol fraction, its simplicity and possibility of ambulatory sampling in normal populations in everyday life, has made salivary cortisol an attractive alternative; since its discovery psychoneuroendocrinological research has been predominantly based on saliva analyses (Kirschbaum & Hellhammer, 1994). Collection of saliva over the day involves demanding protocols and adherence issues and in light of the dynamics of the cortisol sensitivity-response, there are several controllable and uncontrollable factors that affect momentary assessment of cortisol. The next section focuses specifically on the procedural challenges in the assessment of salivary cortisol.

2.3 Methodological issues in salivary cortisol assessment

2.3.1 Measurement issues, sampling factors and adherence

Cortisol from saliva reflects a dynamic fluctuating system of ultradian (repeated oscillation < 24 hours) and circadian (repeated oscillation of 24 hours) patterns and is additionally influenced by stress reactivity. Consequently there are several methodological complications that can hamper the collection of reliable assessments of the cortisol profile and findings can be confounded by limitations inherent in the study designs (Spiga et al., 2014; Vancauter, 1990). These challenges might not only lead to measurement errors and invalidate cortisol values, but may also mask potential associations between the variables of interest; especially in between-subject studies. An example of the difficulties faced by researchers comes from the study of the cortisol awakening response (CAR) and the cortisol diurnal slope. The CAR and the slope have been shown to be only weakly intercorrelated (Golden et al., 2013; Schmidt-Reinwald et al., 1999), suggesting that they might be regulated differently. Although this highlights the importance of studying each of these measures as distinct entities, doing so contributes to heterogeneous findings, as studies frequently focus on only one of the different types of the diurnal cortisol profile, either the CAR, the area-under-the-curve (AUC) or the diurnal cortisol slope.

Cortisol levels vary throughout the day and 62–72% of this variation is attributed to time of day, highlighting the importance of accurate adherence to the sampling protocol (Adam & Gunnar, 2001). The morning assessments of salivary cortisol especially hinge on accurate time adherence, as variation in waking time and in the exact timing of samples may generate quite different results for the CAR.

As the morning acrophase is very prompt, delaying the awakening sampling (first sampling point) generates a false value as it assesses cortisol on the ascendant curve, leading to a correspondingly reduced overall CAR. Participants' adherence therefore represents a significant confounding factor in saliva collection (Dockray, Bhattacharyya, Molloy, & Steptoe, 2008; Kudielka, Broderick, & Kirschbaum, 2003; Smyth, Clow, Thorn, Hucklebridge, & Evans, 2013). In fact, an experimental study by Kudielka and colleagues (2003) showed a considerable amount of non-adherence to the specified sampling protocol for saliva collection. Using electronic monitoring devices (e.g. MEMS Track Caps) that record collection time, the experimenters either did or did not inform participants about the objective monitoring. Participants recorded time of sampling and the objective and subjective ratings were compared. While non-informed participants showed a higher discrepancy between subjective and objective adherence than informed participants, non-adherence also resulted in a significantly reduced CAR and a flatter diurnal slope, invalidating the diurnal cortisol profile. Another study showed similar findings of non- adherence biasing a flatter slope (Broderick, Arnold, Kudielka, & Kirschbaum, 2004). There are certain factors that have been found to contribute to higher non-adherence rates, such as lower socioeconomic status and education as reported in a multi-ethnic population of 935 middle-aged adults, and also psychosocial factors such as lower social support over a 3-day sampling period as found in 83 middle- and older-aged adults (Golden et al., 2014; Kudielka, Hawkley, Adam, & Cacioppo, 2007). One study used random time sampling, indicated by signalling at ten random moments using a digital wristwatch, rather than fixed time samplings, in a sample

of 59 twin and non-twin sisters aged 18–52 years (Jacobs et al., 2005). They found that this method reduced non-adherence in participants and the exerted influence on the cortisol diurnal profile and the parameters.

Further, lack of precise definition of 'waking' might contribute to inconsistent findings. Objective assessments of waking time with the help of Actigraphs have assisted with this methodological drawback. A review that has produced recommendations in methods for optimising adherence in salivary cortisol research designs in large-scale studies included the use of objective measures of Actigraph (Adam & Kumari, 2009). Although the use of track-capped salivettes and objective waking assessments are costly, they are highly valuable as time awareness of objective monitoring devices produces adequate adherence rates and accurate time of waking allows for the correct computation of the different cortisol parameters. However, the majority of research continues to use self-report measures of waking and of sample timing.

The issue of sampling delays has also produced a controversy as to how genuinely the so-called 'CAR non-responders' exist. It is estimated that approximately 75% of participants elicit the typical post-awakening morning response under ambulatory conditions when performed at home, with several studies corroborating this pattern, but others argue this figure to be distorted by non-adherence (Wilhelm et al., 2007; Wust, Wolf, et al., 2000). Efforts to explain the phenomenon of non-responders have suggested methodological aspects, sleep-related aspects, psychosocial factors and the complexity of the HPA-axis to be possible causes. However, in a sleep laboratory study under experimental

conditions, using polysomnographical readings, it has been reported that 100% of participants elicited a positive CAR (Wust, Wolf, et al., 2000). Possible causes for the higher response rate under laboratory conditions might, however, include transient awakenings and therefore missed awakening rise values leading to smaller or flatter CARs or unfamiliar environments which produce a stronger awakening stimulus and thus a positive increase in otherwise non-responders. Yet, the most predominant view is that non-responders are genuine, as conclude by Dockray et al. (2008), using objective measures.

2.3.2 Stability issues

Another important characteristic of the salivary cortisol parameters, especially in the CAR, is that there is not only high between-person variability but also within-person variability when measurements are taken across multiple days. The stability of salivary cortisol measures over time is variable owing to the influence of a number of situational factors such as time of waking, sleep patterns, environmental conditions, smoking, diet and acute psychological states (Adam & Kumari, 2009; Hansen, Garde, & Persson, 2008). Two studies have tested the stability of the different salivary cortisol parameters over extended time periods. A study with three multi-wave studies analysed the stability of the distinct cortisol parameters (CAR, AUC and slope) over a period of 8-24 months and concluded the CAR to be the least stable parameter, followed by the slope and the AUC (Ross, Murphy, Adam, Chen, & Miller, 2014). In fact, between 78-85% of the variability in the CAR is attributable to day-to-day fluctuations, demonstrating more state-like

rather than stable characteristics. Another study assessed stability over periods of up to 6 years (Wang et al., 2014). In this case, the greatest stability was reported for measures of slope, and the least for the CAR.

Despite these concerns, it should be pointed out that the vast majority of research on cortisol and psychosocial factors, and on cortisol and health outcomes, detailed below has used single days of cortisol assessment. Additionally, most of this research did not involve electronic measures of sample timing or of waking time. So important discoveries can be made irrespective of these methodological refinements.

2.3.3 Implications for studies of psychosocial factors

A body of research supports the impact that psychosocial factors play in shaping HPA-axis functioning. Especially in the CAR, psychosocial stressors account for the highest amount of day-to-day variability (Adam & Gunnar, 2001; Golden et al., 2013; Schmidt-Reinwald et al., 1999). This high variability would indeed explain the suggestion that the CAR presents a distinct entity of the circadian cortisol rhythm. A higher CAR is observable on weekdays compared to weekend days, controlling for time of waking (Kunz-Ebrecht, Kirschbaum, Marmot, & Steptoe, 2004), endorsing the role that work and daily stress and psychological factors play in shaping the CAR. One theoretical explanation about the function of the CAR comes from the anticipation hypothesis, an assumption that the CAR is responsible for coping with anticipated demands and challenges for the forthcoming day (Powell & Schlotz, 2012; Wetherell, Lovell, & Smith, 2015). This could partly explain

the difference in CARs between weekdays and weekend days: higher stress anticipations regarding the forthcoming working day. A series of five studies with competitive ballroom dancers reported a greater CAR on competition days, highlighting the amplified anticipatory effect on the CAR (Rohleder, Beulen, Chen, Wolf, & Kirschbaum, 2007). Several findings support the notion that mood and negative cognitions affect morning awakening responses, however, feelings prior to the sampling day seem to be of higher relevance than feelings on the same day. For instance, a greater CAR was associated with prior-day feelings of threat, sadness and lack of control in 156 older adults (Adam, Hawkley, Kudielka, & Cacioppo, 2006), and prior-day feelings of loneliness in 108 young adults (Doane & Adam, 2010).

However, not only the variations in the magnitude of the CAR might be partly explained by daily social and emotional dynamics, but also variations in the other aspects of diurnal cortisol patterns, including the AUC and the slope. Highlighting the impact social context and perception can have on neuroendocrine functioning, studies demonstrate associations between cortisol and fluctuations in mood states and loneliness. Reporting higher levels of tension and anger predicted a flatter diurnal cortisol rhythm over the same day (Adam et al., 2006). Negative affect and agitation were associated with higher AUC and appeared to mediate the relationship between daily stressful events and cortisol excretion, suggesting negative affectivity to be an important potential confounder (vanEck, Berkhof, Nicolson, & Sulon, 1996). A study investigating the day-to-day emotional variations in relation to cortisol found that momentary feelings of loneliness throughout the

day predicted related transitory increases in cortisol. These findings emphasise the role loneliness plays in HPA-axis functioning with trait, state and momentary experiences of loneliness separately affecting different cortisol parameters (Doane & Adam, 2010).

Further, some relationships might be concealed by lack of control for certain factors. One study reported that when daily hassles and stressors were included in analyses, relationships between depressive symptoms, rumination and HPA-axis activity emerged that were not significant when these factors were not controlled (Huffziger et al., 2013). In a sample of 40 young men, Pruessner and colleagues (Pruessner, Hellhammer, Pruessner, & Lupien, 2003) showed the potential mediating role stressful experiences and mood can play in associations between depression and the CAR; higher levels of perceived stress were positively related to both depression and the CAR. Also negative affect has been shown to mediate the relationship between stressful life events and higher AUCs and flatter cortisol slopes in 92 middle-aged males (vanEck et al., 1996). This suggests that affect is directly related to cortisol but also acts as a confounder of cortisol secretion in relation to certain variables of interest. The effect of mood and daily stressors on cortisol parameters highlights the diverse factors that would need to be taken into account when studying laboratory stress research, environmental stressors and momentary psychological states (Fries, Dettenborn, & Kirschbaum, 2009; Hansen et al., 2008).

2.3.4 The impact of sleep on cortisol parameters

Sleep duration and quality have been shown to be related to disturbed cortisol rhythms (Castro-Diehl et al., 2015). In their sample of 600 adults aged 54-93 years with a 7-day Actigraph-based sleep assessment and a 2-day salivary sampling period, shorter sleep duration and lower sleep efficiency (more time spent in bed relative to the actual sleeping time) and more sleeping problems were associated with a smaller CAR and a flatter cortisol slope. Analyses from a 10-year follow-up study with an occupational cohort also suggests recurring shorter sleep duration to be associated with a flatter diurnal slope and the number of symptoms of insomnia with a steeper CAR, demonstrating the adverse long-term effects sleep problems can have on HPA-axis functioning (Abell, Shipley, Ferrie, Kivimaki, & Kumari, 2016). Using a within-subjects study design, Bostock and Steptoe (2013) were able to see the impact of early and late work shifts on the diurnal cortisol rhythm in 30 male pilots. Compared to late shifts or rest days, early shifts were associated with a higher CAR, a higher AUC and a flatter slope, even after the shorter sleep duration and the lower mood ratings were adjusted for. Also chronotype, the propensity of an individual's sleeping patterns or timings, has been shown to be associated with the CAR in 112 young men, independent of awakening time and sleep length (Kudielka, Federenko, Hellhammer, & Wust, 2006). Morning chronotypes compared with evening chronotypes were associated with a greater CAR.

In a sample of 188 working women, higher frequency of nightmares was shown to be associated with a blunted CAR, implying that nightmares and disrupted sleeping patterns might be an indicator of affective distress which disturb HPA-axis

functioning or vice versa (Nagy et al., 2015). However, it remains unclear as to whether the psychological components of nightmares or abrupt awakenings, as triggered by these vivid negative dreams, explain these associations or a combination of both. Interventions of forced waking did not alter subsequent CARs on the following day in 13 adult women (Dettenborn, Rosenloecher, & Kirschbaum, 2007), however, the reasons for waking assessed in this study were exogenously produced rather than endogenously (as would be the case for emotional stress related processing during sleep) and therefore do not provide evidence for this hypothesis.

2.3.5 Confounding variables and situational factors

Other potential sources leading to interindividual variability in the assessment of the cortisol parameters are dietary patterns, acute food consumption, exercise and smoking (Maina, Bovenzi, Palmas, Rossi, & Filon, 2012). Midday meals have been shown to activate the HPA-axis, depending on macronutrient proportions. Over two separate test days, midday meals high in protein proportion consistently induced acute cortisol increases (Gibson et al., 1999). However, another study found the effect of macronutrient content on cortisol secretion to depend on the individual's body fat distribution (Vicennati, Ceroni, Gagliardi, Gambineri, & Pasquali, 2002). While several studies support the effect of caffeine consumption in stimulating cortisol secretion, one intervention study demonstrates that caffeine also amplifies the effect of meal-inducing cortisol rises (Lovallo, Farag, Vincent, Thomas, & Wilson, 2006). Interestingly dietary

behaviours also affect cortisol irrespective of psychosocial factors; calorie restriction was found to be related to an increased AUC, an association that could not be explained by increased perceived stress (Tomiyama et al., 2010). There are inconsistencies in the impact of exercise on cortisol levels, which might be attributable to methodological issues of both sampling and type and duration of exercise and participant physical fitness (e.g. Hayes et al., 2013; Kirschbaum & Hellhammer, 1994). In a within- and between-participant design, a 22-week intervention of physical activity in inactive, obese participants resulted in increased cortisol levels up to 6 months after the intervention (Foss, Sæterdal, Nordgård, & Dyrstad, 2014), in line with similar findings from another shorter intervention study (Alghadir, Gabr, & Aly, 2015). Acute cortisol induced effects of exercise have also been studied. In particular, the effects of high-intensity exercise on cortisol levels suggest that vigorous physical activity should be limited before studies (Jacks, Sowash, Anning, McGloughlin, & Andres, 2002). Smoking is related to higher CARs even without having smoked during the morning saliva sampling period (Steptoe & Ussher, 2006), demonstrating the enduring effects smoking has on HPA-axis dysregulation. Interestingly, differentiating between low and high nicotine content in 20 young men, there also seems to be a dose-dependent relationship between smoking and acute cortisol secretion (Mendelson, Sholar, Goletiani, Siegel, & Mello, 2005), suggesting as well that smoking should be avoided 30 minutes prior to saliva sampling.

Finally, further potential confounding factors are medications containing hydrocortisone, oral contraceptives and the phase of the menstrual cycle (Hansen

et al., 2008; Kirschbaum, Kudielka, Gaab, Schommer, & Hellhammer, 1999). Specifically, the phase of the menstrual cycle (menstrual, follicular, ovulation or luteal phase) significantly predicted first morning urinary cortisol levels in a longitudinal study, with changes occurring between the follicular and luteal phase (Nepomnaschy et al., 2011). Cortisol is also altered during pregnancy and breastfeeding, affecting methodological concerns in developmental research (Clements, 2013). Although findings are inconsistent, especially with studies using varying cortisol parameters and calculations (Wust, Wolf, et al., 2000), these findings highlight the complexity of methodological aspects in assessing the HPA-axis.

Together, these findings on psychology- and stress-related dynamic patterns of cortisol secretion support the need for daily life research to better understand psychoneuroendocrinological mechanisms related to disease (Myin-Germeys et al., 2009). The fact that there are both state-related (within-person) and trait-related (between-person) variability in cortisol levels increases the probability of analytical challenges (Saxbe, 2008).

2.4 The need for a measure of long-term cortisol output: the development of hair cortisol analysis

Assays of cortisol from saliva and blood can show deviations from the typical diurnal cortisol cycle, explore dynamics and might hence reveal a disturbed HPA-axis function. Yet, these only reflect momentary cortisol concentrations at the time of sampling rather than sustained levels. Each salivary cortisol parameter, especially

the CAR, is a state-dependent phenomenon due to large intraindividual differences and there are several methodological aspects that impede simple measurements of the profile (Hellhammer et al., 2007; Maina et al., 2012). A number of methodological and biological sources acting as confounding variables have been identified in the literature which complicate study designs, assessments, analyses and interpretations and ultimately lead to heterogeneity of findings (Hansen et al., 2008). More importantly, falsified cortisol levels and parameters, as might be produced by non- adherence, can significantly impact the ability to detect relationships between cortisol and the variables in question or the phenomenon being investigated, potentially leading to concealing existing associations or falsely accepting non-existing associations. Smaller scale studies, as predominantly used in psychobiological stress research have not enough power and degrees of freedom to control for a cluster of potential covariates that might obstruct potential associations (Adam & Kumari, 2009). Epidemiological data might be more suitable in this regard with their higher samples sizes and more representative population samples. However, these, in turn, are limited in being able to take more detailed, repeated assessments, to control for adherence to sample collection protocols and also risk higher attrition rates which might mask important information (Adam & Kumari, 2009).

Associations between health, stress and cortisol are abundant and convincing. However, chronic health is of concern and salivary-derived cortisol assessment may prove inadequate when evaluating long term HPA-axis functioning. Longitudinal studies using state-dependant measures (such as salivary cortisol) can

be problematic and interpretations of causal directions cannot be drawn accurately with short-term stress parameters. In light of the health-effects of chronic stressors and the pathways that link chronic stress to disease, a measure or biomarker for long-term stress is needed. Since the detection of cortisol in human hair in 2004, there has been a steep rise in studies using hair as a specimen for cortisol analyses and its validation as a measure of long-term cortisol exposure (Raul, Cirimele, Ludes, & Kintz, 2004). Based on hair growth of approximately 1 cm per month, a hair segment of 1 cm closest to the scalp represents net cortisol output for the month preceding sample collection (Hayashi, Miyamoto, & Takeda, 1991; Kirschbaum, Tietze, Skoluda, & Dettenborn, 2009). Analysis of scalp hair is not influenced by the pulsatility and circadian rhythm of the HPA-axis and therefore is thought to represent a retrospective index of cumulative cortisol output (Kirschbaum et al., 2009; Raul et al., 2004). The major practical advantage of hair testing is that it covers a larger time-frame from weeks to month depending on the length of the hair shaft, highlighting the utility of this way to assess long-term cortisol secretion. Since hair cortisol shows high short-term intraindividual stability, with correlations ranging from 0.68 to 0.79 in repeated hair cortisol measurements in two-month intervals, it is thought to provide a robust trait estimate of cortisol exposure (Stalder, Steudte, Miller, et al., 2012; Staufenbiel, Penninx, Spijker, Elzinga, & van Rossum, 2013). Its rather standardised sampling method is another methodological advantage over the traditional specimens to obtain cortisol. Different laboratories with diverse analysis techniques have progressively shown

more findings in different contexts that support the use of hair cortisol as a measure of HPA-axis function (Russell et al., 2015).

However, in order to maximise the potential that hair cortisol analyses might offer in the future to investigate robust HPA-axis functionality in longitudinal designs, which offer more valid interpretations and conclusions about certain possible associations, the method of cortisol analysis in hair must be further validated and fully understood. In recent years, studies have evaluated the validity of hair cortisol analyses and findings will be presented in the following sections, after an introduction of the structure of hair.

2.5 Hair

2.5.1 The structure of hair and its life cycle

Human hair consists of two distinct structures. One part is the hair follicle, invisible and below the skin surface. The second part is the hair shaft, which is the visible structure that protrudes through the epidermis. The hair follicle, located 3-4 mm below the skin's surface, is an agglomeration of cells that actively grow and create the visible hair. Abundant processes occur inside the hair follicle itself and also in interaction with its cutaneous environment, determining hair activity, growth and renewal. The hair follicle contains the most rapidly proliferating bodily cells (producing cell division of around 0.4mm/ day); from this cell division, gradual hardening of cells (called keratinisation) takes place, pushing older cells upwards, leading to the production of hair (Harkey, 1993). Generated during embryonic development, all 5 million hair follicles are established before birth with no new

hair follicle being produced after birth (Paus & Cotsarelis, 1999). Between 500 and 1,000 hair follicles are present in a $1cm^2$ skin section (Boumba, Ziavrou, & Vougiouklakis, 2006).

The hair follicle consists of several structures, as can be seen in Figure 2.1. The outside shield of the hair follicle develops from the germinating layer that grows into the dermis, creating the base of the hair follicle, the hair bulb, which contains a small hole, the hair papilla (Sperling, 1991). A capillary loop is connected to the hair papilla, which is responsible for the interaction between the hair and the dermis and sub-cutaneous layers of the skin, giving it nourishment. The papilla is the part of the hair follicle that is highly sensitive to hormones which moderate the cycle of hair growth, a process described later on in this section. The germinal matrix is surrounding the papilla where stem cells are retained (Tiede et al., 2007). Keratinisation, a process of hyalinisation and stabilisation of the protein keratin within the hair, takes place within the keratogenous zone, which is located above the papilla and one-third below the skin surface. Figure 2.1 depicts the structure of one single hair strand.

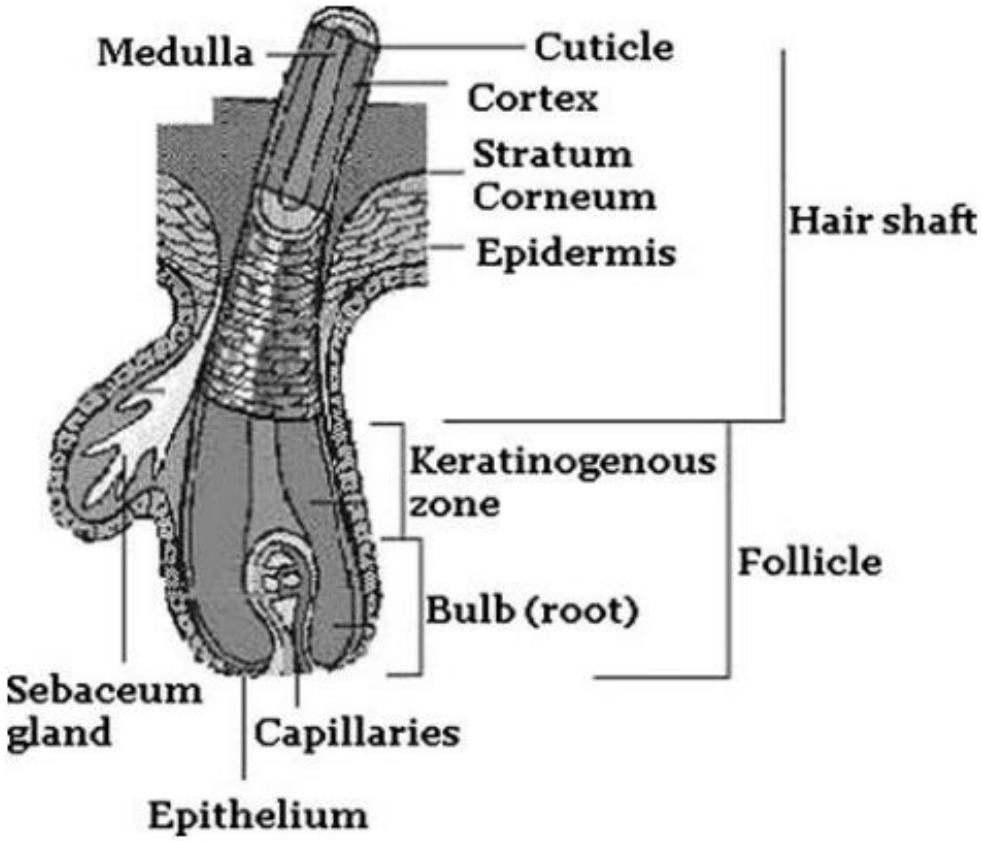

Figure 2.1 Hair structure (from Boumba et al., 2006).

The hair shaft is composed of keratinised, dead cells. From a cross-section perspective, it consists again of different layers: the cuticle, the cortex and the medulla (Hashimoto, 1988). The first and outermost layer, the cuticle, functions as a hair sheath. It consists of flat, overlaid cells, directed towards the hair tip. It is this layer that is an important indicator of hair health; healthy hair has a flat-lying cuticle. The cortex, the middle layer, is the biggest proportion of the hair shaft and the most important one, as it contains the numerous fine keratinous fibres and the colour-determining melanin granules and forms the actual structure of the visible hair. It is this layer that is responsible for the hair's strength and elasticity. Finally, the medulla is the innermost layer, a central canal running throughout the hair,

which contains transparent cells and air spaces. Two different glands are surrounding the hair follicle: the sebaceous and the sweat glands (Krause & Foitzik, 2006). The sebaceous glands, which attach to the hair follicle, produce an oily substance called sebum, which serves as lubrication and protection and makes the hair pliable. Sebum production is moderated by sex hormones; it is increased by androgens and decreased by oestrogen (Paus & Cotsarelis, 1999). Hair density is positively associated with the number of sebaceous glands (Smith & Thiboutot, 2008). Sweat glands produce salt-water, allowing perspiration and thermoregulation.

The human hair follicle is a complex mini-organ that undergoes a cyclic activity of regeneration and regression, maintaining a natural balance between growing hair and shedding hair (80-100 hairs per day) (Stenn & Paus, 2001). During its life course, a single hair follicle produces between 20 and 25 hairs, each lasting several years. The hair growth cycle entails three distinct and successive stages – the anagen or active growth phase, catagen or transition phase and telogen or final/ resting phase (Alonso & Fuchs, 2006). At any given time around 85% of all scalp hair is in the anagen phase, whilst most of the remaining hair is in the telogen phase and a tiny proportion in the catagen phase. In the anagen phase, lasting between 2 and 6 years (a time span that has been shown to be genetically determined), the hair is actively growing (Krause & Foitzik, 2006). It is the key phase for healthy hair formation and is very sensitive to increased exposure to stress and nutritional deficiencies, both factors that can inhibit hair growth (Botchkarev, 2003). The longer the hair stays in this phase the faster, stronger and longer the

hair will grow. Once the signal for the anagen phase to stop has been sent (it is still unknown what causes the follicle to switch from the anagen to the catagen phase), the hair enters the catagen phase, which is the changing transitional phase and lasts between 1-3 weeks. In this phase, hair shaft production ceases as the hair is detached from the blood supply and the follicle shrinks to one sixth of its size (Stenn & Paus, 2001). In the telogen phase, the hair remains dormant for around three to four months, which serves as a preparative period for future growth or shedding; intercellular signalling exchange between the papilla and the cutaneous environment is minimal. The old hair (characterised by a visible bulb of keratin at the end of the root) is pushed out by the new one or falls out naturally, signalling the anagen phase to begin again (Krause & Foitzik, 2006). This mutual duration and cycling between the phases determines hair growth and the individual hair length. Genetic analyses have identified a transcriptional regulation of this dynamic cycling process, with certain genes being activated during the anagen phase and inactive during the resting phase (Tornqvist, Sandberg, Hagglund, & Carisson, 2010). Different growth factors, hormones and cell adhesion molecules have been suggested to influence this regulation and ultimately hair growth (Hardy, 1992).

Genetic factors play a major part in determining inhibited hair growth, increased hair loss and age-related balding, caused by levels of the androgen hormones testosterone and dihydrotestosterone. Yet, dermatological research has identified stress as one of the main culprits, affecting the movement of the stages in the hair's life cycle (Cotsarelis & Millar, 2001). Specifically, during extreme stress, the switch from the anagen phase to the telogen phase can happen prematurely

(with even up to 70% of hair being in the telogen phase), which can result in inhibited hair growth and excessive hair loss, exceeding the natural daily hair loss limit of 100 hairs (Arck et al., 2003). This leads to telogen effluvium, a state in which hair follicles stay in this dormant, telogen stage indefinitely as no new hair is substituted, visibly producing general thinning of the hair. Stress-related telogen effluvium is a phenomenon that is still poorly understood, however, research has identified multifactorial triggers for telogen effluvium. Substance invasions such as vaccinations, excessive exposure to toxins or specific medicines (e.g. certain types of anti-depressants and cancer-treating cytostatic drugs), metabolic imbalances as induced by dieting, and also postpartum and post-surgery hair loss are all examples of telogen effluvium-inducement that can be explained by adaptive physiological processes taking place involving the immune and the endocrine system (Arck et al., 2003).

Telogen effluvium often manifests itself one or two months after suffering the stressful event. This is a reversible process as the hair follicle is not permanently damaged; once the stressful period has ceased hair follicles start producing new hair fibre and thus hair growth re-initiates; with complete restoration occurring at around 6 months after stress termination. While stress related to existing illness could be a contributing factor, telogen effluvium can also be a comorbid condition to inflammatory illnesses, thyroid disorders, and Addison's disease (hypocortisolism) (Wiedemeyeer, Schill, & Oser, 2004).

2.6 Hair analyses and incorporation mechanisms

Looking at its history, hair analyses originated with the development of detection techniques which enabled drugs, medicines and psychoactive substances in hair to be identified and also with the establishment of different extraction procedures, such as radioimmunoassays (Sachs, 1997). In 1979, the first animal analyses revealed hair to be suitable to obtain an account of specific drugs, i.e. opiates (Baumgartner, Jones, Baumgartner, & Black, 1979). This study also established that differences in the concentration of the relevant substances in the length of the hair shaft (from closest-to-scalp to the more distal section) correlated with the time duration the substance had been administered, thus initially declaring hair as an index of dosage history. This index was evaluated based on the postulation of an approximate hair growth rate of 1 cm per month (Chase, 1954). Since then, hair analyses have been extensively used and improved for drug diagnosing and forensic analytical purposes (including post-mortem toxicology). Substance identification in hair analysis allowed increased clarity, precision, duplication capability and prevention of sample substitution in toxicological examinations that used conventional specimens, not only as hair provides a record of rapidly excreted drugs (those which are untraceable after 72 hours in urine) but also of certain substances that are not excreted in urine (Kintz, 2004; Midio, Moreau, & Silva, 2001). Drug analyses also permitted the establishment of approximate time windows at which substances can be detected in the different specimens after administration: i.e. 1 hour - 24 hours in saliva, 3 hours -2 days/ 1 week in sweat, 3 hours – 2 days in plasma, 6 hours – 3 days in urine and 1 week – 1

month in hair (Wennig, 2000). In the last two decades, technical advances have enhanced chromatographic–mass spectrometric techniques for hair analyses, resulting in better sensitivity and limits of quantitation and detection (initial detection units at ng/mg have improved to detection units at pg/mg) (Pragst & Balikova, 2006).

Together these established methods exemplify the methodological advantage for hair analyses over traditional specimens, i.e. urine and blood for detecting various substances. Apart from the increased time-window coverage as a valuable benefit, ease of collection (including non-invasiveness), storage techniques and increased sample stability facilitate detection of selected substances in this specimen. The recognition of corticosteroids in hair in 2004 prompted much interest and led to a plethora of research into the psychoneuroendocrinological pathways, research that will be presented in the next sections. Prior to this, an outline of the incorporation of endogenous substances into the growing hair will be given. Also, basic principles of hair sampling and the analytical methodology will be described.

2.6.1 Incorporation mechanisms

It is generally understood that endogenous substances are incorporated into the hair via passive diffusion at the capillary loop within the hair papilla. The amount of the relevant substance and the time it lasts in the bloodstream influences the absorption rate by hair follicle cells and hence the quantity detected later via hair analyses. However, the multi-compartment model (as shown in Figure

2.2) proposes that incorporation occurs at several times throughout the hair growth cycle and over multiple sites (Henderson, 1993). Specifically, incorporation via capillaries, skin tissue and the glandular system might occur during the process of cell growth and hair creation within the keratogenous zone, after hair creation, and after the hair has protruded through the epidermis. Assuming ideal (steady and uniform) hair growth, segmental hair analysis has been thought to provide information about the time course of substance use or exposure. Although several studies have supported a dose-concentration correlation after drug administration, these have not been consistent and the pharmacokinetic profile in the participants' plasma could not explain the observed variability of the substance content within the different segments of the hair (Henderson, 1993). Experimental studies have demonstrated that sweat and sebum disturb the blood-hair concentration correlation, supporting that several mechanisms are involved (Boumba et al., 2006).

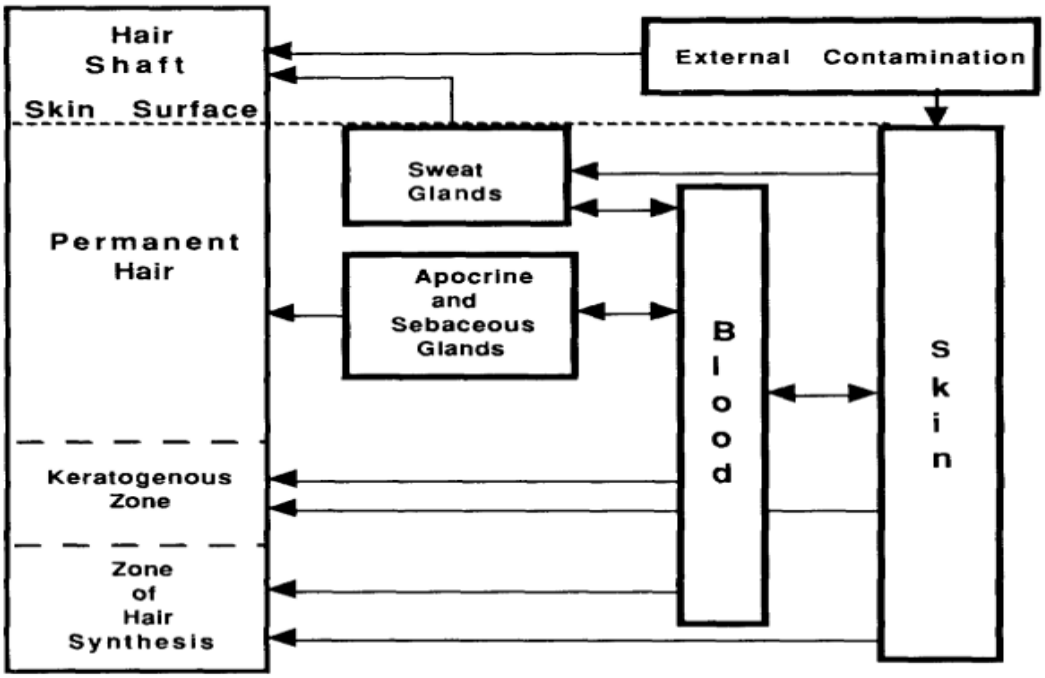

Figure 2.2 The multi-compartment model of substance incorporation into hair. Incorporation might occur during the process of cell growth and hair creation within the keratogenous zone, after hair creation, and after the hair has protruded through the epidermis; via capillaries, skin tissue and the glandular system. From Henderson (1993).

Uncertainty about substance incorporation and hair growth physiology, but also ambiguity about the importance of molecular binding abilities and exogenous contamination factors, have been held responsible for these discrepant findings. As a result, since then, the model of multifactorial mechanisms of incorporation has been the most accepted view with regards to hair analyses (Boumba et al., 2006; Pragst, Rothe, Spiegel, & Sporkert, 1998), and specifically for the assessment of cortisol (Gow, Thomson, Rieder, Van Uum, & Koren, 2010). An experimental study with guinea pigs found that hair cortisol content might indeed be 'contaminated' (confounded) by locally produced glucocorticoids at the hair follicle site (Keckeis et

al., 2012). After sequential radiolabelled-glucocorticoid injections, radiolabelled and unlabelled glucocorticoid metabolites were analysed in urine, faeces and hair. Compared to faecal and urine samples, hair contained smaller quantities of radioactive substance and relatively large quantities of unlabelled glucocorticoid metabolites. This study suggests that radioactive glucocorticoids were metabolised and subsequently deposited in hair; however, the unlabelled cortisol present in the hair shaft indicates locally produced glucocorticoids. The study was based on eight guinea pigs and used a relatively short hair growth period of 7 days to establish the incorporation mechanism. A review on non-human hair cortisol studies concluded that there is currently insufficient evidence around the incorporation mechanisms into the hair shaft and further research is needed to ascertain to what extent hair cortisol is a reflection of systemically produced cortisol and to what extent locally produced cortisol (Burnard, Ralph, Hynd, Hocking Edwards, & Tilbrook, 2016). While local production of cortisol at the hair shaft might indeed play a role in endogenous cortisol concentration, the proposed pathways of incorporation and the correlations with cortisol in different body tissues support hair cortisol as an adequate measure of chronic systemic cortisol production.

It has been suggested that cortisol's main pathway of incorporation into the hair shaft occurs via the bloodstream. Figure 2.3 depicts these pathways graphically: Pathway A indicates incorporation via the bloodstream, pathway B via sebum and pathway C via sweat glands (Russell, Koren, Rieder, & Van Uum, 2012). As has been outlined above, the human body has abundant corticoid receptors, permitting cortisol its multifaceted actions. Cortisol is carried through the

bloodstream to multiple receptor sites and also to the hair follicles where it is permanently entrapped into the newly created hair shaft. Cortisol enters the stem cells via the blood capillaries at the germinal matrix surrounding the hair papilla and also subsequently the growing hair during keratogenesis; therefore getting sealed in the creating cells and also in the keratinised cells of the growing hair strand.

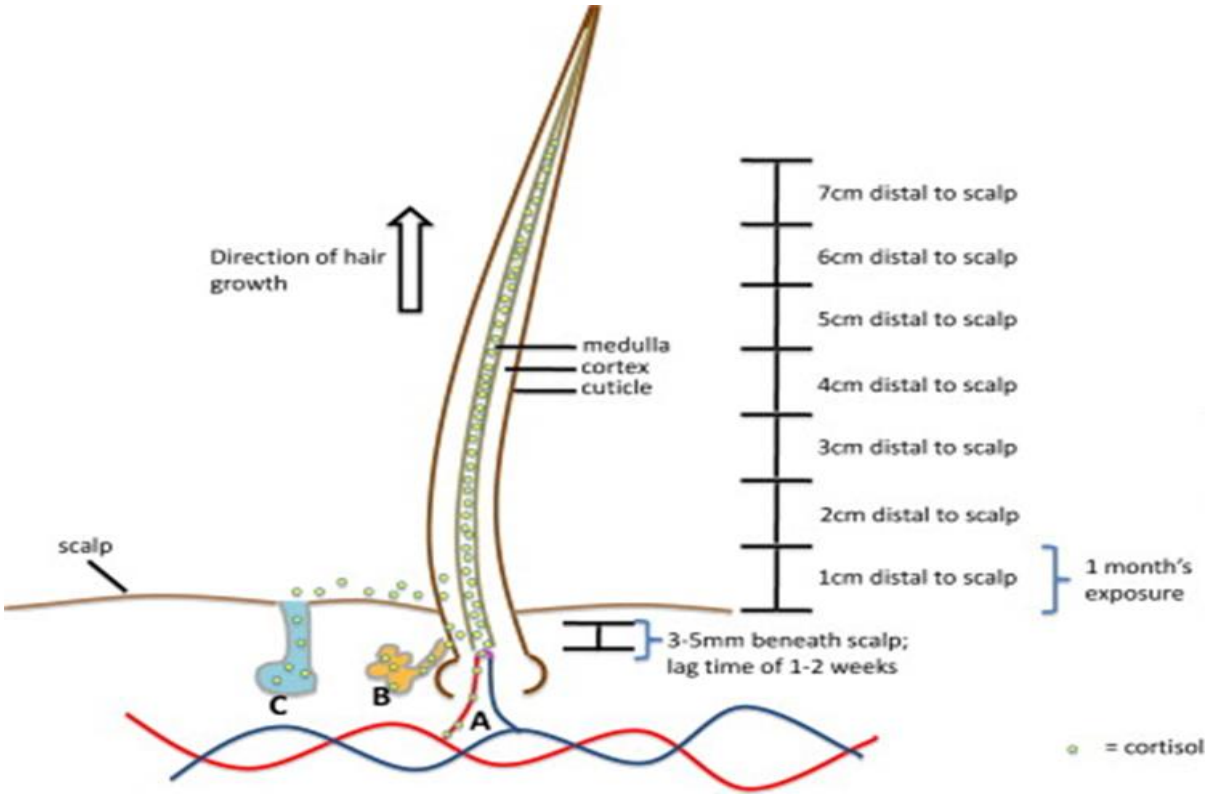

Figure 2.3 Suggested incorporation pathways of cortisol into the growing hair. From Russell et al. (2012).

The other two pathways (B and C) have been thought to be of less importance in the aetiological significance of cortisol content in hair. However, evidence in the last few years indicates that these might need to be investigated in more detail before assumptions about their exact contribution can be drawn. Indeed, several studies have shown that cortisol has been identified from human

eccrine sweat samples and that these levels correlate with salivary cortisol concentration (Grass et al., 2015; Jia, Chew, Feinstein, Skeath, & Sternberg, 2016; Russell, Koren, Rieder, & Van Uum, 2014). If the pathway for cortisol via sweat incorporation proves right and confirms a significant contribution, this would endorse sweating as a confounding factor for hair cortisol concentration. Two studies report on higher hair cortisol levels with increasing degree of physical activity, which might partly or indirectly be accounted for by increased sweating. In one study with 304 amateur endurance athletes and 70 controls, athletes had higher hair cortisol levels (Skoluda, Dettenborn, Stalder, & Kirschbaum, 2012). However, hair cortisol was also related to athletic training volume and the authors could not rule out psychological stress due to competitive races as a confounding factor. In another study with 46 university students, vigorous physical activity, as assessed by accelerometer data, was positively related to hair cortisol controlling for age, gender and perceived stress (Gerber et al., 2012). Whilst physical activity has been related to increases in circulating cortisol levels based on bodily fluids (Gatti & De Palo, 2011), a direct relationship seems plausible, yet the role of sweating in the contribution to endogenous cortisol levels in the hair strand remains somewhat unclear.

In fact, using an in-vitro experiment, Russell et al. (2014) showed that exposing hair to a solution that mimics sweat significantly increased hair cortisol concentrations in a time-dependent relationship, which was not affected by washing procedures prior to analyses. However, two independent in-vivo intervention experiments (one with and one without acute cortisol reactivity),

suggest hair cortisol is not affected by sweating (Grass et al., 2015). The first intervention induced sweating with a treadmill challenge in 42 regular runners, which lead to acute cortisol reactivity as observed by salivary cortisol measures. The second intervention induced sweating with a sauna bathing challenge in 52 regular adult sauna visitors in which acute cortisol reactivity was absent. Sweat cortisol was not related to hair cortisol concentration in either of the two interventions. However, the researchers measured the effect of the intervention on hair cortisol within one day only, assuming diurnal variation in the hair segment. It could indeed be the case that chronic sweating over a month might contribute to cortisol incorporation into the hair shaft and would need to be assessed in a systematic intervention study that controls for an applicable hair growth period.

2.6.2 Hair sampling and analytical methodology

The proportion of anagen to telogen hair and growth rate has been shown to differ according to anatomical bodily and also scalp sites (Pragst & Balikova, 2006). The posterior vertex position in the back of the scalp has been shown to have the lowest proportion of hair in telogen growth. Also this region has been identified as most constant regarding hair growth and as least influenced by age and sex (Villain, Cirimele, & Kintz, 2004). In fact, a study comparing hair samples from different head regions (the posterior vertex, nape, temporal, anterior vertex, and frontal sections) resulted in a high coefficient of variation of 30.5% in hair cortisol levels (Sauve, Koren, Walsh, Tokmakejian, & Van Uum, 2007). Comparing hair samples confined to the posterior vertex position resulted in a considerably

lower coefficient of variation of 15.6% (Sauve et al., 2007). Hence this region has been identified as most suitable for hair sampling.

Hair collection methods have long been established since the detection of substances in hair in 1979 (Baumgartner et al., 1979). However, the procedure of the analyses to determine cortisol levels were only developed in 2004 and studies on hair cortisol that have been subsequently published have based their analytical procedure on these (Raul et al., 2004). As previously mentioned, based on hair growth of approximately 1 cm per month, a hair segment of 1 cm closest to the scalp represents net cortisol output for the month preceding sample collection (Hayashi et al., 1991; Kirschbaum et al., 2009). The first and the majority of hair cortisol studies have analysed 3 cm hair strands which represent the average cortisol accumulated over an approximate timespan of three months prior to sampling. Later studies have performed segmental analyses with hair strands of only 1 cm in length.

The scalp hair strand is collected from the posterior vertex position. The number of strands collected is not as important; however, at least 10 mg ± 0.5 mg of hair needs to be available for the final analyses of the segment (Kirschbaum et al., 2009). The hair in the posterior vertex position is separated from the rest of the upper scalp hair. A hair strand is placed through a loop of cord which is locked as close as possible to the scalp. The hair strand is cut close to the scalp with fine medical scissors, and is placed onto aluminium foil, labelled with the identification number of the participant and stored in a dry, dark place. Several laboratories world-wide have established their own method to determine cortisol concentration

in hair resulting in different methodologies and variations between laboratories and ultimately leading to non-comparable levels of cortisol concentration. One laboratory analysed hair samples with different processing conditions and found that absolute levels of cortisol concentration differed remarkably among some, i.e. powdered vs. minced hair samples, extraction at 50°C vs. at room temperature) (Xiang, Sunesara, Rehm, & Marshall, 2016). Nevertheless, an international inter-laboratory round robin test, which is a standardised method of independent inter-laboratory testing, compared four leading laboratories in their measurements and analytical procedures to assess different ranges of hair cortisol concentration (low, intermediate, and high range) of the same hair samples (Russell et al., 2015). The different immunoassay and liquid chromatograph–mass spectrometry (LC–MS) methods across all four laboratories showed strong positive intercorrelation of hair cortisol levels and the study proposed a correction factor that can be applied if immunoassays methods are used to convert concentration levels into standard LC–MS equivalents.

The hair samples collected for the present PhD studies were all analysed by Kirschbaum's lab at the Technical University of Dresden, Germany. Figure 2.4 depicts the process of hair sample preparation, extraction and analysis. The wash procedure and steroid extraction is undertaken using high performance LC-MS, as described by Kirschbaum and colleagues (2009). Isopropanol solution is added to the cut hair segment and washed in two repeated cycles with the help of an overhead rotator, after which the hair is left to dry for 12 hours. Then, the hair segments are milled until powdered. Methanol is added to the hair powder in a vial

which is allowed to rotate with a microcentrifuge for 24 hours for steroid extraction. Thereafter, under nitrogen stream, the alcohol is evaporated and the samples are left to dry after which they are swirled in a phosphate buffer. Finally, with help of ELISA (enzyme-linked immunosorbent assay) and the LC–MS, cortisol content can be determined.

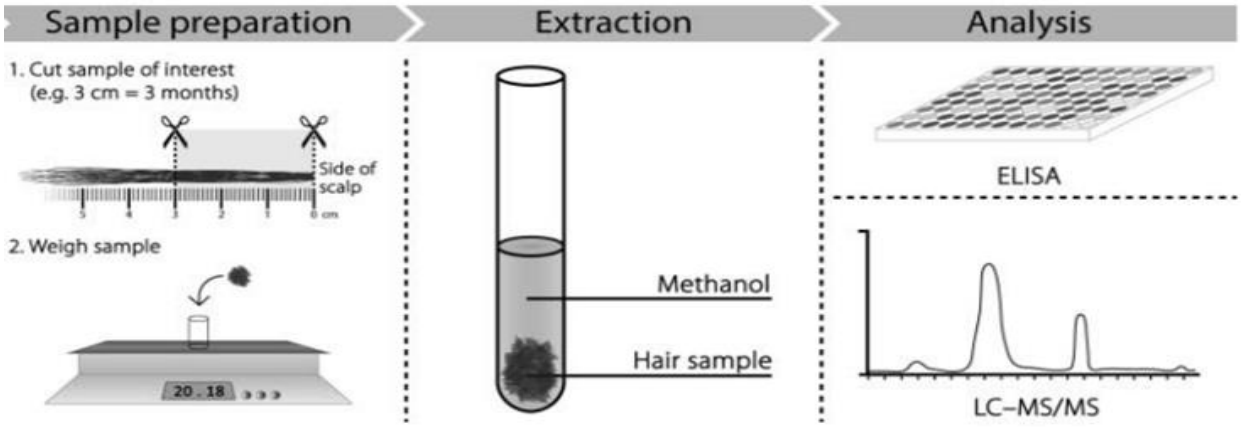

Figure 2.4 Overview of hair sample preparation, extraction and analysis. From Wester & van Rossum (2015).

2.7 Early validation studies

In 2004, the detection of endogenously produced glucocorticoids using liquid chromatography and mass spectrometry in hair by Raul et al. (2004) lead to the emergence of hair cortisol analyses. Following this quantification discovery, there has been much interest in evaluating and advancing the technique that allegedly could estimate long-term retention of cortisol exposure. Several research groups around the world are now contributing to this rapidly-growing research area. Hair cortisol has subsequently been promoted as providing novel

opportunities for research in the field of stress research. In an attempt to validate hair cortisol analysis as a measure of HPA-axis function and of chronic stress, studies have been conducted to compare hair cortisol against traditional specimens and to replicate previous findings that were based on these (Stalder, Steudte, Miller, et al., 2012).

2.7.1 Hyper-and hypocortisolemic conditions

Known endocrine disorders leading to extreme hyper- and hypocortisolemic conditions provided a great opportunity for using hair as a measure of long-term cortisol exposure. In one study the clinical course and treatment response of Cushing's syndrome could be observed in the relevant hair sections (Thomson et al., 2010). Using segmental hair analyses that provided historical information about the disease, the authors found that hair cortisol levels corresponded with severity of symptoms, being higher before and lower after tapering hydrocortisone therapy and treatments. In a different study, hair cortisol levels corresponded with symptomatic periods of cyclic Cushing's syndrome and hair analysis was suggested to aid the diagnosis of cyclic Cushing's syndrome, a condition characterised by alternating episodes of hypercortisolism and normal cortisol secretion (Manenschijn, Koper, et al., 2012).

With these findings, hair cortisol was not only recognised as a method of identifying aberrant endogenous cortisol levels (and thus HPA-axis activity) in patients with endocrine disorders but also to record the process of exogenous cortisol administration as through hydrocortisone replacement therapy. In fact, hair

cortisol analyses have been found to enable long-term screening and identification of potential over-substitution of hydrocortisone treatment in mitotane-treated adrenocortical cancer patients (Manenschijn, Quinkler, & van Rossum, 2014). This over-treatment of exogenous glucocorticoids has also been identified in patients with adrenal insufficiency, which might lead to increased morbidity and mortality risk if sustained (Gow, Koren, Rieder, & Van Uum, 2011). Later research has also supported hair as a useful monitoring specimen for glucocorticoid replacement therapy for different endocrine diseases such as congenital adrenal hyperplasia, characterised by severe hypocortisolism (Noppe, de Rijke, Koper, van Rossum, & van den Akker, 2016).

Another important finding on exogenously administered glucocorticoids and the effects on HPA-axis functioning comes from traditional cortisol assessments in relation to asthma which has recently been supported by hair cortisol analyses (Wlodarczyk, Gibson, & Caeser, 2008). Hair cortisol has been found to be lower in pre-pubertal children with asthma compared to matched controls (Kamps et al., 2014) and also in pregnant women suffering from asthma compared to non-asthmatic pregnant women (Smy, 2015; Smy et al., 2016). Asthmatic pharmacotherapy used as anti-inflammatory agents contains corticosteroids and supposedly lead to elevated cortisol content in the body. It has been suggested that prolonged use of asthmatic medication leads to increased risk of adrenal suppression/ insufficiency by adrenal fatigue, impeding the ability to produce sufficient quantities of cortisol, with potentially fatal consequences (Ahmet, Kim, & Spier, 2011). A study with 18 asthmatic children compared hair cortisol levels prior

and during asthmatic pharmacotherapy and reported inhaled corticosteroids to be related to lower hair cortisol concentration, indicating that HPA suppression might be associated with corticosteroid therapy (Smy et al., 2015). This highlights the clinical importance of hair cortisol analyses as a treatment monitoring tool; hair cortisol can aid controlling asthma symptomatology and treatment by increasing awareness and identification of vulnerable asthmatic patients.

Elevation in steroid hormone production during pregnancy is well known and this function favours, for example, lipogenesis and fat storage. The increase in production of cortisol across trimesters has also provided a good opportunity to investigate the time-related validity of hair cortisol analyses. Kirschbaum and colleagues (2009) were among the first to investigate the decline in cortisol in 103 women with a neonate child from the proximal to the more distal scalp hair segments. Their study found a difference in cortisol levels in all three 3cm hair segments during each trimester in pregnancy. The same findings were observed in a different study on pregnant women, in which hair cortisol levels from the third trimester were higher than the second and the first trimester, while post-partum hair cortisol levels were again lower than in the third trimester (D'Anna-Hernandez, Ross, Natvig, & Laudenslager, 2011). Another study found a positive association between hair cortisol concentration and gestational age in 117 pregnant women (Kramer et al., 2009). These findings were corroborated in a later study with 90 women, in which hair cortisol levels most strongly predicted preterm birth, controlling for covariates such as gestational age, perceived stress during pregnancy and physiological pregnancy-related factors (Hoffman, Mazzoni, Wagner,

Laudenslager, & Ross, 2016). This study demonstrates the potential of this analytical method for identification of preterm birth and potential targets for interventions to attenuate stress-related physiological responses in pregnancy. Hair cortisol's utility has also been demonstrated in predicting clinical pregnancy in 88 women undergoing in-vitro fertilisation treatment whilst salivary cortisol has not been a significant predictor variable (Massey et al., 2016).

Based on findings from the traditional specimens, age has been positively associated with cortisol and positive associations would be expected from a measure of long-term cortisol exposure. Whilst the findings are not consistent, most of the null findings are due to small sample sizes and age not being the main interest of investigation. Nevertheless, studies with appropriate age ranges (1–91 years) support a positive association between age and hair cortisol levels (Dettenborn, Tietze, Kirschbaum, & Stalder, 2012; Feller et al., 2014; Raul et al., 2004; Staufenbiel et al., 2013). These associations are of dual importance: as evidence for age-related HPA-axis functioning in relation to mental and physical health, and also as a confounding factor in between- and within-subjects designs.

2.7.2 Comparison studies

The most direct validation of hair cortisol analysis has been derived from direct comparisons between cortisol measures from hair and saliva (but also urine) to establish whether hair cortisol indeed reflects integrated cortisol output over a prolonged time period. A crucial factor for the validity and usefulness of segmental hair cortisol analyses is to understand which aspect of HPA-axis activity is presented

by hair cortisol. As outlined above, it is still not fully understood how cortisol incorporates into the hair shaft, and whether production of hair cortisol merely reflects the same HPA-axis processes as those observed in salivary and sebum cortisol production or only to a certain extent. Specifically, to some extent hair cortisol might indeed reflect adrenal cortex secretion but hair cortisol values might be distorted by intrafollicular cortisol production. Correlational studies between different cortisol specimens have tried to investigate this issue. While the absolute concentration in the two tissues is not comparable, between-person correlations can establish whether individuals show similar ranking of levels in hair and saliva.

Studies have been conducted sampling both salivary and hair cortisol concurrently or over corresponding time-intervals. Most of the early studies sampled cortisol concurrently in the different tissues, i.e. saliva/ blood/ urine cortisol were taken at the same time point or within the same week, thus the body fluid cortisol levels did not represent the same time period as reflected in the analysed hair segment. Although, many of these studies do not specify the time window in which the two sampling points took place, it is evident that they were not sampled so that the two tissues reflect the same period of cortisol exposure. Only a few studies have sampled both tissues in a more systematic design, in which salivary cortisol mirrors the same time period as hair cortisol. Table 2.1 (ordered by method of assessment and publication date) depicts the comparison studies that have been conducted in humans between salivary, blood and urine cortisol and hair cortisol. Mixed findings have emerged in both types of approaches, in concurrent assessment and also in systematic assessment of corresponding time-intervals,

however, the latter comprises a more rigid methodology and results seem to be more consistent in this approach.

Table 2.1 Comparison studies between salivary, blood and urine cortisol and hair cortisol.

Author (Year)	Sample	Method a) Concurrent vs corresponding time-intervals b) Saliva sampling c) Salivary cortisol calculation d) Hair sampling	Findings
Sauve et al. (2007)	39 adults (aged 20-76)	a) Concurrent assessment b) 1 saliva sample (7.30-10am) 24-hour urine cortisol 1 blood sample (7.30-10am) c) One value per tissue d) 1 hair sample of 1 cm	HCC x morning SCC n.s. HCC x 24-hour UCC r = 0.33; *p* = 0.041 HCC x PCC n.s.
Steudte et al. (2011)	15 GAD patients and 15 controls (aged 25-45)	a) Concurrent assessment b) 6 saliva samples (at awakening, +30 min, 12pm, 16pm, 8pm and bedtime) on 2 consecutive weekdays c) 2-day mean values (summing all 12 saliva samples to calculate mean) and mean AUCg d) 3 hair samples of 3cm each, but correlational analyses based on first 3cm segment	HCC x SCC AUCg on combined group n.s.
Vanaelst et al. (2012)	39 children (aged 5-11)	a) Concurrent assessment b) 4 saliva samples per day (at awakening, +30 min, +60min, 7-8pm) on 2 consecutive weekdays c) AUC of CAR (awakening to +60 min) and cortisol decline (evening sample – awakening sample) d) 1 hair sample of 6cm	HCC x SCC decline n.s. HCC x +30 min SCC r = 0.398, *p* = 0.022 HCC x SCC AUC of CAR r = 0.398, *p* = 0.024

Author (Year)	Sample	Method a) Concurrent vs corresponding time-intervals b) Saliva sampling c) Salivary cortisol calculation d) Hair sampling	Findings
van Holland et al. (2012)	29 production workers (aged 36-56)	a) Concurrent assessment b) 6 saliva samples per day (9am, 11am, 1pm, 3pm, 5pm, 8pm) over a 3-day period c) 3-day mean value (summing all 18 saliva samples to calculate mean) d) 1 hair sample of 3 cm	HCC x mean SCC r = 0.41, *p* = 0.03
Ouellette (2015)	60 children (aged 7 at follow-up)	a) Longitudinal assessment (SC at age 3, HCC at age 7) b) 7 saliva samples during induced stress period between 12-3.30pm (30min prior to stress task, immediately prior to stress task, +10 min, +20min, +30min, +40min, and +50min post-stressor c) AUC for stress reactivity d) 1 hair sample of 3cm	HCC x AUC stress reactivity r = 0.30, *p* = 0.021
Flom et al. (2016)	Infants (aged 1 year)	a) Concurrent assessment b) 2 saliva samples per day (at awakening, at bedtime) over a 3-day period c) Averaged waking value, bedtime, value, slope, AUC d) 1 hair sample of 3cm	HCC x Waking SCC r = 0.304, *p* < 0.01 HCC x Bedtime SCC r = 0.529, *p* < 0.001 HCC x AUC r = 0.454, *p* < 0.001 HCC x Slope n.s.

Author (Year)	Sample	Method a) Concurrent vs corresponding time-intervals b) Saliva sampling c) Salivary cortisol calculation d) Hair sampling	Findings
D'Anna-Hernandez et al. (2011)	21 pregnant women (ages 18-45)	a) Corresponding time-interval b) 3 or 6 sampling days with 3 samples (+30min post-awakening, before lunch, and +10 hours post-awakening) c) AUC d) 4 hair samples of 3cm each from 3 trimesters (at 15, 26 and 36 weeks gestation) and 3 months postpartum.	HCC x AUC SCC of 6 days second trimester: r = 0.43 third trimester: r = 0.57; both *p*'s < 0.05
Xie et al. (2011)	32 male graduates (aged 22–30)	a) Corresponding time-interval b) 1 saliva sample (+30 min post-awakening) at 3 time points in one-week intervals over a period of a month c) Highest peak value (+30 min post-awakening) per time point (1/2/3) and mean highest peak value (summing all 3 time points) d) 1 hair sample of 1 cm	HCC x SCC (+30min) time 2 r = 0.399, *p* < 0.05 HCC x mean SCC (+30min) r = 0.383, *p* < 0.05
Van Ockenburg et al. (2016)	10 adults (aged 21-58)	a) Corresponding time-interval b) 24-hour urine samples for 63 consecutive days (2 months) c) Mean values for 1st month and for 2nd month d) 1 cm hair sample after 2nd month analysed in two 1cm segments representing 1st and 2nd months, retrospectively	HCC1 x UCC time 1 r= 0.467, *p* = 0.060 HCC2 x UCC time 2 r= 0.200, *p* = 0.421 Mean HCC x Mean UCC r= 0.422, *p* = 0.089

Author (Year)	Sample	Method a) Concurrent vs corresponding time-intervals b) Saliva sampling c) Salivary cortisol calculation d) Hair sampling	Findings
Short et al. (2016)	17 adults (21-53)	a) Corresponding time-interval b) 3 saliva samples (at awakening, +30 min post-awakening, at bedtime) daily over a period of 30 days, with objective time assessment c) Monthly average of three salivary cortisol parameters (summing all 30 daily parameters): AUC, CAR, Slope d) 1 hair sample of 1 cm	HCC x mean SCC (AUC) r = 0.61, *p* = 0.01 HCC x SCC (AUC prior 4 weeks) r = 0.56, *p* = 0.02 HCC x SCC (AUC prior 3 weeks) r = 0.50, *p* = 0.04 HCC x SCC (AUC prior 2 weeks) r = 0.40, n.s. HCC x SCC (AUC prior 1 week) r = 0.38, n.s. HCC x mean SCC (CAR) r = 0.37, n.s. HCC x mean SCC (Slope) r = 0.35, n.s. HCC x mean UCC r = 0.27, n.s.

Note. HCC = hair cortisol concentration; SCC = salivary cortisol concentration; PCC = plasma cortisol concentration; UCC = urinary cortisol concentration; AUC = area-under-the-curve; Pearson's r value is given to 3 decimal places where possible, to allow accurate comparison.

The first correlational studies were based on concurrently assessed cortisol levels from the tissues, i.e. salivary cortisol not representing the same time period as reflected in the analysed hair segment. One early study with 39 healthy middle-aged adults found a positive moderate correlation between hair and 24-hour urine cortisol levels (r = 0.33), but not between concurrently measured hair and salivary cortisol levels, nor between hair and serum cortisol (Sauve et al., 2007). However, the null associations are not surprising given that a single sampling point (e.g. sampling between 7.30am and 10am) was used to obtain saliva/ serum cortisol levels. Another study with 39 school-aged girls found a positive moderate correlation (r = 0.39 and r = 0.39) between concurrently measured hair and salivary AUC of the CAR and the highest peak value (30 minutes post-awakening), respectively, but no association between hair and serum cortisol levels (Vanaelst et al., 2013). Again, the blood sample was based on a single morning sample point, while the salivary cortisol measure was derived from a 2-day sampling period. Another study with 29 production workers, using a 3-day salivary sampling period with 6 daily samples, also reported a positive slightly stronger association (r = 0.41) between mean cortisol levels and hair cortisol (van Holland, Frings-Dresen, & Sluiter, 2012). A study by Steudte et al. (2011) with 15 GAD patients and 15 controls reported no association between two-day salivary mean values and hair cortisol (Steudte, Stalder, et al., 2011). However, their correlational analyses were based on the combined sample of patients and controls with significantly different hair cortisol concentrations, apart from having a relatively small sample size. A recent study with 60 infants aged 12 months analysed 2 daily saliva samples over a

three-day period and hair cortisol (Flom, St John, Meyer, & Tarullo, 2016). The authors found that the average waking value, bedtime value and the AUC based on these two measures correlated positively with infant's hair cortisol levels; surprisingly, the correlation coefficient with the bedtime value reached r = 0.529. The fact that infants' values seem to correlate relatively high with a single value of the diurnal cortisol profile might point towards lower variability at this younger age and endorses the role of HPA-axis changes throughout the life span (Almeida, Piazza, & Stawski, 2009b; Jessop & Turner-Cobb, 2008).

Even though cortisol samples from hair and saliva reflect different time periods in studies based on the concurrent assessment method, positive moderate relationships have been found in the two tissues. Studies that have systematically compared hair and salivary cortisol samples obtained over a corresponding time-interval have produced similar strength coefficients. A saliva-hair cortisol correlation analysis in 32 healthy young men analysed three salivary cortisol samplings methodically in one-week intervals over a period of a month with a 1 cm hair segment taken at the end of the month (Xie et al., 2011). They found a significant correlation between salivary and hair cortisol levels at just one of the three sampling time points (time point 2: r = 0.398), and also when using an average score from all three sampling time values (r = 0.383). Surprisingly, the association was based on salivary cortisol concentration from a single sampling 30 min. post-awakening without controlling for baseline waking cortisol levels. Although reflecting the early morning diurnal zenith, which theoretically presents a strong indicator for accumulated long-term cortisol output in hair, the AUC is a

more robust indicator of total diurnal salivary cortisol, and associations might be stronger between hair and salivary cortisol if salivary cortisol is based on diurnal profile measurements.

Another study with 21 pregnant women sampled salivary cortisol in each trimester and 3 months postpartum and hair cortisol (3 cm) at the end of all four periods, i.e. 1st, 2nd, 3rd trimester, 3months postpartum (D'Anna-Hernandez et al., 2011). In each period, saliva sampling occurred on three (1st trimester, 3 months postpartum) or six sampling days (2nd and 3rd trimester; 3 sampling days twice within each trimester with a 4-5-weeks interval). Three samples were taken over a sampling day and the AUC was calculated. The authors found that only those salivary samples that were repeatedly taken with AUC computations based on 6 salivary sampling days, as opposed to 3 sampling days, significantly correlated with cortisol extracted from the relevant hair section (2nd trimester: r = 0.43; 3rd trimester: r = 0.57). Hellhammer and colleagues (2007) highlighted that it needs up to 5 sampling days to provide a reliable indicator of total cortisol exposure. However, recent studies indicate that this is more relevant for cortisol parameters such as the CAR and the diurnal slope rather than the AUC (Golden et al., 2014; Ross et al., 2014). The fact that only the 6-sampling-day salivary AUCs were associated with their respective cortisol output as captured in hair, supports Hellhammer et al.'s (2007) conclusions and reinforces the notion that the salivary cortisol pattern is most robustly reflected with several sampling days. In light of saliva-hair cortisol associations, considering that the AUC is the most relevant in terms of total daily output, this methodological aspect of saliva sampling is of high

importance and could elicit stronger associations if conducted properly. More systematic designs based on healthy individuals could yield even stronger direct positive associations.

A recent study that systematically sampled 24-hour urine for 63 consecutive days in 10 adults and hair cortisol in two segments representing the same two months, only revealed a marginally significant positive moderate associations (r = 0.467 and r = 0.422, respectively) between the two tissues in the first month and also when averaging the 2-months urinary cortisol and hair cortisol accordingly (van Ockenburg et al., 2016). While the study was underpowered, it highlights that only a small proportion of the variability in hair cortisol can be explained by systemic urinary cortisol and many other factors that contribute to this gap still need to be explored. A methodologically strong study is the recent study by Short et al. (2016). The authors assessed 3 daily salivary cortisol samples in 17 adults over a period of 30 days and four weekly 24-hour urinary samples and took a 1 cm hair segment after this period. From these 3 daily salivary measures they computed the three distinct parameters, the CAR, the AUC and the slope. They found that hair cortisol was positively associated with the monthly averaged salivary AUC values (r = 0.61) and also with the prior 3 and the 4 weekly averaged AUC values (r = 0.50 r = 0.56, respectively) but not with the prior 1 and 2 weekly averaged AUC values and not with averaged urinary cortisol levels. The integrated monthly salivary AUC measure showed the strongest correlation with hair cortisol concentration, which further supports the cumulative nature of hair cortisol content. This study has a small

sample size but reports in a systematic design the highest correlation coefficient up to date.

Lastly, one longitudinal study with 60 children aged 7 assessed the association between stress reactivity (AUC) in a controlled stress-induced task (time-pressured matching task) and hair cortisol levels measured 3-years later (Ouellette et al., 2015). The findings revealed that higher stress reactivity correlated with higher hair cortisol levels at follow-up. While this study assessed stress reactivity rather than diurnal levels, it was still deemed highly relevant in relation to the salivary-hair cortisol relationship as these findings highlight a degree of stability in stress-related HPA-axis functioning in relation to long-term cortisol secretion. One aim of the present PhD thesis is to investigate whether individual differences in the different aspects of the salivary diurnal cortisol profile (total output indexed by area-under-the-curve, the diurnal slope and the cortisol awakening response) show long-term associations with hair cortisol concentration in an adult population. This will be outlined and explored in Chapter 4 using Study I and II.

An important issue in the correlational studies based on corresponding time-intervals is ensuring that the hair is allowed to grow appropriately. Specifically, it has been suggested that a time lag effect of hair growth occurs (LeBeau, Montgomery, & Brewer, 2011). Reviewing the literature on drug incorporation methods and analyses, these authors concluded that new hair takes around 2 weeks to be formed in the follicle and to appear at the hair shaft. Thus, hair cortisol analyses that examine corresponding time intervals between salivary cortisol and hair cortisol would need to allow for these 2 weeks for permitting corresponding

hair growth. This study also assessed the ability of 14 individuals with different levels of experience of hair sampling (ranging from novices to experts) to uniformly cut and obtain hair and concluded that on average a section of approximately 0.8 ± 0.1 cm of hair (equivalent of a period of 3 weeks of corresponding hair) was left after cutting closest to the scalp, even for the expert hair collectors. Therefore, the exact time frame that is measured from the saliva sampling may not accurately be reflected in the hair segment. In particular, segmental hair analysis could suffer from this methodological bias if retrospective stressors are taken into account in an attempt to be 'mapped' on to the corresponding hair segment.

None of these correlational studies have taken this time lag effect into account. Employing a design for a correlational analysis in which salivary cortisol is repeatedly assessed within a time frame that the hair section covers, by allowing appropriate hair growth (of 2-3 weeks after last salivary sampling) and a careful hair cutting method, could potentially help to overcome this timing issue. Further, it would be of interest to investigate the associations between salivary and hair cortisol in two different periods of stress exposure as individual psychosocial factors might play a major role in affecting fluctuating salivary cortisol levels throughout the assessed month. Comparing the associations within the same individuals in a systematic correlational design, using corresponding time intervals, in a period under high stress and under relatively low stress might be informative in relation to the strength of the variability and correlation. Such a methodological study design is the aim in Study II which will be outlined in Chapter 5.

2.8 Disease-related findings

Hair cortisol has also been associated with various diseases, risk factors for disease and also with certain health conditions. Whilst these findings are no validation for hair cortisol per se, they highlight the usefulness and the value of hair cortisol and its relevance in health research.

2.8.1 Cardiometabolic parameters and CVD

As outlined above, cortisol's involvement in blood pressure regulation, glucose and lipid metabolism, and immune responses makes it impossible to negate its role in certain risk factors for CVD, such as hypertension, adiposity and insulin resistance (Girod & Brotman, 2004). Elevated hair cortisol levels have been linked with adverse cardiometabolic parameters, such as elevated glycated haemoglobin, in 1258 aerospace employees (Stalder et al., 2013). Diabetes has also been associated with elevated hair cortisol levels in 654, in 760 and in 3507 middle-aged adults, respectively (Abell, Shipley, et al., 2016; Feller et al., 2014; Staufenbiel, Penninx, de Rijke, van den Akker, & van Rossum, 2015). With regards to the development of weight-related diseases, the central indicators of disease risk are waist circumference, which estimates fat topography, and body mass index. Hair cortisol levels have been positively associated with waist circumference, waist-to-hip ratio and body mass index in distinct studies with children and adults (Manenschijn, Koper, Lamberts, & van Rossum, 2011; Papafotiou et al., 2016; Stalder, Steudte, Alexander, et al., 2012; Wester et al., 2014).

As with traditional cortisol specimens the findings have not been consistent (Kirschbaum et al., 2009; Manenschijn, Koper, et al., 2011). Nevertheless, methodologically strong studies have repeatedly found more robust associations. Hair cortisol has been shown to be elevated with markers of metabolic syndrome, i.e. triglycerides, systolic blood pressure and waist circumference (Chan, Sauve, Tokmakejian, Koren, & Van Uum, 2014; Kuehl et al., 2015; Stalder et al., 2013). Interestingly, one such study assessed hair cortisol and the CAR from saliva sampling simultaneously in 41 healthy middle-aged adults and 44 patients with major depression (Kuehl et al., 2015). In both groups it was found that these two biomarkers associate with criteria for the metabolic syndrome in opposite directions: hair cortisol was positively associated with certain markers (e.g. triglycerides and systolic blood pressure), while the CAR was negatively associated with blood pressure. This is congruent with previous literature in which blunted cortisol activity in the morning is related to metabolic risk factors (Lasikiewicz, Hendrickx, Talbot, & Dye, 2008). Yet, most importantly, it highlights the usefulness of exploring short-term and long-term cortisol secretion simultaneously (as with saliva and hair samples, respectively).

Furthermore, hair cortisol levels have been found to be positively related to heart failure severity and functional limitations in 44 congestive heart failure patients (Pereg et al., 2013). Research studying the mechanisms of acute cardiovascular events indicates that psychological and physiological stressors play a role in triggering the onset of such events (Tofler & Muller, 2006). Studies employing hair cortisol could potentially further elucidate the effect of acute versus

long-term stressors on cardiac functioning, as retrospective evaluation of stressors are prone to bias, with previous stressors being over- or underestimated (Dimsdale, 2008). Studies have reported independent associations between salivary cortisol and cardiovascular mortality, such as a flatter diurnal slope being related to a 1.87-fold increased risk in 4047 civil servants (Kumari et al., 2011). A cross-sectional study with 283 elderly participants found that hair cortisol levels were positively associated with history of CVD but not with other chronic non-cardiovascular diseases such as lung disease, cancer and osteoporosis (Manenschijn et al., 2013). A prospective case control study, comparing 56 acute myocardial infarction patients at admission with 56 patients hospitalised for other reasons, found that hair cortisol concentration before admission (as seen retrospectively in the 3-cm hair segment taken at admission) was the strongest predictor for acute myocardial infarction, after controlling for known risk factors for the disease (i.e. age, cholesterol, BMI, smoking status, previous occurrence of myocardial infarction) (Pereg et al., 2011). These new studies employing hair specimens provide important insights into the development of heart disease, its rehabilitation process and the possible role of chronic stress. Nevertheless, some studies are methodologically weak and need to control for important confounding factors.

2.8.2 Pain

Chronic pain patients often show higher levels of salivary cortisol than control groups (Vachon-Presseau et al., 2013). This hyperactivity of the HPA-axis relating to pain has also been found in the hair cortisol literature. Compared with

matched controls (N = 39), chronic pain patients (N = 15) had elevated levels of hair cortisol that corresponded with their perceived stress caused by their condition (Van Uum et al., 2008). This association with pain was supported by another study in a sample of acute trauma patients, where reported pain catastrophizing (an indicator of distress caused by the pain) was also linked to elevated hair-normalised salivary cortisol waking responses (Walton, Macdermid, Russell, Koren, & Van Uum, 2013). However the authors did not report any associations between pain symptomatology and hair cortisol levels, but rather with 'hair-normalised' salivary cortisol. Hair-normalised salivary cortisol, calculated by a ratio of salivary cortisol to hair cortisol levels, has been suggested by the authors as an indicator of state to trait HPA-axis activity as a ratio (salivary to hair cortisol, retrospectively). The lack of association between pain and hair cortisol is unclear, however, the fact that hair-normalising the CAR strengthened the association between pain catastrophising and salivary cortisol suggests that this combined index may be a more robust indicator of state HPA-axis activity. However, this index measure has not been evaluated and used in the literature prior to this study and therefore warrants further research. Overall, there is some evidence for pain to be related to elevated hair cortisol levels, however, methodologically stronger case-control studies with bigger sample sizes and inclusion of important covariates are needed that enable more reliable conclusions.

2.8.3 Psychiatric disorders

There is increasing interest in using hair cortisol analyses in populations with psychiatric disorders; an approach that is particularly convenient for assessing the longitudinal clinical course of such disorders. Findings have been inconsistent when using cortisol samples taken from other body fluids, i.e. saliva or blood samples, in relation to psychiatric disorders such as depression (Harris et al., 2000). However, findings in the hair cortisol literature with psychiatric disorders are also mixed. Dettenborn and colleagues (2012) have found increased hair cortisol content in 23 medicated clinically depressed patients compared to 64 age- and sex-matched healthy controls. A large population-based cohort (the Whitehall II cohort) with 3507 middle-aged to older adults also reported positive associations between depressive symptoms and hair cortisol concentration, controlling for medication and physical disease (Abell, Shipley, et al., 2016).

Another study assessed hair cortisol levels in 13 recurrent and 22 first-episodic patients with depression and 30 controls (Wei et al., 2015). They found that first-episodic patients had higher hair cortisol levels during the disease episode than recurrent patients and controls, despite there being no baseline differences prior to the disease episode. These results suggest that disease episodes and onset of disease might drive cortisol secretion differently. Surprisingly, recurrent depressed patients had similar hair cortisol levels to controls. Further, severity of the disorder and number of past episodes did not associate with hair cortisol. However, this study did not differentiate between medicated and non-medicated patients. A medication-induced HPA-axis dampening has been reported in

depressed patients (Pariante & Lightman, 2008; Vythilingam et al., 2004), which makes assessing patient's medication a critical factor. Another study of 121 CHD patients found no difference in hair cortisol between those who were depressed and non-depressed (Dowlati et al., 2010). However, the time of cardiac event between the groups differed by about a month, and this was not included as a covariate in the analyses. Furthermore, a proportion of the depressed patients also reported the use of concomitant psychotropic medications and seemed to have comorbid anxiety disorders, which might affect possible relationships with hair cortisol. This potential comorbidity-driven impact is in line with previous research with the traditional cortisol specimens, in which comorbid depressed-anxious patients show different patterns of salivary cortisol secretion (Young et al., 2004). The depression-cortisol relationship has been shown to differ depending on the intensity, onset and episode frequency of the disease (Cowen, 2002; Hardeveld et al., 2014; Mangold et al., 2011). Together, these might present potential overlooked aspects that might lead to heterogeneity in findings.

Consistent with this, a study with bipolar disorder patients found no differences in hair cortisol compared to healthy controls, but hair cortisol levels were higher in those diagnosed with psychiatric comorbidities (Manenschijn, Spijker, et al., 2012). Hair cortisol was differentially associated with disease onset. Elevated hair cortisol was observed in patients with a late-onset of disease, suggesting aetiologic heterogeneity in bipolar disorder. This is in line with a review on onset age in bipolar disorders that report onset of disease to predict homogeneous sub-groups of bipolar disorder patients (Leboyer, Henry, Paillere-

Martinot, & Bellivier, 2005). Their clinical course and symptomatology was differently influenced by pathogenic and genetic influences at different ages, which genetic factors playing a stronger role for early onset and stressful experiences for late onset of the disease.

A later study reported on higher hair cortisol levels in bipolar disorder patients compared to controls with a corresponding positive relationship between manic symptoms and hair cortisol (Streit et al., 2016). Using data from the Psychiatric Genomics Consortium, this is in fact the first to report an association between genetic risk for bipolar disorder and hair cortisol in healthy controls. This link between genetic risk and elevated hair cortisol proposes the effect of HPA-axis dysregulation to be present before the disorder is manifested. Whilst this substantiates the difference in pathogenesis of bipolar disorder by onset of disease, it also supports the notion that sustained HPA-axis dysregulation is involved in the aetiology of the psychiatric disorder as opposed to resulting from its manifestation. Nonetheless, the cause-effect relationship and direction in several psychiatric disorders remains uncertain and longitudinal studies using genomic data and hair cortisol might allow for more practical investigations of potential cause-effect relationships. The experience of negative life events impacts the course of bipolar disorder and this relationship might be to some extent mediated by cortisol (Havermans, Nicolson, Berkhof, & deVries, 2011). Recent negative life events have been shown to be related to elevated hair cortisol in 71 bipolar disorder patients (Staufenbiel et al., 2014). Interestingly, social support was also assessed which attenuated this association, thus supporting the buffering effect of social support.

The findings relating to PTSD and hair cortisol are somewhat mixed but revealing. An early study found higher levels in 10 traumatised individuals with post-traumatic stress disorder (PTSD) compared to 17 traumatised individuals without PTSD (Steudte, Kolassa, et al., 2011), a result that is rather inconsistent with salivary cortisol findings (Wessa, Rohleder, Kirschbaum, & Flor, 2006). In a later study, Steudte and colleagues (2013) found lower hair cortisol levels in both PTSD and non-PTSD traumatised participants when being compared to controls. A study with 43 women found that childhood sexual abuse was related to lower salivary cortisol reactivity and elevated hair cortisol but that number of childhood traumatic experiences and depression seem to play a role in driving this relationship (Schalinski, Elbert, Steudte-Schmiedgen, & Kirschbaum, 2015). Also in 70 community-based healthy children, hair cortisol content was positively related to the number of lifetime exposures to traumatic events such as accidents, crime, bereavement, mother-child separations, domestic violence and abuse (Simmons et al., 2016). In another cross-sectional study with a sample of 84 middle-aged healthy adults, reported history of childhood maltreatment (such as emotional, sexual, physical abuse or neglect) predicted lower hair cortisol concentrations in adulthood (Hinkelmann et al., 2013). In line with this, childhood adversity (abuse, neglect or household dysfunction) was associated with lower hair cortisol levels in 55 healthy college students (Kalmakis, Meyer, Chiodo, & Leung, 2015).

As already suggested by salivary cortisol analyses, trauma type, exposure and duration present potential confounding factors that might affect relationships with the biomarker. Interestingly, however, with its longitudinal nature, hair

cortisol seems more suitable to track individual's development of PTSD, because baseline levels prior to the traumatic event can be reliably assessed. A study found the experience of a disaster (an earthquake in China) to be detectable in hair, with higher cortisol levels in 32 traumatised individuals with PTSD and in 32 traumatised individuals without PTSD, compared to 20 controls (Luo et al., 2012). This elevation in hair cortisol in both groups seems to suggest a proper response to stress. However, further segmental hair analyses revealed that among individuals experiencing the disaster hair cortisol content was different in the long-term (several month after the disaster). Those that suffered subsequently from PTSD compared with individuals not developing PTSD had lower hair cortisol levels in the hair segments of 2-4 months (3 month hair segment) and 5-7 months (3 cm hair segment). Segmental hair cortisol analyses in relation to certain outcomes have not been investigated in detail. Methodological factors such as the well-known 'wash-out' effect of the hair segment (decreased hair cortisol content after a 6 cm hair segment) (Dettenborn, Tietze, Bruckner, & Kirschbaum, 2010; Gao et al., 2010; Vanaelst et al., 2013) would need to be taken into account; in fact, this study showed this decreased effect in the whole study sample (each of the three groups). Whilst segmental analyses needs to be interpreted with caution, this study might potentially provide some first insights into the development and potential long-term HPA-axis functioning in PTSD. This long-term blunted response has long been suggested to result from an exhaustion of the HPA-axis activity (Miller et al., 2007). Interestingly, in a study with 90 male soldiers, PTSD symptomatology after military deployment could be predicted by lower hair cortisol levels and lower salivary

cortisol stress reactivity, suggesting that attenuated HPA-axis functioning might be a risk marker for subsequent PTSD development (Steudte-Schmiedgen et al., 2015). Whilst this is at odds with previous findings that baseline cortisol levels (prior to traumatisation) seem to be similar to controls, if substantiated this might give insights into HPA-axis driven vulnerability factors. Inconsistencies in the trauma-hair cortisol relationship (similar to traditional cortisol specimens) seem to be related to the type and severity of the trauma and time since traumatisation (Schreier et al., 2016; Steudte-Schmiedgen, Kirschbaum, Alexander, & Stalder, 2016; Vives et al., 2015). Segmental hair analyses highlight the potential of hair cortisol to elucidate certain unclear or inconsistent associations that have prevailed.

Another study reported that 24 psychosis patients had higher hair cortisol levels compared with 27 healthy controls (Andrade et al., 2016). Additionally, segmental hair analyses in patients revealed that segments prior to hospitalisation were associated with severity of psychopathology, e.g. disorientation and ambivalence. These findings corroborate findings based on salivary cortisol measurements; reviews have reported consistently higher salivary cortisol levels in patients at clinically high-risk for psychosis with salivary cortisol that correspond to increased symptom severity and progression (Karanikas & Garyfallos, 2015; Walker et al., 2013). The retrospective nature of hair cortisol analyses aids in understanding the disease aetiology as findings indicate that HPA-axis abnormalities were already present prior to disease manifestation, indicated by an elevation prior to psychotic conversion.

Hair cortisol has also been associated with addictive disorders (substance use). For example, heavy ecstasy users, but not light ecstasy users, had significantly elevated hair cortisol compared to controls/ non-users (Downey et al., 2015). Also, heroin abusers were found to show elevated hair cortisol levels compared to matched controls (Xie et al., 2016). A further study confirmed this finding; heroin addicts on methadone maintenance had higher hair cortisol levels than healthy matched controls (Yang et al., 2016). However this relationship was explained by perceived stress and symptoms of anxiety and depression. Another study also reported the experience of stressful life events prior to admission in treatment-seeking drug-addicts to be positively associated with hair cortisol levels (Grassi-Oliveira et al., 2012). Alcoholics in an acute withdrawal phase were found to have elevated hair cortisol levels when compared with abstinent alcoholics and controls (Stalder et al., 2010), mirroring evidence from salivary cortisol research (Badrick et al., 2008). Further studies need to confirm to what extent HPA-axis stimulation, and hence higher cortisol content, is caused by substance use itself or by the psychosocial elements (stress and anxiety) related to the addictive condition and withdrawal aspiration. A study including 30 adolescents with mental health problems (depression, anxiety, bipolar disorder, psychosis, suicidality, long-term care need) found elevated hair cortisol levels compared with 27 matched controls, controlling for psychiatric medication (Heinze, Lin, Reniers, & Wood, 2016). While the clinical sample reported significantly higher levels of perceived stress, subjective stress experience did not moderate this relationship. Hair cortisol was not related to any of the clinical measures taken with regard to the mental

disorders. However, this study comprised a heterogeneous and comorbid sample of varying mental disorders and might therefore lack power due to small sub-group sizes. The findings on perceived stress or stressful life events and hair cortisol are somewhat inconsistent. A stronger relationship has repeatedly emerged in clinical settings, whilst the relationship in healthy individuals is somewhat unclear; findings that will be outlined in the following section.

2.9 Associations between hair cortisol, stress-related and psychosocial factors in healthy samples

2.9.1 Stressful conditions

In line with findings from salivary cortisol research (Chandola, Heraclides, & Kumari, 2010), research using hair cortisol has found that individuals exposed to adverse working conditions, have higher hair cortisol levels than healthy matched controls. In a study with 31 unemployment and 28 employed controls, unemployment was related to higher levels of hair cortisol (Dettenborn et al., 2010). Also shift workers (N = 33) at a young adult age had higher hair cortisol levels compared to day workers (Manenschijn, van Kruysbergen, de Jong, Koper, & van Rossum, 2011). Living conditions can be regarded as a chronic exposure to stress. In a study with 132 middle-aged adults the lack of natural environment (area density of green spaces, woodland, farming land water bodies) in the neighbourhood area was associated with elevated hair cortisol levels; also overall deprivation (assessing several domains of deprivation such as income, employment, education skills and

training, crime and living environment) was related to higher hair cortisol levels (Gidlow, Randall, Gillman, Smith, & Jones, 2016).

Dementia caregivers have been shown to have higher cortisol content in hair, with hair cortisol corresponding positively to caregiving burden and depressive symptomatology (Stalder et al., 2014). Another study found caregivers' hair cortisol levels to be associated with characteristics of the disability of their child. Whilst perceived stress as measured by the Perceived Stress Scale (PSS) was not associated with caregivers' hair cortisol, the relationship with features of the disability might indicate increased stress exposure that might not have been covered by the questionnaire (Chen et al., 2015). Own experience of disability (such as limited physical and social functioning or lack of concentration) was also found to be linked to elevated hair cortisol levels in a pooled database of diverse community samples of 324 middle-aged individuals (Wells et al., 2014).

As mentioned above, pregnancy itself is associated with higher cortisol secretion. There are, however, several studies reporting a relationship between higher hair cortisol levels and perceived stress and depressive symptoms during pregnancy and also after delivery (Hoffman et al., 2016; Kalra, Einarson, Karaskov, Van Uum, & Koren, 2007), although not all results are consistent (Braig et al., 2016; Kalra et al., 2005). Potential confounding factors, such as single motherhood, type of delivery (natural birth versus C-section), and season of delivery (summer versus winter) were identified in these studies and should be assessed for better controlled analyses in future work (Braig et al., 2015). In a sample of 209 infants, early detrimental prenatal psychosocial vulnerability (a composite score of several

aspects such as unemployment the year before pregnancy, single motherhood or maternal experience of life events) was related with higher hair cortisol content at age 1 in a cumulative manner; further these vulnerabilities predicted the number of common childhood diagnoses (e.g. acute upper respiratory infections, asthma, urticaria) in a dose-response relationship (Karlen et al., 2015).

2.9.2 Perceived stress, psychosocial factors and hair cortisol

While the effects of major adverse life events and stressful conditions on cortisol secretion seem to be noticeable in hair in certain stress-exposed groups, the findings for self-reported stress and hair cortisol are less consistent. A few studies have found positive correlations between perceived stress and hair cortisol in specific populations. Perceived stress was also positively correlated with hair cortisol in chronic pain patients, which might be attributable to the condition and the subsequent physical limitations (Van Uum et al., 2008). Also in male middle-aged patients with adrenal insufficiency, perceived stress was positively related to their hair cortisol content (Gow et al., 2011).

However, not all studies with population groups either living under chronic stressful conditions or with a disease diagnosis, found an association between perceived stress and hair cortisol content. For instance, in CVD patients, although having higher hair cortisol content than controls, no relationship between cortisol and perceived levels of stress could be found (Dowlati et al., 2010). Also perceived stress in the unemployed adult sample in Dettenborn et al.'s (2010) study did not correspond to the hair cortisol concentration. This lack of association might be

attributed to the fact that these population groups already have elevated hair cortisol levels and they might be perceiving stress relatively homogenously, resulting in little variation in the stress values preventing correlations between hair cortisol and stress to emerge. Equally, this might be due to confounding by other variables known to be important to both CVD and unemployment such as socioeconomic factors.

The associations between perceived stress and hair cortisol in healthy individuals without any stressful condition or under stressful circumstances are even more inconsistent (Dettenborn et al., 2010; Stalder, Steudte, Alexander, et al., 2012). As this PhD focuses on stress-related factors and perceived stress in relation to hair cortisol in *healthy* samples (without any chronic physical illness), these findings are of particular interest. Table 2.2 shows the findings of studies that have measured perceived stress and stressful life events in healthy population groups. Some studies that are based on a clinical sample but have included a control group are also incorporated as some of these findings are deemed relevant. Different instruments have been used to assess subjective stress levels such as the Perceived Stress Scale (PSS; Cohen, 1986), the Depression, Anxiety and Stress Scale (DASS; Lovibond & Lovibond, 1995) and the Trier Inventory for the Assessment of Chronic Stress (TICS; Schulz & Schlotz, 1999); however, the majority have employed the commonly used Perceived Stress Scale.

Table 2.2 Studies assessing perceived stress in healthy participants.

Author	Sample	Method of subjective stress assessment	Hair cortisol assessment	Findings
van Holland et al. (2012)	29 middle-aged production workers	3-item stress screener; time-period not specified	Concurrently assessed hair sample of 3 cm	HCC x Stress screener n.s.
Chan et al. (2014)	57 adults (39 non-obese and 18 obese)	PSS over last 1-2 month	Concurrently assessed hair sample of 3 cm	HCC x PSS n.s.
Chen et al. (2015)	86 caregivers aged 35-55	14-item PSS over last 1 month	Concurrently assessed hair sample of 3 cm	HCC x PSS n.s.
Dettenborn et al. (2010)	31 unemployed and 28 employed adults	14-item PSS over last 1 month	Concurrently assessed hair sample of 3 cm	HCC x PSS n.s.
Faresjö et al. (2013)	124 Greek and 112 Swedish young adults	10-item PSS; time-period not specified	Concurrently assessed hair sample of 3 cm	HCC x PSS n.s.
Milam et al. (2014)	27 adolescents	10-item PSS over last 1 month 65-item Stressful Life Events checklist over last 3 month	Concurrently assessed hair sample of 1 cm	HCC x PSS n.s. HCC x Stressful Life Events n.s.

Author	Sample	Method of subjective stress assessment	Hair cortisol assessment	Findings
Olstad et al. (2016)	70 middle-aged women from socioeconomically disadvantaged neighbourhoods	4-item PSS over last 1 month	Concurrently assessed hair sample of 3 cm (hair sample collected by participant with home kit)	HCC x PSS n.s.
Smyth et al. (2016)	88 young adult females (aged 18-27) 27 elderly females (aged 67-91)	4-item PSS over last 1 month 21-item Depression, Anxiety and Stress Scale (DASS) over last 1 month	Concurrently assessed hair sample of 3 cm	HCC x PSS (combined and separate groups) n.s. HCC x DASS (combined and separate groups) n.s.
Skoluda et al. (2012)	395 middle-aged adults (319 athletes and 76 controls)	PSS over last 1 month	Concurrently assessed hair sample of 3 cm, 6 cm or 9cm	HCC x PSS n.s
Stalder et al. (2010)	23 alcoholics in acute withdrawal, 25 abstinent alcoholics and 20 controls	PSS; time-period not specified	Concurrently assessed hair sample of 3 cm	HCC x PSS n.s.

Author	Sample	Method of subjective stress assessment	Hair cortisol assessment	Findings
Steudte et al. (2011)	15 GAD patients and 15 controls	10-item PSS over last 1 month Trier Inventory for the Assessment of Chronic Stress (TICS) over over last 1 months	Concurrently assessed 3 hair samples of 3 cm each	HCC x PSS n.s. HCC x TICS n.s.
Braeckman et al. (2015)	108 production workers	PSS; time-period not specified Stress outside work scale; time-period not specified	Hair sample; length not specified	HCC x PSS Positive association, $p < 0.05$ (r no specified) HCC x Stress outside work scale n.s.
Faresjö et al. (2014)	112 middle-aged female nurses and librarians	10-item PSS; time-period not specified	Concurrently assessed hair sample of 3 cm	HCC x PSS r = 0.20, $p = 0.041$
Gerber et al. (2013)	42 exercise and health science university students	10-item PSS over last 1 month	Concurrently assessed hair sample of 3 cm	HCC x PSS r = –0.18, $p = 0.052$
Gidlow et al. (2016)	132 middle-aged adults	10-item PSS over last 1 month 16-items Appraisal of Life Events Scale (3 months retrospective perception), consisting of threat, challenge and loss subscales	Retrospectively/ 3 month follow-up assessed hair sample of 3 cm	HCC x PSS n.s. HCC x Appraisal of Life Events Scale (Loss subscale) r = 0.168, $p = 0.057$ HCC x Appraisal of Life Events Scale (Challenge subscale) r = 0.164, $p = 0.063$

Author	Sample	Method of subjective stress assessment	Hair cortisol assessment	Findings
Karlen et al. (2011)	99 university students	Life events: dichotomous (yes/no) 1-item question 14-item PSS over last 3 months	Concurrently assessed hair sample of 3 cm	HCC x Life events Positive association, $p = 0.045$ (ANOVA) HCC x PSS r = -0.061 $p = 0.025$ In regression model including life events, PSS and perceived health, PSS was n.s. but Life events was sig. predicting HCC
Lambert et al. (2014)	45 patients with temporomandibular disorder and 71 controls	14-item PSS over last 3 months	Concurrently assessed hair sample of 3 cm	HCC x PSS in whole sample r= − 0.188, $p = 0.044$ HCC x PSS in separate groups (i.e. patients or controls) n.s.
O'Brien et al. (2013)	135 adults (aged 18–66)	PSS over last 3 month 15-item Confusion, Hubbub, and Order Scale (CHAOS) 18-item City Stress index	Concurrently assessed hair sample of 3 cm	HCC x PSS n.s. HCC x CHAOS n.s. HCC x City Stress index n.s. HCC x Overall stress score (combining PSS, CHAOS, City Stress index) r = 0.19, $p < 0.05$

Author	Sample	Method of subjective stress assessment	Hair cortisol assessment	Findings
Oullette et al. (2015)	60 mothers aged 35-45	UCLA Life Stress Interviews: High or low maternal chronic stress over the past 6 months (stress in eight domains: quality of intimate relationship, close friendships, relationships with children, social life, finances, work, health of self, and health of family members)	Retrospectively/2 year follow-up assessed hair sample of 3 cm	HCC x UCLA Life Stress Sum Score Negative association, $p = 0.035$ (ANOVA)
Rietschel et al. (2016)	109 adolescents and young adult twins (12-21)	PSS over past 1 months (aged >16 years); 30-item Daily Life and Stressors Scale (DLSS) over past 1 month (aged <16 years)	Concurrently assessed hair sample of 3 cm	HCC x PSS high cortisol value group/ median split $r = 0.47$, $p < 0.05$ HCC x PSS whole sample $r = 0.22$, $p < 0.05$

Author	Sample	Method of subjective stress assessment	Hair cortisol assessment	Findings
Stalder et al. (2012)	Study I: 45 adults (38 amateur endurance athletes and 7 controls) Study II: 64 students	Study I: PSS and Screening Scale of Chronic Stress of the Trier Inventory for the Assessment of Chronic Stress (SSCS-TICS) Study II: SSCS-TICS over last 2 months	Study I: 2 hair sample of 3 cm (1 year apart) Study II: 3 hair sample of 3 cm at two-month intervals	Study I: HCC change x PSS r = −0.33, *p* = 0.03 HCC x SSCS-TICS n.s. Study II: HCC x SSCS-TICS n.s.
Stalder et al. (2012)	Study I: 155 students Study II: 58 students	Study I: SSCS-TICS over the last 2 months Study II: TICS over the last 2 months	Study I: Concurrently assessed hair sample of 2 cm Study II: Concurrently assessed hair sample of 2 cm	Study I: HCC x SSCS-TICS n.s. HCC x TICS (general) n.s. HCC x TICS (social overload subscale) r = 0.29, *p* = 0.03
Steudte et al. (2011)	27 traumatised individuals (10 with PTSD and 17 without PTSD)	4-item PSS over last 1 month Lifetime traumatic events (34 traumatic event types)	Concurrently assessed hair sample of 3 cm	HCC x lifetime traumatic events (combined sample) r = 0.41, *p* < 0.05

Author	Sample	Method of subjective stress assessment	Hair cortisol assessment	Findings
Vanaelst et al. (2012)	39 children aged 5-11	Coddington Life Events Scale for Children (frequency and timing of 36 positive and negative life events) over last year. Calculation of life event within last 3,6,9,12 months	Concurrently assessed hair sample of 6 cm	HCC x Life Event within last 3,6,9,12 month r = 0.398, $p < 0.05$
Wells et al. (2014)	Pooled data (N = 324) from five diverse community samples: 70 adults 44 young adults 78 mental health treatment seekers 49 family members of people treated for mental health 83 community volunteers	10-item PSS over last 1 month 17-item Chronic Stress Scale (financial, work, relationship, parenting and family stress)	Concurrently assessed hair sample of 2 cm	HCC x Chronic Stress Scale r = 0.114, $p = 0.045$ HCC x PSS Curvilinear relationship/ inverse U relationship, $p < 0.05$ HCC increased with higher PSS but decreased at the highest PSS

Note. PSS = Perceived Stress Scale

Several studies report no association between perceived stress and hair cortisol in different healthy population groups: in adolescents and young adults (Faresjo et al., 2013; Karlen et al., 2011; Milam et al., 2014; Skoluda et al., 2012; Smyth et al., 2016), in working middle-aged adults (Chan et al., 2014; Dettenborn et al., 2010; Kramer et al., 2009; Lambert et al., 2014; Stalder et al., 2010; Steudte, Stalder, et al., 2011; van Holland et al., 2012), in elderly people (Smyth et al., 2016), in athletes (Skoluda et al., 2012), in caregivers (Chen et al., 2015) and in socioeconomically disadvantaged women (Olstad et al., 2016). Table 2.2 outlines the sample size of each of these groups, the instrument used to assess perceived stress with its respective time frame, the length of the hair segment measured and the findings for each of the instruments.

Several studies do report a relationship between perceived stress and hair cortisol; however, the direction of the association is mixed. A study of middle-aged healthy female nurses or librarians found a positive association between hair cortisol and perceived stress and also depressive symptoms, and an inverse association with self-rated health (Faresjö et al., 2014). Another study with 109 adolescents and young adult twins reported positive associations between hair cortisol and three risk factors for psychopathology, i.e. perceived stress, depressive symptoms and neuroticism (Rietschel et al., 2016). Some parent-infant studies have been conducted to evaluate the impact of parental psychosocial factors in related to hair cortisol in their offspring. For instance, in a study with 297 infants, parenting stress and maternal psychopathological aspects such as depression were positively

associated with their 1-year-old infants' hair cortisol levels and disrupted socioemotional development (Palmer et al., 2013).

Conversely, negative associations between perceived stress and hair cortisol have been reported in, for instance, 42 health science university students (Gerber et al., 2012) and in a study of 38 amateur endurance athletes and 7 controls (Stalder, Steudte, Alexander, et al., 2012). In this latter study, the fact that these individuals were highly physically active might contribute to this relationship. Exercise has been previously linked to elevated saliva cortisol and also hair cortisol (Gerber et al., 2012; Skoluda et al., 2012) and might therefore act as a stress-buffer or confound the relationship between stress and cortisol. Interestingly, O'Brien and colleagues (2013) found that measures of global stress (i.e. perceived general stress, city stress and the extent of chaos in the home environment), but not single-stress evaluations, were associated with hair cortisol levels in an older adult sample. Finally, another study with a diverse community-based pooled database sample reported a curvilinear relationship between subjective stress and hair cortisol; whilst a positive association between hair cortisol and perceived stress was observed, hair cortisol levels decreased at higher levels of stress (Wells et al., 2014).

In 108 production workers, perceived stress was positively associated with hair cortisol; however reported stress outside work was not related, which indicates that the perceived stress actually reflected work-related stressors (Braeckman et al., 2015). Overall, stress scales have been employed to evaluate different aspects of stress. For example, Stalder et al. (2012) utilised the Screening Scale of Chronic Stress of the Trier Inventory for the Assessment of Chronic Stress (SSCS-TICS; Schulz

et al., 2000), which includes stress aspects in relation to work overload and performance pressure, worries and social overload. The authors did not find any association between hair cortisol and the stress-related measures, or between hair cortisol and dispositional aspects (i.e. self-efficacy) in two separate studies of 155 and 58 healthy individuals, respectively. Work-related stress has been inconsistently associated with hair cortisol; literature that will be discussed in detail in Chapter 3. Living under financial strain can be a major stressor and only a few studies have investigated income levels in relation to hair cortisol levels, with a lower socioeconomic status and higher financial strain being negatively associated with hair cortisol (Henley & Koren, 2014; Vaghri et al., 2013; Vliegenthart et al., 2016). Again this literature specific to finance-hair cortisol associations will be discussed in Chapter 3, as this is the focal point of the first study of this PhD.

Stressful live events seem to associate with hair cortisol differently than perceived stress. As outlined above traumatic experiences and early life adversity in healthy children, adolescents and adults is related to aberrant hair cortisol levels, be it either elevated or reduced levels, depending on trauma type and duration of the exposure (Schreier et al., 2016; Simmons et al., 2016; Steudte-Schmiedgen et al., 2016). The experience of stressful life events in healthy individuals has been consistently related with elevated hair cortisol levels. In university students, Karlen et al. (2011) assessed perceived stress plus stressful life events; perceived stress was negatively related to hair cortisol content and the experience of life events was positively related to hair cortisol content. When including both factors in a regression model, only life events remained a significant predictor for hair cortisol.

In an aforementioned study with middle-aged healthy female nurses or librarians, upon interviewing two cases with extremely elevated hair cortisol levels, it emerged that they had experienced serious life events in the previous weeks (Faresjö et al., 2014). Steudte and colleagues (2011) assessed hair cortisol content in a case-control study of PTSD patients and traumatic experiences were related to higher hair cortisol when analysing the complete sample including the control group, an effect that was not driven by the traumatic experiences in PTSD patients despite their even higher elevated hair cortisol content.

In 39 children aged 5-11, Vanaelst et al. (2012) assessed the frequency and timing of 36 positive and negative life events (such as parental job attainment, family move, death, illness, school entry) over one year while analysing a hair segment over the last half year (6 cm) and life events were positively associated with hair cortisol. This is in line with another study which found that children's first entry into elementary school could be objectively determined by comparing pre- and post-entry hair segments (Groeneveld et al., 2013). The effect was largest for fearful school children, who may have perceived the transition as more stressful. While this study did not assess life events or perceived stress, school entry is deemed as a relatively positive and relatively negative life event, depending on the child's psychosocial characters and perceptions (Coddington, 1972).

Lastly, one study found a negative association between the experience of life stressors and hair cortisol in 60 middle-aged mothers (Ouellette et al., 2015). Interestingly, the UCLA Life Stress instrument was employed in this study to evaluate acute and chronic stressors over the past 6 months in several domains

such as family, friendships, social life, finances, work and health. The authors took hair samples in a 2-year follow-up assessment, thus the associations are longitudinal in nature. The negative associations might be explained by the exhaustion-cortisol relationship as discussed earlier and in relation to PTSD.

There is not much research on potential associations between hair cortisol and well-being. Despite evidence for a negative relationship between well-being and cortisol based on traditional specimens, the findings employing hair cortisol are puzzling. In a study with 654 middle-aged and older adults, better mental health (perceived social functioning, personal accomplishments, vitality) was associated with higher hair cortisol levels; however this association disappeared once age, sex and smoking were taken into account (Feller et al., 2014). In a study with a younger and an older sample, in the older sample (N = 27), a positive association between hair cortisol and well-being could be found (Smyth et al., 2016). More research needs to be conducted to elucidate these findings, controlling for potential covariates.

A meta-analysis of salivary cortisol showed that timing of the stressor is a vital aspect of shaping the HPA activation with higher activation at stressor onset, but reduced or normalised activation when the stressor is no longer existent (Miller, Chen, & Zhou, 2007). Timing of perceived stress exposures or stressful life events in the hair cortisol literature has not been considered carefully and a potential time lag might lead to methodological issues. As can be seen in Table 2.2, most studies employ a questionnaire design with a retrospective window of the previous 1 month; yet they concurrently assess a hair sample of 3 cm length,

representing the previous 3 months of cortisol exposure. Inconsistencies in the stress-hair cortisol relationship in healthy population groups might be attributable to a biased reflective time window between the two measures. Specifically, the hair samples and measures and the examined time frames vary within studies hindering proper evaluation. Likewise, the possibility exists that perceived stress, dispositional psychological and affective factors show no such relationship in healthy individuals. Overall, there is some tendency for stress and life events to be "objectively mapped" in hair tissue, although further research is warranted. In healthy individuals, hair cortisol levels might not always relate to single stress indices, a notion that is worth further investigating. There is a body of research based on salivary cortisol analyses showing the protective (or moderating) effects of certain psychological attributes, such as coping and positive affect, on stress-related HPA-axis activity (Leserman et al., 2000; O'Donnell, Badrick, Kumari, & Steptoe, 2008; Steptoe et al., 2009). Yet, there is lack of research from hair analyses that corroborate such effects.

2.10 Correlates and confounds

2.10.1 Hair-related characteristics and environmental factors

Human hair is subjected to various chemical substances for hygienic (and also cosmetic) purposes. Several studies including reviews have reported that frequency of hair washes and also hair treatment (colouring) decreased endogenous levels of cortisol in hair (Abell, Stalder, et al., 2016; Feller et al., 2014;

Meyer & Novak, 2012; Russell et al., 2012; Stalder, Steudte, Miller, et al., 2012; Staufenbiel et al., 2013) and should be assessed and controlled for. Interestingly, an experimental study analysed the effect of different standard chemical treatments on resulting hair cortisol levels and found that cortisol content was altered by the chemical treatment processes differently (Hoffman, Karban, Benitez, Goodteacher, & Laudenslager, 2014). Bleaching, demi-permanent colouring and shampoo washes significantly decreased hair cortisol level; however depending on the percentages of peroxides that hair cortisol was exposed to, colouring could lead to an increase in cortisol. Not all studies have found that treatment lowers hair cortisol, which might be explained by the fact that treatment type and percentages of peroxide substance (if relevant) might confound these associations.

Furthermore, water alone significantly decreases hair cortisol content, which might imply that substances common in water, such as chalk variation, might also play a potential role as a confounding factor (Hamel et al., 2011). Segmental analyses demonstrated a wash-out effect over the more distal segments from the scalp (hair washes, exposure to toxins etc.) and the possibility of inter-segment loss, thus retrospective cortisol assessment might be limited to 6 cm hair sections, representing the preceding 6 months prior to sampling (Dettenborn et al., 2010; Gao et al., 2010; Vanaelst et al., 2013). Association analyses using segmental hair, such as in pregnancy and also with PTSD patients, might be confounded by the wash-out effect. With the more distal section, hair cortisol levels were shown to decrease. This implies that any decreases over the segments might be

underestimated or any increases might be overestimated, respectively, depending on the direction of the hypotheses in the target population.

Most studies report no associations between hair cortisol and hair colour and hair structure (e.g. Feller et al., 2014; Kirschbaum et al., 2009; Noppe et al., 2014). However, in some studies hair colour was indeed associated with hair cortisol, with darker colours being associated with higher cortisol levels (Rippe et al., 2016; Staufenbiel et al., 2015). Also ethnic groups seem to differ in hair cortisol, with black people showing higher hair cortisol content than white people (Abell, Stalder, et al., 2016; Wosu, Valdimarsdottir, Shields, Williams, & Williams, 2013), which might be partly due to their black-coloured hair. However, this is unclear and more research is needed to evaluate the true effect of hair colour and ethnicity on hair cortisol.

Season also seems to play a role in affecting hair cortisol levels, as identified in human and in animal research (Staufenbiel et al., 2015). This seasonal variation in hair cortisol might be explained by external light factors. Ultraviolet (UV) radiation has been found to reduce hair cortisol in all experimentally tested hair samples with artificial UV, but also after exposure to natural sunlight for a period of 40 hours (Grass et al., 2016; Li et al., 2012; Wester, van der Wulp, Koper, de Rijke, & van Rossum, 2016).

The impact of some of these factors, such as external water exposure and UV light, on hair cortisol concentration is still uncertain and the vast majority of research on hair cortisol have not taken these factors into account. More

methodologically strong studies need to be conducted before reliable conclusions can be drawn.

2.10.2 Health behaviours and socio-demographic factors

Health behaviours have been little studied in relation to hair cortisol. Reviews evaluating quality of research methods have found that smoking use is linked to elevated hair cortisol (Feller et al., 2014; Russell et al., 2012; Wosu et al., 2013), consistent with the evidence from traditional cortisol specimens. However, not all studies report that tobacco use is related to hair cortisol (e.g. Chen et al., 2015; Dettenborn, Muhtz, et al., 2012; Skoluda et al., 2012). Yet, a reduction in smoking, as has been studied in a pilot study in a 7-week smoking cessation intervention based on mindfulness and cognitive elements, was shown to be related to pre-to-post intervention hair cortisol reduction (Goldberg et al., 2014). Exercise has been shown to increase momentary cortisol (assessed via saliva or blood sampling) and also hair cortisol has been shown to be positively correlated with amount of physical activity (Gerber et al., 2012; Skoluda et al., 2012). Sleep has not been well studied, however, there is some evidence for daytime sleeping to be positively associated with hair cortisol (Feller et al., 2014).

The relationship between age and hair cortisol has been described earlier (see section 2.7.1). These associations not only indicate HPA-axis functioning in relation to ageing processes but also highlight age as a confounding factor in between- and within-subjects designs. Similarly, the strong positive relationship between BMI and hair cortisol highlights the importance of taking BMI into account

in all analyses (Feller et al., 2014; Staufenbiel et al., 2013; Wosu et al., 2013). Sex is a potential confounding factor in associations between variables of interest and hair cortisol. Men have been found to have higher hair cortisol content than women in several studies (Feller et al., 2014; Manenschijn et al., 2013), despite not consistently (Gow et al., 2010; Karlen et al., 2011) .

Socioeconomic status is a common confounding factor in epidemiology-based research. In the cortisol literature based on traditional specimens, there has been strong evidence for lower socioeconomic positions, lower income groups and financial stress to be related to elevated cortisol. This has been studied as a stressor and also as a confounding factor in associations between cortisol and disease. Also in the hair cortisol literature, there have been some findings in relation to socioeconomic factors which will be discussed in the next Chapter as part of Study I.

2.11 Chapter summary

This chapter provided an overview of the traditional cortisol specimens, saliva, blood and urine with a particular focus on salivary cortisol and the methodological drawbacks associated with its analysis, such as measurement and sampling issues, adherence, stability issues and importantly, the impact of momentary psychosocial factors. This has provided a rationale for the development of hair cortisol analyses as a measure of long-term cortisol output that is not affected by these methodological shortcomings. Nevertheless, hair cortisol analyses have their own methodological issues, in particular the uncertainty surrounding the mechanism by which cortisol is incorporated into the hair shaft. Systematic

correlational studies are needed to assess salivary cortisol and hair cortisol over corresponding time intervals. This literature review has shown that hair cortisol is associated with several well-known hypercortisolemic conditions and physical and mental diseases, such as CVD, diabetes, depression, anxiety and PTSD. While the effects of major adverse life events and stressful conditions on cortisol secretion seem to be noticeable in hair in certain stress-exposed groups, the findings for self-reported stress and hair cortisol are less consistent. Methodological aspects such as the potential time lag in the measures (stress measures and hair cortisol content) and low variability might account for some inconsistencies, yet, hair cortisol analyses warrant further research in order to be established and further validated as a chronic measure of HPA-axis activity and stress. This PhD will look at the effect of certain stress-related factors in relation to hair cortisol in healthy samples and also at the associations between salivary and hair cortisol levels.

CHAPTER 3. ASSOCIATIONS BETWEEN HAIR CORTISOL, SOCIO-ECONOMIC, WORK-RELATED AND PSYCHOSOCIAL FACTORS (STUDY I)

3.1 Chapter overview

Findings of lower socioeconomic factors being related to adverse health outcomes could potentially be partly explained by similar results using neuroendocrine markers. This chapter is interested in the associations between hair cortisol concentration and socioeconomic factors, work-related stress and social support. It makes use of the Daytracker Study, a study conducted at UCL and at Semmelweis University in Budapest (Hungary). The study uses data from two time points and therefore looks at income, financial stress and work stress as a dynamic factor, besides its cross-sectional effects, assessing whether changes (deteriorations or improvements) in such stressors might predict hair cortisol levels distinctively. Social support has often been considered as a moderating factor in the relationship between stress and health markers.

3.2 Introduction

3.2.1 Income and financial strain

During the past three decades, widening income inequality in many developed countries has become a major problem (Dabla-Norris, Kochhar, Suphaphiphat, Ricka, & Tsounta, 2015). Highlighting the importance of socioeconomic factors in relation to health, research in various European countries

demonstrates that financial disadvantage leads to many types of health inequality, including higher mortality and morbidity rates (Marmot, 2002). Nevertheless, data analysing gross domestic product per capita and life expectancy suggest that there seems to be a level of income after which increased levels cease to be an important predictor for health, i.e. the higher the income group the less improvement can be observed in health per income unit, displaying a concave relationship or the so-called Preston curve (Mackenbach et al., 2005; Preston, 2007; Wilkinson, 1994). At an individual level, financial stability is a critically important life domain as many essential daily activities and opportunities for education, realisations and achievements are dependent on existing financial resources (Peirce, Frone, Russell, & Cooper, 1996). Similarly, socioeconomic status (SES) is an indicator of relative rank that an individual holds in society and can thus be implicated in social predicament.

Self-report measures of health outcomes and status have often been used to evaluate the link between income and health, but they might underestimate the true negative socioeconomic inequalities of health (Baker, Stabile, & Deri, 2004); although self-report might be also related to overestimation of inequalities due to common-method variance (Eriksson, Unden, & Elofsson, 2001; Podsakoff, MacKenzie, Lee, & Podsakoff, 2003). Research has shown that there can be striking discrepancies between subjectively reported health outcomes and objectively measured biological markers or health conditions, and that education and income may contribute to erroneous reporting of health outcomes (Johnston, Propper, & Shields, 2009; Mackenbach, Looman, & van der Meer, 1996). The use of more

objective measures, such as endocrine, metabolic or immune markers as proxies for health may improve the trustworthiness of such findings and provide information about the pathways mediating these associations.

Further, most studies have examined income as a static phenomenon, making use of cross-sectional data (Gunasekara, Carter, & Blakely, 2011). However, income status can also be regarded as a dynamic entity, and the effect of changes over time might further inform the relationship between accumulated economic adversity, exposure to stressors and health. Cumulative socioeconomic disadvantage and social downward mobility over the life course have been shown to be related to cardiovascular disease mortality, but the underlying pathways are poorly understood (Johnson-Lawrence, Galea, & Kaplan, 2015). Moreover, changes in the labour market, including job insecurity and downward social mobility, have created the phenomenon of status incongruity. People whose occupational position is lower than might be expected from their educational attainment and those whose status is greater than might be expected from their education can be regarded as experiencing status incongruity. Status incongruity has long been thought to be linked to health outcomes such as cardiovascular risk factors, myocardial infarction and stroke and also self-rated health (Braig et al., 2011; Dunlavy, Garcy, & Rostila, 2016; Honjo et al., 2014). To date, no study has assessed status incongruity, as the mismatch in education and income, in relation to cortisol. Long-term cortisol is especially of interest as status incongruity can present as a chronic stressor.

Supporting the involvement of psychoneuroendocrinological pathways in the link between income and health, a body of research has suggested lower income to be associated with higher salivary and urinary cortisol levels (Dowd, Simanek, & Aiello, 2009; Jimenez, Osypuk, Arevalo, Tucker, & Falcon, 2015). Lower income and education have also been associated with elevated diurnal cortisol values and with flatter diurnal rhythms in a graded fashion (Cohen, Doyle, & Baum, 2006). Although low income has been found to be predominantly associated with elevated cortisol, there are contradictory findings, partly due to variations in methodology and cortisol assessments, i.e. cortisol reactivity levels, diurnal level or morning values (Dowd et al., 2009).

The relationship between financial status (income) and hair cortisol has been little studied. One study reported elevated hair cortisol in 108 individuals earning less than the minimum wage in different communities in sub-Saharan Africa (Henley et al., 2014). Another study by O'Brien et al. (2013) with 135 adults analysed hair cortisol in relation to socioeconomic status, calculated as a composite score (z-score subsequently divided into tertiles) of income and education together. While socioeconomic status did not predict hair cortisol by itself, an interaction of socioeconomic status by race (as minority or non-minority based on 13 ethnic categories) on hair cortisol emerged. Specifically, for minority groups both low and high socioeconomic status was related to higher hair cortisol levels than at mid-socioeconomic status, which corresponded with perceived stress measures. These high levels of perceived stress and hair cortisol in higher socioeconomic status respondents might indicate underlying stress due to discrimination against these

groups. Nevertheless, the question remains why income alone was not used in the analyses rather than the composite score.

A study by Dettenborn and colleagues assessed the difference in hair cortisol levels between employed versus unemployed individuals and found unemployment to be related to elevated hair cortisol concentration (2010). Evaluating the effect of varying income levels was not the aim of the study and hence not analysed nor acknowledged in the interpretations of the findings. However, income levels were different among the two groups, with 80.6% of the unemployed group versus 5.5% of the employed group receiving only minimal monthly income (1000 €, equivalent to around £860/ month). These findings might therefore relate to a link between hair cortisol and actual income levels that differed according to employment status - though unemployment is known to be a very serious stressor.

Some mixed evidence also exists for the effect of parental income on children's hair cortisol levels. One study found a tendency (borderline significance) for a negative relationship between parental income and hair cortisol levels in 33 children aged 10-12 years (Bosma et al., 2015). Another study found lower maternal and paternal education but not income per se to be linked with elevated hair cortisol levels in 339 pre-schoolers (Vaghri et al., 2013), supporting the notion that different measures of SES might associate differently and that methodological variations lead to heterogeneous findings (Duncan, Daly, McDonough, & Williams, 2002; Henley & Koren, 2014). A different study with 270 children and adolescents reported higher hair cortisol levels according to lower neighbourhood

socioeconomic status as indexed by postal code, controlling for age, sex and ethnicity (Vliegenthart et al., 2016). Lastly, a recent study evaluated hair cortisol levels in 2484 children aged 6 years in different parental household income groups and found lower income groups to be linked to elevated hair cortisol among the children (Rippe et al., 2016).

Trajectories over time in income have not been studied at all, although previous research has shown changes in salivary cortisol in relation to changes in financial strain over 3 years (Steptoe, Brydon, & Kunz-Ebrecht, 2005). The protective effect of upward social mobility on different health outcomes, including cardiovascular disease mortality risk, is well documented, but no study has evaluated this phenomenon in relation to hair cortisol. There is therefore a sound rationale for exploring socioeconomic factors in relation to this long-term cortisol marker. Financial stress, especially debts, can cause pressure and emotional distress, a concept that has not been studied in relation to hair cortisol levels. There are findings of financial stress being associated with salivary cortisol levels and psychosocial ill-health (Essex, Klein, Cho, & Kalin, 2002; Starrin, Aslund, & Nilsson, 2009), making the dynamic aspects of financial strain together with income relevant to be assessed in relation to chronic secretions of cortisol.

3.2.2 Work stress

In the UK, recent Health and Safety Executive research statistics quantified the extent to which subjective stress exists, and identified money and work-related issues as the main source of stress (HSE, 2014). Stress caused by finances, as seen in

low-income families, can be a direct causal factor linked to increased work-load, poorly tolerated complex work schedules and workplace flexibility challenges, and dual-earning aspects involving managing multiple home- and work-related responsibilities and roles. Employees under financial stress, such as in a study with 7200 credit counselling clients, are more likely to experience work stress, often take their financial personal issues to the workplace and are at increased risk for workplace absenteeism (Kim & Garman, 2003). Nevertheless work-stress and financial-related strain does not necessarily go hand in hand and need to be analysed separately.

According to the European Commission, more than one in every three employees in Europe suffer from inordinate work stress (European Commission, 2011). The globalisation and increasing pressure to perform in society has led to a shift in the physical and psychological strain to which employees are subjected. A body of research in the field of organisational management, occupational psychology, and vocational behaviour has been conducted with the purpose of improving organisational outcomes, such as absenteeism and optimising productivity. This has provided valuable insight into the complexities of occupational responsibilities and the subsequent behavioural outcomes (Ganster & Schaubroeck, 1991). However, more importantly, over the last 20 years, research in occupational health, health promotion practice and public health has become increasingly noteworthy, considering the impact adverse psychosocial work environments have on mental and physical health (Ganster & Rosen, 2013; Melchior et al., 2007). The most important findings relating income but also

stressful work environments to different health outcomes and biomarkers will be presented after introducing the most extensively used models to estimate work stress.

In 2004, the Trade Union Trends Survey (representing workers from all industries and sectors within the UK) identified workload as the most pervasive factor in causing work stress among varying work-related aspects, i.e. workloads, staff cuts, work change, hours, bullying, shiftwork, working conditions, redundancies and sexual or racial harassment (Congress, 2004). However, occupational stress research maintains that not only workload, but underlying work-related aspects such as control, decision making and work-related satisfaction, play a crucial role too.

Two theoretical frameworks that are widely used in occupational health studies to capture the interrelation between work-related factors and health outcomes are the Demand-Control model (DCM) by Karasek and Siegrist's Effort-Reward Imbalance (ERI) model (Karasek, 1979; Siegrist et al., 1990). Both models are based on the concept that work stress arises from a combination of two fundamental dimensions of the work experience.

The Demand-Control model (DCM) suggests that job-associated stress levels are determined by demand-control combinations, so that people with persistent high job demands and low control over work eventually suffer from stress-induced health issues. Job demand encompasses the psychological and emotional effort involved in performance in terms of workload and pressures to accomplish tasks. Job control refers to the extent of decision making autonomy over detailed aspects

of performance and skill utilisation; skill discretion in particular focuses on repetition and variability of the applied skills. High levels lead to growth, learning and motivation, and therefore satisfaction (Karasek, 1979). Some of the weaknesses of the DCM that have been identified in the literature are that it was constructed in the context of stable work environments that were typical in previous decades, so fails to include challenges of opportunities, career fragmentations and other career-thriving paths, more commonly taking place in the current global economic environment, as well as the individual's personality-related characteristics and resources to cope with the assumed work stain (Siegrist, 2001).

With these limitations in mind, Siegrist developed the ERI model as an extension to the DCM (Siegrist, 2001). The ERI model proposes that strain occurs when there is an imbalance between the effort spent at work and work-related gratification. This model can be regarded as a cost-benefit formulation with a focus on the extrinsic efforts in the work environment, for instance workload, responsibility, time pressures and commitments, and the perceived rewards in social, emotional, intellectual or financial aspects or in terms of promotion prospects and job security. The imbalance of high effort over low reward is thought to be stressful as it violates core expectations about reciprocity (Siegrist et al., 1990). It is this model with its concept of effort and reward that is at the core of the exploration of work stress and hair cortisol output in this chapter.

3.2.3 The psychoneuroendocrine pathway linking SES, work stress and health

One mechanism potentially linking SES, work stress and health is the HPA-axis pathway and the involvement of stress-related cortisol responses (Lupien, King, Meaney, & McEwen, 2001). The Whitehall I and II study in the UK are epidemiological cohorts prospectively investigating the biological, psychosocial, behavioural and socioeconomic determinants of health in 8,000 male civil servants and in 10,308 civil servants (two thirds male), respectively. Analyses from the first cohort served to demonstrate a social gradient of health, i.e. lower grade levels of the male civil servants' employment were related to a three-fold increase in mortality rate from CHD (Marmot et al., 1978). More recently, a meta-analysis conducted in 2006, which included studies from the Whitehall II cohort, found that work-related stress is associated with a 50% excess risk of CHD (Kivimaki et al., 2006). The second cohort, specifically, was designed to investigate the underlying pathways and mechanisms for this relationship in a sample of both male and female workers and established that there is an interconnectedness of socioeconomic position, work-related characteristics, psychosocial factors, and health outcomes, controlling for varying socio-demographic factors and health behaviours. The adverse effects of work strain, in terms of having low control over the accomplished tasks, and also of low work social support, were highlighted as moderators of this relationship and in being related to poor mental and physical health and also increased sickness absenteeism at work (Stansfeld, 2000). Likewise, work-related reward aspects such as skill discretion and decision authority with their protective role, and at the same time negative stressful aspects with their

destructive impact, have both been found to mediate the association between income and self-reported ill-health in a Swedish dataset with 5982 working adults (Hemstrom, 2005).

Work stress has been established as a risk factor for a variety of health disorders, predominantly cardiovascular disease, the metabolic syndrome and diabetes (Chandola et al., 2006; Heraclides et al., 2012; Kivimaki et al., 2012). Studies have identified blood pressure, neuroendocrine pathways, lipoproteins and various immunomarkers as the underlying biological correlates of work stress (Catalina-Romero et al., 2013; Liao et al., 2013; Nakata, 2012; Steptoe, Siegrist, et al., 2004).

There is ample evidence linking work-related stress in different contexts with cortisol output. A meta-analysis by Chida and Steptoe (2009) evaluated the CAR in relation to various psychosocial factors. Using 9 studies focused on work-related aspects (totalling 20 effect sizes) concluded that the CAR showed strong positive associations with job stress. Reported differences in the CARs based on whether the measurements were taken on a work or leisure day also strongly reflect the effect work-related tensions, anticipation of the work load and pressures have on the HPA-axis (Kunz-Ebrecht et al., 2004). Another study took detailed CAR measurements with dummy electronic monitoring salivettes over a day with a stressful demonstration lesson and a control day in 21 teachers (Wolfram, Bellingrath, Feuerhahn, & Kudielka, 2013). The CAR itself did not differ between the two days; conflictingly with previous findings, but the CAR and the ERI showed a relationship. However, remarkably, higher ERI was associated with a blunted CAR

on the control day, whilst there was no effect on the stressful workday. As outlined in Chapter 2, the function of the CAR is assumed to be related to anticipatory aspects about the day ahead; a notion that might explain this difference in individuals who experience low levels of control over their work in general settings but might assume more personal responsibilities and internal locus of control when being monitored by external services. Additionally, an observational study, measuring salivary cortisol over one day during a self-reported high stress and a low stress work-week in 55 white-collar workers in a counterbalanced design, found a flattened diurnal cortisol pattern over the high stress work-week sampling day (Dahlgren, Kecklund, & Akerstedt, 2005).

This body of evidence offers a sound rationale to explore work stress in relation to a long-term cortisol marker, overcoming some of the issues related to momentary changes in salivary cortisol over the day. Only a few studies have yet been conducted to investigate hair cortisol output in relation to work-related stress. There are findings on positive associations with hair cortisol in certain populations under adverse work conditions, such as shift work, which however might be attributable to several confounding factors affecting the HPA-axis, such as general stress and also fatigue and sleep deprivation (Manenschijn, van Kruysbergen, et al., 2011).

Three studies assessed hair cortisol in relation to different aspects of work stress not making use of the two major work stress models, the ERI model and the DCM. A study with 175 workers in a ready-made garment factory in Bangladesh assessed work-related demands and values and interpersonal resources and hair

cortisol (Steinisch et al., 2014). While these overall work-related aspects did not associate with hair cortisol, work-related values, in particular prospective positive work-related promotion, were linked to elevated hair cortisol content. These positive associations might indicate underlying pressure, or anticipatory stress related to the forthcoming promotion which might lead to an elevation in the HPA-axis. Another study with 58 adults affiliated to a higher education institution investigated work overload, overtaxing and discontent and performance pressure with the Trier Inventory for the Assessment of Chronic Stress work-subscale in relation to hair cortisol and did not find any association between the variables and hair cortisol (Stalder, Steudte, Alexander, et al., 2012). Need for recovery after work (measured by a 11-item inventory with questions such as 'I feel empty at the end of a working day,' and 'I feel mentally exhausted by my work') was analysed in 29 middle-aged workers in the meat-processing industry in relation to hair cortisol and no relationship could be found (van Holland et al., 2012).

Only one research group assessed the two work stress models in two distinct studies. A study with 43 female teachers from different kindergartens in China assessed need for recovery after work together with the job demand-control model (assessed with the Job Content Questionnaire, which is a validated Chinese questionnaire to assess work-related demand and control with similar items used in the DCM) (Qi et al., 2015). Hair cortisol was associated with neither need for recovery after work nor with demand-control. In another sample of 39 female kindergartens teachers, this research group found the effort–reward imbalance scale and an imbalance in the ratio between effort and reward to be positively

associated with hair cortisol content (Qi et al., 2014). Teachers that experienced more effort and less reward from their work-related tasks had higher cortisol content in their hair.

Both models are appropriate in assessing chronic work stress and therefore relevant in relation to a long-term HPA-axis marker. However, the analyses in the present study will mainly focus and present data on the ERI. In the light of this limited literature, I thought it was of interest to see whether ERI at work or the respective components (effort and reward) would show an association with hair cortisol in a healthy adult female population from two different cities. Work stress can be regarded as a dynamic process. The effects of higher work stress and changes over time might be related to worse HPA-axis functioning and therefore dynamic work-related factors as assessed in a longitudinal design are of interest as well.

3.2.4 Social support

As outlined in the literature review, underlying psychosocial factors play a crucial role in shaping physical and psychological health outcomes (Holt-Lunstad, Smith, & Layton, 2010). Research has suggested that neuroendocrine processes underlie the relationship between low quality social relationships and risk for morbidity and mortality. This has provided new avenues for research. Studies relating social support and cortisol generally indicate a positive effect of social support on the HPA-axis and potential buffering role of the negative impact of stress on health (Turner-Cobb et al., 2000). Since the focus of this study was on the

effect of low income, high financial strain and general work stress on long-term HPA-axis functioning, the buffering hypothesis of social factors was also of interest. Social support outside the work place might play a crucial role in moderating the deleterious effect of high work strain on physical and mental well-being.

Social aspects have not been well studied in relation to hair cortisol. A study with two different samples of 155 and 58 adults, found inconsistent (only in the second sample) positive associations between hair cortisol and social overload (with items such as 'I must frequently care for the well-being of others') (Stalder, Steudte, Alexander, et al., 2012). In the study by Dettenborn and colleagues (2010) comparing hair cortisol in unemployed and employed adults, social stress, lack of social recognition and social isolation were not associated with hair cortisol in either of the two groups.

3.2.5 The present study

The present chapter is based on the Daytracker Study, a project funded by the US National Institute on Aging and the UK Economic and Social Research Council. The Daytracker Study was a prospective study investigating the associations between psychosocial factors, everyday life experiences and biological reactivity in the work environment in two contrasting cultures. A female sample was chosen as women were rather underrepresented in the literature on work stress at the time the study was conducted. The data were collected in London and Budapest during 2007/08; by researchers from University College London (London, UK) and the Semmelweis University (Budapest, Hungary). At baseline, the

participants underwent two 24-hour monitoring periods (over a working and a leisure day), involving diurnal salivary cortisol assessment and various psychosocial and affective measures. Many measures were assessed but only those relevant for this PhD will be described and analysed. Four years later, participants were re-contacted via email, telephone, LinkedIn and Facebook and invited for a follow-up study at both sites. Hair was collected for hair cortisol analysis and certain psychosocial measurements of interest were repeated. I carried out the follow-up study, including contacting participants at both sites, data collection and data analysis. However, the analyses for this study incorporate analyses on data collected at both time points. Chapter 4 is using the same dataset as well as an additional dataset and relevant aspects of the methodology of the study will be described in either chapter.

3.2.6 Aims and Hypotheses

Aims

The aims of the current study were to investigate hair cortisol concentration in relation to socioeconomic factors (concurrent income, income change over a 4 year period indicating a shift in income group, accumulated low income over 4 years and status incongruity), financial strain, work stress and social support in a healthy sample of women. Hair cortisol was measured only once, but repeat measures of the psychosocial factors allowed several aspects of this relationship to be investigated: the longitudinal associations between hair cortisol and the

different psychosocial variables using aggregated and change measures and the cross-sectional associations between hair cortisol and the different variables.

Hypotheses

The following hypotheses will be tested in this study:

1. Lower income (cross-sectionally) and a negative shift in income over four years or prolonged low income (longitudinally) will predict higher hair cortisol levels.

2. Higher financial strain (cross-sectionally), an increase in financial strain over the 4 years or prolonged financial strain (longitudinally) will be associated with elevated hair cortisol.

3. Negative status incongruity will be associated with elevated hair cortisol (cross-sectionally).

4. Work stress (an 'unfavourable' ERI) or specific components of adverse dimensions of the model (high effort or low reward; cross-sectionally), and a deterioration in work stress (longitudinally) will be positively related to hair cortisol concentration.

5. Social support will have a moderating effect on the potential relationship between work stress and hair cortisol (cross-sectionally and/or longitudinally) and potentially a direct negative association with hair cortisol (cross-sectionally and longitudinally).

3.3 Method

3.3.1 Participants

At baseline, 199 women employed at University College London (UCL) and neighbouring higher education institutions in London, and 202 women employed at Semmelweis University in Budapest were recruited via email and poster advertisements. The eligibility criteria were as follows: aged 18 to 65 years, in full-time paid employment, not taking any medication except for oral contraception, not being pregnant, and not having any serious illness. At follow-up, participants were excluded if they were pregnant, had a chronic or acute condition e.g. CVD or cancer, or were on regular glucocorticoid-containing medication, as these factors have been shown to affect cortisol secretion (Hellhammer, et al., 2009).

Figure 3.1 depicts the recruitment process. Of the London sample (N = 199), 68 women took part in the follow-up assessment; 33 were excluded due to incomplete baseline cortisol samples or missing contact details, 14 were excluded due to ineligibility upon contact (5 illness; 9 pregnancy/ maternity leave), 4 refused, 10 could not be scheduled and 70 were otherwise not contactable (at baseline many participants were junior research and clerical staff who had since moved away from UCL and London, often leaving no contact information). Of the Budapest sample (N = 202), 97 women took part in the follow-up assessment; 20 were excluded due to ineligibility upon contact (8 illness; 12 pregnancy/ maternity leave), 7 refused, 11 could not be scheduled, 1 person had died and 66 were

otherwise not contactable. Participants received financial compensation of £10 for participating. The study was approved by the UCL Research Ethics Committee.

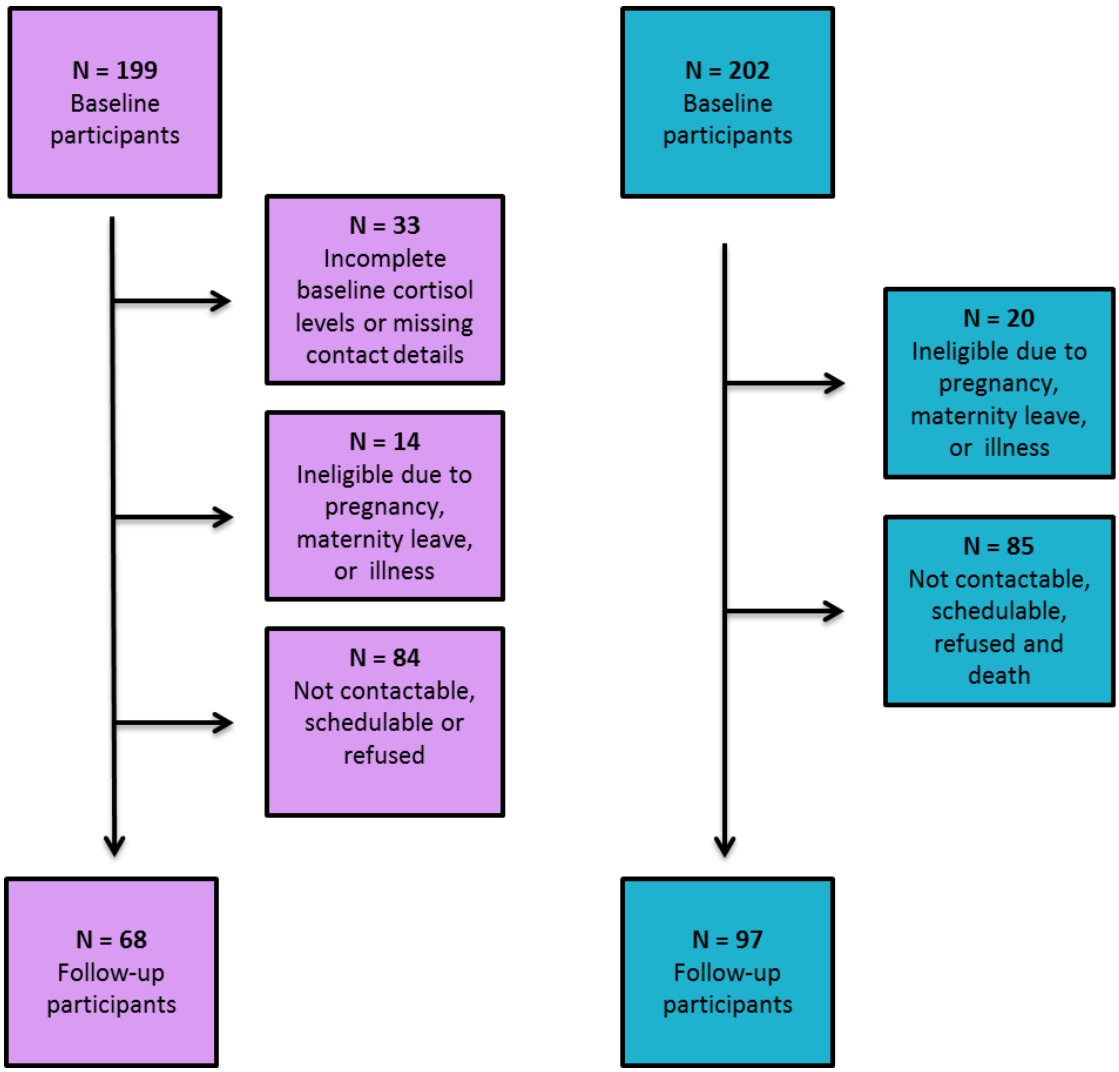

Figure 3.1 Flow chart of recruitment process in London (purple) and Budapest (blue).

3.3.2 Procedure

Data were collected in a research laboratory at UCL and at the Semmelweis University, respectively. At baseline participants provided self-report data on work-related factors and on social support (Appendix 4). Participants' socio-demographic characteristics were then recorded and anthropologic measures were taken with height and weight measures. Body Mass Index (BMI) was calculated using the standard formula kg/m^2. At the follow-up session four years later, each participant was provided with an information sheet (Appendix 2) upon arrival, explaining that the study involved providing a hair sample. Hair samples were taken from the posterior vertex and BMI measurements were repeated. An online questionnaire containing demographic and work-related aspects (Appendix 4) was sent to the participants and was requested to be filled in within a week following hair sampling.

3.3.3 Measures

Hair cortisol

Hair collection took place at the follow-up assessment four years later. A scalp hair strand of 3 cm was collected from the posterior vertex position (all participants were able to provide a hair strand length of 3 cm). Hair strands were cut close to the scalp with fine medical scissors, were placed onto aluminium foil and labelled with the identification number, following the hair protocol described by Kirschbaum et al. (2009). Upon collection, they were stored in a dry, dark place for a maximum period of six months, until shipped to the Technical University of Dresden, Germany. The wash procedure and steroid extraction were undertaken

using high performance liquid chromatography–mass spectrometry (LC-MS), as described by Kirschbaum and colleagues (2009), with a minimum of 10 mg ± 0.5 mg of hair, cut from each 3 cm hair segment. Based on an average monthly hair growth of approximately 1 cm, the scalp-nearest hair segment of 3 cm represents averaged cortisol accumulated over an approximate timespan of three months prior to sampling. Forty-seven samples were assayed in duplicates with a different LC-MS technique and showed high intraindividual stability (r = 0.816, $p < 0.001$). Hair-specific factors that could affect hair cortisol concentration (washing frequency, hair colour and curvature, hair treatment) were assessed by self-report (Appendix 4).

Demographic factors and health-related behaviours

Age, ethnicity and educational attainment were assessed at baseline. As educational systems varied between the countries educational level was classified according to whether the participant had a University degree or not. At baseline and follow-up, information on marital status (married, co-habiting, relationship, single, divorced, widowed – coded into married or not married), children (binary variable) and health-related behaviours, i.e. smoking (binary variable), alcohol consumption (weekly) and physical activity (light, moderate, vigorous weekly level) were collected (Appendix 4). Physical activity was measured by self-report, asking the participants how often ("Never/ hardly ever", "1-3/ month", "1-2/ week", "3 times or more/ week") they engaged in weekly moderately energetic activities such as cycling, scrubbing, decorating, and dancing, and in vigorous activities such as

running, hard swimming, tennis, and cycle racing. The total moderate and vigorous activities engaged in were summed (range: 0-6), with higher scores indicating greater engagement in physical activity.

Income

Both at baseline and at follow-up, participants reported income using an 8-point categorical measure in each country. Due to absolute differences in personal annual income ranges between the UK and Hungary, these variables were harmonised into low, medium and high income categories. The threshold for the three groups in London were: <£25,000, £25,000-£34,999, and >£35,000, while the corresponding cut-offs in Budapest were <1,080,000 HUF, 1,080,000-1,559,999 HUF, and >1,560,000 HUF. Income was therefore referred to as low/ medium / high in the main analyses on income and as a binary variable where applicable for instance for status congruence computation (low: <£35,000 and high: >£35,000, based on sample sizes between the three income groups).

Exposure to stressors/ work-related factors

Work stress was assessed by the ERI model, the components effort and reward were assessed with the Effort-Reward Imbalance Scale (Siegrist et al., 2004). This questionnaire (Appendix 4) consisted of two components: job effort (5 items, Cronbach's $\alpha = 0.75$ – all Cronbach's α presented are for the inventories assessed at follow-up) and job reward (5 items, Cronbach's $\alpha = 0.80$), with reward based on three different schemes: financial/ job promotion, prestige/ respect and control of

job status/ security. The ratio for ERI was calculated by effort/reward. A value above 1 indicates an adverse balance (in which the numerator, i.e. effort, is higher than the denominator, i.e. reward) indicating a higher degree of work stress. The scale shows high internal consistency and discriminant validity based on epidemiologic studies from five European countries, making it suitable to use for two samples from different countries (Siegrist et al., 2004).

Financial strain was assessed with an adaptation of the Economic Strain Model by Pearlin et al. (1981) (Appendix 4). Of the 9 original items, 7 were used to assess difficulty in paying one's bills, the degree of being able to provide for one's family and replacement for items (e.g. car), and the amount of money left at the end of the month. The other two items specified family provision to medical care and clothing and were omitted. The questionnaire has been previously used by other researchers (e.g. Kidd et al., 2014) and the Cronbach's α for this scale in the present study was 0.88.

Social support

Social support was evaluated with the short form of the Interpersonal Support Evaluation List (ISEL–SF; Peirce, Frone, Russell, & Cooper, 1996) (Appendix 4), with 12 questions such as "I feel that there is no one I can share my most private worries and fears with" (4-point scale: "Never"/ "Often"). It measures three dimensions of perceived social support: appraisal, belonging and tangible support. After relevant items being reverse coded, sum scores were computed so that higher

level of social support correspond to a higher score on the scale [range: 0-36]. The Cronbach's α for this scale was 0.84.

3.3.4 Statistical analyses

Data analysis was performed using SPSS 23.0. An alpha level of $p < 0.05$ was considered significant for all analyses. From the hair cortisol values, one outlier (defined as three standard deviations above the mean; raw hair cortisol concentration = 59.9 pg/mg), was identified and removed from statistical analyses. Hair cortisol and salivary cortisol data were skewed and therefore logarithmically (ln) transformed to normalise the distributions. Associations between the work-related factors and social support and hair cortisol levels were examined using linear regression analyses adjusted for age, BMI, smoking status and hair treatment, as these factors have been identified in the literature as covariates for hair cortisol (Russell et al., 2014). Analyses were performed separately within each country and with the full sample, adjusting for country. However, only the regression models on the full sample (adjusted for country) will be presented in this chapter as the results of the analyses by country were not different to those with the combined sample. Financial strain, work-related factors and social support were computed in three different ways, all based on the continuous scale; income was also computed in these three distinct way, based on its categorisation. First, cross-sectional analyses were based on values obtained at the follow-up assessment only (cross-sectional, concurrent level). Further, an aggregate level of scores from baseline and follow-up assessment was computed for each variable, as this may provide a more robust

measure of each factor. Lastly, a change score (follow-up score minus baseline score) was calculated to assess whether shifts in certain factors predicted cortisol.

Results are presented as unstandardised regression coefficients (B) and 95% confidence intervals (C.I.). Socio-demographic differences between the two groups and hair-related characteristics were analysed with t-tests and Chi² tests for continuous and categorical variables, respectively.

3.4 Results

3.4.1 Sample characteristics, descriptive statistics

Participants who completed the follow-up did not differ from those who did not take part on any of the socio-demographic factors (all p's > 0.264), apart from age (t = -3.732, df = 399, $p < 0.001$); follow-up participants were slightly older (M = 37.7, SD = 9.8) than those who did not take part (M = 34.3, SD = 10.2). Table 3.1 summarises the characteristics of the participants that were assessed at follow-up in the two countries (descriptives are presented for data from follow-up assessment only). The age range was similar [29-65 vs. 26-65], but participants from London were significantly younger than participants from Budapest (t = -2.311, df = 162, $p = 0.022$) and had fewer children ($\chi 2 = 15.247$, df = 1, $p < 0.001$). The UK and Hungary samples did not differ in marital status, BMI and smoking status, education, income and status incongruity (all p's > 0.109). Mean +/- SD hair cortisol concentration in the whole sample was 8.39 +/- 6.35 pg/mg [1.92 +/- 0.63 ln(pg/mg)], with no difference between countries (t = -0.326, df = 162, $p = 0.745$).

Table 3.1 Socio-demographic, health-related characteristics and hair cortisol values of study participants at follow-up.

Follow-up Characteristics	Mean (SD) /frequency (%)			Group diff. p-value
	Combined sample (N = 164)	**London (N = 67)**	**Budapest (N = 97)**	
Age	43.6 (9.8)	41.5 (9.3)	45.0 (10.0)	0.022
Body mass index (kg/m^2)	24.1 (4.4)	24.1 (3.7)	24.1 (4.8)	0.952
Current smoker				0.139
Yes	21 (14.0)	4 (8.3)	17 (17.5)	
No	124 (85.5)	44 (91.7)	80 (82.5)	
Children				0.001
Yes	74 (45.1)	18 (26.9)	56 (57.7)	
No	90 (54.9)	49 (73.1)	41 (42.3)	
Marital status (Married)	94 (57.3)	41 (61.2)	53 (54.6)	0.404
Education (degree)				0.234
Yes	117 (72.2)	51 (77.3)	66 (68.8)	
No	45 (27.8)	15 (22.7)	30 (31.3)	
Personal income[a]				0.163
Low	26 (15.9)	15 (22.4)	11 (11.3)	
Medium	50 (30.5)	19 (28.4)	31 (32.0)	
High	88 (53.7)	33 (49.3)	55 (56.7)	
Status incongruity group[b]				0.256
Low	22 (13.6)	10 (15.2)	12 (12.5)	
Negative	52 (32.1)	23 (34.8)	29 (30.2)	
Positive	23 (14.2)	5 (7.6)	18 (18.8)	
High	65 (40.1)	28 (42.4)	37 (38.5)	
Hair cortisol				
(pg/mg)	8.39 (6.3)	8.52 (7.3)	8.30 (5.7)	0.827
ln(pg/mg)	1.92 (0.6)	1.90 (0.7)	1.93 (0.6)	0.745

Note. [a] Income groups are equivalent to <£25,000/ £25,000–35,000/>£35,000 and <1,080,000 HUF/1,080,000–1,559,999 HUF/>1,560,000 HUF for London and Budapest, retrospectively.
[b] Status incongruity: Low (lower income status; lower education group/ no degree), negative (income status < education), positive (income status > education), high (higher income status; higher education group/ degree).

3.4.2 The influence of potential confounders on hair cortisol: cross-sectional analyses

Univariate analyses revealed no associations between hair cortisol and age, education, marital status, children, BMI, smoking status or regular alcohol consumption (see Table 3.2; all *p*'s > 0.168). Physical activity was significantly associated with hair cortisol levels (β = 0.097, C.I. 0.037/ 0.156, *p* = 0.002) with higher levels of physical activity being related to higher levels of hair cortisol. Figure 3.2 illustrates the relationship between hair cortisol and physical activity by dividing the population into tertiles of physical activity. This relationship withstood adjustment for covariates that have previously been suggested to be associated with hair cortisol levels, namely age, BMI, smoking and hair treatment. Physical activity was positively associated with hair cortisol; in line with previous research. The analyses were performed initially without adjustment of this variable as it is not regarded as a covariate, nevertheless, analyses were repeated controlling for physical activity and results were unchanged.

Analyses regarding the hair-related characteristics revealed that hair treatment in the 3 months prior to sampling was associated with hair cortisol; individuals with treated hair had lower cortisol levels than those with untreated hair (β = -0.233, C.I. -0.436/ -0.029, *p* = 0.026; 1.84 vs. 2.07 ln [pg/mg]). Hair colour, number of hair washes per week or use of hair products did not show any associations with hair cortisol (*p*'s > 0.143).

Table 3.2 Association between hair cortisol and demographic, health-related characteristics and hair-related factors (Regressions and ANOVAs, respectively).

Variable	B (95% C.I.)	p-value
Age	-0.007 (-0.017/0.003)	0.168
BMI	0.001 (-0.022/0.024)	0.926
Physical Activity	0.097 (0.037/0.156)	0.002

Unstandardised regression coefficients (B) and 95% confidence intervals (C.I.).

Variable	F (df)	p-value
Marital status	0.834 (1, 162)	0.363
Children	0.322 (1, 162)	0.571
Smoking	0.274 (1,143)	0.601
Alcohol consumption	0.080 (1, 143)	0.778
Hair-related factors		
Hair treatment	5.067 (1,162)	0.026
Hair colour	0.766 (3,160)	0.515
Hair washes (weekly)	1.584 (2,161)	0.208
Hair product use	2.172 (1, 116)	0.143

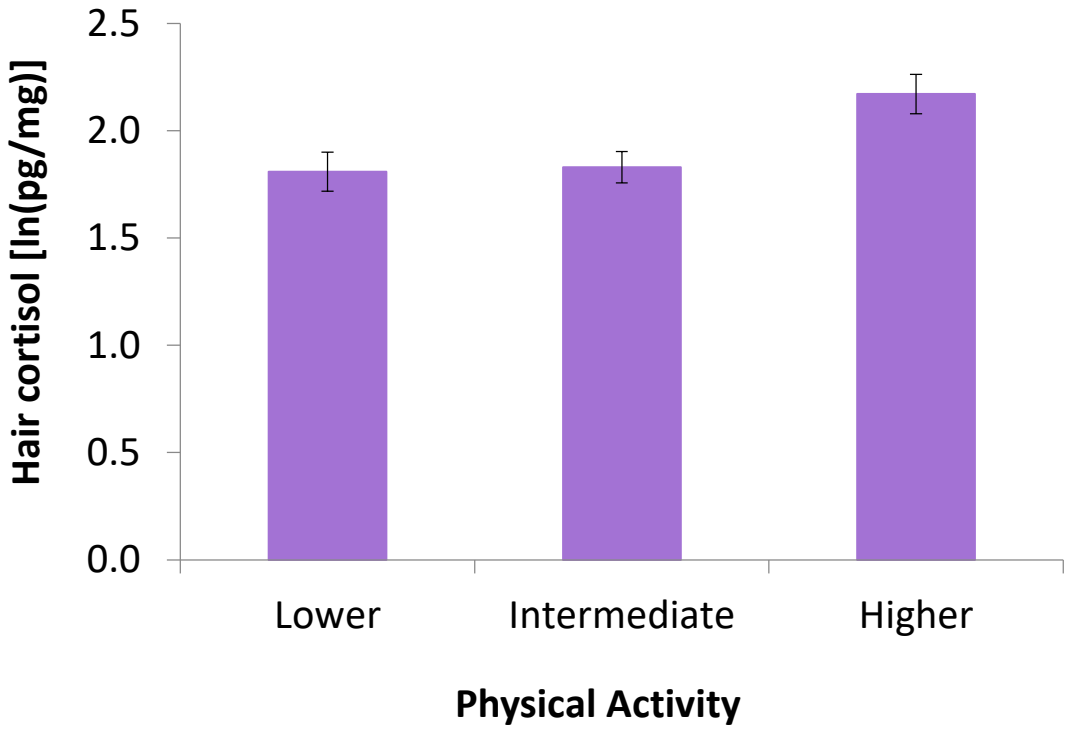

Figure 3.2 Mean hair cortisol levels across physical activity tertiles (for illustrative purpose only). Error bars are SEM.

3.4.3 Socioeconomic factors and status incongruence in relation to hair cortisol

Analysis of covariance (ANCOVA), including polynomial contrast analysis for linear and quadratic terms, were conducted with concurrent income group (income at follow-up), aggregated income group and change in income group in relation to hair cortisol. The two countries did not differ in income level categorisation, therefore these analyses were conducted with the whole sample, adjusting for country as a covariate. There was a linear association between concurrent income group and hair cortisol levels, adjusting for age, BMI, smoking status, hair treatment and country, which withstood also adjustment of physical activity ($F_{2,129} = 3.356$, $p = 0.038$). Figure 3.3 depicts the negative gradient between income group and hair cortisol levels in the three income categories. Post-hoc analyses with the least-significant multiple comparison test adjusting for Bonferroni correction indicated that hair cortisol levels in the lower income group were significantly higher compared to the higher income group (mean difference: 0.402 ln [pg/mg], C.I. 0.013/ 0.791, $p = 0.040$). This confirms that cortisol concentration is greater in lower income participants.

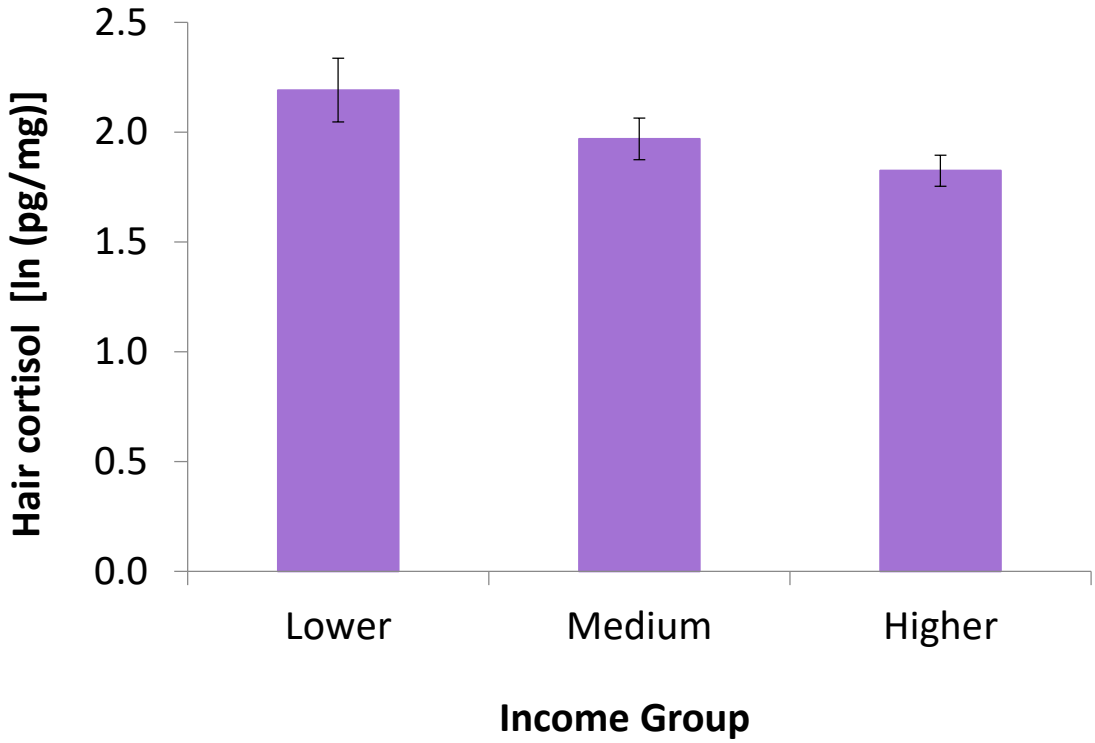

Figure 3.3 Mean hair cortisol levels by income group. Error bars are SEM.

Repeated measures ANOVA showed there was a significant change in income ($F_{1,161}$ = 45.404, p < 0.001), with overall income being higher after 4 years. Figure 3.4 displays the change in frequencies from baseline to follow-up across income groups. At follow-up there were fewer individuals in the lower and middle income groups and more in the higher income group. There was no country-time interaction in income ($F_{1,160}$ = 0.008, p = 0.843). The analysis for the aggregated measure of the income group (aggregation created a wider range of responses in income group, i.e. 1, 1.5, 2, 2.5, 3, which were subsequently analysed in multiple regression) showed a marginal significant negative effect on hair cortisol, adjusted for age, BMI, smoking status, hair treatment and country, and with or without

physical activity (β = -0.159, C.I. -0.319/ 0.001, p = 0.052; β = -0.136, C.I. -0.297/ 0.024, p = 0.096, respectively).

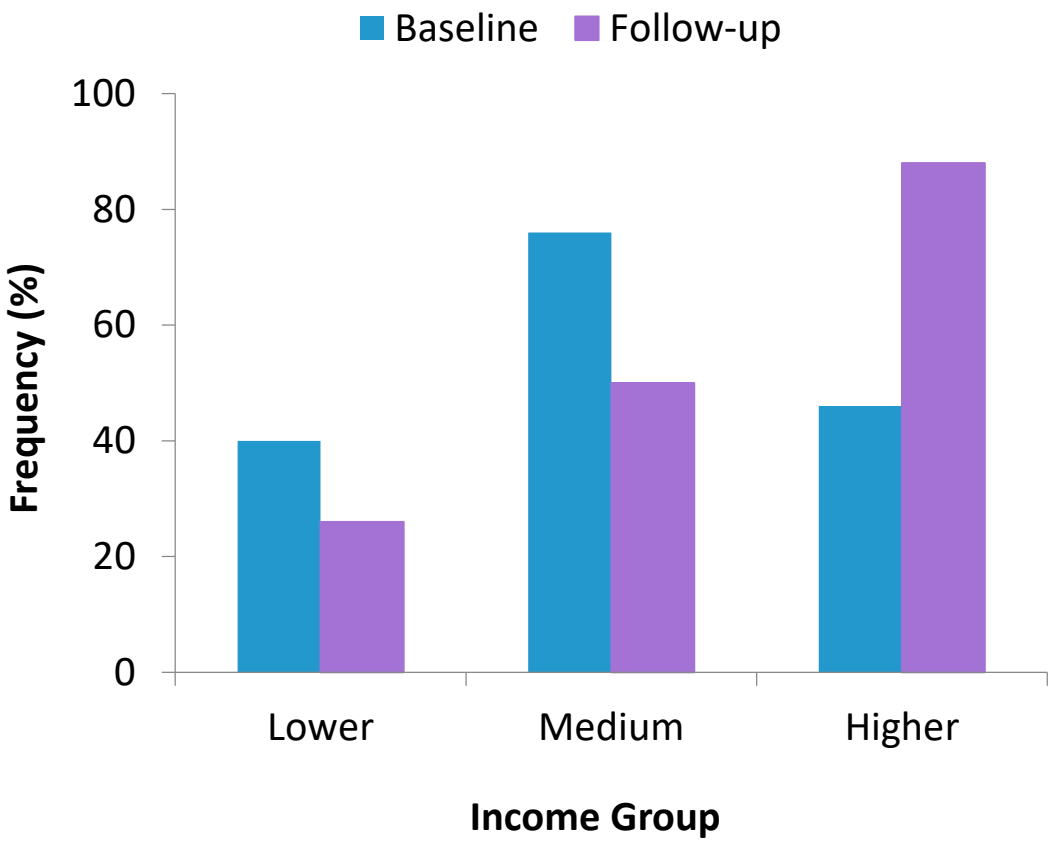

Figure 3.4 Frequency of income group at baseline and follow-up.

Change in income group was linearly associated with hair cortisol; a deterioration in income group was related to higher hair cortisol levels and an improvement in income group to lower hair cortisol, adjusting for age, BMI, smoking status, hair treatment and country, which withstood also adjustment of physical activity ($F_{2,159}$ = 3.067, p = 0.049; Figure 3.5). Post-hoc analyses with the least-significant multiple comparison test adjusting for Bonferroni correction revealed that a deterioration in income was significantly associated with higher hair

cortisol levels, compared to an improvement in income over the four years (mean difference: 0.483 ln [pg/mg], C.I. 0.010/ 0.955, $p = 0.043$).

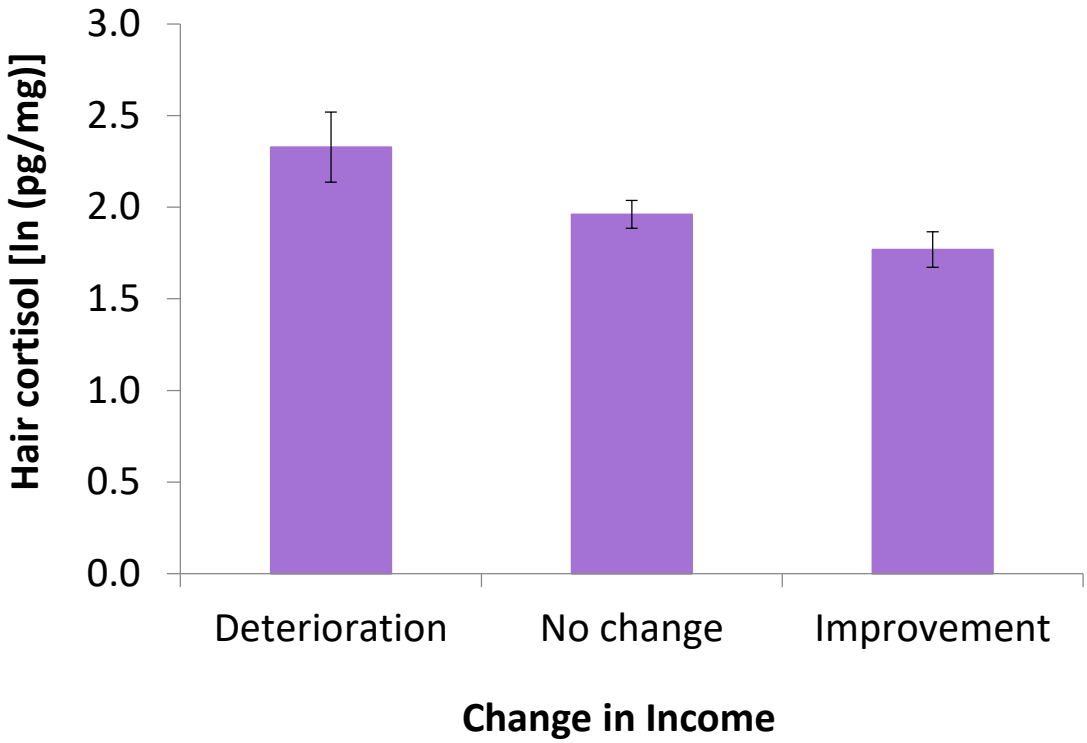

Figure 3.5 Mean hair cortisol levels by change in income over the last 4 years. Error bars are SEM.

Education was unrelated to hair cortisol levels ($F_{1,131} = 0.053$, $p = 0.818$), adjusting for age, BMI, smoking status, hair treatment and country, which did not change if physical activity was included. An interaction term between income (binary variable) and education was computed to assess status incongruity, with four income status/ education categories: low-status congruent group (low income status, low education), negative incongruent group (income status < education), positive incongruent group (income status > education) and high-status congruent group (high income status, high education). I hypothesised that negative status

incongruity would be associated with greater hair cortisol concentration. There was a significant interaction between income and education on hair cortisol concentration, adjusting for age, BMI, smoking status, hair treatment and country, which withstood also adjustment of physical activity ($F_{3,127}$ = 3.610, p = 0.015). Post-hoc analyses with the least-significant multiple comparison test adjusting for Bonferroni correction indicated that hair cortisol levels in the negative incongruent group were higher than in the high congruent status group (mean difference: 0.371 ln [pg/mg], C.I. 0.042/ 0.701, p = 0.018; Figure 3.6).

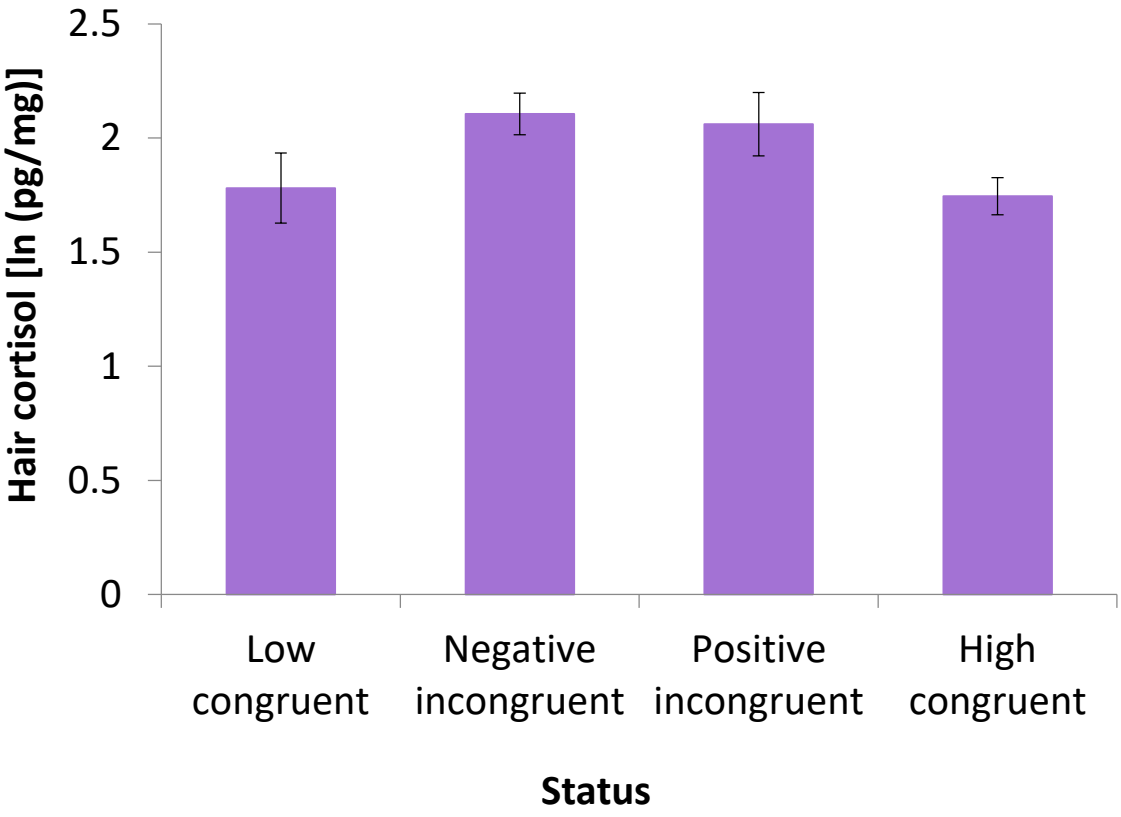

Figure 3.6 Mean hair cortisol levels by status incongruence group. Error bars are SEM.

3.4.4 Associations between hair cortisol and financial, work-related and psychosocial factors

Analyses of country differences in the stress factors

Separate ANOVAs analysed whether the country differed in work-related factors assessed either concurrently with hair cortisol, aggregate level across 4 years, and change scores between baseline and 4-year follow-up. Table 3.3 shows the means and standard deviations for the psychosocial and work-related factors. The two countries differed in financial strain at baseline and follow-up (and hence also at the aggregate level), with participants from London reporting significantly less financial strain than participants from Budapest (follow-up analysis: $F_{1,161}$ = 50.547, $p < 0.001$).

Table 3.3 Work-related and psychosocial factors of study participants.

Characteristics	Level	Mean (SD) /frequency (%)		p-value
		London (N = 67)	Budapest (N = 97)	
Exposure to stressors				
Financial strain	Baseline	4.34 (2.63)	8.10 (3.50)	0.001
	Follow-up	3.80 (2.80)	7.63 (3.71)	0.001
	Aggregate level	4.00 (2.27)	7.86 (3.38)	0.001
	Change	-0.47 (2.66)	-0.47 (2.51)	0.992
ERI	Baseline	0.53 (0.35)	0.79 (0.58)	0.001
	Follow-up	0.63 (0.39)	0.66 (0.40)	0.642
	Aggregate level	0.57 (0.27)	0.73 (0.41)	0.007
	Change	0.12 (0.43)	-0.14 (0.59)	0.003
Effort	Baseline	10.29 (3.98)	12.57 (4.35)	0.001
	Follow-up	12.18 (3.76)	11.72 (4.06)	0.469
	Aggregate level	11.21 (3.17)	12.20 (3.30)	0.061
	Change	1.94 (4.51)	-0.97 (5.24)	0.001
Reward	Baseline	21.51 (4.04)	18.94 (5.58)	0.002
	Follow-up	21.20 (4.30)	20.16 (5.09)	0.182
	Aggregate level	21.41 (3.14)	19.53 (4.77)	0.007
	Change	-0.42 (4.94)	1.09 (5.04)	0.066
Social factors				
Social support	Baseline	25.41 (7.28)	27.84 (6.01)	0.021
	Follow-up	25.34 (7.04)	26.87 (5.69)	0.128
	Aggregate level	25.35 (6.62)	27.42 (5.17)	0.027
	Change	-0.10 (5.55)	-0.84 (5.20)	0.389

Mean values of the ERI scale (Table 3.3) indicate that participants did not experience an 'unfavourable' imbalance between effort and reward on average, as the ratio is below the value 1; perceived work-related rewards were relatively higher than the effort put into the job. Scorings on the ERI scale did not differ on follow-up. However, there were differences at baseline and hence the aggregate level and the change level differed (driven by baseline values) between the two countries; participants from London reported significantly higher 'unfavourable' imbalance (suggesting greater strain) in effort-reward than participants from Budapest at baseline and at the aggregate level. As for the change in the ratio from baseline to follow-up, while the London sample had a deterioration in the imbalance ratio (imbalance score of 0.82 versus 0.98 for baseline and follow-up, respectively), the Budapest sample showed a slight average improvement over the four years (imbalance score 1.02 versus 1.00 for baseline and follow-up, respectively) (*p*'s < 0.01). An 'unfavourable' effort-reward imbalance, i.e. values > 1.0 at follow-up was observed only in 14% of participants (London: 10.9% and Budapest: 16.1%).

There were no differences in social support between the two countries at follow-up ($F_{1,162}$ = 2.336, p = 0.128). However, the two countries differed at the baseline and aggregate level (driven by baseline values) in social support, with participants from London reporting significantly less social support than participants from Budapest (aggregate level analysis: $F_{1,160}$ = 4.955, p = 0.025).

Regression analyses of stress factors on hair cortisol

Several hierarchical regression analyses were performed with the work-related and psychosocial factors as independent variables and hair cortisol concentration as the outcome variable adjusting for age, BMI, smoking and hair treatment. As income and physical activity were found to predict hair cortisol levels, all models were analysed including income as a covariate and also repeated with including physical activity; however, this did not affect the results. Also, the country differed significantly according to whether they had or did not have children, this was tested as another covariate, which again did not affect the results. Hence, the results reported below were based on the models not adjusting for income, physical activity or children. These regression analyses were performed in separate analyses by country (not reported as no different results emerged) and in the analyses with the whole sample (to increase statistical power). Table 3.4 depicts the different regression models with hair cortisol as the dependant variable and the different predictor variables at the three levels for the combined sample.

Financial strain and the ERI model (or ERI components; not reported) were not associated with hair cortisol levels (all *p*'s > 0.107) in either country (not reported) or in the combined country sample. Regression analyses showed that social support at follow-up was positively associated with hair cortisol levels, controlling for age, BMI, smoking status, country and hair treatment (β = 0.024, C.I. 0.007/ 0.041, *p* = 0.005); adjusting also for income and physical activity did not affect this relationship. Reporting higher levels of social support was associated with higher hair cortisol concentration. This was the case for the follow-up

assessment social support score (concurrently assessed measure) and the aggregate level (both *p's* < 0.016). This association emerged both in the whole sample and in separate analyses for each country (not reported). Exploring the three different sub-types of social support at follow-up (appraisal, belonging and tangible) were positively and significantly (tangible; marginally significant) associated with hair cortisol (Table 3.4). Figure 3.7 depicts the gradient relationship between mean hair cortisol levels (with error bars representing SE of the mean) and social support after division into tertiles (for illustrative purpose only). Regression models on financial strain and the work-related factors on hair cortisol were repeated including social support as an interaction term and no differences in findings could be observed. As income was related to hair cortisol and also social support, an interaction term between these two variables was created and assessed in relation to hair cortisol, but there was no significant association, adjusting for age, BMI, smoking status, country and hair treatment (β = -0.054, C.I. -0.006/ 0.003, *p* = 0.567); adjusting also physical activity did not change the findings.

Table 3.4 Regression models of various psychosocial and work-related factors in relation to hair cortisol levels in the combined sample (N = 164).

Psychosocial factor	Level	β (standard error)	B (95% C.I.)	p-value
Exposure to stressors				
Financial strain	Follow-up	-0.134 (0.016)	-0.022 (-0.053 - 0.009)	0.170
	Aggregate level	-0.089 (0.018)	-0.016(-0.051 - 0.019)	0.371
	Change	-0.153 (0.020)	-0.038 (-0.077 - 0.001)	0.107
ERI	Follow-up	0.059 (0.128)	0.092 (-0.162 - 0.345)	0.476
	Aggregate level	0.043 (0.141)	0.072 (-0.207 - 0.351)	0.609
	Change	0.031 (0.098)	0.035 (-0.158 - 0.229)	0.718
Social factors				
Social support	Follow-up	0.251 (0.008)	0.025 (0.009 - 0.041)	0.003
	Aggregate level	0.237 (0.009)	0.025 (0.008 - 0.043)	0.005
	Change	0.080 (0.010)	0.009 (-0.010 - 0.028)	0.330
Social support				
Appraisal	Follow-up	0.053 (0.022)	0.220 (0.010 - 0.096)	0.015
Belonging	Follow-up	0.059 (0.021)	0.243 (0.017 - 0.102)	0.006
Tangible	Follow-up	0.039 (0.022)	0.156 (-0.005 - 0.083)	0.082

Adjusted for age, BMI, smoking and hair treatment, country

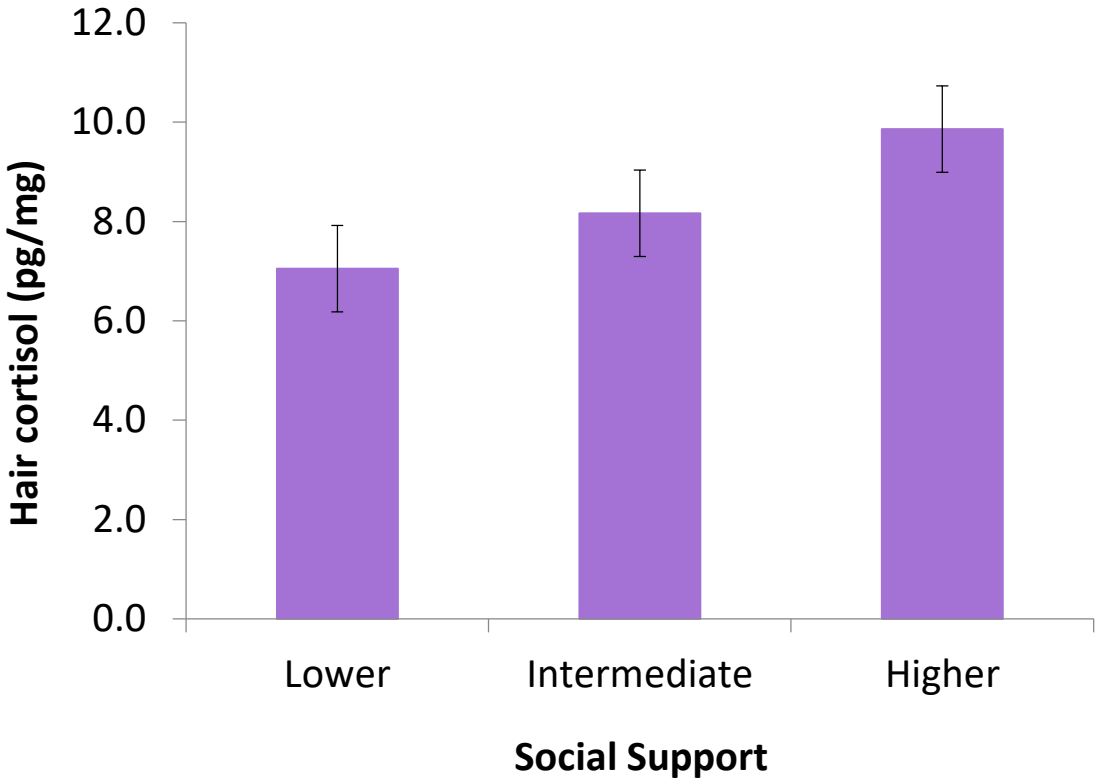

Figure 3.7 Mean hair cortisol levels across social support tertiles (for illustrative purpose only).

Exploratory analyses: Regression analyses by income group

As income was significantly related to hair cortisol levels, using the combined sample (to increase power), separate regression models were run by income group (as a binary variable based on low income as <£35,000 and high income as >£35,000) with the financial strain and the work-related factors. Financial strain and the ERI model were not associated with hair cortisol levels in the low or in high income group (all *p*'s > 0.197). Further exploratory analyses were performed to assess the individual components of the ERI (effort, reward) in relation to hair cortisol, split by income. Four separate regression models were performed with the two individual components as the IV, respectively, and hair cortisol as the DV, adjusting for age, BMI, smoking and hair treatment, country (and including and excluding physical activity). These analyses revealed that higher degree of effort (as a subscale of the ERI model) at work was associated with elevated hair cortisol levels in the high income group (β = 0.247, C.I. 0.001/ 0.074, *p* = 0.043) but not in the low income group (β = -0.042, C.I. -0.048/ 0.036, *p* = 0.766). In the higher salary category, reporting higher amounts of effort at work was associated with elevated hair cortisol levels; there was no difference in hair cortisol based on low or high work effort in the lower income group. Figure 3.8 depicts the relationship between work effort and hair cortisol by income group, showing lower hair cortisol in individuals reporting low effort in the high income group compared to the low income group (mean difference: -0.310 ln [pg/mg], C.I. -0.603/ -0.018, *p* = 0.038).

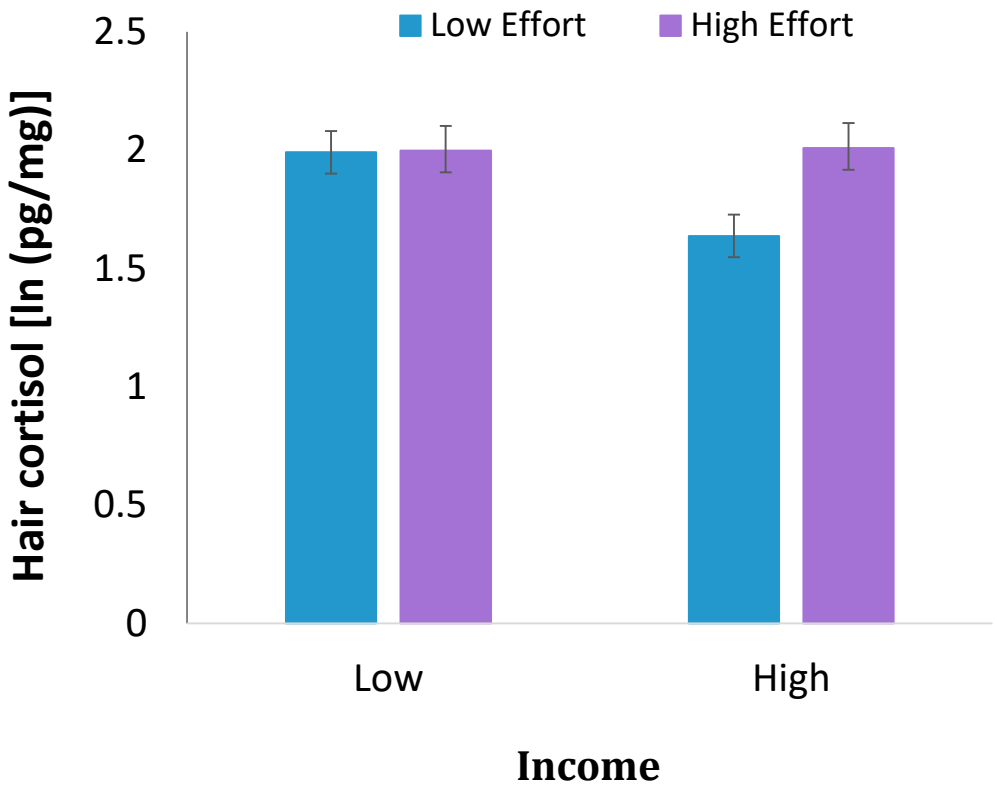

Figure 3.8 Mean hair cortisol levels in low and high work effort by income group.

3.5 Discussion

Hypotheses: i-iv) adverse socioeconomic factors (low concurrent income, income deterioration over a four year period and status incongruity), financial strain and work stress would be associated with elevated hair cortisol levels, v) social support would have a moderating effect on the potential relationship between work stress and hair cortisol.

3.5.1 Summary of findings

The present study investigated the associations between hair cortisol concentration and socioeconomic factors and work-related stress in a healthy sample of working women. All predictor variables were analysed in three distinct forms, concurrently assessed variables (at follow-up assessment), an aggregate score using the mean of the baseline and follow-up assessment and a change score to assess shift over time. A relationship between income and income change over the last four years and hair cortisol concentrations was found. There was a linear association between concurrent income group and hair cortisol levels. Change in income group was linearly associated with hair cortisol; a deterioration in income group was related to higher hair cortisol levels and an improvement in income group to lower hair cortisol. Also negative status incongruity (lower income in relation to level of education) was related to higher hair cortisol levels. Financial strain and work stress factors as assessed with the Effort-Reward Imbalance Model were unrelated to hair cortisol, however, one subscale of the Effort-Reward

Imbalance inventory, namely job effort, was positively associated with hair cortisol in the higher income group only. Social support was positively associated with hair cortisol.

3.5.2 Income

Income and income change over the last four years were associated with hair cortisol concentrations. The pattern indicated that lower income was associated in a graded fashion with higher hair cortisol. Similarly, hair cortisol levels in individuals experiencing an unfavourable change in income over the last four years were higher than in those with no or a favourable change in income. Further, an incongruent status (a mismatch between education and income), for both over- and undereducated individuals in relation to their income group, was related to higher cortisol levels compared with a congruent status (low education/ low income and high education/ high income).

The results are in line with previous studies focusing on traditional cortisol specimens from saliva and blood that have reported lower income to be associated with disturbed cortisol regulation (Cohen et al., 2006; Dowd et al., 2009). Nevertheless, they are novel in terms of showing this association in adults in the Western world in relation to hair cortisol analyses. The findings on income and hair cortisol levels are consistent with recent studies using hair cortisol analyses regarding others aspects and measures of socioeconomic status. Lower income as a measure of deprivation was related to higher hair cortisol levels in women from sub-Saharan African communities (Henley et al., 2014). Further, lower parental

income has been linked to elevated hair cortisol levels in the offspring, in children aged 10-12 years (Bosma et al., 2015) and in children aged 6 (Rippe et al., 2016). Higher hair cortisol levels have also been observed in children from families living in lower compared to higher socioeconomic status neighbourhoods by indication of postal code areas (Vliegenthart et al., 2016), which might be an indication of income. The present study adds to these findings by showing a clear dose-response relationship between income and hair cortisol.

The income measure in the present study was derived from personal salary levels. Interestingly, there are several features of low income and material deprivation that have been linked with detrimental health outcomes, namely absolute income, relative income (comparisons with relevant others at an individual level) and income inequality (comparisons with relevant others at a community level) (Miething, 2013). In the present study, the two samples did not differ in their average hair cortisol levels despite their remarkable differences in overall income; the Hungarian currency cut off points correspond to UK's absolute income values of <£2,400, £2,400-3,600 and £3,600+ per annum. However, we found a gradient relationship *within* the different levels of income and hair cortisol after controlling for country. The associations point therefore to a strong relative income effect, but not an absolute income effect. In fact, relative income seems to have a substantial additive effect on absolute income in relation to mortality (Miething, 2013). And although income levels seem to be useful, objective representations of concrete circumstances, perception of financial resources, interpretations and importance may all be significant if not even bigger contributing factors towards ill-health.

The present study did not find a relationship between financial strain and hair cortisol. However, the inventory for financial strain assessed the capacity for being able to afford certain items rather than asking about perceptions or satisfactions about these capacities. It therefore does not capture how stressful individuals find not being able to afford certain items. Living under financial pressure is a stressor and some individuals might perceive this as more stressful than others. Making use of subjective assessments about income satisfaction and income perceptions in relation to context-dependent relative income might be of higher comprehensive value as they fully capture experiences and evaluations (Veenhoven, 2002). Future studies might want to distinguish between different aspects of financial strain and subjective views of income to investigate this further.

An unfavourable change in income over the last four years was related to higher hair cortisol levels. Dynamic aspects of socioeconomic and psychosocial circumstances seem to be fundamental in studying life course and disease development. In fact, one study found that income trajectory predicted cardiovascular disease mortality more than income per se, thus implying that the dynamics, especially downward trends, have a larger impact on health than chronic financial disadvantage (Johnson-Lawrence et al., 2015).

Other than neuroendocrine pathways, studies have identified further underlying biological correlates of adverse socioeconomic circumstances, such as SNS-PNS (Sympathetic Nervous System- Parasympathetic Nervous System) dysregulation, systemic inflammation and weakened immune functions, adverse metabolic function components as potential mechanisms (Hickson et al., 2012;

Steptoe et al., 2003). Evidence also exists linking income with various immunomarkers and cardiovascular disease risk factors (including cardiovascular disease incidence), such as leukocyte telomere length, CRP, triglyceride and fibrinogen levels; all mechanisms that also accelerate biological ageing (Albert, Glynn, Buring, & Ridker, 2006; Carroll, Diez-Roux, Adler, & Seeman, 2013).

The causal mechanisms of income and income dynamics and these health-related outcomes is not well understood, but increased stress exposure and underlying stress reactions, such as frustration, a deterioration of life quality due to limited access to resources, a disjuncture between actual and expected status and resulting feelings of deprivation are thought to mediate this relationship (Lynch et al., 2004). The present study only focused on a relatively short time period to assess income change, but provides some preliminary findings on the relationship between social mobility and neuroendocrine functioning. A further distinction between income mobility and occupational status mobility could be a fruitful area of research, as they have been shown to co-occur in both a diverging and converging manner over the life-course (Breen, Mood, & Jonsson, in press). The effect of intergenerational mobility (between parent and child) on hair cortisol, considering the pivotal mediating role education might play in the ability of social mobility, also warrants further study (Goldthorpe, 2014). Hair cortisol is a biomarker that is very suitable for future longitudinal studies assessing sustained effects of socioeconomic variations and adjustments.

3.5.3 Education and Income/ education interactions

Hair cortisol concentration was recently found to be negatively associated with education in a sample of young male adults, with education being divided into three subgroups: junior high school/ high school/ above high school (Boesch et al., 2015). Also lower maternal and paternal education has been linked to elevated hair cortisol levels in 339 pre-schoolers (Vaghri et al., 2013). No relationship between education and hair cortisol could be found in the present study. However, it included a group of relatively well-educated women, employed at higher-education institutions in varying positions, in which a certain level of education is a prerequisite. This sample is not representative of the complete population. It is possible that comparing people across the whole spectrum of education would have shown more direct associations with hair cortisol. Although we did not find an effect of education on hair cortisol levels per se, assessing education in relation to income group, status incongruity appeared to have an effect on hair cortisol. In support of this, it has been suggested that various socioeconomic indicators (income, education, occupational position, social class or household area) should be assessed simultaneously as they present specific interdependent interactions and stratification of the different indices of SES has shown to have a specific additional effect on health (Torssander & Erikson, 2010).

A discrepancy between educational attainment and level of income indicates that the individual's status characteristics are not congruent, whether in an inferior (negative incongruent) or superior (positive incongruent) direction. Status incongruity, or also termed status inconsistency and status crystallization,

has been studied in various contexts, ranging from sociology, economic and political environments to health settings (Braig et al., 2011; Lenski, 1954). For example, data from population cohort studies indicate that overqualified individuals have a higher cardiovascular disease risk than congruent qualified individuals, although this effect has not been observed in underqualified individuals (Braig et al., 2011; Honjo et al., 2014). In line with these findings, a recent 19-year longitudinal study found a higher mortality risk only in individuals who were over-educated in relation to their occupational status (negative incongruent) but not in under-educated individuals (Garcy, 2015). The present study did not show this protective effect (i.e. lower hair cortisol levels) in the positive incongruent group, but rather hair cortisol levels that were comparable with those of the negative incongruent group. Only the negative incongruent was significantly associated with higher hair cortisol levels compared to the high status group.

Psychological strain and role conflict might be generated as a result of the economic compensation not corresponding to the individual's intellectual skills and competencies. The underlying aspects for such conflict and dissatisfaction have been suggested to stem from expectancy discrepancy and cognitive dissonance (Sampson, 1963). For instance, shaming experiences but also negative emotions and depressive symptoms have been suggested as negative emotional consequences of status incongruity (Bracke, Pattyn, & von dem Knesebeck, 2013; Lundberg, Kristenson, & Starrin, 2009). Epidemiological and especially psychobiological research would benefit from detailed distinctions between objective and subjective accounts of status incongruity in order to identify the

pathways through which status incongruity affects psychoneuroendocrinological functioning.

3.5.4 Work stress

The different measures of work stress did not show an association with hair cortisol, except work effort being positively related to hair cortisol in the high income group. Nevertheless, the work stress inventories indicated that only a small proportion of the female sample experienced work stress. It might be that this sample was fairly homogenous in not experiencing high amounts of work stress; therefore, not finding any associations between work-related factors and hair cortisol is not surprising. Indeed, it might be the case that the present sample has experienced stress in other work-related aspects, aspects that were not covered by this inventory. Yet, the second work stress model DCM, which assess different work-related stress components (not main focus of this PhD and therefore not reported) was also analysed and no associations with hair cortisol levels could be found.

Nevertheless, only a few studies have investigated hair cortisol in relation to work-related stress in healthy samples and evidence for a relationship has not been strong. Certain populations under adverse work conditions seem to have higher hair cortisol levels compared to individuals not exposed to these stressful conditions, such as has been observed in unemployed individuals and in shift workers (Dettenborn et al., 2010; Manenschijn, van Kruysbergen, et al., 2011). However, unemployment involves stress-associated aspects such as lower financial

gains and the emotional instability that might be responsible for this effect; similarly, in shift workers it is unclear whether stress related to the work schedule and its uncontrollability or fatigue and sleep deprivation drive this increased HPA-axis activity.

Actual work-related stress components such as work-related demands and values and interpersonal resources did not associate with hair cortisol in ready-made garment factory workers in Bangladesh (Steinisch et al., 2014). Garment factory workers might be exposed to similar types of work routines and practices, potentially resulting in relatively similar levels of perceived work-related demands. Similarly to the present study, this might leave a relatively small range and variety of work stress. Work overload, overtaxing and discontent and performance pressure did not associate with hair cortisol in a different sample of 58 adults affiliated to a higher education institution (Stalder, Steudte, Alexander, et al., 2012). Recovery after work, which can be a consequence of work overload and performance pressures, also failed to be associated with hair cortisol in industry workers (van Holland et al., 2012) and in kindergarten teachers (Qi et al., 2015). In the group of kindergarten teachers, an imbalance in job demand-control also did not predict hair cortisol. Yet, hair cortisol was positively associated with an imbalance in the ratio between effort and reward in another sample of kindergarten teachers assessed by the same research group (Qi et al., 2014).

Future studies might aim to target a sample with more divergent work positions in terms of, for instance, job responsibility and demands, job aspirations and security, allowing for a larger variability in work stress. Equally, work stress can

be regarded as a dynamic process, which does not necessarily translate into HPA-axis alterations that can be captured in a long-term cortisol biomarker. The simultaneous assessment of salivary and hair cortisol measures might be informative to investigate this possibility.

3.5.5 Social support

Social support was assessed to investigate potential moderating effects on the relationships between income, financial strain, work stress and hair cortisol. No moderating effect could be found, neither for the income-hair cortisol relationship. The present study found a positive association between social support and hair cortisol concentration. This is surprising and in opposition to what was expected. It was hypothesised that low as opposed to high social support would be related with elevated long-term secretion, in line with previous findings based on salivary cortisol measures and other health-relevant markers and outcomes (Wang et al., Uchino et al., 1996; 2003). To date, social aspects have not been assessed well enough in relation to hair cortisol. Only one study has assessed several psychosocial factors including social support (Stalder, Steudte, Alexander, et al., 2012). Perceived social support was assessed as emotional support (with the 5-item ENRICHD Social Support Index; Kendel et al., 2011) but no association with hair cortisol concentration could be found. The present study assessed social support using three dimensions: appraisal, belonging and tangible support, which all associated with hair cortisol levels independently (tangible support; borderline significance).

The reason for this positive association is unclear and it may be a chance funding. In Study II, I therefore tested the relationship in more detail.

3.5.6 Other associations

Physical activity was positively associated with hair cortisol levels, in line with previous findings (Gerber, Jonsdottir, et al., 2013; Skoluda et al., 2012). This is not a variable that is typically taken into account as a covariate – however, in the present study all analyses were evaluated including physical activity as a covariate, which did not affect the results. Other covariates such as age, BMI and smoking did not associate with hair cortisol. This is not surprising given the limited range of these measures. These associations seem to emerge in studies with a systematic or primary focus on these variables, allowing for wider ranges and bigger sample sizes (Feller et al., 2014; Staufenbiel et al., 2015; Staufenbiel et al., 2013).

3.5.7 Study advantages and limitations

One of the study's main strengths is that it is based on two samples from two different capital cities with remarkable differences in overall income, allowing for analyses of a relative income effect and an absolute income effect on hair cortisol. Further, it makes use of an established work stress model, the Effort-Reward Imbalance Scale. The longitudinal design allowed assessing income and working stress as dynamic processes. The changes over time in income seem sensible to be evaluated in relation to a long-term cortisol biomarker, aspects that are novel in the hair cortisol literature.

Nevertheless, given the exploratory nature of the study, a number of limitations need to be acknowledged. A key limitation is that no hair samples were collected at baseline. This is because the sampling of cortisol in hair was not widely known in 2007 when this study was designed. This means that it is not known whether the differences in hair cortisol in relation, for example, to income seen at follow-up were present at the baseline as well. The sample was limited to young and middle-aged women in the two countries; therefore it is unclear whether the results can be generalised to the wider population. The measure of income reflects personal income and not household income. This was due to a great part of the sample not being married and reporting personal income only. A proportion of the sample may have been living in households in which their personal income only made a partial contribution, hence personal income may not be a good indication of economic resources. However, when marital status and also having children were included in the model, results remained unchanged. Only women were studied in this investigation, but men might be expected to be more sensitive to status incongruity since income has a strong influence on prestige and social status among men (von Rueden, Gurven, & Kaplan, 2011). A more pronounced association between status incongruity and hair cortisol is therefore possible.

With regards to income change, a simple change score of income category was computed which made no distinction between magnitude of income change, meaning that small and large increases in income were treated the same. A bigger sample size allowing for more detailed change estimates would be more informative regarding the gradient of the relationship. Financial strain was assessed

as capacity to be able to purchase essential and non-essential items, but did not evaluate the subjective experience about this and satisfaction with income per se. A more thorough measure of financial strain would be interesting in relation to objective measures of financial situation. The analyses on status incongruity were based on division into four groups of education and level of income. Post-hoc analyses revealed that hair cortisol levels in the negative incongruent status group were significantly higher compared to the high congruent status group but not compared to the other status incongruity groups; it is possible that these analyses suffer from a loss of power and a bigger sample size would have allowed to establish more trustworthy findings.

Further, only a subset of individuals could be re-contacted for the follow-up assessment mostly due to missing, invalid or non-updated contact information. Follow-up and non-follow-up participants did not differ in income and in any other baseline characteristics apart from age. For many younger individuals London is a transient place and participants that could be reached were more settled inhabitants, a factor which might or might not be related to income dynamics and also stressors including financial strain and work stress. Younger individuals might leave London or the UK because of income-, job- or stress-related factors. It is not certain whether this might have played a role in the loss to follow-up. If the younger individuals that were lost at follow-up were included, a different pattern of income dynamics might have emerged which might have impacted the results. Likewise, work stress and financial strain might have been present differently in these individuals which might have been reflected in hair cortisol concentration.

Hair cortisol differences among certain independent variables were significant but relatively small. This is not surprising, since income and status are two of many factors that might affect cortisol output. But if the differences reflect sustained variations in cortisol output over extended periods of months and years, they might contribute to differences in health risk. Other variables that might need to be considered when exploring these associations are other psychosocial factors such as stress, anxiety and depression. Stressful life events have been shown to be related to hair cortisol levels in some studies. At the design phase of the Daytracker study the assessment of life events was not planned. It would have been useful to assess life events, anxiety and subjective stress perceptions in relation to possible life events as these might moderate potential relationships between different stress exposures and hair cortisol. In general, depressive symptoms may present a confounding factor for the analysed relationships, as they can bias self-reports and as they can directly affect both psychosocial variables and hair cortisol. Depressive symptoms were assessed at baseline and at follow-up, however, no associations emerged and were therefore omitted from the analyses.

3.5.8 Conclusion

To conclude, a dose-response association between income, income dynamics on hair cortisol was found. Negative status incongruity was associated with elevated hair cortisol concentration. Quantifying the degree of these socioeconomic elements appears to be relevant to health and underlying biological mechanisms. Clearly, there are multiple pathways by which socioeconomic factors

determine biological functioning and ultimately health; therefore future studies should focus on more comprehensive designs including macro- and microeconomic contexts, social, psychological, behavioural and biological factors. No relationship could be found in relation to work stress and financial strain, however the range of these constructs was relatively narrow. Exploratory analyses revealed that effort (one subscale of Effort-Reward Imbalance) was positively associated with hair cortisol in the higher income group only. Social support was positively associated with hair cortisol levels – a relationship that needs to be explored in more detail in future research.

3.6 Chapter summary

This chapter investigated the associations between hair cortisol concentration and socioeconomic factors, work-related stress and social support. It made use of a study with two time points four years apart and therefore was able to evaluate psychosocial factors cross-sectionally and as a dynamic entity. Income was negatively associated with hair cortisol levels in a dose-response fashion, and also an association between income change (worsening) over the last four years and elevated hair cortisol concentrations was found. Moreover, an effect of status incongruity on hair cortisol was found. Social support was positively associated with hair cortisol levels; an association that was unexpected and warrants further research. No relationship could be found in relation to general work stress and financial strain.

CHAPTER 4. LONG-TERM CONSISTENCY OF CORTISOL: LONGITUDINAL ASSOCIATIONS BETWEEN SALIVARY AND HAIR CORTISOL (STUDY I AND II)

4.1 Chapter overview

This chapter looks at the long-term consistency of cortisol in two distinct tissues, salivary cortisol and hair cortisol by comparing salivary cortisol parameters, specifically the CAR, the AUC and the slope with hair cortisol levels several years later. It makes use of two data-sets. Firstly, it utilises the same dataset that was used in the previous chapter, the Daytracker Study. Secondly, it draws on a bigger population-based cohort, the English Longitudinal Study of Ageing (ELSA), which includes both women and men.

4.2 Introduction

4.2.1 Cortisol long-term consistency

As discussed in Chapter 2, the correct interpretation of hair cortisol as a long-term cortisol marker relies on proper understanding of the time-related features of hair cortisol concerning the associations with other cortisol specimens. Further, longer term consistency and stability of cortisol in general is of interest, regardless of specimen type. The stability of measures over time is variable owing to the influence of a number of situational factors such as time of waking, sleep patterns, environmental conditions, smoking, diet and acute psychological states (Adam & Kumari, 2009; Hansen et al., 2008). Two studies have tested the stability

of different salivary cortisol parameters over extended time periods. In three multi-wave studies involving measurement over a period of 8-24 months, Ross et al. (2014) concluded the CAR to be the least stable parameter, followed by the slope and the AUC. Another study assessed stability over periods up to 6 years (Wang et al., 2014). In this case, the greatest stability was reported for measures of slope, and the least for the CAR. Using hair as a specimen, a few studies have evaluated intraindividual stability of hair cortisol in varying intervals of up to 1 year and report high degree of stability (Liu, Snidman, Leonard, Meyer, & Tronick, 2016; Sauve et al., 2007; Stalder, Steudte, Miller, et al., 2012).

There are mixed findings between the associations between hair and salivary cortisol, with some correlational studies reporting positive associations and some reporting no associations. While these differ in their methodological designs, as either concurrently assessing cortisol in or ensuring corresponding intervals in both tissues, all rely on cross-sectional designs. To date, the longest interval between saliva and hair sampling in the literature was up to 3 months (with a correlation coefficient of r = 0.57 for salivary cortisol and hair cortisol; D'Anna-Hernandez, et al., 2011). In this study, saliva was sampled up to 9 months (1st trimester of pregnancy) before the final (post-partum) hair segment sampling, however the authors do not report any findings about this relationship. Nevertheless, the study was conducted on pregnant women and the known increase in cortisol during the three trimesters might have confounded any longitudinal associations between the two tissues.

Associations between cortisol in saliva and hair have therefore not been used to show longer term consistency so far. To explore long-term associations between different aspects of cortisol diurnal profiles and concentrations in hair two longitudinal datasets were used that had data on salivary cortisol and hair cortisol sampled in the same people several years apart.

4.2.2 The present study

The present study aims to evaluate the long-term consistency of cortisol in two distinct tissues. It compares different salivary cortisol parameters (the CAR, the AUC and the slope) with hair cortisol levels several years later. These analyses are based on two datasets. Firstly, the Daytracker Study, the same study that was outline and used in Chapter 3 and secondly, the English Longitudinal Study of Ageing (ELSA).

Daytracker Study

The Daytracker Study was outlined in the previous chapter in relation to the relevant objectives. For the purpose of the analyses in this chapter, some relevant materials will be added. Briefly, the Daytracker Study was a prospective study initiated at 2007/2008 investigating the associations between psychosocial factors, everyday life experiences and biological reactivity in two contrasting cultures London (UK) and Budapest (Hungary). At baseline, the participants underwent two 24-hour monitoring periods, involving diurnal salivary cortisol assessment and various psychosocial and affective measures. For the analyses on this chapter only

the cortisol measures are of relevance. Four years later, participants were re-contacted and invited for a follow-up study at both sites at which hair was collected for hair cortisol analysis.

ELSA

To further evaluate the longitudinal associations between hair cortisol and salivary cortisol levels, the English Longitudinal Study of Ageing (ELSA) was analysed (Steptoe, Breeze, Banks, & Nazroo, 2013). ELSA is a longitudinal panel study of a representative cohort of men and women aged 50 years and older, formed by a subset of respondents from the Health Survey for England. The cohort was initiated in 2002 with an original sample of 11,391 participants, with follow-ups being conducted every two years since then. It collects objective and subjective data relating to economic, social, psychological, cognitive, health and biological factors. The dataset used for the present analyses involved a subset of ELSA participants who provided hair samples as well as saliva samples.

4.2.3 Aims and Hypotheses

Aims

The aims of the current study were to investigate cortisol long-term consistency of cortisol in two distinct tissues, by comparing salivary cortisol parameters, specifically the CAR, the AUC and the slope with hair cortisol levels measured several years later.

Hypothesis

Hair cortisol concentration will be associated with salivary cortisol parameters collected 4 and 8 years before.

4.3 Method

4.3.1 Participants

Daytracker Study

Participants of this study as well as the recruitment process were described in Chapter 3. Briefly, at baseline, 199 women employed at University College London (UCL) and neighbouring higher education institutions in London, and 202 women employed at Semmelweis University in Budapest were recruited with the following eligibility criteria: aged 18 to 65 years, in full-time paid employment, not taking any medication except for oral contraception, not being pregnant, and not having any serious illness. At follow-up, participants were excluded if they were pregnant, had a chronic or acute condition e.g. CVD or cancer, or were on regular glucocorticoid-containing medication, as these factors have been shown to affect cortisol secretion (Hellhammer, et al., 2009). Of the London sample (N = 199), 68 women took part in the follow-up assessment. Of the Budapest sample (N = 202), 97 women took part in the follow-up assessment. There was no difference in baseline cortisol parameters from individuals who took part in the follow-up and those who did not take part (p's > 0.05).

ELSA

As part of wave 2 (2004/5), a subset of participants (≤80 years old) was asked to take four saliva samples at home over a single day, and to complete a log book at the same time. The number of individuals who provided saliva samples was 4,732, but funding was only available for the analysis of 610 individuals. Samples of hair were assayed from 2,583 participants in wave 6 of ELSA (2012/13). The selection of individuals for saliva analysis was not based on the availability of hair samples or vice versa, so the number of people with both salivary and hair cortisol assays is 394. There was no significant difference in the hair cortisol concentration of participants who were included and excluded from this comparison with salivary cortisol (*p*'s > 0.05).

4.3.2 Procedure

Daytracker Study

Data were collected in a research laboratory at UCL and at the Semmelweis University, respectively. At baseline participants provided saliva samples on two days. Seven saliva samples for each 24-hour period (5pm, at bedtime, at waking, 30 minutes after waking, 10am, 12pm and 3pm) were collected over the two days, a weekday and a leisure day with the order of starting day counterbalanced. The sampling procedure was demonstrated by a researcher in the lab and participants were instructed to abstain from smoking, food and fluid intake, teeth brushing and exercise for a period of 30 min prior to saliva sampling. Participants recorded abstention times before sampling and adherence to the time points of sampling in a

diary. Participants' demographic characteristics (age and smoking) were then recorded. At the follow-up session four years later, each participant was provided with an information sheet upon arrival, explaining that the study involved providing a hair sample of 3 cm length. Hair samples were taken from the posterior vertex. Hair-specific factors that could affect hair cortisol concentration (washing frequency, hair colour and hair treatment) were assessed by self-report.

ELSA

In ELSA, in wave 2 (2004/5), participants were instructed to take four saliva samples: at waking, 30 minutes later, then at 7:00 pm and just before getting into bed. Participants were also asked not to eat, drink or brush their teeth in the 15 minutes before taking the sample. As in the Daytracker Study, saliva was collected using Salivettes (Sarstedt, Numbrecht-Rommelsdorf, Germany) and the procedure was explained to participants by a research nurse during a home visit. Respondents also completed a logbook in which they indicated time of sample collection and gave mood ratings; the latter have been analysed in previous publications (Steptoe, Leigh, & Kumari, 2011; Steptoe & Wardle, 2011). Participants were asked to carry out the assessments on a week day, and returned the samples and logbooks in a prepaid envelope. The hair samples were collected in wave 6 (2012/13) of ELSA by a research nurse during a home visit, using the same procedures as described above, except that 2 cm rather than 3 cm of hair was cut. Information about hair treatment (combining hair dyeing and chemical treatment), smoking and age was also collected. The assays to analyse cortisol in both saliva and hair samples were

conducted by the same research team (Technical University of Dresden, Germany) as for the Daytracker Study and followed the same procedures (see Section 4.3.3 for details).

4.3.3 Measures

Salivary cortisol

Saliva was collected with Salivette devices (Sarstedt, Numbrecht-Rommelsdorf, Germany). Samples were stored in a freezer at −20 °C until they were sent by courier to the Technical University of Dresden, Germany, where steroid extraction was performed. Cortisol levels were assessed using a time resolved immunoassay with fluorescence detection, and the intra- and inter-assay coefficients of variation were less than 4%. To calculate the different diurnal cortisol parameters, the following computational indices were used. The cortisol awakening value was calculated as the difference between cortisol levels at waking and 30 min after waking. The AUC was computed by trapezoidal calculation of the diurnal values and represents the AUC with respect to the ground (Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003). The cortisol slope was defined by regressing cortisol values from waking until 3:00 pm, excluding the 30 min after waking sample. Participants with a delay of more than 15 min between waking and saliva sampling were excluded from the CAR analyses (Dockray et al., 2008).

Hair cortisol

Hair collection took place at the follow-up assessment four years later (Daytracker Study) and eight years later (ELSA), respectively. A scalp hair strand of 3 cm and 2 cm, respectively, was collected from the posterior vertex position. Hair strands were cut close to the scalp with fine medical scissors, were placed onto aluminium foil and labelled with the identification number, following the hair protocol described by Kirschbaum et al. (2009). Upon collection, they were stored in a dry, dark place for a maximum period of six months, until shipped to the Technical University of Dresden, Germany. The wash procedure and steroid extraction were undertaken using high performance liquid chromatography–mass spectrometry (LC-MS), as described by Kirschbaum and colleagues (2009), with a minimum of 10 mg ± 0.5 mg of hair, cut from each 3 cm / 2 cm hair segment. Based on an average monthly hair growth of approximately 1 cm, the scalp-nearest hair segment of 3 cm represents averaged cortisol accumulated over an approximate timespan of three months prior to sampling. In the Daytracker Study, 47 samples were assayed in duplicates with a different LC-MS technique and showed high intraindividual stability ($r = 0.816$, $p < 0.001$).

4.3.4 Statistical analyses

Data analysis was performed using SPSS 23.0. An alpha level of $p < 0.05$ was considered significant for all analyses. In both datasets, hair cortisol and salivary cortisol data were skewed and therefore logarithmically (ln) transformed to normalise the distributions. In the Daytracker Study, one outlier of hair cortisol

(defined as three standard deviations above the mean; raw hair cortisol concentration = 59.9 pg/mg), was identified and removed from statistical analyses. Analyses were performed using the full sample to increase sample size (N = 164), and controlled for country. The two days of saliva collection were combined to provide a more robust measure of the profile. In the Daytracker Study, the salivary cortisol AUC and slope were log-transformed as they were not normally distributed. In the ELSA, only the salivary cortisol AUC was log-transformed.

Multivariate linear regression analyses were used to analyse the association between saliva and hair cortisol concentration, adjusting for covariates of interest (age, smoking and hair treatment). Results are presented as unstandardised regression coefficients (B) and 95% confidence intervals (C.I.).

4.4 Results

4.4.1 Sample characteristics, descriptive statistics and main analyses

Daytracker Study

Participants who completed the follow-up did not differ from those who did not take part on any of the baseline salivary cortisol parameters (all *p*'s > 0.05). As described in Chapter 3 age was significantly different between these two groups age (t = -3.732, df = 399, $p < 0.001$); follow-up participants were slightly older (M = 37.7, SD = 9.8) than those who did not take part (M = 34.3, SD = 10.2). Table 4.1 summarises the characteristics, hair cortisol concentration and different salivary cortisol parameters of the participants that were assessed at follow-up of the

sample combined across countries. Mean +/- SD hair cortisol concentration in the whole sample was 8.39 +/- 6.35 pg/mg [1.92 +/- 0.63 ln(pg/mg)], with no difference between countries (t = -0.326, df = 162, p = 0.745). There were also no country differences in the salivary cortisol parameters (CAR: t = -0.268, df = 148, p = 0.789; AUG: t = -0.737, df = 149, p = 0.462; Slope: t = 1.434, df = 160, p = 0.154, respectively). Repeated-measures ANOVA yielded a main effect of sampling time from the saliva cortisol samples ($F_{1, 150}$ = 25.817, p < 0.001), confirming significant changes in saliva cortisol concentrations over the day and the expected diurnal profile. Figure 4.1 depicts the cortisol profile over the two days combined.

Table 4.1 Characteristics and cortisol values of the combined sample (N = 164).

Variable	Mean (SD) /frequency (%)
Age (years)	43.60 ± 9.8
Current smoker	21 (14.5%)
Hair treatment	54 (32.9%)
Hair cortisol (pg/ml)	8.39 (6.35)
Hair cortisol [ln (pg/ml)]	1.92 ± 0.63
Cortisol CAR (nmol/l)	6.44 ± 7.4
Cortisol AUC (nmol/l)	5177.9 (1925.6)
Cortisol AUC [ln (nmol/l)]	8.48 ± 0.34
Cortisol slope (nmol/l/hr)	-4.61 (1.22)
Cortisol slope [ln (nmol/l/hr)]	0.01 ± 0.02

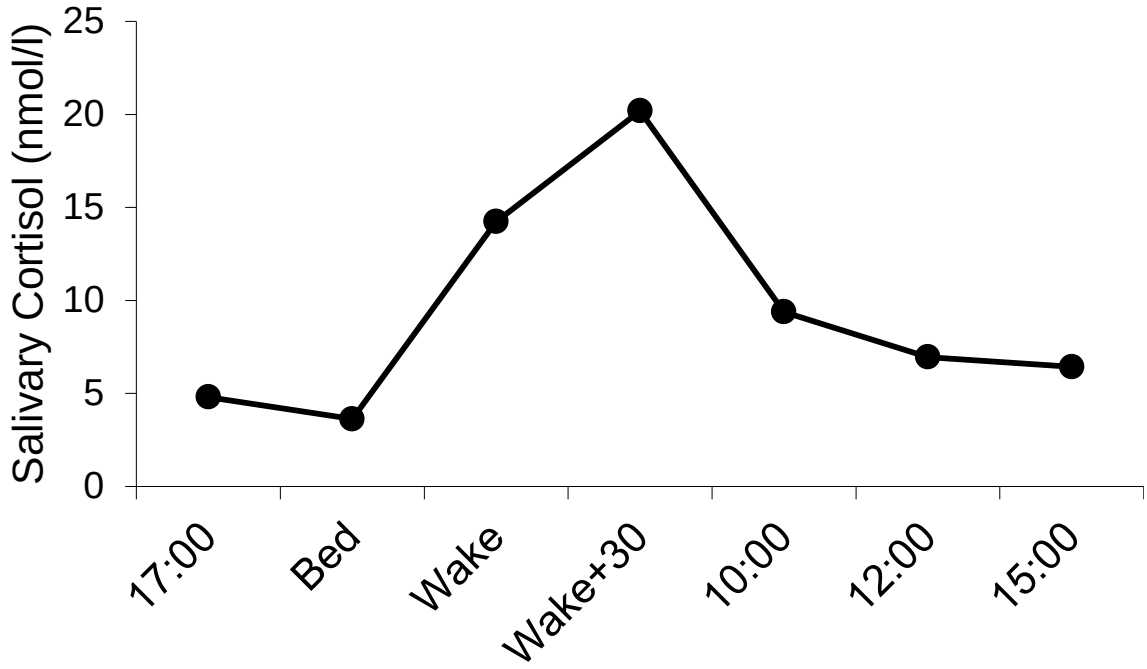

Figure 4.1 Salivary cortisol profile of the combined sample (N = 164).

Multivariate linear regression analyses were performed on hair cortisol concentration (as the DV) with each of the cortisol parameters (as IVs in separate analyses), controlling for the covariates that have previously been deemed important (age, smoking and hair treatment). Additionally, for the analysis using the CAR, waking time was included in the model but did not change the results. Table 4.2 shows the full regression models of the hair cortisol on salivary cortisol slope and other measures. Regression analyses showed that flatter salivary cortisol slope (at baseline) across the day predicted higher hair cortisol levels 4 years later after adjustment for age, smoking, hair treatment and country (p = 0.028). Neither the salivary CAR nor AUC significantly predicted hair cortisol levels (p's > 0.753).

Figure 4.2 graphically depicts hair cortisol concentration in relation to steeper and flatter salivary cortisol following a binary split in slope values.

Table 4.2 Multivariate regression of hair cortisol on the CAR, the AUC and the cortisol slope adjusted for age, smoking, hair treatment and country.

		Regression coefficient B	95% CI	Standardised coefficient β (standard error)	p
a)	Cortisol CAR	-0.00	-0.02 – 0.01	-0.23 (0.01)	0.753
	Age	-0.00	-0.01 – 0.01	-0.06 (0.01)	0.497
	Smoking status	-0.05	-0.36 – 0.26	-0.03 (0.16)	0.750
	Hair treatment	-0.12	-0.37 – 0.13	-0.09 (0.13)	0.286
	Country	0.10	-0.14 – 0.35	0.08 (0.12)	0.397
b)	Cortisol AUC	-0.00	0.00 – 0.00	-0.03 (0.00)	0.739
	Age	-0.00	-0.02 – 0.00	-0.13 (0.01)	0.167
	Smoking status	-0.10	-0.41 – 0.19	-0.13 (0.01)	0.488
	Hair treatment	-0.14	-0.38 – 0.11	-0.10 (0.12)	0.268
	Country	0.11	-0.13 – 0.35	0.08 (0.12)	0.370
c)	Cortisol slope	-7.81	-14.37 – -0.84	-0.20 (3.42)	0.028
	Age	-0.01	-0.02 – 0.00	-0.12 (0.01)	0.202
	Smoking status	-0.06	-0.36 – 0.23	-0.04 (0.15)	0.675
	Hair treatment	-0.13	-0.37 – 0.10	-0.10 (0.12)	0.256
	Country	0.08	-0.15 – 0.31	0.06 (0.12)	0.500

Figure 4.2 Hair cortisol and salivary cortisol slope, adjusted for age, smoking status, hair treatment and country. Error bars are SEM.

ELSA

The analyses of ELSA involved 116 men and 278 women, as detailed in Table 4.3. Respondents were aged 69 years on average, ranging from 52 – 79 years. Fewer than 10% were smokers, but just over half of women in the study had had recent hair treatments (dyeing or chemical treatment). Diary entries in the logbooks indicated that overall the four saliva samples obtained in 2004/5 were taken according to schedule, with mean times (standard deviation) of 7:14h (0:54), 7:44h (0:54), 19:05h (0:24), and 22:59h (0:55) for waking, waking + 30 minutes, 7:00pm and bedtime samples. The profile of salivary cortisol is summarised in Figure 4.3, showing a typical pattern of high levels on waking, an increase 30

minutes later, declining to low values in the evening. We found a near significant difference in cortisol AUC between men and women with greater values in men, but no difference in cortisol slope over the day. Hair cortisol concentrations were significantly greater in men than women (Table 4.3). There was, however, no difference in hair cortisol concentration between women who did and did not have hair treatments (p = 0.643).

Table 4.3 Characteristics and cortisol values by sex.

	Men (N = 116)	**Women (N = 278)**	***p* difference**
Age (years)	69.0 ± 5.6	68.7 ± 5.4	0.618
Current smoker	9 (7.8%)	26 (9.4%)	0.704
Hair treatment	0 (0.0%)	150 (54.0%)	0.001
Hair cortisol [ln (pg/ml)]	2.50 ± 1.47	1.91 ± 1.31	0.001
Cortisol CAR (nmol/l)	8.65 ± 13.9	7.69 ± 14.0	0.548
Cortisol AUC [ln (nmol/l)]	4.16 ± 0.18	4.11 ± 0.22	0.053
Cortisol slope (nmol/l/hr)	1.36 ± 0.77	1.31 ± 0.68	0.517

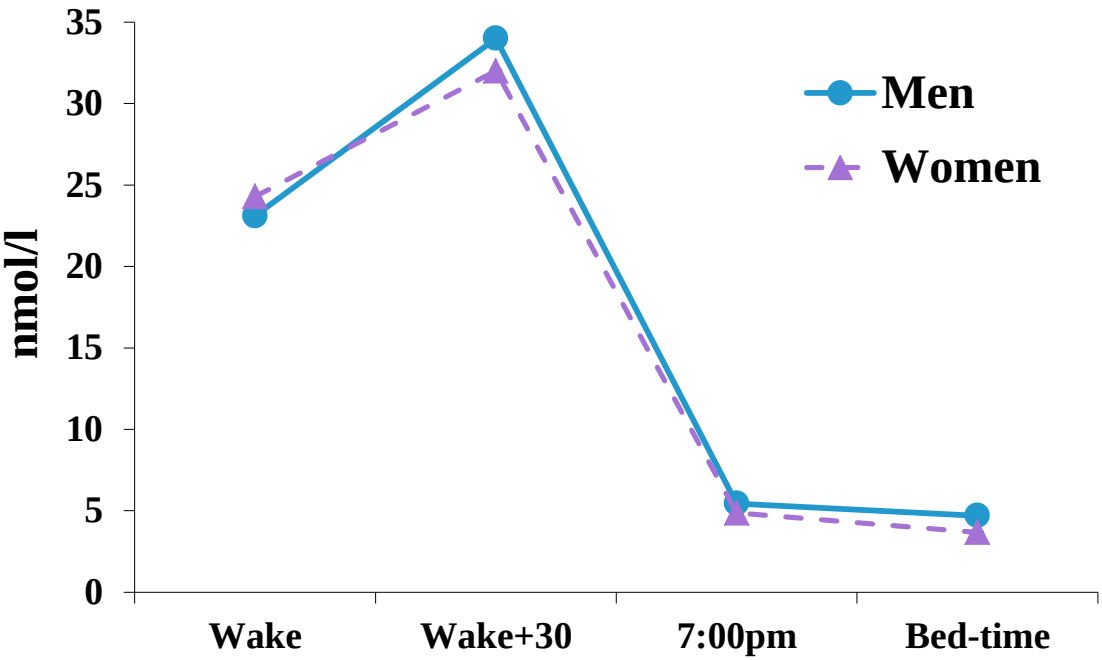

Figure 4.3 Salivary cortisol profile in the ELSA by sex.

The regressions of salivary cortisol measured 8 years earlier on hair cortisol concentrations are summarised in Table 4.4. Hair treatment was not included as a covariate in these analyses, since it was not associated with the outcomes. In men, there was a significant negative association between cortisol diurnal slope and hair cortisol concentration (p = 0.028). Men with flatter slopes of cortisol decline over the day had significantly higher hair cortisol concentrations. Figure 4.4 depicts this relationship between hair cortisol and the slope as a binary variable. In addition, age was positively associated with hair cortisol, with higher concentrations in older participants (p = 0.004). There was no association between cortisol AUC or CAR (with or without waking time included as a covariate) and concentration in hair among men, and no significant associations in women. The results for women were

unchanged when hair treatment was included in the model, or when analyses were limited to those who did not report any hair treatments.

Table 4.4 Multivariate regression of hair cortisol on the CAR, the AUC and the cortisol slope adjusted for age and smoking in men and women.

		Regression coefficient B	95% CI	Standardised coefficient β (standard error)	*p*
Men					
a)	Cortisol CAR	0.006	-0.002 – 0.02	0.14 (0.09)	0.148
	Age	0.03	0.01 – 0.05	0.27 (0.09)	0.006
	Smoking status	0.43	-0.04 – 0.89	0.17 (0.09)	0.070
b)	Cortisol AUC	-0.22	-0.88 – 0.45	-0.06 (0.09)	0.517
	Age	0.03	0.01 – 0.05	0.27 (0.09)	0.004
	Smoking status	0.38	-0.06 – 0.82	0.16 (0.09)	0.092
c)	Cortisol slope	-0.16	-0.02 – -0.31	-0.20 (0.089)	0.028
	Age	0.30	0.01 – 0.50	0.26 (0.091)	0.004
	Smoking status	0.33	-0.10 – 0.76	0.09 (0.093)	0.350
Women					
d)	Cortisol CAR	-0.001	-0.006 – 0.004	-0.31 (0.06)	0.621
	Age	0.01	-0.002 – 0.02	0.10 (0.06)	0.104
	Smoking status	0.07	-0.17 – 0.30	0.04 (0.06)	0.563
e)	Cortisol AUC	-0.10	-0.41 – 0.21	-0.04 (0.06)	0.542
	Age	0.01	-0.00 – 0.02	0.10 (0.06)	0.114
	Smoking status	0.07	-0.17 – 0.30	0.03 (0.06)	0.577
f)	Cortisol slope	0.07	-9.20 – 9.38	0.001 (0.06)	0.980
	Age	0.73	-0.45 – 1.92	0.07 (0.06)	0.231
	Smoking status	-3.61	-25.59 – 18.37	-0.02 (0.06)	0.750

Figure 4.4 Hair cortisol and salivary cortisol slope in men only, adjusted for age and smoking status. Error bars are SEM.

4.5 Discussion

Hypothesis: Hair cortisol concentration will be associated with salivary cortisol parameters collected 4 and 8 years before.

4.5.1 Summary of findings

This study assessed the long-term consistency of cortisol in two distinct tissues, salivary cortisol at baseline and hair cortisol at follow-up in two independent studies. A relationship between the rate of decline in salivary cortisol over the day and hair cortisol concentrations several years later could be found. Specifically, the slope was negatively associated with hair cortisol, indicating that individuals with a flatter slope of cortisol decrease over the day had higher hair cortisol concentrations. This pattern was observed in two independent studies of healthy younger and middle-aged working women (Daytracker Study) and in older men (ELSA). Interestingly, the association between salivary cortisol slope and hair cortisol was not found in the older women of the ELSA. The CAR and the AUC did not show any associations with hair cortisol concentration in either study.

4.5.2 Links to previous work and interpretation of findings

A flatter slope was associated with elevated hair cortisol concentration 4 and 8 years ago (in men only) in two distinct datasets, respectively. Previous studies comparing cortisol in hair with saliva have reported associations with overall cortisol values or AUC rather than specific components of the diurnal rhythm such

as the slope over the day (D'Anna-Hernandez et al., 2011; Steudte, Stalder, et al., 2011; van Holland et al., 2012; Xie et al., 2011). However, these investigations focused on assessments in the two tissues over the same time period, not the longer term associations explored here. The present study differentiated between three parameters, the CAR, the AUC and the slope of decline over the day. It is notable that the cortisol slope rather than the AUC showed an association with hair cortisol and that this finding was replicated in two independent studies with very different participants and time intervals between saliva and hair measurement. It might have been expected that the AUC which provides an estimate of total diurnal cortisol output would be the strongest indicator of accumulated long-term cortisol as captured in hair.

The reasons for the association with cortisol slope rather than AUC are not clear. One possibility relates to the stability of the two parameters. There are mixed findings on the stability of the various salivary cortisol parameters. In Ross et al.'s (2014) study, AUC was more stable than other salivary cortisol parameters over a two year period. However, this study predominantly involved children and young adults, with only 47 middle-aged participants. By contrast, the larger study of middle-aged adults by Wang et al. (2014) showed stronger stability over a 6-year period for the slope across the day rather than AUC. So it is plausible that stronger associations with cortisol slope emerged because it shows stronger long-term stability.

A second possibility is that longer-term associations with hair cortisol concentration were observed for cortisol slope because this reflects individual

differences in impaired regulatory processes and diminution in normal diurnal variation. As noted in Chapter 1 (section 1.3), a body of research has linked a flattening of the diurnal cortisol rhythm to adverse mental and physical health outcomes (Bower et al., 2005; Kumari et al., 2011; Liao et al., 2013). As the slope is an indicator of the trajectory of change across the day, and might therefore indicate circadian disruption, individuals with maladaptive cortisol decreases across the day repeated over long periods could show elevated cortisol levels in hair several years later. This association might be particularly marked at older ages when chronic diseases associated with inflammation and neuroendocrine dysregulation become more common, and chronic allostatic load leaves its mark on health outcomes (Gruenewald, Seeman, Ryff, Karlamangla, & Singer, 2006).

An additional possibility relates to nocturnal cortisol output. The secretion of cortisol into hair presumably continues in the night as well as the day, while saliva samples are only taken in the day and evening. The overall output over the day reflected in AUC provides limited information about 24-hour output, whereas a flatter slope could be more closely associated with individual differences in nocturnal output. However, this explanation is speculative and cannot be explored directly in the present datasets.

In the present study hair cortisol concentrations were remarkably similar in women from the two studies, despite the difference in age. While previous findings have been mixed regarding the associations between hair cortisol and age, inconsistencies are partly due to the limited age ranges tested, with age itself not being the main focus of the research (Dettenborn, Tietze, et al., 2012; Feller et al.,

2014). A more systematic study by Feller et al. (2014) investigated 654 adults aged 47-82 years and reported that hair cortisol concentration increases with age. Why this was not the case in the present study is unclear. However, the older samples had higher salivary cortisol values on waking and 30 minutes later compared to the younger samples. Based on the salivary cortisol literature, age-related changes of the HPA-axis function have been commonly associated with features of neurocognitive ageing, psychological well-being and physical capacities. Indeed, HPA-axis dysfunction has been suggested as one of the distinct pathways contributing to both physiological and pathological ageing, resulting from impaired HPA feedback and reduced glucocorticoid receptor sensitivity (Gupta & Morley, 2014; Sapolsky, 1999; Straub et al., 2001). A body of studies has reported a flattened diurnal cortisol pattern with increasing age, with higher nocturnal nadir concentrations, resulting in overall elevated daily levels (Deuschle et al., 1997; Heaney, Phillips, & Carroll, 2012). Some studies have described reduced cortisol upon awakening and a less pronounced CAR in older than younger people (Heaney et al., 2012; Kudielka & Kirschbaum, 2003) though this has not been a consistent finding (Wust, Wolf, et al., 2000). In the present study, the older sample had higher morning cortisol levels than the younger participants, while evening levels did not differ. Individual variability and the complexity of cortisol rhythmicity and medical comorbidities associated with ageing may contribute to the mixed findings.

The explanation of why associations between saliva and hair emerged in ELSA only in men and not in women is uncertain. While some studies using salivary cortisol samples have shown no sex differences in the age-related variation in

cortisol (Wust, Wolf, et al., 2000), others have reported an increased CAR with age only in men (Almeida, Piazza, & Stawski, 2009a). No sex difference in the salivary cortisol slope or the CAR could be found, although the AUC tended to be greater among men (Table 4.3). But it is noticeable that hair cortisol concentration was higher in men on average than women. Previous studies including the work by Feller et al. (2014) have also reported that men have a higher hair concentration than women (Dettenborn, Tietze, et al., 2012; Manenschijn et al., 2013). It is possible that this sex difference in hair cortisol levels, together with a slightly wider range of hair cortisol values in men, might have driven this sex-related association seen in ELSA.

4.5.3 Study advantages and limitations

The strengths of the study are that two independent data-sets with different age groups were employed and that three distinct salivary cortisol parameters were assessed in relation to hair cortisol simultaneously. The fact that associations emerged in one of the salivary cortisol parameters but not in others endorses the value of obtaining several markers of the diurnal profile within the same study. If feasible and/ or data is available, future studies should incorporate different aspects of cortisol diurnal profiles simultaneously.

Due to the exploratory nature of the study, several limitations must be acknowledged. First, as outlined already above only a subset of individuals could be re-contacted in the Daytracker Study. Nevertheless, apart from age, follow-up and non-follow-up participants did not differ in their baseline characteristics. Diurnal

profiles were based on two sampling days in the Daytracker Study and only one sampling day in ELSA. Aggregating salivary cortisol measures obtained from an increased number of study days would have provided a more robust estimate and might have strengthened the findings. Moreover, participant adherence to the saliva sampling protocol was based on self-report. The use of objective measures such as electronic monitoring devices would have enhanced the reliability of salivary cortisol values and the resulting profiles of change over the day (Kudielka et al., 2003).

4.5.4 Conclusion

To conclude, two independent studies with different age groups were analysed, and provided consistent associations between the diurnal cortisol slope and hair cortisol levels several years later. No evidence for a long-term association between hair cortisol and either the AUC or the CAR was found. The fact that associations emerged in slope across the day endorses the value of obtaining several markers of the diurnal profile within the same study. Hair cortisol analysis has emerged as a suitable method for assessing long-term cortisol exposure. The present findings support the notion that hair and saliva reflect common fundamental processes in HPA regulation, and also suggest enduring stability in cortisol dysregulation.

4.6 Chapter summary

This chapter evaluated the long-term consistency of cortisol in two distinct tissues, salivary cortisol and hair cortisol. The findings indicate that a flatter rate of decline in salivary cortisol over the day was associated with elevated hair cortisol concentrations several years later, 4 and 8 years ago (in men only) in two distinct datasets, respectively. No relationship could be found between hair cortisol in relation to the AUC and the CAR. Several limitations were acknowledged such as assessment of cortisol, time-frame of assessment and loss at follow-up and lack of inclusion of certain stress-related aspects such as the experience of life events; factors that will be addressed more thoroughly and systematically in the next chapter.

CHAPTER 5. ASSOCIATIONS BETWEEN SALIVARY CORTISOL AND HAIR CORTISOL – A SYSTEMATIC COMPARISON (STUDY III)

5.1 Chapter overview

Correlational studies between hair cortisol and different cortisol specimens have been conducted to understand the correspondence of systemic cortisol production with cortisol output found in hair. This chapter makes use of a dataset called the Academic Stress Study, in which medical and law students are assessed in two distinct phases: during average academic stress and during high academic stress. In both phases diurnal salivary cortisol was taken three times over a period of a month and hair cortisol is assessed at the end of this period, addressing some of the methodological shortcomings of previous work (e.g. ensuring corresponding time intervals and appropriate hair growth). This assessment over corresponding time-intervals of both tissues allows more reliable evaluation of hair cortisol as an index of actual systemic or central HPA-axis function.

5.2 Introduction

Chapter 2 has described the development of hair cortisol analyses. The most direct validation of hair cortisol analysis has been derived from direct comparisons between cortisol measures from hair and saliva (also urine and blood cortisol). These comparison studies have been conducted to establish whether hair cortisol truly reflects integrated cortisol output over a prolonged time period. All correlational studies that have been conducted so far were outlined in Chapter 2

(see Section 2.7.2). Some studies have sampled both salivary and hair cortisol concurrently or over corresponding time-intervals, with most of the early studies using the former design and some later studies the latter. Concurrent assessment (at the same time point) of the different tissues, i.e. saliva/ blood/ urine cortisol implies that systemic cortisol levels do not represent the same time period as reflected in the analysed hair segment. Assessment of corresponding time-intervals in which systemic cortisol levels are assessed is a stronger approach methodologically. However, there is still some uncertainty as to the exact time period to which hair cortisol corresponds. The present chapter describes a study in which salivary measures were taken at several time points before hair samples, so as to investigate this issue in more detail.

There are only a few studies that assessed the saliva-hair cortisol correlation with saliva sampling in the weeks before hair measures. These were described in Chapter 2 but will briefly be outlined again to highlight the different methodologies and correlation coefficients. In the first, Xie et al. (2011) reported a correlation coefficient of $r = 0.383$ in 32 healthy young men between one single salivary sample (30 min. post-awakening) taken at one-week intervals three times over a period of a month, followed by a 1 cm hair segment at the end of the month. The investigators found hair cortisol to be correlated with salivary cortisol only at one single time point and when averaged. The study by D'Anna-Hernandez et al. (2011), with 21 pregnant women, reported correlation coefficients of $r = 0.43$ and $r = 0.57$ between 3 cm hair segments and salivary cortisol AUC values based on six sampling days spread out over the corresponding trimester. Pregnancy is an exceptional

state with elevated systemic cortisol production. While this might provide a further context in which hair cortisol can be validated, these saliva-hair cortisol associations may not reflect normal patterns.

The more recent study by Short et al. (2016) was strong from the methodological point of view with similarities to the present study. In this study, 17 adults took three salivary cortisol samples (at awakening, +30 min post-awakening, at bedtime) every day over a period of 30 days, with a 1 cm hair segment taken after this period. Three parameters of cortisol output were computed, the CAR, the AUC and the slope, but only the AUC showed positive correlations with hair cortisol: the monthly averaged salivary AUC values had a correlation coefficient of r = 0.61 and the prior 3 and the 4 weekly averaged AUC values correlation coefficients of r = 0.50 r = 0.56, respectively; the AUC measures based on the 1 and 2 weeks before hair sampling were not associated with hair cortisol at all. This study had a small sample, and the saliva sampling protocol was not ideally suited to the assessment of AUC, since no measures were obtained between 30 minutes after waking and bedtime, and a linear change in output over this period is probably not an accurate representation of the true pattern of change. It is interesting that salivary concentrations in the two weeks before hair sampling were not significantly associated with hair.

My plan in this study was to evaluate correspondence between salivary and hair cortisol by systematically studying three time periods - two weeks, four weeks, and six weeks - before hair sampling. I wished to differentiate the three salivary parameters (AUC, slope and CAR), and investigate in a systematic design how these

associate with hair cortisol. The AUC is a marker of total diurnal output and might therefore be expected to show high correlations. Further, the 30 minutes post-awakening value, reflecting the early morning diurnal zenith of the diurnal cortisol profile, might present a strong indicator for accumulated peak cortisol output. Additionally, the previous study (Chapter 4) showed associations between salivary diurnal slope and hair cortisol, providing rationale for assessing the different cortisol parameters simultaneously. It was also of interest to study these associations under conditions of higher and lower stress, rather than just the ordinary period analysed by Short et al. (2016).

The study described in this chapter makes use of a naturalistic stressor 'academic stress' and therefore assesses students in two distinct phases, one under average academic stress and one under high academic stress. Comparisons between the days surrounding academic examinations and control periods have shown reliable changes in psychological and physiological processes (Amir et al., 2010; Glaser, Pearl, Kiecolt-Glaser, & Malarkey, 1994; Steptoe, Wardle, Pollard, Canaan, & Davies, 1996). Specifically, in periods of stress exposure individual psychosocial factors might play a major role in affecting fluctuating salivary cortisol levels throughout the assessed month (Weekes et al., 2006b; Chapter 6 mainly focuses on the psychosocial factors in relation to these two time periods). Comparing the associations within the same individuals in a systematic correlational design, using corresponding time intervals, in a period under high stress and under relatively low stress might be informative in relation to the

strength of the variability and correlation between salivary and hair cortisol samples.

An important issue in the correlational studies based on the corresponding time-intervals is ensuring that the hair is allowed to grow appropriately, as it has been suggested that a time lag effect of hair growth occurs. A review on general hair cortisol collection methods and analyses concluded that new hair takes around two weeks to be formed in the follicle and to appear at the hair shaft (LeBeau et al., 2011). Thus, hair cortisol analyses that examine corresponding time intervals between salivary cortisol and hair cortisol would need to consider these two weeks. Therefore, it might be the case that the hair segment does not accurately reflect the exact time frame that is measured in the saliva sampling period. None of the correlational studies have taken this time lag effect into account. The present study is designed to allow appropriate hair growth (of 2 weeks after last salivary sampling) to overcome this timing issue.

5.2.1 The present study

The present study, called the Academic Stress Study, is based on a within-subject design, studying medical and law students in two distinct phases: during average academic stress and during high academic stress. Medical (less predominantly law students) students are often targeted for such research as students from this/these degrees generally experience a higher workload and face greater responsibilities compared to other student groups (Glaser et al., 1994). As in the previous study, the Daytracker Study, there are two main focal points of

research. The first aim is methodological in nature: analysing the saliva-hair correlation. This is the focus of the present chapter. Secondly, I was interested in studying hair cortisol levels as an index of stress reactivity to chronic naturalistic stressors, by comparing hair cortisol between the two periods. This is the focus of Chapter 6.

5.2.2 Aims

Aims

The aim of this study was to evaluate the temporal relationship between hair cortisol and saliva samples. Comparisons were made between salivary and hair cortisol levels under average and under high academic stress conditions (the impact of stress will be examined in the next chaper).

Hypothesis

Hair cortisol concentration will be associated with the four salivary cortisol parameters throughout the preceding six weeks (at the different time points) and most strongly with the averaged (monthly) salivary AUC.

5.3 Methodology

5.3.1 Study design

The study had two phases, six months apart: one under average academic stress, which took place in October – December 2013, and the second under high academic stress, which took place in April – May 2014 (Figure 5.1). Each phase lasted 7 weeks and meetings were scheduled at the beginning and end of each. Participants completed three diurnal salivary cortisol profiles at week 1 [termed salivary cortisol collection (SCC) Time 1], week 3 (SCC Time 2) and week 5 (SCC Time 3), equivalent to 6, 4 and 2 weeks, respectively, prior to hair cortisol sampling, with five diurnal saliva samplings at each collection point. Hair samples were taken in week 7, two weeks after the last salivary sampling to ensure corresponding hair growth [termed hair cortisol collection (HCC)].

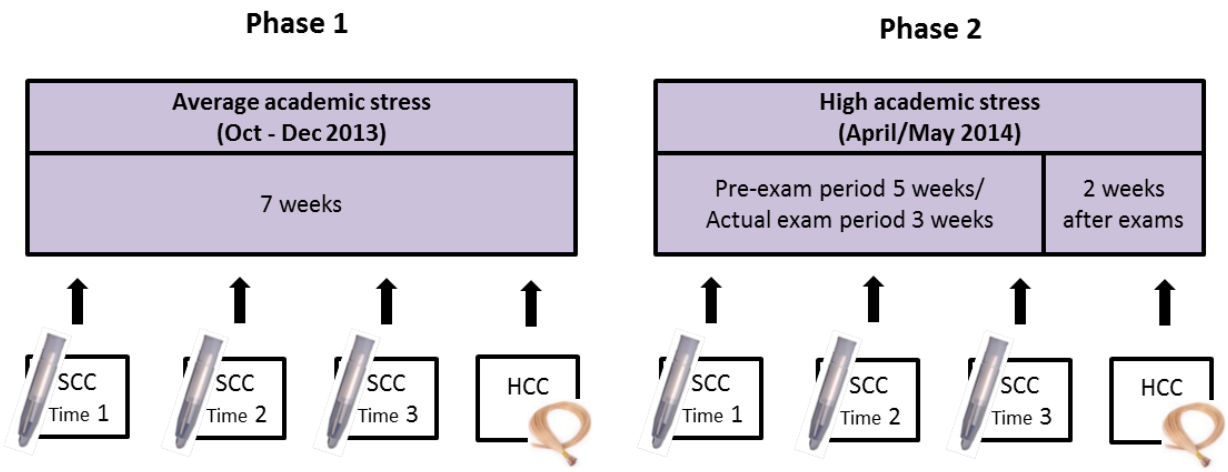

Figure 5.1 Study design and temporal distance of sampling. Salivary cortisol concentration (SCC) sampling at two-week intervals with hair cortisol concentration (HCC) sampling at the end of each period. Each SCC consisted of 5 saliva sampling points [11am, 3pm, at bedtime, at awakening (the following day) and 30 minutes after awakening].

5.3.2 Participants

Participants were 77 second-year students (aged 18-25) from the Medical and Law School at UCL, invited via emails and flyers to take part in the study (Appendix 5). Only second-year students were invited, as first-year students are assumed to be more stressed about adjustments to university life rather than degree-related stress (e.g. adjustment to independent learning, new teaching styles, first time away from home etc.). Due to the limited sample size, only female participants were selected to obtain a more homogenous group. Therefore inclusion criteria were being female, a second-year medical or law student at UCL, not being pregnant, not taking any medications and not having any chronic disease. Participants needed to be able to commit to all assessments and they were compensated with £50 once all assessments were completed (at the end of Phase 2).

5.3.3 Procedure

Data collection took place in a research laboratory at UCL. Participants were seen twice in each phase (average and high academic stress; see Figure 5.1 for a detailed outline of the sampling times). At the first visit, each participant was provided with an information sheet outlining the study purpose and procedure (Appendix 6). After informed consent was obtained, demographic factors (age, ethnicity) and anthropometric measures (height and weight), smoking status and alcohol consumption were assessed. Next, participants were shown how to sample their saliva and were given a pack containing five labelled Salivette tubes (Sarstedt,

Numbrecht-Rommelsdorf, Germany) to take home with them. They were also given the packs for the two following sampling points, at week 3 and 5. Psychosocial factors were assessed several times and this will be outlined in Chapter 6. At the second meeting (at week 7), hair was taken from the posterior vertex and participants were asked about any hair treatment they had had in the preceding 1-2 months. Half a year later, participants were invited by email to return to the lab and to repeat the exact same procedure as in the first phase, with sampling and measures being taken at analogous times. It is noteworthy that the actual exam period in Phase 2 took 3 week; however, the period before the actual examinations was assumed to be most stressful and pressuring (this literature will be outlined in Chapter 6). The two week period after the exams was used as the appropriate time for hair growth in this phase (see Figure 5.1).

5.3.4 Measures

Salivary cortisol

Saliva was collected with Salivette devices (Sarstedt, Numbrecht-Rommelsdorf, Germany). Samples were collected on three full days per phase: at week 1, week 3 and week 5 (6, 4 and 2 weeks prior to hair cortisol sampling; see Figure 5.1). Participants started the saliva sampling the day after they had been seen by the researcher, and carried out the collection protocol over a 24-hour period [11am, 3pm, at bedtime, at awakening (the following day) and 30 minutes after awakening]. Reminder emails and text messages were sent before 11am to encourage participants to adhere to the sampling protocol (Appendix 10).

Participants were asked to abstain from smoking, food, medication and alcohol intake, brushing teeth, and exercise 30 minutes prior to saliva sampling (see Appendix 9 for the salivary cortisol dairy). All samples were returned to UCL in the following 3-6 days and collected by the researcher. Samples were stored in a freezer at −20°C until they were sent to the Technical University of Dresden, Germany, where steroid extraction was performed. Cortisol levels were assessed using a time resolved immunoassay with fluorescence detection, and the intra- and inter-assay coefficients of variation were less than 4%.

The cortisol awakening value was calculated as the difference between cortisol levels at waking and 30 minutes after waking. Participants with a delay of more than 15 min between waking and saliva sampling were excluded from the CAR analyses (Dockray et al., 2008). The 30 minutes post-awakening value (termed Wake +30) was used adjusting for the same delay procedure as in the CAR calculation. The AUC was computed by trapezoidal calculation of all five samples over the 24-hour period diurnal values [mean (11am and 3pm divided by time interval) + mean (3pm and bedtime divided by time interval) + mean (wake and wake +30 divided by time interval)]. The cortisol slope was defined by regressing cortisol values from 11am until bedtime.

Hair cortisol

Hair collection took place in both periods at the end of the 7-week study period. A scalp hair strand of 1 cm was collected from the posterior vertex position. Hair strands were cut closely to the scalp with fine medical scissors, were placed

onto aluminium foil and labelled with the identification number, following the hair protocol described by Kirschbaum et al. (2009). Upon collection, they were stored in a dry, dark place for a maximum period of eight months. All hair samples were shipped to the Technical University of Dresden, Germany after the end of Phase 2 (to ensure identical hair analytical procedure). The wash procedure and steroid extraction were undertaken using high performance liquid chromatography–mass spectrometry (LC-MS), as described by Kirschbaum and colleagues (2009), with a minimum of 10 mg ± 0.5 mg of hair, cut from each 1 cm hair segment. The hair segment of 1 cm nearest to the scalp represents the averaged cortisol accumulated over an approximate timespan of 2-6 weeks prior to sampling.

Hair-related questions

Hair-specific factors that could affect hair cortisol concentration were assessed by self-report and verified at the time of hair sampling (Appendix 8). These factors included the frequency of hair washes per week, hair colour and curvature, use of hair products and any type of hair treatment within the period of investigation.

5.3.5 Statistical analyses

Data analysis was performed using SPSS 23.0. An alpha level of $p < 0.05$ was considered significant for all analyses. Separate analyses were carried out relating salivary and hair cortisol during Phase 1 (reference period) and Phase 2 (examination period). Hair cortisol and AUC of the salivary cortisol data were

skewed and therefore logarithmically (ln) transformed to normalise the distributions. Of the sample of N = 77, one outlier was identified with hair cortisol concentration of three standard deviations above the overall mean (raw hair cortisol concentration at Phase 1: 252.25 pg/mg and at Phase 2: 308.50 pg/mg of the same individual) and was therefore removed prior to analyses. Where possible analyses were performed using the full sample with both student groups to increase sample size (N = 76) with student group included as a covariate. However, separate analyses per student group were of interest for certain aspects. Therefore, precise Ns are reported throughout.

Aggregated measures were calculated by averaging the three salivary measures (Time 1, Time 2, Time 3) to provide a more robust measure of the three parameters (AUC, slope, CAR and the 30 minutes post-awakening value). Where only two values were available these were averaged, while people who only had one set of salivary measures were excluded for the aggregated analyses. The calculation of the CAR involved sampling time between the first morning sample and the sample 30 minutes post-awakening. Individuals with a delay of more than 15 minutes were excluded from the analyses resulting in a reduction of sample sizes (Time 1: of 75, 7 were excluded; Time 2: of 75, 1 was excluded; Time 3: of 76, 9 were excluded for Phase 1; Time 1: of 68, 2 were excluded; Time 2: of 70, 5 were excluded; Time 3: of 68, 3 were excluded for Phase 2).

ANOVAs and Chi^2-tests were performed to assess any differences among the two student groups. Repeated-measure ANOVAs were conducted to assess whether the cortisol parameters differed across the three sampling time points.

Correlational analyses were conducted on the different salivary cortisol parameters and hair cortisol concentration to facilitate comparison with correlation coefficients from previous studies. This has been done in both the separate student groups and in the combined sample to see whether strength of the associations might differ, in case some between-group differences could not be accounted for. For the combined sample, the analyses were repeated in linear regression analyses controlling for covariates of interest (i.e. age, BMI, smoking, hair treatment) and also student group.

5.4 Results

5.4.1 Sample characteristics

From the initial 77 participants, 71 completed both phases. There were 57 medical students and 20 law students in Phase 1 and 52 medical students and 19 law students in Phase 2. Participants who completed Phase 2 (N = 71) did not differ from those who dropped out after Phase 1 (N = 6) on hair cortisol levels (t = 0.904, df = 75, p = 0.369), or on the salivary cortisol parameters at baseline (CAR: t = 0.398, df = 72, p = 0.692; Wake +30: t = 0.659, df = 71, p = 0.490; AUC: t = 1.114, df = 74, p = 0.269; Slope: t = -0.317, df = 75, p = 0.752). The correlational analyses in both phases have been conducted on the sample available at each phase, i.e. in Phase 1, there were 77 salivary and hair cortisol measures available and in Phase 2, there were 71 salivary and hair cortisol measures available. One outlier was removed

prior to analyses resulting into a sample size of N = 76 at Phase 1 and N = 70 at Phase 2.

Table 5.1 summarises the socio-demographic and health-related characteristics measured at the baseline at the start of the study. Mean age of the whole sample was 21 years (range: 20-24) and 88.2% had a BMI in the normal range (BMI < 25), 7.9% were overweight (BMI between 25 and 30) and 3.9% were obese (BMI > 30). ANOVAs and Chi^2-tests were performed to assess any differences among the two student groups (Table 5.1). There were no differences between medical and law students in terms of age (t = 0.549, df = 74, *p* = 0.585), ethnicity (χ2 = 0.314, df = 2, *p* = 0.855), BMI (t = 0.916, df = 74, *p* = 0.363) and smoking status (χ2 = 0.081, df = 1, *p* = 0.776) and alcohol consumption (χ2 = 3.385, df = 2, *p* = 0.184) at baseline. The comparison between the two degrees in the different cortisol measures is presented in the relevant sections.

Table 5.1 Demographic characteristics and health-related factors of the combined sample and per student group.

Variable	Combined sample (N = 76)	Student group		Group diff. p-value
		Medical students (N = 56)	Law students (N = 20)	
	Mean (SD) /frequency (%)	Mean (SD) /frequency (%)	Mean (SD) /frequency (%)	
Age	20.8 (1.07)	20.8 (1.10)	20.7 (0.99)	0.585
Ethnicity				
White	44 (57.7)	32 (57.1)	12 (60.0)	0.855
Asian	26 (33.8)	20 (35.7)	6 (30.0)	
Other (black/ mixed)	6 (8.4)	4 (7.1)	2 (10.0)	
Body mass index (kg/m^2)	22.28 (4.07)	22.53 (4.51)	21.56 (2.38)	0.363
Current smoker				0.776
Yes	10 (13.2)	7 (12.5)	3 (15.0)	
No	66 (86.8)	49 (87.5)	17 (85.0)	
Alcohol drinker				0.184
Frequent/ regular	23 (30.3)	19 (33.9)	4 (20.0)	
Occasional	36 (47.4)	23 (41.4)	13 (65.0)	
No	17 (22.4)	14 (25.0)	3 (15.0)	

5.4.2 Salivary cortisol profile and hair cortisol

Phase 1

Figure 5.2 depicts the cortisol profile over the three sampling points. Repeated-measures ANOVA were all significant (all *p*'s < 0.05), confirming significant changes in salivary cortisol concentrations over each 24-hour period and thus the expected diurnal profile. Repeated-measure ANOVAs showed that the cortisol parameters did not differ significantly across the three sampling time points (CAR: $F_{2, 112} = 0.856$, $p = 0.428$; Wake +30: $F_{2, 112} = 0.256$, $p = 0.775$; AUC: $F_{2, 140} = 0.130$, $p = 0.412$; Slope: $F_{2, 142} = 0.798$, $p = 0.452$).

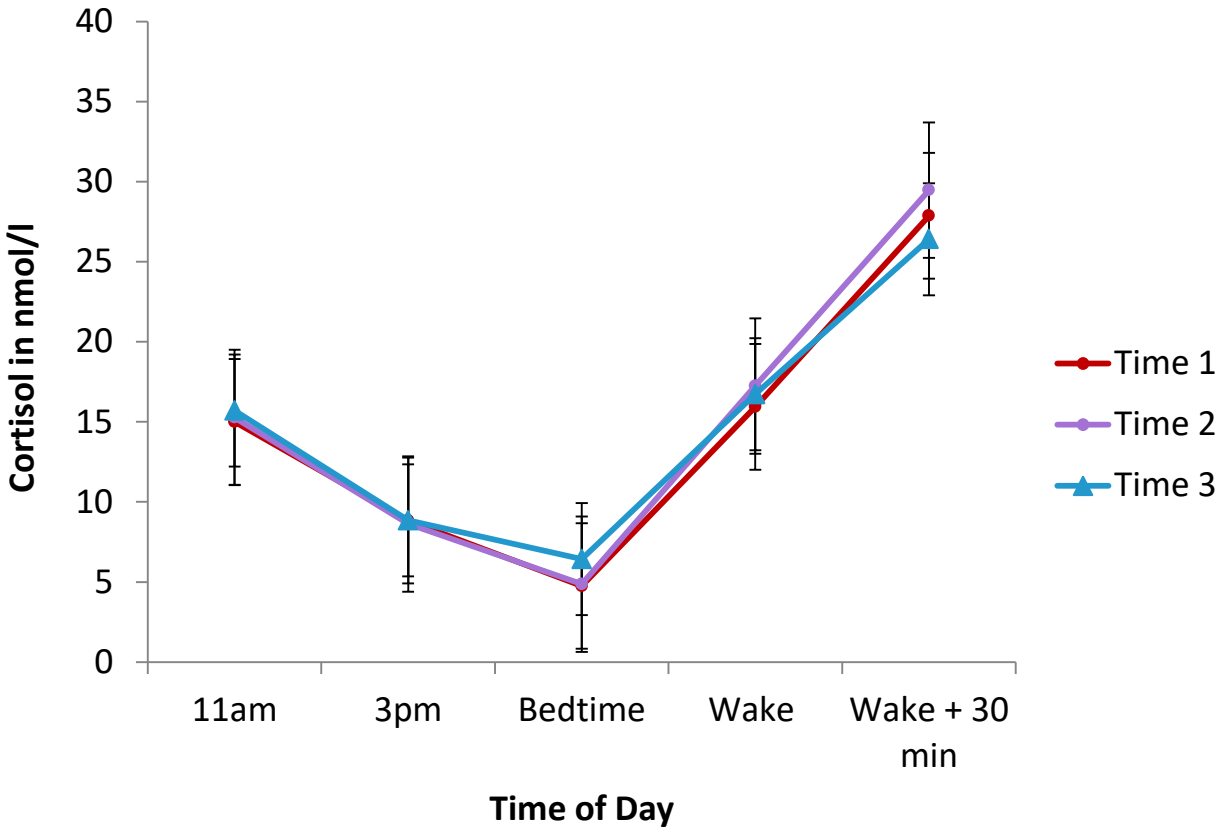

Figure 5.2 Diurnal salivary cortisol profile over the three sampling points; Phase 1.

Table 5.2 shows the mean values for the different cortisol parameters for each time point throughout Phase 1 and the aggregated measure with all relevant sample sizes. The student groups did not differ in any of the salivary cortisol parameters within Phase 1 and also at the aggregated measure (all *p*'s > 0.05). There were also no differences between the student groups in hair cortisol concentration (t = -1.346, df = 74, *p* = 0.183). Hence, there was no reason why the two groups could not be combined to have a larger sample size. In all regression analyses, degree was additionally controlled for, to investigate whether this made affected the results.

Table 5.2 Mean values for the different salivary cortisol parameters (at each time point and the aggregated measure) and hair cortisol concentration in Phase 1.

Cortisol parameter	Combined sample		Student group				Group diff. p-value
			Medical students		Law students		
	Mean (SD)	N	Mean (SD)	N	Mean (SD)	N	
CAR 1 (nmol/l)	11.98 (11.49)	67	11.76 (12.08)	51	12.68 (9.69)	16	0.784
CAR 2 (nmol/l)	11.27 (14.70)	73	11.22 (14.87)	53	11.34 (14.59)	20	0.963
CAR 3 (nmol/l)	9.53 (10.51)	66	10.53 (9.27)	50	6.42 (13.59)	16	0.175
CAR agg (nmol/l)	11.03 (9.37)	73	11.34 (8.81)	55	10.16 (11.03)	19	0.639
Wake +30 1 (nmol/l)	28.08 (13.26)	67	28.62 (13.60)	51	26.34 (12.60)	16	0.553
Wake +30 2 (nmol/l)	28.60 (15.42)	72	28.97 (15.48)	52	27.64 (15.62)	20	0.745
Wake +30 3 (nmol/l)	26.34 (12.56)	67	28.11 (11.83)	50	21.11 (13.53)	17	0.460
Wake +30 agg (nmol/l)	27.99 (10.55)	73	28.95 (9.90)	54	25.26 (12.08)	19	0.192
AUC 1 [ln(nmol/l)]	8.83 (0.42)	75	8.87 (0.40)	55	8.71 (0.47)	20	0.166
AUC 2 [ln(nmol/l)]	8.76 (0.51)	74	8.71 (0.53)	55	8.89 (0.47)	19	0.207
AUC 3 [ln(nmol/l)]	8.85 (0.50)	73	8.83 (0.52)	55	8.93 (0.44)	18	0.450
AUC agg [ln(nmol/l)]	8.81 (0.78)	75	8.81 (0.39)	56	8.84 (0.34)	19	0.705
Slope 1 (nmol/l/h)	0.78 (0.74)	77	0.73 (0.77)	57	0.91 (0.67)	20	0.346
Slope 2 (nmol/l/h)	0.84 (0.91)	74	0.75 (0.87)	55	1.10 (1.00)	19	0.144
Slope 3 (nmol/l/h)	0.74 (0.85)	74	0.70 (0.84)	56	0.85 (0.88)	18	0.521
Slope agg (nmol/l/h)	0.79 (0.57)	77	0.72 (0.58)	57	0.96 (0.50)	20	0.105
Hair cortisol [ln(pg/ng)]	2.54 (0.78)	76	2.47 (0.78)	56	2.74 (0.74)	20	0.183

Note. agg = aggregated (average of Time 1, Time 2 and Time 3).

Phase 2

There were 71 participants who completed Phase 2 and who had viable hair cortisol. The same outlier as before was removed prior to analyses, leaving a sample of 70. Figure 5.3 depicts the cortisol profile over the three sampling points. Repeated-measures ANOVA were all significant (all *p*'s < 0.05), again confirming significant changes in salivary cortisol concentrations over each 24-hour period and thus the expected diurnal profile. Repeated-measure ANOVAs showed that the parameters did not differ significantly across the three sampling time points (CAR: $F_{2, 118} = 0.061$, $p = 0.941$; Wake 30+: $F_{2, 118} = 0.610$, $p = 0.545$; AUC: $F_{2, 126} = 0.610$, $p = 0.545$; slope: $F_{2, 128} = 1.583$, $p = 0.209$).

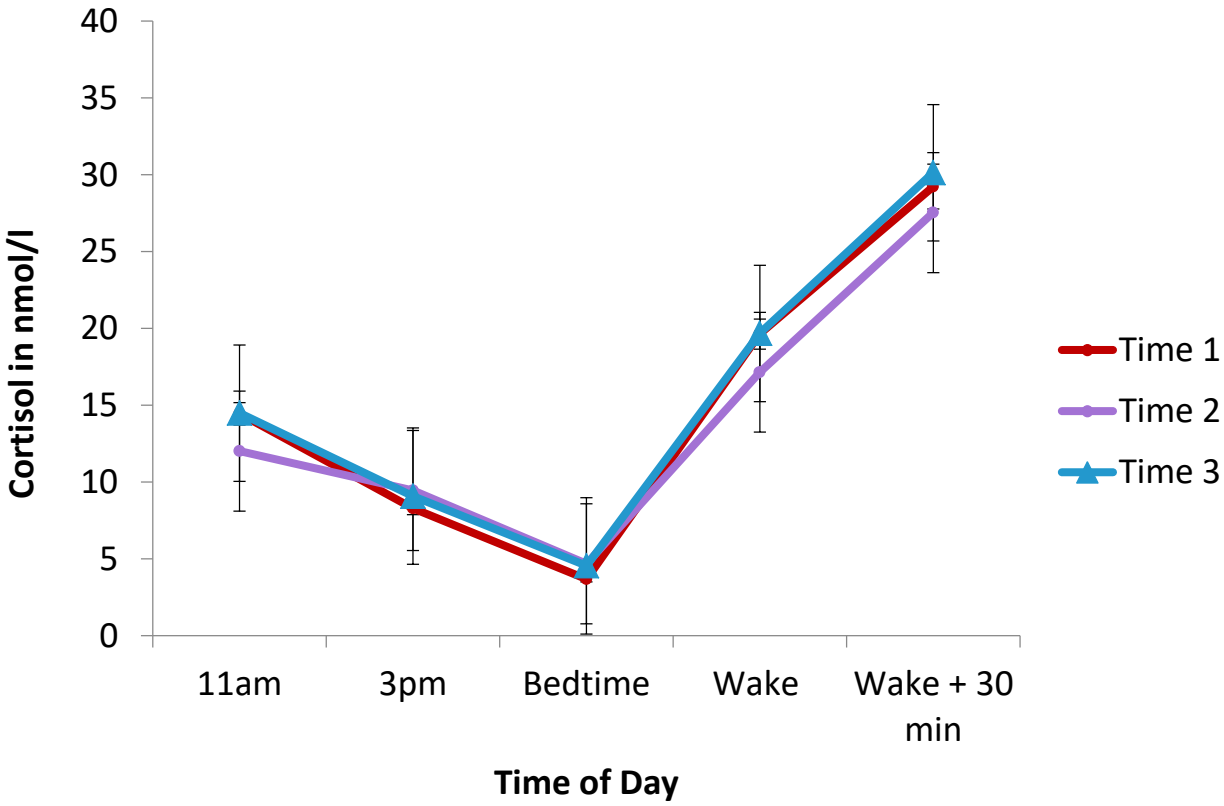

Figure 5.3 Diurnal salivary cortisol profile over the three sampling points; Phase 2.

Table 5.3 shows the mean values for the different cortisol parameters for each time point throughout Phase 2 and the aggregated measure, again with all relevant sample sizes. The student groups did not differ in any of the salivary cortisol parameters within Phase 2 and also at the aggregated measure (all *p*'s > 0.05). There were also no differences between the student groups in hair cortisol concentration (t = 0.671, df = 68, *p* = 0.504). Therefore, again in this Phase there was no reason why the two groups could not be combined. Once more, in all regression analyses, degree was additionally controlled for, to investigate whether this affected the results.

Table 5.3 Mean values for the different salivary cortisol parameters (at each time point and the aggregated measure) and hair cortisol concentration in Phase 2.

Cortisol parameter	Combined sample		Student group				Group diff. p-value
			Medical students		Law students		
	Mean (SD)	N	Mean (SD)	N	Mean (SD)	N	
CAR 1 (nmol/l)	9.75 (10.95)	66	8.71 (10.38)	49	12.74 (12.30)	17	0.194
CAR 2 (nmol/l)	10.79 (13.64)	65	9.37 (13.72)	47	14.52 (13.07)	18	0.175
CAR 3 (nmol/l)	10.47 (14.10)	65	10.09 (14.85)	47	11.46 (12.27)	18	0.730
CAR agg (nmol/l)	10.50 (9.35)	66	9.55 (9.42)	48	13.03 (11.15)	18	0.209
Wake +30 1 (nmol/l)	29.40 (13.34)	66	28.90 (12.71)	49	30.86 (15.34)	17	0.605
Wake +30 2 (nmol/l)	28.03 (15.23)	65	27.07 (15.71)	47	30.54 (14.02)	18	0.417
Wake +30 3 (nmol/l)	30.02 (13.17)	65	31.11 (12.56)	47	27.16 (14.66)	18	0.283
Wake +30 agg (nmol/l)	29.09 (10.94)	66	27.97 (10.24)	48	29.42 (12.93)	18	0.884
AUC 1 [ln(nmol/l)]	8.71 (0.50)	68	8.72 (0.54)	50	8.71 (0.40)	18	0.974
AUC 2 [ln(nmol/l)]	8.76 (0.55)	68	8.69 (0.57)	50	8.95 (0.46)	18	0.099
AUC 3 [ln(nmol/l)]	8.78 (0.44)	66	8.82 (0.47)	47	8.70 (0.37)	19	0.326
AUC agg [ln(nmol/l)]	8.76 (0.37)	68	8.75 (0.39)	50	8.79 (0.31)	18	0.674
Slope 1 (nmol/l/h)	0.76 (0.61)	69	0.82 (0.62)	50	0.61 (0.56)	19	0.198
Slope 2 (nmol/l/h)	0.58 (0.72)	69	0.62 (0.63)	50	0.39 (0.92)	19	0.219
Slope 3 (nmol/l/h)	0.70 (0.69)	67	0.73 (0.76)	48	0.60 (0.50)	19	0.496
Slope agg (nmol/l/h)	0.67 (0.50)	70	0.72 (0.50)	51	0.53 (0.47)	19	0.164
Hair cortisol [ln(pg/ng)]	2.52 (0.73)	70	2.56 (0.67)	51	2.43 (0.87)	19	0.504

5.4.3 Potential hair cortisol confounding factors

Correlational analyses showed that in this sample, age and BMI were not significantly associated with hair cortisol levels (r = -0.106, *p* = 0.181 and r = 0.016, *p* = 0.447, respectively). Ethnicity was not related to hair cortisol levels ($F_{2, 67}$ = 0.277, *p* = 0.759). An independent t-test revealed that smokers [M = 3.07 ln(pg/mg), SD = 0.73] had significantly higher hair cortisol concentration than non-smokers [M = 2.44 ln(pg/mg), SD = 0.70; t = -2.521, df = 68, *p* = 0.014] with hair cortisol taken in Phase 2 but not in Phase 1 (t = -0.499, df = 74, *p* = 0.620). Hair treatment (N = 5) was significantly associated with lower hair cortisol content taken in Phase 1 [M = 1.86 ln(pg/mg), SD = 0.45], compared to hair cortisol content in individuals without hair treatment [M = 2.59 ln(pg/mg), SD = 0.78; t = 2.055, df = 73, *p* = 0.043], but not in Phase 2 (N = 4; t = -0.427, df = 68, *p* = 0.671). Yet, all of these variables were included in the regression models as covariates.

5.4.4 Associations between salivary cortisol and hair cortisol

Phase 1 (low stress)

Table 5.4 shows the correlation coefficients between each of the salivary cortisol parameters and hair cortisol concentration in Phase 1. Hair cortisol was significantly positively correlated with the AUC at all three sampling points (Time 1: r = 0.362; Time 2: r = 0.427; Time 3: r = 0.461, all *p*'s < 0.001; Table 5.4). When the AUC was averaged the correlation between hair and salivary cortisol levels became stronger (r = 0.543, $p < 0.001$). This was also the case when analysing the student groups separately. Hair cortisol was also significantly positively associated with aggregate wake 30+ values (r = 0.326, $p = 0.005$) and also at two sampling points (Time 1: r = 0.362; $p = 0.001$ and Time 2: r = 0.426; $p = 0.071$). Hair cortisol was also positively associated with the CAR at Time 1 (r = 0.252, $p = 0.040$). Figure 5.4 shows the correlation between hair cortisol levels and the AUC averaged from the three sampling points.

Table 5.4 Correlation coefficient for the different salivary parameters and hair cortisol concentration in Phase 1.

Salivary cortisol parameter	Combined sample	Student group	
	(N = 76)	Medical students (N = 56)	Law students (N = 20)
CAR 1 (nmol/l)	r = 0.252; *p* = 0.040	r = 0.258; *p* = 0.067	r = 0.212; *p* = 0.431
CAR 2 (nmol/l)	r = -0.036; *p* = 0.765	r = -0.054; *p* = 0.702	r = 0.014; *p* = 0.954
CAR 3 (nmol/l)	r = 0.034; *p* = 0.787	r = 0.086; *p* = 0.555	r = -0.026; *p* = 0.924
CAR agg (nmol/l)	r = 0.072; *p* = 0.546	r = 0.102; *p* = 0.462	r = 0.021; *p* = 0.932
Wake +30 1 (nmol/l)	r = 0.410; *p* = 0.001	r = 0.417; *p* = 0.002	r = 0.439; *p* = 0.089
Wake +30 2 (nmol/l)	r = 0.214; *p* = 0.071	r = 0.259; *p* = 0.064	r = 0.132; *p* = 0.580
Wake +30 3 (nmol/l)	r = 0.130; *p* = 0.295	r = 0.231; *p* = 0.107	r = -0.033; *p* = 0.900
Wake +30 agg (nmol/l)	r = 0.326; *p* = 0.005	r = 0.421; *p* = 0.002	r = 0.172; *p* = 0.481
AUC 1 [ln(nmol/l)]	r = 0.362; *p* = 0.001	r = 0.338; *p* = 0.012	r = 0.556; *p* = 0.011
AUC 2 [ln(nmol/l)]	r = 0.427; *p* < 0.001	r = 0.429; *p* = 0.001	r = 0.362; *p* = 0.128
AUC 3 [ln(nmol/l)]	r = 0.461; *p* < 0.001	r = 0.417; *p* = 0.002	r = 0.606; *p* = 0.008
AUC agg [ln(nmol/l)]	r = 0.543; *p* < 0.001	r = 0.504; *p* < 0.001	r = 0.688; *p* = 0.001
Slope 1 (nmol/l/h)	r = 0.010; *p* = 0.928	r = -0.018; *p* = 0.894	r = 0.031; *p* = 0.898
Slope 2 (nmol/l/h)	r = 0.135; *p* = 0.255	r = 0.116; *p* = 0.406	r = 0.098; *p* = 0.691
Slope 3 (nmol/l/h)	r = -0.109; *p* = 0.359	r = -0.146; *p* = 0.288	r = -0.042; *p* = 0.869
Slope agg (nmol/l/h)	r = 0.025; *p* = 0.828	r = -0.021; *p* = 0.881	r = 0.061; *p* = 0.800

Note. agg = aggregated (average of Time 1, Time 2 and Time 3).

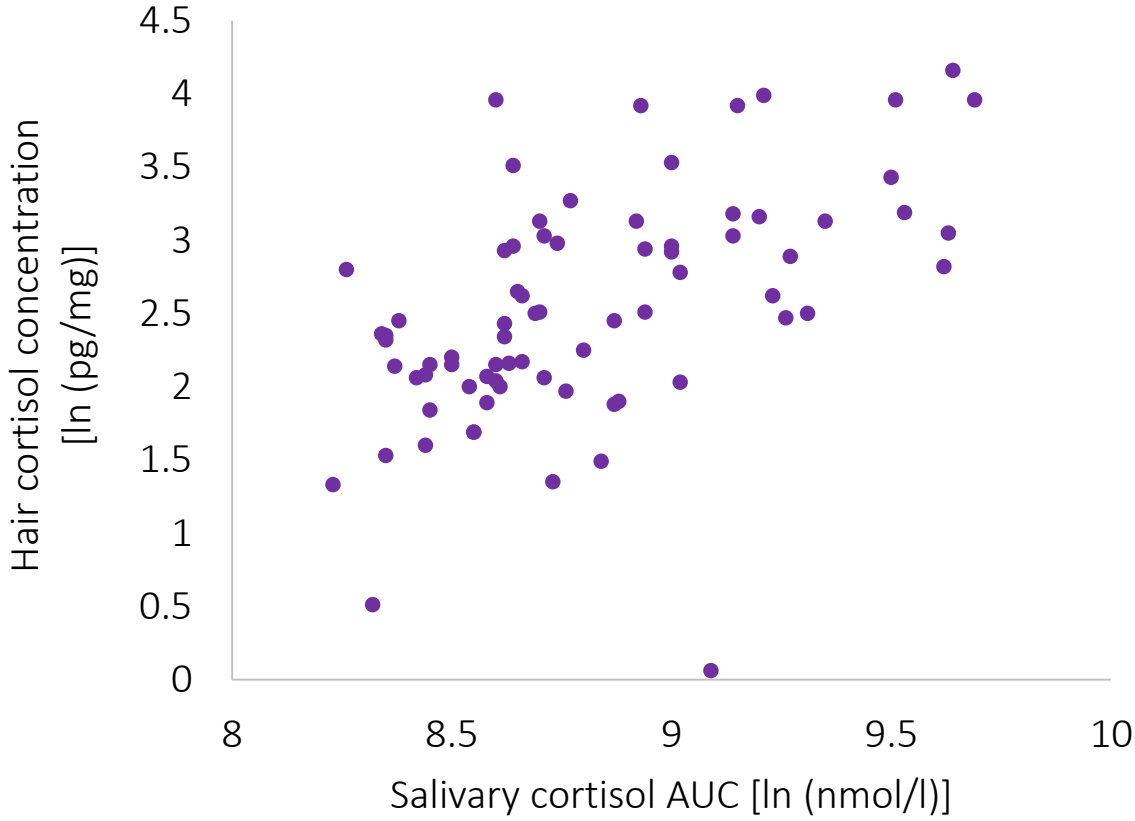

Figure 5.4 Correlation between averaged AUC and hair cortisol in Phase 1.

Regression models were performed with the averaged salivary cortisol parameters, (CAR, Wake 30+, AUC, slope) controlling for covariates of interest (age, BMI, smoking, hair treatment and student group). The relationship between hair cortisol and the AUC and the Wake 30+ remained significant when adjusting for the covariates (Table 5.5).

Table 5.5 Multivariate regression of hair cortisol on the averaged CAR (N = 71), the Wake 30+ (N = 71), the AUC (N = 73) and the cortisol slope (N = 74) adjusted for age, BMI, smoking, hair treatment and student group.

		Regression coefficient B	95% CI	Standardised coefficient β (standard error)	p
a)	Cortisol CAR	0.047	-0.016 – 0.024	0.004 (0.010)	0.699
	Age	-0.095	-0.246 – 0.109	-0.068 (0.089)	0.446
	BMI	0.000	- 0.046 – 0.046	-0.007 (0.023)	0.997
	Smoking status	0.017	-0.545 – 0.627	0.041 (0.293)	0.891
	Hair treatment	-0.215	-1.399 – 0.091	-0.654 (0.373)	0.084
	Student group	0.079	-0.291 – 0.569	0.139 (0.215)	0.521
b)	Cortisol Wake 30+	0.025	0.008 – 0.042	0.337 (0.008)	0.005
	Age	-0.054	-0.220 – 0.113	-0.075 (0.083)	0.521
	BMI	0.005	-0.038 – 0.048	0.026 (0.022)	0.823
	Smoking status	0.023	-0.525 – 0.571	0.010 (0.274)	0.933
	Hair treatment	-0.587	-1.289 – 0.115	-0.193 (0.351)	0.100
	Student group	0.245	-0.166 – 0.656	0.139 (0.206)	0.238
c)	Cortisol AUC	1.188	0.762 – 1.614	0.567 (0.213)	0.000
	Age	-0.021	-0.169 – 0.126	-0.029 (0.074)	0.773
	BMI	0.018	-0.021 – 0.056	0.092 (0.019)	0.362
	Smoking status	-0.221	-0.714 – 0.271	-0.093 (0.247)	0.373
	Hair treatment	-0.621	-1.237 – -0.004	-0.199 (0.309)	0.049
	Student group	0.196	-0.157 – 0.549	0.110 (0.177)	0.272
d)	Cortisol slope	0.005	-0.338 – 0.348	0.004 (0.172)	0.976
	Age	-0.068	-0.245 – 0.110	-0.092 (0.089)	0.450
	BMI	0.002	-0.044 – 0.048	0.010 (0.023)	0.937
	Smoking status	0.036	-0.548 – 0.620	0.015 (0.293)	0.903
	Hair treatment	-0.660	-1.405 – 0.085	-0.212 (0.373)	0.081
	Student group	0.201	-0.223 – 0.626	0.115 (0.213)	0.347

Adjusted for age, BMI, smoking, hair treatment and student group.

Phase 2 (high stress)

Table 5.6 shows the correlation coefficients between each of the salivary cortisol parameters and hair cortisol concentration in Phase 2. Hair cortisol was significantly positively correlated with the AUC at Time 3 (r = 0.295, p = 0.016) and marginally at Time 1 (r = 0.228, p = 0.061). The averaged AUC was also positively correlated with hair cortisol levels (r = 0.305, p = 0.012). The CAR, the Wake 30+ and the slope were not correlated with hair cortisol at either time point. The associations were not therefore as consistent during the stress compared with control periods of the study. Figure 5.5 shows the correlation between hair cortisol levels and the AUC averaged from the three samples.

Table 5.6 Correlation coefficient for the different salivary parameters and hair cortisol concentration in Phase 2.

Salivary cortisol parameter	Combined sample	Student group	
	(N = 70)	Medical students (N = 51)	Law students (N = 19)
CAR 1 (nmol/l)	r = 0.056; *p* = 0.656	r = 0.074; *p* = 0.613	r = 0.086; *p* = 0.744
CAR 2 (nmol/l)	r = 0.020; *p* = 0.872	r = -0.120; *p* = 0.420	r = 0.370; *p* = 0.131
CAR 3 (nmol/l)	r = 0.076; *p* = 0.548	r = 0.024; *p* = 0.870	r = 0.232; *p* = 0.355
CAR agg (nmol/l)	r = 0.081; *p* = 0.520	r = -0.009; *p* = 0.949	r = 0.292; *p* = 0.239
Wake +30 1 (nmol/l)	r = 0.096; p = 0.441	r = 0.123; p = 0.401	r = 0.075; p = 0.776
Wake +30 2 (nmol/l)	r = 0.038; p = 0.761	r = -0.047; p = 0.754	r = 0.266; p = 0.287
Wake +30 3 (nmol/l)	r = 0.136; p = 0.281	r = 0.056; p = 0.707	r = 0.246; p = 0.325
Wake +30 agg (nmol/l)	r = 0.119; p = 0.341	r = 0.048; p = 0.746	r = 0.246; p = 0.324
AUC 1 [ln(nmol/l)]	r = 0.228; *p* = 0.061	r = 0.235; *p* = 0.100	r = 0.239; *p* = 0.339
AUC 2 [ln(nmol/l)]	r = 0.172; *p* = 0.161	r = 0.245; *p* = 0.086	r = 0.087; *p* = 0.730
AUC 3 [ln(nmol/l)]	r = 0.295; *p* = 0.016	r = 0.152; *p* = 0.309	r = 0.664; *p* = 0.002
AUC agg [ln(nmol/l)]	r = 0.305; *p* = 0.012	r = 0.283; *p* = 0.046	r = 0.419; *p* = 0.084
Slope 1 (nmol/l/h)	r = -0.030; *p* = 0.805	r = 0.055; *p* = 0.705	r = -0.275; *p* = 0.255
Slope 2 (nmol/l/h)	r = 0.139; *p* = 0.253	r = 0.217; *p* = 0.129	r = 0.000; *p* = 0.999
Slope 3 (nmol/l/h)	r = -0.200; *p* = 0.105	r = -0.140; *p* = 0.344	r = -0.455; *p* = 0.051
Slope agg (nmol/l/h)	r = -0.020; *p* = 0.866	r = 0.067; *p* = 0.638	r = -0.269; *p* = 0.266

Note. agg = aggregated (average of Time 1, Time 2 and Time 3).

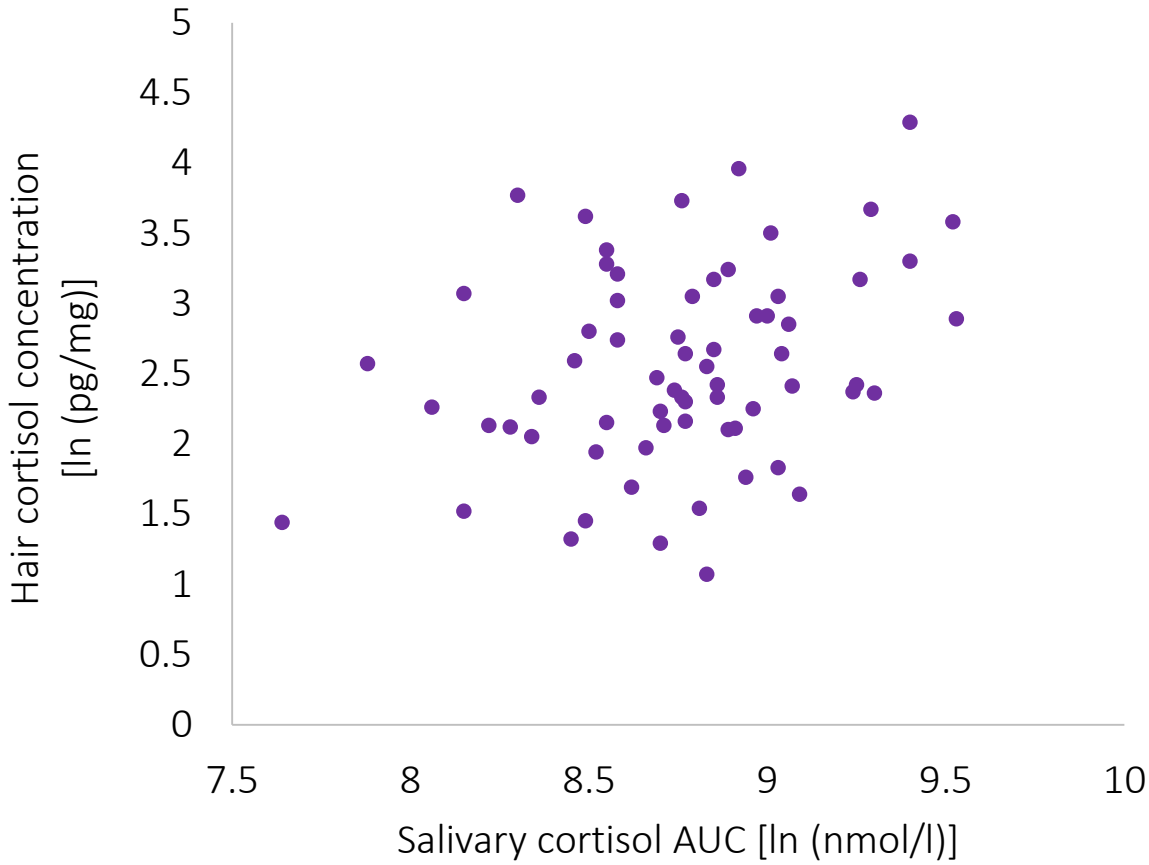

Figure 5.5 Correlation between averaged AUC and hair cortisol in Phase 2.

Again, regression models were performed with the averaged salivary cortisol parameters (CAR, Wake 30+, AUC, slope), controlling for the covariates age, BMI, smoking, hair treatment and student group). The AUC remained significantly associated with hair cortisol concentration when adjusting for the covariates (Table 5.7).

Table 5.7 Multivariate regression of hair cortisol on the averaged CAR (N = 65), the Wake 30+ (N = 65), the AUC (N = 67) and the cortisol slope (N = 69) adjusted for age, BMI, smoking, hair treatment and student group.

		Regression coefficient B	95% CI	Standardised coefficient β (standard error)	p
a)	Cortisol CAR	0.008	-0.010 – 0.025	0.103 (0.009)	0.374
	Age	-0.081	-0.243 – 0.080	-0.120 (0.081)	0.316
	BMI	0.059	0.019 – 0.099	0.338 (0.020)	0.005
	Smoking status	0.724	0.222 – 1.227	0.337 (0.251)	0.005
	Hair treatment	0.253	-0.470 – 0.376	0.082 (0.361)	0.486
	Student group	-0.169	-0.559 – 0.220	-0.108 (0.195)	0.388
b)	Cortisol Wake 30+	0.008	-0.008 – 0.023	0.115 (0.008)	0.318
	Age	-0.089	-0.249 – 0.071	-0.131 (0.080)	0.271
	BMI	0.059	0.019 – 0.099	0.342 (0.020)	0.004
	Smoking status	0.697	0.193 – 1.201	0.324 (0.252)	0.008
	Hair treatment	0.204	-0.517 – 0.924	0.066 (0.360)	0.574
	Student group	-0.151	-0.537 – 0.235	-0.091 (0.193)	0.437
c)	Cortisol AUC	0.527	0.064 – 0.990	0.266 (0.231)	0.026
	Age	-0.040	-0.194 – 0.115	-0.059 (0.077)	0.609
	BMI	0.060	0.022 – 0.098	0.345 (0.019)	0.003
	Smoking status	0.564	0.063 – 1.065	0.262 (0.251)	0.028
	Hair treatment	0.345	-0.357 – 1.047	0.111 (0.351)	0.329
	Student group	-0.145	-0.513 – 0.222	-0.088 (0.184)	0.433
d)	Cortisol slope	-0.021	-0.356 – 0.313	-0.015 (0.167)	0.899
	Age	-0.075	-0.234 – 0.084	-0.110 (0.080)	0.352
	BMI	0.056	0.017 – 0.096	0.323 (0.020)	0.006
	Smoking status	0.694	0.194 – 1.193	0.322 (0.250)	0.007
	Hair treatment	0.210	-0.504 – 0.924	0.068 (0.357)	0.559
	Student group	-0.123	-0.502 – 0.255	-0.076 (0.189)	0.518

Adjusted for age, BMI, smoking, hair treatment and student group.

5.5 Discussion

Hypothesis: Hair cortisol concentration will be associated with the four salivary cortisol parameters (the CAR, the Wake 30+, the AUC and the slope) throughout the preceding six weeks (at the different time points) and most strongly with the averaged (monthly) salivary AUC.

5.5.1 Summary of findings

The present study assessed salivary cortisol on three days over a period of 5 weeks (week 1, week 3 and week 5) and hair cortisol secretion corresponding to the same period of time, allowing for hair growth of approximately 2 weeks. The study involved two different time periods, one under average academic stress (serving as a control period) and the second during a period of high academic stress (serving as a naturalistic stress period). Significant associations were found between the AUC (at each time point and when averaged) and hair cortisol under control conditions (Phase 1) and between the averaged AUC and hair cortisol levels under high academic stress (Phase 2). These associations withstood adjustment for covariates that have been found to be related to hair cortisol, i.e. age, BMI, smoking and hair treatment. This association was significant in both student groups. Further, in Phase 1, the 30 minutes post-awakening value was positively associated with hair cortisol concentration. There was no consistent association between either the CAR or the rate of cortisol decline over the day and hair cortisol in either phase.

5.5.2 Links to previous work and interpretation of findings

Short et al. (2016) reported a correlation coefficient of *r* = 0.61 between hair cortisol and integrated salivary cortisol AUC over 1 month in a sample of 17 individuals. This coefficient is similar in strength to the one in the present study. The averaged AUC in Phase 1 was *r* = 0.543 (which ranged from r = 0.504 to 0.688) in the combined sample. In Phase 2 this association was weaker and these did not emerge consistently over the sampling period as in Phase 1. This might suggest the possibility of heightened reactivity due to acute transient stress (that students were supposedly exposed to in Phase 2), which resulted in fluctuations in circadian cortisol throughout the period. This circadian cortisol variation may have impacted the overall concentration of cortisol within the hair as relative low and high values balance out over the time in which cortisol is incorporated into the hair shaft, potentially reducing the ability to detect an association with salivary cortisol. One possible explanation for fluctuations in cortisol is that there may be accompanying fluctuations in the psychological factors that impact salivary cortisol levels at a subtle level which might not be assessed by the current statistical analyses. The next chapter will investigate the impact of the naturalistic stressor 'examination stress' in relation to values from both cortisol tissues, and also cortisol in relation to psychosocial factors throughout both phases.

In Phase 1, there were also small positive associations between the averaged 30 minutes post-awakening value and hair cortisol (*r* = 0.326) and between the CAR at Time 1 and hair cortisol (*r* = 0.252). The direction of these associations seem sensible. The 30 minutes post-awakening cortisol value, and to

some extent the CAR, reflect the early morning zenith of the diurnal cortisol profile. This theoretically might present an indicator for accumulated long-term cortisol output in hair as peak levels might accumulate and therefore be captured in the relevant hair segment. This would be in line with the findings by Xie et al. (2011). The authors only assessed the 30 minute post-morning level from the diurnal profile and found it to be significantly correlated with hair cortisol levels at one of three sampling points over the month (r = 0.398), and also when using an average score from three sampling values (r = 0.383). It is interesting that the present study also showed these associations at one time point and when aggregating the three values. It is possible that the post-awakening response and also the CAR are more irregular over a day-to-day basis. In fact, there is strong evidence that short-term psychosocial factors influence the morning cortisol levels substantially, leading to day-to-day variability (Adam & Gunnar, 2001; Golden et al., 2013; Schmidt-Reinwald et al., 1999). However, no changes could be observed between the three sampling periods in these two parameters in the current study. The next chapter will focus on psychosocial influences and aims to understand this variation in cortisol in more detail.

In the present study, a period of 2 weeks was incorporated to allow for appropriate hair growth. The fact that earlier time points (Time 1 and Time 2) rather than later time points (Time 3) seemed to associate more robustly with hair cortisol levels, suggests that cortisol in the hair segment might indeed reflect the earlier time period. This would support the notion that the period for hair growth needs to be considered. New hair takes around 2 weeks to be formed in the follicle

and to appear at the hair shaft. Hair cutting methods were shown to be imprecise, leaving 1-2mm of hair at the scalp (LeBeau et al., 2011). Hair collection in the present study was conducted with high precision; however, the possibility of remains of hair cannot be rejected. It would be interesting to know whether an assessment of salivary cortisol even further in advance of hair cutting (for example at 7 or 8 weeks) would be associated with hair cortisol concentration. This is speculative, and the problem is that salivary assessments over time are intercorrelated, so it is difficult to identify a specific association with a particular point in time.

Evening values were assessed at bedtime and therefore varied greatly in timings. This variability might weaken potential associations with hair cortisol, so more rigid time assessments might be useful. Yet, this leaves the question as to what extent *actual* timing might influence the diurnal cortisol profile. Salivary cortisol assessments vary greatly between studies. Many researchers take morning assessments (which depend on the individuals' waking times) and then vary whether the assessments throughout the day are taken at a specific time point (e.g. 12pm, 3pm, 5pm) or at intervals in relation to waking time (i.e. 4 hours post-awakening, 8 hours post-awakening). Individuals differ in waking times and lifestyle, which might generate substantial differences in their cortisol concentration present at the corresponding time points. No study has yet compared these approaches to evaluate the impact on parameter calculations.

In the study by Short et al. (2016) the authors assessed salivary cortisol everyday over a period of a month and hair cortisol at the end of this period. The

correlation coefficient of this relationship between the aggregated AUC and hair cortisol concentration was *r* = 0.61. Despite a small sample size (i.e. 17 individuals), this daily assessment for one full months is a strong methodological design. The fact that the current study produced a comparable correlation coefficient of similar strength suggests that only a certain proportion of the variability in hair cortisol can be explained by systemic cortisol levels and some other factors that contribute to this gap still need to be explored. Short et al. (2016) did not account for time for adequate hair growth after last saliva sampling, i.e. the 2-week time lag of hair growth (for the relevant hair segment to appear at the hair shaft). Yet, to some extent nocturnal cortisol secretion might be a plausible factor contributing to this variability. Further, physiological differences in the growth of the hair might also be present a confounding factor in saliva-hair correlations (Loussouarn, El Rawadi, & Genain, 2005). Else, other unknown factors and also follicle-related HPA-axis influences might drive these differences between the tissues. As already highlighted, making use of dermatological research strategies might be informative to investigate this issue.

5.5.3 Study advantages and limitations

The present study is novel in that it investigates saliva-hair correlation analyses in two distinct periods, i.e. under average (serving as a control period) and under high academic stress conditions (serving as a naturalistic stress period). The different strength of associations between the tissues suggests that under academic stress (Phase 2) there seems to be a greater degree of fluctuation in

circadian cortisol throughout the period. Further, incorporating time for appropriate hair growth of approximately 2 weeks is an innovative approach to reduce margins of errors and a possible time lag effect.

One major limitation of the present study is that it assessed the salivary cortisol over a 24-hour period that was not all on the same day. The first sampling point was at 11am with two further sampling points on the same day, with two morning samples after awakening taking place on the following day. This was planned in order for students to become accustomed to the sampling procedure to ensure appropriate morning samples. Furthermore, this approach was based on the Daytracker Study (Chapter 3 and 4), in which working women were explained how to collect the first saliva sample at similar times. At a later stage during data collection this was alleged not to be a favourable tactic to assess appropriate diurnal cortisol profile. The calculations for the AUC were calculated based on the 11am to bedtime values. Most AUC measures start at waking, so include the much higher values present early in the day.

Due to financial limitations and the difficulty of obtaining compliance only three diurnal salivary profiles could be measured. Although the parameters were also aggregated, the fact that they were spaced out in two-week intervals might lead to higher levels of variation. Nevertheless, Short et al. (2016) obtained daily samples and still observed a correlation coefficient of similar strength to that within Phase 1 in the current study. Increasing the sampling days would be substantial improvement to the design and might inform more about potential fluctuations in cortisol levels in the stressful period.

Further, a homogenous sample group would have been preferable (i.e. one group of medical students). However, due to recruitment size limitations in this student group, the data collection needed to be extended to law students. While some of the associations relating to the AUC and the 30 minutes post-awakening values were evident in both student groups and also when combining the two samples, it would have been valuable to investigate these associations in a group with less variation. Finally, I was only looking at these effects in a young, predominately white sample. It needs to be acknowledged that different effects may be observed in different samples and contexts.

5.6 Chapter summary

This chapter investigated saliva-hair correlations over corresponding intervals in two different time periods, under average academic stress and under high academic stress. Positive moderate significant associations were found between the AUC and hair cortisol in both phases but under high academic stress the strength of the relationship decreased. The integrated monthly salivary AUC measure showed the strongest correlation with hair cortisol concentration, which further supports the cumulative nature of hair cortisol content. Under average academic stress the 30 minutes post-awakening value was also positively associated with hair cortisol concentration. The correct interpretation of hair cortisol as a long-term cortisol marker relies on proper understanding of the time-related features of hair cortisol concerning the associations with other cortisol

specimens. Understanding under which circumstances associations between hair cortisol and corresponding salivary cortisol become stronger might be valuable.

CHAPTER 6. CORTISOL OUTPUT IN ACADEMIC STRESS (STUDY III)

6.1 Chapter overview

This chapter explores the impact of academic stress on cortisol and tries to assess the value of hair cortisol as a stress marker, making use of a naturalistic stressor-design. It utilises the same dataset as in the previous chapter, the Academic Stress Study. Data from two phases with varying degrees of stress exposure will establish whether academic stress induces increases in hair and salivary cortisol concentrations and in psychosocial factors. Further, it investigates whether different coping mechanisms, such as adaptive coping processes, act as moderators of stress-related cortisol output.

6.2 Introduction

6.2.1 Academic stress and cortisol

The initiation of university is a milestone into a new, exciting and interesting period of life. While it is a period that offers opportunity for growth and adaptation (Arnett, 2007), it is also a challenging phase with different demanding transitions and stressors, such as academic pressure, independent work, responsibility, financial strain and adapting to changing social contexts, i.e. changing support networks within the new environment (Robotham & Julian, 2006). Attending university has been found to be associated with an increased engagement in risky or unhealthy behaviours, such as increased tobacco and alcohol consumption,

physical inactivity and poorer eating behaviours and sleep (Keating, Guan, Pinero, & Bridges, 2005; Nguyen, Walters, Wyatt, & DeJong, 2011; Steptoe et al., 2002; Velez et al., 2016). Being in higher education, with the pressure of meeting the requirements of academia, successful progression and completion, has been shown to generate increases in psychological distress and anxiety. Medical students in particular seem to suffer from the academic pressures; systematic reviews report higher prevalence rates of anxiety, distress and burnout in medical students compared with other student groups and age-matched peers (Dyrbye, Thomas, & Shanafelt, 2006; Lyndon et al., 2014). While all degrees have their challenges, especially in medical school, performance early-on shapes future professional pathways. Also law students seem to be under more pressures relative to other degrees (Krantz, 1985). Their degree requires immense effort in recalling clauses and case law; further, the class of a law degree often serves as a screening tool for law companies, again critical for influencing students' personal and professional lives.

The impact of stress on biological processes can be investigated by monitoring responses to naturalistic stressors. Academic examination is a well-established naturalistic stressor (Malarkey, Pearl, Demers, Kiecoltglaser, & Glaser, 1995; Steptoe et al., 1996). A systematic review with 23 studies using academic examinations as a stress and anxiety source in medical students concluded that examinations and assessments evoked substantial psychological distress in students (Lyndon et al., 2014). Comparisons between the days surrounding academic examinations and control periods have shown reliable changes in blood pressure,

heart rate periods, vagal tone, latent antibody titres, salivary immunoglobulin-A and other hormones (Amir et al., 2010; Glaser et al., 1994; Lacey et al., 2000; Lovallo, Pincomb, Edwards, Brackett, & Wilson, 1986; Murphy, Denis, Ward, & Tartar, 2010; Segerstrom & Miller, 2004; Spangler, 1997). Further, other health outcome changes have been observed by exposure to examination stress, such as dental plaque or gingival inflammation (Johannsen, Bjurshammar, & Gustafsson, 2010), suggesting that exams, acting as brief naturalistic stressors, are significant risk factors for immune-related processes (Segerstrom & Miller, 2004). Factors mediating the relationship between academic stress and alterations in immune functioning include neurohormonal changes, including excessive cortisol secretion (Murphy et al., 2010). In line with increased health complaints by students during stressful study periods (El Ansari, Oskrochi, & Stock, 2013), it highlights the impact of HPA-driven multisystem modulations occurring with immediate and long-term health implications.

There is a body of evidence that shows changes in cortisol measured in saliva or blood in response to academic stress (Evans, Bristow, Hucklebridge, Clow, & Pang, 1994; Hewig et al., 2008; Johannsen et al., 2010; Lovallo et al., 1986; Lucini, Norbiato, Clerici, & Pagani, 2002; Murphy et al., 2010; Ng, Koh, & Chia, 2003). Comparing students' dynamic HPA-axes (two assessments months apart prior to examinations) to community matched control samples showed that students had higher levels of stress that corresponded to different cortisol secretion patterns, i.e. elevated plasma cortisol levels or a flattened salivary CAR, respectively (Lacey et al., 2000; McGregor, Murphy, Albano, & Ceballos, 2016). Some studies have measured

variations in cortisol secretion on the day of examination using salivary cortisol assessments within the hours surrounding the exams (before, immediately after and at a certain intervals after the examination) (e.g. Ng et al., 2003; Preuss, Schoofs, Schlotz, & Wolf, 2010; Takatsuji et al., 2008). Evidence from studies employing this design is quite consistent in that the examination experience serves as an acute naturalistic stressor on the day, generating an increase in cortisol concentration. These immediate effects of examination stress on cortisol not only establish the expected rise in cortisol at examination but also anticipatory HPA-responses (Preuss et al., 2010), allowing insights into the temporal dynamics of the HPA-axis when exposed to examination stress, similar to stress-induced laboratory paradigms (Dickerson & Kemeny, 2004).

Other studies have compared cortisol levels and patterns on the days surrounding academic examinations or the day of examination itself to actual control periods, i.e.one or a few week(s) before and/or after the examinations. Most of the evidence suggests that mean cortisol values or AUC measures are increased during the days surrounding the examination, together with perceptions of stress and anxiety (Al-Ayadhi, 2005; Evans et al., 1994; Hellhammer, Heib, Hubert, & Rolf, 1985; Lovallo et al., 1986; Lucini et al., 2002; Pierceall & Keim, 2007). For example Evans et al. (1994) assessed four daily salivary cortisol measures on three days within the two weeks leading to an oral examination in science students. The results show a steady increase of AUC levels leading up to the examination day, parallel to the increasing pattern of perceived stress and arousal. Also performance or test anxiety, as measured right before examinations, has been

associated with increases salivary cortisol reactivity (Herbert, Moore, Delariva, & Watts, 1986; Mattarella-Micke, Mateo, Kozak, Foster, & Beilock, 2011). However, results have been inconsistent. Some studies find no difference between baseline and examination periods in mean day and evening plasma cortisol levels (e.g. Glaser et al., 1994). Also a reduction in levels of cortisol has been reported, however, these results are based on a between-subject design rather than comparing the same student group in distinct phases (Loft et al., 2007).

Most studies have assessed a limited number of salivary samples, not allowing simultaneous assessment of the different parameters of the cortisol profile, the CAR, the AUC and the slope. One study assessed the cortisol profile on five days throughout a period of four months including study/ revision days, mock-examination, examination (single-point salivary assessment), and relaxation days, in 120 medical graduates preparing for a medical specialty training position (Gonzalez-Cabrera, Fernandez-Prada, Iribar-Ibabe, & Peinado, 2014). The AUC was found to be higher on study/ revision and mock-examination days compared to the relaxation day. Also the CAR was significantly elevated on the mock-examination day compared to the relaxation day, supporting that size of the CAR is affected by anticipation of future challenge – in line with the anticipation hypothesis (Adam et al., 2006). Unfortunately, the authors did not analyse any changes in relation to the slope. Another study analysed the AUC and the slope from cortisol assessments based on eight saliva samples at 2-hourly intervals for a total of 6 days within two time periods (Hulme, French, & Agrawal, 2011), presenting a strength with regards to the robustness of the cortisol measure. They found the slope to be steeper on

the phase surrounding the examination day compared to the phase two weeks prior to the examination, which is not what might be predicted from the literature on cortisol slope. However, throughout this examination phase the pattern of the slope was not steady, displaying fluctuations of increase and decrease rather than the expected steady decrease. This might indicate a degree of involuntary or voluntary non-adherence to the sampling protocol or higher levels of daily stressors leading to fluctuations, which is not inconceivable given the period of high stress exposure. Furthermore, this study was based on only 15 medical students and did not include any measure of perception of stress.

The examination phase might last several weeks including the stressful study and revision period. Stress during examination phases might be sustained rather than being apparent on a day-to-day basis. As hair cortisol is a more reliable measure of long-term cortisol activity than the traditional sources of cortisol assessment, it might be suitable for use in examination phases with durations of a few weeks. The inconsistent findings based on salivary cortisol analyses might partly be due to the complexity of HPA-axis function and the pulsatile nature of cortisol release, but also due to methodological factors, such as adherence to sampling times, the sampling interval or the intensity of the stressor, as discussed previously (Kudielka et al., 2003; Malarkey et al., 1995; Stowell, 2003). Employing hair cortisol analyses is a novel approach to studying examination stress and to date no study has applied this analytical method in these settings. It would therefore be of interest to investigate whether academic stress induces increases in cortisol concentrations in hair. This can be tested by comparing concentrations in samples

collected soon after a putative demanding period such as during academic examinations (measuring the hair segment that reflects the period under stress exposure) and a control period of minimal academic stress. The simultaneous inclusion of salivary cortisol measures from which the different cortisol parameters can be derived additionally allows for comparison of the impact of stress in cortisol from different tissues.

Much of the research on academic examination stress was carried out some years ago, and the procedures for academic assessment have changed markedly over recent decades. In earlier times, the entire year of study was assessed during a single short period of unseen written examinations, but this is no longer the case. At UCL Medical School, for example, there are now in-course assessments, course work and portfolio requirements in addition to the summative examination held at the end of the year. In Years 1 and 2, MBBS students sit only two two-hour unseen examinations and a practical, compared with the six or seven examinations of earlier times. This means that the stress of contemporary academic examinations may be much lower than it was 10-20 years ago, when many of the biological stress studies were conducted.

6.2.2 Coping in relation to academic stress and cortisol

The appraisal of an event as a challenge or a threat and possible coping resources determines whether it is perceived as stressful or not and whether physiological stress responses take place (Lazarus & Folkman, 1987). Coping is a

dynamic process, which involves adaptation to new situations and contexts of varying degrees of stress. Cognitive, emotional, and behavioural processes determine whether homeostasis is restored, and whether the individual successfully handles the stressor.

As outlined in Chapter 1, Lazarus and Folkman's categorisation into problem-focused and emotion-focused coping has since been refined by several researchers. Skinner et al. (2003) suggested running exploratory factor analysis and subsequent rational sorting of items into factors in each separate study sample, rather than computing composite scores based on pre-determined scoring inventories. Yet, the broad coping style classifications seem to remain. A problem-focused, task- or action-oriented approach is an active form of dealing with the situation and is thought to reduce the stress exposing stimulus if the stressor is controllable (hence termed an adaptive coping strategy). Adaptive coping mechanisms have been linked to stress-reducing strategies with long-term rather than short-term well-being, gratification and pleasure (Everly & Lating, 2013). Conversely, an emotion-oriented and avoidant-oriented approach involves dealing with the emotional state itself, specifically, decreasing emotional distress by using blaming, distraction or wishful thinking when the stressor is controllable and could be dealt with direct action. Some coping aspects such as seeking social support might relate to both problem-focused and emotion-focused coping (Vitaliano, Maiuro, Russo, & Becker, 1987).

Individual differences of perceptions such as personal beliefs, values, goals, self-esteem, motivation, available resources and social support, determine the

cognitive elements and hence the use of adaptive coping or maladaptive coping strategies (Carver, 1997). Differences in capacity for self-regulation and coping have been shown to play a key role in mitigating the enduring detrimental effects of stress (Baum & Posluszny, 1999). Meta-analytic reviews report avoidant coping styles such as denial or distancing and also emotion-focused coping to be related to negative overall subjective and physical health (Aldwin & Park, 2004; Cheng et al., 2014; Penley et al., 2002). Adaptive coping strategies seem to act as 'stress-buffers' for both psychological and physiological processes (Cohen & Wills, 1985). Researchers have applied different methodologies to assess coping, such as established questionnaires, interviews and observational methods (Compas, Connor-Smith, Saltzman, Thomsen, & Wadsworth, 2001). The most commonly used inventories are the Ways of Coping Questionnaire by Folkman and Lazarus (1988) and the COPE/ brief COPE by Carver (1997; 1989).

Coping with stress and anxiety has long been of interest in academic settings in relation to well-being, efficiency, adaptation/ adjustment, performance or drop-out rates (Baker, 2003; Halamandaris & Power, 1999; Leong, Bonz, & Zachar, 1997), and attempts have been made to develop interventions to reduce stress in students (Regehr, Glancy, & Pitts, 2013). In a study with 475 undergraduates, avoidant coping was positively related to perceived levels of stress, while higher engagement in problem-focused coping was shown to buffer the effects of stress on subjective well-being (Austin, Saklofske, & Mastoras, 2010). The impact of academic stress on subsequent performance has attracted particular interest (Lyndon et al., 2014). A study with 238 undergraduates assessed stress, coping style (Endler & Parker, 1999)

and trait characteristics in relation to academic performance (measured by end-of-year marks). The authors found that poor performance could not be predicted by levels of stress but by adaptive coping, emotion regulation and personality aspects (Saklofske, Austin, Mastoras, Beaton, & Osborne, 2012). Although not assessed in this study, adaptive coping might have involved actions related to study skills, work load and time pressures and management, reducing perceived stress which may have led to better ability to focus on the challenges and problems at hand, i.e. assignments and examinations. This is in line with other studies, assessing the change in stress throughout the academic year, which found that increases in stress and also anxiety (and ultimately suboptimal academic performance) across the year were largely explained by the use of maladaptive coping styles, which relate to rumination, postponing and other inefficient behaviours (Watson, Deary, Thompson, & Li, Spangler, Pekrun, Kramer, & Hofmann, 2002; Struthers, Perry, & Menec, 2000; 2008). The association between poor performance and coping might be due to differences in approaches to studying. Compared to avoidant coping, problem-focused coping has been linked with more profound strategic (versus superficial) approaches to studying (Moneta, Spada, & Rost, 2007). This suggests that adaptive coping styles produce attitudes and behaviours in the revision stage that allows for deeper cognitive encoding and therefore better retrieval in exams which is reflected in higher exam marks.

This sequential pattern of stress and cognitive abilities can be described by the Yerkes-Dodson Law, an empirically-derived inverted-U model, which proposes that with increasing arousal, performance rises up to an optimum level; after this

optimum level, performance is impaired (Teigen, 1994; Yerkes & Dodson, 1908). According to this model, not only performance but also other cognitive functions decrease, such as adaptive coping mechanisms, which might ultimately be reflected in poor performance (Teigen, 1994). Glucocorticoid secretion has been shown to play a major role in this relationship as cognitive abilities function optimally in mild elevation of glucocorticoid levels but decrease under excessive glucocorticoid exposure (Lupien, Maheu, Tu, Fiocco, & Schramek, 2007). Performance is highest at optimal levels of physiological arousal which allow for optimal cognitive abilities, affecting brain structures involving learning, selective attention and memory but also emotion regulation and decision-making.

Adaptive coping strategies have been linked to more favourable cortisol profiles, such as lower diurnal cortisol values over the day (O'Donnell et al., 2008) and lower reactivity to laboratory-based stress paradigms (Houtman & Bakker, 1991). Only a few studies have investigated coping in the academic environment in relation to cortisol. An early study by Hellhammer et al. (1985) with just 10 male students assessed salivary cortisol 10 min before several individual examinations and one week after the last examination and asked students about their coping behaviours prior to examinations. They found that while salivary cortisol levels increased during examinations by approximately 69%, maladaptive coping behaviours (passive-withdrawal and rumination) were associated with elevated cortisol concentration values. A recent study assessed cortisol activity in relation to loneliness and coping strategies in 70 first-year undergraduates (Drake, Sladek, & Doane, 2016). They found that higher engagement in adaptive coping strategies

was related to a lower CAR. Further, students who reported an increase in loneliness over a period of several months and also engaged in maladaptive coping showed flatter diurnal cortisol slopes compared with students who engaged in adaptive coping, supporting the role of coping as a protective factor against social isolation. Lastly, another recent study with 63 students that assessed perceived daily diary-reported stressors and coping in relation to salivary cortisol over a period of three days found that both individual-related (between-person) coping variation and situational-related (within-person) coping variation moderated the relationship between perceived stress and cortisol increases (Sladek, Doane, Luecken, & Eisenberg, 2016). Cortisol responses to daily stressors were positively associated with perceived stress only in students that reported engagement in maladaptive coping strategies as a trait measure and low beliefs in the ability to successfully cope and concomitantly only in situations in which excessive problem-solving was applied.

There is a body of evidence that suggests coping is a protective factor against stress which seems to apply to academic-related stress in the same way as other stressors, resulting in more positive functioning and adaptive outcomes. Research has also provided compelling data contributing to an understanding of the discrepancies in coping mechanisms in relation to cortisol; however, hair cortisol has not been utilised in this way. Therefore, I thought it would be interesting to investigate whether engagement in adaptive coping strategies serves as a protective factor for university-related and examination stress.

This chapter describes the second study of my PhD, the Academic Stress Study, whose data has been described and analysed in relation to methodological aspects in the previous chapter (Chapter 5). This study is based on a within-subject design, studying medical and law students in two distinct phases: during average academic stress and during high academic stress. As in previous academic stress research, medical and law students were selected because they generally experience a higher workload and face greater responsibilities compared to other student groups (Segerstrom & Miller, 2004). Analysing the impact of a naturalistic stressor (examination stress) in relation to hair cortisol in a repeated-measures design allows for further validation of hair cortisol as a biomarker of psychological stress. Similarly, it enables the analysis of the relationship between hair cortisol and stress-related psychological attributes such as perceived stress and anxiety. Employing these psychological measures, I will investigate whether the effect of stress is visible in both tissues, salivary and more importantly hair cortisol, and whether adaptive psychological coping mechanisms have a protective effect on elevated stress-related cortisol output.

6.2.3 The present study

The present study utilises the Academic Stress Study, the same dataset as described in the previous chapter. As outlined formerly, it is based on a within-subject design, studying medical and law students in two distinct phases: during average academic stress and during high academic stress. It makes use of variations of stress exposure and perception, the dynamics of the cortisol sensitivity-response

(salivary cortisol) and cumulative hair cortisol. By comparing hair cortisol levels between the two periods it is possible to study hair cortisol as an index of stress reactivity to chronic naturalistic stressors (stress exposure over a few weeks).

Academic stress can be perceived as more stressful by some individuals and less stressful by others. Perception of stress might therefore be an important moderating factor in the stress-cortisol relationship. Likewise, maladaptive coping mechanisms might strengthen any possible relationships. Therefore, this study examines whether cortisol secretion in saliva and hair specimen reflect individual differences in coping styles under stressful periods.

6.2.4 Aims and Hypotheses

Aims

This study has three main aims. The first aim is to test the value of hair cortisol as a stress marker. Data from both phases will establish whether academic stress induces increases in cortisol concentrations in hair specimens (and in salivary specimens). This will be tested by comparing samples taken from university students collected during/ shortly after academic examinations with those collected during a control period of minimal academic stress. Perceived stress and anxiety (due to the forthcoming examinations) might act as moderating factors in the stress-cortisol relationship. The second aim is therefore to investigate the impact of different levels of perceived stress and anxiety on hair cortisol levels. Lastly, different psychosocial attributes might function as a stress buffer. Thus, a third aim

is to investigate psychological coping processes as moderators of cortisol responses to stress.

Hypotheses

The following hypotheses will be tested in this study:

1. Hair cortisol content will be higher in the high academic stress compared with the average academic stress period
2. Perceived stress and anxiety in the high academic stress period will be positively associated with salivary cortisol and hair cortisol concentration.
3. Adaptive coping responses, such as problem-focused coping, will be associated with lower cortisol levels in both hair and saliva samples compared with maladaptive coping styles or will mediate the impact of perceived stress/ anxiety on cortisol.

6.3 Methods

6.3.1 Study design

The study was described in the previous chapter. However, its design and method will be outlined briefly again as there are additional measures that were assessed and which are the focus of this chapter. The two phases were six months apart. Phase 1 took place under average academic stress exposure and Phase 2 took place under high academic stress exposure (with each phase lasting 7 weeks).

Figure 6.1 depicts the design of the study and the relevant assessments at each time point throughout each phase. At three time points at week 1, week 3 and week 5 (6, 4 and 2 weeks prior to hair cortisol sampling) participants completed three diurnal salivary cortisol profiles (SCC). At all sampling points, participants were also asked to complete a battery of self-report questionnaires (Appendix 8) assessing psychological attributes, i.e. perceived stress (PSS), anxiety (STAI) and coping (COPE). Hair samples (HCC) were taken in week 7, two weeks after the last salivary sampling (SCC Time 3) to ensure corresponding hair growth.

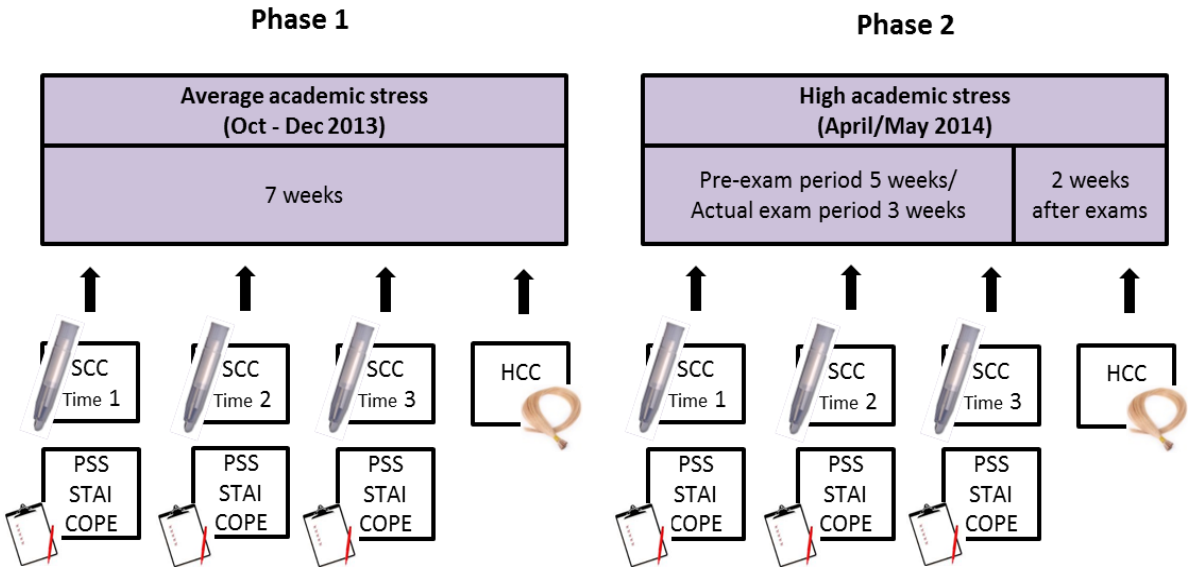

Figure 6.1 Study design and temporal distance of sampling. Salivary cortisol concentration (SCC) sampling at two-week intervals with assessment of perceived stress (PSS), anxiety (STAI) and coping (COPE). Hair cortisol concentration (HCC) was sampled at the end of each 7-week period.

6.3.2 Participants

Participants were 77 second-year students (aged 18-25) from the Medical and Law School at UCL, invited via emails and flyers to take part in the study. Participants needed to be able to commit to all assessments and they were compensated with £50 once all assessments were completed (at the end of Phase 2).

6.3.3 Procedure

The data collection procedure was explained in the previous chapter. Briefly, participants were seen twice in each phase in a research laboratory at UCL. At the first visit, socio-demographic factors and anthropometric measures were taken. Participants were instructed on how to take saliva samples and were given packs containing five salivette tubes for each time point assessment (week 1, week 3 and week 5). Participants were reminded that they would be sent several online questionnaires to fill in; an initial comprehensive questionnaire at baseline (week 1) was followed by repeated assessments of relevant psychosocial factors at weeks 3 and 5. Participants were given the opportunity to ask questions before they were thanked and dismissed. At the second meeting (at week 7), hair was taken from the posterior vertex and participants were asked about any hair treatment they had had in the preceding 1-2 months. Half a year later, participants were invited by email to return to the lab and to repeat the exact same procedure as in the first phase, with sampling and measures being taken at analogous times. All questionnaires are presented in Appendix 8.

6.3.4 Measures

Salivary cortisol

Saliva was collected with Salivette devices (Sarstedt, Numbrecht-Rommelsdorf, Germany). Samples were collected on three full days per period: at week 1, week 3 and week 5 (6, 4 and 2 weeks prior to hair cortisol sampling; see Figure 6.1). Participants started the saliva sampling the day after they had been seen by the researcher, and carried it out over a 24-hour period [11am, 3pm, at bedtime, at awakening (the following day) and 30 minutes after awakening]. Reminder emails and text messages were sent before 11am to ensure that participants adhered to the sampling protocol and in the evening to prepare the two salivettes for the coming morning. Participants were asked to abstain from smoking, food, medication and alcohol intake, brushing teeth, and exercise 30 minutes prior to saliva sampling. A diary was provided for each salivary sampling day, which assessed time of sample (and therefore adherence to the 30 minutes post-awakening interval). All samples and diaries were returned to UCL in the following 3-6 days and collected by the researcher. Samples were stored in a freezer at −20 °C until they were sent to the Technical University of Dresden, Germany, where steroid extraction was performed. Cortisol levels were assessed using a time resolved immunoassay with fluorescence detection, and the intra- and inter-assay coefficients of variation were less than 4%. The cortisol awakening value was calculated as the difference between cortisol levels at waking and 30 minutes after waking. Participants with a delay of more than 15 min between waking and saliva sampling were excluded from the CAR analyses (Dockray et al., 2008). The

AUC was computed by trapezoidal calculation of the diurnal values (11am, 3pm and bedtime) and represents the AUC with respect to the ground (Pruessner et al., 2003). The cortisol slope was defined by regressing cortisol values from 11 am until bedtime.

Hair cortisol

Hair collection took place in both periods at the end of the 7-week study period. A scalp hair strand of 1 cm was collected from the posterior vertex position. Hair strands were cut closely to the scalp with fine medical scissors, were placed onto aluminium foil and labelled with the identification number, following the hair protocol described by Kirschbaum et al. (2009). Upon collection, they were stored in a dry, dark place for a maximum period of eight months. All hair samples were shipped to the Technical University of Dresden, Germany, after the end of Phase 2 to ensure identical hair analytical procedure. The wash procedure and steroid extraction were undertaken using high performance liquid chromatography–mass spectrometry (LC-MS), as described by Kirschbaum and colleagues (2009), with a minimum of 10 mg ± 0.5 mg of hair, cut from each 1 cm hair segment. The hair segment 1 cm nearest the scalp represents the averaged cortisol accumulated over an approximate timespan of 2-7 weeks prior to sampling.

Psychosocial measures

The questionnaire included measures of psychological distress, anxiety and coping (Appendix 8). Questions on psychological distress, anxiety and coping were repeated at 2-week intervals when saliva was sampled. At time point 3 (week 5), specific stressor questions were administered to assess stressful experiences.

i) Perception of stress

The Perceived Stress Scale (PSS; Cohen, Kamarck, & Mermelstein, 1983) was employed to measure the degree of perceived stress covering the period of two weeks prior to administration. The 10-items include items such as “In the last two weeks, how often have you been angered because of things that were outside of your control? “. Answers were given on a 5-point Likert scale ranging from 0 (never) to 4 (very often) and sum scores were calculated. The Cronbach’s alpha for this scale was 0.85 (based on Phase 1 values).

ii) Anxiety

The 20-item state scale of the Spielberger State-Trait Anxiety Inventory (STAI; Marteau & Bekker, 1992) measured anxiety, e.g. “I am tense”. Responses were given on a 4-point Likert scale (not at all/ somewhat/ moderately/ very much). Sum scores were calculated, with higher scores corresponding to greater levels of anxiety. The Cronbach’s alpha for this scale was 0.94 (based on Phase 1 values).

iii) Coping

The brief COPE (Carver, 1997) assesses coping strategies with stress. With its 28 items, it assesses 14 conceptually different coping subscales: self-distraction, active coping, denial, substance use, emotional support, instrumental support, behavioural disengagement, venting, positive reframing, planning, humour, acceptance, religion and self-blame. Participants were asked to give responses ranging from “I haven't been doing this at all” to “I've been doing this a lot” on a 4-

point Likert scale for questions such as “I gave up trying to deal with it.” The subscale self-distraction was eliminated as it showed extraordinarily low internal reliability (Cronbach’s α= 0.20; all Cronbach’s α are based on Phase 1). The Cronbach’s alphas for the other scales ranged from 0.74 (venting) to 0.98 (religion). Although the different subscales roughly categorise into problem-focused and emotion-focused coping styles, aggregate variables from these subscales should depend on the factor loadings in each dataset (Carver, 1997). Thus a factor analysis with varimax rotation was performed with the 13 subscales to identify the underlying dimensions of distinct coping styles (Table 6.1).

Table 6.1 Factor loadings for the 13 subscales per phase.

	Factor loadings								
	Phase 1				Phase 2				
	F1	F2	F3	F4	F1	F2	F3	F4	F5
Active coping	0.802				0.863				
Denial				0.804				0.753	
Substance use				0.703			0.516	0.388	-0.322
Emotional support		0.931				0.932			
Instrumental support		0.894				0.924			
Behavioural disengagement			0.495	0.591			0.662	0.407	
Venting		0.488	0.511			0.358	0.655		
Positive reframing		0.474	0.412		0.575			0.321	
Planning	0.903				0.855				
Humour			0.774						
Religion	0.577								0.945
Self-blame			0.648				0.806		
Acceptance	0.590			-0.459	0.756				

Different factors emerged for both phases (four factors for Phase 1 and 5 factors for Phase 2) and some subscales loaded onto distinct factors, thus several subscales that were not normally distributed were removed for this factor analysis and (denial, substance use, behavioural disengagement and religion). One subscale that had high cross-loadings was removed (positive reframing). From the factor analysis performed with the remaining 8 subscales, three factors emerged with Eigenvalues >1 accounting for 69% of the variance in Phase 1 and 73% in Phase 2 (Table 6.2). Factor loadings and also Cronbach's alpha were similar in strength for both phases. Factor 1 consisted of active coping, planning and acceptance, and was labelled problem-focus coping (Cronbach's α = 0.81 and 0.83). Factor 2, labelled socially-supported coping, comprised instrumental support and emotional support with Cronbach's α = 0.89 and 0.93. Factor 3 consisted of self-blame, venting and humour and was named avoidant coping; the Cronbach's alpha was low for this scale (α = 0.34 and 0.48).

Table 6.2 Final factor extraction with the 8 subscales.

	Factor loadings		
	Problem-focused coping (Phase 1/Phase 2)	**Social support coping (Phase 1/Phase 2)**	**Avoidant coping (Phase 1/Phase 2)**
Planning	0.910/0.903		
Active coping	0.892/0.916		
Acceptance	0.767/0.728		
Instrumental support		0.927/0.937	
Emotional support		0.920/0.960	
Humour			0.359/0.821
Venting			0.746/0.576
Self-blame			0.804/0.661

iv) Specific stressors/ experience of negative life events

At the end of each phase, a self-developed questionnaire was administered to assess levels of general stress including those outside the academic domain over the last 7 weeks, e.g. "In the past 7 weeks has your relationship with your parents been stressful?" and responses were given on the following 5-Likert-type response scale: Not stressful at all/ very little stressful/ slightly stressful/ quite a bit stressful/ very stressful. Stress due to family, financial issues, feeling fatigued, neighbourhood/ environment, lack of organisation (e.g. running late to lectures, forgetting things) and university was assessed. Sum scores were calculated for the former five items with higher scores indicating a higher degree of stress. University-related stress was assessed with one item (which should serve as an indicator that Phase 2 was perceived as more stressful than Phase 1 because of university and not

due other factors). Finally, one item assessed the experience of negative life events over the past 7 weeks as a binary variable (Yes/ No).

Health behaviours

At the beginning of each phase several health behaviours were assessed. These included physical activity, smoking (binary variable) and weekly alcohol consumption. Physical activity was measured by asking the participants how often ("Never/ hardly ever", "1-3/ month", "1-2/ week", "3 times or more/ week") they engaged in weekly moderately energetic activities such as cycling and dancing, and in vigorous activities such as running, hard swimming, tennis, and cycle racing. The total moderate and vigorous activities engaged in were summed (range: 0-6), with higher scores indicating greater engagement in physical activity.

Hair-related questions

Hair-specific factors that could affect hair cortisol concentration were assessed by self-report and verified at hair sampling. These included frequency of weekly hair washes, hair colour and curvature, use of hair products and any type of hair treatment within the period of investigation.

6.3.5 Statistical analyses

All analyses were conducted on participants who completed both phases (N = 71) of the study. One outlier in hair cortisol concentration was identified and removed from the analyses (Phase 1: 252.25 pg/mg and at Phase 2: 308.50 pg/mg),

resulting in a final sample of 70 students. As stated in the previous chapter, hair cortisol and AUC of the salivary cortisol data were skewed and therefore logarithmically (ln) transformed to normalise the distributions.

T-tests and Chi^2-tests were performed to assess any differences among the two student groups. Repeated-measure ANOVAs were conducted to assess whether the cortisol parameters differed across the three sampling time points. Dependent t-tests were conducted to analyse the differences between the two phases and repeated-measures ANOVAs were conducted to explore changes throughout each phase (i.e. time 1, time 2 and time 3). Linear regression analyses with and without controlling for covariates of interest (i.e. age, BMI, smoking, hair treatment) and also student group were performed for the different variables.

6.4 Results

6.4.1 Sample characteristics

As outlined in the previous chapter, from the initial 77 participants, 71 completed both phases. There were 57 medical students and 20 law students in Phase 1 and 52 medical students and 19 law students in Phase 2. T-tests revealed that participants who completed Phase 2 (N = 71) did not differ from those who dropped out after Phase 1 (N = 6) in any of the cortisol measures or perceived stress and anxiety at baseline (all *p*'s > 0.05). All following analyses are based on the sample who completed both phases, excluding one individual/ outlier with extremely high hair cortisol values at both phases (N = 70). Table 6.3 summarises

the socio-demographic and health-related characteristics of the sample. Briefly, t-tests and Chi^2-tests yielded no differences between medical and law students in any of these aspects.

Table 6.3 Demographic characteristics and health-related factors of the combined sample and per student group.

Variable	Combined sample	Student group		Group differences p-value
	N = 70	Medical students (N = 51)	Law students (N = 19)	
	Mean (SD) /frequency (%)	Mean (SD) /frequency (%)	Mean (SD) /frequency (%)	
Age	20.7 (1.07)	20.8 (1.14)	20.5 (0.84)	0.306
Ethnicity				
White	41 (58.6)	29 (56.9)	12 (63.2)	0.760
Asian	23 (32.9)	18 (35.3)	5 (26.3)	
Other (black/ mixed)	6 (8.6)	4 (7.8)	2 (10.5)	
Body mass index (kg/m^2)	22.34 (4.17)	22.56 (4.68)	21.75 (2.29)	0.470
Current smoker				0.655
Yes	9 (12.9)	6 (11.8)	3 (15.8)	
No	61 (87.1)	45 (88.2)	16 (84.2)	
Alcohol drinker				0.202
Frequent/	22 (31.4)	18 (35.3)	4 (21.1)	
regular	32 (45.7)	20 (39.2)	12 (63.2)	
Occasional	16 (22.9)	13 (25.5)	3 (15.8)	
No				

Tables 6.4 and 6.5 summarise the mean values for the different salivary cortisol parameters (at each time point and the aggregated measure) and hair cortisol concentration in Phase 1 and in Phase 2, in the combined sample and by student group. T-tests yielded a significant difference between students in Phase 1 for salivary cortisol AUC at time point 2 and in hair cortisol. Law students had higher AUC values [M = 8.94 ln(nmol/l), SD = 0.41] than medical students [M = 8.66 ln(nmol/l), SD = 0.51] at time point 2 (t = -2.107, df = 66, p = 0.039), but not at the aggregated level (t = -0.734, df = 66, p = 0.466). Law students had higher hair cortisol values [M = 2.81 ln(nmol/l), SD = 0.68] than medical students [M = 2.38 ln(nmol/l), SD = 0.68 , t = -2.232, df = 68, p = 0.029]. Even though there were no differences in cortisol measures at baseline between individuals that dropped out and participants who completed both phases, the drop-out impacted on the difference between the student groups in the sample that completed both phases. Analyses are presented for the whole sample to increase sample size and therefore power; however, degree course was included as a covariate. There were no differences between medical and law students in any of the other cortisol measures at either phase (all p's > 0.05).

Table 6.4 Mean values for the different salivary cortisol parameters (at each time point and the aggregated measure) and hair cortisol concentration in Phase 1.

Cortisol parameter	Combined sample	Student group		Group differences p-value
	N = 70	Medical students (N = 51)	Law students (N = 19)	
	Mean (SD)	Mean (SD)	Mean (SD)	
Phase 1				
CAR 1 (nmol/l)	11.65 (11.30)	11.31 (11.77)	12.68 (10.03)	0.687
CAR 2 (nmol/l)	11.12 (14.39)	10.96 (14.30)	11.53 (14.98)	0.884
CAR 3 (nmol/l)	9.50 (10.00)	10.19 (8.63)	7.41 (13.47)	0.462
CAR agg (nmol/l)	10.88 (9.36)	11.03 (8.69)	10.48 (11.26)	0.834
AUC 1 [ln(nmol/l)]	8.81 (0.42)	8.84 (0.40)	8.71 (0.48)	0.243
AUC 2 [ln(nmol/l)]	8.74 (0.50)	8.66 (0.51)	8.94 (0.41)	0.039
AUC 3 [ln(nmol/l)]	8.85 (0.47)	8.80 (0.49)	8.98 (0.40)	0.174
AUC agg [ln(nmol/l)]	8.80 (0.35)	8.77 (0.36)	8.88 (0.31)	0.280
Slope 1 (nmol/l/h)	0.80 (0.76)	0.76 (0.78)	0.90 (0.69)	0.506
Slope 2 (nmol/l/h)	0.82 (0.88)	0.71 (0.80)	1.15 (1.00)	0.062
Slope 3 (nmol/l/h)	0.77 (0.82)	0.73 (0.80)	0.88 (0.90)	0.512
Slope agg (nmol/l/h)	0.80 (0.58)	0.73 (0.59)	0.98 (0.51)	0.100
Hair cortisol [ln(pg/ng)]	2.50 (0.73)	2.38 (0.73)	2.81 (0.68)	0.029

Note. agg = aggregated (average of Time 1, Time 2 and Time 3).

Table 6.5 Mean values for the different salivary cortisol parameters (at each time point and the aggregated measure) and hair cortisol concentration in Phase 2.

Cortisol parameter	Combined sample	Student group		Group differences p-value
	N = 70	Medical students (N = 51)	Law students (N = 19)	
	Mean (SD)	Mean (SD)	Mean (SD)	
Phase 2				
CAR 1 (nmol/l)	9.75 (10.95)	8.71 (10.38)	12.74 (10.30)	0.194
CAR 2 (nmol/l)	10.79 (13.64)	9.37 (13.72)	14.52 (13.07)	0.175
CAR 3 (nmol/l)	10.47 (14.10)	10.10 (14.85)	11.46 (12.27)	0.730
CAR agg (nmol/l)	10.50 (9.95)	9.55 (9.42)	13.03 (11.15)	0.209
AUC 1 [ln(nmol/l)]	8.72 (0.50)	8.72 (0.54)	8.72 (0.40)	0.974
AUC 2 [ln(nmol/l)]	8.76 (0.55)	8.70 (0.57)	8.95 (0.46)	0.099
AUC 3 [ln(nmol/l)]	8.78 (0.44)	8.82 (0.47)	8.70 (0.37)	0.326
AUC agg [ln(nmol/l)]	8.76 (0.37)	8.75 (0.39)	8.79 (0.31)	0.674
Slope 1 (nmol/l/h)	0.76 (0.61)	0.82 (0.62)	0.61 (0.56)	0.198
Slope 2 (nmol/l/h)	0.56 (0.72)	0.62 (0.63)	0.38 (0.92)	0.219
Slope 3 (nmol/l/h)	0.70 (0.69)	0.73 (0.76)	0.60 (0.50)	0.496
Slope agg (nmol/l/h)	0.67 (0.50)	0.72 (0.50)	0.53 (0.47)	0.164
Hair cortisol [ln(pg/ng)]	2.52 (0.73)	2.56 (0.67)	2.43 (0.87)	0.504

Note. agg = aggregated (average of Time 1, Time 2 and Time 3).

6.4.2 Impact of stress exposure on hair cortisol and salivary cortisol

Hair cortisol

Table 6.6 shows the hair cortisol concentration and salivary cortisol parameters for both phases. Paired-samples t-tests analysed whether there were significant differences in levels between the low academic stress and the high academic stress phase. Hair cortisol did not differ significantly between the two phases [t = -0.293, df = 69, p = 0.770, Phase 1: M = 2.50 ln(pg/mg), SD = 0.74; Phase 2: M = 2.52 ln(pg/mg), SD = 0.73].

Table 6.6 Mean values for hair cortisol and for the different salivary cortisol parameters for Phase 1 and Phase 2.

Cortisol parameter	Phase 1 Mean (SD)	Phase 2 Mean (SD)	p-value
Hair cortisol [ln(pg/ng)]	2.52 (0.74)	2.52 (0.73)	0.770
CAR (nmol/l)	10.99 (9.52)	10.48 (9.82)	0.675
AUC [ln(nmol/l)] old	8.80 (0.35)	8.76 (0.37)	0.302
Slope (nmol/l/h) new	0.80 (0.58)	0.67 (0.50)	0.089

However, repeated-measures ANOVA with student group as a between-group factor revealed a significant interaction; $F_{1, 68}$ = 7.445, p = 0.008. Direction of change in hair cortisol levels across the two phases differed by student group. Two separate repeated-measures ANOVAs for each student group showed tendencies but did not reach significant levels in both analyses; medical students: $F_{1, 50}$ = 3.102, p = 0.084 and law students: $F_{1, 18}$ = 3.628, p = 0.073. Figure 6.2 depicts this

interaction, indicating that medical students had a tendency for increased hair cortisol levels at Phase 2 compared with Phase 1 [M = 2.38 ln(pg/mg), SD = 0.73 and M = 2.56 ln(pg/mg), SD = 0.67, respectively], whilst law students had a tendency for decreased hair cortisol at Phase 2 compared to Phase 1 [M = 2.81 ln(pg/mg), SD = 0.68 and M = 2.43 ln(pg/mg), SD = 0.87, respectively]. As seen in Table 6.4 hair cortisol levels were significantly different between the student groups at Phase 1 but not at Phase 2 (Table 6.5); the interaction therefore seem to be mainly driven due to the differences in the low academic stress period (Phase 1) rather than the high academic stress period (Phase 2).

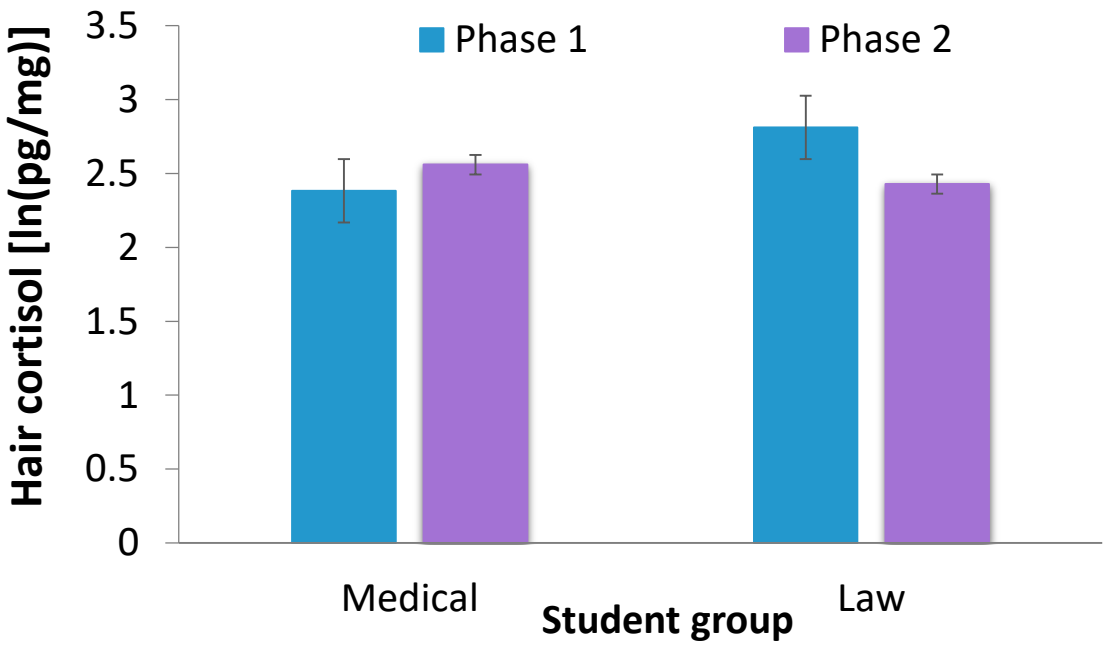

Figure 6.2 Hair cortisol concentration in both phases by student group (significant interaction), indicating that medical students had a tendency for increased hair cortisol levels at Phase 2, whilst law students had a tendency for decreased hair cortisol at Phase 2.

Salivary cortisol

To be able to compare the salivary cortisol parameters between the two phases, the aggregated measure (average of the three assessments) was used in the analyses; see Table 6.6. From the averaged salivary cortisol parameters, there was a tendency for the slope to be flatter in Phase 2 (M = 0.67 nmol/l/h, SD = 0.50) compared to Phase 1 (M = 0.80 nmol/l/h, SD = 0.58; t = 1.726, df = 69, p = 0.089). The CAR and the AUC did not differ significantly between the two phases (t = 0.421, df = 63, p = 0.675 and t = 1.039, df = 67, p = 0.302, respectively).

Repeated-measure ANOVAs were conducted on the salivary cortisol parameters with student group as a between-group factor to investigate whether there were any interactions. There was a significant interaction between phase and student group for the slope, $F_{1, 68}$ = 7.431, p = 0.008. Two separate repeated-measures ANOVAs for each student group revealed that only law students had a significantly flatter slope in Phase 2 compared with Phase 1 ($F_{1, 18}$ = 8.463, p = 0.009) (Phase 1: M = 0.98 nmol/l/h, SD = 0.51; Phase 2: M = 0.53 nmol/l/h, SD = 0.47), whilst medical students' slope values did not differ significantly between the two phases, ($F_{1, 50}$ = 0.016, p = 0.900) (Phase 1: M = 0.73 nmol/l/h, SD = 0.59; Phase 2: M = 0.72 nmol/l/h, SD = 0.50; Figure 6.3). There were no interactions between change in cortisol between the two phases and student group for the CAR ($F_{1, 62}$ = 1.678, p = 0.200) or the AUC ($F_{1, 66}$ = 0.350, p = 0.556).

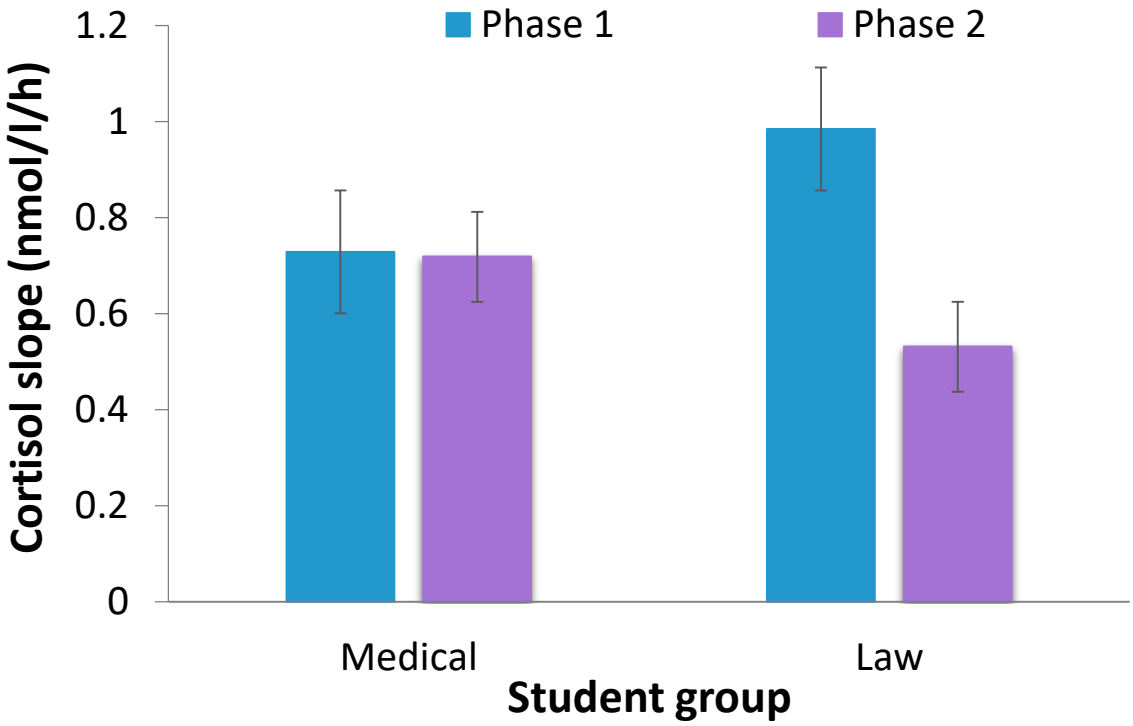

Figure 6.3 The salivary slope in both phases by student group (significant interaction), indicating that only law students had a significant decrease in the slope from Phase 1 to Phase 2.

Repeated-measures ANOVAs were conducted on the salivary cortisol parameters in each phase to see whether these differed throughout these two periods. Analyses showed that the cortisol parameters did not differ significantly across the three sampling time points at Phase 1 (CAR: $F_{2, 100} = 0.669$, $p = 0.515$; AUC: $F_{2, 128} = 1.111$, $p = 0.332$; Slope: $F_{2, 130} = 0.260$, $p = 0.771$). In Phase 2, cortisol parameters did not differ significantly either across the three sampling time points (CAR: $F_{2, 118} = 0.061$, $p = 0.941$; AUC: $F_{2, 126} = 0.610$, $p = 0.545$; Slope: $F_{2, 128} = 1.583$, $p = 0.209$).

6.4.3 Impact of stress exposure on the psychological measures

As there were only some slight differences between the two phases in the cortisol measures and not significantly in hair cortisol as expected, it was of interest to evaluate whether the purportedly stressful examination phase was an appropriate stressor to induce observable changes. For this I tested whether Phase 2 was perceived as more stressful than Phase 1, induced greater levels of anxiety and whether there were also any changes or fluctuations in perceived stress and anxiety throughout each of the two phases.

Prior to this, t-tests were conducted to evaluate whether there were differences in the student groups in perceived stress and anxiety. Table 6.7 shows the descriptive statistics of perceived stress and anxiety of the combined sample and per student group. There were no significant differences in perceived stress at Phase 1 [t = -1.150, df = 68, p = 0.254) or at Phase 2 [t = -1.391, df = 68, p = 0.169). However, there were differences in anxiety at Phase 1 [t = -2.894, df = 68, p = 0.005) and slightly missed the significance level at Phase 2 [t = -1.892, df = 68, p = 0.063). Law students reported higher levels of anxiety at both phases compared to medical students (Table 6.7).

Table 6.7 Mean values for the different psychosocial variables in the combined sample and per student group in both phases.

Psychosocial variable	Combined sample	Student group		Group differences p-value
	N = 70	Medical students (N = 51)	Law students (N = 19)	
	Mean (SD)	Mean (SD)	Mean (SD)	
Phase 1				
Perceived stress	17.97 (5.54)	17.50 (5.86)	19.21 (4.44)	0.254
Anxiety	37.12 (8.76)	35.37 (8.35)	41.84 (8.27)	0.005
Phase 2				
Perceived stress	21.74 (4.99)	21.24 (5.29)	23.09 (3.86)	0.169
Anxiety	50.06 (9.97)	48.71 (9.51)	53.68 (10.53)	0.063

Paired-samples t-tests analysed the change between phases in the different psychosocial factors. As can be seen in Table 6.8, results showed that there was a significant increase from Phase 1 to Phase 2 in perceived stress (t = -6.848, df = 69, $p < 0.001$; 21% increase) and in anxiety (t = -13.286, df = 69, $p < 0.001$; 35% increase), indicating that Phase 2 indeed acted as a stress-inducing experience, simultaneously serving as a manipulation check. The experience of stressful stressors other than university stress (i.e. stress due to family, financial issues, feeling fatigued, neighbourhood/ environment and lack of organisation) within the last six weeks did not differ between the two phases (t = -0.648, df = 68, $p = 0.519$). By contrast, university-related stress did increase from Phase 1 (M = 3.74, SD = 0.78) to Phase 2 (M = 4.59, SD = 0.71; t = -6.694, df = 68, $p < 0.001$), pointing

towards the fact that the increase in perceived stress and anxiety could be attributed to actual university-related pressure rather than outside sources of stressors. Chi^2 analysis showed that the experience of negative life events was not different between the phases [$\chi 2 = 0.337$, df = 1, $p = 0.562$; Phase 1: Yes N = 22 (40.6%) and Phase 2: Yes N = 17 (57.1%)]. Repeated-measures ANCOVAs with student group as a covariate revealed the same results.

Table 6.8 Psychosocial factors between Phase 1 and Phase 2.

Psychosocial factors	Phase 1 Mean (SD)	Phase 2 Mean (SD)	p-value
Perceived stress	17.97 (5.54)	21.74 (4.99)	0.000
Anxiety	37.12 (8.76)	50.06 (9.97)	0.000
Specific stressors	10.36 (2.83)	10.61 (3.18)	0.519
University-related stress	3.74 (0.78)	4.59 (0.71)	0.000

Repeated-measures ANOVAs were conducted on perceived stress and anxiety in each phase to see whether these differed throughout the two periods (Table 6.9). In Phase 1, there were no significant differences in either of the two psychosocial factors between the time points ($F_{2, 138}= 0.084$, $p = 0.920$ and $F_{2, 138}= 0.533$, $p = 0.588$, respectively). In contrast, in Phase 2, there were differences throughout the period ($F_{2, 138}= 3.522$, $p = 0.032$ and $F_{2, 138}= 10.954$, $p < 0.001$, respectively). Post-hoc analyses with Bonferroni correction revealed that perceived stress was non-significantly higher at Time 3 compared to Time 1 (mean difference: 1.557, $p = 0.062$) but anxiety increased significantly between Time 1 and the

assessments made closer to the actual examination at Time 2 and Time 3 (mean difference: -4.386, $p = 0.001$ and mean difference: -5.771, $p < 0.001$, respectively).

Table 6.9 Change in psychosocial factors during Phase 1 and Phase 2.

Variable	Time 1 Mean (SD)	Time 2 Mean (SD)	Time 3 Mean (SD)	p-value
Phase 1				
Perceived Stress	18.10 (5.68)	17.84 (6.76)	17.96 (6.45)	0.920
Anxiety	36.44 (10.92)	37.94 (11.76)	36.99 (11.14)	0.588
Phase 2				
Perceived Stress	21.01 (6.08)	21.63 (6.09)	22.57 (4.99)	0.032
Anxiety	46.67 (10.42)	51.06 (12.19)	52.44 (12.53)	0.000

6.4.4 The influence of potential confounders on hair cortisol

Univariate analyses revealed no associations between hair cortisol and age, BMI, smoking status, regular alcohol consumption and physical activity (see Table 6.10; all p's > 0.280). Ethnicity was unrelated to hair cortisol levels ($F_{2,67} = 0.277$, $p = 0.759$). Analyses regarding the hair-related characteristics revealed that hair treatment in the 7 weeks prior to sampling was associated with hair cortisol; individuals with treated hair had lower cortisol levels than those with untreated hair ($F_{1,67} = 4.189$, $p = 0.045$; 1.86 vs. 2.55 ln [pg/mg]). Hair colour, number of hair washes per week and use of hair products did not show any associations with hair cortisol (p's > 0.086). As the variables age, BMI and smoking have been identified as confounders in the literature, they were used as potential covariates in all analyses.

Table 6.10 Association between hair cortisol and demographic, health-related characteristics and hair-related factors (Regressions and ANOVAs, respectively).

Variable	B (95% C.I.)	p-value
Age	-0.048 (-0.214/0.117)	0.562
BMI	0.001 (-0.042/0.043)	0.979
Physical Activity	0.059 (-0.050/0.169)	0.280

Unstandardised regression coefficients (B) and 95% confidence intervals (C.I.).

Variable	F (df)	p-value
Smoking	0.470 (1,68)	0.496
Alcohol consumption	1.028 (1,67)	0.363
Hair-related factors		
Hair treatment	4.189 (1,67)	0.045
Hair colour	1.207 (4,64)	0.317
Hair washes (weekly)	0.103 (2,66)	0.902
Hair product use	2.553 (2.65)	0.086

6.4.5 Associations between hair cortisol and psychosocial factors in each phase

Associations between the perceived stress, anxiety and hair cortisol levels were analysed per phase. For this, several hierarchical regression analyses were performed with the two psychosocial factors (as monthly average measure) as independent variables and hair cortisol concentration as the outcome variables, respectively, without and with adjustments for age, BMI, smoking, hair treatment and student group. Experience of specific stressors (as a sum score) in relation to hair cortisol levels was assessed with regression analysis and the experience of a negative life event was assessed using ANOVA/ ANCOVA in each phase, using unadjusted and adjusted models, respectively.

Phase 1 (low stress)

There were no associations between hair cortisol and perceived stress (β = 0.003, C.I. -0.030/ 0.035, *p* = 0.877) and between hair cortisol and anxiety (β = 0.003, C.I. -0.024/ 0.017, *p* = 0.751). Specific stressors were not related to hair cortisol (β = 0.037, C.I. -0.026/ 0.100, *p* = 0.247). The experience of a negative life event did not impact on hair cortisol levels ($F_{1, 67}$ = 0.222, *p* = 0.639). Adjustment for the covariates did not change these results.

Phase 2 (high stress)

In Phase 2, there were also no associations between hair cortisol and perceived stress (β = -0.002, C.I. -0.038/ 0.032, *p* = 0.892) or anxiety (β = 0.001, C.I. -0.017/ 0.018, *p* = 0.931). Specific stressors did not associate with hair cortisol (β = -0.051, C.I. -0.106/ 0.003, *p* = 0.062). The experience of a negative life event did not impact on hair cortisol levels ($F_{1, 68}$ = 0.521, *p* = 0.473). Adjustment for the covariates did not change these results.

6.4.6 Associations between salivary cortisol and psychosocial factors in each phase

Further, associations between perceived stress, anxiety and the different salivary cortisol parameters were analysed per phase. For this, several hierarchical regression analyses were performed with the three psychosocial factors (the monthly average measures) as independent variables and the three salivary cortisol parameters (the monthly average measures) as the outcome variables, respectively, without and with adjustment for student group and also age, BMI and smoking.

To enable analyses of perceived stress and anxiety in relation to salivary cortisol output throughout each phase, and therefore to account for the fluctuations in the psychosocial factors within each phase, the time points where the two psychosocial factors were highest were assessed in relation to the three salivary cortisol parameters at the respective time point. Hierarchical regression analyses were performed with the respective psychosocial factor as the independent variable and the three respective salivary cortisol parameters as the outcome variables.

Phase 1 (low stress)

There was a significant association between perceived stress and the slope (β = 0.027, C.I. 0.003/ 0.052, *p* = 0.030), which withstood adjustment for age, BMI, smoking and student group (β = 0.026, C.I. 0.001/ 0.050, *p* = 0.039); higher levels of perceived stress were associated with a steeper slope. There were no associations between perceived stress and the CAR (β = 0.135, C.I. -0.274/ 0.545, *p* = 0.512) or the AUC (β = 0.004, C.I. -0.011/ 0.020, *p* = 0.592). There was a significant association between anxiety levels and the slope (β = 0.020, C.I. 0.004/ 0.035, *p* = 0.012), which withstood adjustment for age, BMI, smoking and student group (β = 0.017, C.I. 0.001/ 0.033, *p* = 0.033); higher levels of anxiety were associated with a steeper slope. There were no associations between anxiety and the CAR (β = -0.109, C.I. -0.368/ 0.150, *p* = 0.405) or the AUC (β = 0.002, C.I. -0.008/ 0.012, *p* = 0.717).

There were no associations between the highest time point of perceived stress and the respective CAR (β = -0.062, C.I. -0.675/ 0.551, *p* = 0.841), the respective AUC (β = 0.008, C.I. -0.012/ 0.028, *p* = 0.414) or the respective slope (β =

0.029, C.I. -0.005/ 0.063, *p* = 0.090). There was a significant association between the highest time point of anxiety and the respective slope (β = 0.027, C.I.-0.012/ 0.041, *p* = 0.001), which withstood adjustment for age, BMI, smoking and student group (β = 0.026, C.I. 0.010/ 0.043, *p* = 0.002); higher levels of highest time point anxiety were associated with a steeper slope. There were no associations between the highest time point of anxiety and the respective CAR (β = -0.018, C.I. -0.311/ 0.275, *p* = 0.904) or the respective AUC (β = 0.004, C.I. -0.005/ 0.014, *p* = 0.386).

Phase 2 (high stress)

In Phase 2, there was a significant association between perceived stress and the slope (β = -0.024, C.I. -0.048/ -0.001, *p* = 0.044); higher levels of perceived stress were associated with a flatter slope. This, however, became non-significant after adjustment for student group and non-significant when also adjusting for age, BMI, smoking (β = -0.019, C.I. -0.044/ 0.005, *p* = 0.123). There were no associations between perceived stress and the CAR (β = 0.000, C.I. -0.486/ 0.487, *p* = 0.999) or the AUC (β = -0.002, C.I. -0.021/ 0.016, *p* = 0.799). There were no significant associations between anxiety levels and the slope (β = -0.010, C.I. -0.022/ 0.002, *p* = 0.093), the CAR (β = 0.003, C.I. -0.247/ 0.247, *p* = 1.000) or the AUC (β = -0.002, C.I. -0.011/ 0.007, *p* = 0.694).

There were no associations between the highest time point of perceived stress and the respective CAR (β = -0.299, C.I. -0.883/ 0.286, *p* = 0.310), the respective AUC (β = -0.023, C.I. -0.047/ 0.001, *p* = 0.063) or the respective slope (β = -0.024, C.I. -0.059/ 0.010, *p* = 0.160). Highest time point of anxiety was not related

to the respective CAR (β = -0.111, C.I. -0.541/ 0.319, *p* = 0.605) or the respective AUC (β = 0.008, C.I. -0.003/ 0.019, *p* = 0.164) or the respective slope (β = -0.010, C.I. -0.030/ 0.010, *p* = 0.310).

6.4.7 Coping and its association with cortisol in each phase

Table 6.11 shows the descriptive statistics of the coping mechanisms of the combined sample and per student group. There were no significant differences in problem coping, avoidant coping or support coping at either phase between the two student groups (*p*'s > 0.575).

Table 6.11 Mean values for the different coping mechanisms in the combined sample and per student group in both phases.

Coping mechanism	Combined sample	Student group		Group differences p-value
	N = 70	Medical students (N = 51)	Law students (N = 19)	
	Mean (SD)	Mean (SD)	Mean (SD)	
Phase 1				
Problem-focused coping	16.87 (3.51)	16.96 (3.73)	16.61 (2.86)	0.719
Avoidant coping	20.61 (4.34)	20.64 (4.29)	20.53 (4.58)	0.927
Social support coping	10.52 (2.37)	10.54 (2.50)	10.46 (2.03)	0.904
Phase 2				
Problem-focused coping	16.96 (3.67)	17.11 (3.73)	16.56 (3.57)	0.581
Avoidant coping	21.22 (4.60)	21.18 (4.30)	21.34 (5.44)	0.894
Social support coping	10.59 (2.59)	10.69 (2.74)	10.30 (2.16)	0.575

Associations between coping and the different cortisol parameters were analysed per phase. For this, several hierarchical regression analyses were performed with the respective coping mechanism (problem-focused coping, avoidant coping and support coping as the monthly average measure) as the independent variable and the respective cortisol parameters (hair cortisol and the three salivary cortisol monthly average measure) as the outcome variable, without and with adjustment for age, BMI, smoking and student group (and hair treatment for hair cortisol analyses).

To assess whether dominant coping mechanisms were related to cortisol (hair cortisol and the three salivary cortisol parameters), in each phase participants were allocated to a coping mechanism group which reflected the style they most frequently attributed to (i.e. problem, avoidant or support coping). This computation allows for exploring whether a particular dominant coping mechanism style rather than the scope of different coping mechanisms are related to cortisol. ANOVAs/ANCOVAs with the cortisol parameter as the dependant variable and coping style as the independent variable were run, using both unadjusted and adjusted models.

Table 6.12 depicts the frequency in each phase of the most reported coping mechanism. Avoidant coping was the most predominantly reported and support coping was the least used. In Phase 1 no individual predominantly reported support coping; in Phase 2 just one participant predominantly reported support coping which was made it unsuitable for ANOVAs/ANCOVAs and was therefore removed

prior to analyses. Table 6.12 Frequency of the dominant coping mechanism per phase.

Coping mechanism	Frequency (%)
Phase 1	
Problem-focused coping	16 (23.2)
Avoidant coping	53 (76.8)
Social support coping	0 (0)
Phase 2	
Problem-focused coping	12 (17.1)
Avoidant coping	57 (81.4)
Social support coping	1 (1.4)

Phase 1 (low stress)

There was no association between hair cortisol and averaged problem-focused coping (β = -0.006, C.I. -0.057/ 0.045, p = 0.809), avoidant coping (β = -0.003, C.I. -0.045/ 0.038, p = 0.879) or support coping (β = 0.050, C.I. -0.025/ 0.125, p = 0.185) in Phase 1. However, ANOVA revealed that individuals who predominantly used avoidant coping approaches had higher hair cortisol levels [M = 2.61 ln(pg/ng), SD = 0.77] than individuals who predominantly used problem-focused coping approaches [M = 2.11 ln(pg/ng), SD = 0.48; $F_{1, 67}$ = 6.043, p = 0.017 unadjusted] which withstood adjustment for the covariates age, BMI, smoking status, hair treatment and student group ($F_{1, 61}$ = 2.074, p = 0.047). This also withstood adjustment for perceived stress and anxiety. Figure 6.4 depicts the association between dominant coping style and hair cortisol in Phase 1.

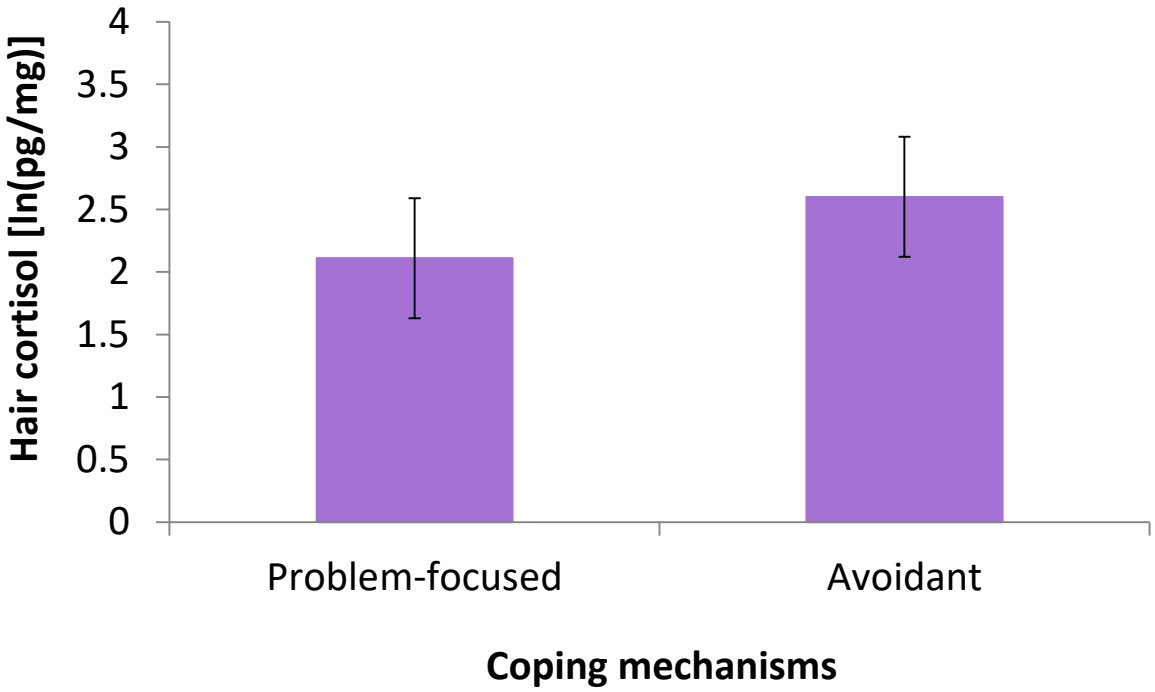

Figure 6.4 Hair cortisol concentration and dominant coping style in Phase 1, indicating that individuals who reported predominantly using avoidant coping approaches had higher hair cortisol levels than individuals who reported predominantly using problem-focused coping approaches.

Regression analyses revealed that there was a marginal significant association between averaged problem-focused coping and the CAR (β = -0.640, C.I. -1.283/ 0.003, *p* = 0.051); lower average engagement in problem-focused coping was associated with a higher average CAR. This withstood adjustment for the covariates age, BMI, smoking status and student group (β = -0.645, C.I. -1.315/ 0.025, *p* = 0.059). There was no association between average problem-focused coping and the AUC (β = -0.011, C.I. -0.036/ 0.013, *p* = 0.365) or the slope (β = -0.034, C.I. -0.074/ 0.005, *p* = 0.088). There was no association between average avoidant coping and the CAR (β = -0.008, C.I. -0.558/ 0.541, *p* = 0.975), the AUC (β = -0.004, C.I. -0.024/ 0.016, *p* = 0.663) or the slope (β = 0.003, C.I. -0.029/ 0.036, *p* =

0.835). There was no association between average support coping and the CAR (β = -0.260, C.I. -1.257/ 0.738, *p* = 0.605), the AUC (β = 0.019, C.I. -0.017/ 0.056, *p* = 0.287) or the slope (β = 0.036, C.I. -0.023/ 0.096, *p* = 0.223); adjustment for covariates did not change these results.

Regarding the dominant coping styles, ANOVA revealed a marginally significant association between coping and the AUC ($F_{1, 66}$ = 3.813, *p* = 0.055). Individuals who reported predominantly using avoidant coping approaches had higher AUC levels [M = 8.84 ln(nmol/l), SD = 0.36] than individuals who reported predominantly using problem-focused coping approaches [M = 8.65 ln(nmol/l), SD = 0.29; Figure 6.5]. There was a tendency for individuals who reported predominantly using avoidant coping approaches to have higher CAR levels [M = 11.89 nmol/l, SD = 9.73] than individuals who reported predominantly using problem-focused coping approaches [M = 7.39 nmol/l, SD = 7.61; ($F_{1, 64}$ = 2.856, *p* = 0.096]. There was no significant association between coping and the slope ($F_{1, 67}$ = 1.402, *p* = 0.241).

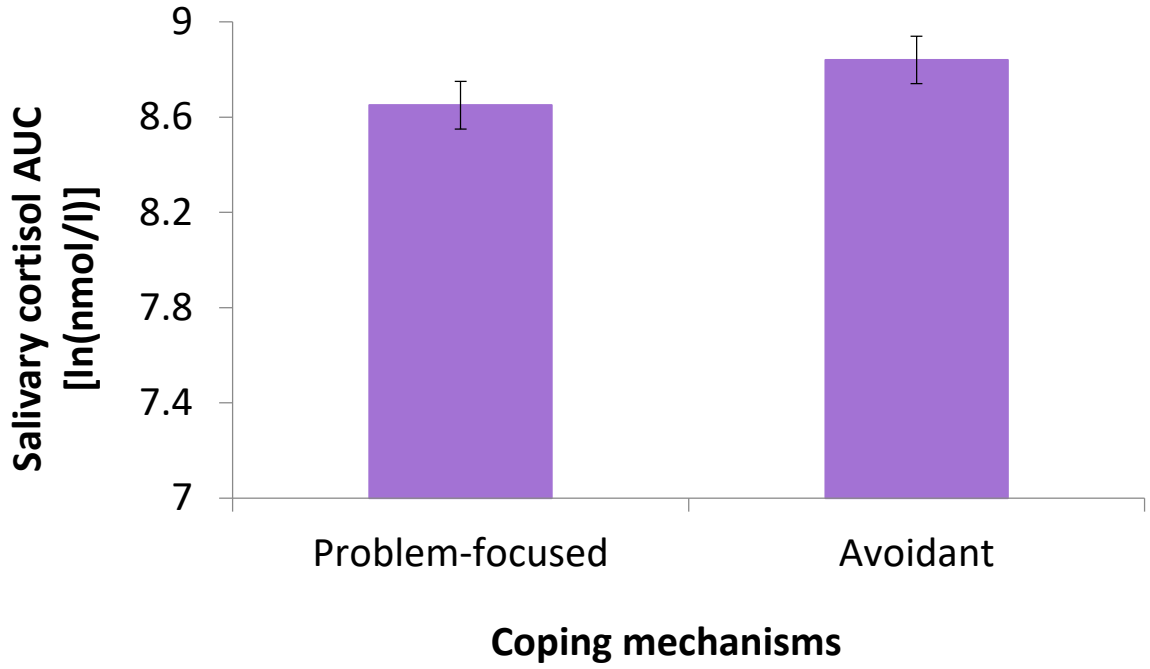

Figure 6.5 Salivary cortisol AUC and dominant coping style in Phase 1 (marginally significant association), indicating that individuals who reported predominantly using avoidant coping approaches had higher salivary cortisol AUC levels than individuals who reported predominantly using problem-focused coping approaches.

Phase 2 (high stress)

In Phase 2, there was also no association between hair cortisol and average problem-focused coping (β = -0.037, C.I. -0.084/ 0.010, *p* = 0.120), avoidant coping (β = -0.004, C.I. -0.043/ 0.034, *p* = 0.816) or support coping (β = 0.050, C.I. -0.016/ 0.117, *p* = 0.137).

Regression analyses revealed that there was a significant association between average problem-focused coping and the AUC (β = -0.030, C.I. -0.054/ -0.006, *p* = 0.014; lower average engagement in problem-focused coping was associated with a higher average AUC. This withstood adjustment of the covariates age, BMI, smoking status and student group (β = -0.024, C.I. -0.048/ 0.000, *p* =

0.046). There was also a significant association between average problem-focused coping and the slope (β = -0.036, C.I. -0.068/ -0.005, *p* = 0.025); lower average engagement in problem-focused coping was associated with a steeper average slope, which withstood adjustment of the covariates (β = -0.037, C.I. -0.069/ -0.004, *p* = 0.028). There was no association between average problem-focused coping and the CAR (β = -0.191, C.I. -0.865/ 0.482, *p* = 0.573). There was no association between average avoidant coping and the CAR (β = -0.374, C.I. -0.903/ 0.155, *p* = 0.163), the AUC (β = -0.012, C.I. -0.031/ 0.008, *p* = 0.244) or the slope (β = 0.019, C.I. -0.045/ 0.007, *p* = 0.142). There was no association between average support coping and the CAR (β = -0.333, C.I. -1.265/ 0.498, *p* = 0.477), the AUC (β = -0.008, C.I. -0.043/ 0.026, *p* = 0.634) or the slope (β = -0.029, C.I. -0.075/ 0.017, *p* = 0.209); adjustment for covariates did not change these results.

ANOVAs/ANCOVAs showed that dominant coping style was not related to hair cortisol levels ($F_{1, 67}$ = 1.459, *p* = 0.231). There were no significant associations between dominant coping and the CAR ($F_{1, 63}$ = 0.149, *p* = 0.701), the AUC ($F_{1, 65}$ = 0.206, *p* = 0.651) or the slope ($F_{1, 67}$ = 1.684, *p* = 0.199). Adjustment for the covariates did not change these results. Table 6.13 summarises the associations between the different cortisol measures and the coping styles in each phase.

Table 6.13 Summary table of findings: coping mechanism and cortisol per phase.

Coping mechanism	Problem-focused coping (PFC)	Avoidant coping (AC)	Social support coping (SSC)	Dominant coping (AC vs. PFC)
Phase 1				
Hair cortisol [ln(pg/ng)]	n.s.	n.s.	n.s.	↑ in ↑ AC*
CAR (nmol/l)	inverse ass.*	n.s.	n.s.	↑ in ↑ AC
AUC [ln(nmol/l)]	n.s.	n.s.	n.s.	↑ in ↑ AC^{T}
Slope (nmol/l/h)	n.s.	n.s.	n.s.	n.s.
Phase 2				
Hair cortisol [ln(pg/ng)]	n.s.	n.s.	n.s.	n.s.
CAR (nmol/l)	n.s.	n.s.	n.s.	n.s.
AUC [ln(nmol/l)]	inverse ass.*	n.s.	n.s.	n.s.
Slope (nmol/l/h)	inverse ass.*	n.s.	n.s.	n.s.

*$p < 0.05$ Tp = marginally significant
inverse ass. = inverse association

6.5 Discussion

Hypotheses: i) hair cortisol content would be higher in the period under high academic stress compared to the period under average academic stress, ii) psychosocial factors would be positively associated with salivary cortisol and hair cortisol concentration, iii) adaptive coping responses would be associated with lower hair and salivary cortisol levels compared with maladaptive coping styles, or would mediate the impact of perceived stress/ anxiety on cortisol.

6.5.1 Summary of findings

The present study investigated the impact of academic stress on cortisol in two tissues, hair and salivary cortisol by comparing cortisol and psychosocial factors between two phases with varying degrees of naturalistic stress exposure. The analyses revealed that students responded differently towards the stressor, with medical students showing a non-significant tendency for an increase in hair cortisol levels when facing examination, whilst law students showed a non-significant tendency for a decrease in hair cortisol (Figure 6.2). Further, only law students had a significantly flatter slope in the examination phase compared with the low stress period (Figure 6.3). The naturalistic stressor was generating substantial increases in stress and anxiety, in both student groups, which could be attributed to actual university-related pressure rather than outside sources of stress. No associations between hair cortisol and stress and anxiety or other life events could be found; however higher levels of perceived stress (and a tendency for higher levels of anxiety) were associated with a flatter slope in the academic stress period.

Predominantly using avoidant coping approaches was associated with elevated hair cortisol in the low academic stress phase. Further, lower engagement of problem-focused coping and/ or higher engagement in avoidant coping approaches was associated with aberrant salivary cortisol levels, higher CAR and AUC values and flatter slopes, more consistently in the low academic stress phase than the high academic stress phase.

6.5.2 Interpretation of findings and links to previous work

Hair cortisol concentration differed between the student groups and somewhat between the phases. Medical students showed a tendency for an increase in hair cortisol levels when facing examination and law students showed a tendency for a decrease in hair cortisol. Hair cortisol levels were significantly different between the student groups at Phase 1, with law student showing higher levels of hair cortisol, but not at Phase 2. The interaction (and differential pattern that did not reach significance level) therefore seem to be mainly driven due to the differences in the low academic stress period (Phase 1) rather than the high academic stress period (Phase 2). It is uncertain why the student groups differed in their baseline hair cortisol levels. The student groups differed in anxiety; law students reported higher levels or anxiety in both the low academic stress period and the high academic stress period. Whilst this difference would be congruent with the observed difference in hair cortisol concentration and salivary AUC levels (higher concentration in law students), the decrease in hair cortisol levels in law students in the examination phase is puzzling given an increase in anxiety and stress

levels. Also only in law students the salivary slope was flatter in the examination phase compared to baseline; medical students' salivary slope values did not differ between the phases.

There might be degree-related differences in the student groups that have generated this differential pattern. It is possible that academic commitments or pressures were present in law students that were not considered. Likewise, the difference in perceived anxiety between medical and law students might be due to personality differences. Yet, there were no observed differences in coping mechanisms between the two student groups. It might be the case that other differences in coping not captured by the measure or other not assessed personality aspects lead to differences in biological responses. One obvious difference between groups is that medical students are more familiar with human experimental studies (since they often have to test each other's biological responses). They may have been more relaxed at baseline, which might have generated between-group differences.

The inconsistencies in the literature on whether examination successfully generates observable changes in cortisol might also lead to other explanations. In the present study law students showed a decrease in hair cortisol. It is possible that a physiological pattern of adaptation or habituation to the stressor takes place in some individuals. A meta-analysis reviewing 208 laboratory-based studies has established that cortisol reactivity as well as recovery time upon stress exposure depends on several factors, such as novelty, uncontrollability and social-evaluative threat (Dickerson & Kemeny, 2004). It might be that for some individuals the

examinations were not perceived as novel but rather as expected, predictable and well-known. It would be interesting to compare the sorts and familiarity of exams that law students sit in relation to medicine students. Similarly, academic stressors involving social-evaluative threat elements such as in oral examinations or presentations, including anticipation of such events, might elicit substantial increases in cortisol concentration, whilst written examination might generate a smaller reactivity. However, this is merely a possibility and has not been tested in a naturalistic environment. It would be of interest to investigate whether these aspects show differential psychobiological response patterns.

Stowell (2003) proposed recommendations for using academic examinations as a source of stress in psychoneuroendocrinological research (focusing on traditional cortisol specimens); examination difficulty, timing of repeated assessments and incorporation of analyses of interindividual variability emerged as vital aspects to reliably assess the impact of academic stress on students. A study by Malarkey et al. (1995) highlighted the need for evaluations of between-group differences. The authors assessed psychological stress and ACTH and cortisol in medical students and did not find that mean serum cortisol levels differed between the pre-, peri- and post-examination periods overall. However, they performed further sub-group analyses dividing students into whether they followed the expected incremental and decremental pattern of perceived stress or not (pre- to peri- and peri- to post-perceived stress values). For students that followed the expected pattern serum cortisol levels significantly increased from the pre- to the peri-examination period. This suggests that not only the nature of the stressor

(examination phase) but also perception of the stressor (perceived stress) is driving cortisol responses. In the present study, only a minor number of students had a slight decrease in perceived stress from baseline to examination phase (N = 6), therefore, these sub-analyses could not be performed. Overall, there was an increase in perceived stress and anxiety levels in the examination phase, suggesting that it effectively induced this change. However, it is unclear whether this increase in stress levels and anxiety was really big enough to induce changes in biology. Much of the research on academic examination stress was carried out some years ago; stress of contemporary academic examinations may be much lower than it was 10-20 years ago as the procedures for academic assessment have changed markedly over this time. Perhaps earlier biological stress studies have stimulated much larger differences.

Hair cortisol was unrelated to stress and anxiety levels. Perception of stress and anxiety was assessed at three time points throughout a five-week period, reflecting the period evaluated from the 1cm hair segment. These levels were averaged and in this were thought to provide a more robust measure of stress and anxiety. Having only limited assessments of perceived stress, anxiety and respective coping hinders the assessment of the complex transaction occurring between individuals and the stressors. However, it is possible that more repeat assessments of momentary or state perceptions similar to the Ecological Momentary Assessment (EMA) method are required to provide enough variability for an association to emerge. Specific stressors, an aggregate of stress due to family, financial issues, feeling fatigued, neighbourhood/ environment and lack of

organisation, did not associate with hair cortisol. The experience of a negative life event [Phase 1: Yes N = 22 (40.6%) and Phase 2: Yes N = 17 (57.1%)] also did not show any relationship with hair cortisol at either phase. Nevertheless, it might have been valuable to know the sources of negative life events and whether level of impact and seriousness might even out potential associations.

The findings on perceived stress or stressful life events and hair cortisol are inconsistent, as explored in Chapter 2. While the effects of major adverse life events and stressful conditions on cortisol secretion seem to be noticeable in hair in certain stress-exposed groups, the findings for self-reported stress and hair cortisol are less consistent (Grassi-Oliveira et al., 2012; Meyer & Novak, 2012; Staufenbiel et al., 2014; Staufenbiel et al., 2013; Wells et al., 2014). A stronger relationship has repeatedly emerged in clinical settings, whilst the relationship in healthy individuals is rather unclear. Karlen et al. (2011) studied life events and perceived stress in relation to hair cortisol in 99 university students. While the experience of stressful life events was related to elevated hair cortisol, perceived stress was negatively associated with hair cortisol. However, the authors assessed stress perception (three months perceived stress using the PSS) and hair cortisol levels (measured in a 3cm hair segment) concurrently, which might introduce bias. Yet, the authors report that when including life events, perceived stress and perceived health in a regression model, perceived stress was no longer significant whilst life events significantly predicted hair cortisol.

Interestingly, in the present study, levels of stress and anxiety fluctuated in the examination phase and seemed to be highest towards the end of the period.

This fluctuation was not observed at low academic stress. Examinations were mainly taking place throughout the weeks between the third to the fifth weeks (Time 2 - Time 3). Students reported their levels of stress, anxiety and coping only at 2-weeks intervals; it would have been interesting to record actual timings of examinations taking place with real-time stress- and anxiety-related factors throughout this period rather than retrospective evaluation. No significant fluctuation in salivary cortisol throughout each phase could be observed, although mean values seemed to show higher variability in the high stress period compared to the low stress period. It is plausible that the fluctuations in the psychosocial measures could not be captured in the limited assessment of cortisol and more repeated assessments would have yielded different results.

An important aspect that has not been considered is students' differences in perceived significance of their exams. Causes of student distress that seem to be involved in stress regulation and HPA-axis responses include time pressures and students' perceptions of the significance or impact of exams for their professional development (Murphy et al., 2010). Likewise, preparation for examinations and expectations might moderate potential differences and even-out possible associations. Whilst changes in perceived stress and anxiety levels were observable, perceived importance might have generated intraindividual differences in these psychosocial factors, in coping and hence also in cortisol secretion. In fact, a critical review of the literature around students' stress concluded that most researchers focus on quantitative approaches (Robotham & Julian, 2006). Individuals stress experiences in the student and academic environment might need to be explored in

a more thorough way to properly understand what aspects need to be considered when evaluating its impact on physiological and psychological health.

Stress and anxiety were related to salivary cortisol measures. Higher levels of perceived stress (and a tendency for higher levels of anxiety) were associated with a flatter slope in the academic stress period; this, however, did not withstand adjustment for student group. This might be due to between-group differences in the slope and the psychosocial measures. Conversely, at baseline higher levels of perceived stress and also anxiety (including highest time point of anxiety throughout the phase) were associated with a steeper slope, which is puzzling.

The findings revealed that predominantly using avoidant coping approaches was associated with elevated hair cortisol in the low academic stress phase, which withstood adjustment for the covariates and also perceived stress and anxiety. This association did not emerge in the high academic stress phase. Further, lower engagement in problem-focused coping and/or higher engagement in avoidant coping approaches was associated with aberrant salivary cortisol levels, namely higher CAR and AUC values and flatter slopes. Again this was more consistent in the low academic stress phase. These findings are in line with previous studies that assessed coping in students facing academic stress in relation to cortisol output. Maladaptive coping behaviours were associated with elevated mean salivary cortisol levels in an early study by Hellhammer et al. (1985) and also with high CAR levels (Drake et al., 2016). There is a body of evidence that suggests coping to be a protective factor against stress and the present study found that adaptive coping

was related to both positive cortisol profiles as assessed from salivary and hair specimens.

The coping behaviours in this study were orthogonal, and not at opposite ends of a single spectrum. Although it was possible to identify the most frequently used strategies, each student had a combination of scores. What might be of interest is the relative balance of exam-specific adaptive and maladaptive coping strategies used by students. While the present study tried to look at dominant coping style used throughout each phase, coping in relation to exam-specific cognitive and behavioural aspects would provide more insight into the coping mechanisms used in that context. What would also be of interest is the distinction between dispositional coping style and situational coping response as only a limited amount of shared variance has been found between daily-reported coping mechanisms and retrospective recall (Smith, Leffingwell, & Ptacek, Sladek, 2015; Sladek et al., 2016; 1999).

6.5.3 Study strengths and limitations

The present study is novel in that it investigated the impact of a naturalistic stress (examination stress) in relation to hair cortisol in a repeated-measures design. This provided an opportunity for further validation of hair cortisol as a biomarker of psychological stress. In medical students an increase in hair cortisol was observed, pointing towards its potential in capturing the shift and elevation of stress caused by the exams, although effects were small. Examination phases, including the stressful study and revision period, might last several weeks rather

than a short period of time. As hair cortisol is a more reliable measure of long-term cortisol activity than the traditional sources for cortisol assessment, it might be suitable for use in examination phases with durations of a few weeks. In the present study, the inclusion of the different parameters of the cortisol profile in addition to the hair specimen allowed for comparison of the impact of stress in cortisol from different tissues. Psychological coping under stressful experiences has been under-researched in the hair cortisol literature and this study adds value with the findings that avoidant coping approaches are associated with elevated hair cortisol levels under low-stress conditions.

Several limitations need to be acknowledged in the present study. Perhaps most importantly, the key exposure measure (examination stress) may not have been sufficiently intense. Although differences between phases were recorded in perceived stress and anxiety, they may not have been great enough to stimulate clear-cut differences in cortisol output. As noted earlier, changes in the significance of examinations in student assessments may have undermined the study design. Widely-used and established inventories were utilised in the present study (i.e. the PSS, STAI and the COPE). Nevertheless, given the context, the measures used may not have accurately reflected the sources of student-related stress. Future research might investigate other academic stress and degree-related aspects, potentially drawing on familial, social, cultural and ethnic factors to incorporate different assessments of beliefs and expectations in the academic environment.

There is a plethora of instruments that assess academic-related sources of stress. For example the Academic Expectations Stress Inventory (AESI; Ang & Huan,

2006) assesses expectations by different entities (parents, society, self) as a source of academic stress. The Academic Stress Scale (ASS; Kohn & Frazer, 1986), the Higher Education Stress Inventory (HESI; Dahlin et al., 2005) and the Perceived Medical Student Stress Scale (PMSSS; Vitaliano, Russo, Carr, & Heerwagen, 1984) assess general stress relating to demanding situations in the academic environment. Although the PSS is a validated inventory, and has been often applied in examination stress studies, the academic-related stress inventories might have been more suitable in this context.

With regards to anxiety, the present study used the Spielberger State-Trait Anxiety Inventory (Marteau & Bekker, 1992; STAI; Spielberger, Gorsuch, Lushene, Vagg, & Jacobs, 1983) which is an established questionnaire to differentially assess trait (chronic) anxiety and state (temporary) anxiety and has been majorly used in psychoneuroendocrinological research (Boudarene, Legros, & Timsit-Berthier, 2002; Weekes et al., 2006a). Another self-report scale, also constructed by Spielberger, is the Test Anxiety Inventory (TAI; Spielberger & Gonzalez, 1980; Taylor & Deane, 2002), a widely used tool in research that assesses anxiety specifically in relation to academic exams and these anxiety subscales have been distinctly associated with coping strategies (Chapell et al., 2005; Stober, 2004). Fear of failure or over-ambition generate higher levels of anxiety when facing examinations (Alam, 2013). As this scale differentiates between different aspects of anxiety, with the subscales worry, emotionality, interference and lack of confidence, it might have provided a more comprehensive and reliable reflection of anxiety confronting the aversive occurrence of examinations.

Likewise, there are several exam-specific coping strategy inventories. For instance, the Strategic Approach to Coping Scale (GSACS-Exam/ SACS; Buchwald & Schwarzer, 2003) incorporates sub-constructs such as individual/ communal, prosocial/ antisocial, supportive/ delegated dyadic coping efforts. The Differential Performance Anxiety Inventory (DAI; Rost & Schermer, 1997) evaluates coping in terms of threat control through productive study behaviours, distraction and trivialization, relaxation techniques (after identification of physiological arousal) and avoidance and deception. The Coping with Pre-Exam Anxiety and Uncertainty (COPEAU; Stober, 2004) is an adaptation of the DAI and the COPE and therefore contains relevant subscales specifically adjusted to the context of examinations. Again, none of these measures are as well validated and widely used as the COPE measure by Carver et al. (1997) but might have given a richer assessment of students' exam and degree-specific interpersonal resources.

The present study followed the suggestions by Skinner et al. (2003) and Carver et al. (Carver, 1997) to run exploratory factor analysis for the coping measure with subsequent rational sorting of items into factors in each separate study sample. As two phases of varying degrees of stress were employed (potentially generating engagement in different coping mechanisms), the factor analysis with all sub-scales included many cross-loadings and a mixed pattern of factor extraction. The exclusion of certain sub-scales based on low variability and high cross-loadings yielded a cleaner factor loading pattern across both phases (with similar factor loadings). Three factors were retained termed problem-focused coping, avoidant coping and social support coping, following the broad coping style

classifications. Yet, it might be that a differential way of extraction and aggregation would have been more appropriate such as combining the coping styles across the two phases or solely focusing on the low academic stress phase (Phase 1). Further, the computation method of dominant coping mechanism style might have been too simplistic and there might be more advanced analytical procedures that could explore the association between coping and cortisol. Avoidant coping was the most reported dominant coping strategy and compared to problem-focused coping, avoidant coping was associated with elevated hair cortisol, a higher AUC and CAR in Phase 1 (during low academic stress), which is in line with previous findings. However, given the limitation of this computation method, results should be interpreted with caution and require further exploration.Two sets of student groups were incorporated due to recruitment difficulties. This induced inter-individual differences and might have introduced bias. It is possible that academic commitments and pressures were different between the students groups that were not considered. Medical and law degrees differn in length of the course and have different types of assessment – factors that might contribute to between-group differences. Limiting the sample to one student group would have minimised potential confounding factors. Further, no data were available on academic performance. Given the association between stress and performance, it might have been interesting to explore the impact of stress, HPA-axis functioning (incorporating both salivary and hair cortisol measures) and academic performance.

Another limitation relates to the timings of cortisol assessment and the calculation of the different profiles. Due to the sampling strategy, salivary cortisol

was assessed over a 24-hour period split over two days. The first sampling point was at 11am with two further sampling points on the same day, following two morning samples after awakening the next day. This was intended to allow students to become accustomed to the sampling procedure and to ensure appropriate morning samples; however, this strategy had a negative impact on the calculation of the diurnal cortisol profile. The calculations for the AUC were calculated based on the 11am to bedtime values. Most AUC measures include the waking sample which contain the much higher values present early in the day. Therefore the parameter of the AUC might not fully reflect cortisol output over the day. This issue relates also to the calculation of the diurnal slope which was calculated for the specified day period (11am until bedtime) excluding any values prior to 11am, limiting its value to reliably yield the decline throughout the whole day. Despite these limitations most results relating to the salivary cortisol parameters are in line with previous findings and were able to capture associations with the psychosocial factors.

A study exploring the seasonal effect on hair growth found that there is faster hair growth in the summer months compared to the winter months (Randall & Ebling, 1991). Although both phases of the study took place in Autumn/Winter and Spring, respectively, seasonal effects might have contributed to a difference in cortisol concentration. If in Spring hair growth rates increase (preceding the summer months) this would imply cortisol concentration in the hair segment may be less 'concentrated' and not cover the full month's cortisol exposure, generating variability in the analysed hair segments. The analytical procedure for hair cortisol

analysis is based on the weight of hair supplied and not on the absolute amount of hair (or of cortisol). It would therefore be important to understand whether pattern of hair growth and what other factors impact actual hair weight. Interestingly, it has been shown that there are racial differences in hair weight (based on evidence from very early studies as the area of hair weight is underresearched; e.g. Bernstein & Robertson, 1927; Kneberg, 1936), which might partly explain the differences in hair cortisol depending on ethnicity (not considering other socioeconomic or stress-related factors that minority groups might experience) (O'Brien et al., 2013; Schreier, Enlow, Ritz, Gennings, & Wright, 2015). In the present study hair cortisol concentration was not associated with ethnicity; however, there the sample predominantly included white (59%) and Asian (33%) students and only a minor percentage (8%) having a mixed or black ethnic background. These hair-analytical methodological aspects still need to be explored in more detail before assumptions and recommendations can be drawn to what factors need to be considered when using hair cortisol as markers in longitudinal designs.

Future research might include female and male students. A female sample was selected to avoid sex differences, increase power and to facilitate hair collection. An issue in a female sample is the possible effects of menstrual cycle and use of contraceptives on biological measures. While there have been inconsistencies in the literature based on traditional cortisol specimens as to whether menstrual cycle phase and oral contraceptives exert influences on HPA-axis functioning and reactivity (Boisseau et al., 2013; Kirschbaum et al., 1999; Liening, Stanton, Saini, & Schultheiss, 2010) most hair cortisol studies did not find

any association with oral contraceptives (Dettenborn, Tietze, et al., 2012; Stalder, Steudte, Alexander, et al., 2012) and menstrual cycle has not been assessed as a potential confounding factor. Nevertheless, analysing a segment of hair that reflects one months prior to sampling would entail that all female participants have undergone all of the distinct phases throughout this time, i.e. the follicular, the luteal (premenstrual) and the menstrual phase.

It would be of interest to see whether male and female students showed a differential response in hair cortisol, given the evidence from previous stress research using traditional specimens. It has been repeatedly reported that female students seem to be more stressed than male students (Dahlin et al., 2005; Pierceall & Keim, 2007), with higher feelings of discomfort and failure (Frankenhaeuser et al., 1978). A study, evaluating methodological issues with respect to using academic examination as sources for stress in psychoneuroendocrinological studies, reported sex differences in biological responses towards stress (Weekes et al., 2006a). In their study with 57 undergraduates (33 males, 34 females) only males had elevated salivary cortisol levels in the examination stress phase. Although this study compared a relatively low academic stress period to a high stress period, the latter period included either examinations or assignment deadlines. The inclusion of assignment deadlines rather than limiting these to examinations might have generated differences in levels of stress in the student groups. Yet, this is not the only study that reports sex differences in examination stress related biological responses, with stronger elevations in cortisol in males compared to females (Frankenhaeuser et al., 1978).

Future studies are warranted that incorporate hair cortisol analyses to corroborate these gender-specific effects.

6.6 Chapter summary

This chapter explored whether academic examinations would generate observable changes in hair cortisol. Two student groups (medical and law students) were included. The student groups differed slightly in their hair cortisol levels at baseline and also in levels of anxiety, impeding proper conclusive findings. Compared to the low stress period, hair cortisol levels were slightly higher (not reaching significance) in medical students only and the diurnal cortisol slope was flatter in law students only in the stress period, endorsing the role of academic stress as a potential precursor to aberrant HPA-axis functioning. Perceived stress and anxiety were not related to hair cortisol but they were to salivary cortisol. The findings on perceived stress or stressful life events and hair cortisol are somewhat inconsistent and the present study did not add clarity to the existing literature. There is a body of evidence that suggests coping to be a protective factor against stress. In the present study the use of adaptive coping mechanisms was associated with lower hair cortisol levels, corroborating this line of evidence. Future studies are warranted that incorporate contextual and cultural factors in academic stress and coping.

CHAPTER 7. OVERALL DISCUSSION

7.1 Overview

This PhD thesis made use of a novel analytical method to investigate cortisol concentration, which is the quantification of cortisol from hair specimen. It incorporated three studies that tried to explore the validity of hair cortisol analyses from two different perspectives. The first perspective was to evaluate the associations between individual differences in relation to stress and hair cortisol and this was explored in two studies (Study I and Study III). One study (Study I) investigated the associations between hair cortisol concentration and socio-economic factors, work-related stress and social support in working women. A dataset of two time points four years apart enabled assessment of psychosocial stress factors cross-sectionally and as a dynamic entity. The other study (Study III) explored the impact of academic stress on hair cortisol (and also salivary cortisol) in undergraduate students by using data from two phases with varying degrees of academic stress. Core elements included perceived stress, anxiety and coping mechanisms to assess individual differences in relation to stress responses. The second perspective focused on the methodological aspects of hair cortisol concentration. Using three studies, both long-term and temporal associations (Study III) between salivary and hair cortisol were examined. Drawing from two independent studies with two time points four years (Study I) and eight years (Study II) apart, it was possible to explore the long-term consistency of cortisol in saliva and hair. Study I was based on a sample of middle-aged working women and

Study II drew on a larger population-based cohort, the English Longitudinal Study of Ageing (ELSA), which included both women and men.

All three studies were based on observational designs. Observational/ naturalistic studies are based on a method of investigation that allow the researcher to analyse the impact of stress on biological functioning without intervening in participants' daily lives (Steptoe, 2005). They provide practical means to assess physiological functioning in the individual's natural settings. Further advantages are that naturalistic designs make the study of the covariation between psychological experience and biology over time in real life environments possible. Similarly, these assessments are possible in situations of real relevance to people, such as during periods of work stress. A major drawback of naturalistic studies is that they often involve small sample sizes and therefore lack power to control for a wide range of possible confounding factors or covariates. Study III incorporated two student groups which produced intraindividual differences and although the initial sample size of 80 would have sufficed for the planned analyses, having had a greater number in the law student group (N = 19) would have strengthened possible separate analyses. It is also important to note that these were observational studies so cannot prove causation.

7.2 Main findings and their implications

7.2.1 Study I: Associations between hair cortisol, socio-economic, work-related and psychosocial factors

Study I (the Daytracker Study) was based on a longitudinal design in two contrasting cultures (London and Budapest). The baseline data were collected in 2007/08, investigating the associations between psychosocial factors and biological functioning in the work environment in a female sample. Diurnal salivary cortisol assessments and various psychosocial measures were taken. The follow-up data collection took place four years later (2012/13), in which psychosocial measures were re-assessed and hair was collected for hair cortisol analysis. The aim was to investigate the associations between hair cortisol concentration and socio-economic factors, work-related stress and social support. The analyses were performed using the variables in three distinct forms: concurrently assessed variables (at follow-up assessment), an aggregate score using the mean of the baseline and follow-up assessment, and a change score to assess the shift over time. This allowed cross-sectional analyses and also the evaluation of the dynamic process of the variables.

It was found that concurrent income was negatively associated with hair cortisol levels in a dose-response fashion. There was also a relationship between income change over the last four years and hair cortisol concentrations; a deterioration in income group was related to higher hair cortisol levels and an improvement in income group to lower hair cortisol. The two contrasting cultures showed remarkable differences in overall income (with higher income being

reported by participants based in London compared to participants based in Budapest) but did not differ in their average hair cortisol levels. The gradient relationship *within* the different levels of income and hair cortisol emerged in the whole sample after controlling for country, pointing to a strong relative income effect, but not an absolute income effect. Although there was no effect of education on hair cortisol levels per se, assessing education in relation to income group, status incongruity appeared to have an effect on hair cortisol. Negative status incongruity (lower income in relation to level of education) was related to higher hair cortisol levels. This may indicate that when the economic compensation does not correspond to the individual's intellectual skills and competencies then psychological strain and role conflict generates, leading to higher secretion of stress hormones. Financial strain and work stress factors as assessed with the Effort-Reward Imbalance Model were unrelated to hair cortisol; however, job effort, one subscale of the Effort-Reward Imbalance inventory, was associated with hair cortisol. In the higher income group only higher job effort was related to elevated hair cortisol compared to lower job effort. Social support was positively associated with hair cortisol.

The study's strengths lied in assessing income and working stress as dynamic processes, which seems a sensible approach to take in relation to a long-term cortisol biomarker. Despite the limitation that hair cortisol was not assessed at baseline (generating ambiguity as to whether the differences in hair cortisol in relation to income and status incongruity would have been present at baseline as well), the linear associations provide ground for future research in this area. It

would be interesting to replicate these findings in different samples assessing income and perception of financial hardship in more detail.

Further, drawing on two samples from different countries with remarkable differences in overall income, allowed for potential analyses of a relative income effect and an absolute income effect on hair cortisol. Although there were no observable differences in hair cortisol between the samples, these findings highlight the utility of hair cortisol analyses in cross-national designs with varying degrees of stress-related exposure. Epidemiological studies in particular might benefit from hair cortisol analyses as it limits the individual, methodological and situational factors involved in the analytical methods of the traditional cortisol specimens, i.e. measurement and sampling issues, adherence, stability issues and importantly, the impact of momentary psychosocial factors and lifestyle behaviours.

The hair cortisol literature has demonstrated that certain populations under adverse work conditions (such as shift work) were shown to have higher hair cortisol levels compared to individuals not exposed to these stressful conditions (Manenschijn, van Kruysbergen, et al., 2011). Only a few studies have investigated hair cortisol in relation to work-related stress in healthy samples and evidence for a relationship has not been strong (Qi et al., 2014; Stalder, Steudte, Alexander, et al., 2012). Work stress and also socioeconomic factors have repeatedly been linked to worse health outcomes, e.g. cardiovascular disease, the metabolic syndrome and diabetes and higher mortality rates (Chandola et al., 2006; Heraclides et al., 2012; Kivimaki et al., 2012). A body of evidence suggest that stress-related cortisol responses, based on traditional cortisol specimens, seem to underlie these

relationships (Chida & Steptoe, 2009; Dahlgren et al., 2005; Kunz-Ebrecht et al., 2004; Lupien et al., 2001).

There was no evidence for an association between work stress and hair cortisol. Despite this lack of association, it is possible that certain work-related factors in certain sub-groups or population groups might be related to worse HPA-axis functioning. The change or accumulation of work-related stress was thought to add a dynamic feature to these factors in this study. Nevertheless, work stress can be regarded as a dynamic process which does not necessarily translate into HPA-axis alterations that can be captured in a long-term cortisol biomarker. Repeated measurements assessing these stress-related fluctuations and the simultaneous assessment of salivary and hair cortisol measures might be informative to investigate this possibility.

7.2.2 Study I and II: Long-term consistency of cortisol - longitudinal associations between salivary and hair cortisol

To assess the long-term consistency of cortisol, salivary cortisol and hair cortisol data were analysed in two studies with varying interval length between assessments. Study I (Daytracker Study) was based on a sample of middle-aged working women and assessed salivary cortisol at baseline and hair cortisol four years later. Study II (ELSA) drew on a bigger population-based cohort, which included both women and men, and assessed salivary cortisol at baseline and hair cortisol eight years later. Associations between several salivary cortisol parameters, specifically the CAR, the AUC and the slope with hair cortisol levels were analysed. Longer term consistency and stability of cortisol in general is of interest, regardless

of specimen type. The associations between saliva and hair have only previously been tested over relatively short intervals of a few months and no study has investigated associations of values obtained several years apart.

It was found that the rate of decline in salivary cortisol over the day was associated with hair cortisol concentrations several years later. Individuals with a flatter slope of cortisol decrease over the day had higher hair cortisol concentrations. This pattern was observed in two independent studies (in older men only in Study II) with very different participants and time intervals between saliva and hair measurement. The CAR and the AUC did not show any associations with hair cortisol concentration in either study. The fact that the slope rather than the AUC related to hair cortisol is a finding that was unexpected.

A flattening of the diurnal cortisol rhythm has been linked to various adverse mental and physical health outcomes (Bower et al., 2005; Kumari et al., 2011; Liao et al., 2013). It is possible that the cortisol slope reflects individual differences in impaired regulatory processes and diminution in normal diurnal variation. Individuals with maladaptive cortisol decreases across the day repeated over long periods might show elevated systemic cortisol levels several years later, as assessed by hair cortisol. It is also conceivable that a flatter slope is more closely associated with individual differences in evening and nocturnal cortisol output and that this nocturnal secretion of cortisol is reflected in hair. With regards to the stability of the different parameters that have been computed from salivary cortisol output, there are inconsistent findings. Yet, Wang et al. (2014) showed strong stability over a 6-year period for the slope across the day relative to the other

parameters. It is plausible that stronger associations with cortisol slope emerged because it shows stronger long-term stability.

7.2.3 Study III: Associations between salivary cortisol and hair cortisol – a systematic comparison

The most direct validation of hair cortisol analysis and best method to explore whether hair cortisol indeed reflects integrated cortisol output over a prolonged time period is by direct comparisons between cortisol values from hair and saliva. Study III examined the temporal association between salivary and hair cortisol in a systematic design, addressing some of the methodological short-comings of previous correlational studies. To understand the correspondence of systemic cortisol production with cortisol output found in hair, this study was designed in a way that the two tissues reflected corresponding time-intervals, meaning that salivary cortisol mirrored the same time period as hair cortisol. This association was assessed in two distinct phases: during average academic stress and during high academic stress. In both phases (each lasting 7 weeks) diurnal salivary cortisol was collected three times over a period of a month (at week 1,3 and 5) and hair cortisol was assessed from a 1cm hair segment at the end of this period (week 7). One of the methodological shortcomings of previous work is that a time lag effect of hair growth occurs, meaning that appropriate hair growth needs to be considered. It has been suggested that new hair takes around two weeks to be formed in the follicle and to appear at the hair shaft (LeBeau et al., 2011). If not taken into account, the hair segment most probably does not accurately reflect the exact time frame that is measured in the saliva sampling period. The present study

was designed to allow appropriate hair growth (of 2 weeks after last salivary sampling) to overcome this timing issue and therefore ensure corresponding hair growth.

Significant associations were found between the AUC (at each time point and when averaged) and hair cortisol under low academic stress (Phase 1) and between the averaged AUC and hair cortisol levels under high academic stress (Phase 2), that withstood adjustment for covariates that have been found to be related to hair cortisol, i.e. age, BMI, smoking and hair treatment. This association emerged in both law and medical student groups. In the high academic stress phase the associations were considerably weaker that in the low academic stress phase. Further, under low academic stress, the 30 minutes post-awakening value was positively associated with hair cortisol concentration. The CAR, or the rate of cortisol decline over the day, and hair cortisol in either phase were not associated.

The AUC provides an estimate of total diurnal cortisol output and it is plausible that in corresponding time-intervals it presents the strongest indicator of accumulated long-term cortisol as captured in hair. This is in line with previous findings generating similar correlation coefficients (Short et al., 2016). In Phase 2 the associations between the AUC and hair cortisol were weaker and did not emerge consistently over the sampling period (of the forth-weekly salivary cortisol measurements) as in Phase 1. The exposure to the examination period (Phase 2) successfully induced psychological stress and anxiety. Although the relationship between these psychosocial factors and cortisol were not very clear and consistent, it is possible that heightened reactivity due to acute transient stress (that students

were supposedly exposed to in Phase 2), resulted in fluctuations in circadian cortisol throughout the period. This circadian cortisol variation may have impacted the overall concentration of cortisol within the hair as relative low and high values balance out over the time in which cortisol is incorporated into the hair shaft, potentially reducing the ability to detect an association with salivary cortisol. Although there were fluctuations in psychosocial stressors in the exam period, no strong fluctuations in the salivary cortisol parameters could be found. It is plausible that the accompanying fluctuations in the psychological factors that impact salivary cortisol levels at a subtle level might not have been adequately assessed by the current statistical analyses; more frequent assessment might have been valuable.

Assessment of corresponding time-intervals in which systemic cortisol levels are assessed is a stronger methodological approach. While the absolute concentration in the two tissues is not comparable, between-person correlations can establish whether individuals show similar ranking of levels in hair and saliva. These associations by direct comparisons suggest that hair cortisol indeed reflects integrated cortisol output, validating its use as a reliable cortisol measure and a biomarker of stress.

7.2.4 Study III: Cortisol output in academic stress

Study III, the Academic Stress Study, relied on a causality-sensitive design making use of variations of stress exposure and perception, the dynamics of the cortisol sensitivity-response and lastly the cumulative cortisol measure in hair. Data in medical and law students from two phases (during average academic stress and

during high academic stress) were gathered to establish whether academic stress induces increases in hair and salivary cortisol concentrations and in psychosocial factors and whether coping mechanisms would show an effect in this relationship.

It was found that the impact of examination stress impacted cortisol levels differently in medical and law students. There was a non-significant tendency for medical students to have increased hair cortisol levels when facing examination (comparing hair cortisol levels between Phase 1 and Phase 2) and law students to have decreased hair cortisol levels. A significantly flatter slope in the examination phase compared with the low stress period was observed only in law students. Higher levels of perceived stress (and a tendency for higher levels of anxiety) were associated with a flatter slope in the academic stress period. Predominant use of avoidant coping approaches was associated with elevated hair cortisol in the low academic stress phase and lower engagement of problem-focused coping and/or higher engagement in avoidant coping approaches was associated with aberrant salivary cortisol levels, higher CAR and AUC values and flatter slopes, more consistently in the low academic stress phase than the high academic stress phase.

The associations were somewhat mixed and larger differences in cortisol were expected. It is plausible that the key exposure measure (examination stress) may not have been sufficiently intense. Although there was an increase in self-reported distress (perceived stress and anxiety) between the two phases, these may not have been great enough to stimulate substantial differences in cortisol output. Earlier studies evaluating the impact of examination stress on biological responses have stimulated much larger differences and this might be due to

methodological factors or changes in the significance of examinations in student assessments.

7.3 Importance of findings

On the whole, not all of the hypotheses proposed in my studies were supported by the analyses. Even so, the findings have provided some insight into the associations between certain psychosocial stress factors and cortisol and also into the relationship between salivary and hair cortisol. Traditional specimens for cortisol include saliva, blood and urine; hair has only recently been used as a cortisol measure. The studies used for this PhD thesis were valuable in the quest of further validating hair cortisol analyses as a suitable tool to reliably quantify cortisol concentration. The strong associations between salivary cortisol and hair cortisol in a design that reflects corresponding time-intervals indeed suggests that hair reflects integrated cortisol output. Interestingly, during reported high levels of stress the association between the two tissues is weaker. Long-term consistency of cortisol needs to be explored in more detail but the findings indicate that a diminution in normal diurnal variation and regulatory processes might be reflected in long-term cumulative cortisol secretion.

Some of the findings from the present studies support also the utility of hair cortisol analyses as a biomarker of stress. The findings of an association between socioeconomic factors and hair cortisol are in line with previous findings in different population groups using other methods of assessment. Dynamic processes and long-term exposure to strain might be particularly relevant for the use of a chronic

stress marker, given that long-term stress exposure and long-term health consequences are of interest. Making use of a naturalistic stressor-design allows for the assessment of the impact of stress on long-lasting cortisol levels. There was some evidence for stress-related cortisol responses (for the salivary cortisol parameters) although the associations were weak and not consistent (Phase 1 versus Phase 2). It is plausible that a stressor that is more intense might produce more observable biological changes. Likewise, the possibility exists that perceived stress, dispositional psychological and affective factors merely show no such relationship in healthy individuals. The findings on income (Study I) relate to very long-term stress exposure, probably over months or years. The exam stress study (Study III) involved an episodic, relatively short-term stressor. It is indeed plausible that hair cortisol is more suited to the first of these experiences, capturing enduring and resistant stress exposure. Finally, the fact that associations emerged in separate (one or several) of the salivary cortisol parameters but not in others endorses the value of continuously obtaining several markers of the diurnal profile within the same study.

7.4 Methodological issues, limitations and suggestions for future research

7.4.1 Main issues and limitations relevant to the present studies

The findings need to be interpreted in light of their limitations. A key limitation when comparing longitudinal data is to include the same measures at baseline and at follow-up. Study I was designed and conducted in 2007/8 and the sampling of cortisol in hair was not widely known at that time. The associations

between the socioeconomic factors, concurrent income and status incongruity and hair cortisol were therefore cross-sectional. It is unclear whether the associations would have been present at the baseline as well. It was aimed to assess the dynamic aspect of income by evaluating income deterioration over a four year period and this was associated with elevated hair cortisol levels. Incorporation of hair sampling at baseline would have strengthened the confidence in the interpretation of the findings and would have permitted analysis of hair cortisol change in relation to these factors. Further, magnitude of income change could not be reliably assessed, which would have been informative regarding the gradient of the relationship.

Study I was based on a sample of young and middle-aged educated women working in higher education institutions. The null findings in the work-related stressors in relation to hair cortisol might be attributable to different confounding and methodological factors. Future studies might target a sample with more divergent work positions in terms of, job responsibility and demands, job aspirations and security, allowing for a larger variability in work stress, using inventories that cover stress in varying work-related aspects. The analyses in Study I revealed that low income and a negative incongruent status (lower income in relation to level of education) was related to elevated hair cortisol levels. It would be interesting to replicate these findings in different samples including males. It has been suggested that men might be more sensitive to financial hardship and the effect of status incongruity due to the strong influence of financial aspects on social status among men (von Rueden et al., 2011). A more pronounced association

between status incongruity and hair cortisol is therefore possible and future research is warranted to investigate potential sex differences in these associations.

In Study III, the key exposure measure, examination stress, may not have been sufficiently intense in generating observable changes in the cortisol measures. It would be interesting to use other naturalistic stressors in a mixed-method design. Although widely-used, established and the most validated inventories were utilised in the present study (i.e. the PSS, STAI and the COPE), in the given context some other measures (e.g. TAI, GSACS-Exam, DAI) may more accurately reflect the sources of student-related stress, anxiety and exam-specific coping strategies. Future studies might evaluate whether certain inventories better capture context-dependant stress and anxiety in relation to hair cortisol levels, given that the findings on perceived stress in the hair cortisol literature is quite inconsistent in healthy samples. A few studies suggest that multiplicity of adversities should be analysed in relation to hair cortisol rather than individual sources of stress and adversity. A study by O'Brien et al. (2013) found that an overall stress score, in which three different stress measures (each assessing different sources of stress) were aggregated, was positively associated with hair cortisol concentration, while the measures separately did not relate to hair cortisol. Further, the findings by Karlén et al. (2015) suggest that single early life adversities show no association with hair cortisol levels, while a cumulative measure indeed predict hair cortisol levels and also physiological health outcomes (disease incidence). This highlights the complexity of psychoneuroendocrinological research and the need to incorporate various factors rather than assessing independent effects. It is possible

that several stress factors would need to be assessed and aggregated to increase the possibility for a more reliable and potent measure of stress. Further, given the association between stress and performance, future studies might investigate the impact of stress, HPA-axis functioning (incorporating both salivary and hair cortisol measures) and academic performance. Two sets of student groups were incorporated due to recruitment difficulties. This induced inter-individual differences and might have introduced bias. Limiting the sample to one student group would have minimised potential confounding factors.

All studies investigated the association between salivary and hair cortisol (Study I and II: longitudinal associations; Study III: temporal associations with corresponding time-intervals). It is still not fully understood how cortisol incorporates into the hair shaft, and whether production of hair cortisol merely reflects the same HPA-axis processes as those observed in salivary cortisol production or might be disturbed due to intrafollicular cortisol production (Sharpley, McFarlane, & Slominski, 2012). The strong saliva-hair correlations (as observed in Study III) suggest that hair cortisol indeed reflects integrated cortisol output, validating its use as a reliable cortisol measure. Recent research assessing daily cortisol levels over a month in a similar design as used in Study III (Short et al., 2016) produced correlation coefficients of comparable strength. It is important to understand what other aspects explain the lack of shared variance between the two tissues. Nocturnal cortisol output is a plausible explanation as this is not captured in the salivary cortisol parameters that are computed from the diurnal cortisol profile. Further, incorporation of endogenous substances into the hair shaft has been

shown to occur over multiple sites (based on the multi-compartment model, shown in Figure 2.2 in Chapter 2) (Henderson, 1993) and cortisol content has been shown to be 'contaminated' (confounded) by locally produced glucocorticoids at the hair follicle in animals (Keckeis et al., 2012). It is therefore plausible that there are individual differences in intrafollicular cortisol production (Sharpley et al., 2012). Together, these factors may also somewhat explain the lack of shared variance and inconsistencies in findings. More research needs to be conducted to explore these aspects in more detail.

7.4.2 Factors associated with inconsistencies in cortisol assessment

Cortisol follows a dynamic diurnal pattern complicating research in assessing HPA-axis functioning. Salivary cortisol assessment is subject to several methodological, situational and context-dependant influences, such as time of waking, sleep patterns, environmental conditions, smoking, diet and acute psychological states (Adam & Kumari, 2009; Hansen et al., 2008). Despite these methodological concerns, the vast majority of past research using salivary cortisol measures have generated important discoveries in relation to the psychoneuroendocrinological processes involved in health and disease. Hair cortisol analyses have provided major advances to the assessment of long-term cortisol output. Considering the impact of *chronic* stress exposure, it is of interest to assess *long-term* psychobiological processes. Whilst the literature on salivary cortisol assessment has provided compelling evidence for the cortisol-health link and the

role that psychosocial factors play in this, understanding the *cumulative* effect of excessive cortisol secretion is of importance.

There have been inconsistencies in findings in the salivary cortisol literature, many which might be due to situational and methodological factors. Several review papers and procedure recommendations were published in the last three decades trying to contend with these methodological drawbacks (e.g. Adam & Kumari, 2009; Wust, Wolf, et al., 2000). In 1999/2000, guidelines have been set by the MacArthur Research Network (comprising pioneers and experts in the cortisol literature), dealing with conceptual issues and the protocol for saliva sampling (MacArthur Research Network on SES and Health, 2000). Since then, research methodologies have improved substantially, yet studies have been inconsistent in the assessment of cortisol and methodological refinements were not stable. Studies differ in their sample collection times (Matsuda, Yamaguchi, Okada, Gotouda, & Mikami, 2012). Some authors instruct to collect saliva at specific timings of the day (e.g. 10am/ 12am/ 3pm/ 6pm, with variation regarding timings); some other authors instruct sampling at certain time intervals after waking (30min/ 1h/ 4h/ 9h after waking). It is unclear what method is more advantageous as no study has ever assessed whether these two approaches differ in their ability or strength with regards to assessing potential associations and both within- and between-subject variability. It would be of interest to evaluate whether sampling at particular timings of the day without reference to time of awakening or whether sampling at time intervals after waking enables better comparison between individuals.

The variation in cortisol quantification strategies also leads to incompatibility of findings between different research groups. A review of the commonly used point indices of cortisol, especially for the purpose of stress responsivity measurements, concluded that a variety (and redundancy) of indices of the cortisol profile or stress-induced cortisol rise measurements exist and are continually being used in the literature, without justification for the chosen method, contributing to heterogeneous findings (Khoury et al., 2015). The authors identified single time-point cortisol values, non-standardised calculations of indices or different calculations of the exact same parameter as producing the most mixed findings. Many studies employ salivary cortisol analyses, not taking specific confounders and the analytical challenges of multiple sampling into account, potentially spreading the contention of findings, which might have been flawed, or inconsistent findings, which lead to the formation of false interpretations and conclusions (Saxbe, 2008). More current guidelines on adequate assessments, time-related factors, confounding factors are therefore needed to promote best practice and hence homogenise the methods in the salivary cortisol literature. The reliable assessment of the CAR has received more attention recently. Expert consensus guidelines have been recently published specifically for the assessment of the CAR, specifying sampling instructions, protocols, accuracy and adherence, inclusion of covariates and quantification strategies, to encourage methodologically stronger research designs and thus more reliable and replicable results (Stalder et al., 2016). Within the UK, quality of plasma cortisol extraction is controlled by the National External Quality Assurance Scheme (Makin, Honour, Shackleton, & Griffiths, 2010).

Given that studies in stress research and epidemiology is cross-national, such a scheme would be of value at an international level, for steroid extraction procedures within the different tissues.

Hair cortisol analyses are not subjected to the same methodological issues as salivary cortisol assessments (e.g. differences in instructions, adherence to protocols and accounting for situational factors) due to the nature of the tissue and its collection method. However, they do need to involve accurate sampling and quantification methods and inclusion of other important covariates. An international inter-laboratory round robin test, which is a standardised method of independent inter-laboratory testing, compared four leading laboratories in their measurements and analytical procedures to assess different ranges of hair cortisol concentration (low, intermediate, and high range) of the same hair samples (Russell et al., 2015). While absolute ranges of values differed by three-fold, the different immunoassay and liquid chromatograph–mass spectrometry (LC–MS) methods across all four laboratories showed strong positive intercorrelation of hair cortisol levels. This highlights that actual hair cortisol values from different studies and laboratories cannot be compared but are rather limited to evaluations in relation to their own sample. To account for differences in methods within the same laboratory, Russell et al. (2015) proposed a correction factor that can be applied if immunoassays are used to convert concentration levels into standard LC–MS equivalents. Yet, it would be valuable if consensus guidelines for hair analytical methods and reporting could be established to promote more interpretable, reliable and replicable results.

In the last two decades, technical advances have enhanced chromatographic–mass spectrometric techniques for hair analyses, firstly in the field for drug detection and in the last decade for endogenous cortisol, resulting in better sensitivity and limits of quantitation and detection (initial detection units at ng/mg have improved to detection units at pg/mg) (Pragst & Balikova, 2006). Several methodological concerns surround the use of hair cortisol analysis and its reliability as a stress marker, coupled to the inconsistencies in the literature. Despite considerable evidence of its use in relation to certain population groups, hair cortisol analysis is still in its infancy and methodological aspects need to be explored to ensure quality of future studies and best practise.

7.4.3 Issues related to hair cortisol analyses

7.4.3.1 Time lag effect of stress factors

The findings on perceived stress and also stressful life events and hair cortisol are somewhat inconsistent. While low variability or impact of stress might account for some inconsistencies, it is also plausible that there is a potential time lag in the measures, in which the evaluation of stress measures and hair cortisol content, respectively, do not match. Timing of perceived stress exposures or stressful life events and the responsivity to stressor type in the hair cortisol literature have not been considered carefully (Sharpley et al., 2012). Inconsistencies in the stress-hair cortisol relationship in healthy population groups might be attributable to a biased reflective time window between the two measures. Most hair cortisol studies employ a questionnaire design with a retrospective window of

the previous 1 month; yet they concurrently assess a hair sample of 3 cm length, representing the previous 3 months of cortisol exposure. Study I in the present PhD employed a similar design, with hair cortisol analysis of a 3cm hair segment and concurrent assessment of stress sources (retrospective reflection of up to the previous 3 months). This method of self-report assessment of stress over a period of 3 months, is subject to recall bias, possibly generating individual differences in under- or overestimation of (work-related) perceived stress levels.

Study III assessed stress and anxiety levels in a prospective (within-subject) design and still failed to find an association between these factors and hair cortisol. The literature based on the traditional cortisol specimens provide some insight into the lag time effect between exposure and appearance. A meta-analysis of salivary cortisol showed that timing of the stressor is a vital aspect of shaping HPA-axis activation with higher activation at stressor onset, but reduced or normalised activation when the stressor is no longer existent (Miller et al., 2007). It is possible that some stressors (as reflected by higher levels of reported perceived stress) are not enduring, potent and robust enough to produce observable changes in cortisol from hair specimen as incorporation of cortisol into the growing hair shaft occurs steadily. More detailed (intensity and duration) and repeated assessments of perceived stress over the time interval that the hair segment is capturing might provide clarity about this.

7.4.3.2 Hair growth and weight

Dermatological research has identified stress as one of the main factors involved in hair growth inhibition, affecting the movement of the stages in the hair's life cycle (Cotsarelis & Millar, 2001). The hair's life cycle is composed of different stages of growth; the active growing phase (the anagen phase) and the resting phase (the telogen phase). During extreme stress, the switch from the anagen phase to the telogen phase can happen prematurely (Arck et al., 2003). This can result in inhibited hair growth with hair follicles staying dormant. Once the stressful period has ceased hair follicles start producing new hair fibre and thus hair growth re-initiates. Complete restoration of ordinary hair growth patterns might take place at around 6 months after stress termination (Arck et al., 2003). The active growing phase of the hair is also very sensitive to metabolic imbalances as induced by dieting and to nutritional deficiencies (Botchkarev, 2003), the use of drugs and excessive exposure to toxins or specific medicines (Harkey, 1993). Although it was assumed that Study III was based on a healthy sample, no information was collected on substance use (which is not uncommon in university students; Pickard, Bates, Dorian, Greig, & Saint, 2000) and dietary behaviours; factors that might not only play a role in cortisol secretion but also hair growth.

Depending on follicular growth cycle stage, different associations with stress factors might emerge as during high levels of stress the hair collected segment might progressively consist of telogen stage hair. The fact that stress is related to hair growth inhibition presents an interesting aspect for cumulative cortisol estimation. This could imply that people exposed to stressful conditions (and also to

the other factors related to hair growth inhibition) have slower (stagnating) hair growth rates and the analysed hair segment represents a combination of the purported time interval and the period anterior to this interval, potentially weakening the overall strength of accumulation of hair cortisol. It would be interesting to measure real-time hair growth rates in different stress conditions or case studies and to evaluate whether multifactorial triggers for inhibited hair growth relate to hair cortisol content.

Most studies report no associations between hair cortisol and hair colour and hair structure (e.g. Feller et al., 2014; Kirschbaum et al., 2009; Noppe et al., 2014). However, in some studies hair colour was indeed associated with hair cortisol, with darker colours showing higher cortisol levels (Rippe et al., 2016; Staufenbiel et al., 2015). Also ethnic groups seem to differ in hair cortisol, with black people showing higher hair cortisol content than white people (Abell, Stalder, et al., 2016; Wosu et al., 2013), which might be partly due to their black-coloured hair. However, although the established estimate of 1cm hair growth per one month is being predominantly used for hair analytical methods, it has been shown that there is variability in hair growth, with morphological parameters of hair growth being related to ethnicity (Loussouarn et al., 2005). Drug content in hair (when exposed to identical amount of drugs) has been shown to differ depending on ethnicity (Wennig, 2000). However, also thicker hair fibres (which are equally diverse in all ethnic groups) are linked to faster growth rates (Baque et al., 2012; Franbourg, Hallegot, Baltenneck, Toutain, & Leroy, 2003). It is still unclear what

causes these differences and more research is needed to evaluate the exact effect of hair colour and ethnicity on hair cortisol.

A study exploring the seasonal effect on hair growth found that there is faster hair growth in the summer months compared with the winter months (Randall & Ebling, 1991). Increasing hair growth rates (in opposition to hair growth inhibition) would imply that the analysed hair segment not fully/ only partially captures the assumed time interval and hence the full month's cortisol exposure and cortisol concentration in the hair segment may be less 'concentrated'. The analytical procedure for hair cortisol analysis is based on the weight of hair supplied and not on the absolute amount of hair (or of cortisol). The question arises as to whether factors related to hair growth impact hair weight. Interestingly, it has been shown that there are racial differences in hair weight, which might partly explain the ethnic differences in hair cortisol (not considering other socioeconomic or stress-related factors that minority groups might experience) (O'Brien et al., 2013; Schreier et al., 2015). These hair-analytical methodological aspects still need to be explored in more detail before assumptions and recommendations can be drawn to what factors need to be considered when using hair cortisol as markers in longitudinal designs.

Research has identified locally produced glucocorticoids at the hair follicle site (Keckeis et al., 2012), therefore hair cortisol values might be distorted by intrafollicular cortisol production. In particular, the follicular stress response and the hair growth cycling behaviour is of relevance to explore its impact on cortisol in the hair shaft. To date, no study has been conducted to analyse both hair follicle

functioning and hair cortisol in the grown hair. Research into this with multidisciplinary approaches (including dermatological research methodologies) would elucidate to what extent hair cortisol content is impacted by the follicular peripheral HPA-axis and what factors might impact the hair growth-hair cortisol relationship.

7.4.3.3 External influences

Human hair is subjected to various chemical substances for hygienic and cosmetic purposes. Several studies including reviews have reported that frequency of hair washes and also hair treatment (colouring) decreased endogenous levels of cortisol in hair (Abell, Stalder, et al., 2016; Feller et al., 2014; Meyer & Novak, 2012; Russell et al., 2012; Stalder, Steudte, Miller, et al., 2012; Staufenbiel et al., 2013). In both Study I and Study III, hair treatment was associated with lower hair cortisol levels, in line with previous evidence. However, there might be an issue around (inadvertent) false reporting of presence of hair treatment. It is possible that the individual's hair was indeed treated, but the area from which the hair section is taken (i.e. the section closest to the scalp at the posterior vertex position) was untouched/ untreated during the treatment visit. It was noted during data collection of Study III that some participants verbally reported having had hair treatment within the previous weeks (time interval relevant for the hair collection), however, when visually inspecting the collected hair segment together with the participant, treatment has not been applied (and participant's recording has been corrected). As the most suitable region for hair sampling, researchers have

identified the posterior vertex position in the back of the scalp. This hair region is the most constant in hair growth, with lowest proportion of hair in telogen stage (the non-active growing phase) (Villain et al., 2004) and with the lowest coefficient of variation in hair cortisol levels (Sauve et al., 2007). However, this region is covered by the rest of the scalp hair (especially the hair segment closest to the scalp) and therefore might be omitted for certain treatments. An effect of hair treatment on hair cortisol levels was visible in the present studies (which might have been reinforced by revision of records based on visual observation together with the participants in Study III). In previous studies, including individuals in the treatment group that have not been exposed to hair treatment (inadvertent false reporting) might underestimate the true effect of hair treatment and potentially falsify its validity as a covariate. Accurate recording of treatment timing when analysing hair segments of varying lengths and actual objective evaluation of the segments is therefore important when attempting to control for these covariates. Studies that rely on meticulous detail and accurate time intervals might need to consider the precision of hair treatment records.

Interestingly, an experimental study analysed the effect of different standard chemical treatments on hair cortisol levels and found that cortisol content was differentially affected by the chemical treatment processes (Hoffman et al., 2014). Bleaching, demi-permanent colouring and shampoo washes significantly decreased hair cortisol level; however different percentages of peroxide levels in colouring lead to an increase in cortisol. Not all studies in the hair cortisol literature have found that treatment lowers hair cortisol (e.g. Stalder, Steudte, Alexander, et

al., 2012), which might be attributed to varying levels of substances involved in the chemical processing or alternatively to the issue of inadvertent false reporting of hair treatment or a combination of both. Experimental designs provide robust findings for the impact of chemical treatment. Ideally avoiding chemically processed and extremely shampooed hair in recruitment for hair cortisol studies (by asking participants not to get exposed to chemical hair treatment in the relevant months prior to sampling or between baseline and post sampling periods) would avoid issues around the impact of hair treatment on endogenous cortisol concentration.

Water exposure significantly decreases hair cortisol content (Hamel et al., 2011). Segmental analyses demonstrated that the more distal segments from the scalp yield reduced hair cortisol levels due to a wash-out effect (accumulated hair washes and exposure to toxins). Retrospective cortisol assessment is therefore limited to a 6 cm hair segment, representing the preceding 6 months prior to sampling. Association analyses using segmental hair analyses to retrospectively evaluate cortisol exposure over several months (e.g. as has been conducted with traumatised individuals and pregnant women) might be confounded by this wash-out effect (Dettenborn et al., 2010; Gao et al., 2010; Vanaelst et al., 2013). Sunlight exposure also seems to play a role in affecting hair cortisol levels, as identified in human and in animal research (Staufenbiel et al., 2015). In fact, the seasonal variation in hair cortisol might be explained by external light factors. Ultraviolet (UV) radiation has been found to reduce hair cortisol in all experimentally tested hair samples with artificial UV, but also after exposure to natural sunlight for a period of 40 hours (Grass et al., 2016; Li et al., 2012; Wester et al., 2016). The

impact of some of these factors, such as external water exposure, UV light but also external contamination (pollution and other exposure to toxins), on hair cortisol concentration is still uncertain and these elements are under-researched. More methodologically strong studies need to be conducted in relation to both hair cortisol and hair follicle functioning before reliable conclusions can be drawn on the covariates that need to be included in future analyses.

7.4.3.4 Lifestyle factors

Health behaviours have been little studied in relation to hair cortisol, which might present potential reasons for inconsistencies. Health behaviours were only briefly considered in this PhD, e.g. physical activity, smoking and alcohol consumption. Exercise has been shown to increase momentary cortisol (assessed via saliva or blood sampling); also hair cortisol has been positively correlated with amount of physical activity (Gerber et al., 2012; Skoluda et al., 2012). A positive association between physical activity and hair cortisol was also found in Study I but not in Study III.

Reviews have found that smoking use is linked to elevated hair cortisol in methodologically strong studies (Feller et al., 2014; Russell et al., 2012; Wosu et al., 2013), consistent with the evidence from traditional cortisol specimens. In Study III, smoking was unrelated to hair cortisol levels. However, reported smoking between the phases differed. Some students (medical students; N = 5) who reported being current smokers (or ex-smokers) in Phase 1 said that they had never smoked before when re-assessed in Phase 2 (several months later), indicating some unreliability in

assessments. It is possible that social desirability is particularly present in medical students (Motl, McAuley, & DiStefano, 2005). Alcohol consumption was unrelated to hair cortisol in the present study (Study III).

Sleep duration and quality have been shown to be related to disturbed cortisol rhythms as derived from salivary sampling, with shorter sleep duration, lower sleep quality and more sleeping problems being associated with a smaller CAR and a flatter cortisol slope (Abell, Stalder, et al., 2016; Bostock & Steptoe, 2013; Castro-Diehl et al., 2015). Sleep has not been well studied in relation to hair cortisol. There is some evidence for daytime sleeping to be positively associated with hair cortisol (Feller et al., 2014). Exploring the reasons for daytime sleeping (due to increased levels of stress) might elucidate this relationship; nevertheless future research is necessary to provide clarification of the impact of sleep in general. Measuring accumulated sleep patterns or sleep changes over the period of a month might be particularly relevant in relation to a long-term cortisol measure.

Other potential sources leading to interindividual variability in the assessment of the cortisol parameters are dietary patterns and coffee consumption (Maina et al., 2012). Dietary behaviours have been shown to be related to salivary cortisol concentration (Tomiyama et al., 2010; Vicennati et al., 2002). Caffeine intake has been linked to increased salivary cortisol levels and stress reactivity in a dose-response relationship (Harris, Ursin, Murison, & Eriksen, 2007; Lovallo et al., 2006). Coffee consumption has not been studied at all in relation to hair cortisol, despite the evidence of its HPA-axis stimulating effects. It would be interesting to

explore the relationship between these nutritional aspects and long-term cortisol output.

7.4.4 Future advances to the hair cortisol literature

7.4.4.1 Advances for stress research and utility as a biomarker

Hair cortisol analysis has the potential to expand our current knowledge about endocrine changes and the long-lasting effect of cumulative cortisol exposure. The simultaneous assessment of salivary cortisol and hair cortisol allows to explore the dynamic and long-term cortisol pattern of certain diseases and therefore provide interesting complementary resources. There are heterogeneous findings in the salivary cortisol literature in relation to depression, anxiety and PTSD (Chida & Steptoe, 2009; Hek et al., 2013; Meewisse et al., 2007; Vreeburg et al., 2010; Yehuda et al., 2005). While inconsistencies have also been found in the hair cortisol literature (Meyer & Novak, 2012; Russell et al., 2012; Stalder & Kirschbaum, 2012; Staufenbiel et al., 2013; Steudte-Schmiedgen et al., 2017), hair cortisol seems to be able to provide some critical insights into the factors that generate these mixed findings.

For instance, segmental hair analytical studies have increasingly been conducted with PTSD patients in the attempt to understand the development of the mental disorder (Luo et al., 2012; Steudte, Kolassa, et al., 2011). The literature based on the traditional cortisol specimens have produced mixed findings, with PTSD being related to both increased and decreased cortisol output (Meewisse et al., 2007). Interestingly, in a mixed-method design, Luo et al. (2012) found that

individuals exposed to a traumatic event (earthquake in China) had higher hair cortisol concentration compared to a control group. However, segmental hair analyses revealed that among individuals experiencing the disaster, those who suffered subsequently from PTSD (compared to traumatised individuals without PTSD) had lower hair cortisol levels several months after the trauma. These findings are in line with longitudinal studies based on traditional cortisol specimens, that support the phenomenon of adrenal exhaustion by suppression or inhibition of HPA-axis activity (Kellner, Yehuda, Arlt, & Wiedemann, 2002).

The concurrent use of both salivary and hair cortisol can be of significant value. In one study, patients with anxiety disorder had lower hair cortisol values compared with matched healthy controls, while no differences could be found in diurnal cortisol profiles (Steudte, Stalder, et al., 2011). Also paradoxical findings emerged in simultaneous assessments of salivary and hair cortisol with traumatised individuals, showing lower salivary cortisol but higher hair cortisol levels (Schalinski et al., 2015). Frequency, severity and duration of traumatic events have been linked to differences in HPA-axis pattern which might generate contradictory findings. Future studies employing simultaneous assessments of short-term and long-term cortisol controlling for vital covariates and strong methodological designs might provide insight into the complexity of these mental disorders. Incorporating stress reactivity in studies using hair cortisol analysis can further inform about the short-term and long-term specificity of HPA-axis changes (Steudte-Schmiedgen et al., 2017).

Further, studies employing hair cortisol analyses suggest that this method might serve as a marker to differentiate between subtypes of several mental disorders, episodes of disease and inform about aetiology and onset of mental disorders. Patients with a later onset of bipolar disorder have been showed to have higher hair cortisol levels whilst patients with comorbid psychiatric disorders (e.g. panic disorder) have lower hair cortisol levels (Spijker et al., 2014). Another study found that patients with first-episodic depression have higher hair cortisol concentration than recurrent depressive patients (Wei et al., 2015). Most of these studies have small sample sizes and do not include sufficient distinction between pure and comorbid mental disorder groups; future studies might enhance our current knowledge in these areas and potentially aid diagnosis and assessment of the clinical course of mental disorders.

Hair analysis allows for quantification of a range of hormones (Gao et al., 2013). Analysing hair hormone profiles and also the ratio of different steroids, including androgen or oestrogen hormones and their precursors, might inform and develop the field on the interaction of the endocrine system in relation to psychopathology. For example, cortisol-testosterone ratio has been linked to incident ischemic heart disease (Smith et al., 2005) and has also been studied in social contexts (Terburg, Morgan, & van Honk, 2009). Future work in the hair cortisol literature might focus on cortisol-cortisone, cortisol-DHEA and also testosterone-cortisol ratios (Terburg et al., 2009).

7.4.4.2 Diagnostic, monitoring and intervention tool and clinical relevance

Hair cortisol has immense potential for becoming a new indicator for health status and risk factors and as well high exposure to psychological strain (Gow et al., 2010). The detection of endogenous cortisol levels in hair, sparked its potential as a therapy monitoring tool for steroid medication. Specifically, hair cortisol has been found to be an effective monitoring method (as a clinical follow-up parameter) for glucocorticoid replacement therapy in patients with congenital adrenal hyperplasia (Noppe et al., 2016). It has also provided a historical record of cortisol concentration, the clinical course and responsiveness to medication in Cushing's syndrome patients (Thomson et al., 2010).

Hair cortisol levels have also been found to be related to cardiometabolic parameters and CVD. Elevated hair cortisol content has been positively related to heart failure severity and functional limitations in congestive heart failure patients (Pereg et al., 2013) and to history of CVD (Manenschijn et al., 2013). As acute stressors have been renowned for triggering cardiovascular events (Tofler & Muller, 2006), studies employing hair cortisol could potentially further elucidate the effect of acute versus long-term stressors on cardiac functioning (Pereg et al., 2011). New studies employing hair specimens have the potential to provide insights into the development of heart disease, its rehabilitation process and the possible role of chronic stress. Future methodologically strong studies that control for important confounding factors are needed to establish its usefulness as a clinical marker for cardiovascular risk stratification and for predicting health status in cardiovascular disease. Hair cortisol has become a meaningful physiological marker in

psychoneuroendocrinology but further research is needed. However, there are no clinically established reference ranges for hair cortisol values. At presence, comparisons of hair cortisol concentration is limited to evaluations in relation to the study sample as actual hair cortisol values might differ based on laboratory and analytical quantification method (Russell et al., 2015). If clinical reference values are established, the utility of hair cortisol as a diagnostic and monitoring tool and hence its clinical relevance can be potentiated.

Finally, one pilot study with 18 smokers used hair cortisol analyses to evaluate the effectiveness of an intervention. In a 7-week smoking cessation intervention programme based on mindfulness and cognitive elements, pre-to-post intervention hair cortisol levels showed a reduction in response to successful cessation and a decrease in negative affect (Goldberg et al., 2014). The study sample was relatively small, nevertheless, it demonstrates the potential of hair cortisol analysis to serve as a treatment and intervention tool targeted at stress reduction (Wright, Hickman, & Laudenslager, 2015). Although the associations between hair cortisol and work-related stressors are weak, future studies might further explore these and hence enhance the applicability of hair cortisol analyses in the working environment to decrease stress and employee retention and increase well-being, satisfaction and job performance.

7.5 Final conclusion

This thesis set out to investigate the associations between individual differences in relation to stress and hair cortisol and both the long-term and

temporal associations between salivary and hair cortisol. The findings highlight the utility of hair cortisol analyses in relation to chronic and dynamic stress-related exposure. Quantifying the degree of socio-economic elements appears to be relevant to health and the underlying biological mechanisms. The associations between other psychosocial factors and hair cortisol are still unclear. The present studies did not add much clarity to the existing literature as no clear associations emerged between hair cortisol and levels of perceived stress or stress exposure (work stress in Study I and academic stress perception and exposure in Study III). Noticeably, there are multiple pathways by which psychosocial factors determine biological functioning and ultimately health; therefore future studies should focus on more comprehensive designs including macro- and microeconomic contexts, social, psychological, behavioural and biological factors. Conventional cortisol measurements are not suitable as an index of cortisol exposure over longer periods of time (weeks to months), which however is often useful for most studies in stress research. Hair cortisol reflects long-term cortisol exposure and has therefore the potential to further elucidate relationships between psychological stressors and chronic illness. Most of the studies analysing clinical samples with known HPA-axis dysfunction have demonstrated the usefulness of the method; however, studies employing healthy individuals (which predominantly employ questionnaires) show inconsistent results and need further clarification. Nevertheless, hair specimens present an attractive alternative matrix in bioanalysis and a complementary resource for psychoneuroendocrinological research. This method holds great

promise to improve our current knowledge in the stress-health link and to act as an intriguing tool for future clinical practise to predict and prevent disease.

LIST OF APPENDICES

APPENDICES

Appendix 1. Recruitment email sent to participants (Study I)

Email Subject: Daytracker Follow-up Study

Dear _____,

My name is Bianca Serwinski and I am from the Psychobiology Group at the Department of Epidemiology and Public Health. Sometime between 2007 and 2008 you participated in a study entitled 'The biology of everyday life', which involved completing a questionnaire and providing several biological measurements such as taking saliva samples and wearing an Actiheart (attached to your chest) on one work and one leisure day. The study was conducted by a research team based in the Psychobiology Group directed by Professor Andrew Steptoe.

We are very grateful for your participation in the study, and we would now like to invite you to take part in a follow-up assessment. The reason that we would like to repeat some of the measures is to explore any changes in well-being and biological function that occurred since data collection in 2007/08. We are also interested in how measures taken now would relate to those collected in 2007/08.

It would be extremely helpful if you could participate, and this would involve completing a brief questionnaire and providing a small hair sample. You would also be asked to wear a small watch-like device on your wrist for 7 days to measure your levels of physical activity and sleep. The data will be collected by myself – I am affiliated with the Psychobiology Group at UCL. As an incentive we are able to offer you a £10 M&S voucher and provide feedback on the health related measures we take.

Please find attached the Study Information Sheet.

I look forward to hearing from you soon.

Best Regards,
Bianca

Appendix 2. Participant Information Sheet (Study I)

PARTICIPANT INFORMATION SHEET (Confidential)
You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?
Certain psychological states and emotions are associated with biological responses, such as elevated levels of the stress hormone cortisol. When you participated in the Daytracker study (also known as 'The biology of everyday life study') in 2007/08 you provided various psychological and physiological measures including the hormone cortisol taken from your saliva. You also wore a small device on your chest on one week and one leisure day.

In this project, we want to repeat some of the tests we took in 2007/08 to explore how measures taken now would relate to those collected in 2007/08. This would help to establish whether psychological factors drive changes in biological responses, namely cortisol levels, or vice versa. In addition we want to explore any changes in psychological factors and biological function that occurred since data collection in 2007/08. This study is being conducted by a research team from the Psychobiology Group, directed by Professor Andrew Steptoe.

What will happen during the study?
You will be asked to complete a brief questionnaire and we will take some hair samples from you. You would also be asked to wear a small watch-like device on your wrist for 7 days to measure your levels of physical activity and sleep. Finally, we will ask you to record your bed and wake-up times for 7 days. The collected information is completely confidential; results will not be available to anyone outside the study group and will only be used anonymously.

Who can take part?
This study is being carried out solely with women who took part in the Daytracker study conducted between 2007 and 2008. Volunteers should be healthy and they should not be on any regular medications except for oral contraceptives or hormone replacement therapy. If you have suffered from a serious illness such as heart disease or cancer over the past two years, we will not be able to include you in the study. There are no risks to taking part in the study if you are pregnant, however since pregnancy has effects on some of measures, we do not wish pregnant women to participate.

What if I change my mind during the study?
If at any point for any reason you do not want to carry on, then you may stop. There are no consequences of withdrawal from the study, other than forfeiting the voucher (see below).

What happens to the information?
All the information we get from this study about you, including your name, will be confidential and will only be used for research purposes. The data will be collected and stored in accordance with the 1998 Data Protection Act. The data we collect from all volunteers will be combined, and it will not be possible to identify any individual within published results.

What happens at the end of the study?
Provided you have completed the study we will give you an M&S voucher of £10. When the study is complete and all the results are analysed, we will send you a summary of our findings.

We hope you are able and willing to take part in our study. If you have any questions, please contact Bianca Serwinski (bianca.serwinski.11@ucl.ac.uk). Telephone: 020 7679 8248. Psychobiology Group, Research Department of Epidemiology and Public Health, 1-19 Torrington Place, London WC1E 6BT.

Appendix 3. Informed Consent Form (Study I)

Confidential: Informed Consent Form

Project title: Daytracker Follow-Up Study

Have you read the information sheet about this study?	Yes / No
Have you had the opportunity to ask questions and discuss the study?	Yes / No
Have you received satisfactory answers to all your questions?	Yes / No
Have you received enough information about the study? Yes / No	
Do you understand that you are free to withdraw from the study at any time, without giving a reason for withdrawal?	Yes / No
Do you agree with the publication of the results of this study in appropriate outlets?	Yes / No
Do you agree to take part in this study?	Yes / No

Signature of participant:

Signature of investigator:

Date:

If you have any further questions about the study, please contact:

Bianca Serwinski (bianca.serwinski.11@ucl.ac.uk) or
Marta Jackowska (marta.jackowska.09@ucl.ac.uk)
Psychobiology Group,
Research Department of Epidemiology and Public Health,
1-19 Torrington Place,
London WC1E 6BT

Tel. 07593228046

If you wish to complain about any aspect of the way you have been approached or treated during the course of the study, you should email the Chair of the UCL Committee for the Ethics of Non-NHS Human Research (gradschoolhead@ucl.ac.uk) or send a letter to: The Graduate School, North Cloisters, Wilkins Building, UCL, Gower Street, London WC1E 6BT.

Appendix 4. Questionnaires (work stress, financial strain, income, education, socal support, physical activity, hair-related questions)

Appendix 4. Questionnaire (Study I)

Work stress

The next set of questions are about your work. For each question, please indicate the one answer that best describes your job or the way you deal with problems occurring at work. Please answer each question as accurately as you can.

		Often	Sometimes	Seldom	Never
1.	Do you have to work very fast?	○	○	○	○
2.	Do you have to work very intensively?	○	○	○	○
3.	Do you have enough time to do everything?	○	○	○	○
4.	Do you have a choice in deciding HOW you do your work?	○	○	○	○
5.	Do you have a choice in deciding WHAT you do at work?	○	○	○	○
6.	Do different groups at work demand things from you that you think are hard to combine?	○	○	○	○
7.	Does your work demand a high level of skill and expertise?	○	○	○	○
8.	Does your job require you to take the initiative?	○	○	○	○
9.	Do you have the possibility of learning new things through your work?	○	○	○	○
10.	Do you do the same thing over and over again?	○	○	○	○
11.	Is your job boring?	○	○	○	○

About your position at work; how often do the following statements apply?

		Often	Sometimes	Seldom	Never / Almost never
12.	Others make decisions concerning my work.	○	○	○	○
13.	I have a good deal of say in decisions about work.	○	○	○	○
14.	I have a say in my own work speed.	○	○	○	○
15.	My working time can be flexible.	○	○	○	○
16.	I can decide when to take a break.	○	○	○	○
17.	I have a say in choosing with whom I work.	○	○	○	○
18.	I have a great deal of say in planning my work environment.	○	○	○	○
19.	Does your job provide you with a variety of interesting things to do?	○	○	○	○
20.	Do you get praised for your work?	○	○	○	○
21.	Do you consider your job very important?	○	○	○	○
22.	Do your colleagues consider your job very important?	○	○	○	○
23.	How often are your colleagues willing to listen to your work-related problems?	○	○	○	○
24.	How often is your immediate superior willing to listen to your problems?	○	○	○	○

About your job in general; how satisfied have you been with the following?

		Very satisfied	**Satisfied**	**Dissatisfied**	**Very dissatisfied**
25.	Your usual take home pay.	○	○	○	○
26.	Your work prospects.	○	○	○	○
27.	The help and support you get from your colleagues.	○	○	○	○
28.	The help and support you get from your superiors.	○	○	○	○
29.	The way your abilities are used.	○	○	○	○
30.	The interest and skill involved in your job.	○	○	○	○

For each of the following items, indicate the most accurate response for each statement, using the response choices listed.

		No	Yes, but not at all distressed	Yes, somewhat distressed	Yes, rather distressed	Yes, very distressed
31.	I have constant time pressure due to a heavy work load.	○	○	○	○	○
32.	I have many interruptions and disturbances in my job.	○	○	○	○	○
33.	I have a lot of responsibility in my job.	○	○	○	○	○
34.	I am often pressured to work overtime.	○	○	○	○	○
35.	Over the past few years, my job has become more and more demanding.	○	○	○	○	○

36.	I am treated unfairly at work.	○	○	○	○	○
37.	I have experienced or expect to experience an undesirable change in my work situation.	○	○	○	○	○
38.	My job security is poor.	○	○	○	○	○

For each of the following items, indicate the most accurate response for each statement, using the response choices listed just below. Please note that the responses are different to those of the questions above.

		Yes	No, but not at all distressed	No, somewhat distressed	No, rather distressed	No, very distressed
39.	I receive the respect I deserve from my superiors and colleagues.	○	○	○	○	○
40.	Considering all my efforts and achievements, I receive the respect and prestige I deserve at work	○	○	○	○	○

Please indicate how much you agree or disagree with each statement.

		Strongly Disagree	**Disagree**	**Agree**	**Strongly Agree**
41.	As soon as I get up in the morning I start thinking about work problems.	○	○	○	○
42.	When I get home, I can easily relax and 'switch off' work.	○	○	○	○
43.	People close to me say I sacrifice too much for my job.	○	○	○	○
44.	Work rarely lets me go, it is still on my mind when I go to bed.	○	○	○	○

		Strongly Disagree	Disagree	Agree	Strongly Agree
45.	If I postpone something that I was supposed to do today I'll have trouble sleeping at night.	○	○	○	○

Financial strain

The next set of questions concern the types of difficulty that can arise because of economic problems. Please indicate what is true for you at the present time:

At the present time:		**No difficulty**	**With some difficulty**	**Very great difficulty**
1.	Are you able to afford furniture or household equipment that needs to be replaced?	○	○	○
2.	Do you have enough money for the kind of food you and your family should have?	○	○	○
3.	Do you have problems in paying your bills?	○	○	○
4.	Are you able to afford to replace major items (such as a car) when you need to?	○	○	○
5.	Do you have enough money for the leisure activities you and your family want?	○	○	○
6.	Are you able to afford a home suitable for you and your family?	○	○	○
7.	At the end of the month, do you have: *(please circle)*	Some money left over	Just enough to make ends meet	Not enough to make ends meet

8. What is the total current yearly amount you receive from your wage, benefit allowances, annual salary or other sources (e.g. investments) (before tax is deducted)? Please mark one circle.

- ○ Less than £9,999
- ○ £10,000 - £14,999
- ○ £15,000 - £19,999
- ○ £20,000 - £24,999
- ○ £25,000 - £34,999
- ○ £35,000 - £49,999
- ○ £50,000 - £69,999
- ○ More than £70,000

9. How many people (including yourself) contributed to your household finances? (*e.g. partner, children, parents)* _____ *people*

10. What total income (including your own) has your household received in the last 12 months?

- ○ Less than £9,999
- ○ £10,000 - £14,999
- ○ £15,000 - £19,999
- ○ £20,000 - £24,999
- ○ £25,000 - £34,999
- ○ £35,000 - £49,999
- ○ £50,000 - £69,999
- ○ £70,000 - £99,999
- ○ £100,000 - £199,999
- ○ More than £200,000

Education

What educational qualifications do you have?Please mark the circle next to your highest qualification.

- ○ None
- ○ CSEs or equivalent
- ○ GCSEs, O Levels, etc or equivalent
- ○ A levels
- ○ HNC/HND
- ○ GNVQ
- ○ Modern apprenticeship
- ○ Diploma
- ○ Degree
- ○ Postgraduate (e.g. MBA, Ph.D)
- ○ Other (please specify) _____

Physical Activity

1. In general, how would you say that your health has been in the past month?

○	○	○	○	○
Excellent	Very good	Good	Fair	Poor

How often do you take part in sports or activities that are mildly energetic, moderately energetic or vigorous? (Mark one circle only for each item)

		Three times or more a week	Once to twice a week	About once to three times a month	Never / hardly ever
2.	Mildly energetic *(e.g. walking, woodwork, weeding, hoeing, bicycle repair, general housework)*	○	○	○	○
3.	Moderately energetic *(e.g. cycling, dancing, scrubbing, dancing, golf, decorating, lawn mowing, leisurely swimming*)	○	○	○	○
4.	Vigorous *(e.g. running, hard swimming, tennis, squash, digging, cycle racing)*	○	○	○	○

Social Support

For each of the following statements and/or questions, please circle the point on the scale that you feel is most appropriate in describing you.

		Often	**Sometimes**	**Not often**	**Never**
1.	If I wanted to go on a trip for a day (for example, to the seaside), I would have a hard time finding someone to go with me.	○	○	○	○
2.	I feel that there is no one I can share my most private worries and fears with.	○	○	○	○

		Often	Sometimes	Not often	Never
3.	If I were ill, I could easily find someone to help me with my daily chores.	○	○	○	○
4.	There is someone I can turn to for advice about handling problems with my family.	○	○	○	○
5.	If I decide one afternoon that I would like to go to a film that evening, I could easily find someone to go with me.	○	○	○	○
6.	When I need suggestions on how to deal with a personal problem, I know someone I can turn to.	○	○	○	○
7.	I don't often get invited to do things with others.	○	○	○	○
8.	If I had to go out of town for a few weeks, it would be difficult to find someone who would look after my house/flat (the plants, pets, etc.).	○	○	○	○
9.	If I wanted to have lunch with someone, I could easily find someone to join me.	○	○	○	○
10.	If I was stranded 10 miles from home, there is someone I could call who could come and get me.	○	○	○	○
11.	If a family crisis arose, it would be difficult to find someone who could give me good advice about how to handle it.	○	○	○	○
12.	If I needed some help in moving to a new house or flat, I would have a hard time finding someone to help me.	○	○	○	○

Hair-related Questions

1. What is you **natural** hair colour?

○ Blond	○ Dark blond	○ Brown
○ Dark brown/black	○ Red	○ Grey/white

2. What is your hair curvature?

○ Straight	○ Wavy/ a little curly	○ Very curly

3. How often do you wash your hair per week?

○ Every day	○ 3-5 times	○ 1-2 times

4. Did you have any hair treatment in the last months?

○ No ○ Yes

↬ Which one and when (date/colour name)?

○ Hair dye (_____) Current Colour (_____)	○ Tinting (_____) Current Colour (_____)
○ Permanent straightening (_____)	○ Permanent Wave (_____)

○ Other (please specify): _____

5. Do you use hair straighteners or curling tongs?

○ No ○ Yes, straighteners ○ Yes, curling tongs ○ Yes, both

6. Do you use hair products?

○ No ○ Yes, hair spray ○ Yes, gel/wax ○ Yes, other commercial hair products

`

7. Any comments related to your hair?

_____

Are you female?
2-year medical student?

Then take part in our study and get £50

We are trying to understand how self-reported stress and coping relate to biological function, such as cortisol levels.

Participation in the study involves **two** time-periods: September/October 2013 **and again** in May 2014

Cortisol and Academic Stress Study
Email: Bianca.serwinski.11@ucl.ac.uk
Tel. 07552622553

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Appendix 6. Participant Information Sheet (Study III)

PARTICIPANT INFORMATION SHEET (Confidential)

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

The experience of stressful events or periods is associated with increased cortisol levels. However, certain individual characteristics, such as adaptive coping or optimism, seem to play a role in attenuating this effect. This research aims to investigate whether this relationship can be seen in both, salivary and hair cortisol levels. This study is part of a PhD project supervised by Professor Andrew Steptoe from the Research Department of Epidemiology and Public Health, UCL.

Who can take part?

This study is being carried out with healthy students aged 18 to 45 years old. Volunteers should not be on any regular medications except for oral contraceptives. If you have suffered from a serious illness such as heart disease or cancer over the past two years, you will not be suitable for the study.

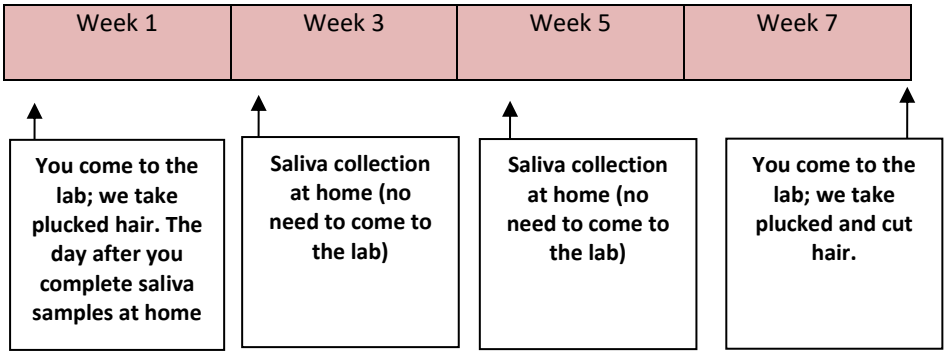
What will happen during the study?

The study will take several measures, and some of them will be taken repeatedly. You will need to be able to commit to all assessments, which includes the period during your revision. We realise that the exam period is a very busy time for you but we can assure you that the meeting in the lab and the subsequent measures you will be required to do will not take a lot of time.

At the beginning of the study we will meet you in our research lab in October. We will ask you to complete an online questionnaire on psychological measures and lifestyle factors such as smoking (you can do this from home). We will meet you two times within a period of 7 weeks; at the beginning and at the end.

At the first meeting, we will explain you the procedure of the study, take anthropometric measurements, hair samples and provide you with a pack for saliva collection. We will explain you how to collect these, as you will need to take 5 saliva samples over a 24 hour period; you will do these by yourself at home on three days within the seven week period at two-week intervals. We take these samples to look at the stress hormone cortisol. At each of the three time-points you will also need to fill in an online questionnaire which will take only a few minutes. This is that we can look for changes in these measures. Then at the end of the period, we meet you again in the lab and take further hair samples.

A couple of months later (in April/ May before and at the end of your exam period), we will repeat exactly the same procedure, meeting you two times in a period of 7 weeks and you will take saliva samples and complete questionnaires at three time points at two-week intervals.



Above, we have mentioned that we will ask you to give us some samples of saliva over a 24 hour period. The saliva samples are taken by chewing gently on a cotton roll for two minutes, then putting the wet cotton roll into a test tube. We want to collect 5 saliva samples over the day. We will ask you to return the saliva samples, but we would be happy to collect them from somewhere around the campus if that is going to be more convenient for you. As for the hair sampling, at each period at the first meeting a few hair strands will be plucked at different areas form the back bone of your head and at the last meeting, some hair will be plucked again and an additional hair sample from the back bone will be cut. This is to investigate hair cortisol content. Plucking hair does not cause considerable pain or distress and has been often used in previous research. Finally, you do not need to worry about the removal of the hair strands, as this is a region below all the hair which is not visible.

The collected information is completely confidential; results will not be available to anyone outside the study group and will only be used anonymously.

What if I change my mind during the study?
If at any point for any reason you do not want to carry on, then you may stop. There are no consequences of withdrawal from the study, other than forfeiting the honorarium payment (see below).

What happens to the information?
All the information we get from this study about you, including your name, will be confidential and will only be used for research purposes. The data will be collected and stored in accordance with the 1998 Data Protection Act. The data we collect from all volunteers will be combined, and it will not be possible to identify any individual within published results.

What happens at the end of the study?
Provided you have completed all the parts of the study we will give you an honorarium of £50. When the study is complete and all the results are analysed, we will send you a summary of our findings.

Can I take part if I am pregnant?
There are no risks to taking part in the study if you are pregnant. However, because pregnancy has effects on some of measures, we do not wish pregnant women to participate.

We hope you are able and willing to take part in our study.

If you have any questions, please contact Bianca Serwinski (bianca.serwinski.11@ucl.ac.uk), Tel. 020 7679 8248 or 07552622553.

Psychobiology Group, Research Department of Epidemiology and Public Health, 1-19 Torrington Place, London WC1E 6BT.

Appendix 7. Informed Consent Form (Study III)

Confidential: Informed Consent Form

Project title: Cortisol Output during Academic Stress

Have you read the information sheet about this study? Yes / No

Have you had the opportunity to ask questions and discuss the study? Yes / No

Have you received satisfactory answers to all your questions? Yes / No

Have you received enough information about the study?
Yes / No

Do you understand that you are free to withdraw from the study at any time, without giving a reason for withdrawal?
Yes / No

Do you agree with the publication of the results of this study in appropriate outlets? Yes / No

Do you agree to take part in this study? Yes / No

Signature of participant:

Signature of investigator:

Date:

If you have any further questions about the study, please contact:

Bianca Serwinski (bianca.serwinski.11@ucl.ac.uk)
Psychobiology Group,
Research Department of Epidemiology and Public Health,
1-19 Torrington Place,
London WC1E 6BT

Tel. 020 7679 8248 (internal 48248).
07552622553

If you wish to complain about any aspect of the way you have been approached or treated during the course of the study, you should email the Chair of the UCL Committee for the Ethics of Non-NHS Human Research (gradschoolhead@ucl.ac.uk) or send a letter to: The Graduate School, North Cloisters, Wilkins Building, UCL, Gower Street, London WC1E 6BT.

Appendix 8. Questionnaire (Study III)

Perceived Stress Scale (PSS)

Please indicate your feelings and thoughts during **the last week**.

		Never	Almost never	Sometimes	Fairly often	Very often
1.	How often have you been upset because of something that happened unexpectedly?	○	○	○	○	○
2.	How often have you felt that you were unable to control the important things in your life?	○	○	○	○	○
3.	How often have you felt nervous and "stressed"?	○	○	○	○	○
4.	How often have you felt confident about your ability to handle your personal problems?	○	○	○	○	○
5.	How often have you felt that things were going your way?	○	○	○	○	○
6.	How often have you found that you could not cope with all the things that you had to do?	○	○	○	○	○
7.	How often have you been able to control irritations in your life?	○	○	○	○	○
8.	How often have you felt that you were on top of things?	○	○	○	○	○
9.	How often have you been angered because of things that were outside of your control?	○	○	○	○	○
10.	How often have you felt difficulties were piling up so high that you could not overcome them?	○	○	○	○	○

Anxiety (The State-Trait Anxiety Inventory; STAI)

Please indicate how you feel **right now**, that is, at this very moment. Do not spend too much time on any one statement but give the answer which seems to describe your present feelings best

		Not at all	A little	Somewhat	Very Much So
1.	I feel calm	○	○	○	○
2.	I feel secure	○	○	○	○
3.	I feel tense	○	○	○	○
4.	I feel strained	○	○	○	○
5.	I feel at ease	○	○	○	○
6.	I feel upset	○	○	○	○
7.	I am presently worrying over possible misfortunes	○	○	○	○
8.	I feel satisfied	○	○	○	○
9.	I feel frightened	○	○	○	○
10.	I feel uncomfortable	○	○	○	○
11.	I feel self-confident	○	○	○	○
12.	I feel nervous	○	○	○	○

13.	I feel jittery	○	○	○	○
14.	I feel indecisive	○	○	○	○
15.	I am relaxed	○	○	○	○
16.	I feel content	○	○	○	○
17.	I am worried	○	○	○	○
18.	I feel confused	○	○	○	○
19.	I feel steady	○	○	○	○
20.	I feel pleasant	○	○	○	○

Coping (Brief Cope)

We are interested in how people respond when they confront difficult or stressful events in their lives. There are many ways to try and deal with stress. The next series of questions asks you what you did and felt in the last week when you experienced a stressful event. Try to rate each item separately from the other items.

1	2	3	4
I didn't do this at all	I did this a little bit	I did this a medium amount	I did this a lot.

1.	I turned to work or other activities to take my mind off things.	1	2	3	4
2.	I concentrated my efforts on doing something about the situation I'm in.	1	2	3	4
3.	I said to myself "this isn't real."	1	2	3	4
4.	I used alcohol or other drugs to make myself feel better.	1	2	3	4
5.	I got emotional support from others.	1	2	3	4
6.	I gave up trying to deal with it.	1	2	3	4
7.	I took action to try to make the situation better.	1	2	3	4
8.	I refused to believe that it has happened.	1	2	3	4
9.	I said things to let my unpleasant feelings escape.	1	2	3	4
10.	I got help and advice from other people.	1	2	3	4
11.	I used alcohol or other drugs to help me get through it.	1	2	3	4
12.	I tried to see it in a different light, to make it seem more positive.	1	2	3	4
13.	I criticised myself.	1	2	3	4
14.	I tried to come up with a strategy about what to do.	1	2	3	4

15.	I got comfort and understanding from someone.	1	2	3	4
16.	I gave up the attempt to cope.	1	2	3	4
17.	I looked for something good in what is happening.	1	2	3	4
18.	I made jokes about it.	1	2	3	4
19.	I did something to think about it less, such as going to movies, watching TV, reading, daydreaming, sleeping, or shopping.	1	2	3	4
20.	I accepted the reality of the fact that it has happened.	1	2	3	4
21.	I expressed my negative feelings.	1	2	3	4
22.	I tried to find comfort in my religion or spiritual beliefs.	1	2	3	4
23.	I tried to get advice or help from other people about what to do.	1	2	3	4
24.	I learned to live with it.	1	2	3	4
25.	I thought hard about what steps to take.	1	2	3	4
26.	I blamed myself for things that happened.	1	2	3	4
27.	I prayed or meditated	1	2	3	4
28.	I made fun of the situation.	1	2	3	4

Specific stressors

Think about the past month. Did anything particularly bad, upsetting or stressful happen during this time? Would you rate that as a stressful life experience?

Yes **No**

_____

		Not at all	**A little**	**Somewhat**	**Very Much So**	**N/A**
1.	In the past month has your relationship with your partner been stressful?	○	○	○	○	○
2.	In the past month has your relationship with your family been stressful?	○	○	○	○	○
3.	In the past month has work been stressful?	○	○	○	○	○
4.	Have you experienced any illnesses in the past month that you have found stressful?	○	○	○	○	○
5.	In the past month have there been any financial issues that have been stressful?	○	○	○	○	○
6.	In the past month have you felt more tired/ fatigued than usual?	○	○	○	○	○
7.	In the past month have you felt stressed by the neighbourhood you live in (e.g. because of litter in the streets, traffic, noises)?	○	○	○	○	○

Hair-related questions

And finally a few question about your hair:

1. What is you **natural** hair colour?

○ Blond ○ Dark blond ○ Brown

○ Dark brown/black ○ Red ○ Grey/white

2. What is your hair curvature?

○ Straight ○ Wavy/ a little curly ○ Very curly

3. How often do you wash your hair per week?

○ Every day ○ 3-5 times ○ 1-2 times

4. Do you daily/weekly/occasionally use hair straighteners or curling tongs?

○ No ○ Yes, straighteners ○Yes, curling tongs ○ Yes, both

5. Have you permed your hair in the last a) 2 weeks, b) 4 weeks?

○ No ○ Yes, straighteners When? _____

6. Have you coloured your hair in the last a) 2 weeks, b) 4 weeks?

○ No ○ Yes, straighteners When? _____

7. What type of shampoo do you normally use?
You may choose more than 1 option
○ Do not use shampoo
○ Beauty shampoo
○ Anti-dandruff shampoo
○ Medicated shampoo
○ Hair and body gel
○ Unknown

Appendix 9. Salivary cortisol diary (Study III)

Cortisol Output during Academic Stress Study Phase 2

Saliva sample diary

Please put your samples in the fridge and return them as soon as possible

If you have any questions, please contact Bianca (bianca.serwinski.11@ucl.ac.uk), 020 7679 8248 or 07552622553

Thank you very much.

THE SALIVA SAMPLES

Over the course of today and tomorrow morning you will be collecting saliva samples at 5 different times, starting with the first sample during your meeting with us in the research lab. Please collect the samples at the times listed in the table opposite. It may be helpful to set an alarm on your watch or phone to remind you beforehand. Each time you collect a sample, please mark the questions in this booklet. There are separate questions (one set per page) for each sample.

Please place the tube for the waking sample (Sample 4) and this booklet next to your bed before you go to sleep tonight.

Your honesty is very important to us in analysing the results. Please write down the actual collection time, even if it is different to the designated time, and answer the questions as accurately as possible.

Instructions:

1. Do not smoke, eat or drink anything for **30 minutes** before you collect the sample.
2. Remove the small plastic cap, and place the cotton swab in your mouth, avoiding touching it with your hands.
3. Gently chew on the swab until it is soaked, this will usually take about 2 minutes. While you are doing this, answer the questions for this sample in this booklet.
4. Once the swab is soaked, place it back in the tube, trying not to use your hands. Put the cap on securely, and place the tube in the plastic bag provided.
5. Store the bagged tubes in a cold place or in a refrigerator until you bring it in.

SALIVA SAMPLE COLLECTION TIMES

Sample Time	Tube No.	Instructions
TODAY: **Please date!**		
11am Today	1	Remember to take this with you if you are going out.
3pm Today	2	Remember to take this with you if you are going out.
Bedtime Today	3	Take this sample just before going to bed. Remember to put **Sample 4** and your saliva sample diary next to your bed, ready for when you wake up.
TOMORROW:		
Waking	4	This sample should be collected as soon as you wake up, and **before** you get out of bed.
30 minutes after waking	5	Take this sample 30 minutes after your waking sample. Do not have any caffeinated drinks, brush your teeth or eat before you collect this sample.

SAMPLE 1 (11 AM)

1. ***What was the exact time you collected the sample?(e.g. 11.10*** (a.m.) / p.m.) a.m. / p.m.

In the last 30 minutes did you feel…..

		Not at all				*Very much*
2.	Happy?	**1**	**2**	**3**	**4**	**5**
3.	Tired?	**1**	**2**	**3**	**4**	**5**
4.	Sad?	**1**	**2**	**3**	**4**	**5**
5.	Stressed?	**1**	**2**	**3**	**4**	**5**
6.	Frustrated or angry?	**1**	**2**	**3**	**4**	**5**

In the 30 minutes prior to collecting the sample did you….

8.	Brush your teeth?	**No**	**Yes**
9.	Drink any tea, coffee or other caffeinated drinks?	**No**	**Yes**
10.	Take any medicines?	**No**	**Yes**
11.	Eat a meal?	**No**	**Yes**
12.	Drink any alcohol?	**No**	**Yes**
13	Do any exercise?	**No**	**Yes**
14.	Smoke any cigarettes?	**No**	**Yes**

SAMPLE 2 (3 PM)

1. ***What was the exact time you collected the sample?*** a.m. / p.m.

In the last 30 minutes did you feel…..

		Not at all				*Very much*
2.	Happy?	1	2	3	4	5
3.	Tired?	1	2	3	4	5
4.	Sad?	1	2	3	4	5
5.	Stressed?	1	2	3	4	5
6.	Frustrated or angry?	1	2	3	4	5

In the 30 minutes prior to collecting the sample did you….

8.	Brush your teeth?	No	Yes
9.	Drink any tea, coffee or other caffeinated drinks?	No	Yes
10.	Take any medicines?	No	Yes
11.	Eat a meal?	No	Yes
12.	Drink any alcohol?	No	Yes
13.	Do any exercise?	No	Yes
14.	Smoke any cigarettes?	No	Yes

SAMPLE 3 (BEDTIME)

1. ***What was the exact time you collected the sample?*** a.m. / p.m.

In the last 30 minutes did you feel…..

		Not at all				*Very much*
2.	Happy?	1	2	3	4	5
3.	Tired?	1	2	3	4	5
4.	Sad?	1	2	3	4	5
5.	Stressed?	1	2	3	4	5
6.	Frustrated or angry?	1	2	3	4	5

In the 30 minutes prior to collecting the sample did you….

8.	Brush your teeth?	No	Yes
9.	Drink any tea, coffee or other caffeinated drinks?	No	Yes
10.	Take any medicines?	No	Yes
11.	Eat a meal?	No	Yes
12.	Drink any alcohol?	No	Yes
13	Do any exercise?	No	Yes
14.	Smoke any cigarettes?	No	Yes

SAMPLE 4 (WAKING SAMPLE)

1. ***What was the exact time you collected the sample?*** a.m. / p.m.

1a. Was there a delay between waking up and collecting your first sample? Yes No

1b. ↳If yes, how long?

↳If yes, in the last 30 minutes did you feel…..

		Not at all				*Very much*
2.	Happy?	1	2	3	4	5
3.	Tired?	1	2	3	4	5
4.	Sad?	1	2	3	4	5
5.	Stressed?	1	2	3	4	5
6.	Frustrated or angry?	1	2	3	4	5

In the time since you woke, but before you collected your sample did you….

8.	Brush your teeth?	No	Yes
9.	Drink any tea, coffee or other caffeinated drinks?	No	Yes
10.	Take any medicines?	No	Yes
11.	Eat a meal?	No	Yes
12.	Drink any alcohol?	No	Yes
13.	Do any exercise?	No	Yes
14.	Smoke any cigarettes?	No	Yes

SAMPLE 5 (30 MINUTES AFTER WAKING)

1. ***What was the exact time you collected the sample?*** a.m. / p.m.

In the last 30 minutes did you feel…..

		Not at all				*Very much*
2.	Happy?	1	2	3	4	5
3.	Tired?	1	2	3	4	5
4.	Sad?	1	2	3	4	5
5.	Stressed?	1	2	3	4	5
6.	Frustrated or angry?	1	2	3	4	5

In the 30 minutes prior to collecting the sample did you….

8.	Brush your teeth?	No	Yes
9.	Drink any tea, coffee or other caffeinated drinks?	No	Yes
10.	Take any medicines?	No	Yes
11.	Eat a meal?	No	Yes
12.	Drink any alcohol?	No	Yes
13	Do any exercise?	No	Yes
14.	Smoke any cigarettes?	No	Yes

That was the last sample. Please put your samples in the fridge and return them as soon as possible.

Appendix 10. Reminders to participants (Study III)

Text message reminding participants to take saliva sample

Dear Participant, it is time to take your next saliva sample - please respond with "OK" and the 6-digit number on the sample, e.g. P2 T1 S3" at the exact time when you have taken it. Thank you

1st Reminding Cortisol Sampling/Questionnaire 1

Hi,

I want to remind you to read the diary and prepare the diary and saliva tubes for tomorrow to have it with you, so you are ready to do the sample at 11am and at 3pm (when you are on the go). Please put the reminder into your phone if you have not done so. Please answer quickly the few questions in the diary after taking each sample. Please remember the morning samples on Thursday (and to stick to the 30min.) - they are very important. Adherence to sampling times is crucial for me to get a proper diurnal cortisol profile – sorry that I say this again.

This is the link to the online questionnaire; it would be great if you could fill it in until Thursday (you can save and return at another point to continue the survey/always save it correctly).

Your ID is: P__

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