The effect of chronic stress on body composition and mineral status among children

Use of hair as a biological matrix

Barbara Vanaelst

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Department of Public Health

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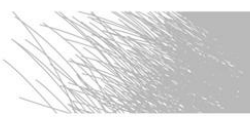

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Department of Public Health

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List of abbreviations

11β-HSD 11beta-hydroxysteroid dehydrogenase

ACTH adrenocorticotropic hormone

ADP air displacement plethysmography

AUC area under the curve

AUCg area under the curve with respect to the ground

AVP arginin vasopressin

BF% body fat percentage

BMI body mass index

Ca calcium

ACN acetonitrile

CAR cortisol awakening response

CBG cortisol-binding-globulin

CEHQ-FFQ Children's Eating Habits Questionnaire – Food Frequency Questionnaire

ChiBS Children's Body composition and Stress

CHS-CUS Children's Daily Hassles and Daily Uplifts Scale

CI confidence interval

CLES Coddington Life Events Scale

Co copper

CRH corticotropic-releasing hormone

DBP diastolic blood pressure

DCM dichloromethane

DEBQ Dutch Eating Behaviour Questionnaire

ECLIA electrochemiluminescence immunoassay

Fe iron

FFQ food frequency questionnaire

FSA familial and social adversities

GR glucocorticoid receptors

GRE glucocorticoid-response elements

HCOOH formic acid

HDL high density lipoprotein

HOMA-IR homeostasis model assessment for insulin resistance

HPA hypothalamus-pituitary-adrenal axis

HRV Heart rate variability

ICP-MS inductively coupled plasma mass spectrometry

IDEFICS Identification and prevention of Dietary- and lifestyle-induced health Effects In Children and infantS

ISCED International Standard Classification of Education

LC-MS/MS liquid chromatography tandem mass spectrometry

LOD limit of detection

LOQ limit of quantification

LPL lipoprotein lipase

MeOH methanol

Mg magnesium

MR mineralocorticoid receptor

MRM multiple reactions monitoring

Na sodium

NLE negative life events

NPY neuropeptide Y

OR odds ratio

P phosphorus

PA physical activity

PES psychosomatic and emotional symptoms

PTSD post-traumatic stress disorder

Q quartile

RRR reduced rank regression

RSD relative standard deviation

S/N signal to noise ratio

SAM sympathetic-adrenal-medullary system

SBP systolic blood pressure

SD standard deviation

SDQ Strengths and Difficulties Questionnaire

UFC urinary free cortisol

UPLC-MS/MS ultra performance liquid chromatography tandem mass spectrometry

WHtR waist to height ratio

YHEI youth healthy eating index

Zn zinc

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PART 1 GENERAL INTRODUCTION

Chapter based on

Vanaelst B, De Vriendt, T, Huybrechts I, Rinaldi S, De Henauw S. ***Epidemiological approaches to measure childhood stress****. Paediatric and Perinatal Epidemiology (2012), 26: 280-297.*

CHAPTER 1.1 DEFINITION AND PREVALENCE OF CHILDHOOD STRESS

1 Definition of childhood stress

In daily life various connotations are given to the term 'stress', mainly reflecting conditions of having too much work, feeling tensed etc. By definition however, stress is described as '*the process in which environmental demands or events are interpreted or appraised by the individual as taxing or exceeding his/her resources and endangering well-being*' (Folkman S. et al., 1986). In this definition three stress components can be distinguished: 1) the sources of stress or so-called 'stressors' which can be of physical (e.g. injury, noise), chemical (e.g. smoke), biological (e.g. viral infection) or psychosocial nature (e.g. family, school), 2) the mental interpretation and affective response given to these stressors, and 3) the biological stress response provoked by exposure to the stressor (Cohen S. et al., 1997a).

Although research already highlighted different stress-related health problems among adults, research over the last decade acknowledged also children as being susceptible to stress. Sandberg defined childhood stress as 'any intrusion into the child's normal physical or psychosocial life experiences that acutely or chronically unbalances the child's physiological or psychological equilibrium, threatens their security or safety, or distorts their physical or psychological growth/development and the psychophysiological consequences of such an intrusion or distortion' (Sandberg S., 2007).

Although childhood stress may sometimes be beneficial (e.g. if the stressor is limited in duration, mastered by the child and results in feelings of self-achievement), prolonged and recurrent exposure to emotionally or physiologically challenging experiences, so-called 'chronic stress', may adversely affect the child's behaviour, personality development and physical health. These stress-related health effects may potentially persist into adolescence and adulthood, as further discussed in *Chapter 1.2. Biology and health consequences of stress* (Cohen S. et al., 2007; Mcewen B. S., 1998; Schneiderman N. et al., 2005; Teicher M. H. et al., 2003; Silber T. J. et al., 2003). In this thesis, childhood is defined as the period between the first life years and adolescence, encompassing preschool years (2 to 5 years old) and middle childhood (6 to 11 years old) (Kliegman R. M. et al., 2011).

2 Prevalence of childhood stressors

The current research interest in childhood stress is reflected by the large number of studies and projects that have been undertaken to study one or more aspects in the broad domain of childhood stress, going from descriptive prevalence research to more mechanistic studies into the adverse health consequences of childhood stress. Examples of large-scale research projects into childhood stress are given in Table 1.1.

Table 1.1 Examples of large-scale research projects into one or more aspects of childhood stress

Name of project	**Involved countries and website**
EuroSTRESS - Stress and Mental Health Project	Pan-European, http://www.esf.org/activities/eurocores/completed-programmes/eurostress.html
CROLS Family Project - Center for Research on Occupational and Life Stress	Ireland, http://crolsfamilyproject.wordpress.com/
SCMHE Project - School Children Mental Health Europe	Bulgaria, Germany, Italy, Lithuania, Netherlands, Romania and Turkey, http://www.scmheproject.com/
BECAN study - Balkan Epidemiological Study on Child Abuse and Neglect	Greece, Albania, Bosnia, Herzegovina, Bulgaria, Croatia, FYR of Macedonia, Romania, Serbia, Turkey, http://www.becan.eu/
the Fragile Families and Child-Wellbeing study	USA, http://www.fragilefamilies.princeton.edu/
the 2010 Stress in America survey - American Psychological Association	USA, http://www.apa.org/news/press/releases/stress/national-report.pdf
the TRAILS Study - TRacking Adolescents' Individual Lives Survey	the Netherlands, http://www.trails.nl/

In a population of American and Mexican adolescents/young adults, parental separation, familial economic adversity and witnessing domestic violence were the most frequently reported adverse childhood experiences (27.5%, 24.5% and 19%, respectively) (Schilling E. A. et al., 2007; Benjet C. et al., 2009). A study in the United Kingdom indicated prevalence percentages of 29%, 41%, 27% and 31% for parental divorce, family discord, financial difficulties and maternal psychiatric illness in childhood and early adolescence (0-14 years old) (Dunn V. J. et al., 2011). Half of the children (aged 9-13 years old) reported previous

trauma exposure in the Great Smokey Mountains Study (North Carolina, USA) (Copeland W. E. et al., 2007), while in the Healthy Passages study (Alabama, California, Texas; USA) 76% of the fifth graders (9-11 years old) reported exposure to at least one family-related stressful life event in the past year (Coker T. R. et al., 2011). Furthermore, one fourth of the participants in a North Carolina adolescent population reported the experience of at least one childhood/adolescent high magnitude event by the age of 16 (Costello E. J. et al., 2002), while this percentage was more than 80% for a population of German children at primary school entry (Furniss T. et al., 2009). For Brazilian fourth-grade elementary school children, a 18% stress prevalence was reported (Sbaraini C. R. et al., 2008). Concerning school-related stressors, 24% of primary school children reported being bullied very frequently (English population) (Wolke D. et al., 2001), and 78% of primary school children continuously felt pressure to perform well at school (Chinese population) (Hesketh T. et al., 2010).

The overall impression from literature is that the prevalence of adverse, stressful life events in children is high all over the world but varies largely between studies and countries because of differences in study participants and methodology used (e.g. the type of stressors being assessed, the age, ethnicity and culture of the children and the data collection methods). Furthermore, the existing literature on childhood stress has indicated a variety of stressors where children may be exposed to, with as main origin the child's everyday surroundings. Table 1.2 presents examples of potential psychosocial stressors originating from the child's everyday environment.

Moreover, the presented prevalence percentages demonstrate that childhood adversity is very often connected to the family environment, highlighting the importance to broaden the childhood stress perspective from single forms of adversity to the larger context of multiple, cumulating adversities in the family environment. After all, family stressors may not be isolated events, but cluster together or give rise to other unfavourable events. For example the increased divorce rates in Western Europe may impact the children through a diversity of everyday life changes such as a changed family structure, decreased economic resources and parental strains, and as a result create a high-risk living context for the children (Allan G. et al., 2001; Coker T. R. et al., 2011; Turner H. A. et al., 2012; Dunn V. J. et al., 2011).

Table 1.2 Potential psychosocial stressors in the child's everyday environment

Family life	socio-economic situation, parent's depression, divorce or unemployment, marital conflict or violence, child abuse or neglect, new baby sibling, death of a family member, fights with siblings, having too many things to do (Gustafsson P. E. et al., 2009; Ram B. L. et al., 2003; Ryan-Wenger N. A. et al., 2005; Coker T. R. et al., 2011; Turner H. A. et al., 2012)
Kindergarten, school and community environment	actual or perceived discrimination by teachers, being bullied by other children at school, peer pressure, losing games, having difficulties making friends or racism, having learning disabilities, moving to another school, bad grades, bad performance at sports, too much homework, violent and criminal neighbourhoods (Brobeck E. et al., 2007; van der Wal M. F. et al., 2003; Wolke D. et al., 2001; Ryan-Wenger N. A. et al., 2005; Pittman T. P. et al., 2012)
Health issues and body image	being obese, being smaller or taller than others, having acne, asthma, severe allergies or diabetes, having motor disabilities, hospital admission (Pop-Jordanova N. et al., 2008; Gibson L. Y. et al., 2008; Silverman W. K. et al., 1995)
Multimedia	cyber-bullying (e.g. on social networking sites), suggestive advertising (Hamer M. et al., 2009; Page A. S. et al., 2010; Strasburger V. C. et al., 2012)

CHAPTER 1.2 BIOLOGY AND HEALTH CONSEQUENCES OF STRESS

1 The biological stress response

The human body reacts to daily events and stress through activation of the biological stress system, in order to maintain stability and to mobilize resources to cope with the stressful situations; a process which is called 'allostasis' or 'the ability to achieve stability through change' (Mcewen B. S., 2007). Allostasis is thus complementary to the process of 'homeostasis' which reflects the elementary maintenance of equilibrium in physiological parameters such as blood pressure, blood pH and body temperature within narrow ranges.

The stress system mainly consists of two pathways: 1) the sympathetic-adrenal-medullary system (SAM) which results in the acute secretion of catecholaminergic hormones (i.e. adrenalin and noradrenalin) and the consequent 'fight or flight response', and 2) the hypothalamus-pituitary-adrenal (HPA) axis resulting in the release of the cortisol hormone by the adrenal glands with longer-term systemic effects (Vanaelst B. et al., 2012b; Cohen S. et al., 1997a). This thesis will focus on the importance of the HPA axis and cortisol for chronic stress research: cortisol is preferred over catecholamines as candidate stress hormone as it is less transient and less responsive to immediate stimuli, less sensitive to exercise, cheaper to analyse, and it can be measured in more biological matrices compared to catecholamine measures (Baum A. et al., 1997).

Cortisol is secreted in a pulsatile, diurnal pattern under the influence of several hormones, as presented in Figure 1.1: Levels are highest on wakening in the morning and decline during the course of the day reaching nadir levels in the evening. The main fraction of circulating cortisol is bound (>90%), predominantly to cortisol-binding globulin (CBG). Only the free fraction of cortisol is available for transport to target tissues and subsequent biological activity: Cortisol performs its function on the genomic level, i.e. by up- or down-regulating gene transcription of glucocorticoid-responsive genes, evoking e.g. the mobilisation of energy stores and numerous other metabolic effects as exemplified in Figure 1.1 (Low M. J., 2011; Stewart P. M. et al., 2011; Vegiopoulos A. et al., 2007).

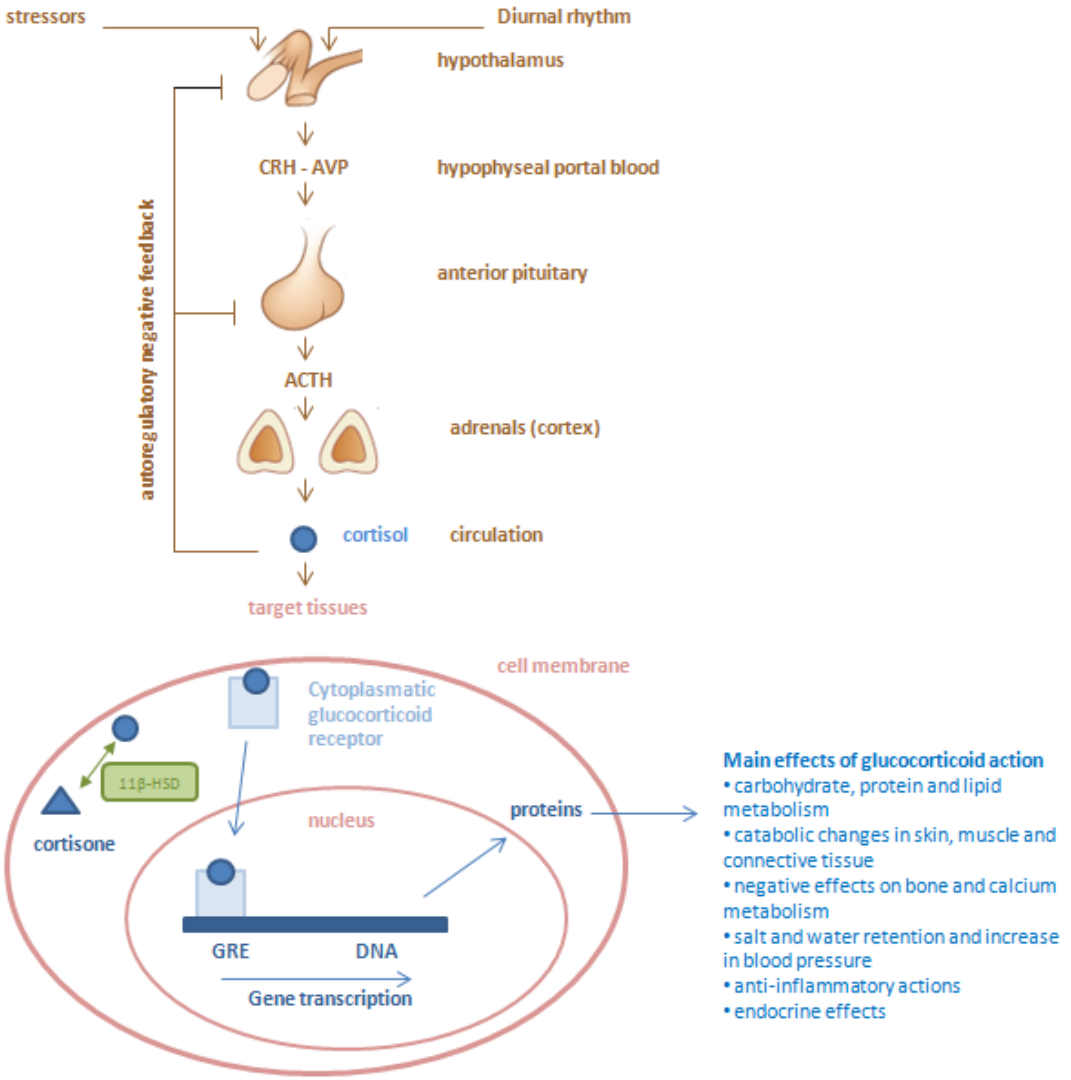


Figure 1.1 Illustration of the hypothalamus-pituitary-adrenal (HPA) axis and the mechanism of cortisol action: Corticotropic-releasing hormone (CRH) and arginin vasopressin (AVP) secretion is regulated by an inbuilt diurnal rhythm and additionally by stressor exposure operating through the hypothalamus. Under the influence of CRH and AVP, the adrenocorticotropic hormone (ACTH) is secreted by the anterior pituitary. Cortisol is produced in the adrenal cortex, which is the main target of ACTH, and is involved in termination of the stress response through a negative feedback control on CRH, AVP and ACTH production (basal HPA activity is regulated through cortisol binding on mineralocorticoid receptors, while stress-related HPA activity is regulated by glucocorticoid receptor binding). Cortisol exerts its physiological effects through binding intracellular glucocorticoid receptors (GR) in target tissue, where it translocates to the nucleus, binds to glucocorticoid-response elements (GRE) in the promoter regions of target genes and up- or down-regulates the expression of glucocorticoid-responsive genes. One of the main pathways

in cortisol metabolism is its conversion into the inactive metabolite cortisone by 11β-hydroxysteroid dehydrogenase (11β-HSD) (Stewart P. M. et al., 2011).

Cortisol is metabolized principally in the liver and kidney by enzymes such as 5α- and 5β-reductase, 3α- and 11β-hydroxysteroid dehydrogenase (HSD), although numerous peripheral tissues are involved in locally regulating glucocorticoid or cortisol hormone action: 11β-HSD is a key metabolizing enzyme mediating the intracellular conversion of cortisol into its inactive metabolite cortisone (11β-HSD2) and vice-versa (11β-HSD1) in peripheral tissues, resulting in tissue-specific regulation of glucocorticoid action (Figure 1.2) (Stewart P. M. et al., 2011).

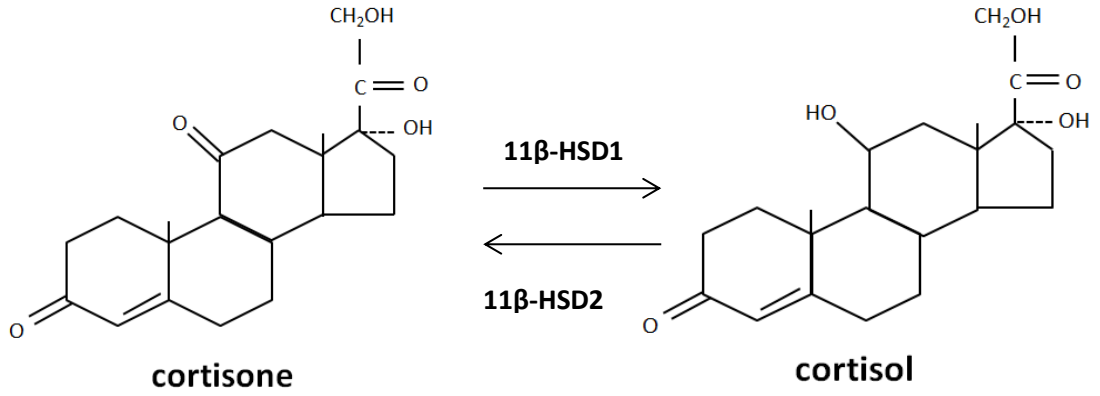


Figure 1.2 Conversion of hormonally active cortisol (containing 11-hydroxyl group) to the inactive cortisone (containing 11-oxo group) by 11β-HSD2, and activity of 11β-HSD1 doing the reverse (Stewart P. M. et al., 2011).

2 Health consequences of childhood stress

Timely activation and de-activation of the stress response system allows an individual to effectively handle a threat and return to normal function: episodic stressful experiences generally stimulate a normal, allostatic cortisol increase with subsequent normalisation of the cortisol levels as the children adapt, without imposing a health burden, as illustrated in the top panel of Figure 1.3. However, if these stressors are prolonged or become chronic and children fail to adapt to their new surroundings, the hormonal changes may no longer be reversible and become maladaptive. These maladaptive stress responses are characterized by an incapability to timely activate or de-activate the stress-system and thus a chronic under- or over-exposure to stress hormones. This state of chronic over- or under-activity of the allostatic stress system has been called ‘allostatic load’, as illustrated in Figure 1.3.

McEwen et al. defined four situations that may be associated with allostatic load. The first is frequent stress, leading to repeated hits from multiple stressors (top left panel in Figure 1.3). In the second type of allostatic load, there is a lack of adaptation to repeated stressors from the same type, resulting in prolonged exposure to stress hormones (top right panel in Figure 1.3). The third type of allostatic load is characterised by a prolonged response due to a delayed shut down after the stress is terminated (bottom left panel in Figure 1.3), while the last type of allostatic load comprises inadequate responses by some allostatic systems which may trigger compensatory increases in others (e.g. inadequate increases in cortisol secretion may lead to elevations in inflammatory cytokines) (bottom right panel in Figure 1.3) (Mcewen B. S., 2007). The nervous, the endocrine and the immune systems are three highly-integrated systems which are involved in allostatic processes or allostatic load and are therefore consequently implicated in numerous physical and mental health disorders, also in children, as described below (Danese A. et al., 2012; Charmandari E. et al., 2005).

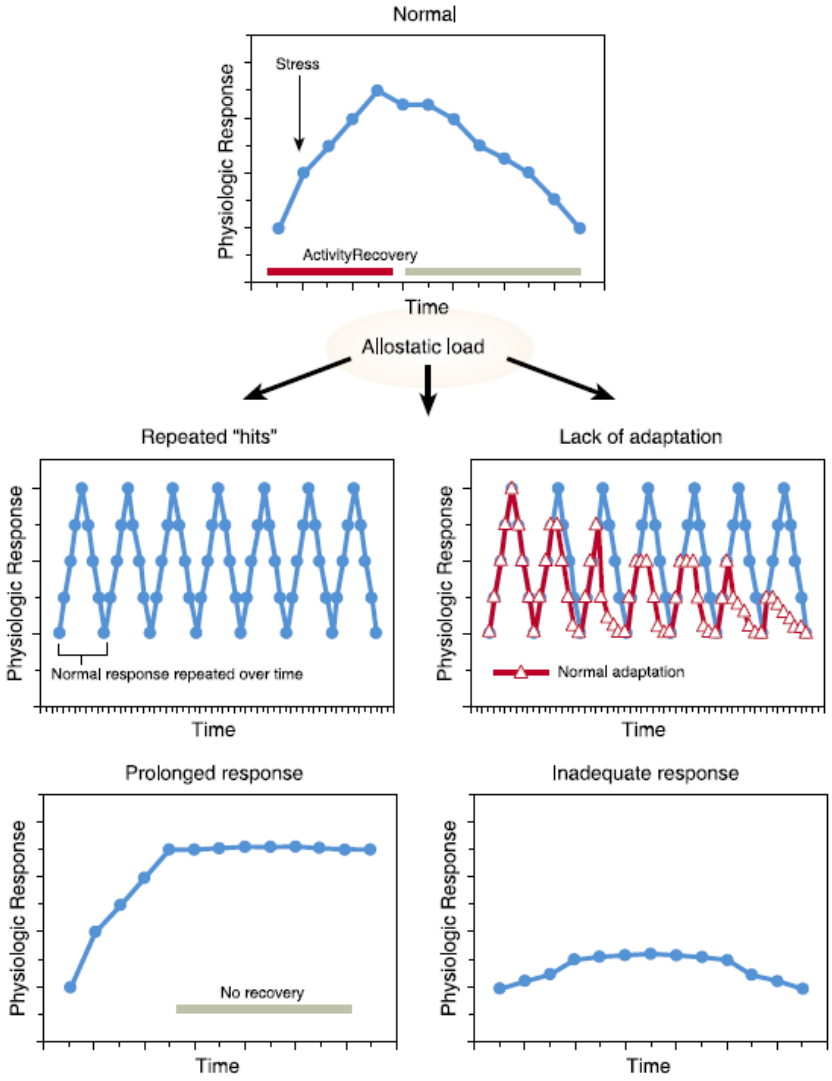


Figure 1.3 Schematic presentation of allostasis and allostatic load - The normal allostatic response is illustrated in the top panel and includes initiation, maintenance and recovery of the stress response. The four panels below present several potential forms of allostatic load. Figure reproduced with permission from (Mcewen B. S., 1998), Copyright Massachusetts Medical Society.

2.1 Developmental interferences

Infancy and childhood are characterized by a process of gradual physical growth and large brain plasticity, and are therefore considered developmental periods with an increased vulnerability to stressors: intense or chronic stress during these periods of life may have long-term and irreversibly effects on children's brain development, neuro-anatomy, cognitive and emotional development, growth and timing of puberty, and metabolism (Danese A. et al.,

2012; Sandberg S., 2007; Teicher M. H. et al., 2003; Sheridan M. A. et al., 2012; Gogtay N. et al., 2004; Pervanidou P. et al., 2011). There is also increasing evidence that experiences in early life may shape a person's stress response and later-life stress reactivity (Hunter A. L. et al., 2011).

2.2 Physical and mental health consequences

Work so far suggests that adverse childhood experiences influence health outcomes throughout the life-course by inducing significant biological changes in children which become apparent in adult life. In particular enduring changes in the nervous, endocrine and immune system have been shown, increasing the risk for cardiovascular disease, age-related metabolic disorders, exacerbations of autoimmune diseases, cellular aging, cognitive decline, etc. (Danese A. et al., 2012; Schneiderman N. et al., 2005; Nielsen N. M. et al., 2012; Pervanidou P. et al., 2012). According to the 'biological embedding of childhood adversity model' of Miller et al., a special role in these stress-related health effects should be reserved for the molecular programming of macrophages (through epigenetic markings, posttranslational modifications and tissue remodelling) which induces a pro-inflammatory phenotype, further exacerbated by stress-induced unhealthy behaviour and hormonal dysregulation. As a result, pathogenic mechanisms are stimulated that ultimately foster chronic disease (Miller G. E. et al., 2011).

Childhood psychosocial stress has been suggested to be a precipitating or provoking factor for certain illnesses such as asthma (Pittman T. P. et al., 2012; Sandberg S., 2007). Furthermore, due to functional abnormalities in the prefrontal cortex, childhood stress may result in psychological sequelae such as behavioural and emotional problems (e.g. affective deregulation, provocative or antisocial behaviour, anxiety, sleep disturbances and even depression in childhood and adolescence) (Danese A. et al., 2012; Schneiderman N. et al., 2005; Laceulle O. M. et al., 2012; Timmermans M. et al., 2010; Harland P. et al., 2002; Schilling E. A. et al., 2007; Benjet C. et al., 2010; Bouma E. M. C. et al., 2008). In this context, the combined increase in the prevalence of both childhood stress and psychosomatic complaints in children is worth mentioning, with headaches, stomach pain and tiredness being frequently observed in stressed children: 20 to 30% of children have been shown to suffer from headaches at least once a week (Hesketh T. et al., 2010; Alfven G. et al., 2008; Petersen S. et al., 2003; Silber T. J. et al., 2003; Milde-Busch A. et al., 2011). Also, childhood stress

has increasingly been recognized to affect the development of obesity (Gundersen C. et al., 2011), as discussed in the section below.

A. Obesity

Given the world-wide epidemic increase of overweight and obesity in young age groups and its numerous co-morbidities (increased risk for hypertension, cardiovascular disease, diabetes mellitus, shortened life expectancy etc.) (Cali A. M. G. et al., 2008; De Vriendt T. et al., 2009; Nieuwenhuizen A. G. et al., 2008; Bray G. A., 2004; Manios Y. et al., 2011), the association between chronic stress and the development of overweight and obesity in children is of further concern.

World-wide at least 110 million children are overweight or obese: In the European Union, the prevalence of childhood overweight and obesity ranges from 10-20% (northern European areas) to 20-40% (Mediterranean Sea countries) and is expected to rise by 1.3 million children per year (Cali A. M. G. et al., 2008; Manios Y. et al., 2011). Obesity is a condition of excessive body fat resulting from a chronic deregulation of the energy balance, with energy intake exceeding energy expenditure. Excessive caloric intake, insufficient physical activity and sleep deprivation are the major lifestyle factors involved in the development of obesity (Moreno L. A. et al., 2011). Recently however, the effects of chronic psychosocial stress as a risk factor for weight gain and adiposity have increasingly been recognized, also in children (Gundersen C. et al., 2011; Holmes M. E. et al., 2010; Pervanidou P. et al., 2011; Sgrenci A. E. et al., 2011; Vamosi M. et al., 2010).

Stress-related over-stimulation of the HPA axis (i.e. excess glucocorticoid exposure) is suggested to be the main driver of this stress-obesity relationship by interacting with energy homeostasis and fat metabolism, as illustrated in Figure 1.4 (Nieuwenhuizen A. G. et al., 2008; Bose M. et al., 2009).

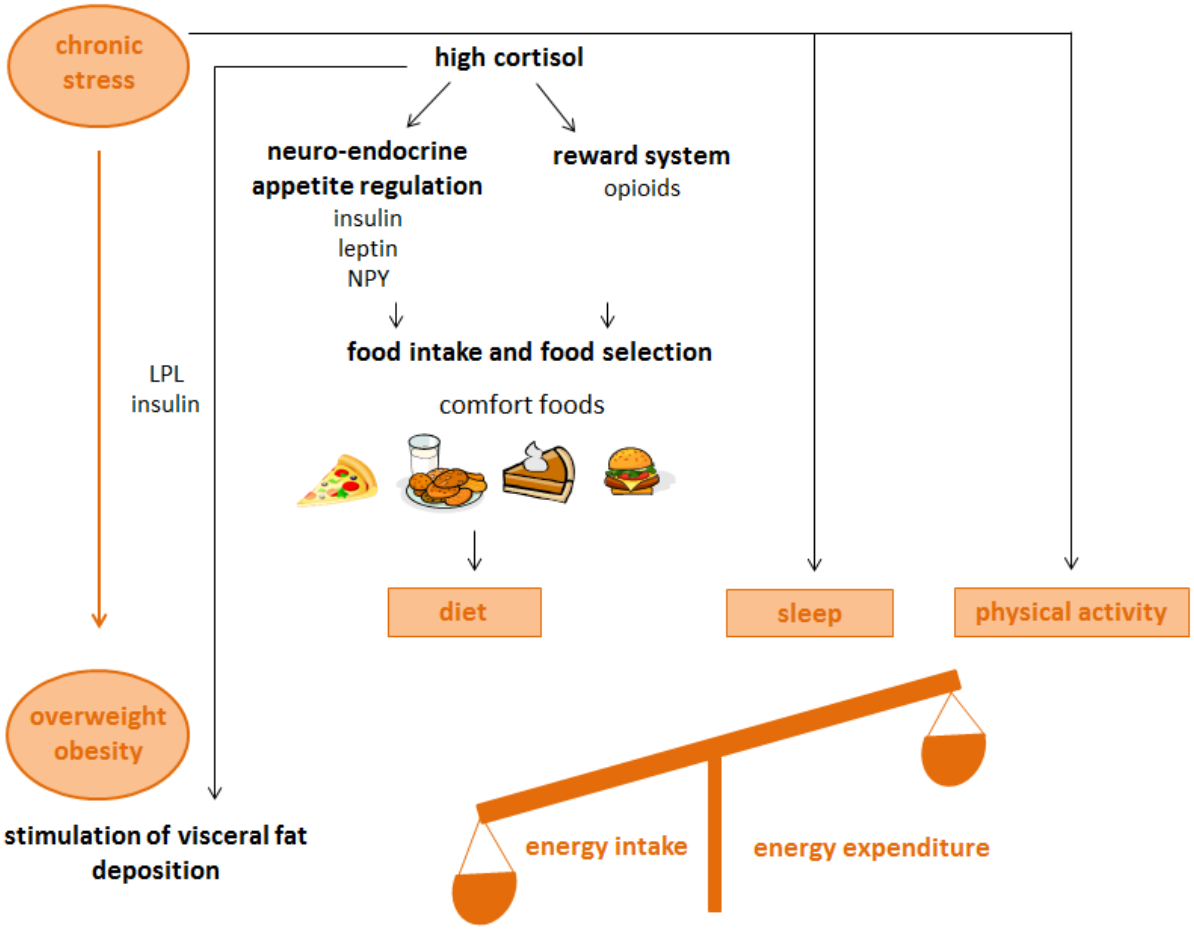


Figure 1.4 Illustration of neuro-endocrinological and behavioural pathways involved in the relationship between stress and the development of obesity.

As illustrated in Figure 1.4, chronic stress exposure may contribute to a positive energy balance and consequently to the development of obesity through the influence of cortisol on food intake regulation: cortisol stimulates food intake by 1) influencing the neuro-endocrine appetite regulation (stimulation of insulin, leptin and neuropeptide Y (NPY)) and impairing sensitization of satiety signals, and by 2) stimulating the brain reward system (e.g. opioid release). This results in an increased food intake with a preference for palatable, energy-dense comfort foods (i.e. fat- and sugar-foods which are abundantly available in our western obesogenic environment), which further stimulates opioid release and relieves the individual from its negative stressful state. Moreover, cortisol favours in particular visceral fat deposition as the density of glucocorticoid receptors is higher in visceral adipocytes compared to the other fat depots: cortisol activates lipoprotein lipase (LPL), which accumulates and retains triglycerides in visceral fat cells, which is further encouraged in the presence of insulin. Stress may also indirectly facilitate the development of obesity by influencing other

lifestyle factors such as physical activity and sleep. After all, stressed persons may be less motivated or have less energy to do physical activity, or may suffer from sleeping problems. Inversely, these lifestyle factors may also influence the stress load: 1) physical activity may be a protective factor against obesity and stress by increasing energy expenditure and by improving mental health and stress coping; 2) on the contrary, lack of sleep may reduce coping capacity and thus resistance against stress (Nieuwenhuizen A. G. et al., 2008; Bose M. et al., 2009; Rosmond R., 2005; Pervanidou P. et al., 2011; Dallman M. F. et al., 2005; Torres S. J. et al., 2007; Wardle J. et al., 2011; Adam T. C. et al., 2007; Holmes M. E. et al., 2010; Tsatsoulis A. et al., 2006; Lam J. C. M. et al., 2010). It is however not yet clear whether stress is causally or co-incidentally related to the development of childhood obesity. Also, it should be noted that depending on the stressor (e.g. type, duration and severity) and factors related to the environment or individual, stress may affect eating in a bidirectional way: in some individuals, stress may influence food intake regulation in the opposite direction, i.e. in the direction of under-eating or weight loss (Wallis D. J. et al., 2009; Epel E. et al., 2004; Stone A. A. et al., 1994).

B. Mineral deficiencies

Micronutrients are nutrients needed by the human body in minimal quantities for proper physiological functioning, and consist of vitamins and trace elements or so-called ‘minerals’. Minerals orchestrate a variety of essential functions (Table 1.3) and are tightly kept within physiological ranges in the body by regulating absorption, distribution and excretion.

Table 1.3 Main biological roles of some essential minerals

Calcium	involved in skeletal mineralization, extra- and intracellular functioning, nerve impulse transmission, hormone secretion, blood-clotting cascade and muscle contraction
Copper	involved in the enzymatic function of metallo-enzymes (e.g. superoxide dismutase, cytochrome oxidase etc.) which regulate diverse metabolic reactions such as the utilization of oxygen during cell respiration, energy utilization and essential component synthesis
Iron	role in erythropoietic function, oxidative metabolism and the cellular immune response
Magnesium	is main intracellular divalent cation and is essential for several enzymatic functions, particularly in carbohydrate metabolism (e.g. insulin action), also involved in DNA transcription and replication, bioelectric-activity and ionic pumps
Sodium	regulates salt- and water-homeostasis, and blood pressure
Phosphorus	implicated in energy metabolism, intracellular signalling, nucleic acid synthesis and cell structure
Zinc	involved in synthesis and degradation of carbohydrates, lipids, proteins and nucleic acids and is involved in a large number of enzymes involved in gene transcription (zinc-finger protein constituent)

(Greenbaum L. A., 2011; World Health Organization et al., 1996; Musso Carlos G., 2009; Peacock Munro, 2010)

Insufficient mineral intakes are not uncommon in the developed world (Vinas B. R. et al., 2011). In particular, iron deficiency in children remains a health problem (Toutain F. et al., 2012). In this chapter, mineral deficiencies are discussed because of 1) their importance for children (i.e. continuous growth and a developing brain create an increased demand for most minerals (Greenbaum L. A., 2011)) and 2) the proposed relationship with stress.

More specifically, prolonged stress has been hypothesized to negatively affect the body's mineral status through a physiological and behavioural pathway, although literature in this regard is scarce and for children even lacking (Seelig M. S., 1994; Grases G. et al., 2006; Moore R. J. et al., 1993). On the physiological level, stress may 1) hinder an adequate absorption, distribution or excretion of minerals, 2) increase the body's need for minerals through changes in metabolism or 3) redistribute the minerals to tissues with higher requirements, although these mechanisms need to be further explored (Yin J. et al., 2004;

Mayer E. A., 2000; Costantini D. et al., 2011; Heshmati H. M. et al., 1998). On the behavioural level, stress may lead to an increased consumption of comfort foods (as presented in Figure 1.4), which is often at the expense of healthy, mineral-rich foods and may on the long-term result in an unbalanced, even deficient dietary mineral intake (Alexy U. et al., 2011; Kaidar-Person O. et al., 2008).

Although the relationship between stress and minerals is not yet well-established, obesity and its comorbidities have more strongly been associated with imbalances or deficiencies in the vitamin or mineral status. For instance, reduced or deficient levels of vitamin B12, vitamin D, magnesium, iron and zinc have been observed in childhood obesity (Kaidar-Person O. et al., 2008; Nead K. G. et al., 2004; Moschonis G. et al., 2012; Zafon C. et al., 2010; Aeberli I. et al., 2009; Jose B. et al., 2012; Marreiro D. D. et al., 2002; Tylavsky F. A. et al., 2010; Pinhas-Hamiel O. et al., 2006; Olson M. L. et al., 2012). This finding is of importance given the complex interactions between stress, diet and obesity, which may altogether influence the body's mineral status.

2.3 Inter-individual differences in stress-outcomes

Two individuals experiencing similar stressful events may highly differ in their emotional reactions and health outcomes. After all, stress-induced health outcomes are not only related to the sole occurrence and the nature of the stressors, but also to individual characteristics such as age, gender, temperament, biological vulnerability (i.e. genetics, constitutional factors), psychosocial resources (e.g. supportive relationships), and the individual's patterns of coping, making individuals less or more vulnerable to stress-induced disease (Danese A. et al., 2012; Schneiderman N. et al., 2005). Furthermore, some individuals are more likely to suffer from adverse health outcomes as life stress accumulates (i.e. so-called 'cumulative stress') (Turner R. J. et al., 1995).

CHAPTER 1.3 EPIDEMIOLOGICAL APPROACHES TO MEASURE CHILDHOOD STRESS

Despite growing research interest in the field of childhood stress over the last years, literature falls short in providing an overview of methods to adequately assess stress in elementary school children. In order to evaluate the impact of stress on children's well-being, it is important to have valid, reliable, safe and relatively inexpensive methods that can be easily implemented in large-scale epidemiological studies. This section discusses common stress assessment methods particularly applicable to epidemiological stress research in elementary school children such as questionnaires and interviews, as well as laboratory measurements of cortisol in biological samples.

1 Methodology in relation to the definition of stress

Measuring stress accurately, is a complicated discipline due to methodological challenges and theoretical issues, such as the various definitions or interpretations given to the term 'stress'. As discussed in *Chapter 1.1 Definition and prevalence of childhood stress*, three components can be distinguished in the definition of stress (i.e. the stressors; the individual interpretation and affective response; the biological stress response). These three components each provide distinct approaches for the assessment and study of stress, namely 1) *the environmental approach*, which is based on the stimulus model of stress and the identification of objective environmental conditions that promote stress and may lead to disease (e.g. study of stressful life events as risk factors for disease) (Holmes T. H. et al., 1967), 2) *the psychological approach*, which focuses on the cognitive, mental interpretation of demanding situations, coping mechanisms and the measurement of a person's emotions, based on the transaction between the individual and the environment ('transactional model of stress') (Folkman S. et al., 1986), and 3) *the biological approach*, which investigates the activation of biological systems that are responsive to physical or psychological demands (Figure 1.5). The psychological response provoked by stressors is assumed to be the linking bridge between stressor exposure and physiological outcomes (Cohen S. et al., 1997a).

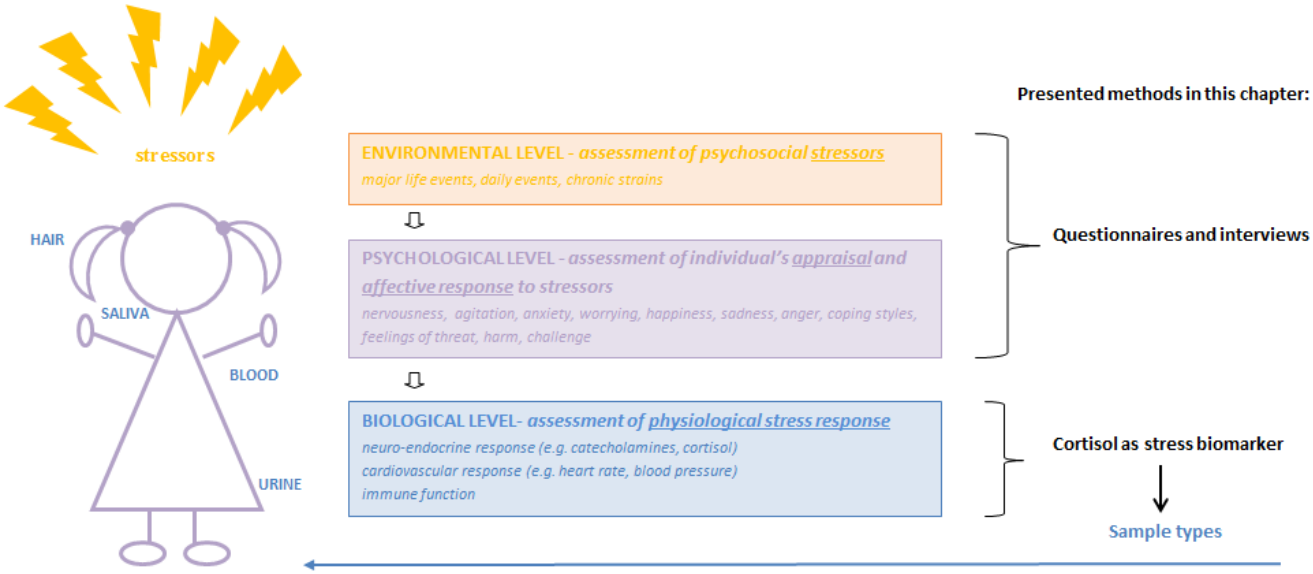


Figure 1.5 Stress assessment methodology in relation to the definition of stress. Depending on which aspect of the stress process one wants to investigate, the selected method will differ. The right side of the figure presents the methods discussed in this section: questionnaires and interviews to measure stressors and the psychological response, and the stress hormone cortisol which can be measured in different sample types (blood, urine, saliva and hair) (Cohen S. et al., 1997a; Pollard T. M., 1997). (Figure reprinted with permission from Epidemiological Approaches to Measure Childhood Stress, Vanaelst B., Paediatric and Perinatal Epidemiology 26, Copyright ©, 2012).

2 Psychological and biological correlates

Stressors elicit an integrated and coordinated response at multiple levels, although these psychological and biological responses may vary individually depending on the type of stressors encountered, genetics and personality characteristics, age, sex, and many other factors that are still unknown (Kudielka B. M. et al., 2009; Michaud K. et al., 2008; Cohen S. et al., 2003; Biondi M. et al., 1999). In this psycho-biological reactivity to stressors, also the type of appraisal, coping strategy and emotions have been shown to determine or evoke specific physiological outcomes (Denson T. F. et al., 2009; Olff M. et al., 2005; Schlotz W. et al., 2011). This might explain why relations between stressors and health outcomes on the individual level do not necessarily extrapolate to the population level, as the factors mentioned above modulate the impact of stressors on the individual level and therefore render detection of a general, univariate impact of stressors on population level more difficult. In this

context, both moderating and mediating effects have been observed for environmental and personal characteristics in the relationship between childhoods stress and its adverse outcomes (a *moderator* influences the direction or the strength of the stress-outcome relationship; a *mediator* accounts for the relationship between stress and the studied outcome) (Grant K. E. et al., 2006).

As both psychological and biological endocrine stress responses are indicators of stress, they are often assumed to be strongly inter-correlated (Campbell J. et al., 2012). However, general correlation studies have indicated inconsistent or contradictory associations between these measures. Only a paucity of studies have addressed this *lack of psycho-endocrine covariance* more thoroughly (Schlotz W. et al., 2008; Oldehinkel A. J. et al., 2011; Campbell J. et al., 2012). Schlotz and colleagues postulated that the different dynamics of the psychological and biological stress response are to a large extent responsible for this lack of psycho-endocrine covariance: the psychological response occurs within seconds and may change dynamically during a prolonged stress situation, while the biological endocrine response lags behind and occurs minutes after stressor exposure and changes less dynamically (Schlotz W. et al., 2008). They indicated both measures of psychological and physiological responses as good stress indicators, but underlined the importance of measurement at similar system-specific stages relative to the onset of the stressors: Schlotz et al. investigated psycho-endocrine cross-correlations at positive and negative time lags (because of the different on-/offsets of the psychological and endocrine stress responses), and demonstrated the highest psycho-endocrine covariance within non-synchronously assessed psychological responses and 10-30 minutes delayed endocrine responses (Schlotz W. et al., 2008). Similarly, Oldehinkel et al. and Hellhammer et al. reported significant but only temporal psycho-endocrine covariation (Oldehinkel A. J. et al., 2011; Hellhammer J. et al., 2012). A summary of possible relevant factors that may affect the correspondence between psychological and biological stress responses was recently provided by Campbell et al., including assessment features, underlying psychological traits and states and physiological dispositions (Campbell J. et al., 2012).

In short, the key message is to account for the different dynamics of the systems and for temporal ordering of the stress experience. In this way, correlations between subjective and biological markers of stress may be elucidated. Moreover, the importance of including multiple stress assessment methods, each assessing a different component of the stress process (as was illustrated in Figure 1.5), is emphasized since measures of stress perception

and appraisal may only provide partial knowledge about the physiological stress responsiveness, and vice versa (Oldehinkel A. J. et al., 2011; Schlotz W. et al., 2008) .

A remaining issue in the discussion about non-comparability or inconsistency of results in stress research, is the critical role of inappropriate, invalid and/or non-standardized methodologies (e.g. diversity in study designs, choice and employment of assessment method in relation to the research question, stressor type or outcome studied, biological sample strategies, conformity in time-scales), which will be discussed in the following sections (Cohen S. et al., 1997a; Hjortskov N. et al., 2004) .

3 Overview of methods for measuring childhood stress

The administration of questionnaires and interviews and laboratory measurements of cortisol in different bio-samples are valuable stress indicators, and are therefore widely performed in childhood epidemiological research (Figure 1.5). These methods are discussed in detail below. Other methods to study stress, e.g. brain function, cardiovascular responses, immune responses and certain stress hormones, are more difficult to implement in large-scale epidemiological studies and thus less frequently used (Dedovic K. et al., 2009; Dhabhar F. S., 2009; Krantz D. S. et al., 1997; Kiecolt-Glaser J. K. et al., 1997). Hence, these were further not considered.

3.1 Questionnaires and interviews

A. Measurement of stressors (environmental approach)

Life events may lead to changes in a person's life. These changes, especially if accompanied by undesirable demands and threats, are assumed to represent a basis for experiencing stress. Therefore, inventories assessing life events (i.e. questionnaires and interviews) are considered as estimates of stress exposure and are based on the magnitude of change, undesirability and contextual threat induced by the event and the personal control over the event (Cohen S. et al., 1997b).

Numerous self-reported checklists and face-to-face interviews are available to assess the occurrence of stressors in elementary school children. Thorough screening of publications

indexed in Web of Science and/or PubMed resulted in a number of child-specific stressor questionnaires and interviews of which examples are given in Table 1.4.

These inventories assess a broad range of childhood stressors in different domains of the child's living environment (e.g. school, family, interpersonal relationships and health) and they vary in scope by comprising either major life events, daily hassles or chronic strains, either short- or long-term exposure to stressors, and either general or more specific types of stressors. The major advantages of this approach are its non-invasiveness and cost-effectiveness. However, no information on the physiological stress response can be obtained via such questionnaires/interviews and unfortunately, there is not always free online access to these inventories (as is indicated in Table 1.4).

Checklists and interviews have evolved from being modified versions of adolescent or adult stressor questionnaires to being specifically designed for and with the input of children (Turner R. J. et al., 1997; Ryan-Wenger N. A. et al., 2005).

Table 1.4 Examples of child-specific stressor questionnaires and interviews

Self-reported stressor checklists	Online *	No. items	Description
Coddington Life Events Scale for Children (CLES-C) (Coddington R. D., 1972)	No	36	major life events in the last year (domains: health and illness, family situation, school situation, social achievements)
Stressful Life Events Scale (SLES) (Yamamoto K., 1979)	Yes	20	unpleasant life events that have occurred on a scale from 'most upsetting' to 'least upsetting'
Life Events Checklist (LEC) (Johnson J.H. et al., 1980)	No	46	Major life events in the past 12 months, indication if item was 'positive' or 'negative' and the degree to which the event was stressful or unpleasant
Children's Hassles and Uplifts Scale (CHS-CUS) (Kanner A. D. et al., 1981)	No	25+25	daily hassles and uplifts that in the past month
Life Stress Inventory (LSI) (Cohensandler R. et al., 1982)	No	x	life events and experiences associated with childhood depression and self-destructive behaviour , over the entire lifespan of the child and for the last 12 months separately
Feeling Bad Scale (FBS) (Lewis C. E. et al., 1984)	Yes	20	discrete life events and on-going stressful processes in the child's life over the last year and assessment of how the children felt
General Life Events Schedule for Children (GLESC) (Sandler I. et al., 1986)	No	20	undesirable life events in the last 3 months
Life Events and Coping Inventory (LECI) (Diselewis J. E., 1988)	Yes	125	stressful life events (traumata and daily hassles) and children's coping strategies
Stressful Life Events (Brown L. P. et al., 1988)	No	17	stressful life events over a long time period
Life Events Questionnaire (LEQ) (Bailey D. et al., 1990)	No	10	life events in the past 3 months, in the second session the child reports freely any 'good or bad things' that have happened in the last 3 months
Daily Life Stressor Scale (DLSS) (Kearney C. A. et al., 1993)	No	30	severity of daily life stressors encountered by children in the past week (home, school, social events, academic and athletic performance)
Everyday Life Events Scale for Children (ELESC) (Jose P. E. et al., 1994)	No	50	common, daily stressful life events in the last month, whether this was a problem and how much of a problem this was perceived
Major Life Events Scale for Children (MLESC) (Jose P. E. et al., 1994)	No	50	major life events, whether this was a problem and how much of a problem this was perceived
Stress and Coping Questionnaire for Children (SCQC): school version (Roder I. et al., 2002)	Yes	4	frequency of occurrence and the experienced stress/emotions for 4 school-related items and the child's coping strategies
Stress in Children Questionnaire (SiC) (Osika W. et al., 2007)	Yes	21	emotional states, psychosomatic symptoms, school-related experiences, social support and cognitive aspects
Child and Adolescent Survey of Experiences (CASE) (Allen J. L. et al., 2009)	Yes	38	life events in the past 12 months, including children's ratings of the events by 'good' or 'bad'

Children's Daily Stress Inventory (CDSI) (Trianes M. V. et al., 2009)	Yes	25	daily life events; in the areas of health, school and family in the last year
Stressor interviews			**Description**
Life Events and Difficulties Schedule for children and adolescents (LEDS) (Monck E. et al., 1985)	No	x	Semi-structured interview to elicit contextual information on events in 10 domains, indication of degree of stress for short- and long-term threats
Child Episodic Life Stress Interview (CELSI) (Adrian C. et al., 1993)	No	x	Semi-structured interview assessing stressful life events in the past year (global questions 12 areas, followed by specific inquiries about the life domains, timing and duration of events, circumstances…) (has been modified to the Youth Life Stress Interview by dr. Hammen-UCLA)
Psychosocial Assessment of Childhood Experiences (PACE) (Sandberg S. et al., 1993)	No	x	Systematic parent and child interview to assess the timing, nature and impact of life events over the preceding 18 months
Daily Diary Interview (DDI) (Walker L. S. et al., 2001)	No	x	Daily interview by telephone in which children are asked about three time periods (morning, school and after school period), interview starts with open-ended questions and is followed by a structured list of possible stressors that may have occurred in the family life, with peers or in school
Stressful Life Events Schedule for Children and Adolescents (SLES) (Williamson D. E. et al., 2003)	No	61	Face-to-face interview about the occurrence of 61 events during the last year prior to the interview, follow-up questions for contextual information and rating of the event
Yale Children's Global Stress Index (YCGSI) (Findley D. B. et al., 2003)	Yes	x	Child and parent interview to determine the 6 most significant life events of the past year, resulting in a global stress score
Life Events Interview for children (LEI) (Monroe S. M., 1983; Alloy L. B. et al., 2000)	No	x	Interview to obtain information on dates, circumstances, short and long term consequences of events and its subjective severity ratings

*Free online availability for the institution Ghent University. Online access and subscription to journals may differ for your institution. Full texts can be bought or can be obtained by contacting the author, X: the number of items could not be retrieved from literature

Questionnaires

Relationships between environmental stressors and other general measures of stress or stress-outcome can be investigated using stressor checklists/questionnaires, which are particularly suitable for large-scale studies.

In self-reported stressor checklists, children are asked to indicate whether the described life events or stressors occurred in a predefined time period. Some stressor checklists additionally assess the 'degree of impact' of the experienced events, by asking how upsetting or stressful these events were perceived by the children ('transactional' checklists) (Table 1.4 - description column) (Turner R. J. et al., 1997; Grant K. E. et al., 2004; Blount R. L. et al., 2008). Although evaluation of this psychological information (appraisal, emotion etc.) may complicate the interpretation of stressor checklists (e.g. by weighing the events by appraisal), the prediction of outcomes may be improved by including the participants' personal ratings of the events in stressor checklists (Stone A. A. et al., 1991). It has been shown that the way in which a child perceives life events may significantly influence its behaviour, adjustment or other outcomes (Jackson Y. et al., 2000; Sheets V. et al., 1996; Ellis A. A. et al., 2009). Assessment of appraisal and affective responses in stress research is described more detailed in the section *3.1.B. Measurement of appraisal and affective responses (psychological approach).*

In contrast to these self-reported checklists, parental-reporting of events was the predominant method used in the early days of childhood stress research. Parent-reporting of events is now considered as less reliable than self-reports by children, since parents may only have a limited awareness of what happens to their children and what kind of daily hassles they have to overcome. Moreover, children might judge the perceived stressfulness of events differently than their parents do (Bailey D. et al., 1990; Muldoon O. T., 2003; Yamamoto K. et al., 2001; Johnston C. A. et al., 2003).

Personal interviews

By using guidelines, definitional rules and interview probes, stressful life events can be characterized more precisely and standardized in personal, face-to-face interviews. In these interviews the importance and impact of life events is assessed objectively by the investigator,

considering additional contextual information (e.g. timing, duration, specific circumstances of the event) which may alter the consequences of these life events. This is in contrast to the self-reported stressor checklists in which only the magnitude of social or environmental change is measured or considered as a basis for stress ('change-readjustment paradigm') (Turner R. J. et al., 1997; Wethington E. et al., 1997).

Although Wagner et al. suggested that checklists and interviews are equally valid approaches to exclusively assess the occurrence of children's life events, interviews are actually more suitable to specifically assess the severity or context of life events and to study the relationship between stressors and the onset of severe physical or mental illness (Wagner C. et al., 2006). Additionally, they are characterized by less mood-related bias in reporting compared to checklists (Grant K. E. et al., 2004; Wethington E. et al., 1997). Disadvantages of interviews are their time-consuming and labour-intensive nature, and the need for highly trained staff for interview administration, all together increasing the costs and making them less applicable for large-scale studies. In addition, embarrassing events or events with possible negative consequences, are less likely to be reported in interviews (Duggal S. et al., 2000).

Checklists and interviews result either in a calculated 'total number of experienced life events' or in a cumulative 'stress-score' based on the event's 'weights'. Additionally, the analysis can focus on a number of specific life events (Turner R. J. et al., 1997; Wethington E. et al., 1997).

B. Measurement of appraisal and affective responses (psychological approach)

The psychological approach focuses on how environmental processes are interpreted by people in relation to their own values and resources and on a person's nature making them less or more vulnerable to stress-induced disease (Folkman S. et al., 1986). These perceptual processes, which are hypothesized to affect the stress-response and to influence health and well-being, may be studied particularly to investigate individual differences in the stress-response and –outcomes. Appraisal and affective responses thus have a central importance in stress research as mediators of the stressor-outcome relationship (Monroe S. M. et al., 1997). After all, two people experiencing similar stressful events may highly differ in their emotional reactions and health outcomes to these stressors.

Analogous to stressor assessment, the individual's appraisal and affective responses can also be evaluated by questionnaires and interviews (Figure 1.5). In essence, these additionally include questions on how the children are feeling, how they are perceiving, interpreting and evaluating the situation and how they believe to cope or deal with the stressful stimulus (Herbert T. B. et al., 1996; Folkman S. et al., 1986). In this way, these measures can assess intra- and inter-individual differences in response to stressors (i.e. particular events can carry a different level of adversity for every child), which may result from differences in family support, the personal importance given to the particular experience, previous stressful life events, the child's age, gender, temperament and socio-economic status (Sandberg S., 2007; Muldoon O. T., 2003; Smeekens S. et al., 2007). The main techniques to assess psychological aspects of the stress response are discussed below, but this overview is not exclusive.

When measuring **appraisal,** either primary appraisal (i.e. interpretation of the environmental situations as irrelevant, positive or stressful), secondary appraisal (i.e. coping, how to deal with the problem and manage emotions), or both can be assessed, using single-item questions on the appraisal of specific stressors or multiple-item inventories measuring the global stress appraisal.

A variety of self-reported methods using multipoint scales, visual analogue scales, graphical representations of cartoon-type faces (especially applicable for children) or mood adjective checklists can be used to measure **affective or emotional responsiveness** in relation to other variables in the stress process. State assessments of affective responses can be used as outcome measure, for instance to study the impact of events on mood, or as a measure of stressor severity, using questionnaires addressing both particular situations and corresponding emotions. Also, observational methods of the individual's emotion can be applied, although these are not feasible in large-scale epidemiological studies (Herbert T. B. et al., 1996; Monroe S. M. et al., 1997; Stone A. A., 1997).

3.2 Cortisol as stress-biomarker (biological approach)

To overcome limitations inherent to the more subjective nature of questionnaires and potential difficulties in implementing checklists and interviews in younger age groups, objective alternatives based on biomarker measurements are increasingly used.

A. Biology of cortisol and reflections on its use as stress-biomarker

Cortisol exhibits a strong diurnal rhythm through which basal homeostasis and metabolic functions are maintained (Kaplan N. M., 1996). This diurnal rhythm is highly important to consider when performing cortisol measurements in stress research. The potential use of cortisol as stress biomarker is an integral part of this feature, as the rise of morning cortisol is particularly sensitive to the influence of on-going life stressors. Alterations in the normal pulsation and rhythmic fluctuation in cortisol production may indicate activation of the (HPA) stress system. However, cortisol measurements are difficult to interpret as many factors, such as physical activity, sleeping, eating, certain medications and illnesses, age, gender, smoking and diet influence basal cortisol levels (Hanrahan K. et al., 2006).

Using cortisol as a biomarker of HPA activity and stress brings along its particular challenges. Psychosocial stressors frequently evolve from being episodic to prolonged and chronic. While acute stress responses are characterized by a clearly increased cortisol secretion, the identification of the chronic stress response and the corresponding HPA axis activity remains disputed. Both hyper- as well as hypo-cortisolism have been demonstrated as deviations from normal HPA function in chronic stress (Miller G. E. et al., 2007; Michaud K. et al., 2008). In a recent meta-analysis of Miller et al., it is stated that a generally assumed 'uniform HPA response' (e.g. cortisol elevations) to chronic stress is no longer appropriate, and that much of the variability in the HPA activity in response to chronic stress is attributable to the features of the stressor, the time since stressor onset (since hormonal activity may reduce as time passes) and the person facing it (Miller G. E. et al., 2007). In addition, more research is needed to illustrate the physiological stress response in children, more specifically to investigate which stressors actually produce a stress response or activate the HPA axis, and consequently alter cortisol output in children. Gunnar et al. have clearly indicated that many studies on cortisol responses in children using stressor models that appeared stressful, failed to induce elevations in salivary cortisol on population level (Gunnar M. R. et al., 2009a). In sum, relations among stress and HPA activity, measured by cortisol output, are complex and depend on many factors (e.g. person and stressor features, existence of non-responders) (Miller G. E. et al., 2007; Baum A. et al., 1997).

B. Biological samples for cortisol analysis

Cortisol can be measured in different biological specimens, including blood, urine, saliva and hair (Figure 1.5), although each type of biological sample is characterized by its specific strengths and limitations as demonstrated in Table 1.5.

Serum

To analyse serum cortisol levels and HPA activity, blood samples in the morning (fasting) or at intervals over periods of up to 24 hours can be collected (the latter for prolonged, circadian hormone profiles). As indicated in Table 1.5, serum is the only biological material in which both total and free cortisol can be measured. Especially in medical settings, cortisol measurements are valuable in examining the diurnal pattern of cortisol and clinical evaluation of adrenal disorders. However, based on findings of previous studies, Levine et al. stated that total cortisol measurements are not always diagnostically useful and that free cortisol might be a preferable approach to assess HPA function (Hamrahian A. H. et al., 2004; le Roux C. W. et al., 2003; Levine A. et al., 2007).

The main logistic differences between measurement of serum cortisol compared to the other biological samples, are the invasive character of the sampling, the need for (para-)medical staff and more stringent clinical and laboratory preparations in which processing (e.g. immediate centrifugation and preservation treatments), storage (e.g. -20°C or -80°C freezers) and transport to the assay laboratory must be carefully planned (Baum A. et al., 1997). Besides, blood sampling is tedious and stressful for children. It has been assumed that venipuncture may elicit a cortisol response and consequently may lead to artificially raised cortisol concentrations. In contrast to catecholamine levels which rise immediately after venipuncture or stressor exposure, cortisol levels respond more slowly and increases in concentrations are not manifest for at least 7 minutes, and peaking 15 minutes, after venipuncture (Levine A. et al., 2007; Baum A. et al., 1997; Levine S. et al., 1985). Since there is a strong positive correlation between free serum cortisol and free salivary cortisol, invasive blood sampling is more often replaced by salivary cortisol (Tunn S. et al., 1992; Kirschbaum C. et al., 1994).

Table 1.5 Main characteristics of biological samples for cortisol analysis in children

	Serum cortisol	**Salivary cortisol**	**24-hour urinary cortisol**	**Hair cortisol**
Measurement of free or total cortisol [a]	free and total cortisol	free cortisol	free cortisol	free cortisol
Invasiveness of sampling	Invasive, induces 'needle' stress	Non-invasive	Non-invasive	Non-invasive
Home or clinical setting	Clinical and laboratory setting	Home sampling, 'naturalistic' field studies	Home sampling, 'naturalistic' field studies	Home sampling, 'naturalistic' field studies
Sampling technique	Blood sampling by medical staff	-Salivette, spitting or passive drooling, cotton or polyester swabs -single morning sample or multiple sampling -good communication with parents for protocol compliance	24 h pooled urine, assisted by parents or teachers, good communication for protocol compliance	Single hair sample from vertex posterior region from head, collection by parents or researcher
Storage	Risk for decomposition, freezing necessary, bio-hazard	Easy storage (temporarily in room or cooling temperature, freezing afterwards)	Easy storage (cooling)	Storage stability at room temperature
Cortisol time frame	Minutes to hours	Minutes to hours	24 hours	Weeks to months
Representativeness of long-term exposure to stressors	No (only if multiple, long-term sampling which is practically unfeasible in children)	Yes, if multiple samples are taken over a longer period or if measurement of the cortisol awakening response	Yes, especially if 24 hour urine sampling is repeated	Yes. Only 1 sample needed to inform about the last months.
Feasibility of sampling in children	Feasible but not recommended for psychosocial stress research in children since availability of alternatives	Feasible but demands standardized protocols and attempts to increase protocol compliance	Feasible but demands standardized protocols and attempts to increase protocol compliance	Feasible and easy in children but the amount of hair needed is substantial, need for additional research
Cost of cortisol analysis per sample (indicative) [b]	Total cortisol: €11.72 [c] Free cortisol: €16.82 [d]	€9 [e] (not validated technique)	€14.91[d]	Not routinely measured

[a] Cortisol circulates in two forms in serum (free and bound). As a result of the 'free hormone hypothesis', interest in total cortisol has gradually been replaced for interest in free cortisol (Mendel C. M., 1989) However, recently this hypothesis has been questioned and might need re-evaluation.(Levine A. et al., 2007) Free cortisol is usually calculated from measurements of total cortisol and CBG plasma levels.(Ho J. T. et al., 2006; Levine A. et al., 2007) Serum free cortisol analysis is therefore pricier since also the concentration of CBG is analysed. [b] To give an indication of the cost of cortisol laboratory analysis five routine clinical laboratories in Flanders (Belgium) were contacted for standard price offers and a mean price was calculated. Not all laboratories offered the cortisol analysis in all types of bio-samples. If the number of samples is large, reductions are often offered. Collection materials are not included in the price [c,d,e] mean cost was based on price offers of respectively 5, 3, 2 laboratories

Saliva

Measurement of salivary cortisol in children has been a standard practice in paediatric stress research for many years as it is less invasive and cheaper than serum cortisol measurements (Jessop D. S. et al., 2008; Hellhammer D. H. et al., 2009; Hanrahan K. et al., 2006; Baum A. et al., 1997). However, collecting salivary cortisol in children brings along challenges related to protocol compliance and more importantly, there is an urgent need for standardized collection procedures and strategies to obtain comparable, accurate and precise results (Hanrahan K. et al., 2006). The most important considerations are a standardized timing for sample collection and the use of consistent materials, protocols and laboratory analytic methods. Important to note is that the collection method may influence the measured salivary cortisol levels (Poll E. M. et al., 2007; Kidd S. et al., 2009). Kidd et al. reported that the use of cotton or polyester swabs as salivary collection method may over- or underestimate salivary cortisol concentrations and therefore suggested that passively drooled saliva may be a more reliable method for salivary cortisol analyses, as it excludes the possibility of assay interference (Kidd S. et al., 2009). This was however in conflict with another study, showing that 'Salivette' salivary cortisol was a better predictor for serum cortisol levels than the drooling method (Poll E. M. et al., 2007). Moreover, this study reported a preference for and easier handling of Salivettes compared to passive drooling. Nonetheless, Salivettes are not always suitable for use in young children (too big for oral cavity, risk for swallowing and choking, insufficient quantities). Similarly, drooling by children may yield insufficient volumes.

In a substantial amount of older epidemiological stress studies, morning cortisol levels have been measured in a single sample, usually taken at or soon after awakening. Alternatively, multiple samples in the morning period (for example immediately, 30 minutes, 45 minutes and 60 minutes after awakening) are now more and more measured to assess the cortisol awakening response (CAR). Since the magnitude of the CAR is associated with psychosocial factors, it has large potential in stress research (Chida Y. et al., 2009; Kudielka B. M. et al., 2010). However, assessment of the CAR in children may impose additional logistic difficulties in collecting samples and may be hampered also by a reduced protocol compliance, especially on schooldays when morning time at home with parents is limited.

Urine

The 24-hour urinary excretion of free cortisol is an accurate measure of adrenal activity and is feasible in children as it is non-invasive. This long-term reflection of circulating cortisol provides an index of the total amount of cortisol released by the adrenals over the circadian cycle (Baum A. et al., 1997). 24-hour urinary free cortisol (UFC) is relatively insensitive to over- or under-estimates of cortisol, which are sometimes present in moment-to-moment sampling due to short-term fluctuations or sudden secretion bursts of cortisol. However, it has several methodological limitations: in addition to analytic difficulties (interference of other urine metabolites in cortisol assays) there are difficulties with protocol compliance, as it is cumbersome, time-consuming and laborious. Consequently, this technique may potentially overburden the children and their supervising adult or teacher, to whom additionally detailed information should be provided. Analyses of urinary creatinine excretions in 24-hour samples may indicate collection errors and serve as verification of protocol compliance (Remer T. et al., 2002). Although UFC measurements bring along logistic difficulties, this technique can be used in childhood (stress) research, as has been done in the past (De Bellis M. D. et al., 1999; Levine A. et al., 1994; Gomez M. T. et al., 1991; Norbury W. B. et al., 2008; Long B. L. et al., 1993; Yehuda R. et al., 2003).

Scalp hair

Although hair analyses have been a common technique in forensic and drug research for years, the first reports on the use of scalp hair for stress research only date back from 2007. Meanwhile, the number of reports showing an association between hair cortisol concentrations and stress has increased rapidly. Table 1.6 presents a chronological overview of stress-related hair cortisol research. Supportive evidence has accumulated on the use of hair cortisol analysis as a valid index of long-term systemic cortisol exposure and thus on its potential as chronic stress biomarker (Stalder T. et al., 2012a; Russell E. et al., 2012; Staufenbiel S. M. et al., 2012). Literature on the use of hair cortisol as a stress biomarker in children is however limited to two publications of Vagri et al. and Groeneveld et al. (Table 1.6) (Vaghri Z. et al., 2012; Groeneveld M. G. et al., 2013).

Table 1.6 Chronological overview of hair cortisol analysis in chronic stress research

2007	Hair cortisol as a potential biologic marker of chronic stress in hospitalized neonates (Yamada J. et al., 2007) The relationship between stress and hair cortisol in pregnant women (Kalra S. et al., 2007)
2008	Elevated content of cortisol in hair of patients with severe chronic pain: a novel biomarker for stress (Van Uum S. H. M. et al., 2008)
2009	Stress pathways to spontaneous preterm birth: the role of stressors, psychological distress, and stress hormones (Kramer M. S. et al., 2009)
2010	Relationship between hair cortisol concentrations and depressive symptoms in patients with coronary artery disease (Dowlati Y. et al., 2010) Higher cortisol content in hair among long-term unemployed individuals compared to controls (Dettenborn L. et al., 2010) Use of hair cortisol analysis to detect hypercortisolism during active drinking phases in alcohol-dependent individuals (Stalder T. et al., 2010)
2011	Hair cortisol and the risk for acute myocardial infarction in adult men (Pereg D. et al., 2011) Decreased hair cortisol concentrations in generalised anxiety disorder (Steudte S. et al., 2011b) Increased cortisol concentrations in hair of severely traumatized Ugandan individuals with PTSD (Steudte S. et al., 2011a) Shift work at young age is associated with elevated long-term cortisol levels and body mass index (Manenschijn L. et al., 2011b)
2012	Hair cortisol and stressful life events retrospective assessment in crack cocaine users (Grassi-Oliveira R. et al., 2012) Measuring short-term and long-term physiological stress effects by cortisol reactivity in saliva and hair (van Holland B. J. et al., 2012) Introducing a novel method to assess cumulative steroid concentrations: Increased hair cortisol concentrations over 6 months in medicated patients with depression (Dettenborn L. et al., 2012a) Hair cortisol as a biomarker for altered hypothalamic-pituitary-adrenal activity in female adolescents with posttraumatic stress disorder after the 2008 Wenchuan earthquake (Luo H. et al., 2012) Elevated hair cortisol concentrations in endurance athletes (Skoluda N. et al., 2012) Cortisol in hair, body mass index and stress-related measures (Stalder T. et al., 2012b) Hair cortisol reflects socio-economic factors and hair zinc in pre-schoolers (Vaghri Z. et al., 2012) Relationship between hair cortisol and perceived chronic stress in a diverse sample (O'Brien K. M. et al., 2012)
2013	High long term cortisol levels, measured in scalp hair, are associated with a history of cardiovascular disease (Manenschijn L. et al., 2013) Hair cortisol as a biomarker of traumatization in healthy individuals and posttraumatic stress disorder patients (Steudte S. et al., 2013) Children's hair cortisol as a biomarker of stress at school entry (Groeneveld M. G. et al., 2013)

Hair is produced by alternating cycles of growth and quiescence in the hair follicles which are located in the epidermal epithelium (Harkey M. R., 1993). It is assumed that cortisol is deposited into hair during the active phase of hair growth, i.e. during the so-called anagen phase, which is characterised by intense metabolic activity. Several mechanisms have been proposed for cortisol incorporation into hair, which are presented in Figure 1.6. Some questions still remain on the physiology, metabolism and incorporation of hair cortisol, however, human research in this context is lacking. In fact, a recent radio-metabolic study in guinea pigs presented strong evidence for metabolisation of cortisol prior to its incorporation in hair (Keckeis K. et al., 2012), which introduces the importance of cortisol metabolites for hair stress research, such as cortisone which may be converted from cortisol by 11β-HSD activity as described in *Chapter 1.2 Biology and health consequences of stress*.

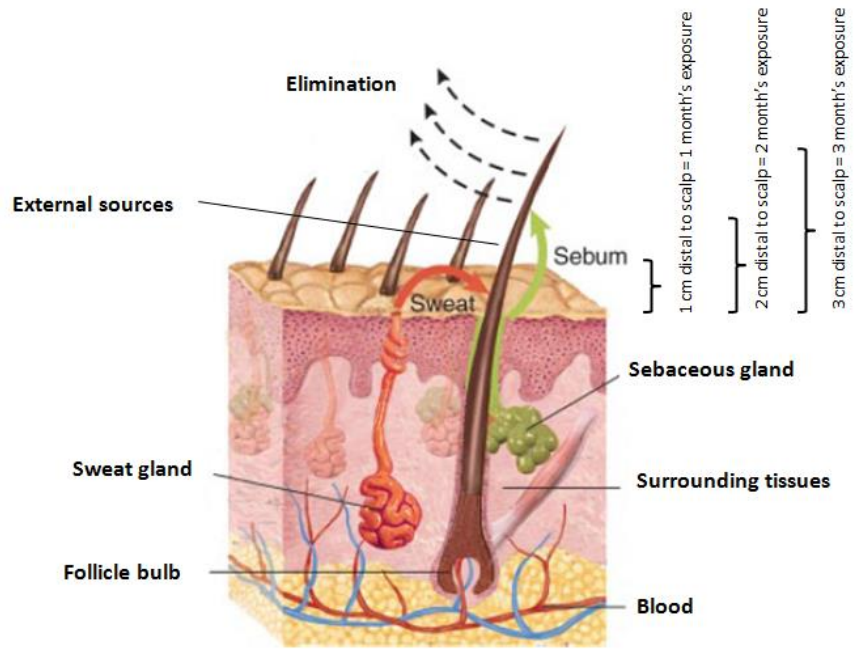


Figure 1.6 Proposed mechanisms for cortisol incorporation into hair and illustration of retrospective, long-term cortisol reflection. Cortisol is assumed to mainly reflect central HPA activity through its passive diffusion from blood, sweat, sebum and surrounding tissues into hair (Meyer J. S. et al., 2012; Russell E. et al., 2012; Stalder T. et al., 2012a; Sharpley C. F. et al., 2012). Moreover, peripheral HPA activity and local cortisol production in the hair follicle may contribute to hair cortisol levels (Ito N. et al., Sharpley C. F. et al.), as may contamination from external sources (e.g. steroid-containing crèmes). Exposure to water and/or shampooing (hair washing), cosmetic hair treatments and UV irradiation may

contribute to elimination of cortisol from hair. (Figure reprinted with permission and modified (Meyer J. S. et al., 2012)).

Hair cortisol has several advantages as biomarker for chronic stress due to its unique characteristics: only one hair sample suffices to obtain information over a prolonged period of months in the past (i.e. based on a general hair growth rate of 1 centimetre per month, a hair sample of 1 cm reflects a 1 month's exposure time) (Figure 1.6); sectioning of hair into fragments allows differentiation between several time periods in the past (i.e. 'month-by-month' measure); hair sampling is non-invasive and for most subjects less stressful than blood sampling; sampling can be performed at any time of the day and at home, reducing the logistic impact; hair does not decompose as other body fluids do, so it can be stored at room temperature and easily sent by postal mailing in a sealed envelope. A limiting factor in the use of hair as a bio-matrix is its availability and length in the vertex posterior region of the head, which is characterized by the lowest intra-individual variation in hair growth rate and hair cortisol content compared to other regions of the head (Li J. et al., 2012; Sauve B. et al., 2007; Harkey M. R., 1993). Although hair sampling might encounter religious, ethical or esthetical objections and hair cortisol does not provide information about single diurnal cycles or day-to-day variations of cortisol, it is especially suitable for the measurement of long-term HPA axis activity and thus chronic stress research (Sauve B. et al., 2007; Van Uum S. H. M. et al., 2008; Gow R. et al., 2010).

Despite the fact that hair cortisol is a promising biomarker for use in children on a large-scale epidemiological level, this concerns a new technique and thus further development and optimization is required. More specifically methodological issues in the use of hair as sampling matrix still need to be addressed (e.g. standardized sampling and storage protocols, processing and extraction procedures, choice of cortisol assay etc.).

Other

Although still preliminary, we would like to mention two pilot studies in which the feasibility of measuring cortisol in a stress-context is demonstrated in human fingernails (Warnock F. et al., 2010; Ben Khelil M. et al., 2011). Since clipping and storage of nails is uncomplicated and nails are rather resistant to decomposition, Warnock et al. introduced fingernail analyses as a practical method to measure hormones over a prolonged time period in human

populations. These pilot studies may initiate further research into the validity and applicability of this technique in epidemiological and clinical research.

C. Cortisol intercorrelations

Cortisol measurements in serum, saliva, urine and hair reflect different cortisol levels from a different time frame (i.e. minutes to hours for serum and saliva, one day for urine, weeks to months for hair). These measurements carry partially different information and thus have different potentials (e.g. studying acute versus chronic stress, short-term versus long-term HPA activity, total adrenal cortisol secretion versus free cortisol effects at binding receptors in target tissue) (Hellhammer D. H. et al., 2009). Consequently, correlations between cortisol measurements in different biological samples have often been contradictory (D'Anna-Hernandez K. L. et al., 2011; Tunn S. et al., 1992; Levine A. et al., 2007; Poll E. M. et al., 2007; Yehuda R. et al., 2003).

3.3 Specific recommendations for the use of questionnaires, interviews and cortisol analyses

Although **stressor checklists and interviews** for children are widely used in epidemiological studies, they can be criticized for several reasons. Some methodological issues may obscure relations between stressor exposure and outcomes if not correctly dealt with. Specific recommendations are therefore formulated below.

The first (major) limitation is the limited event coverage by questionnaires and interviews, as they have a finite number and specific types of life events. The selected life events should be a realistic, balanced and up-to-date representation of events that are of potential relevance to the child's age, living environment and advisably contain both positive and negative life events (Grant K. E. et al., 2004; Turner R. J. et al., 1997; Ryan-Wenger N. A. et al., 2005). It is recommended to use questionnaires developed in cooperation with children, and which clearly discriminate between discrete major life events and on-going daily strains. An item content between 30 and 50 events is optimal in terms of predictive power and logistic use. Also, cultural differences should receive attention, although this implies reduced comparison across diverse populations (Turner R. J. et al., 1997; Ryan-Wenger N. A. et al., 2005).

Secondly, questionnaires and interviews are both prone to recall bias, especially in children since their memory abilities are still immature (Salmon K., 2001; Eisen M. L. et al., 1998). Recall can be influenced by the time since the event, the assessment method used and the age of the respondent, and can be affected in two ways. The first is "memory failure", which results in under-reporting, and the second is the "telescoping effect", which usually results in over-reporting. Telescoping of events refers to the misdating of distant events into a more recent time period and can be attributed to the abstractness of time and location for young children (Wethington E. et al., 1997). To limit recall bias and to increase the accuracy of reporting, calendar events such as birthdays, summer holidays, Christmas or Easter can be included (Garrison C. Z. et al., 1987). Moreover, the use of dolls and toys can be helpful to increase the memory and reporting abilities, although techniques for probing memory and enhancing recall should be used with caution since children are vulnerable to suggestion and their benefits depend on the child's age (Larsson A. S. et al., 2009; Salmon K., 2001).

Thirdly, over- and under-reporting can be the result of personality characteristics or miscomprehension of the items by children. The latter can be avoided by determining the reading level and linguistic abilities of the children in advance or by visualizing the potentially stressful life events with pictures for the youngest age groups of elementary school (5 to 6 years old) or for children with communicative impairments. Another option is to let a trained researcher assist in filling out the questionnaires or to consider face-to-face interview as a valuable alternative, as this method allows to give additional explanations. Children as young as four years of age have been shown to be reliable informants about experienced events, especially when the experiences were highly distinctive (Larsson A. S. et al., 2009). Nonetheless, the reliability of self-reports by children increases with age (Larsson A. S. et al., 2009; Mellor D., 2004).

Fourthly, questionnaires and interviews often have a predefined time frame boundary (days for daily events, months or mostly one year for major life events), which may prevent the reporting of (traumatic) events occurred in a period prior to the time frame of the inventory. Even though these could also have long-term adverse health consequences. It is recommended to incorporate a supplementary checklist of lifetime events or traumas (which some checklists and interviews already do), including the indication of the age at first and last occurrence of the events to obtain a more extensive view on the child's living environment from birth to present (Turner R. J. et al., 1997).

Finally, research groups sometimes develop their own stressor questionnaire or interview, often without reporting the procedure of development or the item content. Therefore, it is recommended to use well-established (preferably reliable and validated) stress assessment methods in a standardized manner and not to develop own questionnaires if good alternatives are available or if this is beyond ones research area to do so (Grant K. E. et al., 2004; Herbert T. B. et al., 1996).

Questionnaires have also been shown to be appropriate assessment tools for **appraisal and emotions**, other core elements of the stress process (Kopp M. S. et al., 2010). Despite being applicable to a variety of research contexts, limitations similar to stressor questionnaires and interviews also apply for appraisal and emotion inventories. A major (and more general) problem is potential confounding by antecedents (of appraisal/emotion), psychological variables (e.g. personality factors, psychopathology, and mood state of the person) and response biases in reporting on appraisal and mood. Moreover, special attention should be given to the children's abilities to report on moods and affection retrospectively and their awareness of distinct psychological feelings and coping strategies. As indicated before, graphical approaches are recommended tools for childhood populations (Herbert T. B. et al., 1996).

Even though **cortisol analysis** may be considered as an objective biomarker of HPA activity and stress, some specific issues and recommendations (dependent to the type of bio-sample) should be considered. As already mentioned, cortisol levels are influenced by many factors (physical activity, diet, circadian rhythm, pulsatility etc.). Within this context, a single cortisol measurement may not be accurate in representing long-term exposure to cortisol (Kaplan N. M., 1996; Miller G. E. et al., 2007). It is therefore advised 1) to increase the frequency of sample collection with multiple measurements per day and on several days (which is hard for serum cortisol from an ethical point of view, especially for children), and 2) to standardize the time of collection of salivary and serum samples between participants to correctly assess the individual stress response over time (Hanrahan K. et al., 2006; Kudielka B. M. et al., 2010). These recommendations do not apply to hair and 24-hour urine samples, as these samples inherently represent cortisol concentrations over a longer period of time by pooling over one or multiple diurnal cycles.

Although it is recommended to measure cortisol in other biological samples, if repeated blood samples are required, it is recommended to apply local anaesthetics, use stress-reducing

butterfly needles and audio-visual distraction (cartoons, music, spoken story via headphones, toys and dolls) (Chambers C. T. et al., 2009; Kettwich S. C. et al., 2007).

Since sampling of saliva, urine or hair may be guided by (or with help of) the parents, detailed information and procedures should be established and provided to the parents to increase protocol compliance (e.g. hand-outs and checklists for home sampling, handling and storage procedures, colour-markings of the different sample tubes, good communication with the parents regarding dates of sampling and collection). Detailed strategies for salivary sample collection and salivary cortisol assays were reviewed by Hanrahan et al. and Gatti et al. (Hanrahan K. et al., 2006; Gatti R. et al., 2009).

4 Conclusions and the selection of an assessment method

Assessing psychosocial stress in children is challenging for both researchers and children and a standardized and meticulous approach is important in the harmonisation of childhood stress research for the sake of comparability and solid progress in our understanding of stress effects in humans. Selection of an appropriate stress assessment method for a particular research question may not be straightforward and should be well-considered. Taking into account the inherent advantages and disadvantages of each method, some broad conclusions can be formulated.

Most importantly, the approach will depend on the research question. Whether one is interested in stressful life events, appraisal, emotions, endocrine changes or combinations thereof, these different aspects of the stress response carry distinct information and thus appropriate methods should be chosen accordingly (Figure 1.5). A key recommendation is to combine multiple stress assessment methods (both questionnaires/interviews and more objective stress hormone measures), since each method has its restrictions but if applied simultaneously, valuable complementary information and a more aggregated view on stress can be obtained. However, this is financially and practically not always feasible, especially in large-scale epidemiological studies, but it could be an option to use both methods in a subsample of the population (e.g. a calibration sample). Moreover, when studying stressors and health outcomes, the time lag between event occurrence and the studied health outcome or stress response should be considered. If researchers are aware of the variation in temporal span between questionnaires (e.g. days, months or years) and cortisol measurements (e.g. minutes to hours for serum or saliva, 1 day for 24-hour urine and weeks to months for hair),

study results may be interpreted more correctly. To measure long-term stressors, multiple measurements with a short interval in between (e.g. months) are recommended.

From a financial and logistic point of view, questionnaires are most feasible, time- and cost-effective in large-scale epidemiological research in children. However, special attention should be given to the youngest age groups since the ability and competence to accurately self-report on stressors and psychological responses might not be fully developed. In contrast, laboratory measurements of stress hormones such as cortisol have a higher logistic burden and are more expensive than questionnaires, as specialized staff and equipment are needed (Table 1.5). As a result, the total cost in large-scale studies could increase rapidly. Moreover, biosample collection may impose a higher burden on the children than questionnaires or interviews do. In addition, still some work is needed to optimize the techniques, standardize sampling protocols and establish reference values in order to obtain comparable research outcomes. Nevertheless, salivary, urinary and hair cortisol measurements are promising biomarkers in epidemiological, stress-free settings.

CHAPTER 1.4 OBJECTIVES OF THIS THESIS

The objectives of this thesis are schematically presented in Figure 1.7 and described in more depth below, including a corresponding reference to the results-chapter. More detailed information on study participants and analytical aspects of this thesis are described in the following methodological Chapters 2.1 to 2.5.

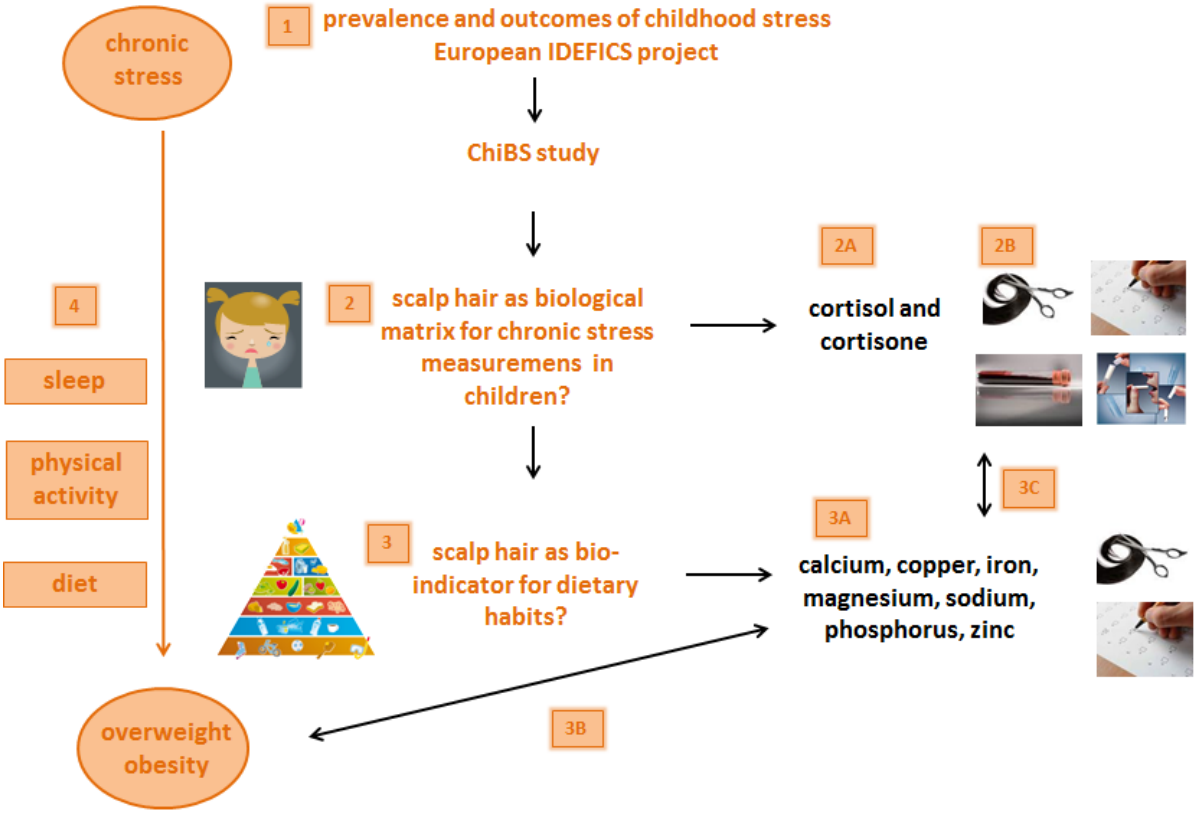


Figure 1.7 Schematic presentation of this thesis' research objectives.

1 Importance of studying childhood stress – European framework

As this thesis is situated in the broad domain of stress, diet and body composition in children, a first aim of this thesis was to indicate the importance of recording and monitoring childhood stress by describing the **prevalence of childhood stress (Chapter 3.1)** and its **relationship with psychosomatic and emotional symptoms in pre- and primary-school children aged 4 to 11 (Chapter 3.2)** in the framework of the European IDEFICS project (Identification and prevention of Dietary- and lifestyle-induced health EFfects In Children and infantS). This objective is presented by box 1 in Figure 1.7.

2 Scalp hair as biological matrix for chronic stress assessments in children

In order to evaluate the impact of stress on children's health and well-being, standardised, valid and reliable stress assessment methods are needed that can be easily implemented in epidemiological childhood research. The second aim of this thesis was therefore to examine the practical utility of scalp hair as a biological matrix for chronic stress assessments in elementary school children. Despite the logistical advantages of scalp hair over other biological sample types for epidemiological research in young children (e.g. its ease of standardised sampling, its non-invasiveness and retrospective character), its application remained uninvestigated in this target group. This objective is presented by box 2 in Figure 1.7.

- In this research, **physiological concentrations of cortisol and cortisone** were measured in children's hair samples; more specifically, in a subsample of children participating in the ChiBS project (Children's Body composition and Stress) (Box 2A in Figure 1.7). As this thesis is situated in the domain of **chronic stress**, it was decided to analyse **hair samples of 6 cm proximally**, as this has generally been assumed to be the maximal length of hair for a reliable estimate of systemic cortisol concentrations in the recent past (Russell E. et al., 2012). These 6 cm hair samples represent a period of 6 months in the past and thus 'chronic' stress (Harkey M. R., 1993). In particular the novelty of the hair cortisone observations should be mentioned, as hair cortisone has never been investigated in relation to psychosocial stress or in children, until now. For this research, it was decided not to apply the commonly used immunoassay technologies for hair cortisol and cortisone quantification, as these commercial kits are not hair-matrix specific and cross-reactivity may occur. Therefore, **a novel analytical method using ultra performance liquid chromatography tandem mass-spectrometry (UPLC-MS/MS) was developed and validated** to simultaneously detect cortisol and cortisone concentrations in children's hair samples **(Chapter 2.3)**.

- In a next step, the **association between hair cortisol and cortisone, and child-reported stress estimates (Chapter 3.3)** was investigated, as were the **inter-correlations of cortisol in different biological sample types (hair, serum, saliva). (Chapter 3.4)**. Also, analyses were performed examining which of the applied stress

assessment methods may **most accurately estimate childhood stress (Chapter 3.4)** (Box 2B in Figure 1.7).

3 Scalp hair as bio-indicator for dietary habits in children and its association with metabolic health and stress

Research has shown that stress and obesity may be associated with changes in the body's mineral status. Apart from being measurable in blood and urine, minerals are also detectable in other biological samples like scalp hair (Kempson I. M. et al., 2011) and evidence suggests that hair mineral levels may reflect dietary habits and/or mineral intake (Chojnacka K. et al., 2010b; Hong S. R. et al., 2009; Jeruszka-Bielak M. et al., 2011), although this topic remained controversial. Within the scope of diet quality in children, a third objective of this thesis was therefore to examine the suitability of hair as a bio-indicator for children's dietary habits via additional measurements of minerals in the obtained hair samples. This objective is presented by box 3 in Figure 1.7.

- More specifically, **long-term calcium, copper, iron, magnesium, sodium, phosphorus and zinc** levels were quantified in 6 cm hair samples (i.e. representing 6 months in the past) using inductively coupled plasma mass-spectrometry (ICP-MS) (Box 3A in Figure 1.7). **Reference values** were calculated and the relationship with **food consumption frequencies** as a marker of the child's dietary pattern was investigated **(Chapter 3.5)**.

- Given the potential relationship between hair minerals and dietary habits on the one hand, and the association between dietary habits and metabolic health or body composition on the other hand, we further examined the potential **relationship between hair minerals, obesity and metabolic health** which has been suggested in literature but remained unexplored in children (Box 3B in Figure 1.7) **(Chapter 3.6)**.

- As stress may directly or indirectly affect the body's mineral status, a last objective in the context of hair mineral research was to investigate the **relationship between hair mineral concentrations and childhood stress estimates (Chapter 3.7)**, which is a novel and unexplored research area (Box 3C in Figure 1.7).

4 Childhood stress and the association with overweight or obesity

As stress has been hypothesized in the development of obesity, also in children, a final aim of this study was to investigate the **relationship between childhood stress and alterations in body composition of the child** (BMI, fat percentage…), taking into account the moderation or mediation **effect of lifestyle factors** (diet, sedentary lifestyle and sleep) **(Chapter 3.8)**. This objective is presented by box 4 in Figure 1.7.

PART 2
METHODOLOGY

Chapter based on

Michels N, Vanaelst B*, Vyncke K, Sioen I, Huybrechts I, De Vriendt T et al.* ***Children's Body composition and Stress- the ChiBS study: aims, design, methods, population and participation characteristics.*** *Archives of Public Health (2012), 70:17.*

Vanaelst B, Rivet N*, Huybrechts I, Ludes B, De Henauw S, Raul JS.* ***Measurement of cortisol and cortisone in children's hair using ultra performance liquid chromatography and tandem mass spectrometry****. Analytical Methods (2013), 5: 2074-2082.*

Vanaelst B, Huybrechts I, Michels N, Vyncke K, Sioen I, De Vriendt T et al. ***Mineral concentrations in hair of Belgian elementary school girls: reference values and relationship with food consumption frequencies.*** *Biological Trace Element Research (2012), 150: 56-67.*

**Joint first authorship*

As this thesis is embedded within the IDEFICS project and its Belgian sub-study, namely the ChiBS project, these projects are presented in this methodological section, including a description of the study population and performed measurements of this research.

A first aim of this thesis was to describe the prevalence and outcomes of childhood stress in the framework of the European IDEFICS project. Chapter 2.1 therefore briefly presents the main objectives, study design and participation characteristics of the IDEFICS project. Chapter 2.1 additionally introduces the position of ChiBS within IDEFICS.

The other research objectives of this thesis relate to the utility and feasibility of scalp hair as biological matrix for measurements of chronic stress and mineral status in children. These research questions were examined in the frame of the ChiBS project, of which Chapter 2.2 describes the objectives, methodology, population and participation characteristics.

Chapter 2.3 and Chapter 2.4 present the development and respectively validation and evaluation of the analytical methods to quantitatively measure respectively cortisol and cortisone, and minerals in scalp hair.

A graphical summary of this thesis' study population for each of the research objectives is given in Chapter 2.5.

CHAPTER 2.1 THE IDEFICS PROJECT

1 Objectives and design

The IDEFICS (Identification and prevention of Dietary- and lifestyle-induced health EFfects In Children and infantS) project is a multicentre longitudinal intervention study of pre- and primary-school children in 8 European countries (Belgium, Cyprus, Estonia, Germany, Hungary, Italy, Spain, Sweden). IDEFICS investigates the aetiology of diet- and lifestyle-related diseases and disorders in children, with a strong focus on overweight and obesity in children and the effects and interactions of diet, lifestyle, psychosocial and genetic factors on children's health. In this project, also community-oriented prevention programmes for obesity are developed and evaluated in a controlled study design (i.e. a non-randomized community trial with geographical separation of intervention and control regions): in each country, one intervention and one control region was selected which were comparable with regard to infrastructural, socio-demographic and socio-economic characteristics. The finality of the IDEFICS intervention program was to induce behavioural changes on three levels, i.e. diet, physical activity and stress, coping and relaxation (De Henauw S. et al., 2011).

The figures below illustrate the timeframe of the IDEFICS survey (Figure 2.1) and a geographical presentation of the participating countries (Figure 2.2). The baseline survey (T0), performed between September 2007 and May 2008, was the starting point for the cohort study and provided baseline data on the prevalence of the disorders under study and their determinants in various European populations, collected in a comparable and standardized manner. Two years later, the children were reassessed in the first follow-up survey (T1) which was performed between September 2009 and May 2010, and which used the same survey modules as in T0. As such, aetiological associations between baseline predictors and selected follow-up end points and effects of the intervention program could be evaluated. The last survey (T2) was limited in scope and included assessment of the sustainability of the intervention program (September-November 2010). More detailed research goals, methodology and instruments of IDEFICS have been described elsewhere (Ahrens W. et al., 2011a).

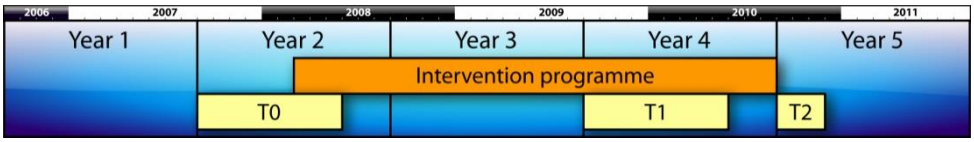


Figure 2.1 Timeline of the IDEFICS survey periods and implementation of the intervention. T0: baseline survey (2007-2008); T1: first follow-up survey (2009-2010); T2: second follow-up survey (2010) (Figure reprinted with permission from IDEFICS).

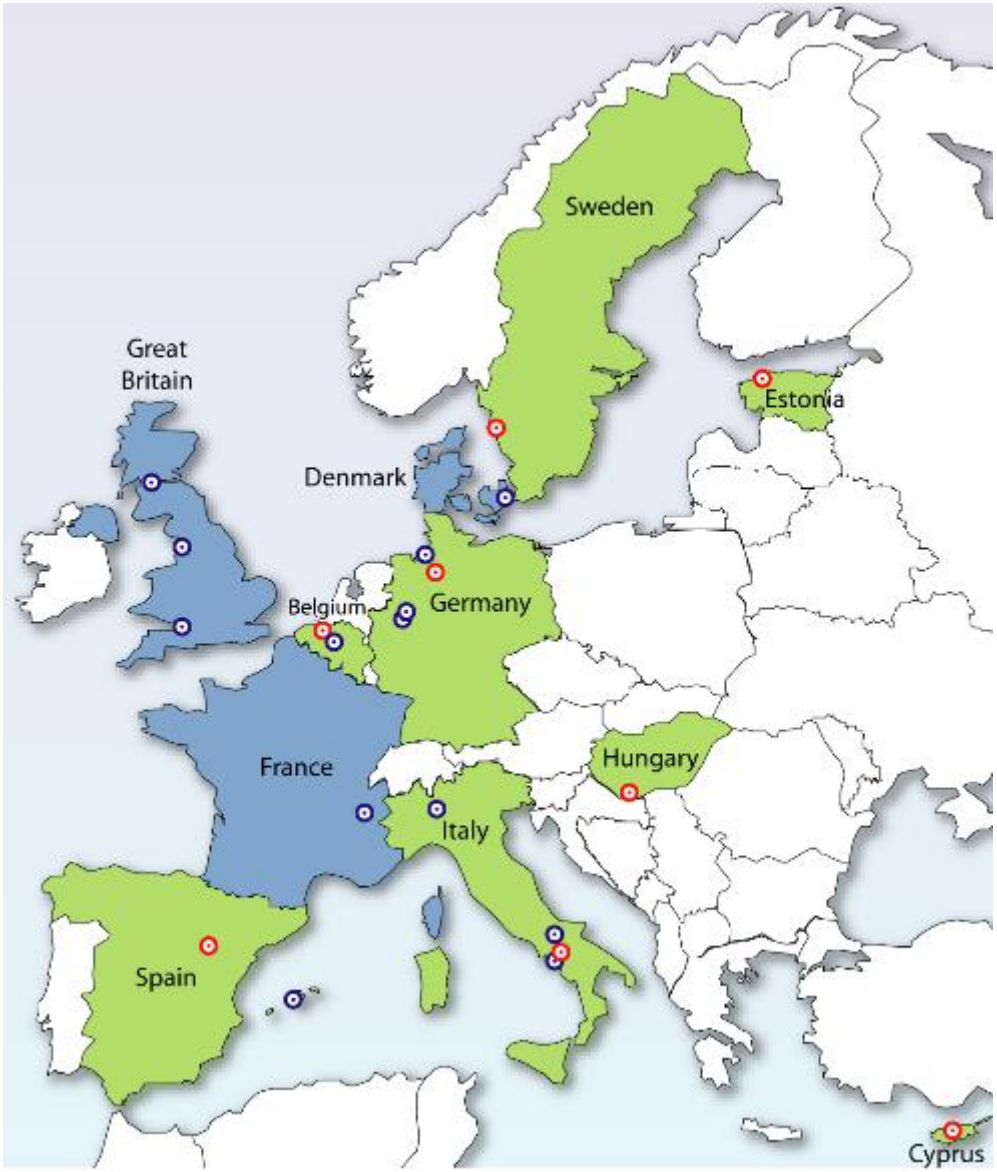


Figure 2.2 Geographical presentation of research institutions and survey centres of the IDEFICS project. The IDEFICS fieldwork was executed in survey centres located in green-coloured countries (C=control region, I=intervention region): Belgium (C: Aalter, I: Geraardsbergen), Cyprus (C: Pafos, I: Strovolos), Estonia (C: Tallinn, I: Tartu), Germany (C: Wilhelmshaven, I: Delmenhorst), Hungary (C: Zalaegerszeg, I: Pecs), Italy (C: Avellino,

Lauro, Quindici, Volturara, Monteforte I, I: Atripalda, Forino, Pratola Serra, Mercogliano), Spain (C: Zaragoza, I: Huesca), Sweden (C: Alingsas/Mölndal, I: Partille) (Figure reprinted with permission from http://www.idefics.eu)

2 Study population and measurements

All children residing in the selected intervention and control regions who were within the defined age group of 2-9 years old at baseline, were eligible for participation in IDEFICS. The baseline survey started with a cohort of 16224 children who were approached through school and kindergarten settings using a letter and leaflet addressed to the parents. The T1 follow-up survey resulted in a total sample size of 13498 children, as presented in Figure 2.3. Children participating in the Belgian control arm of IDEFICS T1 (dark box in Figure 2.3) were eligible for participation in the ChiBS project, a Belgian spin-off project of IDEFICS which is described in the following Chapter.

Examinations in children were performed according to a standardised protocol and included anthropometry, blood pressure, physical fitness, assessment of physiological markers in blood and urine. Socio-demographic, behavioural, medical, nutritional and lifestyle data of the children was collected through parental reported questionnaires (Ahrens W. et al., 2011a).

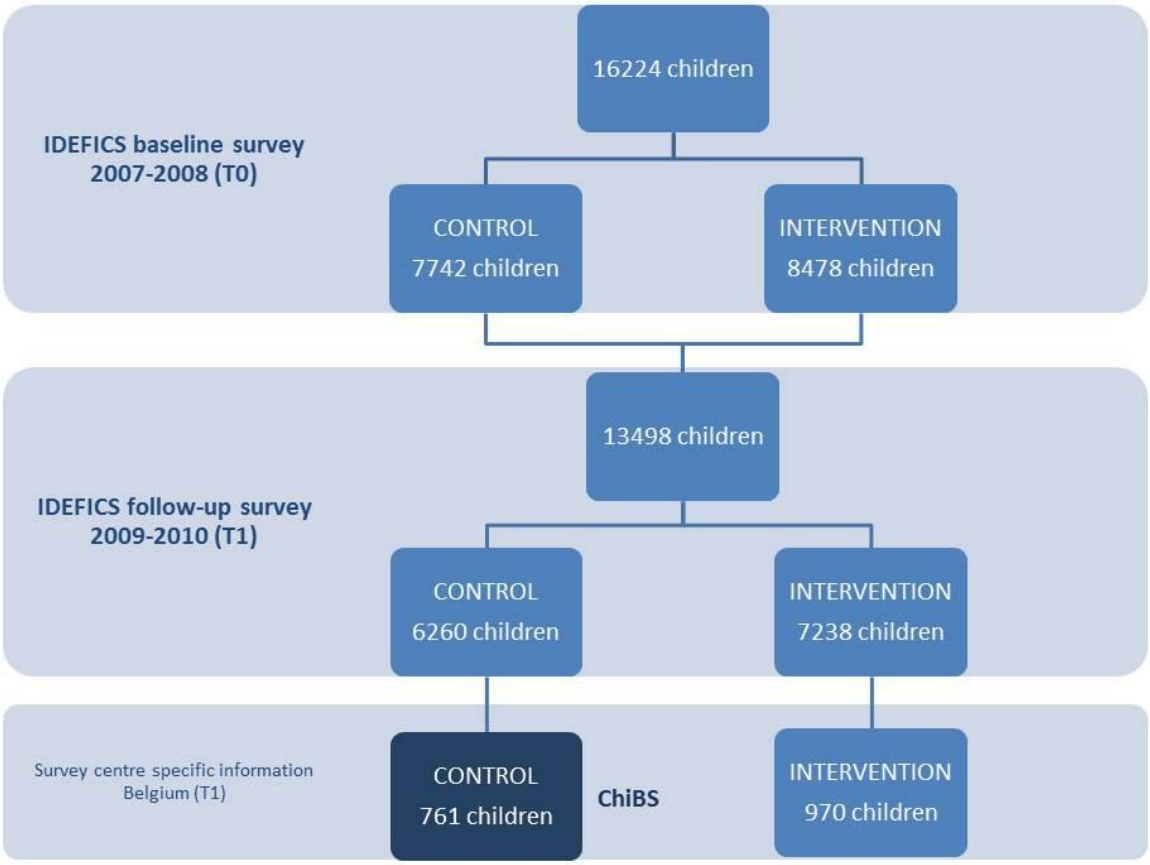


Figure 2.3 IDEFICS study flowchart: total number of participants at baseline (T0) and first follow-up survey (T1) and the T1 survey centre specific information for Belgium. Children participating in the Belgian control arm of T1 IDEFICS (dark blue box) were eligible for participation in the ChiBS project.

CHAPTER 2.2 CHILDREN'S BODY COMPOSITION AND STRESS - THE CHIBS STUDY

1 Objectives

The ChiBS study (Children's Body composition and Stress) was designed at Ghent University to investigate the relationship between chronic psychosocial stress in young children (6-12 years old) and changes in body composition (body fat) over a two-year follow-up period (2010-2012). It is hypothesized that exposure to chronic stressors may affect children's body composition in the long-term by promoting body fatness and hence the development of obesity. More specifically, this study examines the influence of chronic stress on the evolution of different body composition parameters longitudinally, taking into account diet, sleep and physical activity as intermediary factors in this relationship as presented in Figure 2.4.

To accurately measure stress, child- and parent-reported stress questionnaires as well as objective stress biomarkers from different biological matrices are used. A second, parallel aim of this project is to test the feasibility and interrelationships of these different stress measurements in children. Finally, the third aim of this study is to further unravel the impact and mutual relationships of physical activity, diet, sleep and stress. This chapter describes the design of the ChiBS study, its instruments and measurements, population characteristics, and participation and drop-out rates for each examination module.

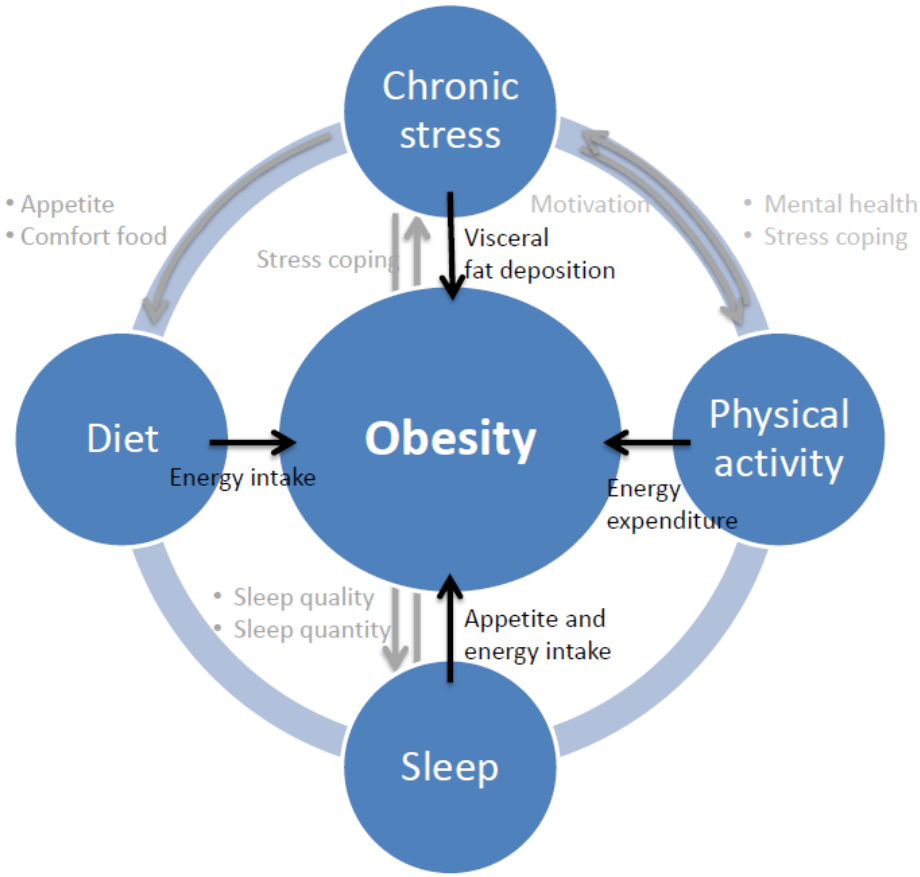


Figure 2.4 Lifestyle factors involved in the development of obesity which are being investigated in the ChiBS project. Grey arrows indicate the study hypotheses, black arrows show the effect of the four lifestyle factors on obesity.

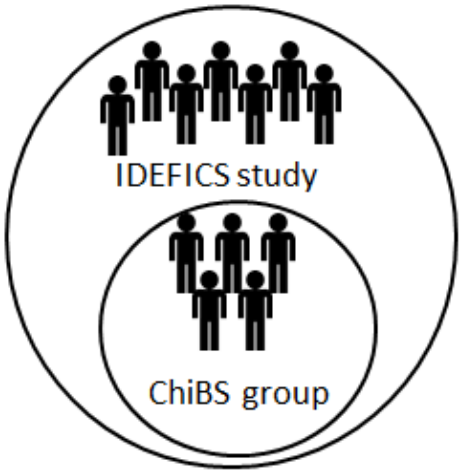
2 Methods

2.1 Study design and sampling

To study the relationship between chronic stress and changes in body composition over a two-year follow-up period, the ChiBS study was designed as a prospective cohort study. Approach and enrolment of the participants for the baseline survey of ChiBS (February 2010) was largely simplified by integrating it in the IDEFICS project (Ahrens W. et al., 2011a). Indeed, the baseline survey of ChiBS coincided with the first follow-up survey (school-year 2009-2010) of the Belgian control cohort (i.e. in the city Aalter) of the IDEFICS study. All children participating in the control section of this IDEFICS survey (N=761) were eligible to join the ChiBS study.

At baseline, the children were between 5 and 11 years old (last year of kindergarten and first four years of elementary school). Their parents were individually contacted by providing a letter via the schools, wherein they were informed and invited to let their children participate in the ChiBS project. Parents were asked to sign a consent form, in which the option was offered to participate in the full ChiBS programme or in a selected set of measurement modules. The children were followed-up in a second and third survey module of the ChiBS study, conducted in February-June 2011 and February-June 2012 to fully cover primary-school age. For both follow-up surveys, the parents of participating children were contacted telephonically and were asked to sign a new consent form, in which options for all separate examination modules are offered. The fieldwork was conducted partly at school and partly at the municipal sports park of the city Aalter (permanent localisation of materials e.g. BODPOD®). The ChiBS study was conducted according to the guidelines laid down in the Declaration of Helsinki and is approved by the Ethics Committee of the Ghent University Hospital.

Figure 2.5 schematically presents the timeline of the ChiBS project and the corresponding measurements and examinations of each survey period. While ChiBS' examinations focus on stress-assessment (i.e. questionnaires and biomarkers), body-composition measurements and eating behaviour; additional information on socio-demographics (e.g. parental education, income, place of birth), sleep, medical conditions, dietary intake and physical activity is obtained as part of the IDEFICS study, collected at the baseline ChiBS survey (Ahrens W. et al., 2011a).



	baseline 2010*	1st follow-up 2011	2nd follow-up 2012
	cross-sectional	longitudinal	
Assessment of			
STRESS BIOMARKERS	Salivary cortisol		
	Hair cortisol		
	Serum cortisol		
	HRV	HRV	HRV
STRESS QUESTIONNAIRES	CLES-C	CLES-C	CLES-C
		CHS-CUS	CHS-CUS
	Emotions	Emotions	Emotions
	Coping	Coping	Coping
	SDQ	SDQ	SDQ
ANTHROPOMETRY	BODPOD	BODPOD	BODPOD
	Routine antropometry		Routine antropometry
DIET	CEHQ-FFQ	CEHQ-FFQ	CEHQ-FFQ
		DEBQ	DEBQ

*Figure 2.5 Timeline of the ChiBS project and corresponding measurements of each survey period. All measurements are conducted at the municipal sports park of Aalter, except for the salivary, hair and serum sampling and routine anthropometric measurements of the baseline ChiBS survey which were conducted at the schools. The questionnaires placed in a frame were administered to the parents, not to the children. *Administration of the stress questionnaires and bio-sampling at the baseline survey were restricted to elementary school children (not children from kindergarten). In the first and second follow-up survey all children were of elementary school age. CEHQ-FFQ= Children's Eating Habits Questionnaire-Food Frequency Questionnaire, CHS-CUS= Children's Daily Hassles and Daily Uplifts Scale, CLES= Coddington Life Events Scale, DEBQ= Dutch Eating Behaviour Questionnaire, HRV= heart rate variability, SDQ= Strengths and Difficulties Questionnaire*

2.2 Measurements and examinations

A. Stress biomarkers

In analysing stress, the two most important stress-pathways are covered using heart rate variability (HRV) representing the autonomic nervous system (Krantz D. S. et al., 1997; Thayer J. F. et al., 2005) and cortisol measurements representing the HPA stress system (Charmandari E. et al., 2005; Baum A. et al., 1997). Cortisol is the most commonly used stress biomarker since both the normal pulsation and rhythmic fluctuation in cortisol production is sensitive to on-going life stressors (Lightman S. L., 2008). As cortisol can be measured in different biological materials which each have their own strengths and limitations (Vanaelst B. et al., 2012b; Baum A. et al., 1997; Levine A. et al., 2007), three different biological samples were collected in the ChiBS study, including serum, salivary and hair samples. This way, short and long-term stress exposure were covered. Furthermore, these three different sample types allow an in-depth investigation of the validity, feasibility and intercorrelations of cortisol measurements in serum, saliva and hair among children.

Salivary cortisol

Saliva was collected into Salivette synthetic swabs especially designed for cortisol analysis (Sarstedt, Germany), of which an illustration is given in Annex 1. The participating children were asked to collect saliva during two consecutive weekdays at four time points: immediately on awakening (T0), 30 minutes after waking-up (T30), 60 minutes after waking-up (T60) and in the evening between 7 and 8 PM (Tev). After all, cortisol secretion has a circadian rhythm with low levels in the evening/night and high levels in early morning and there is also a cortisol awakening response (CAR) showing a quick cortisol increase within 30 minutes after awakening. This collecting scheme allows to obtain information on both the circadian rhythm and the CAR (Fries E. et al., 2009). To standardize sample collection, sampling and storage instructions were provided in a manual (see Annex 1). The parents were also asked to fill in a checklist about instruction compliance (modified from Hanrahan et al. (Hanrahan K. et al., 2006)): (1) awakening time, collection hours, health condition, physical activity, caffeine consumption and medication on the collection days and (2) the compliance to food restriction and teeth brushing one hour before the sampling. Analysis was done using an electrochemiluminescence immunoassay (ECLIA) (Van Aken M. O et al., 2003). Samples

of corticosteroid-users, as well as morning samples collected more than 5 minutes too late/early and evening samples not collected between 7 PM and 9 PM were excluded. The detailed descriptive results were published elsewhere (Michels N. et al., 2012b).

Hair cortisol and cortisone

The ChiBS study functions additionally as a pilot project evaluating the feasibility and validity of hair cortisol and cortisone measurements in healthy children and its applicability for large-scale psychosocial stress research in young children, as this is currently unexplored. Cortisol and cortisone concentrations were analysed in hair samples obtained from the vertex posterior region of the scalp (Gow R. et al., 2010; Russell E. et al., 2012). Sampling was done at school by trained researchers. The hair samples were cut as close to the scalp as possible using clean, stainless steel scissors and tied together with a little cord to mark the proximal side. Only the most proximal 6 cm of the hair strands were analysed (approximately 150 to 200 mg of hair), which is generally assumed to be the maximum length of hair to obtain a reliable estimate of systemic cortisol concentrations in the past (i.e. 6 months) (Russell E. et al., 2012). Hair samples were exclusively taken from girls to maximize the possibility that the hair reached the required length. If the length of hair was shorter than 6 cm, no sample was taken. The hair samples were stored in a folded piece of paper in individual zip-lock bags in a dark, dry place and at constant temperature, to be analysed 12 months later at the University of Strasbourg in the Institute of Legal Medicine (Faculty of Medicine) to which analyses were outsourced. Hair cortisol has been shown to be stable over months to years, justifying the long time lag between sampling and analysing (Webb E. et al., 2010; Sauve B. et al., 2007). An amount of 50 mg of hair was finely minced, after which cortisol was extracted from the hair samples. Cortisol and cortisone concentrations were analysed using a Liquid Chromatography-tandem Mass-Spectrometry technique (Acquity UPLC Waters, Quattro Premier XE and Mass Lynx software). The details of the methods and the validation parameters are described in Chapter 2.3 and photographical illustrations of the performed hair sampling are presented in Annex 2.

Serum cortisol

After an overnight fasting period, blood samples were obtained through venipuncture using local anaesthesia with EMLA® patches (lidocaine+prilocaine). Blood samples were

centrifuged and serum was stored at -80°C. Serum cortisol was assayed using the same technique as salivary cortisol, namely ECLIA.

Heart rate variability

HRV is defined as the variability of the time between consecutive R peaks of the electric QRST interval on the electrocardiogram. In a healthy situation, considerable variability reflects the heart's ability to adequately respond to physiological and environmental stimuli. It is hypothesized that chronic stress might facilitate a decreased HRV (Camm A. J. et al., 1996). A detailed methodological description of the performed HRV measurements is out of this thesis' scope. Therefore, the reader is referred to elsewhere (Michels N. et al., 2012d).

B. Stress questionnaires

If children and parents agreed to provide a saliva or hair sample for cortisol analysis, the children were individually interviewed by a trained researcher to obtain information about their life events *(Coddington Life Events Scale)*, daily hassles and uplifts *(Daily Hassles and Uplifts Scale)*, emotions *(Basic Emotions)* and coping strategy *(Coping Questionnaire)*. Furthermore, parents were asked to report on their child's behavioural and emotional problems (*Strengths and Difficulties Questionnaire*). Only children from primary school were eligible to fill in the questionnaires (not kindergarten children).

Life events

The 'Coddington Life Events Scale' for children (CLES-C) (Coddington R. D., 1972) was used to identify potential physical and mental health problems arising from psychological causalities (reliability: r=0.69; construct validity=0.45). The English questionnaire was translated professionally into Dutch using a translation and back-translation process to ensure identical meaning. This validated 36-item questionnaire measured the frequency and timing of events in the last year relevant for this age group and resulted in a 'life change units' score for the time periods 0-3, 0-6, 0-9 and 0-12 months ago. To limit recall bias and to increase the accuracy of reporting in time, interviewers used calendar events such as birthdays, summer holidays and Easter (Garrison C. Z. et al., 1987). Moreover, drawings and pictures were used to clarify some of the most difficult events, such as 'juvenile court', 'respect' etc. Children

with a score above the age-specific cut-off were considered to be at higher risk to suffer from psychological problems. Apart from the total event score (both negative and neutral events), also a score for exclusively negative life events was calculated.

Daily hassles and uplifts

The children's daily hassles (CHS) and daily uplifts (CUS) scales of Kanner et al. (Kanner A. D. et al., 1981) contain 25 hassles and 25 uplifts, respectively (internal consistency: alpha=0.87). Also for children as young as 5 and 6 years old, an internal consistency of 0.85 was shown and daily hassles correlated with parental reported behavioural problems (Creasey G. et al., 1995). Hassles refer to irritating, frustrating or distressing demands that characterize everyday transactions with the environment. Uplifts refer to positive experiences such as the joy derived from friendship, relief at hearing good news and so on. Children were asked to check which hassles and uplifts occurred during the last month. Furthermore, they were asked to rate whether they felt 'not bad', 'sort of bad', or 'very bad' as a result of the hassle and whether they felt 'OK', 'sort of good' or 'very good' as a result of the uplift. Both a total frequency, a frequency of higher intensity hassles and uplifts ('sort of bad' or 'very bad' and 'sort of good' or 'very good', respectively) and an intensity score can be calculated.

Emotions

Children were questioned about their recent feelings. As in the study of Zimmer-Gembeck (Zimmer-Gembeck MJ. et al., 2009), the feelings anger, anxiety, sadness and happiness were rated on a 0 to 10 Likert-scale (0 'not at all' to 10 'very strong'). To help the children understand these distinct feelings, pictures of a social skills training game for very young children were displayed next to the question (Dupondt L., 2011). These basic emotions are understandable for infants and children (Flavell JH., 1999) and can therefore uncomplicatedly be used in our population.

Coping

The children were asked what they usually do when they are confronted with problems or when they are upset by using an 8 item-questionnaire, with 'never', 'sometimes' or 'often' as

response alternatives. This questionnaire was previously used in the CASE-study (Child and Adolescent Self-harm in Europe) (Madge N. et al., 2008) and translated into Dutch and substantially pilot-tested for a population of Belgian adolescents (Portzky G. et al., 2008). Although no psychometric data on this coping questionnaire was available for our age group, other coping questionnaires have been used with children's self-report (Blount R. L. et al., 2008) and acceptable repeatability was shown in 5 to 6-year old children with open-ended questions (r between 0.67 and 0.77) (Creasey G. et al., 1995). The answers were classified as emotion- versus problem-focused coping, based on the transactional model of Lazarus and Folkman (Folkman S. et al., 1986). Emotion-focused coping is aimed at regulating emotional stress while problem-focused coping deals with the problem and makes changes in the disturbed and stress-inducing person-environment relationship.

Strengths and Difficulties Questionnaire

Parents were asked to complete the standardized 'Strengths and Difficulties Questionnaire' (SDQ) (reliability: ICC=0.80; concurrent validity: r=0.70) (Goodman R., 1997), reporting the emotional problems of their child over the past six months. For each of the 25 statements, parents could answer: 'not true' (0), 'somewhat true' (1) and 'certainly true' (2). The statements were divided in 5 subscales of 5 items each: emotional problems, conduct problems, hyperactivity-inattention behaviour, peer problems, and prosocial behaviour. Subscale scores were computed by summing scores on relevant items (after recoding reversed items). Higher scores on the prosocial behaviour subscale reflect strengths, whereas higher scores on the other four subscales reflect difficulties (Ravens-Sieberer U. et al., 2000).

C. Body composition measurements

Routine anthropometry

The routine anthropometric measurements were carried out by two trained observers at school to improve intra- and inter-observer reliability. Routine anthropometric measurements were performed in accordance with the standardized procedures of the IDEFICS project (Ahrens W. et al., 2011a; Stomfai S. et al., 2011; De Henauw S. et al., 2011). Weight was measured in fasting status with an electronic scale (TANITA BC 420 SMA, Germany) to the nearest 0.1 kg. Height was measured with subjects barefooted on a telescopic height measuring

instrument (SECA 225, UK) to the nearest 0.1 cm. The body mass index (BMI) was obtained as weight(kg)/height(m)², and the SD score of BMI (Z-score BMI) was calculated to adjust for age and sex using the LMS Growth method (abbreviation refers to smooth curve-L, mean-M and coefficient of variation-S; Cole, 1990) and the British 1990 growth reference data (Cole T. J. et al., 1998). Overweight and obesity were determined using the International Obesity Task Force classification (Cole T. J. et al., 2000). Leg-to-leg impedance (ohm) was also measured with the electronic TANITA BC 420 SMA scale (adapted to the small foot size of children). As impedance is dependent on length of the conductor, an impedance index reflecting the fat-free mass was defined as height²/impedance.

Skinfold thicknesses (mm) were measured twice on the right side of the body to the nearest 0.2 mm with a skinfold calliper (Holtain, UK, range 0-40 mm) according to the international standards for anthropometric assessment (ISAK, Marfell Jones 2006) and the mean of both measurements was calculated. The triceps skinfold was taken halfway between the acromion process and the olecranon at the back side of the arm. The subscapular skinfold was measured 20 mm below the inferior angle of the scapula, at an angle of 45° to the lateral side of the body. If the first and second measurement of the skinfolds differed more than 2 mm, a third measurement was performed. Circumferences (cm) were measured once with an inelastic tape (Seca 200, precision 0.1 cm, range 0-150 cm) with the child in a standing position. Circumferences were taken at the following four sites: 1) mid-upper arm, halfway between the acromion process and the olecranon process on a relaxed arm; 2) waist, halfway between the top of the iliac crest and the lower coastal border (10^{th} rib); 3) hip, at the maximum extension of the buttocks; 4) neck, just above the larynx and perpendicular to the long axis of the neck.

Fat mass determination by air displacement plethysmography (ADP)

To obtain reliable and valid body composition measurements, the ADP technique was conducted by the same person over all survey periods. This method is currently considered a good reference technique for body composition measurements with a quick, comfortable, automated, non-invasive and safe measurement process, making it feasible for children (Fields D. A. et al., 2002).

Body volume was measured by ADP (BODPOD®, Software version 4.2.4, Life Measurement Inc., Cranlea and Co, Birmingham, United Kingdom) using standardized procedures

(McCrory M. A. et al., 1995). Children had to refrain from physical activity and food consumption two hours before the measurement. The device was calibrated daily according to the manufacturer's guidelines. Children were measured twice in tight-fitting bathing suit with swim cap to rule out air trapped in clothes and hair. Thoracic gas volume was predicted by the software with a validated child-specific equation (Fields D. A. et al., 2002) and fat mass percentage was calculated using the equation reported by Wells (Wells J. C. K. et al., 2010). If the first and second measurement of the body volume differed more than 150 ml, a third measurement was performed.

D. Diet

Hair mineral analysis

The ChiBS project additionally offered the opportunity to study the suitability of hair as a bio-indicator for the dietary habits of children via additional measurements of minerals in the obtained hair samples and to study its relationship with the children's dietary habits (Kempson I. M. et al., 2011; Chojnacka K. et al., 2010b; Hong S. R. et al., 2009; Jeruszka-Bielak M. et al., 2011): each of the obtained hair samples was split into two fractions, i.e. one fraction for cortisol and cortisone analysis as described above, and one fraction for mineral analysis. Hair minerals, i.e. calcium, copper, iron, magnesium, sodium, phosphorus and zinc, were quantitatively determined with inductively coupled plasma- mass spectrometry (ICP-MS). The details of the methods and the validation parameters are described in Chapter 2.4.

Children's Eating Habits Questionnaire (CEHQ)- Food Frequency Questionnaire (FFQ) (parent-reported)

The CEHQ-FFQ investigates food consumption frequency and behaviours associated with obesity and general health in children. This 43 food-item-containing instrument was developed and validated within the IDEFICS project (Suling M. et al., 2011; Lanfer A. et al., 2011; Huybrechts I. et al., 2011a) and is used as a screening instrument to investigate dietary habits and food consumption frequency in children. Parents were asked to report on the frequency of their child's consumption of selected food items in a typical week during the preceding 4 weeks, outside the school canteen or childcare meal provision settings, using the following response options: 'never/less than once a week', '1-3 times a week', '4-6 times a

week', '1 time per day', '2 times per day', '3 times per day', '4 or more times per day' or 'I have no idea'. Frequencies of intake were assessed without quantifying portion sizes.

Dutch Eating Behaviour Questionnaire (DEBQ) (child-reported)

In the DEBQ, a 33-item questionnaire (reliability: r= 0.87-0.90), three types of eating behaviour can be identified in children: eating in response to negative emotions (emotional eating), eating in response to the sight or smell of food (external eating) and eating less than desired to lose or maintain body weight (restraint eating). In all three types of eating behaviour, the appropriate self-regulating mechanism of food intake is diminished or lost. Children could answer the questions with 'never', 'almost never', 'sometimes', 'often' or 'very often' as response alternatives (Vanstrien T. et al., 1986).

2.3 Sample size calculation

Sample size calculations, performed with the SAS System, indicated that a population of 500 children would allow to calculate correlation coefficients with 95% confidence within a 0.10 confidence interval of the true correlation coefficient of the population. This sample size additionally allows to observe a 10% increase in fat content with 95% confidence and 80% power. Also, this sample size allows to perform multiple regression analysis with BMI as dependent and stress as independent variable, adjusted for 6 covariates (i.e. child's age, sex, socio-economic status, sleep, diet and physical activity) (N=473) (SAS POWER procedure, Type III F Test in Multiple Regression).

3 Population and participation characteristics

3.1 Participation rate

Table 2.1 presents the consent- and drop-out-percentage, as well as the number of cases valid for data-analysis for each measurement module that took place at baseline and first follow-up. In the baseline and first follow-up survey, participation proportions of 68.7% (N=523/761) and 65.8% (N=418/635) were obtained, respectively. Willingness to participate from baseline to first follow-up (i.e. consent percentage) was 71.3% (N=453/635).

3.2 Socio-demographic characteristics

Table 2.2 shows the children's and parental socio-demographic characteristics of participants with a baseline assessment of the stress questionnaires (ChiBS participants) compared to non-participating children in the ChiBS study.

4 Discussion

The ChiBS project is unique in its setting and scope and promising with regard to follow-up percentages (consent percentage from baseline to first follow-up: N=453/635, 71.3%). The representativeness, participation and drop-out proportions of the study population, as well as the feasibility of the examination modules are discussed in depth in *Chapter 4.2 Methodological Considerations* of this thesis.

The ChiBS cohort provides valuable data as will be elaborated at large in this thesis, such as new insights into the influence of chronic psychosocial stress on changes in body composition, and its interaction with food intake regulation, physical activity/sedentary behaviour and sleep in young children. In addition, this study allows an in-depth investigation of the validity of the different methods that were used to assess stress levels in children.

Table 2.1 Participation numbers, separately for baseline survey (2010) and first follow-up (2011) of ChiBS in Aalter, Belgium

	baseline survey (2010) contacted N= 761						first follow-up (2011) contacted N= 635[a]					
	eligible	**informed consent**	**consent percentage[b]**	**participated**	**drop-out proportion[c]**	**valid for analysis[d]**	**eligible**	**informed consent**	**consent percentage**	**participated**	**drop-out proportion**	**valid for analysis**
stress questionnaires	598[e]	563	94.2	523	7.1	523	635	453	71.3	418	7.7	418
salivary cortisol	598[e]	495	82.8	454	8.3	439	n/a	n/a	n/a	n/a	n/a	n/a
hair cortisol and minerals	293[f]	231	78.8	223	3.5	223	n/a	n/a	n/a	n/a	n/a	n/a
serum cortisol	474[g]	406	85.7	357	12.1	272[h]	n/a	n/a	n/a	n/a	n/a	n/a
HRV	761	513	67.4	475	7.4	460	635	453	71.3	437	3.5	412
ADP	761	513	67.4	497	3.1	497	635	453	71.3	453	0.0	453
weight and height	761	761	100	750	1.5	750	635	453	71.3	453	0.0	453

[a] all children who participated to the BODPOD® measurements and/or stress questionnaires in 2010

[b] percentage of informed consents on the total number of eligible children

[c] percentage of children for who consent was given that did ultimately not participate (e.g. fear, test too difficult, defects of equipment or machinery)

[d] number of children of whom data was valid for analysis after data-cleaning

[e] primary school children (boys and girls)

[f] primary school girls

[g] limited to children from whom a salivary and/or hair sample was available

[h] as serum samples were also used to analyse other biological components, an insufficient amount of the sample for cortisol analyses was the largest limiting factor

ADP= air displacement plethysmography; HRV= heart rate variability; n/a= not applicable as salivary, hair and serum sampling were not repeated in 2011

Table 2.2 Socio-demographic characteristics of participating and non-participating children in the ChiBS study (2010, Aalter, Belgium)

	ChiBS participants*		ChiBS non-participants**		X^2-test
	%	N	%	N	p-value
child characteristics					
sex					0.634
male	49.5	259	51.3	117	
female	50.5	264	48.7	111	
age (years)					<0.001
5	0.4	2	55.7	127	
6	11.9	62	19.3	44	
7	24.7	129	7.5	17	
8	26.6	139	6.6	15	
9	24.9	130	7.0	16	
10	11.1	58	3.1	7	
11	0.6	3	0.9	2	
BMI					0.550
underweight	13.6	71	11.8	27	
normal	79.3	415	82.0	187	
overweight	5.5	29	5.7	13	
obese	1.5	8	0.4	1	
parental characteristics					
household income (missing=108)			(missing n=35)		0.377
low to low/medium	5.3	21	5.7	11	
medium	3.3	172	38.2	87	
high/medium	20.7	108	23.2	53	
high	21.5	112	18.4	42	
education (ISCED) (missing n=30)			(missing n=12)		0.163
level 1	0.6	3	0.9	2	
level 2	0.6	3	2.6	6	
level 3	24.7	129	27.6	63	
level 4	20.7	108	18.9	43	
level 5 or higher	47.8	250	44.7	102	
family structure (missing n=32)			(missing n=13)		0.066
traditional	76.9	402	82.5	188	
non-traditional	17.0	89	11.8	27	
migrant status					
father migrant	1.1	6	2.2	5	0.770
mother migrant	3.4	18	3.1	7	0.274

* children participating in the baseline ChiBS stress-questionnaire; ** children participating in the IDEFICS study but not in the ChiBS study; BMI= body mass index with cut-offs as determined by the International Obesity Task Force classification; household income categories based on national statistics; ISCED= International Standard Classification for EDucation (1 'primary education', 2 'lower secondary education', 3 'upper secondary education', 4 'post-secondary non-tertiary education', 5 or higher 'tertiary education'); traditional family structure: children living with both biological parents – non-traditional family structure: all other family structures

CHAPTER 2.3 MEASUREMENT OF CORTISOL AND CORTISONE IN CHILDREN'S HAIR USING ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY

1 Introduction

To evaluate the impact of stress on children's well-being, it is important to have valid and reliable stress assessment methods. Cortisol and its inactive metabolite cortisone are both detectable in human hair (Raul J. S. et al., 2004; Stalder T. et al., 2012a) but have not been applied in relation to childhood stress research, despite its potential value in this complex but relevant discipline. Children's stress research may largely benefit from the establishment of a standardized and validated methodology to measure cortisol and cortisone in hair as this bio-matrix has the advantage of being non-invasive and easily obtainable, both important conditions for childhood research and more practicable indeed in comparison to other commonly used matrices such as saliva or serum.

Immunoassay technologies have been the preferred analytical approach for hair cortisol quantification, despite these commercial kits are not specifically developed for the hair-matrix and cross-reactivity of the kit's antibodies with exogenous glucocorticoids or endogenous cortisol metabolites may occur (Kirschbaum C. et al., 2009; Davenport M. D. et al., 2006; Perogamvros I. et al., 2009; Miller R. et al., 2013). In this context, particularly cortisone has been observed as major source of interference in (salivary) immunoassay research (Miller R. et al., 2013). Although high correlations have been shown for hair cortisol concentrations measured by immunoassay and liquid chromatography tandem mass spectrometry (LC-MS/MS) as reference method (Miller R. et al., 2013; Kirschbaum C. et al., 2009), it has been reported that immunoassay techniques may overestimate cortisol concentrations by 15% compared to LC-MS/MS (Kirschbaum C. et al., 2009). LC-MS/MS thus provides the opportunity to more accurately determine human hormone concentrations, even in small amounts of sample as was previously shown in biological fluids, and for multiple steroid hormones simultaneously (Gosetti F. et al., 2013; Lee S. et al., 2010; Perogamvros I. et al., 2009; Taylor R. L. et al., 2002).

To our knowledge, the first and only report of a LC-MS/MS assay for the simultaneous detection of cortisol and cortisone in human hair samples is attributed to Raul et al. which

dates back to 2004 (Raul J. S. et al., 2004). As this project is situated in the domain of chronic childhood stress research and a consensus on a reference method to measure cortisol and cortisone in hair is currently lacking, the aim of our study was to develop and validate an ultra-performance LC-MS/MS (UPLC-MS/MS) method to simultaneously detect cortisol and cortisone in hair samples for chronic stress research (i.e. hair samples of 6 cm long, representing a period of 6 months in the past and thus 'chronic' stress), based on previous work of Raul et al. (Raul J. S. et al., 2004). An easy and inexpensive method may encourage hair cortisol and cortisone measurements in future childhood stress research and introduce the application of hair cortisol/cortisone ratio measures in stress research.

2 Materials and Methods

2.1 Study participants and hair collection

223 elementary school girls participated to this study as part of the 2010 baseline survey of the ChiBS project ('Children's Body composition and Stress') (embedded within IDEFICS, http://www.idefics.eu). More detailed research goals and participation characteristics are described elsewhere (Michels N. et al., 2012d). Participation to this study was limited to the female participants of the ChiBS project (N=264/523), as hair sampling was only performed in girls to ascertain the required hair length of 6 cm (N=223/264, mean age= 8.44 years, SD=1.11). Agreement for participation was obtained through parental written informed consent. The ChiBS project was conducted according to the guidelines laid down in the Declaration of Helsinki and was approved by the Ethics Committee of the Ghent University Hospital.

One hair sample with a thickness of several millimetres in diameter was cut proximally at the vertex posterior region of the scalp of each participating girl (Russell E. et al., 2012; Li J. et al., 2012) and tied together with a little cord to mark the proximal side. External contamination of the hair shaft by hand-cream and -lotion used by the sampling-investigators, and artificial colouring of the child's hair were ruled out. The samples were individually stored in a folded piece of paper in zip-lock bags, placed in a large box accompanied by desiccant packets (Sorb-It®, Süd-Chemie AG) in the refrigerator at 7°C. Analyses of hair cortisol and cortisone were performed at the University of Strasbourg - Institute of Legal Medicine (Faculty of Medicine) to which analyses were outsourced.

2.2 Analysis of hair cortisol and cortisone

A. Chemicals and calibration standards

Dichloromethane (DCM) and HPLC-grade acetonitrile (ACN) were purchased from Carlo Erba (Val de Reuil, France) and Merck (Darmstadt, Germany), respectively. High pressure liquid chromatography (HPLC)-grade methanol (MeOH) and formic acid (HCOOH) were obtained from VWR (Pessac, France); cortisol, cortisone, prednisone and prednisolone from Sigma (Lyon, France) and deuterated cortisol (cortisol-d4, 9, 11, 12, 12 D) from Steraloids (Newport, USA). Cortisol and cortisone solutions were prepared in MeOH at a final concentration of 0.01, 0.10 and 1.00 mg/L. Deuterated cortisol was prepared in MeOH at a final concentration of 0.1 mg/L. These solutions were stable for at least 6 months at 4°C. Hairs for calibration standards were washed two times with DCM (for one night and two hours) in order to eliminate endogenous steroids and to obtain a pool of blank samples. Calibration standards were prepared at concentrations ranging from 5 to 1000 pg/mg for each analyte.

B. Sample preparation

The most proximal 6 cm of the hair samples were washed twice with DCM for 1 min at room temperature, dried with paper tissue to remove contamination (Raul J. S. et al., 2004) and finely minced using fine scissors. Hair samples were not pulverized as tests in our laboratory did not indicate a higher level of cortisol/cortisone extraction with this procedure, as also indicated by Stalder et al. (Stalder T. et al., 2012c). Fifty mg of minced hair was carefully weighed out in a 5 mL tube (Interchim, Montluçon, France). One mL of MeOH and 50 pg of cortisol-d4 were added, after which the tube was agitated for 24 h for steroid extraction. Samples were centrifuged 15 min at 3000 g and the supernatant was transferred into a new glass tube. The following steps were repeated twice more: One mL of MeOH was added to the minced hair, vortexed and centrifuged 15 min at 3000 g, after which the supernatant was compiled with the supernatant from the previous centrifugation (Ashley N. T. et al., 2011). The alcohol was evaporated at 40°C under a constant stream of nitrogen until the samples were completely dried. Finally, 50 µL of 50% ACN - 50% 0.1% HCOOH solution (mobile phase) was added after which the tube was vortexed for 30 sec. The 50 µL were transferred in a LC-MS/MS insert and centrifuged 5 min at 15000 rpm to eliminate the impurities. Ten µL

of this extract were directly injected into the UPLC-MS/MS system. Photographical illustrations of this methodology are presented in Annex 3.

C. Chromatographic and mass spectrometric conditions

A Waters (Milford, USA) Acquity UPLC system with a column heater and an autosampler were used. Analytes were separated on a Waters Acquity UPLC BEH C18 column (100 x 2.1-mm) with a 1.7 µm particle size and the volume injected was 10 µL. The mobile phase flow rate was 0.4 mL/min and the column temperature was kept at 30°C. A binary mobile phase with a gradient elution was used. Solvent A was milli-Q water with HCOOH at 0.1% (pH 2.6) and solvent B was ACN. The gradient was performed as follows: 10% B increased to 60% B in 6 min, constant for 3 min, and then decreased to 10% in 2.5 min. Under these conditions, the total run was 11.5 min. Detection was carried out by a Quattro Premier XE tandem mass spectrometer (MS/MS) (Waters Micromass, Manchester, UK) using electrospray ionisation in positive mode (voltage of the capillary 3.5 kV). The ion-source temperature was 120°C and the desolvation gas was heated to 400°C at a flow rate of 800L/h. Quantitative results were obtained in MRM (Multiple Reactions Monitoring) mode and Masslynx®4.1 software (Waters) was used for data acquisition.

D. Method validation

The limit of detection (LOD) and limit of quantification (LOQ) were determined in replicate blank hair samples spiked with various concentrations of cortisol and cortisone (N=6) at the signal-to-noise ratio (S/N) of 3 and 10 respectively (Gao W. et al., 2010; Xie Q. Z. et al., 2011). Calibration curves were developed for 9 concentration levels of cortisol and cortisone, i.e. 5, 10, 20, 50, 70, 100, 200, 500 and 1000 pg/mg. Linearity of the method and the proportion of variation explained by the calibration model were expressed by the linear correlation coefficient (between the chromatographic signal and the cortisol concentrations) (r) and the coefficient of determination (R^2, i.e. square of correlation coefficient) respectively.

Precision and accuracy were expressed as RSD% (relative standard deviation percentage) and bias percentage (% Bias) respectively and were evaluated using three solutions of MeOH in blank hair samples spiked with cortisol and cortisone to the nominal concentrations of 5, 50 and 500 pg/mg. The intra-assay precision was assessed by analysing these samples on the same day (N=10 for each sample), while the inter-assay precision was assessed over 6

successive days (N=6 for each sample). Accuracy was assessed by calculating the percentage of difference between the true (spiked, nominal) concentration and the measured concentration (Causon R., 1997; US Department of Health and Human Services - Food and Drug Administration, 2001).

Recovery and matrix effect analyses were performed to study respectively the efficiency of the extraction procedure and the interference or alteration in response due to the presence of matrix (i.e. unintended analytes in hair) (US Department of Health and Human Services - Food and Drug Administration, 2001; Matuszewski B. K. et al., 2003). Analyte recovery of the extraction procedure was determined by comparing the analytical results for extracted samples with unextracted standards that represent 100% recovery in replicate blank hair samples spiked with cortisol and cortisone at 5, 50 and 500 pg/mg, (N=3) (US Department of Health and Human Services - Food and Drug Administration, 2001).

Matrix effects on the other hand were calculated using the formula of Matuszewski et al., i.e. Matrix effect %=B/A x 100, where B = peak areas of blank hair samples enriched with different concentrations of cortisol and cortisone (5, 50 and 500 pg/mg; N=3), extracted and analysed; and where A= peak areas of directly injected standards at the same concentrations (without matrix) (Matuszewski B. K. et al., 2003). Matrix effect values of 100% indicate no matrix effect, values < 100% indicate ion suppression, while values > 100% indicate ion enhancement.

Selectivity of the method was ensured by testing for steroid interferences of cortisol and cortisone analogues (Causon R., 1997). More particularly prednisone and prednisolone interferences were tested by adding a mixture of cortisol, cortisone, prednisone and prednisolone at 50 pg/mg, and by adding prednisone and prednisolone to three blank hair samples.

3 Method validation results

3.1 UPLC-MS/MS characteristics

Quantitative results were obtained in MRM mode. For each compound, the most abundant MRM transition was used for quantification and the minor transition for confirmation, as listed in Table 2.3.

Table 2.3 Multiple reaction monitoring conditions for cortisol and cortisone

Compound	Precursor/parent ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (eV)
Cortisol	363.2	121.1*	35	25
	363.2	309.1	35	17
	363.2	327.1	35	15
Cortisone	361.1	121.0*	35	30
	361.1	163.0	35	25

* quantification ion

Cortisol and cortisone were separated chromatographically under UPLC-MS/MS conditions, with 3.69 ± 0.04 min and 3.75 ± 0.04 min as retention times for cortisol and cortisone respectively (Figure 2.6- Figure 2.7). Figure 2.7 demonstrates distinct retention times for cortisol, cortisone, prednisolone and prednisone and thus excludes errors in identification by these steroid hormones. Analysis of three blank hair samples showed no interfering peaks of prednisone and prednisolone at the retention time of both analytes (data not shown).

3.2 Validation

The LOD and LOQ were 2 pg/mg and 5 pg/mg respectively, for both cortisol and cortisone. The calibration curve obtained from the combination of 3 standard curves showed good linear responses with a R^2 of 0.9928 and 0.9902 for cortisol and cortisone respectively over the range from 5 to 1000 pg/mg.

Table 2.4 displays the recovery, matrix effect, intra- and inter-assay precision and inter-day and intra-day accuracy, tested for each analyte at three different concentrations (i.e. 5, 50 and 500 pg/mg). The extraction recoveries were >84 % and >87% for cortisol and cortisone respectively. The matrix effects were around 130% for both steroids, indicating ion enhancement due to the hair matrix. To overcome this matrix effect, the calibration curve was obtained for the hair matrix and a ratio between steroid area and cortisol-d4 area was calculated for the quantification of steroid concentrations. The intra-assay precision values (N=10) were less than 13.5% and 14.5 % for cortisol and cortisone respectively and inter-assay precision values (N=6) <15% and < 20% at LOQ for both analytes were obtained. The

% Bias was less than 18.9% for intra- and inter-day accuracy for both cortisol and cortisone at LOQ and less than 12.3% at the other concentrations.

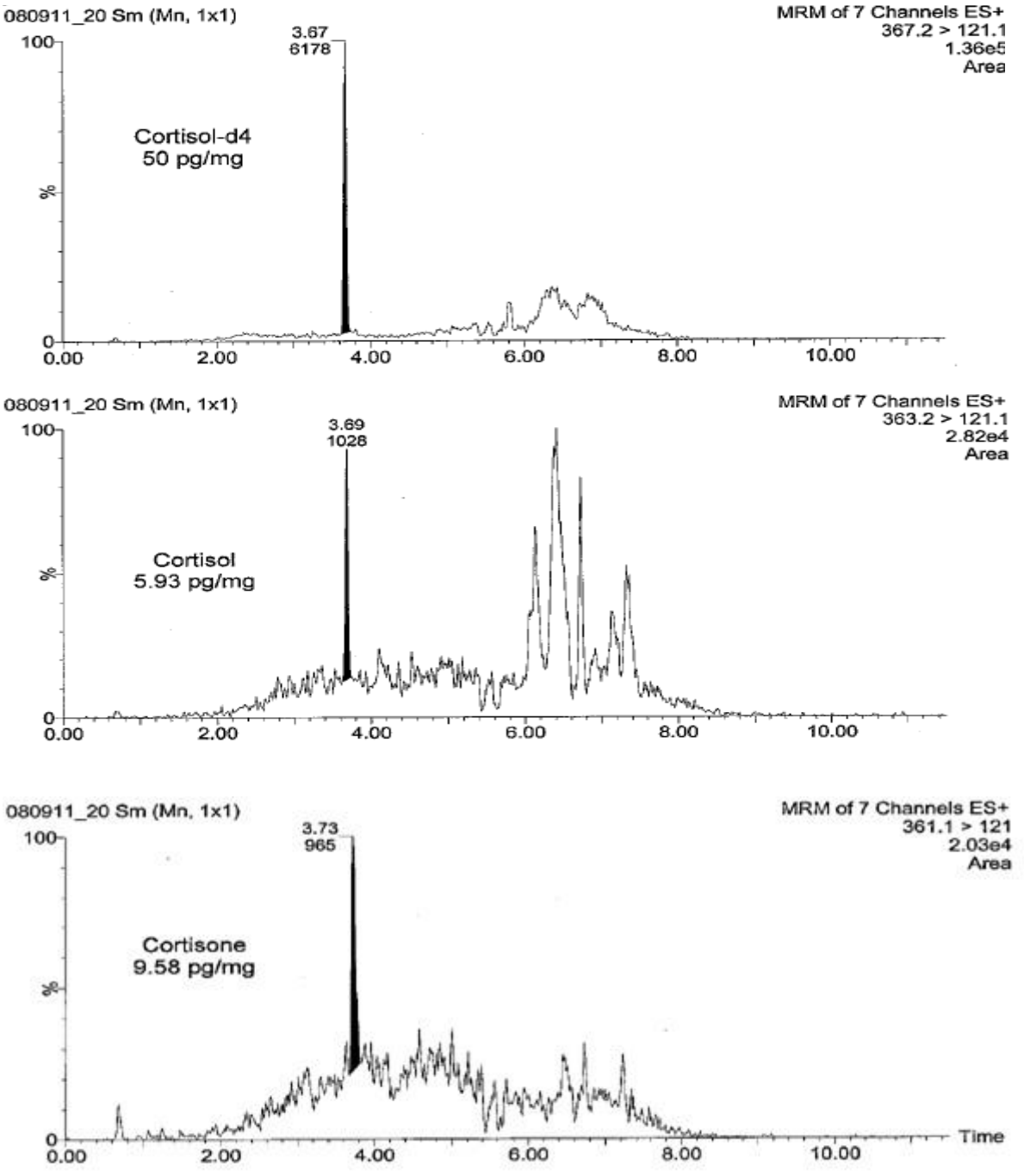


Figure 2.6 Chromatograms for cortisol-d4, cortisol and cortisone in true hair sample

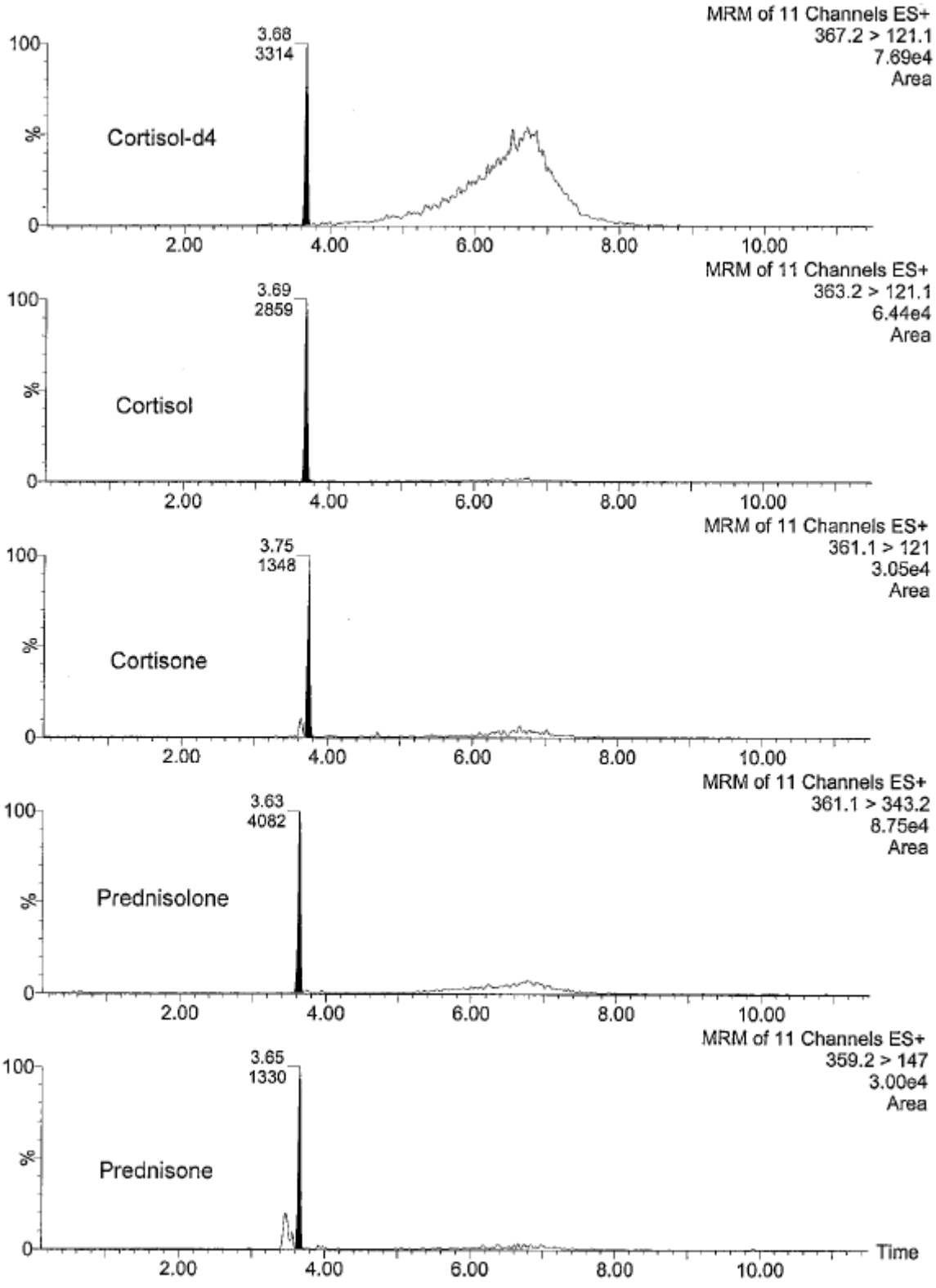


Figure 2.7 Chromatograms of cortisol d-4, cortisol, cortisone, prednisolone and prednisone at 50pg/mg. The four tested hormones show different times of retention and different transitions of quantification.

Table 2.4 Extraction recovery, matrix effect, intra- and inter-day precision and accuracy (RSD%= relative standard deviation percentage)

		Recovery		**Matrix Effect**		**Precision**		**Accuracy**	
						Intra-assay	**Inter-assay**	**Intra-day**	**Inter-day**
		(N=3)		**(N=3)**		**(N=10)**	**(N=6)**	**(N=10)**	**(N=6)**
Compound	**pg/mg**	**%**	**RSD%**	**%**	**RSD%**	**RSD%**	**RSD%**	**%Bias**	**%Bias**
Cortisol	5	92.1	17.8	135.6	21.5	13.4	15.6	+ 17.6	+ 18.9
	50	90.7	7.6	132.4	15.9	7.8	7.5	+ 6.7	+ 10.1
	500	84.9	11.9	129.3	17.1	4.4	4.9	+ 10.7	+ 12.3
Cortisone	5	87.4	15.6	142.0	22.4	14.1	18.8	+ 18.5	+ 18.7
	50	97.3	6.2	130.3	16.1	6.1	14.2	+ 7.8	+ 9.3
	500	89.6	10.9	139.6	19.7	4.4	13.7	+ 9.5	+ 11.6

4 Conclusion

To study cortisol and cortisone in children's stress research, hair may be the preferred matrix as it shows some unique characteristics compared to other biomatrices. This is the first study offering a validated and standardized UPLC-tandem MS methodology to simultaneously detect cortisol and cortisone in children's hair for chronic stress research. The developed method demonstrated good linearity, good precision and accuracy, with an easy and inexpensive extraction procedure (Kushnir M. M. et al., 2011; Wood L. et al., 2008).

However, the inter-assay RSD%'s were somewhat raised and larger compared to some other LC-MS/MS studies (Raul J. S. et al., 2004; Xie Q. Z. et al., 2011; Perogamvros I. et al., 2009), although the observed RSD%'s for the intra- and inter-assay precision experiments were generally within acceptable limits (<15% and <20% for at LOQ) (US Department of Health and Human Services - Food and Drug Administration, 2001). As we may assume that a substantial contribution of this variability arises from (1) the specific hair matrix characteristics which are more complex compared to, e.g., saliva or serum (e.g. variation between hair fragments, influence of colour, treatments and structure of hair, possible passive contamination etc.) (Wennig R., 2000), and (2) from the applied pre-analytical and analytical laboratory procedure, such as differences in storage conditions and in decontamination or extraction procedures (e.g. a triple liquid extraction technique as performed in this study versus solid-phase extraction as performed by Raul et al. and Xie et al. (Xie Q. Z. et al., 2011; Raul J. S. et al., 2004)), the presented method can be considered accurate and reliable for application on children's hair samples.

A more detailed discussion of the presented method and its validation parameters is provided in *Chapter 4.2 Methodological Considerations* of this thesis.

CHAPTER 2.4 MEASUREMENT OF MINERALS IN CHILDREN'S HAIR USING INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY

1 Introduction

Another objective of this thesis is to examine the suitability of hair as a bio-indicator for dietary habits in children via additional measurements of minerals in hair samples obtained in the ChiBS project. This chapter describes the applied methodology for hair mineral analysis of calcium (Ca), copper (Cu), iron (Fe), sodium (Na), magnesium (Mg), phosphorus (P) and zinc (Zn) by the use of inductively coupled plasma - mass spectrometry (ICP-MS). ICP-MS is currently the state of the art technology to measure the elemental content of (hair) samples.

2 Methodology

2.1 Hair sampling

223 elementary school girls participated to this study as part of the 2010 baseline survey of the ChiBS project. Hair samples were obtained from the vertex posterior region of the scalp by trained researchers and split into two fractions to send to different laboratories (cortisol and cortisone analysis versus mineral analysis): from 5 girls, the amount of obtained hair was insufficient to split into two fractions, resulting in 218 hair samples for mineral analysis.

The hair samples were cut as close to the scalp as possible using clean, stainless steel scissors and tied together with a little cord to mark the proximal side. To guarantee that the same time period was investigated in all children, only the most proximal 6 cm of the hair strands was analysed. None of the hair samples was artificially coloured. The samples were stored in a folded piece of paper in individual zip-lock bags in a dark, dry place and at constant temperature until analysis in the Department of Analytical Chemistry (Ghent University) to which analyses were outsourced. The hair contents of Ca, Cu, Fe, Na, Mg, P and Zn were quantitatively determined via ICP-MS, after microwave-assisted acid digestion of the samples. Photographical illustrations of the ICP-MS methodology are presented in Annex 4.

2.2 Washing procedure

Approximately 0.1 g of each hair sample was subjected to a washing procedure prior to the digestion in order to remove any external contamination, such as grease, sweat, dust, etc. This cleaning stage consisted in stirring the samples, first in acetone (8mL) and subsequently in ultrapure water (8mL) (obtained from a Direct Q3 water purification system from Millipore, fed by distilled water) inside an ultrasonic bath for 6 minutes (Branson 5510) followed by further rinsing of the samples with Milli-Q water for two more times. Immediately afterwards, samples were allowed to dry in an oven at a temperature of 60-70°C until complete dryness (overnight).

2.3 Digestion of the samples

Once dried, the samples were weighed, transferred into microwave TFM vessels along with 1 mL of 14 M HNO_3 (Chemlab Belgium, pro analysis, further purified by means of sub-boiling) and 9.8 M of H_2O_2 (Fluka Analytical, Sigma Aldrich Belgium, for trace analysis) and subjected to the following microwave power program for digestion: 2 min at 250 W, 2 min at 0 W, 6 min at 250 W, 5 min at 400 W and 5 min at 600 W (Milestone mls 1200 mega microwave lab station). The digests thus obtained were then allowed to cool down to room temperature before further dilution with Milli-Q water in pre-cleaned PP tubes.

2.4 ICP-MS

Samples were analysed by means of sector field ICP-MS (Thermo Element XR). Simultaneous monitoring of Ca, Cu, Fe, Na, Mg, P and Zn and the internal standard Ge (germanium) (added to a final concentration of 25 μg L^{-1}) was accomplished using the instrument settings and data acquisition parameters summarized in Table 2.5. Sector field ICP-MS was selected for its superior detection power and capability to avoid spectral overlap by measurement at higher mass resolution.

Table 2.5 Instrument settings and data-acquisition parameters used for the measurements with an Element XR sector field ICP - mass spectrometer.

Resolution	Medium (4000)
Cooling gas	15.00 L min^{-1}
Auxiliary gas	1.00 L min^{-1}
Sample gas	0.94 L min^{-1}
RF power	1200 W
Sample uptake rate	0.2 mL min^{-1}
Scan type	E-Scan
Detection mode	Triple
Nuclides monitored	$^{23}Na^{+}$, $^{24}Mg^{+}$, $^{25}Mg^{+}$, $^{26}Mg^{+}$, $^{31}P^{+}$, $^{43}Ca^{+}$, $^{44}Ca^{+}$, $^{56}Fe^{+}$, $^{57}Fe^{+}$, $^{63}Cu^{+}$, $^{65}Cu^{+}$, $^{66}Zn^{+}$, $^{68}Zn^{+}$, $^{74}Ge^{+}$
Runs	5
Passes	3
Sample time	5 ms
Samples per peak	20
Segment duration	0.125 ms
Total time per sample	66 s

2.5 Method evaluation

The internal standard was relied on to correct for matrix effects, instrument instability and signal drift. External calibration was accomplished relying on a series of standard solutions prepared from commercially available 1 g L^{-1} stock solutions. Evaluation of the analytical method developed was carried out by analysing BCR 397 powdered human hair certified reference material. However, certified reference values were available only for Zn and for the rest of the micronutrients considered (with the exception of Na) only informative values were provided. Recovery assays were also carried out for all the target elements as an alternative validation procedure. Analytical parameters such as precision and limits of detection for all the elements of interest were also estimated. With this aim, one sample available in a sufficient amount was selected for evaluating the homogeneity of the hair and method repeatability. Five different locks of that sample were analysed for the same target elements

and the relative variation of the results was taken as an indicator for the precision of the method. The detection limit for each element of interest was calculated as three times the standard deviation on the analysis of ten different procedural blanks, divided by the slope of the external calibration curve.

3 Method evaluation results

Results for both accuracy checks are shown in Table 2.6. While most of the results are in a good agreement with the certified/informative values for the reference material, the value obtained for P attracts attention. However, results for the recovery assays were satisfactory and further analysis by ICP-OES (inductively coupled-optical emission spectrometry) provided a similar result. Moreover, the measured value also seems to be in accordance with the typical values reported in the literature for a normal P hair content.

Table 2.6 Analytical method evaluation figures (RSD: relative standard deviation)

BCR 397 analysis results

Target element	Measured value (μg g^{-1})	Certified value (μg g^{-1})	Informative value (μg g^{-1})	RSD (%)	Recovery (%)
Mg	170		200	13.2	84.8
P	164		3200	7.3	5.1
Ca	1481		1560	4.1	94.9
Fe	523		580	1.8	90.1
Cu	107		110	10.6	97.7
Zn	200	199		9.4	100.5

Digestion recovery assays

Target element	Spike (mg L^{-1})	Recoveries (SD) (%)
Na	0.30	103.1 (1.4)
Mg	1.00	106.1 (2.5)
P	16.00	99.2 (1.5)
Ca	8.30	102.1 (1.0)
Fe	3.00	97.7 (3.9)
Cu	0.50	100.3 (3.5)
Zn	1.40	102.7 (0.9)
K	0.08	100.6 (3.4)

Analytical parameters such as precision and limits of detection for all the elements of interest were also estimated and presented in Table 2.7.

Table 2.7 Evaluation of the precision in terms of the repeatability (expressed as relative standard deviation- RSD) and limits of detection (calculated as three times the standard deviation of the procedural blanks divided by the slope of the external calibration curve) for the analytical method

Target element	RSD (%)	LOD (μg g^{-1})
Na	70.4	1.01
Mg	9.7	0.46
P	2.8	1.44
Ca	10.5	7.30
Fe	4.7	0.27
Cu	3.6	0.03
Zn	2.1	0.54

4 Conclusion

An ICP-MS method was developed and evaluated for quantification of Ca, Cu, Fe, Na, Mg, P and Zn in children's hair samples. Based on the evaluation results, we can conclude a reliable, sensitive, accurate and precise method was developed which can be applied for routine analysis of a large number of children's hair samples.

CHAPTER 2.5 SUMMARY OF STUDY PARTICIPANTS

As this thesis was, depending on the research question, embedded within the T1 IDEFICS survey or baseline ChiBS project, the aim of this chapter is to provide an explicit overview of the included study participants for the different research objectives of this thesis, as presented in Figure 2.8.

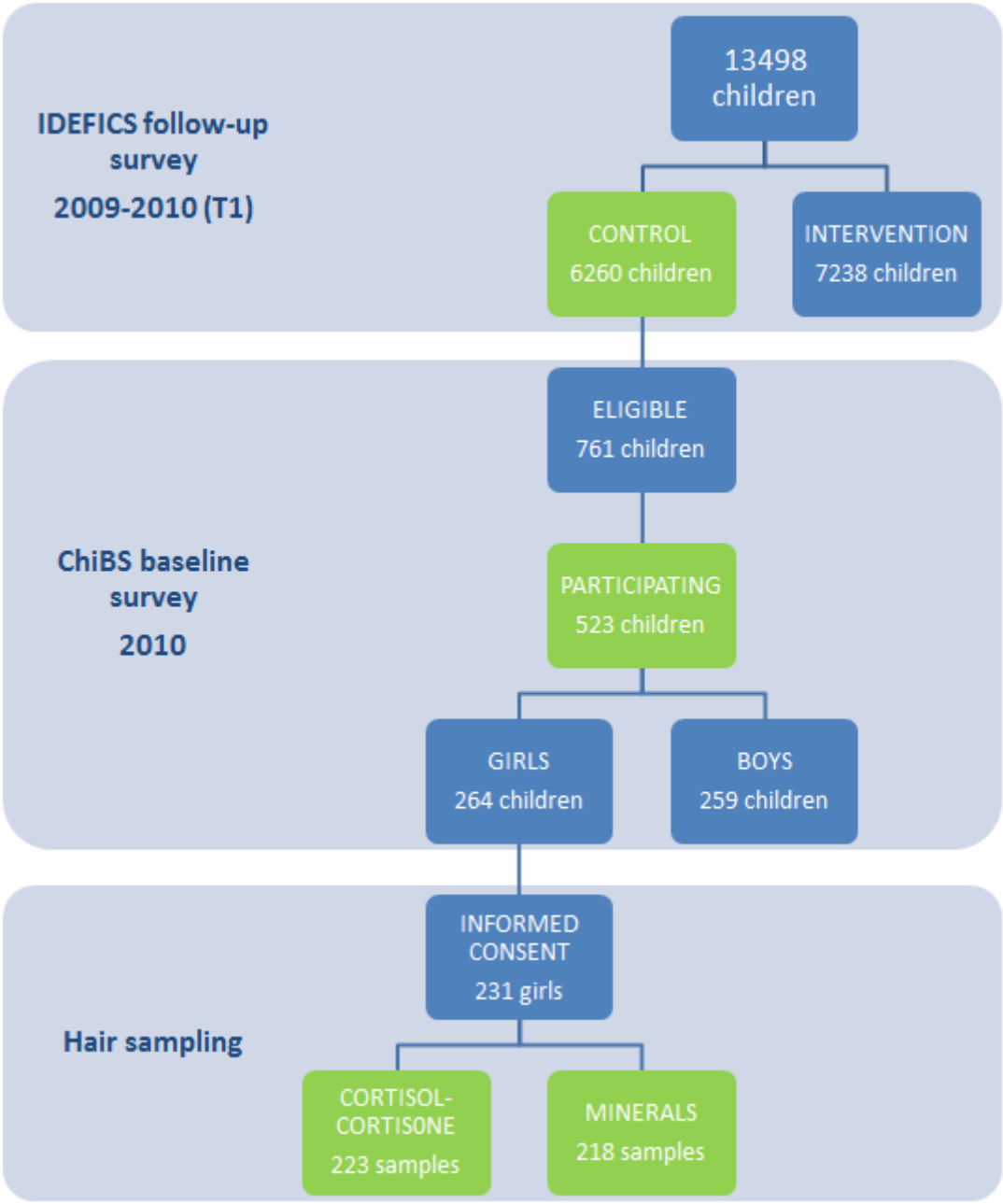


Figure 2.8 Flowchart of included study participants in this thesis

A first aim of this thesis was to examine the prevalence and outcomes of childhood stress on European level. To rule out an intervention bias on the studied variables, this research question was studied only in the control regions of the IDEFICS countries (T1 period, N=6260, top green box in Figure 2.8) (Results in Chapter 3.1 and Chapter 3.2).

The other research objectives were investigated within the ChiBS project, which originated from the Belgian control arm of IDEFICS T1, with a total number of 523 participants at baseline.

While the relationship between childhood stress and body composition was investigated in this complete baseline population of ChiBS (middle green box in Figure 2.8) (Chapter 3.8), the remaining research questions on the use of scalp hair as marker for childhood stress or dietary pattern were examined in girls only to ascertain the required hair length of 6 cm. Parental written informed consent for hair sampling was obtained for 231 girls. From 8 girls no hair sample was taken because of an insufficient hair length (<6 cm), which resulted in 223 hair samples obtained. Each hair sample was split into two fractions, i.e. one fraction for cortisol and cortisone analysis, and one fraction for hair mineral analysis. As for 5 girls, the amount of hair was too limited to split into fractions, the number of samples available for hair mineral analysis was restricted to 218 (bottom green boxes in Figure 2.8). Results on the hair cortisol and cortisone, and hair mineral analysis are presented in Chapter 3.3 to Chapter 3.7.

PART 3 RESULTS

Chapters based on

Vanaelst B, Huybrechts I, De Bourdheaudhuij I, Bammann K, Hadjigeorgiou C et al. ***Prevalence of negative life events and chronic adversities in European pre- and primary- school children: results from the IDEFICS study.*** *Archives of Public Health (2012), 70:26.*

Vanaelst B, De Vriendt T, Ahrens W, Bammann K, Hadjigeorgiou C, Konstabel K et al. ***Prevalence of psychosomatic and emotional symptoms in European school-aged children and its relationship with childhood adversities: results from the IDEFICS study.*** *European Child and Adolescent Psychiatry (2012), 21:253-265.*

Vanaelst B, Michels N, De Vriendt T, Huybrechts I, Vyncke K, Sioen I et al. ***Cortisone in hair of elementary school girls and its relationship with childhood stress.*** *European Journal of Pediatrics (2013),172: 843-846.*

Vanaelst B, Huybrechts I, Bammann K, Michels N, De Vriendt T, Vyncke K et al. ***Intercorrelations between serum, salivary and hair cortisol and child-reported estimates of stress.*** *Psychophysiology (2012), 49:1072-1081.*

Vanaelst B, Huybrechts I, Michels N, Vyncke K, Sioen I, De Vriendt T et al. ***Mineral concentrations in hair of Belgian elementary school girls: reference values and relationship with food consumption frequencies.*** *Biological Trace Element Research (2012), 150:56-67.*

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(continuation)

Vanaelst B, Huybrechts I, Michels N, Florez MR, Balcaen L, Resano M et al. ***Hair minerals and metabolic health in Belgian elementary school girls.*** *Biological Trace Element Research (2013), 151:335-343.*

Vanaelst B, Michels N, Huybrechts I, Clays E, Florez MR, Balcaen L et al. ***Cross-sectional relationship between chronic stress and mineral concentrations in hair of elementary school girls.*** *Biological Trace Element Research (2013), 153: 41-49.*

Vanaelst B, Michels N*, Clays E, Herrmann D, Huybrechts I, Sioen I et al.* ***The association between childhood stress and body composition, and the role of stress-related lifestyle factors – cross-sectional findings from the baseline ChiBS survey****. International Journal of Behavioral Medicine (2013), in press (DOI: 10.1007/s12529-013-9294-1).*

**joint first authorship*

CHAPTER 3.1 PREVALENCE OF NEGATIVE LIFE EVENTS AND CHRONIC ADVERSITIES IN EUROPEAN PRE- AND PRIMARY-SCHOOL CHILDREN: RESULTS FROM THE IDEFICS STUDY

Abstract

Background Children are not always recognized as being susceptible to stress, although childhood stressors may originate from multiple events in their everyday surroundings with negative effects on children's health.

Methods As there is a lack of large-scale, European prevalence data on childhood adversities, this study presents the prevalence of (1) negative life events and (2) familial and social adversities in 4637 European pre- and primary-school children (4-11 years old), using a parentally-reported questionnaire embedded in the IDEFICS project ('Identification and prevention of Dietary- and lifestyle-induced health EFfects In Children and infantS').

Results The following findings were observed: (1) Certain adversities occur only rarely, while others are very regular (i.e. parental divorce); (2) A large percentage of children is shielded from stressors, while a small group of children is exposed to multiple, accumulating adversities; (3) The prevalence of childhood adversity is influenced by geographical location (e.g. north versus south), age group and sex; (4) Childhood adversities are associated and co-occur, resulting in potential cumulative childhood stress.

Conclusions This study demonstrated the importance of not only studying traumatic events but also of focusing on the early familial and social environment in childhood stress research and indicated the importance of recording or monitoring childhood adversities.

1 Introduction

For a long time, stress has incorrectly been assumed to predominantly manifest in adults. Many investigators have however recently turned to the incidence of stress in children (Schilling E. A. et al., 2007; Benjet C. et al., 2009; Alfven G. et al., 2008; Brobeck E. et al., 2007; Costello E. J. et al., 2002; Copeland W. E. et al., 2007; Hesketh T. et al., 2010; Furniss T. et al., 2009; Harland P. et al., 2002; Burke N. J. et al., 2011). Sandberg defined childhood

stress as ‘any intrusion into the children’s normal physical or psychosocial life experiences that acutely or chronically unbalances their physiological or psychological equilibrium, threatens security or safety, or distorts their physical or psychological growth or development’ (Sandberg S., 2007). In this definition, three stress components can be distinguished: 1) the environmental sources of stress or so-called ‘stressors’ (e.g. negative life events or more chronic adversities in the children’s school-, family- or inter-personal environment), 2) the psychological response given to these stressors (e.g. emotions), and 3) the biological stress response provoked by stressor exposure (e.g. the hormonal stress response) (Vanaelst B. et al., 2012b; Cohen S. et al., 1997a). This paper focusses on childhood stressor exposure; more specifically on the occurrence of negative life events and adversities of familial and social nature.

In particular chronic exposure to adverse, stressful situations may affect children’s behaviour and personality development and may have consequences on both their physiological and psychological health, with effects potentially persisting into adolescence and adulthood (e.g. depression, affective disorders, cardiovascular or auto-immune diseases, psychosomatic complaints, substance abuse) (Cohen S. et al., 2007; Schneiderman N. et al., 2005; Vanaelst B. et al., 2012a; Douglas K. R. et al., 2010; Ford E. et al., 2011; Cerel J. et al., 2006; Freeman L. N. et al., 1993). While some children may be relatively shielded from adversities, others may be exposed to a multiplicity of successive hardships or life-course-transitions resulting in cumulative stress (Turner R. J. et al., 1995).

The most obvious demographic change in Western Europe are the increased divorce rates, which may impact on the children’s everyday life through, e.g., a changing family structure (Allan G. et al., 2001). As the family environment may affect the social, emotional and physical health of children, it should be considered an important factor in the child’s well-being (Repetti R. L. et al., 2002; Waldfogel J. et al., 2010). Moreover, stressors from familial origin may not be isolated events, but cluster together or give rise to other unfavourable events (e.g. parental divorce may lead to organizational changes, decreased economic resources and parental strains), all together highlighting the importance of considering the early family and social environment in childhood stress research.

Despite the importance of recording/monitoring childhood adversities, there is a lack of large-scale, international research on the prevalence of negative life events and familial and social conditions which may constitute potential childhood adversity. Moreover, the majority of

previous stress research has focused on rare traumatic events without considering familial and social conditions. Therefore, this study examines the prevalence of (1) negative life events (NLE) and (2) familial and social adversities (FSA) in a large population of European pre- and primary-school children (4-11 years old) cross-nationally, by investigating the following research questions: (1) Is the prevalence of adversity in pre- and primary-school children equally distributed over region, age and sex group (Hatch S. L. et al., 2007; EUROSTAT, 2010)? (2) Can co-occurence and associations between adversities be demonstrated in this young childhood population (e.g. do certain adversities lead to other adversities or tend to co-occur)?

2 Methods

2.1 Participants

Information on NLEs and FSAs in the child's life was parentally reported for 4637 children (aged 4 to 11.8 years, mean (M)=7.91, standard deviation (SD)=1.80, 49.5% boys). This was part of the follow-up survey (September 2009 -May 2010) of the IDEFICS study, an Integrated Project within the 6th Framework Programme of the European Commission ('Identification and prevention of Dietary- and lifestyle-induced health EFfects In Children and infantS', www.idefics.eu).

The IDEFICS project is a multicentre longitudinal intervention study of pre- and primary-school children in 8 European countries (Belgium, Cyprus, Estonia, Germany, Hungary, Italy, Spain, Sweden), investigating the aetiology of diet- and lifestyle-related diseases and disorders in children. In this project, also community-oriented prevention programmes for obesity are developed (working on the level of diet, physical activity and stress reduction) and evaluated in a controlled study design (De Henauw S. et al., 2011). In each country, one intervention and one control region was selected which were comparable with regard to infrastructural, socio-demographic and socio-economic characteristics. All children residing in the selected intervention and control regions who were within the defined age group of 2-9 years old at baseline, were eligible for participation to IDEFICS. Because of budgetary constraints and feasibility considerations, it was not intended to generate a representative sample of a given country or Europe in general.

The baseline survey started in 2007 with a cohort of 16224 children who were approached through school and kindergarten settings using a letter and leaflet addressed to the parents (Figure 3.1). The follow-up survey resulted in a total sample size of 13498 children. More detailed research goals, methodology and instruments of IDEFICS have been described elsewhere (Ahrens W. et al., 2011a).

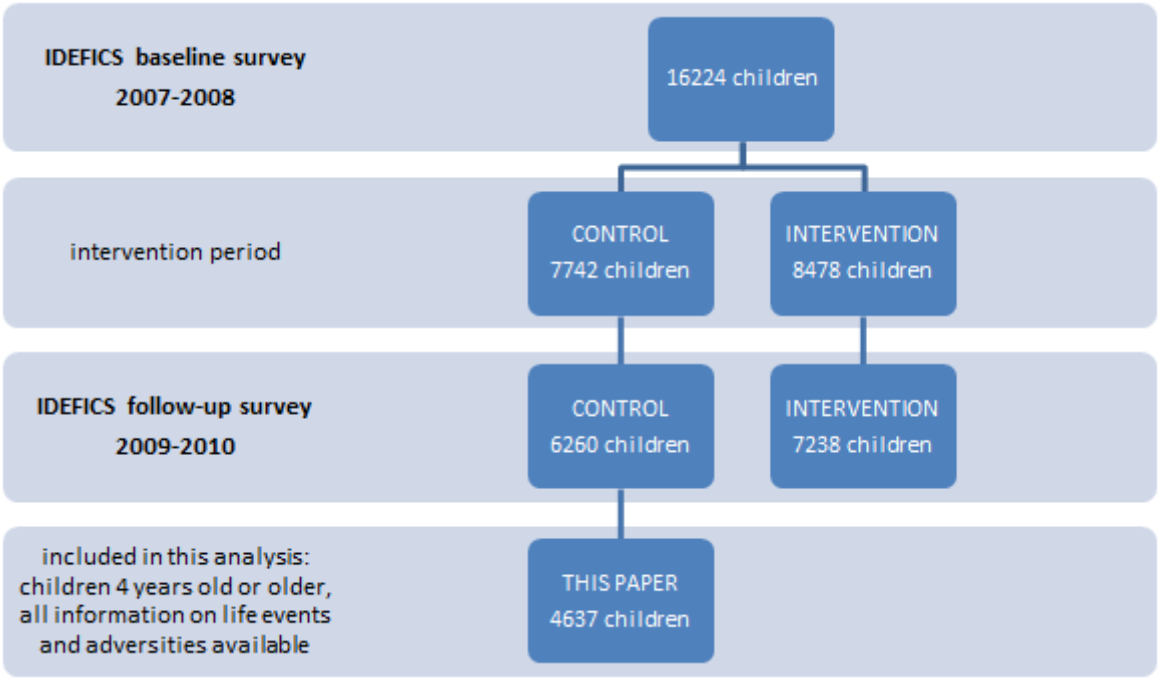


Figure 3.1 Study flow-chart presenting participation information of the baseline and follow-up survey of the IDEFICS project, and the total number of children included in the presented analysis

As one of the IDEFICS intervention modules was directed at stress and stress-coping capacity on community-, school- and family-level (De Henauw S. et al., 2011), we decided to only include the control regions of the participating countries in this study to rule out intervention-bias on the studied variables (N=6260/13504; 46.4%). Statements in this study regarding regional variations thus only relate to the participating control regions and should not be considered as representative for the respective countries. Children younger than 4 years of age (N=69) and children from whom any adversity information was missing were excluded from the analysis (N=1623/6260; 25.9%). This resulted in a final sample size of 4637 participants, which is schematically presented in Figure 3.1. No differences were found between the included and excluded group for sex and age, while low parental education was more prevalent in the excluded group (data not shown). The study was conducted according to the

guidelines of the Declaration of Helsinki and approvals of the local Ethical Committees were obtained for each survey centre.

2.2 Childhood adversities

Life events are generally assumed to represent a basis for experiencing stress as they are accompanied by undesirable demands and threats and lead to changes in a person's life. Therefore, questionnaires assessing life events and adversities are considered estimates of stress exposure (Vanaelst B. et al., 2012b; Cohen S. et al., 1997b). In this study, childhood adversity was studied using a parent-reported questionnaire on adversity and life events, i.e. the 'IDEFICS Parental Questionnaire', including information on socio-demographics, family lifestyle, life events and wellbeing of the children. The quality of the questionnaire and comparability across the survey centres was assured by a translation/back-translation procedure for each local language and by re-administering the parental questionnaire to a convenience sample of study participants (Ahrens W. et al., 2011a; Herrmann D. et al., 2011).

Parental conflicts or divorce (Pryor J. et al., 2001), low supportive or unfavourable family climates (Amato P. R., 2005; Lawson D. W. et al., 2010; Gass K. et al., 2007; Card J., 1981), domestic violence or abuse (Holt S. et al., 2008), parental supervisory neglect (Casper L. M. et al., 2004; Aizer A., 2004), socio-economic disadvantage (Gustafsson P. E. et al., 2009; Conger R. D. et al., 2010; Ram B. L. et al., 2003; Lupien S. J. et al., 2001), serious illness of the child or a family member (Sieh D. S. et al., 2010; Hysing M. et al., 2009), death of a child's parent, grandparent, sibling or pet (Cerel J. et al., 2006), and peer problems or frustrations at school (van der Wal M. F. et al., 2003; Wolke D. et al., 2001; Einfeld S. L. et al., 2006) have in literature all been shown to emotionally and psychologically affect children. Therefore, parents were asked to complete questions on both the life-time occurrence of the above-mentioned **negative life events (NLE)** and the more chronic familial and social situations which may constitute potential childhood adversity **(FSA: familial and social adversities)**, such as ethnicity of the family, education of the mother, employment of the parents, family structure and family relationships. These childhood adversity variables were all of dichotomous nature (occurrence or no occurrence of the event; presence or no presence of the adversity). Figure 3.2 presents an overview of the studied FSA and NLE variables, their assessment and reference to literature. To accurately report on maternal education, family economic hardship and family climate, only data provided by biological-, adoptive-, or

stepparents was included. For the other variables also reporting by foster-parents or family members was allowed.

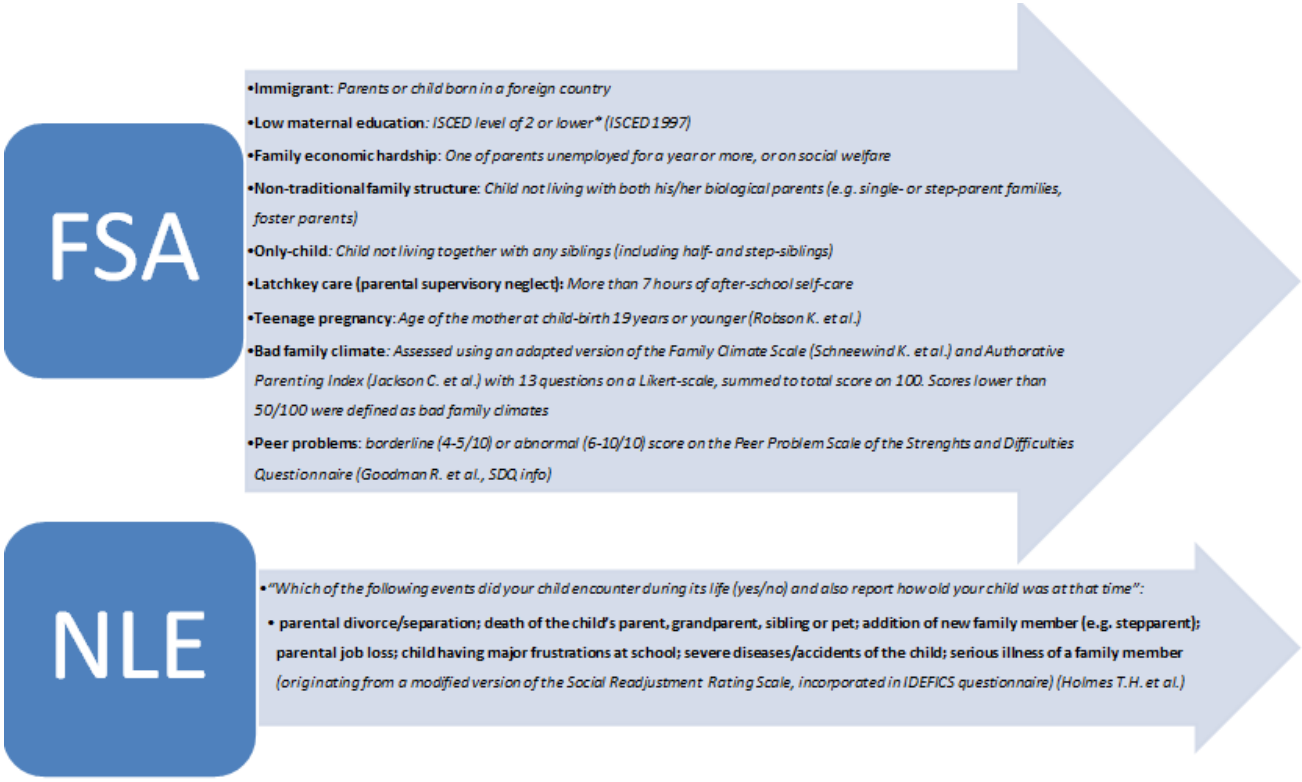


Figure 3.2 Overview of Familial and Social Adversities (FSA) and Negative Life Events (NLE) variables as assessed in the IDEFICS project (2009-2010)

Important to note is that the authors do not consider these variables as **actual** childhood stressors but rather as **potential** stressful conditions during childhood.

2.3 Statistical analysis

Statistical analyses were performed using PASW Statistical Program version 18.0.0 (SPSS Inc., IBM, IL, USA). Each year of age was considered as one age group except children of 10 and 11 years old who were taken together in the age group '10' because of the low number of 11 year olds (N=40). Regional differences were studied by grouping the countries along a north (Sweden, Estonia) - east (Hungary) - south (Italy, Spain, Cyprus) - west cluster (Belgium, Germany), based on the geographical grouping of countries by the United Nations Statistics Division (United Nations Statistics Division, 2011). Cumulative stress from FSAs

and NLEs was studied by summing the number of FSAs and NLEs (Furniss T. et al., 2009; Benjet C. et al., 2010; Schilling E. A. et al., 2008; Schilling E. A. et al., 2007; Forehand R. et al., 1998; Wille N. et al., 2008). To study regional variations and differences among age groups and sexes in the prevalence of FSAs and NLEs, Pearson χ^2 analysis were performed. One-way ANOVA analyses were performed to study continuous variables between groups. Odds ratios (OR) and 95% confidence intervals (CI) were used to report on the co-occurrence of adversities, and the risk (or likelihood) for being exposed to a certain adversity, given another adversity was already present. As we did not aim to determine the unique contribution of each FSA/NLE adjusted for other FSAs/NLEs, unadjusted, univariate (and not multivariate) OR's were presented (which are suitable to demonstrate the associations and co-occurrence between adversities). To correct for multiple testing a Bonferroni correction was applied: p-values <0.002 were considered statistically significant for all tests. P-values between 0.002 and 0.05 were denoted as borderline significant.

3 Results

3.1 Prevalence of FSAs and NLEs

Table 3.1 presents the prevalence of FSAs and NLEs for each survey centre separately and for grouped countries: the three most prevalent FSAs/NLEs are marked numerically and for each FSA/NLE the survey centre or country group with the highest prevalence is indicated in bold and in grey shade. A non-traditional family structure, being only-child or immigrant are the three most reported FSAs overall, while parental divorce/separation, addition of a new family member and parental job loss are the most reported NLEs.

Table 3.1 Prevalence of chronic adversities and negative life events in pre- and primary-school children participating in the IDEFICS study (2009-2010): survey centre and regional variations

		Survey centres									Country groups				
	Total	**Italy**	**Estonia**	**Cyprus**	**Belgium**	**Sweden**	**Germany**	**Hungary**	**Spain**	**P (χ^2)[a]**	**North[c]**	**Easth[d]**	**South[e]**	**West[f]**	**P (χ^2)[a]**
Number of children included (N)	4637	560	787	601	605	543	376	666	499		1330	666	1660	981	
familial and social adversities	*Prevalence % (N)*	*Prevalence %*													
being immigrant	③ **13.0 (604)**	19.6	6.5	**33.1**	2.8	15.8	23.1	2.9	7.0	<0.001	10.3	2.9	**20.7**	10.6	<0.001
low maternal education	11.3 (524)	28.9	3.4	3.0	2.5	4.4	**51.9**	2.9	12.8	<0.001	3.8	2.9	14.7	**21.4**	<0.001
family economic hardship	4.1 (191)	7.1	2.7	2.2	1.2	0.9	**9.3**	5.7	6.4	<0.001	2.0	**5.7**	5.1	4.3	<0.001
non-traditional family structure	① **21.0 (973)**	**30.2**	24.1	28.8	15.4	13.3	22.6	24.2	6.0	<0.001	19.7	**24.2**	22.4	18.1	0.007
single-parent family	13.1 (608)	**25.5**	12.8	23.6	5.0	4.1	14.6	13.2	5.4	<0.001	9.2	13.2	**18.8**	8.7	<0.001
stepparent family	4.1 (192)	0.4	**8.0**	1.2	5.3	3.5	7.4	6.0	0.2	<0.001	**6.2**	6.0	0.6	6.1	<0.001
only-children	② **16.4 (759)**	14.1	**27.2**	8.7	8.3	9.2	24.2	20.7	17.0	<0.001	19.8	**20.7**	13.0	14.4	<0.001
latchkey care	5.6 (261)	1.3	**22.5**	3.3	0.3	7.6	0.8	1.2	0.6	<0.001	**16.4**	1.2	1.8	0.5	<0.001
bad family climate	1.3 (58)	1.3	2.3	1.3	0.5	0.0	**2.4**	0.3	2.2	<0.001	1.4	0.3	1.6	1.2	0.097
teenage pregnancy	2.2 (101)	2.3	**6.1**	3.0	0.5	0.7	2.4	0.6	0.4	<0.001	**3.9**	0.6	2.0	1.2	<0.001
peer problems	8.5 (395)	9.3	7.6	**11.5**	7.8	4.6	9.8	11.0	6.4	<0.001	6.4	**11.0**	9.2	8.6	0.003
negative life events	*Prevalence % (N)*	*Prevalence %*													
parental divorce/separation	① **13.0 (602)**	3.9	**23.0**	7.3	14.7	10.7	18.6	17.4	4.4	<0.001	**18.0**	17.4	5.3	16.2	<0.001
addition of a new family member	② **12.4 (573)**	3.4	14.0	4.5	13.2	**29.8**	13.0	9.5	12.6	<0.001	**20.5**	9.5	6.6	13.1	<0.001
parental job loss	③ **8.7 (403)**	3.2	**14.5**	4.3	4.0	8.8	6.6	13.1	12.2	<0.001	12.2	**13.1**	6.3	5.0	<0.001
severe diseases/accidents of the child	7.2 (333)	5.4	11.3	4.3	4.5	3.9	6.9	**13.4**	5.0	<0.001	8.3	**13.4**	4.9	5.4	<0.001
serious illness of a family member	1.9 (90)	2.3	1.0	0.8	**4.5**	3.1	2.7	1.1	0.6	<0.001	1.9	1.1	1.3	**3.8**	<0.001
major frustration at school	7.4 (344)	5.9	7.5	4.7	8.3	7.0	**11.4**	10.2	5.0	<0.001	7.3	**10.2**	5.2	9.5	<0.001
death of a parent	0.7 (33)	0.7	0.5	1.0	0.7	0.4	0.8	1.4	0.2	0.339[b]	0.5	1.4	0.7	0.7	0.177[b]
death of a sibling	0.6 (26)	0.2	0.6	0.3	1.2	0.4	1.1	0.6	0.2	0.253[b]	0.5	0.6	0.2	1.1	0.034[b]
death of a grandparent	4.8 (221)	6.4	3.6	2.0	5.1	5.2	**7.4**	3.8	6.6	<0.001	4.2	3.8	4.9	6.0	0.121
death of a pet	0.7 (32)	0.0	1.8	0.0	1.0	0.0	**2.4**	0.2	0.4	<0.001	1.1	0.2	0.1	**1.5**	<0.001[b]

[a] Pearson χ^2 test to compare frequencies across countries, [b] Fischer's exact test to compare frequencies between countries, [c] Sweden-Estonia, [d] Hungary, [e] Italy-Cyprus-Spain, [f] Belgium-Germany; ①②③ Top three of most prevalent FSAs/NLEs in total are marked; For each adversity or event, the survey centre or country group with the highest prevalence is indicated in bold and additionally in grey shade if statistically different from the other survey centres or country groups.

A. Influence of region of the prevalence of FSAs and NLEs

Adversity percentages differ significantly between survey centres and country groups. Table 3.1 shows the highest prevalence rates of parental divorce/separation, addition of new family member, stepparent families, teenage pregnancy and latchkey care in the north; being immigrant and single-parent families appear most in the south; low maternal education, illness of a family member and death of a pet in the west; while in the east the following adversities peak: family economic hardship, non-traditional family structure, being only-child, parental job loss, severe diseases/accidents of the child, and peer problems and major frustrations at school. Although family economic hardship has the highest prevalence in the east, it should be marked that this prevalence is comparable to the south percentage. The same is true for the prevalence of only-children which occurs quite equally in the north and the east, and for the prevalence of stepparent families which occurs equal in north, east and west. In summary, Table 3.1 demonstrates large regional variations particularly in family structure: the prevalence of parental divorce/separation and stepparent families is high and comparable for the north, east and west, while being low in the south; single-parent families occur significantly more in the south.

B. Influence of sex on the prevalence of FSAs and NLEs

For boys and girls, no significant differences in FSAs and NLEs are observed, except for severe diseases/accidents of the child (which is more prevalent in boys (8.3% boys, 6.1% girls, $p=0.004$)). When examined for all age groups separately, peer problems are more prevalent in boys, more specifically in the group of 9 year olds (12.1% boys, 7.8% girls, $p=0.015$) (data not shown).

C. Influence of age on the prevalence of FSAs and NLEs

Childhood adversities are more prevalent in older age groups. Significant increases in the prevalence over the age groups are found for low maternal education ($p<0.001$, ranging from 6.2% to 17.1% over the age groups), non-traditional family structure ($p<0.001$, ranging from 14.7% to 25.8%), latchkey care ($p<0.001$, ranging from 0% to 12.7%), parental divorce/separation ($p<0.001$, ranging from 10% to 19.2%), major frustrations at school ($p<0.001$, ranging from 3.9% to 10.4%) and peer problems ($p=0.037$, ranging from 5.8% to 10.0%) (data not shown).

3.2 Cumulative stress from FSAs and NLEs

Table 3.2 demonstrates that 46.6% and 59.7% of the children have not yet experienced any of the studied FSAs or NLEs respectively, or that (vice versa) 53.4% and 40.3% of the children experienced at least one FSA or NLE. With an increasing sum of FSAs or NLEs the percentage of children decreases. Only a small percentage of the children experienced 4 or more FSAs/NLEs. With regard to cumulative stress (from FSAs and NLEs) and age, the percentage of children with no FSAs or NLEs decreases with age (which means that fewer and fewer children are shielded from adversities with increasing age), while the proportion of children with a higher number of stressors increases with age. Furthermore, there is no significant sex difference for cumulative stress from FSAs and NLEs ($p=0.266$ for FSA, $p=0.688$ for NLE).

Table 3.2 Prevalence of cumulative stress from FSAs and NLEs in pre- and primary-school children participating in the IDEFICS study (2009-2010): specifics for country groups, age groups and sex (FSAs: Familial and Social Adversities; NLEs: Negative Life Events)

		Sum of familial and social adversities (FSAs)						**Sum of negative life events (NLEs)**					
			0	**1**	**2**	**3**	**≥ 4**		**0**	**1**	**2**	**3**	**≥ 4**
Country groups	*N*	*Mean sum (SD)*	*Prevalence %*					*Mean sum (SD)*	*Prevalence %*				
North [a]	1330	0.84 (0.989)	46.9	31.4	14.7	5.5	1.5	0.74 (0.901)	50.3	30.6	14.4	4.1	0.7
East [b]	666	0.69 (0.858)	51.7	31.5	13.4	2.9	0.7	0.70 (0.941)	53.9	29.1	11.4	4.1	1.5
South [c]	1660	0.91 (0.975)	41.7	34.9	16.3	5.4	1.7	0.35 (0.615)	71.0	23.7	4.4	0.9	0.1
West [d]	981	0.80 (1.041)	51.1	28.2	12.9	5.8	1.9	0.62 (0.861)	57.4	27.6	10.9	3.4	0.7
Age groups	*N*												
4	258	0.67 (0.906)	55.4	28.3	11.2	3.9	1.2	0.53 (0.739)	60.1	28.3	10.1	1.6	0.0
5	582	0.72 (0.918)	52.1	30.6	11.9	4.3	1.2	0.47 (0.727)	64.8	25.9	7.7	1.0	0.5
6	736	0.79 (1.016)	51.1	28.9	13.2	4.8	2.0	0.52 (0.781)	62.4	26.6	8.4	2.2	0.4
7	605	0.72 (0.898)	51.2	30.9	13.2	3.8	0.9	0.47 (0.710)	63.8	26.9	7.8	1.3	0.2
8	735	0.76 (0.927)	48.6	32.9	13.7	3.5	1.2	0.52 (0.805)	63.0	26.1	7.2	3.1	0.5
9	1113	0.96 (1.016)	40.5	33.3	17.9	6.6	1.6	0.69 (0.910)	54.3	28.0	12.7	4.4	0.7
10	608	1.03 (1.041)	36.5	36.2	17.6	7.4	2.4	0.70 (0.919)	53.5	29.3	12.0	3.9	1.3
Sex	*N*												
Boys	2296	0.82 (0.952)	46.4	32.6	14.6	5.3	2.1	0.58 (0.830)	59.7	27.1	9.5	3.2	0.5
Girls	2341	0.84 (1.007)	46.8	31.4	14.8	5.0	2.0	0.57 (0.819)	59.7	27.4	9.8	2.4	0.7
Total	**4637**	**0.83 (0.980) (range 0-6)**	**46.6**	**32.0**	**14.7**	**5.1**	**1.4**	**0.57 (0.825) (range 0-5)**	**59.7**	**27.3**	**9.6**	**2.8**	**0.6**

[a] Sweden - Estonia, [b] Hungary, [c] Italy-Cyprus-Spain, [d] Belgium-Germany

3.3 Associations and risk for adversities

Table 3.3 demonstrates that variables concerning socio-economic characteristics of the child's life (being immigrant, family economic hardship, parental job loss, teenage pregnancy, low maternal education) are strongly interwoven with each other (e.g. children with low educated mothers are more likely to experience family economic hardship and children with family economic hardship are more likely to be immigrant) but are also associated with the family structure (parental divorce/separation, non-traditional family structure and only-children). A non-traditional family structure is not associated with family economic hardship (in contrast to parental divorce/separation), although single-parent families are 1.8 times more likely to experience economic adversity (data not shown, OR=1.80; 95% CI [1.26,2.59], p=0.001).

Family climate also seems to be associated with socio-economic factors, with bad family climate being more likely with teenage pregnancy, low maternal education, family economic hardship, parental job loss, parental divorce/separation and latchkey care. Similarly, latchkey care is more likely to occur in children from mothers with teenage pregnancy, non-traditional family structure, parental divorce/separation and only-children. Latchkey care is however less likely to occur in children with low maternal education and family economic hardship.

Table 3.3 Risk for co-occurring adversities in pre- and primary-school children participating in the IDEFICS study (2009-2010) (N=4637)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. being immigrant (not being immigrant as RC)															
2. low maternal education (no low maternal education as RC)	1.64*														
3. family economic hardship (no family economic hardship as RC)	1.64**	3.43*													
4. non-traditional family structure (traditional family structure as RC)	NS	1.69*	NS												
5. only-children (children with siblings as RC)	NS	1.34**	NS	2.89*											
6. latchkey care (no latchkey care as RC)	NS	0.36*	0.085*	1.63*	2.09*										
7. bad family climate (no bad family climate as RC)	NS	2.54*	2.74**	NS	NS	2.74**									
8. teenage pregnancy (no teenage pregnancy as RC)	2.01**	4.19*	NS	4.91*	2.31*	3.04*	3.43**								
9. peer problems (no peer problems as RC)	1.68**	1.46**	1.99*	1.53*	NS	NS	6.38*	2.07**							
10. parental divorce/separation (no parental divorce/separation as RC)	NS	1.39**	1.48**	92.5*	3.57*	2.65*	1.96**	5.99*	NS						
11. addition of a new family member (no addition of new family member as RC)	0.67**	NS	NS	4008*	NS	1.61**	NS	4.34*	NS	7.92*					
12. parental job loss (no parental job loss as RC)	NS	NS	5.18*	NS	1.34**	NS	2.22**	2.68*	1.4**	1.99*	1.47**				
13. severe diseases/ accidents of the child (no severe diseases/accidents as RC)	NS	NS	1.83**	NS	1.85*	NS	NS	NS	1.57**	1.53**	1.45**	1.93*			
14. serious illness of a family member (no serious illness family member as RC)	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	2.7*	NS		
15. major frustrations at school (no major frustrations at school as RC)	NS	1.52**	NS	NS	NS	NS	NS	NS	2.86*	1.7*	1.81*	2.27*	2.48*	NS	

Only significant OR (univariate odds ratios) are presented. * significant at p<0.002 level; ** borderline significant (p>0.002 and p<0.05); NS not significant. This table presents the risk for co-occurrence of a specific adversity (first row) if another adversity is already present (first column) vice versa. [a]RC= reference category

4 Discussion

To our knowledge, this study is the first to investigate both chronic and once-only adversities (i.e. FSAs and NLEs) in a large sample of European pre- and primary-school children, allowing us to study the influence of region, age and sex on the prevalence of adversities. Additionally, this study contributed to the knowledge of cumulative stress incidence and adversity-associations in a cross-national setting of young children. It should be noted that the prevalence and the types of reported FSAs or NLEs may vary according to the age of the children, ethnicity or culture, measurement approach and data collection methods and the period of assessment. The aim of the discussion-section below is thus to get an idea how our trends in childhood adversity (i.e. IDEFICS project) fit in the picture known from previous research.

4.1 Prevalence of FSAs, NLEs and cumulative stress

Although a large percentage of the children was shielded from childhood stressors, a small group of children was exposed to multiple, accumulating adversities, which is in line with previous research (Benjet C. et al., 2009; Furniss T. et al., 2009). Exposure to four or more FSAs or NLEs was reported for 1.4% and 0.6% of the children respectively, numbers which are however significantly lower than those reported by Furniss et al. (Furniss T. et al., 2009).

While certain adversities occurred only rarely, others were very regular such as a non-traditional family structure and parental divorce/separation (Benjet C. et al., 2009; Furniss T. et al., 2009). In general, this study indicated that one in five children does not live together with both biological parents. It can be assumed that with increasing age, this percentage increases. Schilling et al. (Schilling E. A. et al., 2007) reported that by the age of young adulthood only one in two will live in an 'intact two parent family'. Also in the IDEFICS project, similar trends were seen over time. In the baseline survey, 82.1% of the children lived in a two-parent-family (Ahrens W. et al., 2011a), a proportion that had already decreased to 79% in the second survey period two years later, and which may further decrease during follow-up.

A. Influence of region

In accordance to EUROSTAT findings (EUROSTAT, 2010), this study indicated regional variations in living arrangements and family formations, with up to five-fold differences in the prevalence of parental divorce and non-traditional family structures. In general, children from northern countries seem to experience more parental divorce/separation and related difficulties (e.g. addition of a new family member, formation of stepparent families, latchkey care), while parents from southern countries reported more socio-economic adversities (e.g. being immigrant, family economic adversity). Remarkably, parental divorce/separation was less prevalent while single-parent families were more prevalent in southern countries compared to northern countries. This may indicate that in southern countries single-parent family structures are not necessarily related to divorce. Possibly, marrying rates may be lower and cohabitation may be more common in the southern survey centres. Fifteen-fold differences were observed for the prevalence of teenage pregnancies, although mean percentages were low. This may indicate that teenage pregnancies are becoming rare in most countries, which is in line with European findings of Robson and Berthoud (Robson K. et al., 2003). Low maternal education prevalence largely varies between survey centres (i.e. high for Italy and Germany, while being low for the other countries) and was previously described by Ahrens et al. (Ahrens W. et al., 2011a) as a possible selection effect at baseline, more specifically as an underrepresentation of low-income groups in some countries at baseline. Also the large difference in immigrant prevalence between Cyprus and Germany (high) compared to the Belgian cohort (low) has been discussed by Ahrens et al. in the context of historical aspects (Ahrens W. et al., 2011a).

Although description of regional variations in this study aimed to be strictly exploratory, cultural, religious and welfare typologies should be considered in interpreting results. Cultural and religious characteristics such as the attitude towards contraception, marriage and divorce, or tri-generational families may affect the observed differences in family formation patterns (e.g. less prevalent divorce in the more Catholic southern countries). Also, differences in perception of 'serious' illnesses, 'major' frustrations and 'bad' family climates due to culture, may have influenced distinct prevalence percentages for some of the studied adversities. Last, the heterogeneity of societal and policy regimes within the studied countries should be considered in interpreting results on socio-economic welfare, educational chances etc.

B. Influence of age

The risk for childhood adversity generally increased with age. This did however not apply for some variables which were more constant over time (e.g. family economic hardship, bad family climates and being immigrant) and can therefore be considered 'chronic', persistent adversities (Benjet C. et al., 2009). Latchkey care increased by 12.7% over the age groups, suggesting that particularly children of the last years of primary-school are more often left alone (after school).

C. Influence of sex

In the literature it has been indicated, although sometimes inconsistently, that sex differences may occur in the types of events experienced, possibly resulting from sex differences in social roles (Hatch S. L. et al., 2007). In this study we could however not demonstrate such sex differences for the studied FSAs and NLEs, except for the occurrence of severe diseases/accidents of the child and peer problems (in the age group of 9 year olds) which were more frequent in boys (borderline significant). Our findings can be explained by significant differences in peer relationships in boys and girls as shown by Rose et al.: girls have been shown to engage in more prosocial interactions with higher self-disclosure in friendships and to empathize with others, while boys have been shown to more frequently engage in organized play (e.g. sports, competitive games, rough-and-tumble play), to emphasize the importance of self-interest and dominance within their peer group and to encounter more peer stress in the form of overt physical or verbal victimization (Rose A. J. et al., 2006). Our findings (i.e. more frequent diseases/accidents and peer problems in boys) thus fit within this described context.

4.2 Associations and risk for adversities

The present findings showed that negative life events and chronic adversities tend to cluster or co-occur (although no statements on direction or causality can be made), i.e. children exposed to a certain NLE or FSA are likely to also be exposed to other socio-economic or familial adversities, all together shaping the living conditions of the child and possibly resulting in cumulative childhood stress. In the context of the indicated connection between socio-economic and familial variables (Table 3.3), teenage pregnancy was (similar to findings of

Robson and Berthoud (Robson K. et al., 2003)) more likely to co-occur with less preferable economic and family situations for the child. Also in line with previous research (Benjet C. et al., 2009), we identified a relationship between parental divorce, single-parent families and family economic adversity. Bad family climates were more likely to occur in families with divorced or separated parents, but not in non-traditional family structures, which may postulate the impact of divorce itself on family tensions and on the parental ability and opportunities to effectively interact with their children (Hines A. M., 1997; Ram B. L. et al., 2003). Furthermore, bad family climates were more likely to take place in families with low educated mothers, which may point to a relationship between the mother's education and the way of interacting with the child and the parent-child relationship (Laosa L, 1982). Children with peer problems were 6 times more likely to experience bad family climates (and vice versa), suggesting an interrelatedness between social and familial relationships. Despite limited financial resources, families with economic hardship and low educated mothers showed less latchkey care, which resembles previous research and may be explained by a more frequent presence of the mother at home due to less frequently being fully-employed (Aizer A., 2004; Casper L. M. et al., 2004). Latchkey care was however more likely in non-traditional family structures speculating that parents from these family structures may receive less help from e.g. a life partner in after-school child-care. Two more remarks relate to only-children. The finding that only-children are more likely to experience latchkey care may be quite obvious since children that are left alone with older siblings are strictly speaking not 'left alone' and may thus be less reported.

4.3 Strengths and limitations

The strength of this study is its large, international sample comprising 8 European countries from north, east, south and west, allowing us to study childhood adversity in a larger context than has previously been done and allowing insightful comparisons across different nations in children younger than 12 years old, by investigating both once-only and more chronic situations. In addition, all survey centres were studied at the same time using the same, standardized protocol. Nevertheless, some weaknesses may lay in some specific methodological aspects: 1) the dichotomous nature of the variables may not consider the complexity of certain issues (e.g. immigration, family structure), 2) only a limited number of NLEs and FSAs were assessed, which were exclusively parent-reported and did not take into account children's perspectives; also the fact that only biological-, adoptive-, or stepparent

reported data on maternal education, family economic hardship and family climate was included, could have excluded the most affected children, 3) measures of NLEs may be underestimated because of their retrospective nature (possible recall bias) and the lack of differentiation between 'no occurrence of the event' or 'missing information' in the NLE questionnaire (although, it is quite likely that serious events such as deaths etc. are reported quite accurate, while other events such as e.g. major frustrations at school are difficult to report by parents and may as well be overestimated), 4) a selection or non-participation bias related to education or income-level, as well as a response bias cannot be ruled out and may thus have influenced prevalence results (since respondents might differ in characteristics from non-respondents and since respondents may have the tendency to give a "morally right" answer) (Ahrens W. et al., 2011a), and to end 5) it is noteworthy that the selected communities are not necessarily representative for each country. Comparisons between countries should therefore be made with caution.

5 Conclusion

Next to showing variations in the prevalence of childhood adversities across regions, age groups and sex, this study demonstrated the co-occurrence and connection between socio-economic adversities and family characteristics, which all together shape the living conditions of the child and which may possibly result in cumulative childhood stress in children younger than 12 years old. Even though family formation change and disadvantage in the early family or social environment may not harm all children equally, they should not be considered risk-free living conditions given their widespread appearance, consequences on family life and long-term health risk (although it should be noted that some family changes may be protective for the children by removing them from conflicted or violent households). The importance of future recording/monitoring potential childhood adversities in pre- and primary-school children lies within the further elucidation of the mental and physical health consequences of childhood adversities and the possibility for short- and long-term prevention of adverse health effects.

CHAPTER 3.2 PREVALENCE OF PSYCHOSOMATIC AND EMOTIONAL SYMPTOMS IN EUROPEAN SCHOOL-AGED CHILDREN AND ITS RELATIONSHIP WITH CHILDHOOD ADVERSITIES: RESULTS FROM THE IDEFICS STUDY

Abstract

Background The prevalence of childhood stress and psychosomatic and emotional symptoms (PES) have increased in parallel, indicating that adverse, stressful circumstances and PES in children might be associated.

Objectives This study describes the prevalence of PES in European children, aged 4 to 11 years old, and examines the relationship between PES, negative life events and familial or social adversities in the child's life.

Methods Parent-reported data on childhood adversities and PES was collected for 4066 children from 8 European countries who participated in the follow-up survey of IDEFICS (2009-2010), by means of the 'IDEFICS Parental Questionnaire'. A modified version of the 'Social Readjustment Rating Scale', the 'KINDL Questionnaire for Measuring Health-Related Quality of Life in Children and Adolescents' and the 'Strengths and Difficulties Questionnaire' were incorporated in this questionnaire, as well as questions on socio-demographics, family lifestyle and health of the child. Chi-square analyses were performed to investigate the prevalence of PES between survey centres, age groups and sex of the child. Odds ratios were calculated to examine childhood adversity exposure between PES-groups and logistic regression analyses were conducted to investigate a) the contribution of the number and b) the specific types of experienced adversities on the occurrence of PES.

Results 45.7% of the children experienced at least one PES, with low emotional well-being during the last week being most frequently reported (38.2%). No sex differences were shown for the prevalence of PES ($p=0.282$), but prevalence proportions rose with increasing age ($p<0.001$). Children with PES were more frequently exposed to childhood adversities compared to children without PES (e.g. 13.3% and 3.9% of peer problems and 25.4% and 17.4% of non-traditional family structure in the PES versus no PES group respectively, $p<0.001$). An increasing number of adversities (regardless of their nature) was found to

gradually amplify the risk for PES (OR=2.85, 95% CI=1.98-4.12 for a number of ≥3 negative life events), indicating the effect of cumulative stress. Last, a number of specified adversities were identified as apparent risk factors for the occurrence of PES such as living in a non-traditional family structure (OR=1.52, 95% CI=1.30-1.79), or experiencing peer problems (OR=3.55, 95% CI=2.73-4.61).

Conclusions Childhood adversities were significantly related to PES prevalence, both quantitatively (i.e. the number of adversities) and qualitatively (i.e. the type of adversity). This study demonstrates the importance and the impact of the child's family and social context on the occurrence of PES in children younger than 12 years old.

1 Introduction

Childhood stressors may originate from multiple events in the child's everyday environment (e.g. school, family, peers) (Washington T. D., 2009). Chronic exposure to adverse, stressful situations may affect the child's behaviour and personality development and may have consequences on both their physiological and psychological health, with effects potentially persisting into adolescence and adulthood, such as the manifestation of depression, cardiovascular or auto-immune diseases, or psychosomatic complaints (Cohen S. et al., 2007; Mcewen B. S., 1998; Schneiderman N. et al., 2005; Teicher M. H. et al., 2003; Silber T. J. et al., 2003).

Psychosomatic complaints are chronic or recurrent somatic complaints without clear physical cause which are related to psychological, emotional or social factors (Silber T. J. et al., 2003; Berntsson L. T. et al., 2000). Headaches, abdominal pain, persistent fatigue and tiredness are frequently observed psychosomatic complaints in children (Silber T. J. et al., 2003). Of all, 17%, 23% and 24% of Swedish adolescents (10-18 years old) (Alfven G. et al., 2008), Swedish schoolchildren (6-13 years old) (Petersen S. et al., 2003) and Chinese schoolchildren (9-12 years old) (Hesketh T. et al., 2010) experience weekly recurring headaches, respectively. In addition, 12% (5-7 year olds) to 14% (7-17 year olds) of German children exhibited signs of mental health problems (Furniss T. et al., 2006; Ravens-Sieberer U. et al., 2008c).

The prevalence of childhood stress and psychosomatic and emotional symptoms (PES) have been increasing in parallel over the last decade, indicating that adverse, stressful circumstances may trigger PES in children (Silber T. J. et al., 2003; McMahon S. D. et al.,

2003; Benjet C. et al., 2010; Tanaka H. et al., 2000; Ostberg V. et al., 2006; Gustafsson P. E. et al., 2009; Harland P. et al., 2002; Furniss T. et al., 2009; Hesketh T. et al., 2010; Schilling E. A. et al., 2007; Forehand R. et al., 1998; Grant K. E. et al., 2004). Moreover, multiple simultaneous or sequential stressors may increase the risk for psychosomatic or emotional problems in a cumulative or additive way (Turner R. J. et al., 1995; Furniss T. et al., 2009; Schilling E. A. et al., 2007; Schilling E. A. et al., 2008; Wille N. et al., 2008; Forehand R. et al., 1998; Anda R. F. et al., 2006; Benjet C. et al., 2010). In this context, familial and social adversities require special attention. These stressors are seldom isolated because they tend to cluster or give rise to other unfavourable events (e.g. parental divorce may lead to decreased economic resources, parental strain and a change in family structure).

To our knowledge, there is a lack of large-scale (international) research on the relationship between PES and negative life events and familial and social adversities in young children. The present study aimed to describe the prevalence of PES in children from 8 European countries (N=4066) and to examine the relationship between PES and childhood adversities cross-nationally by investigating the following research questions: Do children with and without PES differ in their exposure to childhood adversities? Does the number of adversities (regardless of the nature of adversities) influence the occurrence of PES? Is the risk for PES in children affected by specific types of experienced adversities?

2 Methods

2.1 Study design and participants

From September 2009 to May 2010, information on childhood adversities and PES in children was obtained for 4066 children (aged 4 to 11.8 years, mean=7.91, standard deviation (SD)=1.82), 49.7% boys). This was part of the follow-up survey of the IDEFICS study, a Large Integrated Project within the 6th Framework Programme of the European Commission ('Identification and prevention of Dietary- and lifestyle-induced health EFfects In Children and infantS', www.idefics.eu). The IDEFICS project is a multi-centre longitudinal intervention study of pre- and primary school children in 8 European countries (Belgium, Cyprus, Estonia, Germany, Hungary, Italy, Spain and Sweden) investigating the aetiology of diet- and lifestyle-related diseases and disorders in children, in which also a community-oriented prevention programme for primary prevention of obesity is developed and evaluated

in a controlled study design (intervention versus control regions) (Ahrens W. et al., 2011a; De Henauw S. et al., 2011). The baseline survey started in 2007 with a cohort of 16224 children (Figure 3.3). The intervention programme and more detailed aims and methods have been described elsewhere (Ahrens W. et al., 2011a; De Henauw S. et al., 2011). The study was conducted according to the guidelines of the Declaration of Helsinki and approvals of the Ethical Committees were obtained for each survey centre.

Only the control regions of the participating countries were eligible for inclusion in this analysis to rule out intervention-bias on the studied variables (intervention-bias may arise by e.g. the intervention module on creating a family environment that promotes spending time together and a healthy lifestyle) (De Henauw S. et al., 2011; Verbestel V. et al., 2011). Children younger than 4 years of age and children from whom any information on childhood adversities and PES was missing, were excluded from the analysis (N=2194/6260; 35.05%). This resulted in a total number of 4066 children included in this study (Figure 3.3).

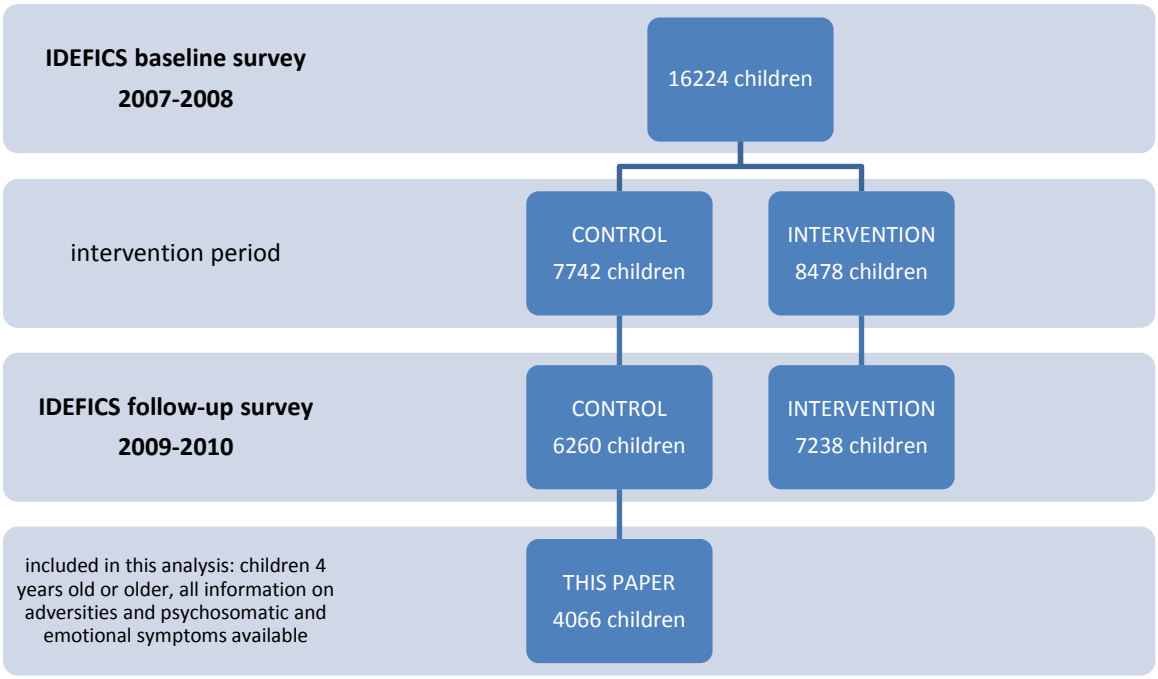


Figure 3.3 Study flowchart

No differences were observed between the included and excluded group for sex (49.7% and 50.8% boys respectively) or age (mean=7.91 (SD=1.82) and mean=7.87 (SD=1.90), respectively). However, low parental education (International Standard Classification of Education level <3) (UNESCO, 1997) was more frequently reported in the excluded group compared to the included group (12.2% versus 6.1% respectively).

2.2 Instruments and variables

In order to obtain information on socio-demographics, family lifestyle, health and mental well-being of the children, parents were asked to complete the 'IDEFICS Parental Questionnaire' and the 'IDEFICS Questionnaire on Health and Medical History' at home and to return them to the schools.. All data in this study on childhood adversities and PES originated from these questionnaires, of which the quality and comparability across the survey centres was assured by a translation/back-translation procedure for each local language and by re-administering the parental questionnaire to a convenience sample of study participants (Ahrens W. et al., 2011a; Herrmann D. et al., 2011).

A. Assessment of childhood adversities

The family environment may strongly affect the social, emotional and physical health of children by shaping the context and the opportunities of children's later lives (Repetti R. L. et al., 2002; Waldfogel J. et al., 2010). Parental conflicts or divorce (Pryor J. et al., 2001), a low supportive or unfavourable family climate (Amato P. R., 2005; Lawson D. W. et al., 2010; Gass K. et al., 2007; Card J., 1981), domestic violence or abuse (Holt S. et al., 2008), parental supervisory neglect (Casper L. M. et al., 2004; Aizer A., 2004), socio-economic disadvantage (Gustafsson P. E. et al., 2009; Conger R. D. et al., 2010; Ram B. L. et al., 2003; Lupien S. J. et al., 2001), serious illness of the child or a family member (Sieh D. S. et al., 2010; Hysing M. et al., 2009) and peer problems or frustrations at school (van der Wal M. F. et al., 2003; Wolke D. et al., 2001; Einfeld S. L. et al., 2006) have all been shown to emotionally and psychologically affect children. Therefore, parents were asked to complete questions on both the life-time occurrence of negative life events (NLE) and the more chronic familial and social adversities (FSA) which may constitute potential childhood adversity, such as ethnicity of the family, education of the mother, employment of the parents, family structure and family relationships. These childhood adversity variables were all of dichotomous nature (occurrence or no occurrence of the event; presence or no presence of the adversity).

Negative life events (NLE) (once-only)

To assess negative life events encountered during the child's life, the parents were asked to complete the following question: "Which of the following events did your child encounter and

also report how old your child was at that time (yes/no): parental divorce or separation, addition of a new family member (e.g. step-parent), parental job loss, severe diseases or accidents of the child, serious illness of a family member, child having major frustrations at school, death of the child's parent, sibling, grandparent or pet". These life event-items originated from a modified version of the Social Readjustment Rating Scale, incorporated in the IDEFICS Parental Questionnaire (Holmes T. H. et al., 1967).

Familial or social adversities (FSA) (chronic)

Next to the above mentioned 'once-only' events (NLE), also conditions with a more chronic character were assessed as these may differently impact PES in children. Ethnicity of the family was based on the birth country of the parents and the child. If one of them was born in a foreign country the child was described as 'being immigrant'. Parental education was evaluated for mothers according to the ISCED classification (UNESCO, 1997). 'Low maternal education' was determined as an ISCED level of 0, 1 or 2 (pre-primary, primary or lower secondary education). Families were identified as suffering from 'family economic hardship' if one of the parents was unemployed for a year or more, or if on welfare (social assistance). If the child did not live with both his/her parents, the family was defined as a 'non-traditional family' (including single-parent families, stepparent families, living with grandparents or foster-parents or in an institution). Children not living together with any siblings (including step- and half-siblings) were defined as 'only-children'. 'Latchkey care' or parental supervisory neglect was presumed if the children were left alone at home for more than 7 hours a week (after-school self-care). If the age of the mother at child-birth was 19 or younger, the pregnancy was considered a 'teenage pregnancy' (Robson K. et al., 2003). The quality of family climate was assessed using adapted versions of the Family Climate Scale and the Authorative Parenting Index (Schneewind K. et al., 1985; Jackson C. et al., 1998). Each of the 13 questions was rated on a 4-point Likert scale, summed to a total score and reversed to a score on 100. Family climates with a score lower than 50/100 were defined as 'bad family climate'. Furthermore, peer problems were defined as a borderline (4-5/10) or abnormal score (6-10/10) on the Peer Problem Scale of the Strengths and Difficulties Questionnaire (SDQ). The SDQ is a 25-item behavioural screening questionnaire on emotional problems, conduct problems, hyperactivity-inattention behaviour, peer problems, and prosocial behaviour that has been validated for its use in several European countries and was incorporated in the IDEFICS Parental Questionnaire (Youth in Mind, 2009; Goodman R.,

1997; Ravens-Sieberer U. et al., 2008a). Important to note is that these variables do not constitute *actual* childhood stressors for all children, but should be considered *potential* stressful conditions during childhood. More detailed information on the rationale, methodology and prevalence of these variables were described previously by our research group (Vanaelst B. et al., 2012d).

B. Assessment of psychosomatic and emotional symptoms

PES in children were described by five different variables: emotional well-being and self-esteem of the child during the last week (the week preceding completion of the questionnaire), emotional problems and frequent occurrence of headaches, stomach-aches or sickness over the last 6 months, and difficulties falling asleep.

Emotional well-being and self-esteem of the child during the last week

Parents were asked to complete the emotional and self-esteem subscales of the 'KINDL Questionnaire for Measuring Health-Related Quality of Life in Children and Adolescents', a questionnaire which assesses the child's quality of life in multiple dimensions (physical well-being, emotional well-being, self-esteem, family, friends and everyday functioning subscale) and which was incorporated in the IDEFICS Parental Questionnaire (Ravens-Sieberer U. et al., 1998). The items of the emotional and self-esteem subscales were scored from 1 (never) to 4 (often or always) with reversals according to the wording of the question, and summed to a total score. These total scores for self-esteem and emotional well-being were transformed to a scale on 100 (mean score on emotional well-being: M=86.93, SD=11.80; mean score on self-esteem: M=86.52, SD=10.75) and dichotomized into 'low' or 'high' scores using sex- and age-specific cut-off scores from the KINDL manual (emotional well-being cut-offs: boys 82.89, girls 83.11; self-esteem cut-offs: boys 66.52, girls 66.68) (Ravens-Sieberer U. et al., 2000; Ravens-Sieberer U. et al., 1998), to obtain a measure of the emotional well-being and self-esteem of the child during the last week.

Emotional problems over the last 6 months

'Emotional problems over the last 6 months' were assessed using the Emotional Symptoms Scale of the SDQ. Each of the 5 items (headaches, stomach-aches, sickness; worries;

unhappiness; loss of confidence; fears) were scored on a three point scale (0 not true, 1 somewhat true, 2 certainly true). This way a maximum score of 10 could be obtained (M=1.65, SD=1.74), with higher scores indicating more emotional difficulties. Cut-off points have been defined, classifying the results into normal (<6/10), borderline (6/10) or abnormal (>6/10) emotional well-being (Youth in Mind, 2009). Borderline and abnormal scores were taken together to represent emotional problems over the last 6 months.

Headaches, stomach-aches or sickness

'Frequent occurrence of headaches, stomach-aches or sickness', one of the items of the Emotional Symptom Scale (SDQ), was also examined separately. The children were classified as having frequent headaches, stomach-aches or sickness if the parents indicated the 'certainly true'-response.

Difficulties falling asleep

Last, the parents reported on the children's general sleeping habits in the 'IDEFICS Questionnaire on Health and Medical History'. The dichotomous variable 'difficulties falling asleep' was used as an indicator of impaired sleep quality.

2.3 Statistical procedures

Statistical analyses were performed with PASW Statistics Program version 19.0.0 (SPSS Inc., IBM, IL, USA). The prevalence of the children's PES was compared between countries, age groups and sex using a χ^2 test. Each year of age was considered as one age group except the children of 10 and 11 years old were grouped together because of the low number of 11-year-olds (N=35). Since the prevalence differed significantly between survey centres, all further analyses were adjusted for survey centre.

To study the difference in childhood adversity exposure between children with and without PES, the children were divided into two groups: those having no PES (sum equal to 0) and those having at least one PES. Independent sample t-tests and odds ratios (OR) were calculated to study age differences and childhood adversity differences between these two groups, respectively.

Logistic regression analyses (OR and 95% confidence intervals (CIs)) were calculated to investigate the contribution of the number of adversities on the occurrence of each PES, and these models were adjusted for survey centre, age and sex of the child, and the sum of FSAs (5 categories) or NLEs (4 categories) as predictors respectively. Because of the low number of children with a sum of NLEs ≥4 (N=23), these children were grouped together in the ≥3 NLEs category.

Further logistic regression analyses were conducted to investigate the independent explanatory value of specific types of adversities as predictors for the occurrence of PES, adjusting for all other adversities, age, sex and survey centre and by using a backward stepwise regression procedure. For each PES, the analyses started with a full model including all adversities, after which the non-significant adversities were eliminated from the model in an iterative process (probability for entry=0.05, probability for removal=0.10). This way, only those predictors with a significant contribution ($p < 0.05$) to the model were reported.

Results from all logistic analyses mentioned above (with adjustments for survey centre) were confirmed by multilevel analyses, more specifically with Generalized Linear Models (Generalized Estimating Equations). P-values <0.05 were considered statistically significant for all tests.

3 Results

3.1 Prevalence of PES

Table 3.4 presents percentages of children's PES for each survey centre, age group and sex separately. 45.7% of the children experienced at least one PES. While the prevalence of most PES was rather rare (percentages below 10%), low emotional well-being in the last week (week preceding completion of the questionnaire) was reported for 38.2% of the children. No sex differences in PES were found. There was a trend for increasing PES prevalence with increasing age, except for difficulties falling asleep which was rather constant across age groups. Additionally, large variations in the prevalence of PES were observed between the survey centres.

3.2 PES and its relation to childhood adversity

A. Differences in exposure to childhood adversity between children with and without PES

Table 3.5 demonstrates a significantly higher prevalence of childhood adversities in children with PES compared to children without PES, with up to two- or three-fold differences in prevalence. More specifically, the following adversities were more frequent in the case of PES (OR > 2): maternal teenage pregnancy, bad family climate, peer problems and major frustrations at school. Still, 25.4% of the children with any form of PES did not yet experience any FSA or NLE (results not shown in table).

B. Contribution of the number of adversities to the occurrence of PES

Except for difficulties falling asleep, the risk for all PES gradually increased with the number of experienced FSAs or NLEs, regardless of the nature of the adversity (Table 3.6).

The number of FSAs or NLEs had the largest impact on emotional problems over the last 6 months, as indicated by the largest ORs. Even though of the occurrence of 3 or 4 adversities resulted in more pronounced increases in the risk for PES, also children experiencing only one FSA or NLE were already two times more likely to experience emotional problems or frequent headaches, stomach-aches or sickness, respectively. The number of FSAs contributed more strongly to the risk for PES compared to the number of NLEs, except for frequent headaches, stomach-aches and sickness for which it was the other way around.

11.8% of the children experiencing ≥4 FSAs did however not exhibit any PES (results not shown in table).

C. Contribution of specific types of experienced adversities to the occurrence of PES in children

Table 3.7 presents the differential contributions of specific adversities on the risk for PES. The importance of specific FSAs or NLEs as predictors for the occurrence of PES depended on the type of PES (e.g. family economic hardship and teenage pregnancy were only positive predictors for the occurrence of low emotional well-being and low self-esteem in last week respectively, without significant contribution to the occurrence of other PES). In general,

living in a non-traditional family structure or in a bad family climate, experiencing peer problems and having major frustrations at school were independent predictors for all studied PES, as demonstrated by sometimes large ORs.

While most of the adversities increased the risk for PES, family economic hardship and latchkey care were negatively associated with difficulties falling asleep. Age was a positive predictor for all PES except for difficulties falling asleep (results not shown).

Table 3.4 Prevalence of psychosomatic and emotional symptoms in children

			KINDL		SDQ		medical questionnaire
		at least one psychosomatic or emotional symptom	**low self-esteem last week**	**low emotional well-being last week**	**emotional problems last 6 months**	**headaches, stomach-aches or sickness**	**difficulties falling asleep**
survey centres	*N of children*			*% of children*			
Belgium	343	42.6	0.6	32.7	7.3	7.3	14
Cyprus	469	42.9	6.4	34.8	3.8	5.8	5.8
Estonia	763	55.8	1.7	48.9	3.5	3	13.9
Germany	337	43.6	1.8	32	3.3	6.8	11.9
Hungary	643	40.3	3.3	34.7	3	2.8	5.1
Italy	520	50.2	3.3	44.2	2.7	4.8	9
Spain	472	49.8	5.7	42.4	4.4	6.8	5.7
Sweden	519	35.5	1	27.9	1.5	3.5	9.6
p-values χ^2		*<0.001**	*<0.001**	*<0.001**	*0.001**	*<0.001**	*<0.001**
age groups	*N of children*			*% of children*			
4	237	38.4	2.1	29.1	0.8	2.5	10.5
5	511	39.3	1.4	30.9	2.9	3.7	10.6
6	640	37.5	1.4	30.8	3.1	3.4	9.1
7	518	43.4	3.3	35.1	2.3	4.1	9.5
8	631	45.2	3.5	36.9	4.4	7.3	8.9
9	991	53.3	4.1	46.6	4.2	4.7	8.1
10+11	538	53.7	3.7	47	4.5	5.6	10.4
p-values χ^2		*<0.001**	*0.009**	*<0.001**	*0.052*	*0.01**	*0.655*
sex	*N of children*			*% of children*			
male	2019	44.9	3.1	38.1	3.4	4.1	9.2
female	2047	46.6	2.8	38.3	3.6	5.3	9.4
p-values χ^2		*0.282*	*0.59*	*0.864*	*0.732*	*0.057*	*0.771*
total	*N of children*				*% of children*		
	4066	**45.7**	**3**	**38.2**	**3.5**	**4.7**	**9.3**

KINDL: KINDL Questionnaire for Measuring Health-Related Quality of Life in Children and Adolescents; SDQ: Strengths and Difficulties Questionnaire

* statistically significant results

Table 3.5 Difference in exposure to childhood adversities between children with and without psychosomatic and emotional symptoms

	no psychosomatic and emotional symptoms (N=2207)	**at least one psychosomatic or emotional symptom (N= 1859)**		
	% of children within 'no psychosomatic and emotional symptoms'	*% of children within 'at least one psychosomatic or emotional symptom'*	*unadjusted OR (95% CI)*	*p-value* χ^2
Familial and social adversity				
being immigrant	12.2	13.1	1.08 (0.90-1.30)	0,423
low maternal education	10.1	13.2	1.36 (1.12-1.65)*	0,002*
family economic hardship	3.4	5.3	1.58 (1.16-2.15)*	0,003*
non-traditional family structure	17.4	25.4	1.62 (1.39-1.88)*	<0,001*
being only-child	15.3	19	1.30 (1.10-1.53)*	0,002*
latchkey care	4.3	8.1	1.93 (1.48-2.51)*	<0,001*
bad family climate	0.1	2.6	29.84 (7.25-122.90)*	<0,001*
teenage pregnancy	1.4	3.1	2.15 (1.39-3.33)*	<0,001*
peer problems	3.9	13.3	3.83 (2.97-4.94)*	<0,001*
Negative life events				
parental divorce/separation	9.8	17.1	1.90 (1.57-2.28)*	<0,001*
addition of a new family member	11.2	14.4	1.33 (1.10-1.59)*	0,003*
parental job loss	7.5	11.4	1.59 (1.28-1.96)*	<0,001*
major frustration at school	4.6	10.7	2.47 (1.93-3.17)*	<0,001*
severe diseases/accidents of the child	6.7	8.2	1.26 (0.99-1.59)	0,056
serious illness of family member	1.9	1.7	0.88 (0.56-1.40)	0,592
death of a parent	0.5	0.9	1.91 (0.86-4.21)	0,104
death of a sibling	0.5	0.6	1.31 (0.55-3.09)	0,539
death of a grandparent	4.7	4.9	1.05 (0.79-1.40)	0,734
death of a pet	0.8	0.5	0.59 (0.27-1.32)	0,195

* statistically significant results

Table 3.6 Contribution of the number of adversities to the occurrence of psychosomatic and emotional symptoms in children

				KINDL				SDQ				medical questionnaire	
		at least one psychosomatic or emotional symptom		**low self-esteem last week**		**low emotional well-being last week**		**emotional problems over the last 6 months**		**headaches, stomach-aches or sickness**		**difficulties falling asleep**	
number of familial and social adversities	*N of children*	*adjusted OR (95% CI)[a], p-value*											
1	1326	1.30 (1.12-1.51)	0.001*	1.48 (0.93-2.37)	0.098*	1.27 (1.09-1.48)	0.003*	2.17 (1.41-3.34)	<0.001*	1.61 (1.13-2.32)	0.009*	1.28 (1-1.65)	0.053
2	597	1.81 (1.49-2.20)	<0.001*	2.11 (1.21-3.66)	0.008*	1.81 (1.49-2.21)	<0.001*	2.50 (1.46-4.31)	0.001*	2.21 (1.43-3.41)	<0.001*	1.43 (1.04-1.97)	0.028*
3	214	3.07 (2.25-4.19)	<0.001*	4.32 (2.30-8.14)	<0.001*	3.28 (2.42-4.45)	<0.001*	5.56 (3.06-10.11)	<0.001*	2.45 (1.36-4.41)	0.003*	0.98 (0.58-1.67)	0.954
≥4	60	7.42 (3.70-14.88)	<0.001*	8.25 (3.37-20.19)	<0.001*	6.98 (3.74-13.02)	<0.001*	10.99 (4.87-24.77)	<0.001*	2.75 (1.04-7.27)	0.042*	1.82 (0.87-3.84)	0.114
number of negative life events	*N of children*	*adjusted OR (95% CI)[b,c], p-value*											
1	1110	1.39 (1.20-1.61)	<0.001*	1.35 (0.87-2.10)	0.182	1.35 (1.16-1.57)	<0.001*	1.39 (0.92-2.09)	0.118	2.14 (1.53-3.01)	<0.001*	1.10 (0.86-1.42)	0.454
2	401	1.88 (1.51-2.35)	<0.001*	2.04 (1.10-3.78)	0.024*	1.72 (1.37-2.15)	<0.001*	2.06 (1.21-3.50)	0.008*	2.64 (1.65-4.21)	<0.001*	1.31 (0.93-1.85)	0.120
≥3	143	2.85 (1.98-4.12)	<0.001*	5.60 (2.84-11)	<0.001*	2.74 (1.92-3.91)	<0.001*	5.47 (3.09-9.66)	<0.001*	4.79 (2.68-8.57)	<0.001*	1.91 (1.18-3.10)	0.008*

KINDL: KINDL Questionnaire for Measuring Health-Related Quality of Life in Children and Adolescents; SDQ: Strengths and Difficulties Questionnaire

[a] Odds ratios (OR) for psychosomatic and emotional symptoms adjusted for age and sex of the child and survey centre; children with no familial and social adversities were taken as reference group (N=1869)

[b] Odds ratios (OR) for psychosomatic and emotional symptoms adjusted for age and sex of the child and survey centre children with no negative life events were taken as reference group (N=2412)

[c] Female sex was a significant predictor for 'headaches, stomach-aches or sickness' if adjusted for the number of NLEs, survey centre and age (OR=1.36; 95%CI=1.01-1.83; p=0.043; boys as reference category)

* statistically significant results

Table 3.7 Contribution of specific types of experienced adversities to the occurrence of psychosomatic and emotional symptoms in children

		KINDL						SDQ				medical questionnaire	
		at least one psychosomatic or emotional symptom		**low self-esteem last week**		**low emotional well-being last week**		**emotional problems over last 6 months**		**headaches, stomach-aches or sickness**		**difficulties falling asleep**	
Familial and social adversities	*N child*	*adjusted OR (95%CI)-p-values*[a]											
family economic hardship	173					1.49 (1.08-2.06)	0.015					0.43 (0.21-0.89)	0.023
non-traditional family structure	858	1.52 (1.30-1.79)	<0.001	1.69 (1.11-2.58)	0.015	1.39 (1.18-1.64)	<0.001	1.91 (1.30-2.80)	0.001	1.77 (1.27-2.47)	0.001		
latchkey care	246					1.38 (1.02-1.86)	0.038					0.37 (0.21-0.65)	<0.001
bad family climate	51	22.77 (5.46-95.02)	<0.001	8.84 (4.15-18.81)	<0.001	22.12 (6.76-72.42)	<0.001	8.2 (4.03-16.71)	<0.001	3.79 (1.78-8.04)	0.001	4.51 (2.40-8.47)	<0.001
teenage pregnancy	89			2.95 (1.27-6.85)	0.012								
peer problems	332	3.55 (2.73-4.61)	<0.001	2.86 (1.80-4.55)	<0.001	3.20 (2.51-4.09)	<0.001	6.34 (4.33-9.29)	<0.001	2.23 (1.49-3.36)	<0.001	1.64 (1.16-2.32)	0.005
being immigrant	513												
low maternal education	467												
being only child	690												
Negative life events	*N child*	*adjusted OR (95%CI)-p-values* [a,b]											
parental divorce/separation	533	1.72 (1.41-2.09)	<0.001			1.72 (1.42-2.09)	<0.001	1.90 (1.24-2.90)	0.003	1.66 (1.09-2.52)	0.018		
addition of a new family member	515			2.07 (1.25-3.43)	0.005					1.63 (1.08-2.46)	0.021		
parental job loss	376	1.43 (1.14-1.78)	0.002	1.98 (1.19-3.30)	0.009	1.34 (1.07-1.67)	0.011						
severe diseases/accidents of the child	300									2.18 (1.38-3.42)	0.001		
serious illness of a family member	75			3.02 (1.15-7.97)	0.025								
major frustration at school	301	2.26 (1.75-2.91)	<0.001	1.76 (1.00-3.11)	0.05	2.08 (1.62-2.66)	<0.001	3.87 (2.53-5.92)	<0.001	2.41 (1.59-3.64)	<0.001	1.59 (1.21-2.26)	0.009
death of a parent	26							5.57 (1.78-17.43)	0.003				
death of a sibling	21												
death of a grandparent	194												
death of a pet	27												

KINDL: KINDL Questionnaire for Measuring Health-Related Quality of Life in Children and Adolescents; SDQ: Strenghts and Difficulties Questionnaire

[a] Odds ratios (OR) for psychosomatic complaints adjusted for age and sex of the child and country; children that did not experience the specific adversity were taken as reference group.
As backward regression analyses were performed, only the predictors with a significant contribution are reported.

[b] Female sex was a significant predictor for 'headaches, stomach-aches or sickness' if adjusted for NLE occurrence, survey centre and age (OR=1.38; 95%CI=1.02-1.86; p=0.035; boys as reference category)

4 Discussion

4.1 Prevalence of PES

In total, 45.7% of the children experienced at least one PES, with low emotional well-being during the last week being the most frequently reported PES. Prevalence proportions of other PES were lower, but rose with increasing age. The latter finding is in line with previous research (Tanaka H. et al., 2000; Petersen S. et al., 2003; Jozefiak T. et al., 2009; Ostberg V. et al., 2006) and may be due to a higher incidence of stressful life events with increasing age (Benjet C. et al., 2009; Wille N. et al., 2008), or to a different perception of reality as the ability to understand, perceive and react to external events increases in children growing older (Washington T. D., 2009). We did not observe general sex differences in the incidence of PES. Despite possible gender differences in the biological and psychological reaction to stressors (Chaplin T. M. et al., 2008; Kajantie E. et al., 2006; Cox S. J. et al., 2010; Rudolph K. D., 2002), the literature has yielded inconclusive results concerning a distinctive prevalence of PES between boys and girls (Alfven G. et al., 2008; Schilling E. A. et al., 2007; Elberling H. et al., 2010; Villalonga-Olives E. et al., 2011; Berntsson L. T. et al., 2000; Petersen S. et al., 2003; Ravens-Sieberer U. et al., 2007; Ravens-Sieberer U. et al., 2008a). The type of the studied stressor and PES and the age of the children may account for these contradictory findings.

The present study demonstrated differences in the prevalence of PES between the survey centres. Despite of the fact that investigating country differences was not the main objective of this study (as the selected communities may not necessarily be representative for each country), differences in the prevalence of PES (more specifically mental health problems) across countries have been shown before (Ravens-Sieberer U. et al., 2008a). Additionally, our results match findings of Elberling et al. and Heiervang et al. (Heiervang E. et al., 2008; Elberling H. et al., 2010); that is lower percentages for PES in more northern countries (see results for Sweden in Table 3.4). Heiervang and colleagues attributed this finding to under-reporting or under-recognition of emotional symptoms by parents from the north due to their more ‘normalizing’ view when filling out questionnaires, rather than representing a real (mental) health advantage for the north. Therefore, cross-cultural differences on

psychosomatics and psychopathology based on questionnaires may be misleading (Heiervang E. et al., 2008).

The mean scores on the self-esteem and emotional well-being subscale of the KINDL questionnaire in this study (mean=86.52, SD=10.75 and mean=86.93, SD=11.80 respectively) were higher compared to those of other studies in children of the same age (Ravens-Sieberer U. et al., 2007; Jozefiak T. et al., 2009; Eser E. et al., 2008; Ravens-Sieberer U. et al., 2008b; Ravens-Sieberer U. et al., 2000). Consequently, only a small percentage of children in this study were categorized as having a low self-esteem (3%). The mean score for the emotional well-being over the last 6 months (SDQ questionnaire) (mean=1.65, SD=1.74) was in accordance to data from different population studies (Youth in Mind, 2009; Mellor D., 2004; Woerner W. et al., 2004); as were our findings on difficulties falling asleep, although some studies show prevalence percentages up to 20 or 30% (Simola P. et al., 2010; Lehmkuhl G. et al., 2008; Fricke-Oerkermann L. et al., 2007; Ostberg V. et al., 2006). However, this PES behaved quite differently compared to the other PES (e.g. no increasing prevalence with increasing age (Table 3.4), no influence of cumulative stressor exposure (Table 3.6)), so difficulties falling asleep (or its current way of assessment) may therefore be less suitable as a psychosomatic outcome in the context of childhood stress research

4.2 PES and its relation to childhood adversity

This study confirmed the previously observed relationship between childhood adversities and PES in school-aged children (Furniss T. et al., 2009; Hesketh T. et al., 2010; Benjet C. et al., 2010; Tanaka H. et al., 2000; Ostberg V. et al., 2006; Gustafsson P. E. et al., 2009; Harland P. et al., 2002; Schilling E. A. et al., 2007; Forehand R. et al., 1998). First, children with PES were more frequently exposed to childhood adversities compared to children without PES. Second, an increasing number of adversities gradually amplified the risk for PES, supporting literature on cumulative stress and PES (Furniss T. et al., 2009; Schilling E. A. et al., 2007; Schilling E. A. et al., 2008; Wille N. et al., 2008; Forehand R. et al., 1998; Anda R. F. et al., 2006; Benjet C. et al., 2010; McMahon S. D. et al., 2003). Last, a number of specified adversities were emphasized as apparent risk factors for the occurrence of PES. So, both quantitative (i.e. the number of adversities) and qualitative effects (i.e. the type of adversities) were observed to be related to PES (although no firm conclusions on causality or directionality of this association can be made).

Even though the exposure to only one FSA/NLE already increased the likelihood of PES, the accumulation of multiple adversities in the child's life more substantially increased the risk for PES. More specifically, the transition from three to four FSAs was associated with a substantial increase in ORs (Table 3.6), as previously suggested by Forehand et al. (Forehand R. et al., 1998). Benjet and colleagues hypothesized a 'ceiling effect' of the number of adversities on PES, meaning that once a certain number of adversities is reached, the impact of any additional adversity on PES may be considerably less (Benjet C. et al., 2010).

Apart from the quantitative effects, the type of experienced adversities was also found to be of importance in the relationship between childhood adversity and PES ('qualitative effect'). This study identified the following familial and social characteristics as apparent predictors for PES: a non-traditional family structure, a bad family climate, peer problems and major frustrations at school. Particularly a bad family climate impacted very strongly on the occurrence of PES (ORs up to 22). However, the low prevalence of this adversity (N=51, 1.2%) may possibly have distorted this relationship. The importance of parental and peer social support, family structure and socio-economic factors in the mental and physical health of children has been shown before (Tanaka H. et al., 2000; Gini G. et al., 2009; Benjet C. et al., 2010; Wille N. et al., 2008; Ostberg V. et al., 2006; Berntsson L. T. et al., 2000; Gustafsson P. E. et al., 2009; Harland P. et al., 2002; Elberling H. et al., 2010; Ravens-Sieberer U. et al., 2008a), although there may be some disagreement on the role of e.g. immigrant status, low parental education, household income and maternal teenage pregnancy on the risk for PES (Berntsson L. T. et al., 2000; Gustafsson P. E. et al., 2009; Elberling H. et al., 2010; Benjet C. et al., 2010).

Concerning the effects of parental divorce and a non-traditional family structure on PES, both stressors increased the risk for all PES (except difficulties falling asleep), although low self-esteem was not affected by parental divorce. It is thus likely that self-esteem is more affected by the 'chronic', continuing change of family structure than by the event of parental divorce itself.

In general, the more consistent or stronger effect of certain specific types of adversities on PES may be due to their higher stressfulness, to their more chronic character, or to their larger impact on behaviour or feelings of self-worth and safety, as previously stated by Benjet et al. (Benjet C. et al., 2010).

A final remark on the independent effects of each adversity on the occurrence of PES is that they should be interpreted in the context of the interrelatedness and clustering of events and adversities (Benjet C. et al., 2010; Schilling E. A. et al., 2008), and by realizing that the occurrence of PES may not be determined by the sole, pure effects of each separate adversity. Instead, all events and adversities together shape the child's living conditions and may contribute to PES as a whole.

Despite the observed relationship between PES and childhood adversity, this study identified children experiencing adversities without exhibiting any PES (i.e. 11.8% of children with ≥4 FSAs), which may be due to the fact that children perceive, evaluate and cope with these adversities in different ways. In short, childhood adversity clearly increases the risk for PES in children but other factors such as coping styles and social support could be involved in this complex relationship (Folkman S. et al., 1986).

4.3 Strengths and limitations

The strength of this study is its large, international sample comprising 8 European countries, allowing studying childhood adversities and PES in a larger context than has previously been done. In addition, the fieldwork in the survey centres was performed at the same time using the same standardized protocol. Nevertheless, there were some specific methodological issues. First, the dichotomous nature of the variables may not consider the complexity of certain events (e.g. family structure). Moreover, this study only assessed a limited number of adversities and psychosomatic and emotional outcomes, which were exclusively parent-reported and did not take into account children's perspectives. Unfortunately, we could not examine the severity of the adversities as the IDEFICS Parental Questionnaire did not allow to study this objective, although Schilling et al. have advised to consider the stressor severity together with the number of adversities in studying cumulative childhood adversity (Schilling E. A. et al., 2008). Also, a selection or non-participation bias related to education or income-level, as well as a response bias cannot be ruled out and may thus have influenced prevalence results in both directions (Ahrens W. et al., 2011a). Finally, this study did not allow investigating causality or directionality in the relationship between adversities and PES.

5 Conclusions

This study described the prevalence of PES in children younger than 12 years old in 8 European countries. We indicated the significance and impact of both quantitative (i.e. the number of adversities) and qualitative (i.e. the type of adversities) effects of negative life events and the child's family and social environment on the occurrence of PES in this cross-national sample of young children. More specifically, an increasing number of adversities gradually amplified the risk for PES. Moreover, children living in a non-traditional family structure or a bad family climate and children experiencing peer problems or major frustrations at school, were more likely to go through PES. These findings emphasize the importance of the child's everyday familial and social environment on its (mental) well-being.

CHAPTER 3.3 CORTISOL AND CORTISONE IN HAIR OF ELEMENTARY SCHOOL GIRLS AND ITS RELATIONSHIP WITH CHILDHOOD STRESS

Abstract

Children may be exposed to stressful situations with adverse effects on their physiological and psychological health. As cortisol and cortisone may be useful additional biomarkers for stress research and as they have been shown to be detectable in human hair, this study measured physiological concentrations of hair cortisol and cortisone in 223 elementary school girls, and explored the relationship with child-reported estimates of stress, more specifically questionnaires on major life events (i.e., Coddington Life Events Scale for Children), emotions (i.e., anger, anxiety, sadness and happiness) and coping strategies (i.e., emotion- versus problem-focused coping). Cortisone concentrations were positively correlated with the overall life event score for the past 6 months (rho=0.223, p=0.004), as well as with the negative event score for this period (rho=0.227, p=0.003) (N=165). Cortisone was not correlated with emotions or coping styles reported by the children. Neither cortisol nor the cortisol/cortisone ratio was associated with child-reported stress estimates. Despite its exploratory nature, this study may suggest elevated hair cortisone concentrations under psychosocial stress in young children. Although the observed findings should be interpreted with prudence, this study may encourage further research elucidating the potential importance and relevance of hair cortisone analysis as an additional or substituting stress biomarker for hair cortisol.

1 Introduction

Childhood stress has been studied extensively over the past years. Chronic exposure to stressful situations in the school, the family and the interpersonal environment may adversely affect a child's physiological and psychological health which may potentially persist into adolescence and adulthood (Danese A. et al., 2012), highlighting the need for valid and reliable stress assessment methods.

Laboratory analysis of cortisol, the primary glucocorticoid in humans, has been widely performed in childhood stress research as it represents the activation of the physiological stress system, i.e. activation of the hypothalamus-pituitary-adrenal axis which results in the release of cortisol by the adrenals (Vanaelst B. et al., 2012b). Cortisone on the other hand mainly origins from cortisol metabolism by enzymatic activity of 11β-hydroxysteroid dehydrogenase, and may be a useful additional biomarker for stress research (Plenis A. et al., 2011), although in this regard many details are still unclear. Particularly for the hair matrix, research on cortisone is completely lacking, although it has been detected in human hair about a decade ago (Raul J. S. et al., 2004).

Therefore, this exploratory study determined physiological concentrations of cortisol and cortisone in the hair of elementary school girls and investigated its relationship with child-reported estimates of stress.

2 Methods

2.1 Study participants

Participants were 223 elementary school girls participating in the ChiBS project (Children's Body composition and Stress) (Michels N. et al., 2012d). Hair samples were collected at the same time point as subjective stress questionnaires for each participant between February and June 2010 at the children's schools and were exclusively taken from the girls to maximize the probability that the hair reached the required length of 6 cm (representing a period of 6 months prior to sampling). No illnesses or cortisol/cortisone affecting medication was reported. Cortisol and cortisone concentrations were measurable in hair samples of respectively 39 and 168 girls. No differences were observed in child-reported stress between the groups of children with measurable versus not-measurable cortisol or cortisone concentrations (Mann-Whitney U test). The study was conducted according to the guidelines of the Declaration of Helsinki and the project protocol was approved by the Ethics Committee of the Ghent University Hospital. More detailed research goals and methodology of the ChiBS project are described elsewhere (Michels N. et al., 2012d).

2.2 Hair cortisol and cortisone analysis

Hair samples, obtained from the vertex posterior region of the scalp, were cut as close to the scalp as possible. External contamination of the hair shaft by hand cream and lotion used by the sampling investigators, and artificial colouring of the child's hair were ruled out. The samples were analysed in the Department of Toxicology, Institute of Legal Medicine, at the University of Strasbourg. In short, the most proximal 6 cm of the hair samples was washed, dried and finely minced. Cortisol and cortisone were extracted from 50 mg minced hair by incubation of methanol for 24 h in the presence of cortisol-d_4 as an internal standard. The incubation medium was separated by centrifugation, after which the following steps were repeated two more times: methanol was added to the minced hair, vortexed and centrifuged. Next, the sample was evaporated to dryness, resuspended with a 50% acetonitrile - 50% of 0.1% formic acid solution and injected into a 2,1 x 100 mm 1,7 µm ACQUITY UPLC BEH analytical column (Waters, Milford, MA, USA). The chromatographic separation was operated on ACQUITY UPLC Waters system, with 0.1% formic acid solution as mobile phase A and acetonitrile as mobile phase B. Detection and quantitative analysis were performed on a tandem quadrupole mass-spectrometer model Quattro Premier XE (Waters, Milford, MA, USA) in positive ionization mode and by using Mass Lynx software (version 4.1). The limits of detection (LOD) and quantification (LOQ) were 2 and 5 pg/mg respectively. Detailed methodology and validation data are described elsewhere (Vanaelst B. et al., 2013e).

2.3 Questionnaires for children

Estimates of child-reported stress were obtained through questionnaires not only on stressors or life events, but also on emotions and coping strategies, as these have been shown to determine or evoke specific biological outcomes (Vanaelst B. et al., 2012b). Completion of the questionnaires was assisted by trained interviewers.

A. Coddington Life Events Scale (CLES)

The Coddington Life Events Scale (CLES) for children is a validated and well-established 36-item questionnaire which measures the frequency and timing of life events relevant for this age group during the past year and results in a 'life change units' score per trimester and for

the time periods of 0-3, 0-6, 0-9 and 0-12 months ago (Coddington R. D., 1972). In this study, only the scores for the periods 0-3, 4-6 and 0-6 months ago were studied in order to correspond with the 6-cm hair sample. Children with a score above the age-specific cut-off (based on 75th percentiles) are at higher risk to suffer from psychological problems.

B. Basic emotions and coping styles

As mood is an essential component of the stress system (Vanaelst B. et al., 2012b), children were asked to report on their recent (described as "lately") feelings of anger, anxiety, sadness and happiness on a 0 to 10 multipoint Likert scale (0 'not at all' to 10 'very strong') in the context of affective responsiveness analogous to a study of Zimmer-Gembeck (Zimmer-Gembeck MJ. et al., 2009). To help the children to understand these distinct feelings, graphical representations of cartoon-type faces from a social skills training game for very young children were displayed next to the question.

Also, the children were asked to report how they usually deal with problems or with being upset in an eight-item questionnaire (previously used and translated to Dutch in the CASE-study (Child and Adolescent Self-harm in Europe study) (Madge N. et al., 2008)) with 'never', 'sometimes' and 'often' as response alternatives. The questionnaire allows classification of the answers into emotion- versus problem-focused coping, based on the transactional model of Folkman et al. (Folkman S. et al., 1986).

2.4 Other variables

Information on the child's age, ethnicity, anthropometry, illnesses and long-term medication use as well as information on the parental education level (International Standard Classification of Education- ISCED (UNESCO, 1997)) were collected in the ChiBS project (Michels N. et al., 2012d).

2.5 Statistical analysis

Statistical analyses were performed with PASW Statistical Program version 19.0.0 (SPSS Inc., IBM, IL, USA). Correlation analyses were performed using Spearman rank correlations, comparison between groups for a continuous variable by Mann-Whitney U-tests. Since neither age, body composition, sociodemographic parameters nor hair colour was associated

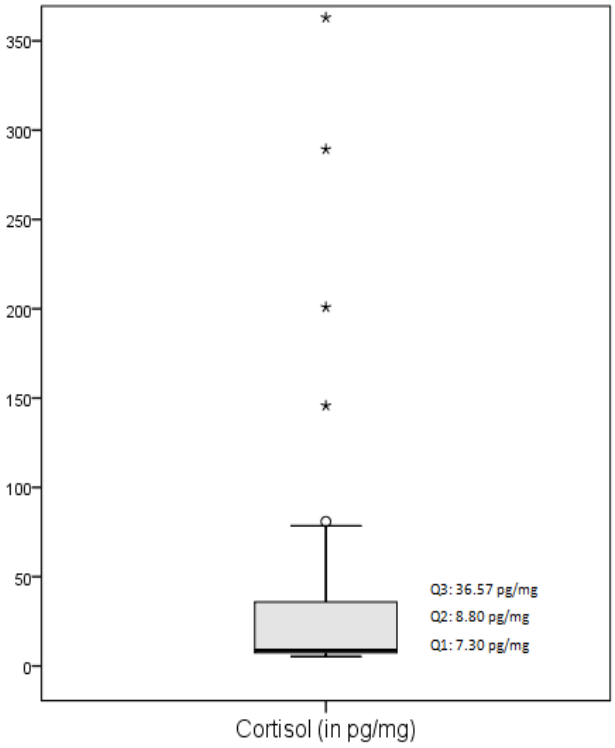
with the hormone concentrations (data not shown), analyses on the relationship between hair hormones and stress questionnaires were not adjusted for these variables. To correct for multiple testing, a Bonferroni correction was applied. P-values ≤0.004 were considered statistically significant.

3 Results

3.1 Population characteristics

The population mainly consists of brown-haired (70.9%), normal weight (74.4%) Caucasian girls (98.2%) between 5 and 10 years old (mean=8.4, SD=1.1) with moderately to highly educated parents (97.1% for ISCED level 3-5) (N=223). Of the girls, 35.4% and 17.5% were identified as being at risk for psychological sequelae by the occurrence of stressful life events the past 3 and 6 months respectively, as assessed by the CLES questionnaire.

The minimum, maximum, median and interquartile concentrations for hair cortisol and cortisone are presented in Figure 3.4.



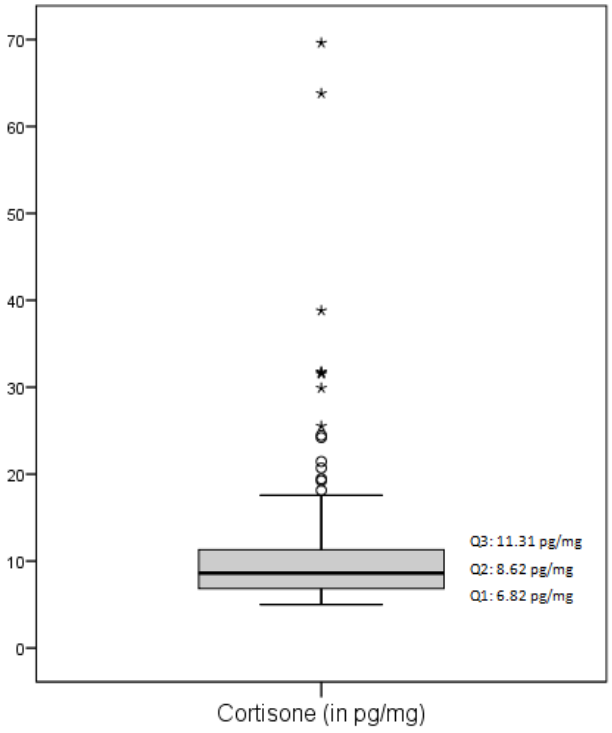


Figure 3.4 Hair cortisol (N=39) and cortisone concentrations (N=168). Cortisol concentrations ranged from 5 to 1330 pg/mg (SE of the mean=35.29). Cortisone concentrations ranged from 5 to 69.6 pg/mg (SE of the mean=0.64). Q1: first quartile, Q2: median, Q3: third quartile. Degree sign and asterix indicate values higher than Q3+1.5(Q3-

Q1) or Q3+3(Q3-Q1) respectively. From the 223 hair samples, 66% (N=147) of the samples and 16% (N=37) of the samples had cortisol concentrations below the LOD or between the LOD and LOQ, respectively. For cortisone, these percentages were 7.6% (N=17) and 17% (N=38). The highest cortisol value (1330 pg/mg) has been excluded from the figure.

3.2 Associations between concentrations of cortisol, cortisone and cortisol/cortisone ratio and questionnaires

Cortisone concentrations were positively associated with the CLES total event score for the past 6 months, as well as with the negative event score for this period (N=165), as presented in Table 3.8. Cortisone was not correlated with emotions or coping styles reported by the children (Table 3.8). Children identified as being at risk for psychological problems by events occurring in the past 3 or past 6 months had no significantly higher cortisone concentrations (p=0.013 and p=0.087 respectively). Neither cortisol, nor the cortisol/cortisone ratio were associated with the child-reported stress estimates (Table 3.8).

Table 3.8 Correlation between concentrations of cortisol, cortisone and the cortisol/cortisone ratio and estimates of child-reported stress

	cortisone		**cortisol**		**cortisol/cortisone ratio**	
	N=165		N=39		N=31	
	spearman's rho	**p**	**spearman's rho**	**p**	**spearman's rho**	**p**
Coddington Life Events Scale						
total event score, months 0-3	0.183	0.019	0.106	0.526	0.185	0.318
total event score, months 4-6	0.09	0.251	0.201	0.219	0.131	0.483
total event score, months 0-6	**0.223**	**0.004**	0.188	0.251	0.211	0.254
negative event score, months 0-3	0.197	0.011	0.103	0.534	0.234	0.205
negative event score, months 4-6	0.082	0.293	0.299	0.065	0.202	0.276
negative event score, months 0-6	**0.227**	**0.003**	0.208	0.204	0.258	0.161
Basic emotions (score 0-10)						
angry	-0.067	0.393	-0.008	0.961	0.036	0.849
anxious	0.055	0.484	-0.184	0.263	-0.029	0.875
sad	-0.022	0.779	-0.27	0.096	-0.172	0.356
happy	-0.008	0.919	0.279	0.085	0.392	0.029
Coping style (score 0-10)						
emotion-based coping score	-0.014	0.858	0.269	0.097	0.154	0.408
problem-based coping score	0.006	0.939	0.064	0.7	0.005	0.977

CLES: Coddington Life Events Scale, p: p-value

4 Discussion

This study was the first to simultaneously measure physiological concentrations of hair cortisol and cortisone in healthy young girls and to study its association with childhood stress.

4.1 Hair cortisol and cortisone concentrations

Cortisol was low or undetectable in the majority of the hair samples. This is to our opinion due to the length of the analysed hair samples and the consequent segmental decline; an issue which is further discussed in detail in *Chapter 4.2 Methodological considerations* of this thesis.

While much is known for hair cortisol in relation to intra-individual or sociodemographic characteristics, hair cortisone research has remained unexplored (except for one study of Raul et al. (Raul J. S. et al., 2004)), limiting the evaluation of our observations to previously reported findings. Therefore, we further refer to hair cortisol research. We did not observe a relationship between cortisol or cortisone concentrations and hair colour or body composition. The latter is in contrast to previous hair cortisol findings in adults (Manenschijn L. et al., 2011a; Manenschijn L. et al., 2011b; Stalder T. et al., 2012b), which may either result from not-manifest body composition differences in our young study population or from a general non-relationship between hair cortisone and body composition; however, this has not been studied before. The non-relationship with hair colour is in line with previous hair cortisone and cortisol research (Dettenborn L. et al., 2012b; Raul J. S. et al., 2004). The observed cortisone concentrations were lower than previously reported findings of Raul et al. (i.e. range 12-163 pg/mg, mean=70 pg/mg, N=44, ages 2-90 years old) (Raul J. S. et al., 2004). As no further data on hair cortisone is available, comparison and interpretation of our observations to previous research is restricted.

4.2 Associations between concentrations of cortisol, cortisone and cortisol/cortisone ratio and questionnaires

No association was observed between hair cortisol (or the cortisol/cortisone ratio) and child-reported stress estimates. This is in contrast to literature indicating a general increase in hair cortisol under stressful situations (Pereg D. et al., 2011; Steudte S. et al., 2011a; Luo H. et al.,

2012; Karlen J. et al., 2011; Dettenborn L. et al., 2010; Manenschijn L. et al., 2011b; O'Brien K. M. et al., 2012; Skoluda N. et al., 2012; Grassi-Oliveira R. et al., 2012), although not all studies have been able to demonstrate this association using stress-related questionnaires (Skoluda N. et al., 2012; Stalder T. et al., 2012c; Stalder T. et al., 2012b; van Holland B. J. et al., 2012; O'Brien K. M. et al., 2012). However, based on the low sample size for cortisol data in this study, no statements on the association between hair cortisol and childhood stress should be made. Future studies should therefore pursuit a minimal sample size of 123 quantifiable hair samples for correlational analyses with an effect size of $\rho=0.250$, $\alpha=0.05$ and a power of 0.80 (G*Power 3.1, according to Faul et al. (Faul F. et al., 2009)).

As this study was the first to examine the relationship between hair cortisone and stress variables, no prior hypothesis was formulated regarding the direction of hair cortisone levels under stress. We have shown positive associations between hair cortisone and stressful events over the past 6 months, although the demonstrated correlations were rather weak. Despite its exploratory nature, this study may thus suggest elevated hair cortisone concentrations under psychosocial stress in young children, which is in line with previous reports on hair cortisol and stress (see above). Although the observed findings should be interpreted with prudence, this study may encourage further research elucidating the potential importance and relevance of hair cortisone analysis as an additional or substituting stress biomarker for hair cortisol.

No association was observed between hair cortisone and emotions or coping style, although this psychological responsivity is generally assumed to be the linking bridge between the occurrence of stressors and the biological stress response. While psychological and biological stress responses are theoretically strongly interconnected through the human stress system, intercorrelations between both have yielded conflicting results, a finding which has been denominated ‘lack of psycho-endocrine covariance’ (Vanaelst B. et al., 2012b).

A major strength of this study is the novelty of the hair cortisone measurements. Nevertheless, some specific methodological issues should be considered. Given the exclusively female population under study, confirmation of our findings is needed in a more heterogeneous population sample (i.e., boys and girls, childhood to adolescence). We did not collect information on pubertal stage (the influence of thelarche or menarche on hair cortisone concentrations can thus not be excluded), nor were hair washing frequencies recorded. Last, we did not divide the full 6-cm segment into fragments of 1 or 3 cm, which would have

allowed to study wash-out effects of cortisone and to study the relationship with the child-reported estimates of stress for each 3-month period separately.

In conclusion, this study suggests elevated hair cortisone concentrations under childhood stress over the past 6 months. However, to fully understand the potential importance of hair cortisone analysis as an additional or substituting tool for large-scale psychosocial stress research, further research should be undertaken in larger and more heterogeneous populations, and into more fundamental aspects such as the influence of intra-individual and sociodemographic characteristics and the influence of wash-out effects and intersegment loss on hair cortisone concentrations.

CHAPTER 3.4 INTERCORRELATIONS BETWEEN SERUM, SALIVARY AND HAIR CORTISOL AND CHILD-REPORTED ESTIMATES OF STRESS

Abstract

To evaluate the impact of stress on children's well-being, it is important to have valid and reliable stress assessment methods. Nevertheless, selection of an appropriate method for a particular research question may not be straightforward, as there is currently no consensus on a reference method to measure stress in children. This paper examined to what extent childhood stress can be estimated accurately by stressor questionnaires (i.e. Coddington Life Events Scale) and biological markers (serum, salivary and hair cortisol), using the Triads (a triangulation) method in 272 elementary school girls. Salivary cortisol was shown to most accurately indicate true childhood stress for short periods in the past (i.e. last three months), whereas hair cortisol may be preferred above salivary measurements for periods more distant and thus for chronic stress assessment. However, applicability should be confirmed in larger and more heterogeneous populations.

1 Introduction

Childhood stress and its effects on children's physical and psychological well-being have been studied extensively over the last years. In particular adverse events with a chronic or cumulative character may strongly affect children's health, with effects potentially persisting into adolescence and adulthood (Schneiderman N. et al., 2005; Teicher M. H. et al., 2003; Schilling E. A. et al., 2007). The combined increase in the prevalence of childhood stress with the prevalence of psychosomatic complaints (Alfven G. et al., 2008; Hesketh T. et al., 2010), obesity (Gundersen C. et al., 2011) and behavioural or mental health problems in children is therefore of special concern (Grant K. E. et al., 2004; Schilling E. A. et al., 2007; Timmermans M. et al., 2010; Vanaelst B. et al., 2012a).

To evaluate the impact of stress on children's well-being, it is important to have valid and reliable stress assessment methods that can be easily implemented in large-scale epidemiological studies. In general, stressor questionnaires and laboratory measurements of

cortisol have been widely performed in childhood epidemiological research, though both approaches measure distinct aspects of the stress response (Vanaelst B. et al., 2012b; Cohen S. et al., 1997a). Stressor questionnaires assess the occurrence of stressful events during a predefined time period, whereas laboratory cortisol measurements in biological samples (e.g. blood, saliva, hair) represent the activation of the physiological stress system provoked by stressor exposure: activation of the hypothalamus-pituitary-adrenal axis, a main pathway of the body's stress system, results in the release of cortisol by the adrenal glands (Figure 3.5). As shown in Figure 3.5, the different biological samples for cortisol measurement reflect cortisol levels of a different timeframe (e.g. serum cortisol for acute stress, salivary cortisol and hair cortisol for longer-term to chronic stress). A more detailed overview of epidemiological approaches to measure (chronic) childhood stress is described elsewhere (Vanaelst B. et al., 2012b).

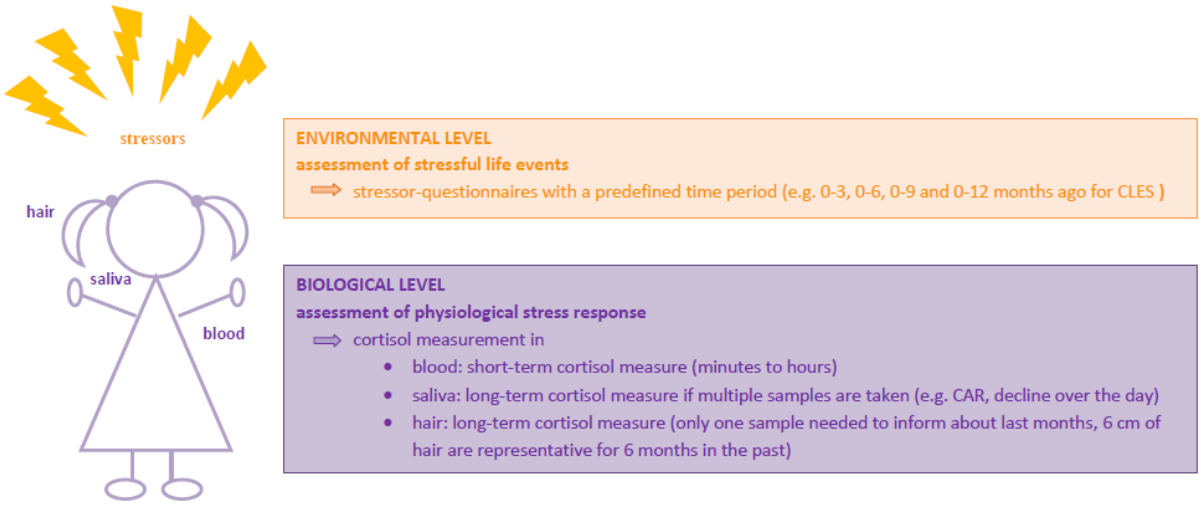


Figure 3.5 Overview of common childhood stress assessment methods at environmental versus biological level. CLES: Coddington Life Events Scale; CAR: cortisol awakening response

As questionnaires and cortisol measurements reflect partially different information, correlations between (1) questionnaires and cortisol measurements and (2) cortisol intercorrelations in different biological samples have often been contradictory (Schlotz W. et al., 2008; Oldehinkel A. J. et al., 2011; Hellhammer D. H. et al., 2009; Vanaelst B. et al., 2012b). Nevertheless, the correlation between salivary and free serum cortisol, both biomarkers of short-term measurement and both representing diurnal cortisol fluctuations, is well supported in literature (Tunn S. et al., 1992; Levine A. et al., 2007; Poll E. M. et al., 2007), also in children (Chou I. C. et al., 2011; Delcorral P. et al., 1994). Hair cortisol, a

measure of long-term cortisol production, has not been shown to correlate well with serum cortisol, but it was observed to be associated with the salivary cortisol awakening response (CAR) or average salivary cortisol (D'Anna-Hernandez K. L. et al., 2011; van Holland B. J. et al., 2012; Xie Q. Z. et al., 2011).

While salivary cortisol has repeatedly been measured in children in relation to childhood stress or behavioural problems (Jessop D. S. et al., 2008; Maldonado E. F. et al., 2008; Lupien S. J. et al., 2001; Smeekens S. et al., 2007; Wolf J. M. et al., 2008; Hatzinger M. et al., 2007; Gustafsson P. E. et al., 2010; Gustafsson P. E. et al., 2006; Gunnar M. R. et al., 2003; Gunnar M. R. et al., 2009b; Ruttle P. L. et al., 2011), hair cortisol analysis as marker of chronic stress is relatively new (Russell E. et al., 2012; Steudte S. et al., 2011b; Steudte S. et al., 2011a; Karlen J. et al., 2011; Manenschijn L. et al., 2011b; Dettenborn L. et al., 2010).

Despite their specific characteristics as regards cost-effectiveness, logistics, invasiveness, bias etc. (Vanaelst B. et al., 2012b), questionnaires and cortisol measurements have both been shown to be valid indicators of childhood stress. Nevertheless, selection of an appropriate stress assessment method for a particular research question may not be straightforward and should be well-considered, as there is currently no consensus on a reference method to measure stress in children. Therefore, this paper will first investigate cortisol intercorrelations in different biological samples (i.e. serum, saliva and hair) and secondly examine to what extent childhood stress can be estimated accurately by stressor questionnaires and biological markers in elementary school girls, using the Triads method.

The Triads method has particularly been applied in dietary validation studies and was developed to get a valid estimation of a true, unknown exposure if a gold standard method is lacking (Kaaks R. J., 1997; Yokota R. T. D. et al., 2010). As the Triads method may also be of value for other research fields, this study expanded its application to stress research. More specifically, through the calculation of ‘validity coefficients’, this study examines which stress assessment method may most accurately indicate true childhood stress by comparing questionnaires and biological markers triangularly.

2 Methods

2.1 Study participants

The ChiBS project (Children's Body composition and Stress) was designed at Ghent University and investigates the relationship between chronic psychosocial stress and changes in body composition in young children (5-11 years old) living in Aalter (a city in Flanders, Belgium), over a two-year follow-up period (2010-2012) (Michels N. et al., 2012d). The ChiBS project offered the opportunity to study the feasibility and interrelationships of different stress assessment methods in children. Parents were asked to sign a consent form in which the option was offered to participate in the full ChiBS programme or in a selected set of measurement modules, resulting in distinct participation numbers for the different measurement modules, as presented in Figure 3.6.

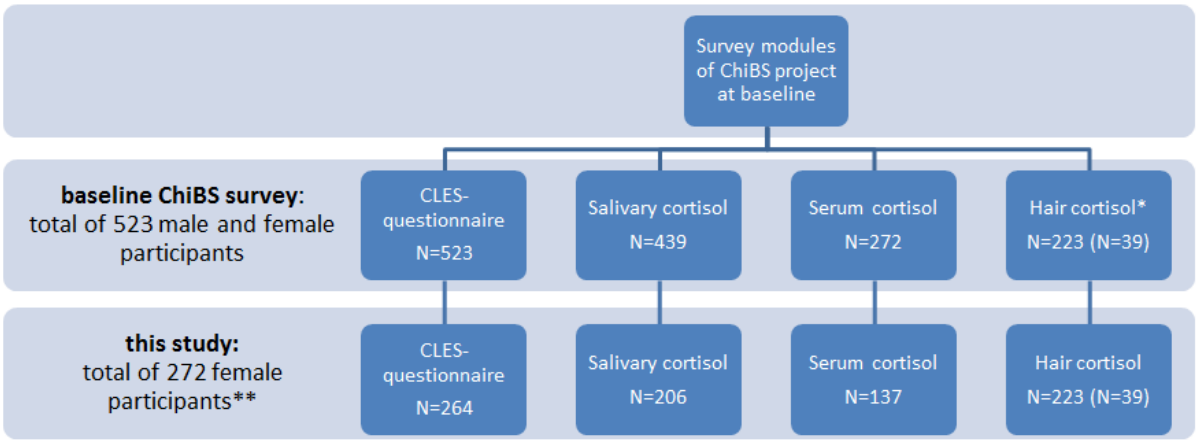


*Figure 3.6 Participation numbers for the different measurement modules in the baseline ChiBS survey and the present study. CLES: Coddington Life Events Scale; * The limit of detection for laboratory cortisol quantification was only reached for 39 hair samples; ** In total 272 girls participated to at least one survey module*

In total, 523 healthy children participated to the 2010 baseline survey of the ChiBS project. Analyses in this study were however limited to the female participants of the ChiBS project as one of the survey modules, more specifically hair sampling, was only performed in girls (N=272/523 or 52%; M age= 8.37 years, SD=1.19) (Figure 3.6). No differences were found between boys and girls for age, BMI, parental education, family structure or migrant status. Detailed socio-demographic information of the ChiBS study population is described elsewhere (Michels N. et al., 2012d). The ChiBS project was conducted according to the

guidelines laid down in the Declaration of Helsinki and was approved by the Ethics Committee of the Ghent University Hospital.

2.2 Instruments

A. Coddington Life Events Scale for children (CLES-C)

Child-reported estimates of stress were collected with the Coddington Life Events Scale for children (CLES-C) of which completion was assisted by a trained researcher. The CLES questionnaires is a validated questionnaire on the occurrence of stressors or life events (Coddington R. D., 1972). It measures the frequency and timing of 36 positive and negative life events relevant for this age group during the last year (four trimesters) and results in a 'life change units' score per trimester and for the time periods of 0-3, 0-6, 0-9 and 0-12 months ago. Apart from the total event score, also a score for only negative life events was calculated.

B. Serum, salivary and hair cortisol

To cover short- and long-term stress exposure, three different biological samples were collected for cortisol analyses, namely serum, saliva and hair samples. Detailed information on the strategies for sample collection and cortisol analyses were previously described (Michels N. et al., 2012d; Michels N. et al., 2012b; Vanaelst B. et al., 2013a).

In short, salivary samples were collected with Salivette swaps during two consecutive weekdays at four time points, i.e. immediately at awakening (T0), 30 minutes after waking-up (T30), 60 minutes after waking-up (T60) and in the evening between 7 and 8 PM (Tev). Salivary area under the curve (AUC) cortisol was calculated on the basis of the morning samples as the total area under the curve between T0 and T60 and cortisol decline over the day as (Tev-T0) divided by the number of hours, taking into account the salivary samples of the two days (mean value). Blood samples were obtained after an overnight fasting period through venepuncture. Hair samples with a diameter of approximately five mm were cut from the vertex posterior region of the scalp. Only the most proximal six cm were used for cortisol analyses. Hair samples were only taken from girls to maximize the probability that the hair reached the required length of six cm. Cortisol was analysed by electrochemoluminescence

immunoassays for serum and salivary samples (nmol/L), and by liquid chromatography-tandem mass-spectrometry for hair samples (pg/mg).

2.3 Statistical procedures

Statistical analyses were performed with PASW Statistics Program version 19.0.0 (SPSS Inc., IBM, IL, USA) and SAS version 9.3 (SAS Institute Inc., USA) for bootstrapping analyses. P-values <0.05 were considered statistically significant for all tests. Because of the specific formulation of the informed consent, different participation numbers for each measurement module were obtained (Figure 3.6). To maximize the sample size and power of calculations, analyses were performed on the largest sample size possible and not restricted to the subsample of children from whom data on all measurement modules was available (as N would only be 19 for this latter approach). The cortisol concentrations in serum, saliva and hair and the CLES scores are presented by their median and interquartile range, as these are not normally distributed (Kolmogorov-Smirnov, Shapiro-Wilk). For all variables Z-scores were calculated to improve the linearity of the distribution. These z-scores were used for all tests.

A. Cortisol intercorrelations in different biological samples

The correlations and agreement between serum, salivary and hair cortisol concentrations in corresponding samples were investigated using Spearman rank correlations and Bland-Altman analyses. Bland-Altman plots were created to graphically present the agreement or comparability between the quantitative measurements of cortisol in (1) hair versus saliva (AUC), (2) hair versus serum, and (3) serum versus saliva (AUC). In these charts, the difference of the paired two measurements is plotted on the vertical axis, against the mean of the two measurements on the horizontal axis. Three reference lines are superimposed on the plot (i.e. the upper limit of agreement (mean+2SD), the average difference between the measurements (mean) and the lower limit of agreement (mean-2SD)). If the two methods are comparable, the mean of the differences will be close to zero. Bland-Altman plots were not created for salivary decline, as no correlation for this variable with hair or serum cortisol was observed (Altman D. G. et al., 1983; Bland J. M. et al., 2010).

B. Correlations between cortisol measurements and child-reported estimates of stress: Triad analyses

This study applied the Triads technique (a triangulation method) to examine which stress assessment method may most accurately indicate true childhood stress. The idea is that, although it is impossible to measure true childhood stress directly, it can be estimated by stressor questionnaires (CLES scores) and biological markers such as salivary and hair cortisol (Michels N. et al., 2012c; Vanaelst B. et al., 2012b). These are therefore the three variables being compared in this triangulation technique, as presented in Figure 3.7.

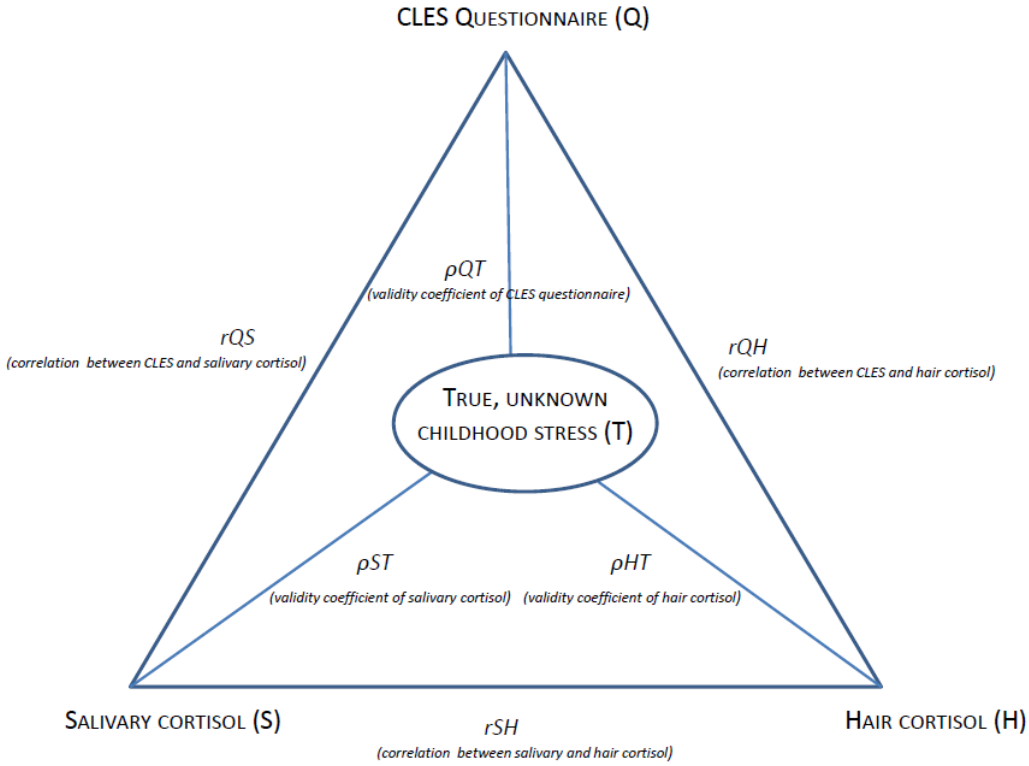


Figure 3.7 Schematic representation of the Triads method comparing the CLES-questionnaire, salivary and hair cortisol triangularly with the true, but unknown childhood stress. Figure adapted from Yokota et al. (Yokota R. T. D. et al., 2010)

The aim of the Triads method is to obtain validity coefficients (ρ) which estimate the correlation between each measurement method and the subject's true but unknown stress, with higher values indicating a better approximation of true exposure (range 0-1) (Kaaks R. J., 1997; Yokota R. T. D. et al., 2010). Serum cortisol was not included in the Triad analysis, because of the absence of a relationship with the CLES scores (data not shown) .

The Triad approach assumes that (1) correlations between the three measurements are explained entirely by the fact that all are linearly related to true stress, and (2) that their random measurement errors are mutually independent (Ocke M. C. et al., 1997; Kaaks R. J., 1997). In a first step, pair-wise Spearman's correlation coefficients (r) are calculated between each of the measurement methods. These correlation coefficients are then used to calculate validity coefficients (ρ) using the formulae

$$\rho QT = \sqrt{\frac{rQS * rQH}{rSH}}$$

$$\rho HT = \sqrt{\frac{rQH * rSH}{rQS}}$$

$$\rho ST = \sqrt{\frac{rSH * rQS}{rQH}}$$

in which Q stands for questionnaire, S for salivary cortisol, H for hair cortisol and T for true childhood stress; and where ρQT, ρHT and ρST are the validity coefficients of respectively the stressor questionnaire, hair cortisol and salivary cortisol in relation to true stress; and rQS, rQH and rSH are the correlation coefficients between respectively the questionnaire and salivary cortisol, the questionnaire and hair cortisol and between salivary and hair cortisol (Figure 3.7). Validity coefficients are always equal to or greater than the sample correlations between that type of measurement and the other two: if all three sample correlations are high, the measurements are expected to have validity coefficients close to 1; likewise, low correlations are expected to result in lower validity coefficients. Validity coefficients higher than 1 are known as 'Heywood cases' and can emerge when the product of two of the three samples correlations is larger than the third and can be explained by two factors: (1) random sampling fluctuations in the observed correlations between measurements, in which case validity coefficients higher than 1 are acceptable; (2) violation of one or more of the model assumptions, in which case the estimated validity coefficients are biased (Ocke M. C. et al., 1997).

The Triads method was applied for four models. In the first model salivary AUC cortisol, hair cortisol and CLES negative event scores were studied as measurement methods. The second model was similar to the first, except for salivary cortisol diurnal decline as new salivary measure. These two models were then repeated after replacing the CLES negative event scores by the CLES total event scores, representing the 3th and 4th model respectively. These

four models were performed for four different time periods in the past (0-3, 0-6, 0-9 and 0-12 months ago, respectively), thus resulting in a total of 16 models that were studied.

For all models, 95% confidence intervals (95% CI) were calculated as the 2.5^{th}- 97.5^{th} percentile for the replicates of estimated validity coefficients from 1000 bootstrap samples of equal size (N=272) (nonparametric bootstrap method). For a number of bootstrap samples validity coefficients could not be estimated because of negative sample correlations coefficients, leading to 95% CI based on less than 1000 samples. When the estimated validity coefficients were higher than 1 (Heywood cases), their value was set to 1 in order to keep the 95% CI within the theoretical range of [0-1] (Ocke M. C. et al., 1997; Kabagambe E. K. et al., 2001; Bhakta D. et al., 2005; Andersen L. F. et al., 2005).

3 Results

3.1 Population characteristics

Table 3.9 presents socio-demographic data of the participating girls and the number of girls included for each measurement module of this study, as well as the serum, salivary and hair cortisol concentrations and the CLES-scores.

3.2 Cortisol intercorrelations in different biological samples

As presented in Table 3.10, serum (free and total) cortisol were positively correlated with salivary cortisol measures. Hair cortisol was correlated with salivary cortisol but showed no correlation with serum cortisol (N=19, data not shown). Neither serum, nor hair cortisol was correlated with the salivary cortisol decline (data not shown).

The Bland-Altman plots in Figure 3.8 present the agreement between serum cortisol, hair cortisol and salivary AUC measurements.

The Bland-Altman plot for hair and salivary measurements (Figure 3.8 top) demonstrated that cortisol concentrations in hair are lower compared to salivary analyses (reference line of the average difference is lower than 0, i.e. -0.38). More specifically, the divergent pattern indicates that the higher the salivary cortisol concentrations, the higher the difference between cortisol in hair versus salivary samples (larger horizontal scattering). Similar findings were

observed in Figure 3.8 - middle representing hair versus serum cortisol concentrations. Figure 3.8 - down illustrates that cortisol concentrations from serum and salivary samples do not agree well on the individual level (large upper and lower limits of agreement, i.e. 2.61 and -2.77), although on the population level these sample types give similar information on the mean cortisol concentration (reference line of the average difference close to 0 and majority of data points nicely located within upper and lower limit of agreement). The disagreement between saliva and serum increases with an increasing mean cortisol.

Table 3.9 Information on socio-demographics and stress measurements in the studied girls (N=272)

	N	%				
Socio-demographic information						
parental education (missing n=12)						
ISCED 1	3	1.2				
ISCED 2	4	1.5				
ISCED 3	74	28.5				
ISCED 4	49	18.8				
ISCED 5	130	50				
age child						
5	5	1.9				
6	29	10.7				
7	67	24.6				
8	77	28.3				
9	68	25				
10	24	8.8				
11	2	0.7				
BMI category of child (COLE) (missing n=1)						
underweight	36	13.3				
normal	208	76.8				
overweight	19	7				
obese	8	2.9				
Stress measurements	**valid N***	**Min**	**Max**	**P25**	**Median**	**P75**
cortisol analyses						
serum total cortisol (nmol/L)	137	105.38	801.65	194.62	257.1	337.51
serum free cortisol (nmol/L)	136	3.01	40.55	5.36	7.61	10.3
salivary cortisol: AUC (nmol/L)	206	6.67	102.62	19.97	22.59	28.93
salivary cortisol: decline (nmol/L)	189	-27.70	-0.19	-1.04	-0.81	-0.63
hair cortisol (pg/mg)	39**	5.34	1330.48	7.30	8.80	36.58
CLES questionnaire						
total event score last 3 months	264	0	281	0	28	61.75
total event score last 6 months	264	0	451	0	39	74.75
total event score last 9 months	264	0	479	17.25	45	88.75
total event score last 12 months	264	0	499	30.25	66	105.75
negative event score last 3 months	264	0	210	0	0	47
negative event score last 6 months	264	0	348	0	21.5	52
negative event score last 9 months	264	0	376	0	28	59
negative event score last 12 months	264	0	396	10	43	75

AUC: area under the curve; CLES: Coddington Life Events Scale; ISCED: International Standard Classification of Education (1 'primary education', 2 'lower secondary education', 3 'upper secondary education', 4 'post-secondary non-tertiary education', 5 'first stage of tertiary education')
* The number of samples/questionnaires valid for analyses is presented. Children could participate to a selected set of measurement modules, resulting in distinct participation numbers. ** The limit of quantification for laboratory cortisol (LOQ= 5 pg/mg) was not reached in the majority of hair samples (N=39/223) (Vanaelst, 2012b)

Table 3.10 Significant cortisol intercorrelations in different biological samples

		N	Spearman's rho	p-value
serum free cortisol	salivary T0 cortisol	122	0.196	0.031
	salivary T30 cortisol	121	0.270	0.003
	salivary T60 cortisol	118	0.274	0.003
	salivary AUC cortisol	114	0.303	0.001
serum total cortisol	salivary T30 cortisol	122	0.272	0.002
	salivary T60 cortisol	118	0.289	0.002
	salivary AUC cortisol	114	0.299	0.001
hair cortisol	salivary T30 cortisol	33	0.398	0.022
	salivary AUC cortisol	32	0.398	0.024

AUC: area under the curve

Figure 3.8

Bland-Altman plots for (free) serum cortisol, salivary (AUC) cortisol and hair cortisol concentrations The horizontal reference lines represent the upper limit of agreement (mean+2SD), the average difference between the measurements (mean) and the lower limit of agreement (mean-2SD), respectively.

3.3 Correlations between cortisol measurements and child-reported estimates of stress: Triad analyses

Table 3.11 presents the correlation and validity coefficients (ρQT, ρHT, ρST) for the different models investigated by the Triads technique, as well as the 95% CI for the estimated validity coefficients. Both models indicated that for elementary school girls, salivary cortisol measurements presented the highest validity coefficient in relation to true (but unknown) stress for a short period in the past (i.e. last 3 months) (ρ=0.74 and ρ=0.63, for salivary AUC and cortisol decline respectively), whereas for periods more distant in the past hair cortisol showed the highest validity coefficients (ρ=0.79, ρ=1, ρ=0.94 and ρ=0.49, ρ=0.55, ρ=0.60 for the last 6, 9 and 12 months in the past for both models respectively). Analyses were repeated with the CLES total event scores and resulted in similar observations (data not shown).

Table 3.11 Triad analyses: Spearman's rank correlation coefficients and validity coefficients for CLES scores, salivary cortisol (AUC and decline) and hair cortisol

	CLES vs. saliva		**CLES vs. hair**		**hair vs. saliva**		**Triad analyses**		
	N	spearman's rho	N	spearman's rho	N	spearman's rho	ρST [95%CI]	ρHT [95%CI]	ρQT [95%CI]
model salivary AUC - hair cortisol - CLES questionnaire									
CLES score negative events last 3 months	202	0.140*	39	0.103	32	0.398*	**0.74** [0.14-1]	0.54 [0.12-1]	0.19 [0.03-0.59]
CLES score negative events last 6 months	202	0.132	39	0.208	32	0.398*	0.50 [0.11-1]	**0.79** [0.19-1]	0.26 [0.05-0.62]
CLES score negative events last 9 months	202	0.099	39	0.259	32	0.398*	0.39 [0.08-1]	**1**** [0.21-1]	0.25 [0.05-0.61]
CLES score negative events last 12 months	202	0.117	39	0.26	32	0.398*	0.42 [0.09-1]	**0.94** [0.22-1]	0.28 [0.06-0.71]
model salivary decline - hair cortisol - CLES questionnaire									
CLES score negative events last 3 months	186	0.188*	39	0.103	32	0.218	**0.63** [0.12-1]	0.35 [0.07-1]	0.30 [0.07-1]
CLES score negative events last 6 months	186	0.191*	39	0.208	32	0.218	0.45 [0.11-1]	**0.49** [0.10-1]	0.43 [0.09-1]
CLES score negative events last 9 months	186	0.186*	39	0.259	32	0.218	0.40 [0.10-1]	**0.55** [0.11-1]	0.47 [0.12-1]
CLES score negative events last 12 months	186	0.157*	39	0.26	32	0.218	0.36 [0.08-1]	**0.60** [0.12-1]	0.43 [0.11-1]

* significant at the p<0.05 level; **Heywood case: original value of 1,02; ρ ST: validity coefficient of salivary cortisol; ρ HT: validity coefficient of hair cortisol; ρ QT: validity coefficient of CLES questionnaire; AUC: area under the curve; CLES: Coddington Life Events Scale

4 Discussion

Although it is quite common to measure childhood stress, this study is to our knowledge the first to compare cortisol measurements in serum, saliva and hair in elementary school girls and to examine their relationship with child-reported stressors in order to identify the most accurate indicator of childhood stress.

4.1 Cortisol intercorrelations in different biological samples

In line with previous research, we have shown a correlation between serum free cortisol and salivary morning cortisol (both short-term measurements, representing diurnal fluctuations and actual cortisol changes) (Tunn S. et al., 1992; Levine A. et al., 2007; Poll E. M. et al., 2007), as well as between serum free cortisol and salivary AUC cortisol (Hellhammer D. H. et al., 2009). Although salivary cortisol reflects the unbound, free cortisol fraction (Levine A. et al., 2007), also correlations with serum total cortisol were observed. This relationship has previously been shown to be of a non-linear nature, depending on the relative saturation of the corticosteroid binding globulin (CBG) protein in blood (Hellhammer D. H. et al., 2009). Our observations also confirmed the previously reported lack of correlation between hair cortisol (long-term measure) with single-point serum or salivary cortisol measurements (Sauve B. et al., 2007; Steudte S. et al., 2011b). However as shown before (D'Anna-Hernandez K. L. et al., 2011; van Holland B. J. et al., 2012), hair cortisol strongly correlated with salivary AUC cortisol which is a longer-term representation of cortisol production/stress (Xie Q. Z. et al., 2011; Chida Y. et al., 2009). However, the Bland-Altman plot did not indicate a good agreement between these two measures, probably due to the small sample size for this analysis (N=32). In summary, this study has demonstrated that the previously observed cortisol intercorrelations are also applicable in this population of young healthy girls.

4.2 Correlations between cortisol measurements and child-reported estimates of stress: Triad analyses

The four models investigated with the Triads method demonstrated that salivary cortisol measurements (both AUC and decline) may more accurately indicate true childhood stress than hair cortisol measurements for short periods in the past (i.e. last three months); hair

cortisol may however be preferred above salivary measurements for periods more distant and thus for chronic stress assessment (i.e. more than three months ago). While single salivary samples represent single-point or short-term cortisol measurements (hours), salivary AUC measurements are assumed to represent cortisol exposure on a longer-term (days, weeks) (Chida Y. et al., 2009), as confirmed by our results (i.e. indicator for stress for up to 3 months ago). For hair cortisol, different research groups have described its potential as a proper chronic stress measure for several months retrospectively (Karlen J. et al., 2011; Manenschijn L. et al., 2011b; Dettenborn L. et al., 2010; Steudte S. et al., 2011b; Steudte S. et al., 2011a; Russell E. et al., 2012), which is in line with our observations.

We limited hair cortisol analyses to the six most proximal cm as this is assumed to be the maximum length of hair being a reliable estimate of systemic cortisol concentrations in the past (Russell E. et al., 2012). Based on an average growth rate of one cm per month (Harkey M. R., 1993), a six cm hair sample thus represents a six months period prior to sampling. Theoretically, our study could thus not report on periods over six months ago, even though hair cortisol presented the highest validity coefficient also for periods of nine and twelve months ago. A possible delay period between stressor exposure and cortisol incorporation in hair may partly explain our observations, although misconceptions and generalizations on hair growth rate may be a more plausible factor involved in this observed associations between 'non-matching periods': LeBeau et al. (2011) have described the influence of genetic and external variables on the hair growth rate and the effect of fluctuations in sample collection. They demonstrated that a one cm hair-segment may correspond to hair formed 1.3 to 2.2 months earlier, which varies considerably from the generally accepted one cm/one month hypothesis. This way, hair hormonal concentrations may be associated with stressor exposure in more distant periods than theoretically possible. Given the small sample size for the hair cortisol analyses (N=39/223), the above-mentioned ('non-matching') results should however be interpreted with caution.

Biological quantification of stress using salivary or hair cortisol has been increasingly used to overcome limitations inherent to the more subjective nature of questionnaires and potential difficulties in implementing checklists in younger age groups. However, salivary and hair cortisol measurements are no clear-cut diagnostic media for childhood stress and measure different aspects of the stress response compared to questionnaires (Figure 3.5). While environmental, psychological and biological stress responses are theoretically strongly interconnected through the human stress system, some differentiation between these

assessment levels should be made because (1) of the different dynamics of the psychological and biological stress system (i.e. the endocrine stress response lagging behind the psychological response) (Oldehinkel A. J. et al., 2011; Schlotz W. et al., 2008), (2) not all stressors conclusively produce a (measurable) psychological or biological stress response (e.g. if it is not perceived stressful) (Gunnar M. R. et al., 2009a) and additionally (3) inter- and intra-individual differences in response to stressors may exist, depending on characteristics of both the stressor and the person facing it (Kudielka B. M. et al., 2009; Michaud K. et al., 2008; Miller G. E. et al., 2007; Cohen S. et al., 2003). Therefore, we want to emphasize the added value of including both stressor questionnaires and biological markers in stress research, as simultaneous application may provide a more aggregated and complementary view on stress in children (Vanaelst B. et al., 2013a). After all, measures of stressor occurrence may only provide partial knowledge about the physiological stress responsiveness, and vice versa (Oldehinkel A. J. et al., 2011; Schlotz W. et al., 2008).

4.3 Strengths and limitations

This study was the first to examine both the environmental (i.e. major life events) and biological (i.e. serum, salivary and hair cortisol) stress dimensions in young girls using a standardized methodology. As there is currently no gold standard method to measure childhood stress and each approach has its strengths and limitations, we examined which studied stress measures could most accurately indicate childhood stress. For this purpose, we used the Triads method which was previously only used in the area of dietary research but now we expanded its application to stress research and gained a clearer insight in the applicability of salivary and hair cortisol measurements for childhood stress research.

Nevertheless, there were some specific methodological limitations. Firstly, results cannot be generalized to boys or children in general, as hair samples were exclusively taken from girls. This implicates that our observations should be confirmed in a more heterogeneous population including boys.

Hair cortisol analyses were performed using LC-MS/MS methodology which is considered the ‘gold standard’ technique for hair analyses (Society of Hair Testing, 2011; Kushnir M. M. et al., 2011). Yet, a large percentage of the hair samples did not reach the limit of quantification (LOQ=5 pg/mg), with only 39 of the 223 hair samples with quantifiable cortisol concentrations. As a result, the sample size for some of the analyses was small which

could have affected the power of the statistical analyses. As the accuracy of the applied LC-MS/MS method was demonstrated in validation experiments (Vanaelst B. et al., 2013a), the low physiological levels of hair cortisol in girls represent true observations rather than a methodological issue. This raises however questions regarding the general utility of hair cortisol analyses for childhood populations. Baring this limitation in mind, we recommend confirmation of our observations in a larger population sample with less drop-out for (laboratory) biological measurements (e.g. hair cortisol).

Concerning the precision of the Triad analyses (i.e. the 95% CI of the estimated validity coefficients), all intervals were very large and included the value 1 (except for the 95% CI for ρQT of model 1), indicating low sample correlations (Yokota R. T. D. et al., 2010). According to Ocké & Kaaks (1997), a sample size of 100 to 200 individuals may in many situations be insufficient to estimate the validity coefficients with reasonable precision, particularly in the view of low sample correlations which often arise using biological markers. So, further research should confirm our observations using a larger and more heterogeneous population to gain a more complete and accurate insight in the applicability of salivary and hair cortisol measurements for childhood stress research.

5 Conclusions

This paper investigated the relationship between cortisol measurements in different biological samples, showing a lack of association and disagreement between measures of single-point, short-term cortisol versus long(er)-term cortisol. In addition, this paper examined to what extent childhood stress can be accurately estimated by stressor questionnaires and biological markers in girls. Salivary cortisol was shown to most accurately indicate true childhood stress for short periods in the past (i.e. last three months), whereas hair cortisol may be preferred above salivary measurements for periods more distant and thus for chronic stress assessment. As a result, we suggest to differentiate the type of biological matrix (i.e. saliva, hair) according to the time period under investigation. Nevertheless, our observations should be confirmed in future research investigating more heterogeneous populations (e.g. including both boys and girls and children from different socio-demographic backgrounds) and in large-scale settings with small drop-out for biological measurements, implicating further improvement of the hair cortisol laboratory analyses. Moreover, analyses should be extended to other age-groups (e.g. adolescent populations) and other stressor questionnaires or forms of self-report should be included into the Triad analyses.

CHAPTER 3.5 MINERAL CONCENTRATIONS IN HAIR OF BELGIAN ELEMENTARY SCHOOL GIRLS: REFERENCE VALUES AND RELATIONSHIP WITH FOOD CONSUMPTION FREQUENCIES

Abstract

Although evidence suggests that hair elements may reflect dietary habits and/or mineral intake, this topic remains controversial. This study therefore presents age-specific reference values for hair concentrations of Ca, Cu, Fe, Na, Mg, P and Zn using the LMS method of Cole, and investigates the relationship between dietary habits (i.e. food consumption frequencies) and hair mineral concentrations in 218 Belgian elementary school girls by Reduced Rank Regression (RRR). Hair minerals were quantitatively determined via inductively coupled plasma - mass spectrometry after microwave-assisted acid digestion of 6 cm long vertex posterior hair samples. The Children's Eating Habits Questionnaire - Food Frequency Questionnaire was used to obtain information on food consumption frequency of 43 food items in the month preceding hair collection. The established reference ranges were in line with data for other childhood or adolescent populations. The retained RRR factors explained 40%, 50%, 45%, 46%, 44% and 48% of the variation of Ca, Cu, Fe, Mg, P and Zn concentrations in hair, respectively. Although this study demonstrated that a large proportion of hair mineral variation may be influenced by food consumption frequencies in elementary school girls, a number of food groups known to be rich sources of minerals did not show a relation with certain hair minerals. Future research should focus on mechanisms and processes involved in mineral incorporation and accumulation in scalp hair, in order to fully understand the importance and influence of diet on hair minerals.

1 Introduction

A large battery of trace elements and minerals are required by the human body for proper physiological functioning (World Health Organization et al., 1996). Apart from being measurable in blood and urine, minerals are also detectable in scalp hair. During the hair growth process, hair is exposed to the blood supply which contains traces of anything consumed by the individual. In this way, minerals are incorporated in the protein structure of

scalp hair and are no longer affected by the prevailing metabolic conditions. Hair mineral concentrations have been shown to be related to a number of (patho-)physiological conditions (e.g. Parkinson's disease) (Kempson I. M. et al., 2011), but there is also evidence suggesting that hair mineral levels may reflect dietary habits. Hair mineral analyses might thus be a valuable tool providing information on an individual's dietary intake of essential elements, particularly for long- or medium-term assessment. After all, bulk hair concentrations present an average over several months and are thus far less affected by daily fluctuations in environmental conditions than, e.g., blood or urine (Wang C. T. et al., 2005a).

Hair mineral levels have been associated with, e.g., the consumption of highly processed foods, slimming and laxative preparations, coffee, tea and vegetables and with malnourishment and vegetarian diets (Ali M. et al., 1997; Chojnacka K. et al., 2010b; Erten J. et al., 1978; Hong S. R. et al., 2009; Weber C. W. et al., 1990; Gonzalez-Reimers E. et al., 2008; Jeruszka-Bielak M. et al., 2011; Rodenas S. et al., 2011). In contrast, some other studies did not observe such relationships (Suliburska J., 2011; Wojciak R. W. et al., 2004; Gonzalez-Reimers E. et al., 2002). It is difficult to determine the reliability of these inconsistent results because of the limited number of studies that have been conducted to specifically address the association between hair mineral concentrations and nutritional status (taking into account food consumption patterns or nutrient intake) (Gonzalez-Reimers E. et al., 2008; Hong S. R. et al., 2009; Jeruszka-Bielak M. et al., 2011; Rodenas S. et al., 2011; Chojnacka K. et al., 2010b), but also because of difficulties in the interpretation of results. For example, hair mineral concentrations may not necessarily represent blood or other endogenous concentrations or ingested mineral doses, as stated by Kempson et al. (Kempson I. M. et al., 2011). Elevated hair mineral concentrations could as well indicate limited use of the mineral by tissues and subsequent elimination in hair as excretory route, as postulated by Chojnacka et al. (Chojnacka K. et al., 2010b), although these hypotheses need to be investigated.

A first aim of this study was to establish age-specific reference values for the minerals calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), phosphorus (P), sodium (Na) and zinc (Zn) for a large sample of healthy, Belgian (Flemish) elementary school girls, a previously unexplored study population (N=218). Also the correlations between these hair minerals was investigated. A next, parallel aim of this study was to examine the relationship between consumption frequencies of different food types and hair mineral concentrations in young children (i.e. elementary school girls), as this has not been studied before. More specifically,

the current investigation studied the proportion of variation in hair mineral concentrations that can be explained by the consumption frequency of specific food groups.

2 Methods

2.1 Study participants

Agreement for participation was obtained through parental written informed consent for 218 elementary school girls participating to the 2010 baseline survey of the ChiBS project ('Children's Body composition and Stress') (mean age= 8.44 yr, SD=1.11 yr; mean BMI z-score=-0.05; SD=1.23). The ChiBS project, designed at Ghent University and embedded within the European IDEFICS study (Ahrens W. et al., 2011a), investigates the relationship between chronic psychosocial stress and changing body composition in children aged 5 to 10 years old (at baseline) over a two-year follow-up period (2010-2012) (Michels N. et al., 2012d). The ChiBS project offered the opportunity to study the utility of hair samples as biological matrix for measurements of stress and mineral status in children and to investigate its interest as a simple and non-invasive alternative for biological sampling (such as blood sampling) in young children. In total, 523 healthy children participated to the 2010 baseline ChiBS survey. Participation to this study was however limited to the female participants of the ChiBS project (N=264/523 or 50.5%), as hair sampling was only performed in girls to ascertain the required hair length of 6 cm (N=218/264 or 82.5%). No differences were found between boys and girls for age, BMI, parental education or food consumption frequencies, except for milk and yoghurt consumption which was higher in boys (p=0.035). Detailed research goals of the ChiBS project and socio-demographic information of the ChiBS study population is described elsewhere (Michels N. et al., 2012d). The ChiBS project was conducted according to the guidelines laid down in the Declaration of Helsinki and was approved by the Ethics Committee of the Ghent University Hospital.

2.2 Hair mineral analysis

Hair samples were obtained from the vertex posterior region of the scalp by trained researchers. The hair samples were cut as close to the scalp as possible using clean, stainless steel scissors and tied together with a little cord to mark the proximal side. To guarantee that

the same time period was investigated in all children, only the most proximal 6 cm of the hair strands was analysed. None of the hair samples was artificially coloured. The samples were stored in a folded piece of paper in individual zip-lock bags in a dark, dry place and at constant temperature until analysis in the Department of Analytical Chemistry of Ghent University. The hair contents of Ca, Cu, Fe, Na, Mg, P and Zn were quantitatively determined via inductively coupled plasma - mass spectrometry (ICP-MS), after microwave-assisted acid digestion of the samples. Approximately 0.1 g of each hair sample was subjected to a washing procedure prior to the digestion in order to remove any external contamination, such as grease, sweat, dust, etc. This cleaning stage consisted in stirring the samples, first in acetone and subsequently in ultrapure water (obtained from a Direct Q3 water purification system from Millipore, fed by distilled water) inside an ultrasonic bath (Branson 5510) followed by further rinsing of the samples with Milli-Q water. Immediately afterwards, samples were allowed to dry in an oven at a temperature of 60-70°C until complete dryness. Once dried, the samples were weighed, transferred into microwave TFM vessels along with 1 mL of 14 M HNO_3 (Chemlab Belgium, pro analysis, further purified by means of sub-boiling) and 9.8 M of H_2O_2 (Fluka Analytical, Sigma Aldrich Belgium, for trace analysis) and subjected to the following microwave power program for digestion: 2 min at 250 W, 2 min at 0 W, 6 min at 250 W, 5 min at 400 W and 5 min at 600 W (Milestone mls 1200 mega microwave lab station). The digests thus obtained were then allowed to cool down to room temperature before further dilution with Milli-Q water in pre-cleaned PP tubes. Samples were analysed by means of sector field ICP-MS (Thermo Element XR). Simultaneous monitoring of Ca, Cu, Fe, Na, Mg, P and Zn and the internal standard Ge (added to a final concentration of 25 μg L^{-1}) was accomplished using the instrument settings and data acquisition parameters summarized in Chapter 2.4.

2.3 Food consumption frequencies

Children's dietary habits were assessed using the self-administered parental questionnaire 'Children's Eating Habits Questionnaire - Food Frequency Questionnaire' (CEHQ-FFQ), which was fully completed for 109 children. The CEHQ-FFQ is a 43 food item-containing questionnaire developed and validated within the IDEFICS project (Suling M. et al., 2011; Lanfer A. et al., 2011; Huybrechts I. et al., 2011a) and is used as a screening instrument to investigate dietary habits and food consumption frequency in children (Willet W., 1990). Parents were asked to report on their child's consumption frequency of selected food items

(e.g. fruits, vegetables, drinks, breads and cereals, milk products, meat, fish, fats, snacks and desserts) in a typical week during the preceding 4 weeks, outside the school canteen or childcare meal provision settings, using the following response options: 'never/less than once a week', '1-3 times a week', '4-6 times a week', '1 time per day', '2 times per day', '3 times per day', '4 or more times per day' or 'I have no idea'. These frequency categories were converted to consumption frequencies per week. Frequencies of intake were assessed without quantifying portion sizes. Additionally, a questionnaire on the use of vitamin supplements was included (type of supplements, formulation, frequency of intake) (completed for N=187).

2.4 Other variables

Within the ChiBS and the IDEFICS project, information was collected on the child's age, body mass index (BMI) (categorization according to the International Obesity Task Force guidelines (Cole T. J. et al., 2000)), physical activity (i.e. hours of playing outdoors and in sports clubs), parental education (i.e. International Standard Classification of Education-ISCED (UNESCO, 1997)) and parental income (relative to the average national net household income).

2.5 Statistical methods

Statistical analyses were performed with PASW Statistical software version 19.0.0 (SPSS Inc., IBM, USA) and SAS software version 9.3 (SAS Institute Inc., USA). P-values <0.05 were considered statistically significant for all tests. Non-normally distributed data were presented by their median and interquartile range (Kolmogorov-Smirnov, Shapiro-Wilk). Spearman correlation analyses were performed to examine the correlation between hair mineral concentrations and continuous variables (age, physical activity, diet score, etc.), while Kruskal-Wallis tests were applied to investigate differences in terms of hair minerals between (categorical) groups.

As hair minerals were previously shown to be influenced by age (Perrone L. et al., 1996; Sakai T. et al., 2000; Kempson I. M. et al., 2011; Rodushkin I. et al., 2000; Senofonte O. et al., 2000; Chojnacka K. et al., 2006), we reported age-specific reference values, using the LMS method of Cole (Cole T. J. et al., 1992) in which each year of age was considered as one age group (age 6 to 10). Only one child had the age of 5 and was therefore excluded from this

analysis. Age-specific, smoothed L (skewness), M (median) and S (coefficient of variation) curves and percentile reference values (3^{th}, 10^{th}, 25^{th}, $50^{th,}$ 75^{th}, 90^{th} and 97^{th} percentile) were obtained for all minerals, except for Na, as the number of samples that exceeded the LOD was too low (N=50) (LMS Chartmaker Pro Software (version 2.54)) (Pan H. et al., 2011). The degrees of freedom for L, M and S respectively, were: 2,2,2 for Ca; 2,3,2 for Cu; 4,4,4 for Fe; 2,4,4 for Mg; 2,3,3 for P and 3,4,4 for Zn. Q tests and detrended Q-Q plots were used to asses goodness-of-fit and normality.

To identify food groups that explain the largest proportion of variation in the hair mineral concentrations, reduced rank regression (RRR) was applied using the partial least square procedure in SAS. RRR determines linear functions of *predictors (i.e. food groups)* and extracts so-called *factors* by maximizing the explained variation in *responses (i.e. hair mineral concentrations)* (Hoffmann K. et al., 2004). *Factor loadings* indicate the relationship between the food groups and the derived factor and thus indicate which food groups load highly onto the factor which explains variation in hair minerals. Data on food consumption frequencies and hair minerals were firstly adjusted for several covariates (children's age, BMI z-score, physical activity level, hair colour and parental income) using linear regression and then entered as residuals into the RRR analyses. As RRR implies that the number of extracted factors equals the number of selected responses, one factor was obtained for each hair mineral. For clarity, only food groups with factor loadings ≥0.15 were reported. RRR analyses were not performed for hair Na as the sample size was too small for these analyses (N=26).

3 Results

3.1 Hair mineral reference values

Population characteristics of the participating girls and age-specific reference values for Ca, Cu, Fe, Mg, Na, P and Zn are presented in Table 3.12 and Table 3.13, respectively.

These reference values refer to healthy, female and predominantly Caucasian girls between 6 and 10 years old living in Flanders (Belgium). Table 3.13 also presents previously reported reference ranges for other childhood and adolescent populations. The child's age and hair Fe concentrations were negatively correlated (Spearman's rho= -0.277, $p<0.001$). For the other minerals, no significant correlation with age was observed (results not shown).

Table 3.14 presents hair mineral contents in relation to parental income and natural hair colour, as Cu and P concentrations in hair were shown to differ significantly according to parental income and natural hair colour respectively (Kruskal-Wallis $p=0.047$ and $p<0.001$ respectively). Parental education and physical activity were not associated with the hair mineral concentrations (data not shown).

3.2 Inter-mineral correlations

As shown in Table 3.15, hair minerals are strongly positively intercorrelated. More particularly, Ca and Cu, Cu and Na, Cu and Mg, Mg and Zn, and P and Zn showed correlation coefficients > 0.3, while Ca and Mg showed an even higher correlation coefficient ($r=0.881$).

Table 3.12 Population characteristics of the studied girls (N=218)

	N	%
Age groups		
5	1	0.5
6	21	9.6
7	55	25.2
8	66	30.3
9	53	24.3
10	22	10.1
BMI categories (COLE)		
underweight	30	13.8
normal weight	163	74.8
overweight and obese	25	11.5
Race		
Asian	1	0.5
Latino	3	1.4
Caucasian	214	98.2
Hair colour		
blond	14	6.4
red	3	1.4
brown	155	71.1
dark brown	43	19.7
black	3	1.4
Physical activity level[a]	**(N=202)**	
low (< 11 hours per week)	69	34.2
medium (11-17 hours per week)	66	32.7
high (> 17 hours per week)	67	33.2
Parental education	**(N=208)**	
ISCED 1	2	1.0
ISCED 2	3	1.4
ISCED 3	62	29.8
ISCED 4	39	18.8
ISCED 5	102	49.0
Parental income	**(N=171)**	
low and low to medium	11	6.4
medium	64	37.4
medium to high	51	29.8
high	45	26.3

[a] hours per week; categorization based on percentiles values (low <P33, medium P33-P66, high >P66)

BMI: Body Mass Index; ISCED: International Standard Classification of Education

Table 3.13 Age-specific L, M, S and percentile values for hair minerals

	age	N	L	S	P3	P10	P25	P50 (M)	P75	P90	P97	Literature reference ranges
								in µg/g				*median [interval] in µg/g [literature reference]*
Ca	6	21	0.27	0.60	135.62	234.51	377.96	576.93	843.41	1190.34	1631.57	212.47 [a] [0.23-1001.00](Park H. S. et al., 2007); 369 [11-3101] (Senofonte O. et al., 2000)
	7	55	0.23	0.66	117.79	211.74	354.81	562.65	853.38	1247.73	1769.10	
	8	66	0.19	0.71	103.47	191.94	333.21	548.36	863.59	1310.83	1928.35	
	9	53	0.16	0.77	91.98	174.76	313.07	534.07	874.18	1381.09	2116.07	
	10	21	0.12	0.82	82.74	159.86	294.32	519.79	885.28	1460.37	2341.78	
Cu	6	21	-0.47	0.48	8.93	11.29	14.68	19.82	28.12	42.78	72.21	15.51[a] [5.82-104.70] (Park H. S. et al., 2007); 10.1 [0.29-280] (Senofonte O. et al., 2000); 19.95 [9.1-59.7][b] (Dongarra G. et al., 2011); 13.7[c] (Sakai T. et al., 2000); 9.7 (Perrone L. et al., 1996)
	7	55	-0.47	0.50	8.38	10.66	13.97	19.04	27.37	42.40	73.58	
	8	66	-0.47	0.52	8.09	10.35	13.67	18.81	27.39	43.21	77.18	
	9	53	-0.46	0.53	7.97	10.25	13.64	18.96	27.96	44.95	82.68	
	10	21	-0.46	0.55	7.82	10.13	13.57	19.04	28.46	46.61	88.41	
Fe[d]	6	21	-1.37	0.30	6.30	7.10	8.17	9.74	12.28	17.27	33.37	12.62[a] [4.16-32.48] (Park H. S. et al., 2007); 12.7 [0.29-206] (Senofonte O. et al., 2000); 43.8 [c] (Sakai T. et al., 2000); 4.8 (Perrone L. et al., 1996)
	7	54	-0.56	0.39	4.51	5.46	6.76	8.61	11.39	15.88	23.90	
	8	66	-0.20	0.40	3.61	4.58	5.88	7.65	10.10	13.54	18.49	
	9	53	-0.53	0.39	3.70	4.49	5.58	7.11	9.41	13.08	19.47	
	10	21	-0.12	0.43	2.81	3.66	4.81	6.39	8.57	11.63	15.97	
Mg	6	21	-0.12	0.51	12.30	16.75	23.07	32.18	45.51	65.33	95.33	12.29[a] [3.30-29.80] (Park H. S. et al., 2007); 20.2 [0.10-313] (Senofonte O. et al., 2000)
	7	55	-0.03	0.58	9.02	13.17	19.31	28.44	42.07	62.51	93.31	
	8	66	0.06	0.65	7.32	11.61	18.18	28.13	43.04	65.15	97.61	
	9	53	0.15	0.67	5.91	10.13	16.69	26.54	40.94	61.50	90.24	
	10	21	0.24	0.71	4.48	8.67	15.33	25.30	39.55	59.20	85.51	
P	6	21	-0.38	0.12	113.05	121.47	130.77	141.09	152.56	165.36	179.69	121.21[a] [65.84-193.90] (Park H. S. et al., 2007); 199 [18-527] (Senofonte O. et al., 2000)
	7	55	0.10	0.15	104.18	115.22	127.32	140.54	154.98	170.76	187.96	
	8	66	0.58	0.17	96.40	110.14	124.64	139.90	155.89	172.61	190.03	
	9	53	1.06	0.17	92.16	108.03	123.76	139.38	154.90	170.33	185.69	
	10	21	1.54	0.16	90.31	108.15	124.54	139.83	154.28	168.04	181.22	
Zn	6	21	-0.28	0.14	166.05	181.51	198.86	218.38	240.42	265.39	293.76	69.99[a][13.82-170.60] (Park H. S. et al., 2007); 149 [24-477] (Senofonte O. et al., 2000); 179.2 [96.86-329.19][b](Dongarra G. et al., 2011); 111 [c] (Sakai T. et al., 2000); 139 (Perrone L. et al., 1996)
	7	55	0.17	0.19	143.21	164.12	187.51	213.62	242.69	274.98	310.77	
	8	66	0.47	0.23	127.65	154.30	183.66	215.76	250.63	288.30	328.79	
	9	53	0.29	0.28	121.22	149.81	182.89	220.86	264.14	313.14	368.29	
	10	21	-0.21	0.28	132.41	156.55	186.25	223.07	269.09	327.15	401.13	
Na[e]		50	(Range: 1.01 - 205.85)		1.01	1.01	2.00	4.26	9.00	37.00	123.25	27.14[a][1.18-153] (Park H. S. et al., 2007)

L (skewness), M (median), S (coefficient of variation) and percentiles with LMS method; [a]mean values; [b] 95% confidence interval instead of min-max interval; [c] geometric mean; [d] An outlier with concentration of 225 µg/g was deleted from LMS analyses as no positive curves could be created; [e] No age-specific reference values could be created because the number of cases was too small (N=50)

Table 3.14 Hair mineral concentrations and population characteristics[a]

	N	Ca	Cu	Fe	Mg	P	Zn
		median (SE of mean) (in µg/g)					
Parental income							
low and low to medium	11	545.9 (114.7)	14.2 (7.16)	8.57 (1.94)	22.4 (5.46)	134.9 (5.46)	222.5 (17.5)
medium	64	574.1 (64.8)	16.2 (1.85)	6.77 (0.54)	28.6 (2.59)	142.8 (2.87)	215.3 (6.29)
medium to high	51	598.3 (78.0)	17.8 (2.61)	6.97 (0.59)	29.1 (2.97)	134.9 (3.00)	210.9 (10.2)
high	45	606.4 (60.4)	22.6 (2.61)	8.27 (4.87)	27.5 (3.83)	139.2 (3.67)	226.4 (6.95)
p-value Kruskal-Wallis		*0.959*	***0.047***	*0.662*	*0.995*	*0.476*	*0.568*
Hair colour							
blond	14	765.0 (110.9)	26.2 (4.09)	6.92 (0.76)	37.04 (5.61)	129.03 (5.97)	209.2 (12.6)
brown and red	158	537.0 (36.9)	17.2 (1.57)	7.40 (1.42)	24.06 (1.72)	137.02 (1.75)	218.5 (4.52)
dark brown and black	46	656.2 (96.8)	18.6 (1.45)	7.97 (0.79)	30.9 (3.15)	150.03 (3.39)	223.7 (9.03)
p-value Kruskal-Wallis		*0.252*	*0.251*	*0.286*	*0.102*	***<0.001***	*0.445*

[a] Na: number of cases too small to report category-specific concentrations, p=0,679 (parental income) and p=0,852 (hair colour)

Table 3.15 Inter-mineral correlations in hair of Belgian elementary school girls (N=217[a]*)*

	Cu		Fe		Na		Mg		P		Zn	
	corr coeff [b]	*p*	*corr coeff*	*p*	*corr coeff*	*p*	*corr coeff*	*p*	*corr coeff*	*p*	*corr coeff*	*p*
Ca	**0.397**	**<0.001**	0.090	0.188	-0.125	0.386	**0.881**	**<0.001**	0.069	0.311	**0.279**	**<0.001**
Cu			**0.163**	**0.016**	**0.378**	**0.007**	**0.395**	**<0.001**	0.079	0.244	**0.140**	**0.039**
Fe					0.163	0.259	**0.134**	**0.048**	**0.135**	**0.047**	0.078	0.250
Na							-0.111	0.442	0.101	0.487	0.239	0.094
Mg									0.065	0.342	**0.318**	**<0.001**
P											**0.331**	**<0.001**
Zn												

[a] correlations with Na are based on 50 cases, [b] Spearman's correlation coefficients

3.3 Food consumption frequencies and hair mineral concentrations

Table 3.16 presents the amount of variation in hair mineral concentrations explained by the retained RRR factor, as well as the loadings of the food groups (based on consumption frequency) for the respective factors (i.e. loadings ≥ 0.15). Relatively large percentages (i.e. at least 40%) of hair minerals were explained by the retained RRR factors.

Examination of food groups that contribute to these factors reveals that the factor explaining Ca mainly consists of milk desserts (ice cream, milk bars), meat, eggs and snacks; the situation is similar for Mg (milk desserts, eggs, sweet snacks). The factor explaining Cu primarily covers snacks, fruits and white bread products. The factor for Fe loads highly on sweetened milk products (sweetened milk, yoghurt, fermented beverages), sweet snacks, chocolate or nut-based spreads and whole meal bread products. Similarly, the factor explaining P mainly consists of sweetened milk products, sweet snacks, white bread products and fish. To end, the Zn factor covers fruits, fish and sweet snacks. Table 3.16 indicates the top 10 of contributing food groups for each hair mineral factor separately. No relationship was observed between hair mineral concentrations and vitamin or mineral supplement use (N=25/187) (data not shown).

Table 3.16 Percentage of variation in hair mineral concentration explained by Reduced Rank Regression analyses and factor loadings of food groups with value ≥0,15[a]

	hair minerals											
	Ca		**Cu**		**Fe**		**Mg**		**P**		**Zn**	
	N=109		N=109		N=109		N=109		N=109		N=109	
percentage variation accounted for by RRR factor	40.45		50.06		45.29		46.43		43.8		48.35	
Food consumption frequency	factor loading	order[b]	factor loading	order	factor loading	order	factor loading	order	factor loading	order	factor loading	order
vegetables												
cooked vegetables, potatoes and beans												
fried potatoes, potato croquettes			0.193	5								
raw vegetables												
fruits												
fresh fruits without added sugar	0.193	9					0.239	6				
fresh fruits with added sugar	0.227	7	0.217	3			0.156				0.523	1
drinks												
water	0.178				0.182	8						
fruit juices												
sweetened drinks												
diet soft drinks			0.166	7								
breakfast cereals and bread												
sweetened or sugared breakfast cereals			0.168	6							0.161	6
porridge, oat meal, gruel, unsweetened cereals												
white bread, white roll, white crispbread			0.2	4					0.174	5		

whole meal bread, dark roll, dark crispbread					0.189	6						
cereal products												
pasta, noodles, rice	0.161						0.226	8				
dish of milled cereal												
milk and yoghurt												
plain unsweetened milk												
sweetened milk					0.481	1						
plain unsweetened yoghurt												
sweetened yoghurt and fermented milk beverages					0.191	5			0.271	1	0.153	7
fish, meat, eggs and meat alternatives												
fish not fried							0.247	5	0.199	4		
fried fish	0.219	8					0.178	10	0.219	3	0.368	2
cold cuts and preserved meat products												
fresh meat, not fried	0.311	2					0.227	7				
fried meat												
fried or scrambled eggs	0.298	4	0.155	8			0.33	3				
boiled or poached eggs	0.306	3					0.4	1				
nuts and seeds and dried fruits												
tofu, tempeh, quorn, soy milk	0.19	10										
cheese												
sliced cheese												
spreadable cheese												
spreadable products												
jam, honey												
chocolate or nut-based spread					0.289	3						

ketchup mayonnaise and mayonnaise based products											0.228	4
fats												
butter, margarine												
reduced-fat products on bread												
snacks and desserts												
pizza hamburger, hot dog, kebab, wrap, falafel snacks like crisps, corn crisps, popcorn etc. snacks like savoury pastries and fritters snacks like chocolate, candy bars	0.269	5	0.419	1	0.185	7	0.2	9				
snacks like candies, loose candies, marshmallow					0.234	4					0.327	3
snacks like biscuits, packaged cakes or pastries, puddings	0.237	6	0.347	2	0.432	2	0.284	4	0.268	2	0.215	5
ice cream, milk or fruit based bars	0.356	1					0.36	2				

[a] adjusted for child's age, BMI z-score, physical activity, hair colour, parental income; [b] indication of factor loading order (highest to lowest), only indicated for positive factor loadings and indication of maximum 10 food items

4 Discussion

As few studies have addressed hair mineral concentrations in relation to specific dietary habits and the significance of this topic has remained controversial, we analysed the relationship between hair minerals (Ca, Cu, Fe, Mg, P and Zn) and food group consumption frequency in a large sample of young girls and established age-specific reference values for Belgian elementary school girls, a previously unexplored study population. In addition we developed reference values for hair minerals in our study population.

4.1 Hair mineral reference values

The data presented are of significance as up to now no reference ranges for hair mineral concentrations were available for Flemish children. The presented reference values are generally in line with previously reported data for other childhood and adolescent populations, although our concentrations of Ca, Mg and Zn were slightly higher, while the observed Fe and Na concentrations were lower than those previously reported (Table 3.13) (Park H. S. et al., 2007; Dongarra G. et al., 2011; Senofonte O. et al., 2000; Perrone L. et al., 1996; Sakai T. et al., 2000). However, comparison of our observations with female specific reference ranges for girls aged 6 to 11 and 7 to 10, published by Perrone et al. and Senofonte et al. respectively, showed that our findings on Ca, Mg and Fe are similar, with even higher concentrations for Mg and lower concentrations for Fe reported in Senofonte et al. and Perrone et al. respectively (Perrone L. et al., 1996; Senofonte O. et al., 2000).

Apart from analytical variability among laboratories (Seidel S. et al., 2001), some inter-individually differing characteristics may account for these varying reference ranges such as ethnicity, geographical and environmental location (urban area, rural area etc.) (Dongarra G. et al., 2012), age, gender, personal habits (use of shampoos, dietary habits etc.) and genetics (Kempson I. M. et al., 2011; Cornelis R., 1973). As our study exclusively consisted of female participants and hair minerals have been shown to differ between genders (Rodushkin I. et al., 2000; Chojnacka K. et al., 2010a; Sakai T. et al., 2000; Perrone L. et al., 1996; Chojnacka K. et al., 2006; Senofonte O. et al., 2000; Forte G. et al., 2005; Dongarra G. et al., 2011), comparison of the reference ranges obtained with those for other general childhood and adolescent populations may not be straightforward. Gender differences in hair minerals have been attributed to differences in metabolism, hormonal balance and physiological role of

minerals between males and females (Sakai T. et al., 2000; Chojnacka K. et al., 2010a). Nevertheless, as our study population was homogenous (i.e. only girls), the presented reference values were thus indirectly normalized for gender.

We observed significant age-specific variations in hair Fe concentrations, although Table 3.13 demonstrates that also Ca and Mg showed a decreasing (but not significant) trend with increasing age (Chojnacka K. et al., 2006). This is in agreement with findings in adult populations (Forte G. et al., 2005; Unkiewicz-Winiarczyk A. et al., 2009) but in contrast with other childhood findings (Senofonte O. et al., 2000): Sakai et al. and Perrone et al. observed age-specific variations for the elements Zn and Cu in hair from birth over childhood to adolescence, representing variations in (hair) minerals with different physiological, developmental periods (Perrone L. et al., 1996; Sakai T. et al., 2000). As the age range included in our study was small (i.e. a 5 year range), it was not possible to observe a clear, significant age-trend for each mineral.

Moreover, we recorded a significant influence of hair colour on hair mineral concentrations, more specifically for P with an increasing trend towards darker hair colours (Kempson I. M. et al., 2011). This is in contrast to findings reported by Chojnacka et al. who did not detect an influence of natural hair colour on the P level, whereas they showed the Cu concentration to be highest in dark hair and those of Ca and Mg in blond hair (Chojnacka K. et al., 2006), findings we could not confirm. No relationship was observed between parental education or physical activity level and hair mineral levels, although parental income was significantly associated with hair Cu concentrations. This may be due to a dietary pattern that varies with socio-economic status (Huybrechts I. et al., 2011b; Bau A. M. et al., 2011); a more elaborate study of this issue is beyond the scope of this manuscript.

4.2 Inter-mineral correlations

Strong positive correlations between hair minerals were observed (Kempson I. M. et al., 2011; Rodushkin I. et al., 2000). In particular, the strong correlation between Ca and Mg (Table 3.15) has been demonstrated previously (Chojnacka K. et al., 2010a; Faghihian H. et al., 2002; Forte G. et al., 2005; Kim O. Y. et al., 2010) and may indicate common chemical properties, common occurrence in nature or common dietary sources (Rodushkin I. et al., 2000; Chojnacka K. et al., 2010a). Indeed, as observed in Table 3.16, the same food items load on the RRR factors for Ca and Mg (except for water and meat alternatives which are only

present for the Ca factor), indicating that consumption frequency of the same food items contribute to the variability in hair Ca and Mg levels. These findings were less apparent for the other inter-mineral correlations. Kempson et al. suggested that mineral relationships may origin or occur at different levels or stages, i.e. at ingestion (e.g. Mg dependence of Ca-absorption (Chojnacka K. et al., 2010a)), during transport, during hair incorporation or after hair formation by way of contamination or substitution (Kempson I. M. et al., 2011).

4.3 Food consumption frequencies and hair mineral concentrations

Few investigators have studied hair minerals in relation to the dietary habits or diet quality. Gonzalez-Reimers et al. analysed the association between hair Fe, Cu and Zn and diets rich in meat, fish or vegetables based on a dietary recall of the last two weeks in healthy adults from the Canary Islands. In contrast to our findings, they could not indicate a relationship between hair Zn and fish, or hair Cu and vegetables and judged the relationships between hair minerals and type of diet to be poor (except for their observed relationship between hair Fe and vegetable consumption) (Gonzalez-Reimers E. et al., 2008). Hong et al. investigated nutrient intakes based on a FFQ in Korean female adults and found that the hair micronutrient content was not directly influenced by each mineral/micronutrient intake: only hair Na correlated positively to dietary potassium and vitamin C intake (Hong S. R. et al., 2009). The relationship between hair Ca and dietary habits in Polish young women has been investigated by Jeruszka-Bielak et al, using four-day dietary records and information on fortified foods and food supplements (Jeruszka-Bielak M. et al., 2011). Hair Ca was found to weakly relate to the diet quality (i.e. meat and dairy consumption), but more importantly synergistic interactions with vitamin D were observed (i.e. higher vitamin D intake from supplements correlated positively with hair Ca). This is in contrast to our study showing no relationship with supplement use or vitamin D supplements in particular, although this could be due to the low number of children taking supplements (N=25/187). The influence of an omnivorous or semi-vegetarian diet on hair minerals and blood pressure has been studied in Spanish postmenopausal women (Rodenas S. et al., 2011), using a 14 day precise weighing method. Rodenas and colleagues found distinct differences in hair mineral contents between omnivorous and semi-vegetarian participants, suggesting the influence of diet on hair minerals; results which are however in contradiction to Wojciak et al (Wojciak R. W. et al., 2004). Last, reduced hair calcium, magnesium, zinc and copper levels were observed in Polish

obese children (10-14 years old) after slimming therapy, indicating an influence of decreased food intake on mineral metabolism and accumulation in hair (Wojciak R. W. et al., 2010).

Our observations are in agreement with the above-discussed findings in other subject groups, indicating a relationship between diet and hair minerals. However, studies in young children are lacking (apart from the study of Wojciak et al. in obese children from 10 to 14 years old (Wojciak R. W. et al., 2010)), limiting detailed comparison of our observations. We did not intend to link hair minerals to healthy or unhealthy diets, or to represent the most important mineral contributors to the child's diet as the administered FFQ was designed to investigate the children's dietary habits only and not to investigate mineral intakes. In contrast, we examined to what level variation in hair minerals may be explained by consumption frequencies of different food groups in elementary school girls, thereby investigating whether or not hair minerals might to a certain extent be related to dietary habits. As expected, we observed that a significant proportion of variation in hair minerals was explained by the consumption frequency of specific food groups, independently from the child's age, BMI, hair colour, physical activity and parental income. This may indicate that high consumption frequencies of specific foods may stimulate the excretion and incorporation of minerals into hair, although further fundamental research into mineral metabolism is needed (e.g. use of minerals by tissues, elimination of minerals into scalp hair as excretory route etc.).

A number of food groups known to be 'rich' sources of minerals did not emerge as contributors to certain hair minerals: e.g. milk did not load to the Ca and Mg factor; and meat products did not appear in the Zn and Fe factor, despite the known high nutrient density and high consumption frequencies of these food groups in the studied population (data not shown) (Subar A. F. et al., 1998). So, despite the observed relationship between hair minerals and food consumption, insufficient evidence is provided to consider hair minerals as direct reflection of dietary habits. We expect other mechanisms and processes to be involved in the mineral incorporation and accumulation in scalp hair (with inter-individually varying rates and extents) which may complicate the direct relationship between diet and hair minerals.

Concerning this complex relationship between nutrient intake and biochemical indicators in general (e.g. serum, urine, hair), it should be noted that nutrient intake is just one of the determinants affecting the nutrient status. Other determinants are, e.g., the homeostatic control mechanisms and a wide array of genetic, environmental and life-style factors (Hunter D., 1990). For these reasons, associations between bio-indicators (in e.g. serum or urine) and

nutrient intake have not always been evident. Although recovery bio-indicators such as 24h-urine samples have sometimes offered valid measures of nutrient intake, the selection and validation of an appropriate method (i.e. matrix) for estimating a particular nutrient intake has been recommended to be made on a nutrient-by-nutrient basis (Hunter D., 1990).

Even though hair mineral determination should not (yet) be considered a clear-cut diagnostic means for assessing the child's dietary habits and/or mineral status, or should not replace serum or urinary nutritional biomarkers, they could be used as complementary means to investigate the minerals status with the possibility to detect deviations from homeostasis or changes between groups of individuals with a different health status, nutrient intake or geographical location over a long-period retrospectively (World Health Organization et al., 1996; Hunter D., 1990).

4.4 Strengths, limitations and further research

To our knowledge, this study was the first to present age-specific hair mineral reference values for Flemish girls and to specifically investigate the relationship between food consumption frequencies on hair mineral variations by the use of RRR analysis, a technique that has gained increasing importance in nutritional epidemiology (Hoffmann K. et al., 2004). Other strong methodological features of this study are the use of the validated and state of the art ICP-MS technique to measure the hair mineral concentrations; application of the LMS method to establish age-specific reference values and its large sample size. However, some limitations should be considered when interpreting results. The CEHQ-FFQ did not include portion sizes or school and canteen meals and was not designed or evaluated for assessing nutrient intakes. Therefore, no mineral intakes could be calculated from the FFQ and a direct comparison between mineral intakes and hair status was not possible. Also, the CEHQ-FFQ assesses children's dietary habits during the last month, while our hair minerals represent a period of approx. 5-6 months in the past (i.e. 6 cm of hair sample). Possibly, results could be stronger or more accurate if a time-matched hair sample would have been analysed (1 cm hair sample), although both measurements represent 'chronic' assessments. As our population only consisted of females in a small age range, findings should be confirmed in a more heterogeneous population sample (i.e. boys and girls, childhood to adolescence), as hair mineral concentrations and dietary habits vary between sexes and age groups which thus influences the relationship between hair minerals and diet. Nevertheless, this study may

initiate further hair mineral research in relation to diet in children, as hair mineral analysis offers considerable advantages for large-scale epidemiological research in children: hair sampling is easy, non-invasive, inexpensive and the samples are easily stored. Moreover, hair minerals are less influenced by short term (hourly or daily) concentration fluctuations in, e.g., food intake or other environmental factors compared to blood or urinary samples. Further research (1) examining intra-individual mineral statuses in different types of biological matrices in parallel (e.g. hair, 24h-urine, blood), (2) investigating detailed mechanisms and processes involved in hair mineral accumulation and (3) further validating hair minerals as bio-indicators for dietary habit and/or mineral intake, may enlarge their use in nutritional epidemiology.

5 Conclusion

This study strengthened previous indications of a relationship between diet and hair minerals. More specifically, we observed that a significant proportion of variation in hair minerals may be explained by the consumption frequency of specific food groups. However, up to now, there is insufficient evidence to consider hair minerals as a direct reflection of dietary habits. Our findings should be confirmed in a more heterogeneous population and future research should investigate the mechanisms and processes involved in the mineral incorporation and accumulation in scalp hair and study the relationship with the total body burden of these minerals or the mineral status in other biomatrices, in order to fully understand the importance and influence of diet on hair minerals.

CHAPTER 3.6 HAIR MINERALS AND METABOLIC HEALTH IN BELGIAN ELEMENTARY SCHOOL GIRLS

Abstract

Literature has repeatedly shown a relationship between hair minerals and metabolic health, although studies in children are currently lacking. This study aims to investigate hair levels of calcium (Ca), copper (Cu), magnesium (Mg), iron (Fe), phosphorus (P) and zinc (Zn) and their association with (1) overweight/obesity and (2) metabolic health in Flemish elementary school girls between 5 and 10 years old. Two hundred eighteen girls participated in this study as part of the baseline ChiBS project. Children were subjected to physical examinations, blood and hair sampling. Hair minerals were quantitatively determined via inductively coupled plasma – mass spectrometry after microwave-assisted acid digestion. Body mass index (BMI) and body fat percentage (BF%) were studied as anthropometric parameters and a metabolic score (including systolic and diastolic blood pressure, insulin resistance and non-high density lipoprotein (non-HDL) cholesterol as parameters) was calculated, with higher scores indicating a more unhealthy metabolic profile. Hair Ca, Ca/Mg and Ca/P positively correlated with the anthropometric parameters. An inverse correlation was observed between Ca, Mg and Ca/P in hair and the metabolic score. Inverse correlations were also observed for individual metabolic parameters (i.e. diastolic blood pressure, homeostasis model assessment for insulin resistance (HOMA-IR), non-HDL cholesterol). In particular girls with a total number of 3 or more metabolic parameters above the age-specific 75th percentile showed significantly reduced hair Ca, Mg and Ca/P concentrations. This study showed reduced hair mineral concentrations in young girls with a more unhealthy metabolic profile. Positive associations were observed between some minerals and BMI and BF%.

1 Introduction

Obesity prevalence has been increasing worldwide the last decades (Ahrens W. et al., 2011b; Cali A. M. G. et al., 2008). Even among children and adolescents at least 110 million children are overweight or obese (Cali A. M. G. et al., 2008). Childhood overweight and obesity is expected to rise by 1.3 million children per year in the European Union (Manios Y. et al., 2011) and is accompanied by a rising incidence of co-morbidities such as dyslipidaemia,

impaired glucose regulation, hypertension and cardiovascular diseases, which have been shown to 'track' into adulthood (Reilly J. J. et al., 2011; Cali A. M. G. et al., 2008; Goran M. I. et al., 2003). A major health concern, particularly among obese children, is the metabolic syndrome, characterized by the clustering of these metabolic abnormalities (Bokor S. et al., 2008; Tailor A. M. et al., 2010).

A sedentary lifestyle and an unbalanced diet are the major factors involved in the development of obesity and its co-morbidities (Moreno L. A. et al., 2011), which have repeatedly been associated with essential mineral imbalances and deficiencies. For instance hypomagnesaemia is a well-described phenomenon in diabetes and hypertension, while deficiencies in magnesium, iron and zinc have frequently been observed in obesity (Vaskonen T., 2003; Sales C. H. et al., 2006; Houston M. C. et al., 2008; Kaidar-Person O. et al., 2008; Bonnefont-Rousselot D., 2012). Moreover, calcium, magnesium and potassium have been suggested to be lipid- and blood pressure lowering and to have an inverse relationship with insulin resistance and body mass index (Vaskonen T., 2003; Sales C. H. et al., 2006; Houston M. C. et al., 2008; Kaidar-Person O. et al., 2008; Bonnefont-Rousselot D., 2012). The mechanisms through which these minerals act on metabolism have been studied extensively, as reviewed elsewhere, although the direction of causality in these associations is not completely clear (Garcia O. P. et al., 2009; Vaskonen T., 2003; Sales C. H. et al., 2006; Houston M. C. et al., 2008; Nielsen F. H., 2010; Barbagallo M. et al., 2007).

Although minerals are commonly measured in serum, hair mineral analysis offers a practical alternative, providing longer-term retrospective information on the body's metabolic activity. Furthermore, it is a less invasive technique affected in a smaller extent by physiological conditions in response to diet and other factors. In general, an inverse relationship has repeatedly been shown for Ca, Mg, Fe, Zn and Cu in hair with obesity, diabetes and the metabolic syndrome, while hair Na has been shown to be increased under these conditions (Wang C. T. et al., 2005a; Wang C. T. et al., 2005b; Skalnaya M. G. et al., 2007; Afridi H. I. et al., 2008; Hong S. R. et al., 2009; Park S. B. et al., 2009; Gonzalez-Reimers E. et al., 2008) (Table 3.17). As other studies were unable to confirm these conclusions, a more complex and nuanced interpretation is needed. Therefore, further analysis of these minerals and their ratios is worth investigating, especially in children given their increasing prevalence of metabolic abnormalities.

Hair minerals have not been studied in relation to metabolic health in children before, for this reason, this study investigates the relationship between long-term levels of calcium (Ca), copper (Cu), magnesium (Mg), iron (Fe), phosphorus (P) and zinc (Zn) in scalp hair and metabolic parameters in elementary school girls between 5 and 10 years old. The study questions read as follows: Are hair Ca, Cu, Mg, Fe, P and Zn concentrations and the ratios Ca/Mg, Ca/P, Fe/Cu and Zn/Cu inversely related to (a) overweight and obesity, and (b) an unhealthy metabolic profile in children?

Table 3.17 Studies investigating hair minerals in the broad context of metabolic disorders

Metabolic condition	Main findings of the study
Obesity (Wang C. T. et al., 2005b)	inverse relationship between hair Ca, Fe, Mg, Zn, Ca/Mg, Fe/Cu, Zn/Cu and BMI categories; positive relationship between Cu, K, Na and K/Na ratio and BMI categories in adult females (N=392)
Obesity (Wang C. T. et al., 2005a)	reduced hair Ca, Cu, Fe, Mg and Zn concentrations in highest BMI category compared to lowest BMI category of adolescent females (N=180)
Obesity (Gonzalez-Reimers E. et al., 2008)	reduced hair Cu in overweight and obese individuals (N=94)
Obesity (Hong S. R. et al., 2009)	positive correlations between hair Na, K, Cr, Cd and BMI in healthy adult women (N=54)
Obesity (Wiechula D. et al., 2012)	increase in (pubic) hair Cr and Mg with increasing BMI; reduced Zn with increasing BMI in 39 overweight and obese females compared to 46 female controls
Obesity and Type 2 Diabetes (Skalnaya M. G. et al., 2007)	increased hair K, Na, Hg and reduced Ca, Mg, Zn, Co in diabetic women (N=93) compared to controls (N=1236) ; increased hair K, Hg, Pb and reduced Ca, Mg, Zn, I in obese women (N=141) compared to controls
Type 2 Diabetes (Afridi H. I. et al., 2008)	reduced hair K, Mg, Ca and increased hair Na in hypertensive type 2 diabetes patients (N=254) compared to controls (N=185)
Cardiovascular disease (Thimaya S. et al., 1982)	reduced hair Se in cardiovascular disease patients compared to controls
Cardiovascular disease (Tan C. et al., 2009)	low hair Se/Fe and high Mn/Cu as risk factors for cardiovascular disease (cardiovascular disease patients N=24, healthy controls N=100)
Myocardial infarction (Bialkowska M. et al., 1987)	increased hair Zn and Zn/Cu ratio in male survivors of myocardial infarction (N=29) compared to male controls (N=23)
Blood pressure (Vivoli G. et al., 1987)	no difference in hair Zn or Cu between early hypertensive adults (N=23) versus matched normotensive controls (N=23)
Coronary atherosclerosis (Fernandez-Britto J. E. et al., 1993)	correlations between hair Pb, Ca/K, Zn/Cu, Fe and coronary atherosclerotic lesions (N=73 autopsy subjects)
Ischemic stroke (Karaszewski B. et al., 2007)	increased hair Mg and K concentrations in male ischemic stroke patients (N=48) compared to male controls (N=24)
Arterial stiffness (Kim O. Y. et al., 2010)	positive relationship between hair Ca and arterial stiffness in 104 healthy adults
Hypertensive and obese patients with insulin resistance (Suliburska J. et al., 2011)	increased hair Ca and reduced hair Zn and Fe (women only) in obese, hypertensive subjects (N=40) compared to controls (N=40); negative association between hair Fe, Zn, Cu and cholesterol, triglycerides and glucose
Hypertension (Durkalec-Michalski K. et al., 2012)	increased hair Ca in hypertension (N=30) compared to controls (N=29), despite reduced dietary Ca intake
Metabolic syndrome (Park S. B. et al., 2009)	reduced hair Ca, Mg, Cu, Ca/P ratio and increased Na, K, Na/Mg ratio and Hg in metabolic syndrome patients (N=73) compared to adult controls (N=270)

Ca : calcium ; Co: cobalt; Cr: chromium; Cd: cadmium; Cu: copper; Fe: iron; Hg: mercury; I: iodine; K : potassium; Mg: magnesium; Mn: manganese; Na: sodium; Se: selenium; Pb: lead ; Zn: Zinc; BMI: body mass index

2 Methodology

2.1 Study Participants

Participants were 218 girls aged 5-10 years old (mean age= 8.43 yr, SD=1.12 yr) taking part in the 2010 baseline survey of the ChiBS project. Blood samples were only taken in 166 girls. The ChiBS project, a study embedded within the European IDEFICS study (Ahrens W. et al., 2011a), investigates the relationship between chronic psychosocial stress and changing body composition in children living in Aalter (Belgium) over a 2-year follow-up period (2010-2012). In this study, also the utility of hair samples as biological matrix for measurements of stress and mineral status in children is examined. More detailed research goals, methodology and participation characteristics are described elsewhere (Michels N. et al., 2012d). Agreement for participation was obtained through parental written informed consent. The ChiBS project was conducted according to the guidelines laid down in the Declaration of Helsinki and was approved by the Ethics Committee of the Ghent University Hospital.

2.2 Physical examinations

Physical examinations were performed in accordance with the standardized procedures of the IDEFICS project (Ahrens W. et al., 2011a; Stomfai S. et al., 2011; De Henauw S. et al., 2011). Weight and height were measured in bare feet and light underwear with an electronic scale (TANITA BC 420 SMA, TANITA Europe GmbH, Sindelfingen, Germany) and a stadiometer (Seca 225, SECA GmbH & Co. KG., Hamburg, Germany) to the nearest 0.1 kg and 0.1 cm, respectively. Age- and sex- specific body mass index (BMI) z-scores were calculated and categorized according to the International Obesity Task Force guidelines (BMI=weight (kg)/height (m^2)) (Cole T. J. et al., 2000). Triceps and subscapular skinfold thicknesses were measured twice with a Holtain skinfold calliper (Holtain Ltd., Croswell, Crymych, UK) and used to calculate body fat percentage (BF%) with the skinfold equations of Slaughter et al. (Slaughter M. H. et al., 1988). Systolic and diastolic blood pressure (SBP and DBP, respectively) were measured on the right arm of the child in sitting position using an electronic sphygmomanometer (Welch Allyn 4200B-E2, Welch Allyn Inc., Skaneateles Falls, NY, USA) (Alpert B. S., 2007). Two consecutive measurements were taken at a 2-min

interval. If these measurements differed more than 5%, a third measurement was performed. Means of consecutive measurements were used in analyses.

2.3 Blood analysis

Blood sampling was performed following the standardized procedures of the IDEFICS project for which detailed procedures are described elsewhere (Peplies J. et al., 2011). Blood glucose, total cholesterol, high density lipoprotein (HDL) cholesterol and non-HDL cholesterol (i.e., total cholesterol minus HDL-cholesterol) were assessed in capillary (N=6) or venous (N=160) blood samples after an overnight fast by point-of-care analysis using a Cholestech LDX device (Cholestech, Hayward, CA, USA) (Panz V. R. et al., 2005). Serum insulin was measured by a luminescence immunoassay technique using an AUTO-GA Immo 2000. Insulin resistance, defined by the homeostasis model assessment for insulin resistance (HOMA-IR) (Matthews D. R. et al., 1985), was calculated from fasting glucose and serum insulin via a standard formula: HOMA-IR = [insulin (μUI/ml) × glucose (mg/dl)]/ 405.

2.4 Metabolic score

A continuous metabolic score was calculated with higher scores being indicative for a more unhealthy metabolic profile. Metabolic parameters included in the calculation of the metabolic score were SBP, DBP, HOMA-IR and non-HDL cholesterol (Camhi S. M. et al., 2010). Age-specific z-scores were calculated for each metabolic parameter, after which the parameter-specific z-scores were summed to obtain an age-specific continuous score of clustered metabolic risk. For these calculations, each year of age was considered as one age group. As only two children had the age of 5, they were included in the age group of 6 year olds, resulting in the following distribution for the age groups of 6, 7, 8, 9 and 10 year olds respectively: N=22, N=55, N=66, N=53 and N=22. Metabolic health was also studied categorically by summing the number of metabolic parameters with a z-score above their age-specific 75th percentile (resulting in a total number of 0 to 4 metabolic risk parameters). Children with a sum of three and four metabolic risk parameters were categorized in a group of '≥3 metabolic risk parameters (because only three children were classified in category 4). Number of children with a total of zero, one, two and more than or equal to three metabolic risk parameters are N=62, N=53, N=25 and N=11 respectively.

2.5 Hair mineral analysis

Hair samples were obtained from all participating girls from the vertex posterior region of the scalp (one sample per person). To guarantee that the same time period was investigated in all children, only the most proximal 6 cm of the hair strands were analysed. To ascertain the required hair length, hair samples were exclusively taken from girls. None of the hair samples was artificially coloured. The samples were stored in a folded piece of paper in individual zip-lock bags in a dark, dry place and at constant temperature until analysis in the Department of Analytical Chemistry of Ghent University. The hair contents of Ca, Cu, Fe, Mg, P and Zn were quantitatively determined via inductively coupled plasma - mass spectrometry (ICP-MS), after microwave-assisted acid digestion of the samples. Detailed procedures and validation data of the applied ICP-MS method are described elsewhere (Vanaelst B. et al., 2012e). The Ca/Mg, Ca/P, Fe/Cu and Zn/Cu ratio were calculated, as suggested in literature to be relevant (Wang C. T. et al., 2005b; Bialkowska M. et al., 1987; Park S. B. et al., 2009).

2.6 Other variables

Within the ChiBS and the IDEFICS project, information was collected on parental education (i.e., International Standard Classification of Education (UNESCO, 1997)) and children's physical activity using a self-administered parentally reported questionnaire. Physical activity was studied as the hours of doing sports in a sports club, which has been shown to correlate to moderate and vigorous physical activity as measured by accelerometers (Burdette H. L. et al., 2004; Verbestel V. et al., 2012).

2.7 Statistical analysis

Statistical analyses were performed with PASW Statistical software version 19.0.0 (SPSS Inc., IBM, USA). P-values <0.05 were considered statistically significant for all tests. Data were presented as mean, SD and median. Insulin, glucose, HOMA-IR, non-HDL cholesterol, BF% and BMI z-scores were skewed and, therefore, logarithmically transformed before z-score calculation. Spearman correlation analyses were performed to examine the correlation between hair mineral concentrations and metabolic parameters, while Kruskal-Wallis tests were applied to investigate differences between groups. Boxplots were created to further

illustrate the relationship between hair minerals and the sum of metabolic risk parameters. As hair minerals were not related to socioeconomic status (i.e., parental education) and physical activity level of the child, and regression models indicated that these variables were no significant predictors in the relationship between hair minerals (dependent variable) and metabolic parameters (independent variable) (data not shown), parental education and physical activity were not included into further analyses.

3 Results

Table 3.18 presents anthropometric and metabolic characteristics of the participants, as well as an overview of their hair mineral concentrations. DBP, HOMA-IR and the metabolic score significantly increased from under- to normal to overweight/obese children, while HDL cholesterol decreased over these BMI categories. Hair minerals did not differ over the BMI categories.

However, hair minerals were significantly correlated with BMI z-scores, BF% and other metabolic parameters, as presented in Table 3.19. Hair Ca, the Ca/Mg ratio and the Ca/P ratio were positively correlated with BMI z-scores, and hair Ca and Ca/Mg also positively with BF%. Inverse correlations were observed between Ca, Mg and the Ca/P ratio and non-HDL cholesterol and the metabolic score indicating lower hair mineral concentrations with an increasing metabolic risk. Also DBP and HOMA-IR were inversely correlated with hair minerals, i.e. Ca/P ratio, and Mg and P respectively.

Figure 3.9 graphically presents the above-mentioned inverse relationship between Ca, Mg, the Ca/P ratio and unhealthy metabolic profile in girls. Girls with a sum of 3 or more metabolic risk parameters above the age-specific 75th percentile (for SBP, DBP, HOMA-IR and non-HDL cholesterol) had significantly decreased Ca, Mg and Ca/P concentrations.

Table 3.18 Anthropometric and metabolic parameters and hair minerals for all participating children and according to BMI categories

	all children N=218				underweight N=29				normal weight N=164				overweight and obese N=25				
	Mean	SD	Median	N	Mean	SD	Median	N	Mean	SD	Median	N	Mean	SD	Median	N	p-value*
Age (years)	8	1	8	218	9	1	8	29	8	1	8	164	8	1	9	25	0.654
Anthropometric parameters																	
Height (cm)	131	9	131	218	130	9	130	29	131	8	131	164	133	7	133	25	0.304
Weight (kg)	28	6	27	218	23	3	22	29	28	5	27	164	37	6	37	25	**<0.001**
BMI (kg/m²)	16	2	16	218	13	1	14	29	16	1	16	164	21	2	20	25	**<0.001**
Body fat (%)	18	6	17	214	13	2	12	29	17	4	17	160	30	6	31	25	**<0.001**
Metabolic parameters																	
SBP (mmHg)	103	8	103	214	100	10	101	28	103	7	103	161	106	7	105	25	0.055
DBP (mmHg)	65	6	66	214	64	7	64	28	65	5	65	161	67	6	69	25	**0.035**
HOMA-IR	1.2	0.9	0.9	158	0.9	0.7	0.7	21	1.1	0.7	0.9	120	2.1	1.2	1.6	17	**<0.001**
HDL-cholesterol (mg/dl)	60	14	61	164	67	14	63	21	60	14	59	125	56	15	52	18	**0.031**
Non-HDL cholesterol (mg/dl)	106	25	103	165	109	27	109	21	105	25	102	125	113	26	110	18	0.415
Metabolic score	-0.1	2.7	0.3	151	-1.0	3.4	-0.5	20	-0.2	2.4	0.0	114	2.0	2.9	1.6	17	**0.007**
Physical activity (hours/week)	16	11	14	202	18	14	13	28	15	10	15	150	19	13	14	24	0.495
Hair minerals (µg/g)																	
Ca	671	508	574	217	486	370	374	29	667	445	603	164	898	856	588	25	0.056
Cu	24	18	18	218	23	21	16	28	25	16	19	164	24	23	17	25	0.318
Fe	9	15	7	218	8	3	7	28	10	18	7	164	9	5	8	25	0.947
Mg	33	21	27	217	25	16	22	29	34	21	29	164	37	30	25	25	0.075
P	140	23	140	217	140	31	140	28	140	22	138	164	142	18	141	25	0.592
Zn	224	57	219	218	230	68	221	28	222	53	218	164	231	69	226	25	0.861
Ca/Mg	21	8	19	217	19	6	17	29	20	8	19	164	24	10	20	25	0.231
Ca/P	5	4	4	217	3	3	3	29	5	3	4	164	6	6	4	25	0.068
Fe/Cu	0.5	0.5	0.4	218	0.5	0.2	0.4	28	0.5	0.5	0.4	164	0.5	0.3	0.5	25	0.245
Zn/Cu	13	7	12	218	15	8	14	28	12	6	12	164	14	11	12	25	0.221

BMI categories based on z-scores and method of Cole. Metabolic score includes SBP+DBP+HOMA-IR+non-HDL. Values were rounded off to the measurement unit except for HOMA-IR, the metabolic score, and Fe/Cu (low values).
SBP: systolic blood pressure; DBP: diastolic blood pressure; HOMA-IR: homeostasis model assessment for insulin resistance; HDL: high density lipoprotein; non-HDL cholesterol: total cholesterol minus HDL cholesterol; * Kruskal Wallis

Table 3.19 Spearman correlations between anthropometric and metabolic parameters (age-specific z-scores) and hair minerals

	Anthropometric parameters				**Metabolic parameters**											
	BMI N=218		BF% N=213		SBP N=213		DBP N=213		HOMA-IR N=158		non-HDL cholesterol N=164		metabolic score N=151	
	r*	p	r*	p	r*	p	r*	p	r*	p	r*	p	r*	p
Hair minerals														
Ca	**0.176**	**0.009**	**0.147**	**0.032**	-0.017	0.809	-0.107	0.119	-0.108	0.177	**-0.169**	**0.030**	**-0.192**	**0.018**
Cu	0.004	0.957	-0.002	0.98	-0.028	0.681	-0.019	0.786	-0.057	0.476	-0.104	0.185	-0.103	0.208
Fe	0.092	0.176	0.052	0.453	-0.059	0.388	-0.070	0.312	-0.051	0.525	0.119	0.130	-0.029	0.728
Mg	0.100	0.144	0.063	0.363	-0.067	0.327	-0.128	0.062	**-0.183**	**0.021**	**-0.170**	**0.030**	**-0.257**	**0.001**
P	0.085	0.209	0.116	0.09	-0.012	0.856	0.128	0.062	**-0.190**	**0.017**	-0.075	0.341	-0.077	0.348
Zn	0.012	0.862	0.025	0.719	-0.074	0.282	-0.018	0.796	-0.146	0.068	-0.087	0.269	-0.117	0.154
Ca/Mg	**0.190**	**0.005**	**0.198**	**0.004**	0.070	0.309	-0.036	0.605	0.107	0.181	-0.072	0.361	0.037	0.655
Ca/P	**0.158**	**0.020**	0.121	0.078	-0.022	0.752	**-0.139**	**0.043**	-0.058	0.467	**-0.160**	**0.040**	**-0.176**	**0.030**
Fe/Cu	0.071	0.297	0.056	0.416	-0.035	0.606	-0.030	0.662	0.014	0.865	0.133	0.090	0.036	0.661
Zn/Cu	-0.012	0.861	-0.012	0.864	0.008	0.905	0.009	0.895	-0.040	0.622	0.076	0.334	0.042	0.609

BMI: Body Mass Index; BF%: body fat percentage; SBP: systolic blood pressure; DBP: diastolic blood pressure; HOMA-IR: homeostasis model assessment for insulin resistance; HDL: high density lipoprotein; non-HDL cholesterol: total cholesterol minus HDL cholesterol; The metabolic score includes SBP+DBP+HOMA-IR+non-HDL; r*: spearman correlation coefficient

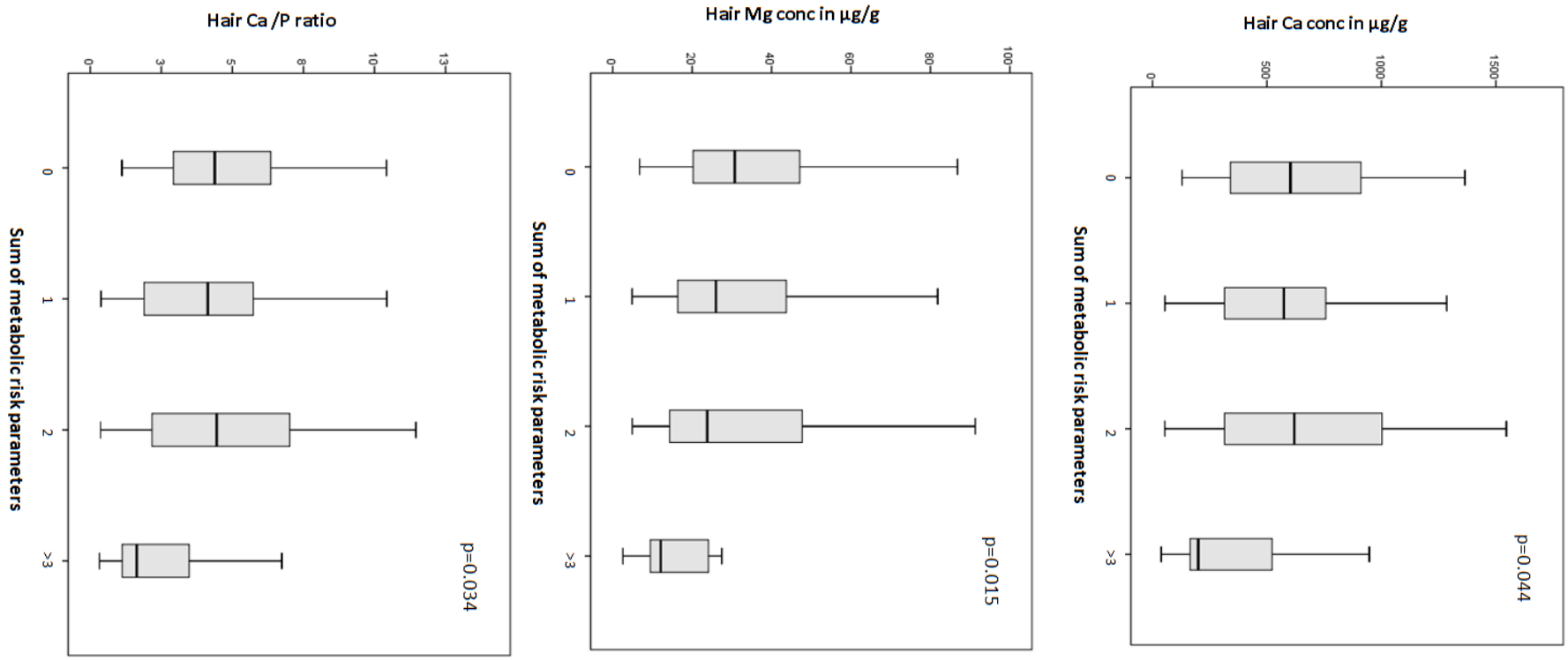


Figure 3.9

Graphical presentation of reduced hair Ca, Mg and Ca/P ratio in children with a more unhealthy metabolic profile. Metabolic health was studied categorically by summing the number of metabolic parameters with a z-score above their age-specific 75th percentile (resulting in a maximal sum of 4). The higher and lower ends of the boxes represent the Q3 and Q1 of the hair mineral concentrations. The Q2 is indicated within the boxes. The whiskers represent the concentration range, excluding outliers and extremes. (Q=quartile, p= p value of Kruskal-Wallis test)

4 Discussion

This study was the first to investigate long-term levels of Ca, Cu, Mg, Fe, P and Zn in scalp hair (and ratios thereof) in relation to overweight/obesity and metabolic health in children, more specifically in a population of Flemish elementary school girls.

Overweight and obese girls presented a higher blood pressure (DBP), reduced insulin sensitivity, reduced HDL-cholesterol and a higher metabolic score (i.e. a more unhealthy metabolic profile) (Table 3.18). Based on these findings, overweight and obesity may thus contribute to an increased metabolic risk in young children (Ho T. F., 2009; Reilly J. J. et al., 2011).

Our observations did not confirm the first research question of an inverse relationship between hair minerals, i.e. Ca, Cu, Mg, Fe, P and Zn, and obesity in this young age group. Positive correlations were observed for hair Ca, Ca/Mg and Ca/P with BMI z-scores and BF% (BF% not with Ca/P) (Table 3.19), but the hair minerals did not vary over the BMI categories (Table 3.18). Our findings are thus inconsistent with the central idea that overweight and obese children are at increased risk for mineral deficiencies due to, e.g., an unbalanced diet (Nead K. G. et al., 2004; Celik N. et al., 2011; Huerta M. G. et al., 2005; Marreiro D. D. et al., 2002; Perrone L. et al., 1998; Kaidar-Person O. et al., 2008), or that mineral deficiencies may contribute to the development of obesity (e.g. Ca deficiency may increase the risk of obesity) (Vaskonen T., 2003; Garcia O. P. et al., 2009). However, as the number of children in the underweight group was higher than the number of overweight and obese children, the observed positive correlations could be interpreted in the context of undernourishment and the presentation of an extremely low mineral status in the underweight children, affecting the direction of the relationship between BMI z-scores, BF% and hair mineral status. Also for the normal weight group, we may hypothesize that diet and energy intake increases with elevating BMI/BF% and that consequently also the mineral intake may rise with increasing BMI/BF%, resulting in positive associations with hair Ca, Ca/Mg and Ca/P, independent from the BMI category of the child. Several explanations for the role of Ca in the regulation of adiposity have been proposed: Dietary Ca may form soaps with fatty acids and thereby prevent their absorption during lipid digestion; it may bind bile acids and reduce lipid absorption; it may increase faecal fat excretion; and it may affect calcitriol-mediated processes involved in thermogenesis and fat deposition (Vaskonen T., 2003; Garcia O. P. et al., 2009). Although childhood obesity has, to our knowledge, not yet been associated with Ca deficiencies, inverse

associations between increased Ca intake and body weight have been observed (Garcia O. P. et al., 2009), also in female children and adolescents (Tylavsky F. A. et al., 2010); thus further contradicting our findings. As this study is the first to examine hair minerals in relation to childhood body composition, our results should be confirmed and the role of minerals in body composition regulation should be further investigated.

Our second research question was partly confirmed: Ca, Mg, P, Ca/Mg and Ca/P levels were inversely related to the occurrence of metabolic risk parameters in the studied girls (Table 3.19) which is in agreement with previous findings (Park S. B. et al., 2009; Skalnaya M. G. et al., 2007), but for the minerals Cu, Fe and Zn no relationship with anthropometric or metabolic parameters was observed, contradictory to other reports (Suliburska J. et al., 2011; Bialkowska M. et al., 1987; Vivoli G. et al., 1987; Fernandez-Britto J. E. et al., 1993; Wang C. T. et al., 2005a; Wang C. T. et al., 2005b; Gonzalez-Reimers E. et al., 2008). In particular girls with three or more metabolic risk parameters showed significantly reduced hair concentrations of Ca, Mg and the Ca/P ratio (Figure 3.9). The inverse relationship between hair Ca and Mg with non-HDL cholesterol strengthens the proposed hypolipidemic effects of Ca and Mg (assumed to operate through inhibition of intestinal absorption of cholesterol, bile acids and fats) (Vaskonen T., 2003) and our data additionally confirm the association between Mg deficiency and insulin resistance during childhood (Mg regulates insulin action through influencing tyrosine kinase activity, and is involved in glucose transportation across membranes) (Celik N. et al., 2011; Huerta M. G. et al., 2005).

Currently there is insufficient evidence to consider hair mineral analysis as clear-cut diagnostic media for the dietary mineral intake. Even though our research group has indicated the influence of food consumption frequencies on hair mineral variation in elementary school girls, specific mineral-rich food groups were not associated with the studied hair minerals (Vanaelst B. et al., 2012e). Therefore, the observed inverse relationship between hair minerals (i.e. Ca, Mg and Ca/P) and metabolic risk should not only be considered in the view of an unbalanced dietary intake but also in the view of an altered mineral metabolism associated with metabolic conditions. Multi-element hair analyses have been proven useful in detecting deviations from homeostasis or changes between groups of individuals with a different health status over a long period retrospectively (World Health Organization et al., 1996; Hunter D., 1990). This study has affirmed the recommendation to not only focus on excessive energy intake but also on deficient mineral intakes in childhood obesity research. Also, based on our

observations, a different imbalance and regulation of essential minerals may be involved in obesity compared to general metabolic risk in children.

4.1 Strengths and limitations

To our knowledge, this study was the first to investigate long-term mineral concentrations in scalp hair to correlate them to anthropometric and metabolic health in children. Application of the ICP-MS technique, which is the state of the art to measure hair mineral concentrations, and performance of anthropometric and metabolic risk measurements according to the standardized procedures of the IDEFICS project are the strongest methodological features of this study. However, some limitations should be considered when interpreting results. As our population only consisted of females in a small age range, analyses should be repeated in a more heterogeneous population sample in order to formulate conclusions also for boys and older children (e.g., adolescents). Also, the cross-sectional design of this study does not allow to investigate the direction of causality in the association between (hair) minerals and obesity and cardiovascular or metabolic health. Last, further validation of hair minerals as bio-indicators for dietary intake would enlarge comprehension in the field and broaden its use in nutritional epidemiology.

5 Conclusions

This study strengthened previous indications of an inverse relationship between essential minerals in hair (i.e., Ca, Mg, Ca/P) and metabolic risk, now observed within young girls. In general, we can conclude that the reduced hair mineral levels may contribute to or result from a poorer metabolic health in young girls. Future research should investigate the relationship between hair minerals and body composition in a large-scale population of healthy, young children in order to explain the unexpected positive associations between hair minerals (i.e., Ca, Ca/Mg, Ca/P) and anthropometry (BMI and BF%) in young girls, as observed in this study.

CHAPTER 3.7 CROSS-SECTIONAL RELATIONSHIP BETWEEN CHRONIC STRESS AND MINERAL CONCENTRATIONS IN HAIR OF ELEMENTARY SCHOOL GIRLS

Abstract

Chronic stress exposure is associated with diverse negative health outcomes. It has been hypothesized that stress may also negatively affect the body's mineral status. This study investigates the association between chronic stress and long-term mineral concentrations of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), phosphorus (P) and zinc (Zn) in scalp hair among elementary school girls. Complete information on child-reported stress estimates (Coddington Life Events Scale (CLES)), hair cortisone and hair mineral concentrations, and predefined confounders in the stress-mineral relationship (i.e. age, body mass index (BMI), physical activity, diet, hair colour and parental education) was provided cross-sectionally for 140 girls (5-10yrs old). The relationship between childhood stress measures (predictor) and hair minerals (outcome) was studied using linear regression analysis, adjusted for the abovementioned confounders. Hair cortisone concentrations were inversely associated with hair mineral concentrations of Ca, Mg, Zn and the Ca/P ratio. Children at risk by life events (CLES) presented an elevated Ca/Mg ratio. These findings were persistent after adjustment for confounders. This study demonstrated an independent association between chronic stress measures and hair mineral levels in young girls, indicating the importance of physiological stress-mineral pathways independently from individual or behavioural factors. Findings need to be confirmed in a more heterogeneous population and on longitudinal basis. The precise mechanisms by which stress alters hair mineral levels should be further elucidated.

1 Introduction

Stress is defined as the process in which environmental demands or events are interpreted and appraised by the individual as taxing or exceeding his/her resources, resulting in psychological and biological changes with risk for disease (Cohen S. et al., 1997a). Acute, adaptational stress responses exert temporal and beneficial effects to cope with the stressful situation, while chronic activation of the stress system may adversely affect health with depression, cardiovascular and auto-immune diseases, and psychosomatic complaints as

potential manifestations (Schneiderman N. et al., 2005; Mcewen B. S., 1998). Furthermore, it has been hypothesized that prolonged stress may negatively affect the body's mineral status. Decreased zinc, selenium, iron and magnesium concentrations have been observed in long-term psychological stress, although literature in this regard is scarce (Pizent A. et al., 1999; Singh A. et al., 1991; Takase B. et al., 2004; Cernak I. et al., 2000; Seelig M. S., 1994; Grases G. et al., 2006; Moore R. J. et al., 1993).

The relationship between stress and minerals may operate through a behavioural and a physiological pathway. On the behavioural side, stress has been shown to alter an individual's dietary pattern with an increase in the consumption of energy-dense "convenience foods" (limiting purchasing/preparing time) or "comfort foods" (rich in sugar and fats), positively stimulating sensations of reward and pleasure (Dallman M. F. et al., 2005; Torres S. J. et al., 2007; Rabinovitz S., 2006). This unhealthy food consumption is often at the expense of healthy mineral-rich foods and may lead to obesity and an unbalanced, even deficient dietary mineral intake (Kaidar-Person O. et al., 2008). The physiological pathway operates through activation of the stress system and the production of stress hormones such as cortisol, shifting the body's metabolism to a catabolic state, thereby increasing oxidative stress and increasing the need for anti-oxidants (e.g. minerals for enzyme function) (Costantini D. et al., 2011; Vertuani S. et al., 2004). Moreover, stress has been associated with common gastro-intestinal disorders and inhibition of nervus vagus activation, perturbing gastric emptying, gastroduodenal/colonic motility and intestinal transit (Kiecolt-Glaser J. K., 2010; Yin J. et al., 2004; Mayer E. A., 2000). As a consequence, nutrients may be less efficiently digested, absorbed and metabolized. Stress hormones may also directly affect mineral absorption, distribution or excretion (e.g. cortisol influences the parathyroid hormone and renal calcium handling, together affecting calcium homeostasis) (Heshmati H. M. et al., 1998). Summarized, stress may hinder an adequate mineral intake, increase the body's need for minerals through changes in metabolism or redistribute the minerals to tissues with higher requirements, although more detailed mechanisms need to be explored.

The last decade has been characterized by an increased interest in the study of stress and its adverse health effects on young children (Teicher M. H. et al., 2003; Michels N. et al., 2012c; Vanaelst B. et al., 2012a; Washington T. D., 2009). In the field of stress and mineral status however, no studies have been undertaken in children. From a methodological point of view, scalp hair may offer opportunities compared to other biological matrices (such as serum or urine) for the study of the stress-mineral relationship in children. Particularly for 'longer-

term' studies the hair matrix may be recommended as it is non-invasive and it presents a retrospective window of stress hormones and mineral levels in the past (Kempson I. M. et al., 2011; Meyer J. S. et al., 2012). Nevertheless, this stress-mineral relationship has not been studied in scalp hair before. This study therefore investigates the association between chronic stress and long-term mineral concentrations of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), phosphorus (P), zinc (Zn) and ratios thereof in scalp hair of elementary school girls by using child-reported stress estimates (life events) and stress hormone measurements in scalp hair. We hypothesize that stress as physiological event may be negatively associated with hair minerals levels in children, independently from individual and behavioural factors such as the child's age, BMI, physical activity, dietary habits, socio-economic status and hair colour (Vanaelst B. et al., 2012e; Chojnacka K. et al., 2010b; Chojnacka K. et al., 2006; Wang C. T. et al., 2005a; Nielsen F. H. et al., 2006; Huybrechts I. et al., 2011b; Montain S. J. et al., 2007).

2 Methodology

2.1 Study participants

140 girls aged 5-10 years old (Mean age=8.46 yr, SD= 1.11 yr) participated in this study as part of the baseline survey of the ChiBS project (Children's Body composition and Stress) (February-June 2010, N=523). The ChiBS project, a study embedded within the European IDEFICS study (Identification and prevention of Dietary- and lifestyle-induced health EFfects In Children and infants) (Ahrens W. et al., 2011a), investigates chronic psychosocial stress and changing body composition in children over a two-year follow-up period (2010-2012), but also examines the utility of hair samples as biological matrix for measurements of chronic stress and mineral status in children (Michels N. et al., 2012d). More detailed research goals, methodology and participation characteristics of the ChiBS project are described elsewhere (Michels N. et al., 2012d).

In this study, the population was limited to the female participants of the ChiBS project, as one of the survey modules, specifically hair sampling, was only performed in girls in order to obtain the required hair length of 6 cm (N=264/523 or 50.5%, Figure 3.10). Parents were asked to sign a consent form in which the option was offered to participate in the full ChiBS programme or in a selected set of measurement modules, resulting in distinct participation

numbers for the different measurement modules (Figure 3.10). Hair samples were obtained from the vertex posterior region of the scalp from 223 girls and hair colour noted. Only the most proximal 6 cm of the hair strands were analysed which is, based on an average hair growth rate of 1 cm per month, representative for a period of 6 months in the past (Harkey M. R., 1993). Each hair sample obtained was split into two fractions and sent to expert-laboratories for determination of cortisone and mineral levels, respectively. For 5 hair samples, the amount of hair was insufficient to split into two fractions, resulting in 218 samples available for hair mineral analysis (more details below). In addition, children were subjected to routine anthropometric measurements and questionnaire administration (e.g. stressful life events, dietary habits etc.) at the same time point as hair sampling, at the children's schools. Data for all measurement modules was completed for 140 girls (Figure 3.10). No differences were observed between girls with complete data (i.e. N= 140) and girls with missing data (i.e. N=223-140=83) for socio-demographic variables (i.e. age, BMI, parental education), hair mineral concentrations (except for P which was higher in the group with complete data) and stress measures (i.e. hair cortisone and negative event score) (Mann-Whitney U test – Fisher's Exact Test). The ChiBS project was conducted according to the guidelines laid down in the Declaration of Helsinki and was approved by the Ethics Committee of the Ghent University Hospital.

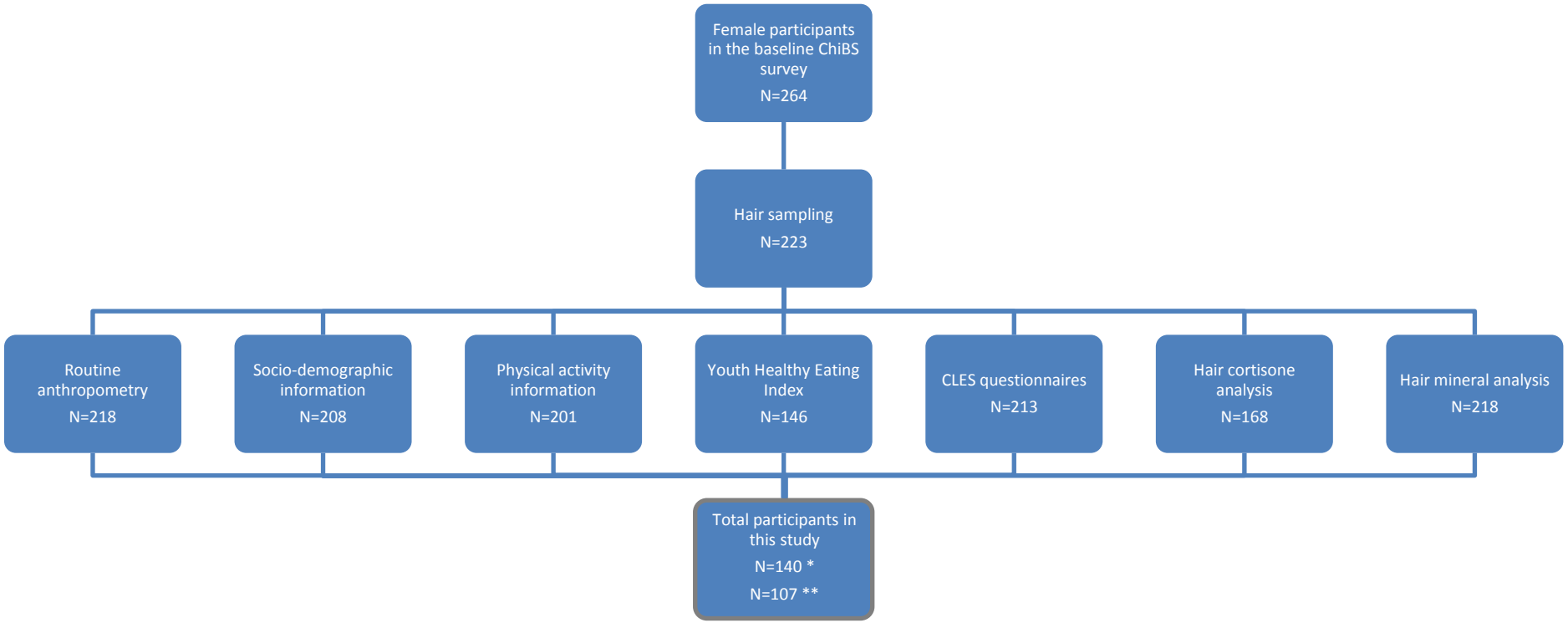


*Figure 3.10 Flowchart of study participants. CLES: Coddington Life Events Scale; Socio-demographic information: International Standard Classification of Education; *Total number of participants if questionnaires (CLES questionnaire or emotion questionnaire) are used as stress measurement; **Total number of participants if hair cortisone concentrations are used as stress measurement*

2.2 Hair mineral analysis

The hair contents of Ca, Cu, Fe, Mg, P and Zn were quantitatively determined via inductively coupled plasma - mass spectrometry (ICP-MS), after microwave-assisted acid digestion of the samples in the Department of Analytical Chemistry of Ghent University. Detailed procedures and validation data of the ICP-MS method applied are described elsewhere (Vanaelst B. et al., 2012e). The Ca/Mg, Ca/P, Fe/Cu and Zn/Cu ratios were calculated, as they were suggested in literature to be relevant mineral ratios (Wang C. T. et al., 2005b; Bialkowska M. et al., 1987; Park S. B. et al., 2009).

2.3 Childhood stress measures

A. Coddington Life Events Scale (CLES)

To investigate the relationship between childhood psychosocial stress and hair mineral concentrations, estimates of child-reported stress were obtained through a questionnaire on stressors or life events. Completion of the questionnaires was assisted by trained interviewers. The Coddington Life Events Scale for children (CLES-C) is a validated and well-established 36-item questionnaire, which measures the frequency and timing of both positive and negative life events relevant for this age group during the last year (four trimesters). By measuring significant life events in terms of life change units, the CLES-C can provide insight into recent events that may be affecting the child's health and results in a 'life change units' score (Coddington R. D., 1972; Villalonga-Olives E. et al., 2008). Children with a score above the age-specific cut-off are considered to be at higher risk to suffer from psychological problems. For this study, a 'negative life events score' and the proportion of children at risk were calculated only for the last 6 months to correspond with the 6 cm hair samples.

B. Hair Cortisone Analysis

Cortisone is a hormone that is only minimally produced by the adrenals but mainly originates from cortisol metabolism, more specifically from the conversion of cortisol into cortisone by 11β-HSD2 (hydroxysteroid dehydrogenase). Cortisone may therefore serve as an additional biomarker for stress research, as exemplified by the involvement of 11β-HSD activity in stress (Welberg L. A. M. et al., 2005; Altuna M. E. et al., 2006; Romer B. et al., 2009; Plenis

A. et al., 2011; Yehuda Rachel et al., 2009). Our research group has previously correlated elevated hair cortisone concentrations with negative life events in the same study sample of elementary school girls (Vanaelst B. et al., 2013a). Therefore, in this study, hair cortisone concentrations were measured as a biological measure of stress. Cortisone was analysed in the most proximal 6 cm of the same hair samples in which minerals were analysed at the Department of Toxicology, Institute of Legal Medicine, Strasbourg University with Ultraperformance Liquid Chromatography-tandem Mass Spectrometry (UPLC-MS/MS). Detailed procedures and validation data of the applied UPLC-MS/MS method are described elsewhere (Vanaelst B. et al., 2013e).

2.4 Other variables

Within the ChiBS and the IDEFICS project, information was collected on parental education (categorized using the International Standard Classification of Education (ISCED) (UNESCO, 1997)) and children's physical activity using a self-administered parentally reported questionnaire. Physical activity was studied as the hours of playing outdoors and doing sports in a sports club, which has been shown to correlate to moderate and vigorous physical activity as measured by accelerometers (Burdette H. L. et al., 2004; Verbestel V. et al., 2012). Children's dietary habits were assessed using the self-administered parental questionnaire 'Children's Eating Habits Questionnaire - Food Frequency Questions' (CEHQ-FFQ). The CEHQ-FFQ is a 43 food item-containing questionnaire developed and validated within the IDEFICS project (Lanfer A. et al., 2011; Huybrechts I. et al., 2011a) and is used as a screening instrument to investigate dietary habits and food consumption frequency in children. Based on these food consumption frequencies, a Youth Healthy Eating Index (YHEI) (Gwozdz W. et al., 2012) was calculated based on Feskanich et al. (Feskanich D. et al., 2004) with higher scores signalling healthier diets. Also standardized routine anthropometric measurements (electronic scale Tanita BC 420 SMA, Tokyo, Japan; stadiometer Seca 225, Birmingham, UK) were performed and used to calculate Body Mass Index (BMI) z-scores (BMI=weight(kg)/height(m^2)) (Bammann K. et al., 2011).

2.5 Statistical analysis

Statistical analysis was performed using the statistical programme PASW version 19.0 (SPSS Inc., IBM, IL, USA). The two-sided level of significance was set at $p<0.05$. To study the

association between childhood stress and hair mineral concentrations, multiple linear regression analysis was performed. The logarithmically transformed hair mineral concentrations and mineral ratios were used as dependent (outcome) variables in 2 models investigating another childhood stress measure: hair cortisone concentrations and the CLES score were consecutively used as independent variables. After performing these analyses without adjustment for confounder variables, the regression analysis was repeated with adjustment for age, BMI z-score, physical activity, YHEI, hair colour and parental education, as these factors have been shown to be associated with hair mineral levels (Wang C. T. et al., 2005a; Vanaelst B. et al., 2012e; Chojnacka K. et al., 2010b; Chojnacka K. et al., 2006; Nielsen F. H. et al., 2006; Montain S. J. et al., 2007; Huybrechts I. et al., 2011b). No multicollinearity between these variables was shown. As the sample size for regression analysis was limited to the children from whom complete information on all variables studied was available (N=140 and N=107, figure 3.10), post-hoc power analysis was performed according to Faul et al. using G*Power 3 (Faul F. et al., 2007; Faul F. et al., 2009). A sample size of 140 and 107 children in a regression model with 7 predictors to detect a medium effect size and with a probability level of 0.05 resulted in a power of 0.92 and 0.80, respectively. Box-plots were created to graphically present significant differences in hair mineral concentrations between children at risk and not at risk for psychological problems by stressful life events in the last 6 months (non-parametrical Mann-Whitney U-tests with non-transformed data mineral concentrations). Also, this stress-variable was used as a dummy variable (at risk versus not at risk) in regression analysis.

3 Results

Descriptive results of the participants' socio-demographic characteristics, hair mineral concentrations and stress measures are presented in Table 3.20.

Table 3.20 Personal characteristics of the participating elementary school girls (N=140)

	Median	**P25**	**P75**
Age	8	8	9
BMI Z-score	-0.19	-0.87	0.66
Physical activity (hours/week)	14	10	19
Youth healthy eating index (score 0-80)	49	42	55
Hair mineral concentrations (µg/g)			
Ca (N=139)	586	293	852
Cu	18	14	30
Fe	7	6	9
Mg (N=139)	28	18	46
P	141	128	155
Zn	222	200	249
Hair cortisone concentration (pg/mg) (N=107)	9	7	11
CLES negative event score (last 6 months)	29	0	53
	N	**%**	
CLES at risk by events last 6 months	24	17.1	
Maximal parental education			
ISCED level 2 and 3	44	31.5	
ISCED level 4	24	17.1	
ISCED level 5	72	51.4	
Hair colour			
blond	7	5	
red	2	1.4	
brown	102	72.9	
dark brown	27	19.3	
black	2	1.4	

Values were rounded off to the measurement unit except for BMI Z-scores.
ISCED= International Standard Classification of Education, 2 'lower secondary education', 3 'upper secondary education', 4 'post-secondary non-tertiary education', 5 'first stage of tertiary education'

Results of regression analysis are described in Table 3.21. Hair cortisone concentrations were inversely associated with hair mineral levels of Ca, Mg, Zn and Ca/P, both with and without adjustment for the confounder variables. These findings indicate reduced levels of certain minerals with increased childhood stress, independently from the child's age, BMI, physical activity, diet, hair colour and parental education.

Table 3.21 Linear regression models investigating the relationship between stress and hair mineral levels in elementary school girls

	Dependent variable*																			
	hair Ca**		hair Cu		hair Fe		hair Mg**		hair P		hair Zn		hair Ca/Mg		hair Fe/Cu		hair Zn/Cu		hair Ca/P	
	B	p	B	p	B	p	B	p	B	p	B	p	B	p	B	p	B	p	B	p
Independent variables Model hair cortisone (N=107)																				
not adjusted	**-0,007**	**0,028**	0,001	0,834	0,003	0,235	**-0,007**	**0,019**	0,001	0,545	**-0,003**	**0,031**	0,000	0,793	0,003	0,389	-0,003	0,254	**-0,008**	**0,010**
adjusted for age and BMI	**-0,008**	**0,013**	0,000	0,897	0,003	0.229 [a]	**-0,006**	**0,027**	0,000	0,655	**-0,003**	**0,031**	-0,002	0.31 [ac]	0,003	0.353 [a]	-0,003	0,285	**-0,009**	**0,005**
adjusted for age, BMI, PA, YHEI, hair colour and ISCED	**-0,009**	**0.007 [d]**	0,001	0,718	-0,004	0.147 [a]	**-0,008**	**0.009 [d]**	0,000	0.649 [d]	**-0,003**	**0,025**	-0,002	0.377 [ac]	0,003	0.319 [ab]	-0,004	0.179 [b]	**-0,010**	**0,003**
Model CLES negative event score (N=140)																				
not adjusted	0,000	0,704	0,000	0,795	0,000	0,663	-0,001	0,118	0,000	0,058	0,000	0,093	**0,001**	**0,042**	$-6.74*10^{-5}$	0,901	0,000	0,675	$6.06*10^{-5}$	0,913
adjusted for age and BMI	0,000	0,656	0,000	0,679	$9.92*10^{-5}$	0.816 [a]	-0,001	0,150	0,000	0,055	0,000	0,074	0,001	0.082 [c]	0,000	0.587 [a]	0,000	0,754	$1.92*10^{-5}$	0,973
adjusted for age, BMI, PA, YHEI, hair colour and ISCED	$6.55*10^{-5}$	0,916	$-1.04*10^{-5}$	0,983	$3.93*10^{-5}$	0.930 [a]	-0,001	0,277	0,000	0.283 [d]	0,000	0.088 [b]	0,001	0.075 [ce]	$4,975*10^{-5}$	0.928 [ab]	0,000	0.5 [b]	0,000	0,869
Model CLES at risk (N=140)																				
not adjusted	-0,007	0,913	0,043	0,427	0,027	0,594	0,080	0,193	0,025	0,129	0,004	0,870	**-0,087**	**0,011**	-0,016	0,804	-0,039	0,48	-0,03	0,615
adjusted for age and BMI	-0,003	0,961	0,050	0,366	0,004	0.928 [a]	0,075	0,231	0,025	0,122	0,005	0,836	**-0,078**	**0.022 [c]**	-0,045	0.474 [a]	-0,045	0,427	-0,029	0,663
adjusted for age, BMI, PA, YHEI, hair colour and ISCED	-0,041	0,572	0,036	0,534	0,009	0.859 [a]	0,042	0,528	0,008	0.645 [d]	0,000	0,985	**-0,083**	**0.021 [e]**	-0,026	0.68 [ab]	-0,036	0.532 [b]	-0,050	0,485

BMI: Body Mass Index z-score; CLES: Coddington Life Events Scale; PA: physical activity; YHEI: Youth Healthy Eating Index; ISCED: International Standard Classification of Education;

*Values were logarithmically transformed, ** for the minerals Ca and Mg the sample size for CLES models is N=139
a age was a significant predictor in the model; b physical activity was a significant predictor in the model; c BMI Z-score was a significant predictor in the model; d hair colour was a significant predictor in the model; e YHEI was a significant predictor in the model

Children at risk for psychological problems by the occurrence of stressful events in the last 6 months demonstrated an elevated Ca/Mg ratio, as presented in Table 3.21 and illustrated in Figure 3.11. The CLES negative events score (as continuous predictor variable) was only associated with the Ca/Mg ratio without adjustment for the individual and behavioural factors (Table 3.21).

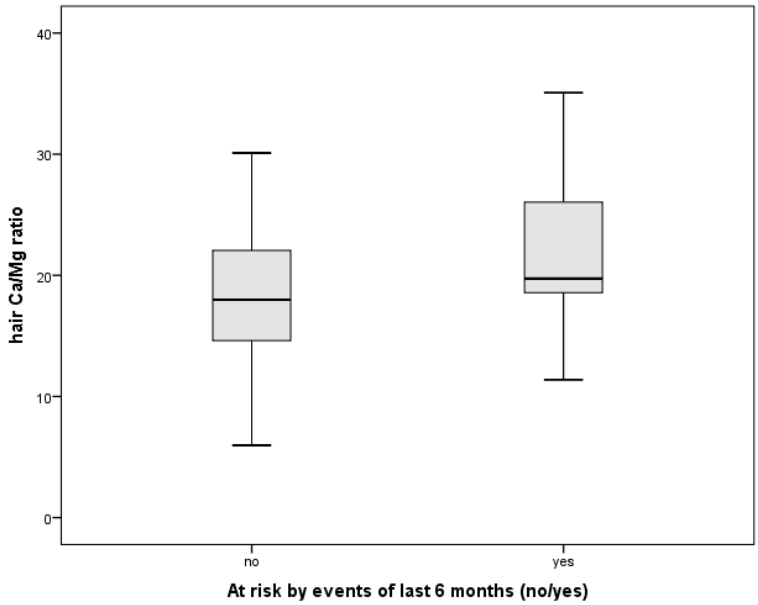


Figure 3.11 Hair Ca/Mg concentrations in children at risk (N=24) and not at risk (N=116) for psychological problems by events of last 6 months. The hair Ca/Mg ratio is higher in children at risk by events of last 6 months (Mann-Whitney U-Test (non-logarithmically transformed data) p=0.016). The higher and lower ends of the boxes represent the Q3 and Q1 of the hair mineral concentrations. The Q2 is indicated within the boxes. The whiskers represent the concentration range, excluding outliers and extremes. (Q=quartile)

Analyses were adjusted for a number of possible confounders (see methodology section). In this study, the following variables were significantly associated with one or more of the dependent variables: (1) age (hair Fe, Fe/Cu and Ca/Mg), (2) BMI Z-score (hair Ca/Mg), (3) physical activity (hair Zn, Fe/Cu and Zn/Cu), (4) hair colour (hair P, Ca and Mg), and (5) the YHEI (Ca/Mg) (Table 3.21).

4 Discussion

This paper was the first to investigate the stress-mineral relationship in children over a long-term in the past, and using scalp hair as biological matrix. We demonstrated an independent

association of chronic stress measures (i.e. hair cortisone and CLES) on hair mineral levels of elementary school girls. As stress-mineral research in scalp hair remained unexplored until now, evaluation of our findings is limited to previous observations in other biological matrices such as serum or urine.

Higher levels of hair cortisone were associated with reduced hair levels of Ca, Mg, Zn and Ca/P, which is in agreement with previous findings in serum and urine (Pizent A. et al., 1999; Singh A. et al., 1991; Takase B. et al., 2004; Grases G. et al., 2006). Since analyses were adjusted for individual and behavioural factors (age, BMI, diet, physical activity, hair colour and parental education), the changes in mineral levels may be ascribed to the unique physiological contribution of increased stress.

Surprisingly however, a relationship between hair mineral concentrations and hair cortisone was observed, while no association was found for the hair minerals with the CLES negative event score (except for the unadjusted relationship with the Ca/Mg ratio). This is unexpected as the CLES score and hair cortisone may be assumed to behave similarly in relation to hair mineral levels based on the following information: (1) environmental stressor exposure (i.e. assessed by CLES questionnaire) is generally linked to a physiological stress response (i.e. assessed by hair cortisone measurements) (Vanaelst B. et al., 2012b; Cohen S. et al., 1997a), (2) our research group previously showed a strong correlation between the CLES questionnaire and hair cortisone concentrations (Vanaelst B. et al., 2013a) and (3) both stress assessment methods represent the same time period in the past (last 6 months). This study may thus indicate that not stressor exposure in general but more specifically the body's physiological stress response is related to hair minerals or mineral metabolism, such as a stress-induced increased excretion of metabolites (such as cortisone and minerals) into hair. Another interpretation could be the more sensitive representation of stress by hair cortisone measurements compared to the child-reported CLES questionnaire, as a result of which the regression analysis with the CLES score may be attenuated compared to the cortisone results. Nevertheless, if CLES life events were studied categorically ('at risk' versus 'not at risk'), a robust relationship was observed with the Ca/Mg ratio, indicating that life events may be associated with minerals only in more extreme cases of repeated stressor exposure, i.e. if the cut-off score for becoming at risk for psychological problems is reached.

Despite the association between stress (particularly cortisone and CLES at risk) and hair mineral levels observed in this study, levels of Cu, Fe and P in hair were not associated with

any of the studied stress estimates, nor with the ‘at risk’ status for psychological problems by stressful events (Table 3.21, Figure 3.11). For Cu, this is in line with previous findings (Singh A. et al., 1991; Pizent A. et al., 1999), while for Fe our results are in disagreement with findings from Singh et al., Moore et al. and Chen et al. (although the latter refers to animal research) (Singh A. et al., 1991; Moore R. J. et al., 1993; Chen J. B. et al., 2009). We may hypothesize that the metabolism or homeostasis of Cu, Fe and P is not, or only to a lesser extent, influenced by stress compared to the other studied minerals.

Although it has been hypothesized that stress may affect the body’s mineral intake or mineral requirements, specific metabolic pathways have largely remained undefined. Nonetheless, some explanations were provided in literature.

Singh et al. partly contributed a decrease in plasma Zn concentrations (in response to stress) to a decrease in Zn-binding proteins (such as albumin) and to a removal of Zn from the circulation by other tissues: glucocorticoids may stimulate hepatic metallothionein synthesis and thereby orchestrate the sequestration of Zn by the liver. No influence of altered urinary Zn excretion was shown by these authors (Singh A. et al., 1991). However, as indicated by Roy et al. (Roy A. et al., 2010), Zn deficiency may activate the hypothalamus-pituitary-adrenal axis, causing glucocorticoid production, suggesting that our observed cortisone-Zn relationship may also operate in the reverse direction.

Reduced Fe levels after stress have been explained by an increase in ferritin concentrations (an intracellular Fe storage protein), indicating a shift from circulating to storage iron (Singh A. et al., 1991). In this context, hair Fe could be considered an excretion or storage pathway. On the other hand, animal studies indicated decreased iron absorption in relation to psychological stress, possibly through changed expression of iron transporters (Chen J. B. et al., 2009).

Grases et al. mainly attributed decreases in Ca and Mg status in response to stress to changes in renal excretion (Grases G. et al., 2006): stress-related cortisol prevents tubular Ca reabsorption mediated by aldosterone, thereby inducing increased urinary Ca. As cortisol inhibits aldosterone activity in renal cells, increases in Mg excretion are also observed. Grases and colleagues associated anxiety to catecholamine production which may also increase urinary Mg excretion and thus lower Mg status in serum and maybe in hair.

Despite the explanations mentioned above, it is clear that further investigations into the effects of psychosocial stress on mineral metabolism are needed. Irrespective of the precise mechanism by which stress is associated with hair minerals (e.g. changes in metabolism, changes in diet), this study has pointed to another health impact of stress exposure, even in young children.

4.1 Strengths, limitations and future research

This study investigated the association between childhood stress and hair minerals, as this association remained uninvestigated. Next to the novelty of the study, other strong methodological features are the use of the validated and state of the art ICP-MS and UPLC-MS/MS technique to measure hair mineral concentrations and hair cortisone levels, respectively, and the adjustment of all analyses for child's age, BMI, physical activity, dietary habits, socio-economic status and hair colour, whereby the unique physiological contribution of stress on hair mineral levels could be studied. A next asset of this study is the assessment of the environmental (i.e. CLES questionnaires) and biological (i.e. hair hormone measurements) stress dimension, which permitted studying the individual associations of these stress-estimates with hair minerals. However, the use of child-reported stress questionnaires may be subject to recall- or reporting-bias. In addition, no corrections for multiple testing were performed (e.g. Bonferroni) as we considered this too stringent for our analysis, although this may have led to an increased likelihood of significant findings because of the high number of regressions analysed. Post-hoc power analysis indicated that our sample size to detect a medium effect size in our regression analysis was sufficient to reach a power of 0.80, although a larger sample size would have allowed studying smaller effect sizes. Another limitation that should be considered is the exclusively female population under study within a small age range, limiting the generalisability of our results. Findings need thus to be confirmed in a more heterogeneous population sample (i.e. boys and girls, childhood to adolescence) and in a longitudinal study design, since the cross-sectional design of this study cannot determine causality. As not much is known in the field of stress and minerals, more specifically hair minerals, evaluation and discussion of our observations to previously reported findings remain limited. Therefore, we again point to the importance of further research. Nevertheless, this study may initiate further hair mineral research in relation to stress and stress-related health effects or behaviour in children. Particularly for large-scale epidemiological research in children, hair mineral analysis may offer considerable advantages

as hair sampling is easy, non-invasive and inexpensive, and the samples are easily stored. For boys on the other hand, a sufficient length of hair should be available to obtain retrospective measures of several months in the past. Last, we recommend further investigation into the detailed mechanisms and processes by which stress influences hair mineral levels/accumulation (e.g. are some minerals more susceptible to stress than others?; is the stress-mineral relationship stressor dependent? etc.) in order to further endorse our findings.

5 Conclusions

This study strengthened previous indications of a relationship between stress and mineral levels. More specifically, we demonstrated an independent association between chronic stress measures (i.e. hair cortisone and life events) and hair mineral levels in young girls, a previously unexplored research area. However, findings need to be confirmed in a more heterogeneous population and on a longitudinal basis. Furthermore, the precise mechanisms by which stress alters hair mineral levels should be further elucidated in order to fully understand the importance of stress on this aspect of health.

CHAPTER 3.8 THE ASSOCIATION BETWEEN CHILDHOOD STRESS AND BODY COMPOSITION, AND THE ROLE OF STRESS-RELATED LIFESTYLE FACTORS – CROSS-SECTIONAL FINDINGS FROM THE BASELINE CHIBS SURVEY

Abstract

Background Stress has been hypothesised to be involved in obesity development, also in children. More research is needed into the role of lifestyle factors in this association.

Purpose This study investigates the cross-sectional relationship between stress and body composition and, more importantly, the possible moderating or mediating role of lifestyle factors.

Methods A total of 355 Belgian children (5-10 years old) participating in the baseline Children's Body composition and Stress' survey were included in this study. The following variables were studied: psychosocial stress (i.e. stressful events, emotions and behavioural/emotional problems, salivary cortisol), stress-related lifestyle factors (high-caloric snack consumption frequency, screen exposure time and sleep duration) and body composition parameters [BMI z-score, waist to height ratio (WHtR)]. Using linear regression analyses (adjusted for sex, age and socio-economic status), the relation between stress and body composition and the possible moderating or mediating role of lifestyle factors was tested.

Results No association was observed between body composition and negative emotions, conduct and emotional problems and salivary cortisol. However, negative life events were positively and happiness negatively associated with BMI z-score and WHtR. Peer problems and WHtR were positively associated in girls only. These associations were not significantly reduced after correction for lifestyle factors. Nevertheless, all lifestyle parameters moderated one or more stress – body composition associations, resulting in even more significant relations after subgroup analysis.

Conclusion Childhood stress was positively related to both overall and central adiposity measures with lifestyle factors acting as moderators but not as mediators. Thus, lifestyle could

be a vulnerability factor in stress-induced adiposity, creating a perspective for multi-factorial obesity prevention, targeting stress and lifestyle factors in parallel.

1 Introduction

The prevalence of childhood obesity significantly increased during the last decades with currently around 110 million overweight and obese children worldwide (Ahrens W. et al., 2011b; Cali A. M. G. et al., 2008). Excessive caloric intake, insufficient physical activity and sleep deprivation are major lifestyle factors involved in the development of childhood obesity (Moreno L. A. et al., 2011). Moreover, a wide array of other - genetic and environmental-factors have been shown to be involved in the development of obesity; a recently identified potential predictor of overweight is chronic stress in children (Pervanidou P. et al., 2011; Gundersen C. et al., 2011).

Although children are not always recognized as being susceptible to stress, chronic exposure to stressful situations in children's school-, family- or interpersonal environment (further defined as 'psychosocial stress') is not uncommon and may adversely affect their physiological and psychological health (Teicher M. H. et al., 2003; Schneiderman N. et al., 2005; Cohen S. et al., 2007). Of special concern is the combined increase in the prevalence of childhood stress with the prevalence of psychosomatic complaints (Vanaelst B. et al., 2012a), behavioural or mental health problems (Timmermans M. et al., 2010) and obesity in children (Gundersen C. et al., 2011).

The relationship between psychosocial stress and childhood obesity has been described both cross-sectionally and longitudinally and is hypothesized to result from direct and indirect pathways. Firstly, direct metabolic changes (such as increased visceral fat disposition and a stimulation of appetite) are mainly caused by a dysregulation of the stress system and the production of stress hormones (mainly cortisol and catecholamine) (Björntorp P., 2001; Pervanidou P. et al., 2011). Secondly, stress may indirectly influence the development of obesity due to behavioural pathways such as maladaptive coping behaviours leading to emotional eating, inactivity and disordered sleep (Biddle S. J. H. et al., 2011; Akerstedt T., 2006; Michels N. et al., 2012a; Dallman M. F. et al., 2005; Torres S. J. et al., 2007; Tsatsoulis A. et al., 2006), possibly mediating this stress-obesity relationship (Pervanidou P. et al., 2011; Gatineau M. et al., 2011). However, there is a need for more focused scientific research into the mechanisms linking psychosocial stress to appetite regulation, energy balance and body

composition in humans and more importantly in children, as children may be particularly vulnerable to the effects of chronic alterations in cortisol secretion influencing brain development, and in the endocrine and metabolic systems.

This cross-sectional study examines the association between psychosocial stress and body composition in young children (5-10 years old). As it is recently hypothesized that psychosocial stress may promote the consumption of so-called comfort foods (rich in sugar and fat), a decreased amount of physical activity and disordered sleep (Michels N. et al., 2012a; Torres S. J. et al., 2007; Lam J. C. M. et al., 2010; Tsatsoulis A. et al., 2006), this study will also contribute to an unexplored domain by investigating to what extent this stress-obesity association is moderated or mediated by diet (high-caloric snacking), sedentary lifestyle (screen exposure time) or sleep (duration) in young children. A particular contribution of this study lies within its stress-assessment methodology, using both questionnaires and salivary cortisol measurements.

2 Methodology

2.1 Study Participants

Children's Body Composition and Stress (ChiBS) a national study embedded within the European Identification and prevention of Dietary- and lifestyle-induced health EFfects In Children and infants study (IDEFICS) carried out in 2007-2008 (Ahrens W. et al., 2011a), investigates the association between chronic psychosocial stress and changing body composition in children over a 2-year follow-up period (2010-2012; Aalter, Belgium) (Michels N. et al., 2012d). Participants were elementary school children aged 5-10 years. Their parents were asked to sign a consent form in which the option was offered to participate in the full ChiBS baseline programme or in a selected set of measurement modules, resulting in distinct participation numbers for the different measurement modules, as presented in Figure 3.12.

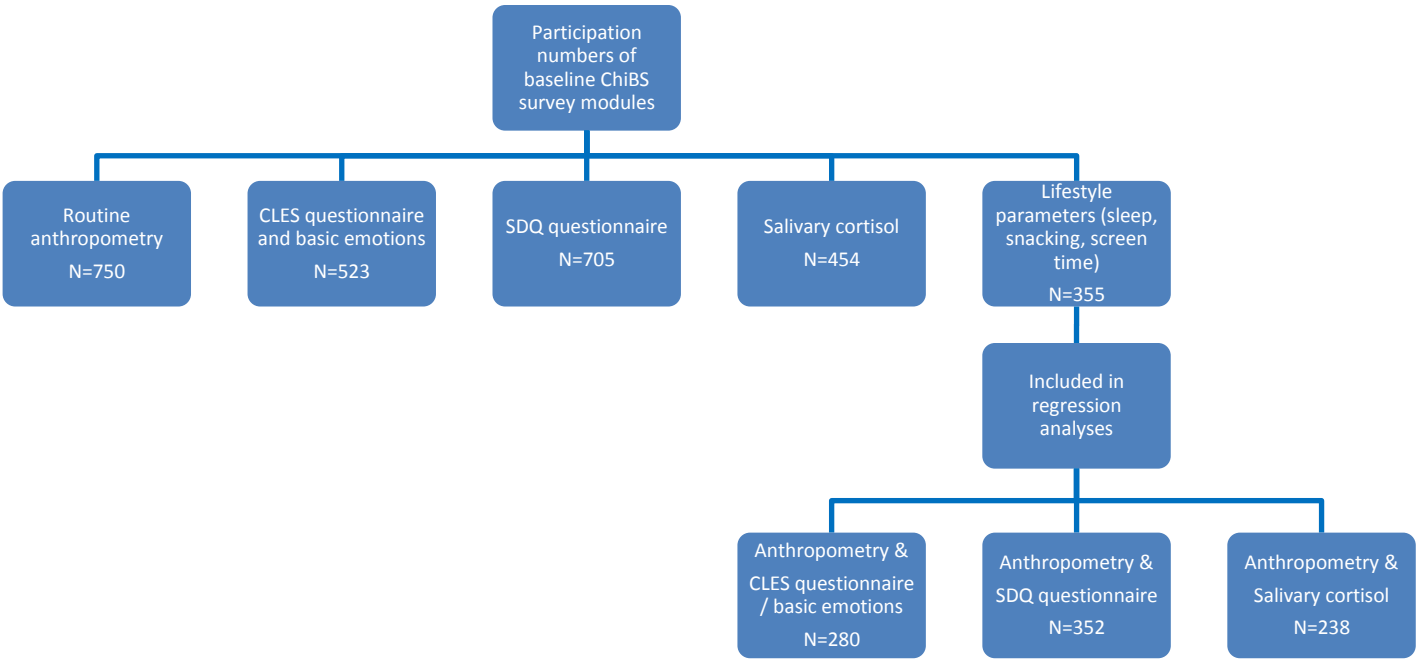


Figure 3.12 Flowchart ChiBS - Children's Body Composition and Stress; CLES: Coddington Life Events Scale; SDQ: Strengths and Difficulties Questionnaire

Analyses in this study were limited to children with complete information for the stress-related lifestyle parameters (N=355) as full information on these parameters was necessary to perform the moderator and mediator analyses. This sample of 355 children however additionally decreased in number depending on the studied stress and body composition variable in analyses, as presented in Figure 3.12. No differences were observed between the included children and children with missing data on age, stress measures and body composition measurements (parametric and non-parametric T-test; data not shown). The ChiBS project was conducted according to the guidelines laid down in the Declaration of Helsinki and was approved by the Ethics Committee of the Ghent University Hospital. More detailed research goals, methodology and participation characteristics were described elsewhere (Michels N. et al., 2012d).

2.2 Anthropometric Examinations

Physical examinations were performed according to the standardized procedures of the IDEFICS study (Bammann K. et al., 2011; Ahrens W. et al., 2011a; Stomfai S. et al., 2011). Weight and height were measured in bare feet and light underwear with an electronic scale (TANITA BC 420 SMA, TANITA Europe GmbH, Sindelfingen, Germany) and a stadiometer

(Seca 225, SECA GmbH & Co. KG., Hamburg, Germany) to the nearest 0.1 kg and 0.1 cm, respectively. Age- and sex- specific body mass index (BMI) z-scores were calculated according to the method from Cole (Cole T. J. et al., 2000). The waist to height ratio (WHtR) was calculated as an indicator of central body fat to investigate central adiposity (Ashwell M. et al., 2005; Nambiar S. et al., 2009).

2.3 Stress Parameters

To cover the complete aspect of stress, not only emotions but also behaviour was examined by questionnaires. Furthermore, salivary cortisol was used as an objective stress biomarker.

A. Coddington Life Events Scale (child-reported)

The Coddington Life Events Scale for children (CLES-C) is a validated and well-established 36-item questionnaire which assesses the frequency and timing of stressful life events relevant for this age group during the last year. By measuring significant life events in terms of Life Change Units (LCUs), the CLES-C can provide insight into recent events that may be affecting the child's health (Coddington R. D., 1972; Villalonga-Olives E. et al., 2008). For this study, the negative life events score was calculated for the previous 6 months.

B. Basic Emotions (child-reported)

As environmental stressors are interpreted by people in relation to own values which may affect the stress response, we additionally included questions on the child's emotional state (Herbert T. B. et al., 1996; Folkman S. et al., 1986; Vanaelst B. et al., 2012b). Children were asked to report on their recent (described as "lately") feelings of anger, anxiety, sadness and happiness on a 0 to 10 multipoint Likert scale (0 'not at all' to 10 'very strong') in the context of affective responsiveness analogous to a study of Zimmer-Gembeck (Zimmer-Gembeck MJ. et al., 2009).

C. Strengths and Difficulties Questionnaire (parent-reported)

Parents were asked to complete the standardized 'Strengths and Difficulties Questionnaire' (Goodman R., 1997), reporting children's behavioural and emotional problems over the past 6 months. For each of the 20 statements, parents could answer: 'not true' (0), 'somewhat true'

(1) and ‘certainly true’ (2), after which the statements were divided in four subscales by summing the scores of the relevant items: peer problems, conduct problems, emotional problems and prosocial behaviour. Higher scores on the prosocial behaviour subscale reflect strengths, whereas higher scores on the other three subscales reflect difficulties (Ravens-Sieberer U. et al., 2000).

D. Salivary Cortisol Analysis

Saliva was collected into Salivette synthetic swabs specifically designed for cortisol analysis (Sarstedt, Germany). The participating children were asked to collect saliva during two consecutive weekdays at four time points: immediately on awakening (T0), 30 minutes after waking up (T30), 60 minutes after waking up (T60) and in the evening between 7 and 9 PM (Tev). Apart from single-point cortisol concentrations, also summary variables have been calculated to represent two cortisol patterns over time: the cortisol awakening response and the diurnal decline. To represent the cortisol awakening response, the area under the curve with respect to the ground (AUCg) was calculated as the total area under the curve between T0 and T60. The diurnal cortisol decline was investigated as the concentration of T0 minus Tev, divided by the number of hours between these sampling periods, with a more positive decline representing a steeper decline (Pruessner J. C. et al., 2003; Michels N. et al., 2012c). Higher cortisol levels and a steeper diurnal decline have been related to more stress in our population (Pruessner J. C. et al., 2003; Michels N. et al., 2012c). Detailed methodology and descriptive results were published elsewhere (Michels N. et al., 2011).

2.4 Stress-Related Lifestyle Parameters

Information on the children’s diet, sedentary behaviour and sleep was collected by a parental-reported questionnaire embedded in the IDEFICS project.

A. Consumption frequency of high-caloric snacks

The Children’s Eating Habits Questionnaire - Food Frequency Questionnaire (CEHQ-FFQ) is a screening instrument to investigate food consumption frequencies associated with overweight, obesity and general health in children, including in total 43 food items (Lanfer A. et al., 2011). Parents were asked to report the child’s consumption frequency of selected food items in a typical week during the preceding 4 weeks. Frequencies of consumption were

assessed without quantifying portion sizes and the frequency categories were converted to consumption frequency per week. The following response options were used: ‘never/less than once a week’ (0), ‘one to three times a week’ (2), ‘four to six times a week’ (5), ‘one time per day’ (7), ‘two times per day’ (14), ‘three times per day’ (21), ‘four or more times per day’ (30) or ‘I have no idea’. To identify dietary patterns related to stress, a ‘high-caloric snack’ food index was calculated by summing up the consumption frequencies of the following food items: chocolate and chocolate bars, candies, biscuits, cake, ice cream, chips and savoury pastries. Also, in additional analyses soft drinks were included.

B. Sleep duration and screen exposure time

Parents reported the typical hours of bedtime and getting up in the morning for weekdays, from which the child’s sleep duration during the week was calculated. The number of screen time hours (e.g. television and computer time) were also reported by the parents, and used as a measure of sedentary behaviour in the analyses.

2.5 Other variables

Parental education level was categorized according to the International Standard Classification of Education (ISCED) (UNESCO, 1997). The maximal ISCED level of the parents was studied and further categorized in two levels of education (low/medium vs. high), with ISCED levels 0-4 being defined as low/medium education, and level 5 being defined as high education (=tertiary education).

2.6 Statistical Analysis

Analyses were performed using PASW Statistical Program version 19.0 (SPSS Inc., IBM, IL, USA). The two-sided level of significance was set at $p<0.05$. Data were presented by their median and interquartile range to handle non-normally distributed data. The difference between boys and girls was examined for continuous variables (independent samples T-test and Mann-Whitney U Test for normally distributed data and skewed data respectively) and for categorical variables (Pearson Chi-Square Test). The association between stress and body composition was analysed using linear regression analyses. All regression analyses were adjusted for sex, age and parental education as sex and age are significantly associated with

body composition measures (age: ß=0.204, p<0.001 and ß=-0.282, p<0.001 for BMI and WHtR respectively; sex: ß=0.093, p=0.077 and ß=0.125, p=0.015 for BMI and WHtR respectively) and parental education is a known factor in influencing children's lifestyle and behaviour. Analyses were only analysed for girls and boys separately if a significant sex interaction factor was present. The distributions of WHtR, high-caloric snack frequency, screen time and sleep duration were skewed and were therefore logarithmically transformed. The regression residuals were normally distributed.

A first set of regressions tested the direct and indirect association between stress (predictor variable) and body composition (outcome variable) and examined the mediating role of lifestyle parameters, as well as sex differences in this association. Mediation was tested according to Baron and Kenny (Baron R. M. et al., 1986). In doing this, the significant reduction of the association between the stress parameter (independent variable) and body composition (dependent variable) after controlling for lifestyle (mediator) was tested non-parametrically by bootstrapping (using 10,000 samples) (Preacher K. J. et al., 2004).

A second set of regressions tested moderation by sex and lifestyle parameters in the association between stress and body composition. According to literature, moderation was tested by including an interaction factor (Frazier P. A. et al., 2004). In those regressions, continuous parameters were transformed in z-scores and the categorical sex variable was effect coded (-1 and 1 for boys and girls respectively). A p<0.10 was used as indicator for moderation as the power to detect interaction effects is lower than for main effects (Frazier P. A. et al., 2004; Selvin S., 1996). If significant, visual representation was done by plotting predicted outcome values (based on the non-standardised coefficients) for three representative groups of the moderator: those at the mean, at one SD below the mean and one SD above the mean. Statistical interpretation was done by testing the significance of the stress predictor for the two groups: for sex, this was boys and girls; for the continuous moderator (the lifestyle parameters) two groups were created based on a median split.

3 Results

3.1 Participant characteristics

Table 3.22 describes the studied anthropometrical, stress and lifestyle parameters for the participating boys and girls separately. Boys and girls did not differ for the studied parameters, except for (1) conduct problems, which were significantly higher in boys; (2) prosocial behaviour which was significantly higher in girls and (3) BMI categories of which the overweight and obese category was significantly more prevalent in girls.

Table 3.22 Participant characteristics (N=355)

	Boys (N=186)			Girls (N=169)			
	Median	**P25**	**P75**	**Median**	**P25**	**P75**	**p-value**
Age (years)	8.00	6.80	9.20	8.00	6.75	9.00	0.475#
Body composition parameters							
BMI z-score	-0.15	-0.74	0.37	-0.11	-0.73	0.66	0.222#
Waist height ratio	0.43	0.41	0.45	0.44	0.42	0.46	0.064
Stress parameters							
Negative events 0-6m	21	0	52	29	0	53	0.476
Happiness (scale 0-10)	8	6	10	8	6	9	0.675
Anxiety (scale 0-10)	1	0	3	1	0	4	0.155
Sadness (scale 0-10)	2	0	4	2	1	5	0.078
Anger (scale 0-10)	2	1	4	3	1	5	0.074
Peer problems (scale 0-10)	1	0	2	1	0	2	0.945
Conduct problems (scale 0-10)	1	0	2	1	0	2	**0.048**
Emotional problems (scale 0-10)	2	1	3	2	1	4	0.251
Prosocial behaviour (scale 0-10)	7	5	8	7	6	8	**0.044**
Salivary cortisol awakening (nmol/l)	11.89	10.09	13.93	11.33	9.51	14.71	0.828
Salivary cortisol AUCg	23.09	17.57	27.54	23.27	17.83	28.39	0.617
Salivary cortisol decline	-0.78	-0.96	-0.58	-0.76	-1.01	-0.62	0.272
Stress-Related lifestyle parameters							
Sleep duration (hours on weekdays)	11.00	10.50	11.25	11.00	10.50	11.50	0.585
High-caloric snack frequency (times/week)	8.50	5.00	13.25	9.00	6.00	13.00	0.284
Screen time (hours/week)	11.50	7.25	15.50	10.25	7.00	14.00	0.051
	N (%)			**N (%)**			
BMI Categories (Cole)							
Underweight	26 (14%)			20 (12%)			**0.021**$
Normal weight	152 (82%)			132 (78%)			
Overweight and obese	8 (4%)			17 (10%)			
Maximal parental education							
ISCED level 2 and 3	50 (27%)			58 (35%)			0.271$
ISCED level 4	41 (22%)			31 (18%)			
ISCED level 5	95 (51%)			80 (47%)			

p-value of Mann-Whitney U Test

p-value of Independent Samples T-test

$p-value of Pearson Chi-Square Test

ISCED= International Standard Classification of Education, 2 'lower secondary education', 3 'upper secondary education', 4 'post-secondary non-tertiary education', 5 'first stage of tertiary education'

3.2 Stress – body composition association and its mediation by stress-related lifestyle parameters

The association between stress and body composition was studied directly and indirectly through the possible mediating role of stress-related lifestyle parameters. Table 3.23 presents the significant results of the linear regression models investigating the association between stress and body composition, with and without adjustment for the studied lifestyle factors. The adjusted linear regression models were repeated with another snack index, i.e. including soft drinks, and resulted in similar results as described below (data not shown).

Table 3.23 The stress-body composition association and its mediation by stress-related lifestyle parameters: results of multivariate linear regression models, unadjusted and adjusted for sleep duration, high-caloric snack frequency and screen time.

	outcome			
	BMI z-score		**WHtR**	
	ß	**p**	**ß**	**p**
predictor				
negative events 0-6m				
unadjusted	0.129	0.033	0.155	0.009
adjusted	0.116	0.050	0.147	0.013
happiness				
unadjusted	-0.134	0.027	-0.143	0.017
adjusted	-0.120	0.044	-0.148	0.014
peer problems (girls only)				
unadjusted	0.077	0.327	0.156	0.042
adjusted	0.066	0.385	0.154	0.044

All analyses were adjusted for age, sex and parental education. Only significant stress-body composition associations are presented.

Neither a direct nor an indirect association was observed between the following stress parameters and body composition: anger, anxiety, sadness, conduct problems, emotional problems, prosocial behaviour, salivary awakening cortisol, salivary AUCg cortisol and salivary decline (data not shown). However, a positive association (both directly (unadjusted βs) and independently (adjusted βs)) was shown for children's BMI z-score and WHtR with the negative events score for the past 6 months, while an inverse association was seen with happiness (Table 3.23). A sex interaction was only observed in the association between peer

problems and WHtR: this association was only significant in girls (Table 3.23 and Figure 3.13).

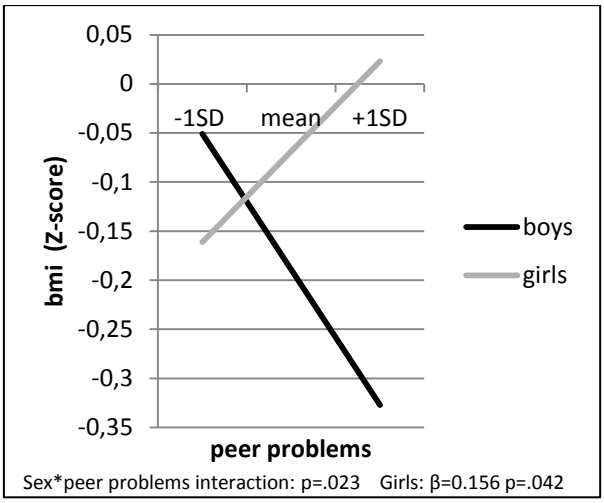


Figure 3.13 Sex as moderator in the stress – body composition relation. The figure shows the stress – body composition relation for boys and girls and for stress levels equal to the mean, one standard deviation above the mean (=high) or one standard deviation below the mean (=low). The significance value of the interaction term for sex - stress in the relation with body composition is given.

After correction for screen time (in the association between peer problems and WHtR in girls) and after correction for high-caloric snack index (in the association between negative events 0-6m and BMI z-score) the stress – body composition association was no longer significant (data not shown), indicating a possible mediating role of these lifestyle factors. However, bootstrapping showed that the stress-overweight association was not significantly reduced after correction for these lifestyle factors [indirect confidence interval (-0.0002;0.0015) and (-0.0004; 0.0012), respectively], demonstrating that these lifestyle factors do not mediate the stress-obesity association and that stress may be an independent risk factor for obesity.

3.3 Moderation by stress-related lifestyle factors

The studied lifestyle parameters were also tested as moderators in the association between stress and body composition, as graphically presented in Figure 3.14. All lifestyle parameters were found to moderate one or more of the stress – body composition associations.

More specifically, the positive association between stress (negative events, cortisol morning levels and cortisol decline) and body composition (BMI and WHtR) was strengthened by

unhealthy behaviour (high-caloric snack frequency, high screen time, low sleep duration), e.g. stress as presented by a large amount of negative events was highly related to a higher BMI z-score in the presence of the unhealthy behaviour of high-caloric snack intake frequency (similar results for snack intake including soft drinks) while almost no association between stress and body composition was found for the healthy behaviour of low snack intake frequency (see Figure 3.14C). Nevertheless, the sleep moderation for sadness was reversed: a positive association between stress and body composition was only seen in children with high sleep duration.

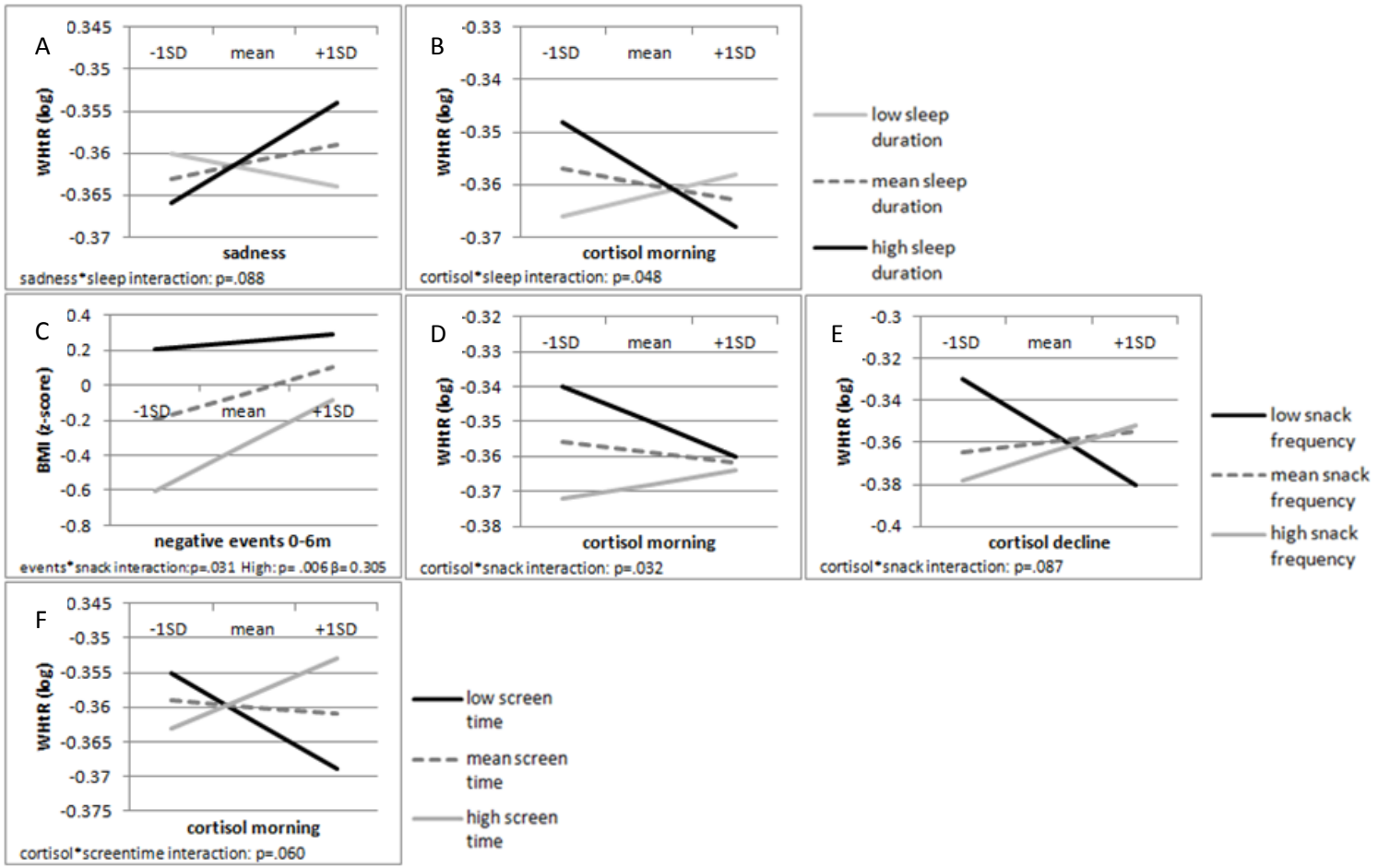


Figure 3.14 Lifestyle factors (sleep duration, high-caloric snack frequency and screen time) as moderator in the stress – body composition relation. 3A-B: sleep duration as moderator; 3C-E: high-caloric snack frequency as moderator; 3F: screen time as moderator. Figures show the stress – body composition relation for moderator (sleep duration, high-caloric snack frequency and screen time) and stress levels equal to the mean, one standard deviation above the mean (=high) or one standard deviation below the mean (=low). The significance value of the interaction term for lifestyle - stress is given. If the stress – body composition relation was significant in one of the two subgroups of the moderator (groups with score above or below the mean of the lifestyle factor), those significance and beta values are also given.

4 Discussion

This study examined the cross-sectional association between psychosocial stress and body composition in young children and investigated the role of high-caloric snacking, sleep and screen time as lifestyle factors mediating or moderating this association.

The main findings of this study were (1) the observed positive association between stress, mainly negative life events, and body composition measures in a direct way, i.e. higher levels of stress were independently associated with increased BMI z-scores or WHtR; (2) the lack of mediation effects for the studied lifestyle factors (i.e. the lifestyle factors did not account for the observed stress-body composition association) and (3) the observation of some moderation: the stress-body composition association was strengthened by the most unhealthy lifestyle patterns (i.e. highest screen time, highest snack consumption or lowest sleep duration).

To our knowledge, this study was the first examining the association between stress and body composition in young children by using multiple stress measures, standardized body composition measurements and by incorporating stress-related lifestyle factors. That is why comparisons of our findings with observations from similar studies were limited. Nevertheless, our general findings on stress-body composition are in agreement with previous studies indicating an association between stress and obesity/body composition in children: Gundersen et al. (Gundersen C. et al., 2011) concluded that in all studies on psychosocial stress and childhood obesity at least one measure of stress was associated with obesity. Also in our study, not all stress measures related to each of the body composition measures: (1) Only negative life events, happiness and peer problems (the latter only in girls) related to BMI z-scores and WHtR; while in the moderation analyses also other stress measures (such as the cortisol measures) were related; (2) No direct significant association was observed between cortisol and body composition, indicating the importance of child-reported stress measures in the stress-body composition association. These observations could point to specificity in the stress-body composition association (i.e. unique, specific relation between a particular risk factor and outcome) (McMahon S. D. et al., 2003). However, our study was not designed to study specificity in the stress-body composition association.

More importantly, in our study both overall (BMI z-score) and central (WHtR) obesity parameters were related to stress parameters (i.e. life events, happiness and peer problems).

Stress has been shown to predominantly stimulate abdominal accumulation of fat since the glucocorticoid receptors (by which cortisol exerts its fat deposition effect) have a high density in the abdominal fat (Björntorp P., 2001). A final specificity in the relation was seen with sex in only one relation, i.e. a significant relation between peer problems and WHtR in girls only. Also previously, sex differences in the stress-related hormonal pathways have been shown (Björntorp P., 2001). A possible explanation in this preadolescent population could be a sex difference in children's experiences of peer interactions as it has recently been reviewed that girls are more sensitive to their friendship status and to receiving higher levels of emotional provisions in their friendships (Rose A. J. et al., 2006). Indeed, sex has been mentioned as a possible moderator on stress and mental health in children (Gatineau M. et al., 2011).

Another research aim was to examine possible underlying pathways in this stress-body composition association. From a physiological point of view, we expected that the association between stress and body composition would operate through the energy balance, i.e. through influencing energy intake (e.g. high-caloric snack consumption) or energy expenditure (e.g. screen time). In previous research, the lifestyle factors diet, activity/sedentary lifestyle and sleep have been related to stress and more consistently to childhood obesity (Torres S. J. et al., 2007; Dallman M. F. et al., 2005; Holmes M. E. et al., 2010; Akerstedt T., 2006; Biddle S. J. H. et al., 2011). Testing moderating or mediating effects of lifestyle in the association between stress or mental health with obesity is a largely unexplored domain (Pervanidou P. et al., 2011; Gatineau M. et al., 2011). In a recent review, low physical activity and an unhealthy diet were mentioned as mediators for the effect of mental health on obesity, while sleep was hypothesised as a mediator in the effect of obesity on mental health. This review also proposed physical activity as mediator regarding the impact of mental health on children's obesity, but not a mediating effect for the other lifestyle factors (Gatineau M. et al., 2011). In a recent stress-obesity study examining all mentioned lifestyle factors in adolescents, stress was related to general and central adiposity, an unhealthier diet and a shorter sleep duration on weekdays but they found no mediating effect of these lifestyle factors on the stress-obesity relation (De Vriendt T., 2012), which is in line with our study: no mediating effects of high-caloric snacking, screen time and sleep duration were observed. This may either point to the importance of non-behavioural pathways in the development of overweight/obesity (e.g. stress-induced hormonal changes causing increased fat deposition), or this may be explained by (1) the cross-sectional design of this study potentially not allowing to observe mediation pathways, or to (2) methodological limitations in the chosen lifestyle variables.

Although the studied lifestyle factors have not been observed as mediators, they acted as moderators of the stress-body composition association. In literature, we have indeed found studies showing e.g. children's physical activity as moderator in the association between stress and overweight or metabolic risk (Yin Z. N. et al., 2005; Holmes M. E. et al., 2010). Consequently, children with an unhealthier lifestyle could be more vulnerable for stress-induced overweight or, vice versa, a healthy life style could be a buffer for the effects of stress on adiposity. Especially in the presence of high consumption frequency of high-caloric snacks, the positive association between stress and body composition was strengthened in our study. To a lesser degree, this was also seen for sleep duration and sedentary behaviour. On the contrary, a moderating effect of sleep on the association between sadness and overweight was observed in an unexpected direction, i.e. the stress-body composition association was strengthened in children with a long sleep duration, which is surprising since in particular sleep deprivation has been associated with obesity. Although we have no clear explanation for this finding, it is possible that children feeling sad have the need to sleep longer. Last, it is assumed that lifestyle may be the underlying mechanism in the stress-obesity association, although from another perspective it is possible that psychosocial stress is clustered with the exposure to an unhealthy lifestyle (e.g. low SES and unhealthy diet patterns), and thereby both have an impact on obesity.

4.1 Strengths and limitations

To our knowledge, this study was the first to examine the association between stress and body composition in young children by using more than one stress measure (stressor questionnaires, salivary cortisol) and standardized measurements of body composition and by considering more than one stress-related lifestyle factor, which are the main strong methodological features of this study. Some limitations should however be considered when interpreting the results. Firstly, we acknowledge the cross-sectional design of this study to be its largest weakness in not allowing to study causality. However, as the ChiBS project progresses, the association between stress and changes in body composition will be further investigated on a longitudinal basis. Also, the directionality of the association between mental health and body composition could be different for youth and adults (Napolitano M. A. et al., 2008). Secondly, the performed anthropometric examinations (BMI and WHtR) are proxy indicators of body adiposity and thus inferior to direct measures of adiposity such as DEXA scanning or air displacement plethysmography technology (BODPOD®). BODPOD

measurements were performed in the ChiBS project but resulted in a smaller sample size compared to the routine anthropometric measurements (BMI and WHtR) because of the additional effort asked from parents in transporting their children to another survey centre where the BODPOD was located. Therefore, we chose to perform analyses with BMI and WHtR to maximize the sample size and power of analyses. Thirdly, a reporting bias in the parent-reported lifestyle parameters (parents may have the tendency to give the morally right answer or may inaccurately estimate their child's lifestyle behaviour) and in the child-reported stress questionnaires (i.e. CLES and basic emotions) cannot be excluded, as a result of which reversed causation may be possible. Concerning the studied lifestyle parameters (which were selected based on their capacity to affect the energy balance), it should be noted that a number of other residual confounders and methodologically more profound measures of the same behavior/lifestyle may have affected the observed associations. Methodologically more profound measurements such as accelerometry/actigraphy for physical activity and sleep quality were available in the ChiBS project, but were not applied in this study because of power issues (i.e. low sample sizes). In this context, it should be noted that the measurement of sleep duration covers the numbers of hours spent in bed and not necessarily the amount of sleep. Next, the CEHQ-FFQ did not include portion sizes and therefore no energy intake could be calculated (Rodriguez G. et al., 2006). As a result, it may not be the most accurate instrument to assess stress-related dietary habits. Nevertheless, using a FFQ has the advantage of showing the habitual diet as dietary recalls can be biased by exceptional days. Examples of residual confounders are sleep quality, total energy intake, eating behavior (e.g. emotional/external/restrained eating), BMI and stress exposure of the parents, psychological appraisal and coping of stress, social support and non-behavioral parameters such as hormonal status of the children (e.g. sex hormones, appetite regulating hormones etc.) and other known or unknown biological factors. Lastly, as the participating children were characterized by a high socio-economic background, we may have missed stronger associations between stress and body composition in lower socio-economic environments. However, no additional selection bias was introduced by including only those children with complete information for the studied variables (as discussed in the "Methodology" section).

5 Conclusion

Children's stress level, characterized by negative life events, happiness and peer problems, was associated with overall and central adiposity. Lifestyle factors (i.e. high-caloric snacking,

screen time and sleep duration) did not mediate this relationship, although they were shown to intervene as moderators. Consequently, an unhealthy lifestyle may make children more prone to stress-induced changes in body composition or, vice versa, a healthy lifestyle may attenuate the effects of stress on adiposity. When framing these results in a public health perspective, it may be recommended to screen for the presence of psychosocial stress (especially negative life events and happiness) and to incorporate education on stress management in obesity prevention programs. Moreover, the moderating effects of lifestyle factors put stress into a new perspective and indicate the importance of multi-factorial obesity prevention programs by focusing concurrently on several lifestyle factors.

PART 4
DISCUSSION

CHAPTER 4.1 SUMMARY AND DISCUSSION OF MAIN FINDINGS

This chapter summarizes the main findings of this thesis in relation to the predefined research objectives. A schematic, graphical summary is provided at the end of this chapter.

1 Importance of studying childhood stress - European framework

A first aim of this thesis was to describe the occurrence of childhood adversity and its relationship with mental health symptoms in European children, as large-scale, international research on this topic is scarce. These research objectives were studied in the framework of the European IDEFICS project, more specifically in pre- and primary-school children between 4 and 11 years old and were based on parental-reported data.

1.1 Prevalence of negative life events (NLE) and familial and social adversities (FSA) in European pre- and primary-school children (Chapter 3.1)

First, the prevalence of NLEs and FSAs was studied cross-nationally in European pre- and primary-school children. The main findings were generally in line with previous research:

- Certain adversities occurred only rarely, while others were very common. A non-traditional family structure (21%), being only-child (16.4%) and being immigrant (13%) were the three most reported FSAs overall, while parental divorce/separation (13%), addition of a new family member (12.4%) and parental job loss (8.7%) were the most reported NLEs (N=4637). In general, childhood adversity was shown to occur quite frequently, indicating the importance of studying stress in young children;
- The prevalence of childhood adversity was influenced by geographical location, age and sex. In particular for family structure, large regional variations between northern, eastern, southern and western European survey centres were observed. Concerning age and sex, childhood adversities were observed to be more prevalent in older age groups, and severe diseases/accidents and peer problems were more prevalent in boys compared to girls;

- Childhood adversities were associated and co-occurred, resulting in potential cumulative childhood stress. Furthermore, a large percentage of children was shielded from stressors, while a small group of children was exposed to multiple, accumulating adversities (e.g. 1.4% of the children experienced 4 or more FSAs).

These findings contribute to the knowledge of (cumulative) stress incidence and adversity associations in a cross-national setting of children younger than 12 years old. Particularly, this study indicated that family formation change and disadvantage should not be considered risk-free living conditions given their widespread presence and potential health risk in children, as described below.

1.2 Prevalence of psychosomatic and emotional symptoms (PES) in European pre- and primary-school children and the association with childhood adversity (Chapter 3.2)

Monitoring and recording childhood stress is above all important for the elucidation of its mental and physical health consequences and opportunities for prevention. This thesis therefore described the prevalence of parental-reported PES in European pre- and primary-school children and examined the relationship among PES, NLEs and FSAs in the child's life, as international research in this context was lacking.

Almost half of the children experienced at least one PES, with low emotional well-being during the last week being the most frequently reported. No sex differences were shown for the prevalence of PES, but prevalence rates rose with increasing age. Children with PES were more frequently observed to have experienced childhood adversity compared to children without PES. Moreover, childhood adversities were significantly related to PES prevalence, both *quantitatively* and *qualitatively* (i.e. an increasing number of adversities gradually amplified the risk for PES, with the number of experienced FSAs contributing more strongly; and some specific adversities were apparent risk factors for the occurrence of PES such as a non-traditional family structure, unfavourable family climate, peer problems and major frustrations at school), although no conclusions on causality or directionality can be made. These findings thus illustrate the importance of the children's everyday family and social environment on their well-being.

However, despite the observed relationship between childhood adversity and the risk for PES, a more nuanced view of this complex relationship is needed, as not all children are harmed

equally by stressor exposure. Other factors such as coping styles and social support are important modulators of this complex relationship (Folkman S. et al., 1986; Olff M. et al., 2005).

1.3 Discussion of the sex differences in the prevalence of FSAs, NLEs and PES (Chapter 3.1 and Chapter 3.2)

Literature has led to inconsistent findings on sex differences in the incidence of childhood adversity and PES, or their association. It is assumed that a potential reporting bias may be involved between males and females (e.g. males and females perceive and report different types of events as 'being important') (Hatch S. L. et al., 2007), and that also potential differences in biological and psychological reaction to stressors between males and females may be implicated (Young E. A. et al., 2008; Chaplin T. M. et al., 2008; Kajantie E. et al., 2006; Cox S. J. et al., 2010; Rudolph K. D., 2002).

In this thesis, no general sex differences were observed in the incidence of NLEs, FSAs or PES, except for the occurrence of severe diseases/accidents and peer problems (in the age group of 9 year olds) which were more frequent in boys compared to girls. These findings can be explained by significant differences in peer relationships in boys and girls as shown by Rose et al.. Girls have been shown to engage in more prosocial interactions with higher self-disclosure in friendships and to empathize with others, while boys have been shown to more frequently engage in organized play, to emphasize dominance within their peer group and to encounter more peer stress in the form of overt physical or verbal victimization (Rose A. J. et al., 2006).

In summary, this study neither demonstrated an overall difference in the prevalence of NLEs, FSAs or PES between boys and girls, nor an overall difference in the association between childhood adversity and psychosomatic or emotional symptoms between sexes. These findings may suggest a comparable psychological experience and processing of stress in our population of prepubertal children, although no direct assessments of this kind were performed.

2 Scalp hair as biological matrix for chronic stress assessments in children

To further study childhood stress and its health effects, it is important to have valid and reliable stress assessment methods that can be easily implemented in large-scale epidemiological studies. As part of this thesis, an extensive literature research was performed to create an overview of commonly applied stress assessment methods in children, with an emphasis on epidemiological research and including a description of the major characteristics, strengths and limitations of these common methods (Vanaelst B. et al., 2012b). Based on this literature review, the application of hair cortisol analysis appeared as a promising and readily applicable technique for stress research, although studies in children had not been performed at that moment.

Therefore, a second aim of this thesis was to evaluate the usefulness and utility of cortisol and cortisone measurements in children's hair samples as a biological marker of stress. This research objective, which was novel and previously unexplored for childhood populations, was studied in a sub-study of the IDEFICS project, i.e. in the ChiBS study, and more specifically in elementary school girls (Michels N. et al., 2012d). In contrast to the previous study objective, preschool children (<5 years old) were now excluded from the investigation to assure accurate and reliable completion of the stress questionnaires which requested child-reporting (and not parent-reporting). Apart from these child-reported questionnaires (i.e. the Coddington Life Events Scale, basic emotions and coping questionnaire), also a serum, salivary and hair sample were collected. In this way, a three-dimensional assessment of stress within one individual was achieved, including stress assessment at the environmental level, psychological level and biological level (see Figure 1.5 of the introductory chapter).

2.1 Cortisol and cortisone in hair of elementary school girls and its relationship with childhood stress (Chapter 3.3)

A. Cortisol, cortisone and cortisol/cortisone ratio concentrations in hair

Immunoassay technologies have been the preferred analytical approach for hair cortisol quantification as they are easy to conduct and affordable. However, these commercial kits are in fact developed for salivary cortisol analysis and cross-reactivity with exogenous

glucocorticoids or endogenous cortisol metabolites may occur (Kirschbaum C. et al., 2009; Davenport M. D. et al., 2006; Perogamvros I. et al., 2009; Miller R. et al., 2013). Therefore, researchers recently turned their attention to LC-MS/MS methodology as it shows higher sensitivity, higher reproducibility and no cross-reactivity compared to immunoassays (Xie Q. Z. et al., 2011; Gao W. et al., 2010; Kushnir M. M. et al., 2011). As LC-MS/MS techniques are considered promising for hair cortisol research, this technology was implemented in this thesis.

The UPLC-MS/MS method which was developed and validated in collaboration with the Institute of Legal Medicine - University of Strasbourg, was applied on the 223 collected hair samples, more specifically on the most proximal 6 cm. The median hormone concentrations (in pg/mg, range) were 8.80 (5-1330.48) and 8.62 (5-69.6) for cortisol (N=39) and cortisone (N=168) respectively. The median cortisol/cortisone ratio was 0.80 (range 0.11-54.4) (N=31). As no further data on hair cortisone and hair cortisol/cortisone ratios is available, evaluation of our observations is restricted. However, cortisol and cortisone concentrations were unquantifiable in a large number of samples (82% and 24% for cortisol and cortisone respectively), which is to our opinion due to the length of the analysed hair samples (i.e. 6 cm) and the consequent segmental decline, as further discussed in *Chapter 4.2 Methodological Considerations*. Therefore, a main recommendation arose from this study, i.e. the recommendation not to analyse segments up to 6 cm long and to limit analyses to the most proximal fragments (e.g. 1 to 3 cm proximal) in order to limit the influence of wash-out effects and inter-segment loss, and to obtain an accurate representation of HPA activity.

Table 4.1 presents an inter-matrix comparison of cortisol/cortisone ratios as observed in previous research. Hair cortisone concentrations were higher than cortisol concentrations, as reflected by the median cortisol/cortisone ratio of 0.80 in our study (Table 4.1). This is in line with previous research in saliva, urine and hair samples (Perogamvros I. et al., 2009; Lee S. et al., 2010; Plenis A. et al., 2011; Taylor R. L. et al., 2002; Raul J. S. et al., 2004) (Table 4.1). In plasma and nails on the other hand, reverse cortisol to cortisone relationships were observed (i.e. higher cortisol as compared to cortisone) (Kushnir M. M. et al., 2004; Ben Khelil M. et al., 2011) (Table 4.1). These distinct cortisol/cortisone levels have repeatedly been discussed in the view of 11β-HSD enzymatic activity which locally regulates glucocorticoid action and the cortisol-cortisone shuttle (as presented in *Chapter 1.2 Biology and health consequences of stress*, Figure 1.2: 11β-HSD1 performs cortisone to cortisol conversion, 11β-HSD2 performs cortisol to cortisone conversion) (Stewart P. M. et al., 2011;

Perogamvros I. et al., 2009; Raul J. S. et al., 2004). Measurement of the matrix-specific cortisol/cortisone ratio may thus serve as an indirect measure of 11β-HSD activity: a cortisol/cortisone ratio < 1 suggests increased conversion of cortisol to cortisone by 11β-HSD2 (as suggested in saliva, urine and hair); while ratios > 1 indicate primarily 11β-HSD1 activity (as suggested in plasma and nails).

The hypothesised increased 11β-HSD2 activity in hair or its surrounding tissues (e.g. skin, sweat glands) could thus account for the higher cortisone concentrations in hair, which is confirmed by a recent radiometabolism study in guinea pigs, in which strong indications are presented for cortisol metabolisation prior to incorporation in hair (Keckeis K. et al., 2012). However, as human studies on the metabolism and incorporation of cortisol and cortisone in different matrices are lacking, more fundamental research into 1) the localization and activity of this enzyme in different tissues and 2) its activity and physiological importance under normal and chronic stressful conditions should be conducted in order to elucidate the significance of cortisol/cortisone ratio assessments.

Table 4.1 Cortisol/cortisone ratios for different matrix types

Biological matrix type	**Assay and unit**	**Cortisol**	**Cortisone**	**Cortisol/cortisone ratio**	**N**	**Reference**
Plasma	APPI-MS/MS					(Kushnir M. M. et
	95% reference interval in µg/l	33-246	8-27	3.33-12.5	120 healthy adults	al., 2004)
Saliva – morning	LC-MS/MS					(Perogamvros I. et
	median [range] in nmol/l	8.3 [3.2-14.8]	34.6 [18.6-47]	0.2 [0.1-0.4]	14 healthy volunteers	al., 2009)
Saliva – evening	LC-MS/MS					
	median [range] in nmol/l	0.6 [0.4-1.9]	5.9 [3.4-18.4]	0.1 [0.1-0.3]	14 healthy volunteers	
Urine	RP-LC-UV					(Plenis A. et al.,
	mean (SD) in ng/ml	31.4 (3.7)	60.9 (7.9)	0.52 (0.09)	20 healthy volunteers	2011)
Nails	UPLC-MS/MS					(Ben Khelil M. et
	median [range] in pg/mg	69.5 [36-158]	65 [32-133]	1.13 [0.98-1.26]	10 adult women	al., 2011)
Hair*	UPLC-MS/MS					Our data
	median [range] in pg/mg	8.80 [5.3-1330]	12.6 [5.48-69.6]	0.80 [0.11-54.4]	31 female children	

*In this table, results are presented only for children with full data on hair cortisol and cortisone (N=31). APPI-MS/MS: atmospheric pressure photoionization tandem mass spectrometry; (UP)LC-MS/MS: (ultra performance) liquid chromatography tandem mass spectrometry; RP-LC-UV: reversed phase liquid chromatography with UV detection

B. Hair cortisol and cortisone and its relationship with childhood stress

To examine the usefulness and utility of scalp hair as a biological matrix for chronic stress measurements in children, the association between hair hormone concentrations (i.e. cortisol, cortisone and cortisol/cortisone ratio) and child-reported stress was investigated.

Table 4.2 presents a literature overview of the types of stressors that have been studied in relation to hair *cortisol* (in primarily adult populations), together with the observed direction of the cortisol response in hair. Based on these previous reports, it can thus be summarized that recent and on-going stressors have generally been associated with increased hair cortisol concentrations in a variety of populations, with (as stated by Staufenbiel et al.) medium to large effect sizes (Staufenbiel S. M. et al., 2012). Therefore, these elevated hair cortisol concentrations could also be hypothesized in stressed children; a finding which was not observed in our study and may have been due to sample size limitations (N=39 for hair cortisol versus N=168 for hair cortisone) and other methodological constraints, as discussed in-depth in *Chapter 4.2 Methodological considerations*.

Table 4.2 Overview of articles addressing chronic physical or psychosocial stress and hair cortisol, and the direction of the observed cortisol response in hair

Authors	Sample	Stressor	Result	Association
(Yamada J. et al., 2007)	60 infants hospitalised for minimum 30 days	Hospitalization in neonatal intensive care unit	Increase in hair cortisol with number of days on ventilation	+
(Kalra S. et al., 2007)	25 healthy pregnant women	Perceived stress during pregnancy	Positive association between hair cortisol and perceived stress	+
(Van Uum S. H. M. et al., 2008)	15 chronic pain patients receiving opioid treatment for at least 1 year – 39 control subjects	Severe chronic pain	Elevated hair cortisol in severe chronic pain patients compared to controls; no relationship with perceived stress	+/No
(Kramer M. S. et al., 2009)	31 'preterm birth' mothers – 86 control women	Chronic stressors (e.g. job, family life, abuse) and psychological distress	No relationship between hair cortisol and stress measures	No
(Dowlati Y. et al., 2010)	121 patients of a cardiac rehabilitation program, of whom 34 depressed patients	Severity of depression and perceived stress	No relationship between hair cortisol and the severity of depression or perceived stress	No
(Dettenborn L. et al., 2010)	31 long-term unemployed and 28 employed adults	Unemployment	Increased hair cortisol in unemployed individuals compared with employed individuals; no relationship with measures of psychological stress	+/No
(Stalder T. et al., 2010)	23 alcoholics in acute withdrawal – 25 abstinent alcoholics – 20 matched controls	Psychological stress measures	No relationship between hair cortisol and psychological stress measures in the studied groups	No
(Karlen J. et al., 2011)	99 university students	Major life events and perceived stress	Increased hair cortisol levels with major life events	+
(Steudte S. et al., 2011a)	10 traumatized individuals with PTSD – 17 traumatized controls without PTSD	Lifetime traumatic events in PTSD patients	Positive association between the number of traumatic events and hair cortisol	+
(Manenschijn L.	33 shift workers and 89 day	Shift-work	Increased hair cortisol levels in shift-workers	+

et al., 2011b)	workers		compared to day-workers	
(Grassi-Oliveira R. et al., 2012)	23 treatment-seeking crack cocaine-dependent women	Stressful life events in crack cocaine users	Positive association between the number of stressful life events and hair cortisol	+
(van Holland B. J. et al., 2012)	29 production workers in meat-processing industry	Self-reported stress and need for recovery after work	No relationship with hair cortisol	No
(Luo H. et al., 2012)	32 adolescent females with PTSD and 32 without PTSD – 20 matched adolescents as control	Earthquake experience	Increased hair cortisol in traumatized individuals in first month after earthquake; increased hair cortisol in non-PTSD traumatized individuals compared to PTSD-traumatized individuals several months later after the same stressor	+
(Skoluda N. et al., 2012)	304 amateur endurance athletes – 70 controls	Endurance training and perceived stress	Elevated hair cortisol in endurance athletes compared to controls, with a dose-response relationship with the training volume; no relationship with perceived stress	+/No
(Stalder T. et al., 2012b)	Study 1: 155 adults – study 2: 58 adults	Psychosocial stress measures	No relationship between hair cortisol and psychosocial stress variables	No
(Vaghri Z. et al., 2012)	339 children from child care centres in different neighbourhoods	Socio-economic situation of children	Inverse association between hair cortisol and parental education (i.e. higher education, lower child hair cortisol)	+
(O'Brien K. M. et al., 2012)	Diverse sample of 135 adults	Perceived chronic stress	Positive correlation between hair cortisol and a global, cumulative stress index, but not with single stress indices	+
(Steudte S. et al., 2013)	28 PTSD patients, 27 traumatized and 32 non-traumatized healthy controls	Trauma-related and chronic stress measures	Inverse relationship between hair cortisol and the number, frequency, time interval and severity of traumatic events	-
(Groeneveld M. G. et al., 2013)	42 healthy children	Entering elementary school as childhood stressor	Higher hair cortisol concentrations two months after school entry than before school entry	+

Ass: direction of the association between hair cortisol and the studied stressor: + (positive association), - (inverse association), no (no association); PTSD: post-traumatic stress disorder

However, in this study, hair *cortisone* concentrations were positively correlated with the life events score for the past 6 months (Coddington Life Events Scale). This finding thus indicates elevated hair cortisone under psychosocial stress in children and suggests hair cortisone as a useful, additional chronic stress marker; a hypothesis which is strengthened by the following theoretical basis: 1) 11β-HSD has been located in human skin cells (Tiganescu A. et al., 2011; Hirasawa G. et al., 1997; Terao M. et al., 2011), and 2) stress and HPA activity have been shown to influence 11β-HSD activity, thereby potentially affecting cortisol bioavailability and hair cortisol/cortisone levels (Plenis A. et al., 2011; Romer B. et al., 2009; Altuna M. E. et al., 2006; Welberg L. A. M. et al., 2005; Yehuda Rachel et al., 2009; Perogamvros I. et al., 2010; O'Donnell K. J. et al., 2012). However, as observations in this area mainly result from animal experiments or human trauma and depression studies, further research should elucidate the importance of glucocorticoid metabolism and incorporation of cortisol and cortisone in hair in response to human psychosocial stressors.

In this study, no associations were found between hair cortisol or cortisone, and emotions or coping style, although these psychological and biological stress responses are assumed to be strongly interconnected through the human stress system (Vanaelst B. et al., 2012b; Cohen S. et al., 1997a; Staufenbiel S. M. et al., 2012). As also demonstrated in Table 4.2, observations between hair cortisol concentrations and self-reported measures of perceived stress have yielded inconsistent results. A first factor to understand this 'lack of psycho-endocrine covariance' are the different dynamics of the psychological and biological stress system (Oldehinkel A. J. et al., 2011; Schlotz W. et al., 2008), making it hard to find an association between 'earlier' affective responses and 'later' endocrine responses (Miller G. E. et al., 2007). In fact, Stalder et al. and Staufenbiel et al. recently discussed some limitations of psychosocial stress assessment as particular shortcomings in establishing hair-stress relationships, such as the reliance on one-time retrospective stress assessments, problems of recall-bias and social desirability, heterogeneous populations, diverse questionnaires and variable lengths of studied hair samples etc. (Stalder T. et al., 2012b; Staufenbiel S. M. et al., 2012). Second, not all stressors conclusively produce a psychological or biological stress response (e.g. because it is not perceived stressful) (Gunnar M. R. et al., 2009a; Stalder T. et al., 2012b) and additionally, inter- and intra-individual differences in response to stressors may exist, depending on characteristics of both the stressor and the person facing it (Kudielka B. M. et al., 2009; Michaud K. et al., 2008; Miller G. E. et al., 2007; Campbell J. et al., 2012). Also, large intra-individual stability has been observed in hair cortisol concentrations, with

only a minimal influence by stress-related or occasion-specific factors (Stalder T. et al., 2012c).

Despite its exploratory nature, this study contributed significantly to psychosocial stress research as it identified hair cortisone as a novel additional or substituting stress biomarker, a subject that had never been investigated in children's hair or in the field of childhood stress up to now. Because little is known about the metabolism or incorporation of cortisol and cortisone in hair and its response to stress, and considering the need to identify and quantify potential confounding variables, this study encourages further chronic childhood stress research.

C. Altered cortisol secretion as chronic stress response

As mentioned above, stressors have generally been associated with increased hair cortisol concentrations (see Table 4.2). However, depending on the biological matrix, and time-, stressor- and person-dependent characteristics, both decreased and increased cortisol may be observed as chronic stress response. This section elaborates in more depth these altered cortisol responses.

In general, chronic exposure to psychosocial stressors is associated with a raised cortisol output: increases in CRH and consequent chronic activation of the HPA axis attenuates morning cortisol levels and elevates afternoon cortisol levels. As a result, the circadian variation is flattened and the daily output of cortisol is increased. However, in the case of persistent chronic stress, this HPA axis hyperactivity has also been shown to flip over to a hypo-active state (Danese A. et al., 2012; Miller G. E. et al., 2007).

As already briefly mentioned in *Chapter 1.3 Epidemiological approaches to measure childhood stress*, it needs to be acknowledged that chronic stress may elicit a variety of HPA responses, depending on a number of features, which are described in more detail below:
1) the time elapsed since stressor onset (stress results in an initial increase in HPA activity which decreases with time since stressor onset), 2) the nature of the threat (e.g. traumatic stressors may result in more pronounced alterations in HPA function compared to non-traumatic events), 3) emotions elicited by the stressor, 4) the controllability of the stressor (e.g. uncontrollable chronic stress may diminish HPA activity), 5) the psychiatric consequences of chronic stress which may influence the magnitude and direction of HPA axis activity (e.g. hypocortisolism and hypercortisolism in respectively PTSD and depression as

key examples), 6) genetics (e.g. polymorphisms in GR or MR), 7) effective coping strategies and social support, 8) previous exposure to stressful circumstances (e.g. early life stress results in lower HPA axis reactivity and blunted cortisol responses to stress in childhood (Danese A. et al., 2012; Hunter A. L. et al., 2011; Ouellet-Morin I. et al., 2011; Badanes L. S. et al., 2011)) and 9) sexual and pubertal maturation stage (e.g. increase in baseline cortisol and stress reactivity with age and pubertal development (Gunnar M. R. et al., 2009b; Miller G. E. et al., 2007).

These time-, stressor- and person-dependent characteristics may thus result in both decreased and increased cortisol as chronic stress response in biological matrices. However, depending on the sample type in which cortisol is measured, the situation may differ in complexity. In particular for the hair matrix, the cortisol response to stress has been shown to be more consistent (see Table 4.2, i.e. increased cortisol). This may particularly be attributable to the unique characteristics of the hair matrix, providing a long-term, retrospective measurement which accumulates cortisol information across multiple diurnal cycles.

Especially for this reason, future hair research should further elaborate and explore the potential of segmental hair analysis, as initiated by Luo et al. (Luo H. et al., 2012): in chronic stress situations, the initial increase in cortisol may be observed in the more distal parts of the hair samples, while a cortisol decrease may be observed in the more (recent) proximal segments of hair samples. This unique ability of the hair matrix to represent a month-by-month reflection of cortisol exposure in response to chronic stress, has up to now been explored insufficiently.

2.2 Intercorrelations between serum, salivary, and hair cortisol and child-reported estimates of stress (Chapter 3.4)

Stressor questionnaires and biological measurements of cortisol have both been shown to be valid indicators of (childhood) stress exposure, although there is currently no consensus on a reference method to measure stress in children. This thesis investigated cortisol intercorrelations between different biological sample types, namely serum, saliva and hair, which together cover short- and long-term stress exposure. Furthermore, a triangulation method was applied to examine to what extent childhood stress can be estimated accurately

by stressor questionnaires (Coddington Life Events Scale) and biological markers (serum, salivary and hair cortisol).

In line with previous research, a lack of association and disagreement were observed between measures of single-point, short-term cortisol versus long(er) term cortisol (Levine A. et al., 2007; Hellhammer D. H. et al., 2009; D'Anna-Hernandez K. L. et al., 2011; van Holland B. J. et al., 2012; Xie Q. Z. et al., 2011): serum cortisol positively correlated with single salivary measures (both short-term), while hair cortisol showed no correlation with serum cortisol, but positively correlated with salivary (AUC) cortisol (both longer-term measures). The Triad analysis showed that salivary cortisol most accurately indicated 'true' childhood stress for short periods in the past (i.e. Coddington Life Events Scale, event score for last 3 months), whereas hair cortisol may be preferred above salivary measurements for periods more distant and thus for chronic stress assessment.

As a result, differentiating the type of biological matrix (i.e. saliva, hair) may be suggested according to the time period under investigation, which is in fact the most contributing finding of this study. The key recommendation from *Chapter 1.3 Epidemiological approaches to measure childhood stress* to combine multiple stress assessment methods (i.e. both questionnaires and stress hormone measures), is therefore unaffected by our findings. After all, combining environmental, psychological and biological measures provides complementary information and a more aggregated view on stress.

3 Scalp hair as bio-indicator for dietary habits and its association with metabolic health and stress

As literature suggests that stress may affect dietary habits and the body's mineral status (Torres S. J. et al., 2007; Grases G. et al., 2006), the scope of this thesis was broadened to also include hair mineral analysis: a third objective of this thesis was to examine the suitability of scalp hair as bio-indicator for specific aspects of children's dietary habits, a topic which remained controversial in literature (Jeruszka-Bielak M. et al., 2011; Rodenas S. et al., 2011; Wojciak R. W. et al., 2004; Wojciak R. W. et al., 2010; Hong S. R. et al., 2009; Ozden T. A. et al., 2012; Contiero E. et al., 1994).

Each of the hair samples collected from the 'ChiBS' elementary school girls was split into two fractions, i.e. one fraction for cortisol and cortisone analysis as described above, and one

fraction for mineral analysis. Then, an ICP-MS technique was validated in the Department of Analytical Chemistry of Ghent University to quantitatively determine the concentration of Ca, Cu, Fe, Mg, Na, P and Zn in the 6 cm hair samples. Reference values were established for these hair minerals, after which the relationship with dietary habits, metabolic health and childhood stress was investigated, as described below.

3.1 Reference values of hair minerals in elementary school girls and their relationship with food consumption frequencies (Chapter 3.5)

First, using the LMS method of Cole (Cole T. J. et al., 1992), age-specific reference values were established for this population of healthy, Flemish elementary school girls. The reference values were in line with data for other childhood or adolescent populations. Second, this study examined to what level variation in hair minerals could be explained by the consumption frequencies of specific food groups in elementary school girls. Using reduced rank regression (RRR), we aimed to investigate whether or not hair minerals might be related to respective dietary intakes. The retained RRR factors explained respectively 40, 50, 45, 46, 44 and 48% of variation in Ca, Cu, Fe, Mg, P and Zn in hair (independently from the child's age, BMI, hair colour, physical activity and parental income), indicating an appreciable influence of food consumption frequencies on hair mineral concentrations. Nevertheless, no relationship was observed with vitamin or mineral supplement use, and a number of food groups known to be 'rich' sources of minerals did not emerge as contributors to certain hair minerals, resulting in insufficient evidence to fully consider hair minerals as direct reflection of dietary habits or intake. Therefore, further elucidation of the mechanisms and processes involved in mineral incorporation and accumulation in scalp hair is needed, as these are largely unknown and may complicate the understanding of the diet-hair mineral relationship. For example, homeostatic control mechanism, genetics, and environmental and lifestyle factors may determine the mineral status (Hunter D., 1990)

3.2 Hair minerals and metabolic health in elementary school girls (Chapter 3.6)

As hair minerals may to an appreciable extent reflect dietary intake (section above) (Jeruszka-Bielak M. et al., 2011; Rodenas S. et al., 2011; Wojciak R. W. et al., 2010; Hong S. R. et al., 2009; Ozden T. A. et al., 2012; Contiero E. et al., 1994), and diet has been shown to be

associated with metabolic health and body composition, this thesis further examined the potential relationship between hair minerals, obesity and metabolic health in elementary school girls. This association has been suggested in literature for adult populations (Wang C. T. et al., 2005a; Wang C. T. et al., 2005b; Skalnaya M. G. et al., 2007; Hong S. R. et al., 2009; Park S. B. et al., 2009; Chung J. H. et al., 2012) but remained unexplored in children. Based on previous studies, we hypothesized inverse associations between hair minerals and a) overweight and obesity, and b) metabolic health in children.

First, this study indicated an increased metabolic risk for overweight and obese girls as they demonstrated a higher blood pressure, reduced insulin sensitivity and HDL cholesterol, and a higher (i.e. more unhealthy) metabolic score (based on systolic and diastolic blood pressure, homeostasis model assessment for insulin resistance and non-HDL cholesterol as parameters).

Second, this cross-sectional study presented an inverse correlation between hair Ca, Mg, and Ca/P and the girls' metabolic score, which is in line with the formulated hypothesis. In particular girls with a total number of three of more metabolic parameters above the 75^{th} percentile showed significantly reduced Ca, Mg, and Ca/P concentrations. It may thus be concluded that reduced hair mineral levels may contribute to or result from a poorer metabolic health in young girls. This finding should not only be viewed in the context of an unbalanced dietary intake, but also in the view of an altered mineral metabolism associated with specific metabolic conditions.

Finally, positive associations were observed between some minerals (Ca, Ca/Mg ratio and Ca/P ratio) and body composition parameters (BMI and BF% as continuous variables), although the minerals did not differ over BMI categories. This is in contrast with the formulated hypothesis and thus inconsistent with the idea that overweight and obese children are at increased risk for mineral deficiencies due to e.g. an unbalanced diet, or that mineral deficiencies may contribute to the development of obesity (Vaskonen T., 2003; Garcia O. P. et al., 2009). However, the observed positive associations may be interpreted in the context of malnutrition and the presentation of low mineral statuses in underweight children, given their higher number compared to overweight or obese children in our population. This may have affected the direction of the relationship between BMI z-scores, BF% and hair mineral status. Also for the normal weight group, we may hypothesize that diet and energy intake increases with elevating BMI/BF% and that consequently also the mineral intake may rise with

increasing BMI/BF%, resulting in positive associations with hair Ca, Ca/Mg and Ca/P, independent from the BMI category of the child.

However, as this was the first study to examine hair minerals in relation to childhood body composition, no overall conclusions should be made in this regard.

3.3 Chronic stress and mineral concentrations in hair of elementary school girls (Chapter 3.7)

Although literature in this regard is scarce, there are indications that stress may negatively affect the body's mineral status through behavioural or physiological pathways (Seelig M. S., 1994; Grases G. et al., 2006; Moore R. J. et al., 1993). As cortisone concentrations in children's hair were associated with child-reported stress (Chapter 3.3), it is possible that the experience of childhood stress may also influence the level of minerals in hair, e.g. through a change in dietary habits or a change in the metabolism and distribution of minerals. Therefore, this thesis additionally studied the association between childhood stress and hair mineral levels, as a first, exploratory study in its field.

Independently from individual and behavioural factors, hair cortisone concentrations were inversely associated with concentrations of Ca, Mg, Zn, and the Ca/P ratio in hair. The CLES negative event score was however not associated with hair minerals levels, which may indicate that that the mechanisms affecting hair mineral levels cannot be detected or reflected by stressor questionnaires.

Nevertheless, if CLES life events were studied categorically ('at risk' versus 'not at risk'), an elevated Ca/Mg ratio was observed in children at risk for psychological sequelae by life events in the past 6 months. This may indicate that life events may be associated with minerals only in more extreme cases of repeated stressor exposure, i.e. if the cut-off score for becoming at risk for psychological problems is reached.

Since analyses were adjusted for individual and behavioural factors, the changes in mineral levels may be ascribed to the unique physiological contribution of increased stress, although many details in this stress-mineral pathway are still unclear. For example, it is unclear if and why some minerals are more susceptible to stress than others, or if the stress-mineral relationship is stressor dependent. Irrespective of the precise mechanism by which stress is

associated with hair mineral levels (e.g. changes in metabolism, changes in diet), this study has pointed to another potential health impact of stress exposure in young children.

3.4 Conclusion on hair minerals in relation to dietary habits, metabolic health and stress

First, this exploratory hair mineral research in children contributed to the field of nutritional epidemiology by demonstrating that hair mineral analyses could, after further validation, be used as a complementary method to investigate the body's mineral status and potentially dietary habits over a long-term in the past (e.g. 6 cm hair sample theoretically represents 6 months in the past). Second, reduced hair mineral levels were observed with a poorer metabolic health and increased stress in young girls, probably due to an altered mineral metabolism or change in lifestyle associated with these conditions (e.g. unbalanced dietary intake). Unfortunately, this study did not allow unravelling more detailed mechanisms in the involved pathways. Also, many variables that influence hair mineral concentrations remain to be accurately described (Kempson I. M. et al., 2011), as further discussed in *Chapter 4.2 Methodological considerations*. Nevertheless, we have shown the opportunities of hair mineral analysis for future epidemiological research, particularly for research objectives demanding long-term, 'chronic' assessments with little temporal or daily fluctuations (e.g. identifying correlations with lifestyles, morbidity and risk factors).

4 Childhood stress and the association with overweight or obesity

The last decades have been characterized by a global growing obesity epidemic, starting already in childhood (Ahrens W. et al., 2011b; Cali A. M. G. et al., 2008). Therefore, a better understanding of the complex aetiology of obesity is needed in order to help developing effective prevention programs. Evidence confirmed the importance of focusing obesity prevention on young age groups, as obese children were found to be at increased risk of becoming obese adults ("tracking phenomenon") (Singh A. S. et al., 2008) and to experience increased metabolic complications in adulthood (Reilly J. J. et al., 2011).

Recently, the effects of chronic psychosocial stress have been increasingly recognized, also in children. The final aim of this thesis was to investigate the **association between childhood stress and alterations in body composition in young children, taking into account the moderation and mediation effect of lifestyle factors (Chapter 3.8)** such as diet, sedentary behaviour and sleep. This research objective was studied on baseline ChiBS data for both

boys and girls. A novel and contributing aspect of this study is its inclusion of multiple stress assessment methods, standardized body composition measurements and the incorporation of stress-related lifestyle factors.

Child-reported negative life events were positively while happiness was negatively associated with body composition indicators (BMI and WHtR), also after adjustment for the lifestyle factors. The lifestyle factors did not mediate the stress-body composition association. This could point to a predominant importance of non-behavioural, neuro-endocrinological pathways in the development of overweight/obesity, as also described in the introductory *Chapter 1.2 Biology and health consequences of stress* (Figure 1.4): i.e. in particular the stress-related over-stimulation of the HPA axis and cortisol excess may be the main drivers of this stress-obesity relationship. However, we did not observe a relationship between salivary cortisol and body composition, which is an unexpected finding as salivary cortisol patterns were previously related to child-reported stress in this population (Michels N. et al., 2012c). We therefore assumed an association between salivary cortisol and body composition, similarly to the observed relationship between child-reported stress and body composition. The lack of an association between salivary cortisol and body composition in this study may be explained by the occurrence of cortisol abnormalities in overweight and obese children only (i.e. abdominal obesity has been associated with hypercortisolemic conditions (Bjorntorp P. et al., 2000; Nieuwenhuizen A. G. et al., 2008)), and the low prevalence percentages of overweight or obesity in our population. Concerning the moderation effects of behaviour in the stress-body composition relationship, the studied lifestyle parameters were shown to moderate one or more stress-body composition relationships: more specifically an unhealthy lifestyle (high snack frequency, high screen time, low sleep duration) strengthened the stress-body composition association.

In this preadolescent population, a sex interaction was observed in the association between peer problems and WHtR, with a significant association demonstrated only for girls. This is in line with an adolescent study in which stress and measures of adiposity were associated in girls only (De Vriendt T. et al., 2012). Sex differences in social roles, brain differentiation and stress responsiveness may be involved in this finding (Rose A. J. et al., 2006; Young E. A. et al., 2008; Gatineau M. et al., 2011; Naninck E. F. G. et al., 2011). More specifically, it has been shown that HPA function is influenced by sex steroids, resulting in a higher sensitivity of the adipose tissue to the stress response in females compared to males (Naninck E. F. G. et al., 2011; Young E. A. et al., 2008; Staiano A. E. et al., 2012). These sex differences may

particularly emerge during adolescence given the major physical and behavioural changes associated with this developmental period. In conclusion however, no general sex differences were observed in the association between childhood stress and body composition, as only one of the studied relationships showed a sex interaction effect.

As no conclusions on causality or direction can be made, these cross-sectional findings are mainly of importance by indicating that an unhealthy lifestyle may make children more prone to stress-induced changes in body composition or, vice versa, that a healthy lifestyle may attenuate the effects of stress on adiposity.

5 Schematic summary of findings

The aim of Figure 4.1 is to provide a schematic overview of the main results of this thesis. Boxes 1 to 4 present the results in relation to the research objectives of this thesis.

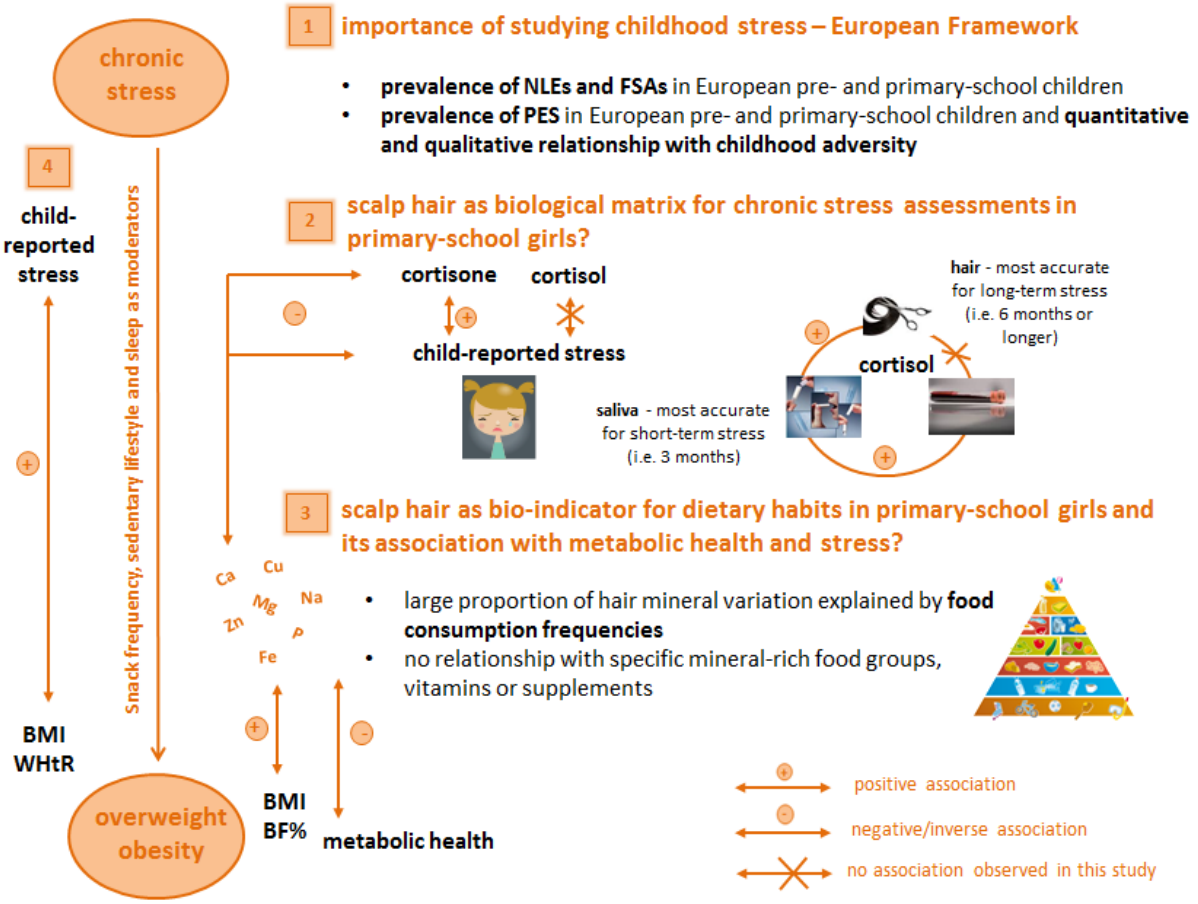


Figure 4.1 Schematic presentation of thesis' objectives and the main results. The lines in the figure illustrate the observed associations in this study. BF%: body fat percentage; BMI: body mass index; Ca: calcium; Cu: copper; FSA: familial and social adversity; Fe: iron; Mg: magnesium; Na: sodium; NLE: negative life event; P: phosphorus; PES: psychosomatic and emotional symptom; WHtR: waist to height ratio; Zn: zinc.

CHAPTER 4.2 METHODOLOGICAL CONSIDERATIONS

1 The IDEFICS and ChiBS research framework

The first research objectives of this thesis (Chapter 3.1 – 3.2) were studied within the framework of the IDEFICS project. A main strength of this approach is the large, international sample comprising 8 European countries, which allowed studying the prevalence of childhood adversity and its relationship with psychosomatic and emotional problems in a larger context than was previously done. Moreover, this allowed insightful comparisons across different nations. However, the regional comparisons were aimed to be strictly exploratory as the survey centres are not necessarily representative for the countries, and cultural, religious and welfare typologies, as well as heterogeneities in societal and policy regimes should be considered when interpreting cross-national comparisons. A second strength of this approach is the standardized research methodology over all survey centres for questionnaire administration, body composition assessment, measurement of metabolic parameters etc., resulting in comparable data.

The remaining research objectives of this thesis were studied within the ChiBS project, which was embedded within the Belgian IDEFICS setting. The ChiBS cohort provided valuable data and new insights into the influence of chronic psychosocial stress on changes in body composition, but also allowed an in-depth investigation of the validity of the different childhood stress assessment methods, such as hair analysis.

1.1 Representativeness of the ChiBS cohort

Embedment of ChiBS within IDEFICS inherently implied the introduction of a selection bias, i.e. parents interested and willing to participate in IDEFICS may also be more motivated to participate in another study, e.g. ChiBS. The high consent percentages of the baseline ChiBS examination modules (ranging from 67% to more than 90%) must be interpreted in this context. On the other hand, embedding ChiBS within IDEFICS, which is already burdening children and parents, may have decreased participation to the additional ChiBS project.

However, we did not find significant socio-demographic differences between ChiBS participants and ChiBS non-participants (χ^2 tests in Table 2.2 of *Chapter 2.2 Children's body*

composition and stress – the ChiBS study), indicating that there was no further participation bias introduced by the subjects included in the ChiBS study in comparison with the existing IDEFICS cohort, as also illustrated in Figure 4.2 below.

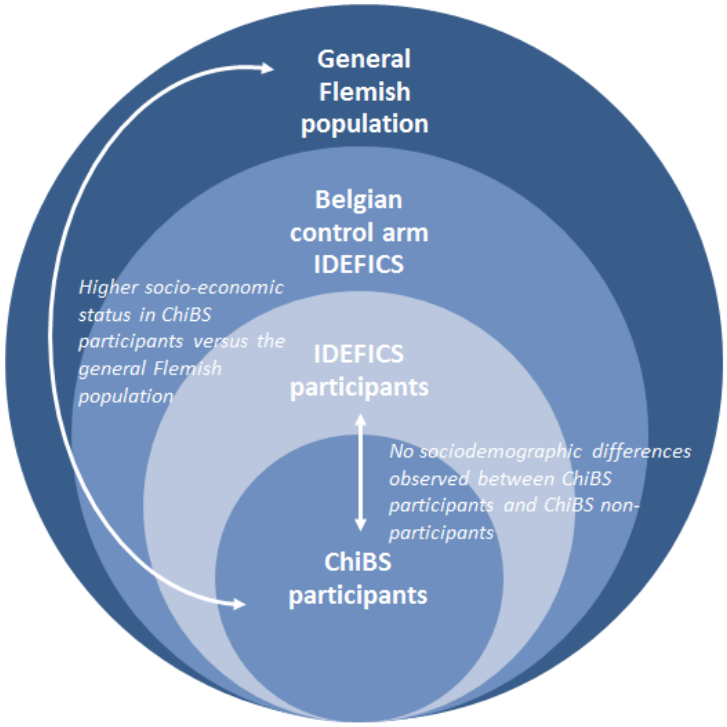


Figure 4.2 Representativeness of the ChiBS cohort. Inner circle: children participating in the Belgian control arm of IDEFICS and additionally participating in ChiBS ('ChiBS participants'); second inner circle: children participating in the Belgian control arm of IDEFICS, without additionally participating to ChiBS ('ChiBS non-participants' or 'IDEFICS participants').

To further examine the representativeness of our study population, we compared the study sample with socio-demographic characteristics of the general population in Flanders. This socio-demographic information was obtained through consulting statistics of the Flemish authorities and 'Child and Family' (Studiedienst van de Vlaamse Regering, 2007; Studiedienst van de Vlaamse Regering, 2011; Kind en Gezin, 2010) and resulted in the following findings. Parents of the children participating to ChiBS were higher educated and less often of migrant origin compared to the general Flemish population (47.8% versus 31.7% of ISCED 5 or higher; 3.4% versus 6.4% of migrants; respectively). Additionally, a traditional two-parent family structure was more prevalent in ChiBS participants compared to the general Flemish population (76.9% versus 66.1% of traditional family structures respectively), indicating that the ChiBS children belong to a higher socio-economic class compared to the

general Flemish population (see Figure 4.2). It should however be noted that these findings are inherently related to the overall higher socio-demographic characteristics of the city of Aalter compared to the general Flemish population (Agentschap voor Binnenlands Bestuur et al., 2011). The overweight and obesity percentages observed in the baseline ChiBS survey were low compared to the most recent reference data available for Flemish children. The Flemish Growth Survey, conducted between 2002-2004 in 16,000 Flemish children and adolescents, demonstrated overweight and obesity percentages of 14.2% and 3% for female participants respectively, and 11.8% and 2.6% of overweight and obesity for male participants respectively (2-18 years old) (compared to 5.5% and 1.5% of overweight and obese children in ChiBS) (Roelants M. et al., 2009). The low overweight and obesity percentages in ChiBS thus limit further investigations, i.e. evolutions and changes in body composition parameters (as continuous variables) should be investigated longitudinally instead of studying differences between obese and non-obese children.

1.2 Feasibility of the examination modules

We experienced all examination modules as feasible techniques in working with young children. However, as blood sampling may be a stressful and invasive procedure, we experienced the need to apply local anaesthetics and to use stress-reducing butterfly needles (Chambers C. T. et al., 2009; Kettwich S. C. et al., 2007). In addition, handling the stringent logistical conditions for serum sampling (e.g. medical staff, processing and storage) may be challenging in large-scale epidemiological research. With regard to the administration of stressor questionnaires, we have experienced a poor or even lack of comprehension of items on the stress questionnaires by the youngest age groups (first year of elementary school), even though children as young as four years of age were previously shown to be reliable informants about experienced events (Larsson A. S. et al., 2009). Therefore, we let a trained researcher assist in filling out the questionnaires, and used pictures and calendar events for clarification and to increase the accuracy of reporting in time (Garrison C. Z. et al., 1987). The saliva sampling protocol was quite intensive to standardize, e.g. with regard to the frequency and timing of saliva collection, the return of saliva samples, the compliance with checklist on restrictions etc. (Michels N. et al., 2012b). In this context, hair sampling was experienced as a more feasible and low burden technique which offered considerable logistical advantages compared to other biological samples: it is non-invasive; only one sample is needed to retrospectively examine cumulative cortisol/cortisone (or mineral) concentrations; samples

can be taken at any time of the day in home-settings and can be stored for a long time without special storage conditions (Russell E. et al., 2012).

2 Hair cortisol and cortisone analysis

2.1 Wash-out and segmental decline

To our knowledge, this study was the first to report a combined UPLC-MS/MS assay for simultaneous cortisol and cortisone detection in children's hair for chronic stress research. Despite the obtained, adequate validation parameters of the UPLC-MS/MS method (Chapter 2.3), cortisol and cortisone were either low or unquantifiable in a large number of samples. As this study was the first to examine hair cortisol and cortisone in healthy children with UPLC-MS/MS, we cannot verify similar problems of non-traceability with the UPLC-MS/MS methodology or simply evaluate our results with previously reported concentrations in literature. However, as the reliability of our method was demonstrated in validation experiments, the cortisol and cortisone results indicate true observations of low (to very low) physiological levels of cortisol and cortisone in 6 cm segments of children's vertex posterior hair, which is in line with recent findings of Vaghri et al. presenting hair cortisol concentrations in preschool children around 20 pg/mg in the first 1-2 cm of proximal hair (immunoassay) (Vaghri Z. et al., 2012). The main factor involved in the observed low concentrations is likely the length of the analysed hair samples and the consequent segmental decline, as described below.

As the presented laboratory method was developed in the context of stress-research (Michels N. et al., 2012d), more specifically chronic stress over a six month period, it was decided to analyse hair samples with a length of 6 cm. This has generally been assumed to be the maximum length of hair being a reliable estimate of systemic cortisol concentrations in the past (Russell E. et al., 2012), although this proposition might need a more stringent revision for the future. Wash-out effects and inter-segment loss have repeatedly (but not always) been shown from scalp-near to distal hair fragments (Dettenborn L. et al., 2010; Kirschbaum C. et al., 2009; Gao W. et al., 2010; Steudte S. et al., 2011b; Stalder T. et al., 2012a; Dettenborn L. et al., 2012b; Hamel A. F. et al., 2011; Xie Q. Z. et al., 2011; Li J. et al., 2012) and have been attributed to repeated exposure to shampooing or to water alone, with the possibility of an additional contribution from UV radiation (Meyer J. S. et al., 2012; Hamel A. F. et al., 2011;

Li J. et al., 2012). More specifically, Dettenborn et al. demonstrated a 16% decline in cortisol from the most proximal 3 cm hair segment to the next 3 cm hair segment (Dettenborn L. et al., 2012b). Furthermore, Xie et al. observed a continuous decline in hair cortisol concentrations along a 5 cm hair shaft (Xie Q. Z. et al., 2011).

As we analysed the full 6 cm hair segment, this inter-segment loss could have contributed to a dilution and underestimation of the cortisol and cortisone concentrations resulting in low total concentrations for the full segment (and thus higher numbers of samples below the LOD). Given our observations, we thus recommend not to analyse segments up to 6 cm long, but to divide the hair samples into fragments of 1 or 3 cm maximum in order to limit these wash-out effects. The most proximal fragments can then be considered the most accurate representation of HPA activity. This inter-segment loss and possible wash-out effects remain an important task for future research to fully understand the usefulness of hair cortisol and cortisone measurements as a valid reflection of cumulative excretion in the more distant past.

2.2 Other methodological aspects

Hair analysis offers considerable advantages for large-scale epidemiological stress research in children compared to other commonly used biomatrices, although standardisation of the sampling and laboratory analysis methods between research groups are prerequisites for comparison between studies. Currently, laboratories differ in the amount of hair needed (e.g. 5 to 50 mg), the duration and method of storage, the applied hair processing technique (e.g. grinding versus mincing), the hair washing procedure (e.g. no washing, isopropanol or dichloromethane washing), the cortisol extraction method and method of analysis (e.g. immunoassays (ELISA, RIA, CLIA), chromatography) (Staufenbiel S. M. et al., 2012; Meyer J. S. et al., 2012; Russell E. et al., 2012; Stalder T. et al., 2012a). Staufenbiel et al. therefore pointed to the importance of assimilating the laboratory techniques and creating a ‘gold standard’ in order to rule out methodological differences in comparing results (Staufenbiel S. M. et al., 2012).

Furthermore, as little information is available on fundamental aspects that may affect hair cortisol/cortisone concentrations, potential confounding variables remain to be identified and quantified. An initiative in this regard was taken by Dettenborn et al. (Dettenborn L. et al., 2012b), demonstrating a lack of effect for natural hair colour, curly or waved hair structure, use of oral contraceptives, cigarette smoking and hair washing frequency on hair cortisol

concentrations (only for proximal fragments). With regard to sex and age, a quadratic relationship was shown between hair cortisol concentrations and age, and higher concentrations were observed in men compared to women. Other potential confounding variables that remain to be investigated are e.g. influence of storage duration on cortisol/cortisone stability in hair samples, puberty, menopause, corticosteroid use, cosmetic hair treatments, hair growth rate, body composition, intra-individual stability (Stalder T. et al., 2012c), local synthesis or metabolism of cortisol and cortisone in the hair follicle etc. Also, it should be clarified if these intra-individual and socio-demographic effects also apply for hair cortisone concentrations.

Last, application of hair analyses in a general population sample will have to deal with the issue of insufficient hair (growth) at the vertex posterior region to reflect long-term cortisol/cortisone production, e.g. boys or men having shorter shaved heads; or people who have religious or aesthetic objections against hair sampling.

3 Hair mineral analysis

As mentioned before, scalp hair is for several reasons an ideal matrix for epidemiological research (non-invasiveness, storage etc.). Apart from hormones, also minerals get sealed into scalp hair during its formation, which thus offers opportunities for hair mineral research.

Hair minerals have been studied extensively in relation to disease states, metabolic disorders, environmental exposures, nutritional status etc., but have remained controversial as it is uncertain what a mineral concentration in hair actually represents (Kempson I. M. et al., 2011). For instance, does an elevated hair mineral concentration reflect a high dietary intake, a high actual endogenous concentration, or an increased elimination in hair as excretory route? Or a complex mixture of these pathways?

As it remains unclear to what extent hair minerals represent the total body availability of minerals, some studies have examined the correlation between hair mineral concentrations and mineral concentrations in other sample types: no or only weak correlations were demonstrated between hair and serum or urine, while correlations were stronger between the hair and nail matrix (Rodrigues J. L. et al., 2008; Sukumar A. et al., 2007; Rodushkin I. et al., 2000; Przybylowicz A. et al., 2012; Carneiro M. F. H. et al., 2011; Ozden T. A. et al., 2012; Gurgoze M. K. et al., 2006; Laitinen R. et al., 1988; Afridi H. I. et al., 2012; Afridi H. I. et al., 2011a; Afridi H. I. et al., 2011b; Afridi H. I. et al., 2008; Dastych M. et al., 2010; Haddy T. B.

et al., 1991). Given the distinct representative time windows between biological fluids and hair samples (i.e. serum or urine generally reflect hours to days, while hair and nails present a larger window of detection) and given the distinct factors involved in mineral homeostasis or incorporation in hair versus other tissues, this finding is not surprising. Therefore, it could be recommended to analyse multiple sample types in parallel: a parallel increase or decrease in multiple samples could then point to respectively a mineral excess or deficiency in the individual (World Health Organization et al., 1996).

Furthermore, there is still a poor understanding about the incorporation mechanisms of minerals in hair and variables altering hair mineral concentrations, such as UV-degradation, hair colour, hair treatments, age, gender, ethnicity, personal habits and seasonal fluctuations, geographical factors, inter-element correlations or competition, genetic polymorphisms and external sources of contamination (Kempson I. M. et al., 2011; Kempson I. M. et al., 2007; Chojnacka K. et al., 2006; Sukumar A. et al., 2007; Oyoo-Okoth E. et al., 2012). With regard to the latter, Morris et al. recently investigated the longitudinal distribution of minerals along the hair strand and demonstrated for most minerals an increase in their concentration from root end to distal end fragments. This may be attributed to continuing contamination from exogenous sources as the hair grows and continues to be exposed, although appropriate washing or decontamination procedures should solve this issue. An additional countermeasure to reduce the impact of these longitudinal differences could be to limit hair analysis to the first most proximal centimetre, although this would increase the number of hair strands needed to obtain an adequate hair mass for laboratory analysis (and thus potentially decrease participation rates in epidemiological research), and inhibit long-term retrospective assessments (Morris J. S. et al., 2012).

Nevertheless, it can be concluded that hair mineral analysis is currently useful as complementary analytical method for mineral assessment, with the unique characteristic of backtracking the time period of assessment to several months ago and being more stable with regard to daily or temporal fluctuations. Hair mineral analysis has in particular been shown valuable in detecting differences between groups of individuals with different body composition, nutrient intake, disease states etc. and in providing information regarding acute and chronic consumption or exposure.

4 Main strengths and limitations of this research

The main strength of this research lies in its strong innovative nature: for the first time, cortisol, cortisone and minerals were studied in girls' hair, more specifically in relation to psychosocial stress, food consumption and metabolic health. Although salivary cortisol analysis has been a commonly used, non-invasive method for childhood stress research (Chida Y. et al., 2009; Hellhammer D. H. et al., 2009), hair analysis additionally offers the unique advantage of a longer-term detection window with standardized sampling, avoiding problems of non-adherence and situational confounding, which is an important feature for research in children. The main contribution of this thesis is therefore the in-depth exploration of this matrix for epidemiological stress research in children. Also, the public health relevance of this thesis should be acknowledged, particularly for studying the relationship between stress and body composition in children, as further discussed in *Chapter 4.3 Public health and future research*. Moreover, some important methodological strengths of this research should be mentioned, such as its international context in studying the prevalence of childhood adversity, its standardized measurements, its assessment of stress at different levels (i.e. environmental, psychological and biological), and its application of state of the art laboratory techniques (i.e. UPLC-MS/MS and ICP-MS). Nevertheless, also some specific methodological issues and limitations require attention.

A first limitation of this research is its cross-sectional study design which does not allow studying causality, nor ruling out reciprocal effects. For example, this thesis indicated that some childhood adversities are apparent risk factors for psychosomatic and emotional symptoms in children (Chapter 3.2). However, a reverse relationship is also plausible: children with sleeping or emotional problems may be at greater risk for disturbed social interactions such as peer problems or bad family relationships and stress sensitivity. Also, we demonstrated an association between childhood stress and body composition (Chapter 3.8), supporting the hypothesis that chronic stress may be involved in the development of childhood overweight or obesity. However, being overweight or obese may reciprocally cause distress, and the observed moderation effects of lifestyle factors may implicate other factors obscuring the stress-body composition relationship. Nevertheless, as some of the research objectives were new and exploratory, our cross-sectional findings may promote further research with, e.g., a longitudinal or interventional design (see *Chapter 4.3 Public health and future research*). Secondly, the thesis objectives related to the hair measurements were

restricted to girls to ensure a minimal hair length of 6 cm and thus a 'chronic' time frame in the past (i.e. the past 6 months). Although a minority of boys might have had a sufficient length of hair to participate, they were excluded to keep the population under study homogenous. Our observations are thus not representative for the childhood population in general. Thirdly, some of the observed relationships (e.g. relationship between childhood stress and hair cortisol or cortisone, or the development of obesity), may be underestimated given the high socio-economic status of the ChiBS study population. A fourth remark concerns the applied FFQ. Although FFQ's are a simple and time-saving method to get an impression on dietary habits or dietary quality, an appropriately adapted FFQ to each research aim is not always available: in this study, the FFQ was not specifically designed to measure nutrient intakes, which limited associations with the hair mineral concentrations. To do so, repeated weighted dietary records or 24-hour recall interviews may be more accurate but are logistically more burdening for the parents and children. Within the IDEFICS project, 24-hour recall interviews were performed but not used in the frame of this thesis because of a too low sample size. Despite the advantages of a parent-reported FFQ compared to children's self-reports on dietary intake (i.e. with regard to accuracy in recalling or estimating dietary intake), the applied FFQ was inherently limited to meals consumed under the parent's control (i.e. excluding meals consumed at schools or childcare) which may have affected the presented results in this research. Fifthly, the child-reported CLES questionnaire is (although validated and well-established) somewhat limited in scope: stressors characteristic for the 21st century such as school- and social media-related stressors (e.g. bullying) were not included in the questionnaire. Although the original American-English CLES questionnaire was professionally translated into Dutch (Flemish) using a forward- and back-translation method, we did not test the psychometric properties and validity of the Dutch version in relation to the original U.S. version (Coddington R. D., 1972; Bullinger M. et al., 1998). Also, with regard to the assessment of NLEs and FSAs (Chapters 3.1 and 3.2), the dichotomous nature of these variables requires attention: NLEs were considered to be 'once-only' events (occurrence versus no occurrence), although these events may arise multiple times in the child's life, an issue which was not be reflected in the IDEFICS parental questionnaire. The calculated 'sum of NLEs' may therefore be an underestimation of actual overall NLE experience of the child. This may have led to underestimated or distorted associations with PES prevalence. Moreover, the absence of an accurate child-reported measure of subjective appraisal and affective response to stressors is another drawback of this research. Incorporation of a questionnaire or interview assessing the stressfulness and appraisal of experienced life events

by children could have led to stronger associations between the three levels of stress experience and stronger psycho-endocrine correlations (i.e. environmental, psychological and biological, see 'psycho-endocrine covariance' as described in Chapter 1.3). In this context, also the controllability of the stressors has been a missing dimension in the performed research as stress controllability may influence HPA responsivity (e.g. uncontrollable stress has been shown to elicit a flattened HPA response) (Miller G. et al., 2007). Judgements of controllability may vary largely among people and may therefore have influenced the observed associations between hair cortisone and child-reported stress. More specifically, stronger associations could be assumed if individual ratings of control would have been available. Also, inherent to the use of retrospective questionnaires, is the possible underestimation because of recall- and response-biases. However, as stress assessment was performed at different levels, the limitations of each method are complementary and may thus be 'attenuated'. A second last limitation relates to the laboratory hair analysis. Although the ICP-MS method was developed and validated for quantification of 7 minerals in children's hair samples, the number of samples that exceeded the LOD for Na (i.e. 1 µg/g) was limited to 50. Because of this low sample size, Na was not included in the analysis investigating the relationship between hair minerals and dietary habits, metabolic health and stress. Despite the fact that Na and for example also potassium are very interesting minerals to study in relation to diet and health, hair mineral analysis using ICP-MS may encounter some difficulties for their quantification. The main issue in hair cortisol and cortisone analysis was the problem of non-detection and potential wash-out in the 6 cm hair samples, which could not be studied in more detail as no segmental analysis was performed and no information on hair washing frequency was gathered. We decided not to perform segmental analysis (e.g. analysis of several one-cm fragments) to limit the amount of hair needed for laboratory analysis, although this would have allowed studying the relationship with childhood stress (for hair cortisol and cortisone) or dietary habits (for hair minerals) more accurately. Last, to increase willingness for participation, the ChiBS informed consent was split such that the participants could refuse participation to single examination modules. However, in combination with the exclusion of boys for hair sampling, this has led to different participation rates for each examination module and studying sub-groups of ChiBS participants in investigating separate research questions, with sometimes small sample sizes. Therefore, we performed post-hoc power analyses on the smallest sample size of this thesis (i.e. 107 children in a regression model with 7 predictors examining the relationship between hair cortisone and hair mineral levels, Chapter 3.7). These post-hoc power analysis indicated that this sample size was sufficient to

detect a medium effect size with a power of 0.80. Nevertheless, a larger sample size would have allowed studying smaller effect sizes. Also in this context, it should be mentioned that due to the low sample size for cortisol data in this research (Chapter 3.3), this research cannot inform about the association between hair cortisol and childhood stress. For this objective, a minimal number of 123 quantifiable hair samples would have been necessary for correlational analyses (as described in Chapter 3.3).

CHAPTER 4.3 PUBLIC HEALTH AND FUTURE RESEARCH

1 The public health dimension

Chronic stress is a well-known problem of the 21st-century western society. We live in an 'a.s.a.p.', 'high-speed', 'more', 'better'...world, in which success and status determine our future. This constant drive to grasp opportunities and to create a successful life may lead to disorientation, isolation and self-doubts. It is therefore not surprising that chronic exposure to psychosocial stress has been associated with deteriorating health and well-being, both physically and mentally. For example sleeping problems, anxiousness, nervousness, constant fatigue, or neck and shoulder pain are often stress-related (Danielsson M. et al., 2012). This poses a major societal and public health challenge. In addition, because of the concomitant economic costs in terms of absenteeism, loss of productivity and health care expenses (Danielsson M. et al., 2012; Kalia M., 2002), the prevention and management of stress have received increasing interest from industries and local authorities (World Health Organization, 2005).

Each life phase is characterized by its own vulnerabilities and difficulties, indicating the need for a differentiated focus and mental health promotion approach for different age groups. Childhood is a life period which may determine the health and well-being in later life, affecting e.g. social or academic achievements. It is a stage of life with great plasticity and cognitive, social and emotional development. Therefore, in particular children are at risk for the high demands of modern society in which performances are no longer judged by one's effort, commitment, cleverness, or creativity, but by speed, marks, school records, capacity to reproduce etc. (Peerlings W., 2008). Therefore, educational programs offer important opportunities in improving the social and emotional skills of children which are needed to identify and manage emotions, relationships and stressful situations. Childhood stress prevention- and management programs can be implemented at several levels, i.e. at the community, the school or family of the children, as schematically presented in Figure 4.3.

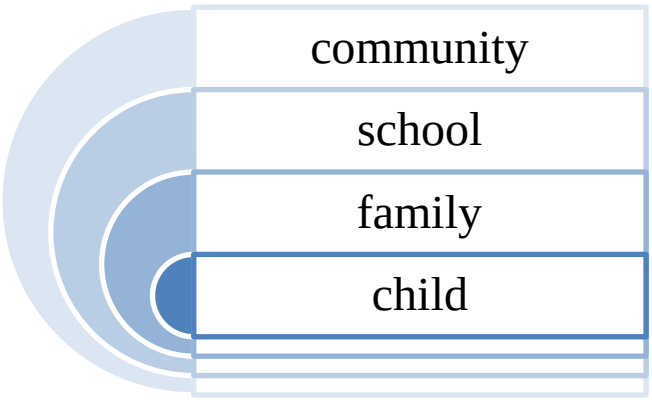


Figure 4.3 Involved parties in childhood stress management- and prevention programs

In Flanders, VIGeZ (the Flemish Institute for Health Promotion and Disease Prevention, http://www.vigez.be) is an important actor in the field of mental health promotion. By strengthening resilience, improving coping skills and encouraging the adoption of a general healthy lifestyle, public health interventions may lead to an improved quality of life and the *prevention* of stress-related mental health disorders, instead of *curing* them (VIGeZ, 2011).

Also school-based approaches for stress prevention and management may be valuable (Atkins M. S. et al., 2010) as the school environment has a long-term influence on the children's development and it reaches children with diverse backgrounds on a large scale. In a '*whole school approach*', the classes, the school environment, the parents and the community altogether take part in the program; while '*curriculum based programs'* are concentrated on the education of communication-, problem solving-, relational- and coping- skills to children. The final aim of school-based programs is to reduce stress symptoms in children, to improve their mental health and social competences, and to suppress e.g. aggression and bullying at schools (Atkins M. S. et al., 2010; Kraag G. et al., 2006). '*Learn Young, Learn Fair*' (the Netherlands) and '*Zippy's Friends'* (implemented in 19 countries) are examples of elementary school-based stress management programs (Kraag G. et al., 2009; Holen S. et al., 2012). In Belgium, the non-profit organization '*Living Keys'* ('*Leefsleutels'*, http://leefsleutels.be) and '*School Without Bullying'* ('*School Zonder Pesten'*, (http://schoolzonderpesten.dev.tnt.be) develop materials and trainings for schools and teachers to improve the social-emotional well-being of children. In addition, they organize interactive school shows for kindergarten and elementary school children. For example, '*Victor and its Feel Good Machine'* ('*Victor en zijn Goedgevoel Machine*') and '*Bram and the OK-Brigade'* ('*Bram en de OK-Brigade')* deal with (non-) acceptable behavior, emotions and thoughts in children. In addition, '*Healthy at*

School' (*'Gezond Op School',* http://www.gezondopschool.be) provides information and methodologies for the implementation of mental health policies at Flemish schools.

Further, parents and the family environment of children are important actors in the prevention of childhood stress and the promotion of children's mental health. *Chapter 3.1* and *Chapter 3.2* of this thesis demonstrated the frequent occurrence of certain familial and social adversities, and their relationship with psychosomatic and emotional symptoms in children. Next to having peer problems and major frustrations at school, a non-traditional family structure and a bad family climate were apparent risk factors for the occurrence of psychosomatic and emotional problems. Therefore, the significance of programs aimed at strengthening parental skills (e.g. with regard to parent-child communication, maintaining positive relationships, providing parental warmth and attachment, and setting consistent boundaries) has increasingly been recognized (Sanders M. R., 2008; Riesch S. K. et al., 2006). In this context, the *Triple-P Positive Parenting Program* was designed in Australia to create a 'family friendly' environment that supports parents in raising their children; by targeting the entire population, as well as high-risk children (Sanders M. R., 2008). This Triple-P Program has been implemented in Flanders, more specifically by the LOGO's (platforms of Local Health Consultation) of Antwerp and East-Flanders (VIGeZ, 2011). Even though the health and well-being of children is at the center of the abovementioned programs, children may still feel lost or misunderstood. '*Awel'*, a children's and youth phone (http://www.awel.be), may offer salvage and/or comfort if support and communication with parents or other primary care givers is unavailable or impossible.

The ultimate objective of childhood stress prevention- and management programs is learning children to identify and recognize feelings and difficulties, providing a supportive environment and increasing the awareness of stress-related behavioral changes. Concerning the latter, *Chapter 3.8* of this thesis showed a relationship between childhood stress and body composition; a relationship which was moderated by unhealthy behavior (snacking, screen time, sleep). An important step towards the prevention of stress and stress-related health effects (such as the development of obesity) is therefore increasing children's, parents' and teachers' knowledge about potential stress-related behavioral changes and providing healthy, alternative stress management strategies. For instance, in addition to health education on the active food guide pyramid, also education on potential stress-related dietary changes in children (such as emotional eating or external eating) should be implemented in stress management– or obesity prevention programs. This way, the awareness on this issue and the

identification of unhealthy dietary behaviour as potential stress consequence in children could be enhanced. Moreover, standard screening for psychosocial stress in children could be considered. The most important public health contribution of this thesis lies therefore in the exploration of a new stress biomarker, i.e. hair cortisol and hair cortisone measurements, and its application in large-scale epidemiological research. However, in order to identify high-risk children and to develop a corresponding personalized approach, more research is needed into the diagnostic potential of hair as matrix for stress assessment.

2 Recommendations for future research

This thesis 1) contributed to the knowledge about childhood stress prevalence and adversity associations, 2) performed exploratory analysis into the use of scalp hair as bio-indicator for childhood stress (i.e. hair cortisol – cortisone measurements) and dietary habits (i.e. hair mineral analysis) and 3) demonstrated an association between childhood stress and body composition. Therefore, this thesis may encourage future chronic childhood stress research on several levels.

First, the application of hair cortisone measurements as a new additional stress biomarker for children should be expanded to larger and more heterogeneous populations (e.g. including both sexes, including more diverse socio-economic environments, including different pubertal stages). Reference values should be established for hair cortisol and cortisone in children, and a greater emphasis on long-term longitudinal and interventional studies would expand the usefulness and utility of hair cortisol and cortisone as stress biomarker. For example, stress reduction and management strategies (e.g. strengthening resilience or coping capacities of children) may influence HPA activity and could be examined by monitoring alterations in children's baseline hair cortisol or cortisone concentrations through hair segmental analysis. Also, longitudinal studies might, in contrast to cross-sectional studies, reveal which stressful situations actually initiate a cortisol release in children and the extent to which hair cortisol and/or cortisone concentrations can be affected. This is an important field of research as literature has indicated that many studied childhood stressors fail to induce a biological stress response or fail to activate the HPA axis (Gunnar M. R. et al., 2009a). Specific methodological recommendations with regard to the measurement of hair cortisol-cortisone were described in *Chapter 4.2 Methodological considerations*: in summary, future research should further investigate hair segmental loss of cortisol and cortisone, establish new guidelines concerning the maximal length of hair to be analysed, further explore the potential

of hair segmental analysis to monitor timely cortisol changes, and standardize laboratory protocols (Miller R. et al., 2013). Also, more research is needed into the mechanisms of cortisol-cortisone formation or incorporation in hair, as well as into 11β-HSD activity and the importance of glucocorticoid metabolism in response to human psychosocial stressors. Lastly, as salivary and hair cortisol are no clear-cut diagnostic media for childhood stress, stress research would benefit from developing a 'childhood stress score' or 'childhood stress index', in which several stress assessment methods are combined to obtain a more accurate view on stress exposure and stress experience in children.

Despite the observed associations between hair minerals and diet, metabolic health and childhood stress, future research should 1) confirm our observations in larger and more heterogeneous populations; 2) elucidate the mechanisms and processes involved in the mineral incorporation and accumulation in scalp hair; and 3) study alterations of these processes associated with a changing lifestyle or stress. Other specific methodological recommendations with regard to hair mineral analysis were described in *Chapter 4.2 Methodological considerations*.

Last, this thesis demonstrated an association between child-reported stress and body composition, which was moderated by lifestyle behaviour of the child. Evidently, this important finding should be further examined on a longitudinal and interventional basis, including investigating the influence of sex- and appetite-regulating-hormones on this relationship. Ultimately, this may lead to a number of public health recommendations, such as screening for the presence of psychosocial stress in children and incorporating education on stress management in schools and obesity prevention programs.

CHAPTER 4.4 GENERAL CONCLUSION

Chronic stress is a well-known problem of the 21st-century western society, to which also children are exposed. This study presented high prevalence percentages for some childhood stressors in European pre- and primary-school children and demonstrated that childhood adversity is very often connected to the broad family context. In addition, childhood adversities were quantitatively and qualitatively related to the prevalence of psychosomatic and emotional symptoms in children.

Given the world-wide epidemic increase of overweight and obesity in young age groups, the association between childhood stress and the development of overweight and obesity in children is of high relevance and further concern: child-reported stress was positively associated with body composition in Flemish primary-school children, an association which was strengthened by an unhealthy lifestyle (high snack consumption, sedentary behaviour and low sleep duration). Further monitoring of childhood stress is therefore strongly recommended.

For this aim, reliable methods are needed for chronic stress assessment in children, which are sensitive, reliable, non-invasive and applicable on a large-scale. No association was observed between hair cortisol and child-reported stress in Flemish primary-school girls, probably because of wash-out effects in the 6cm hair samples. However, a positive association was demonstrated between 6-month hair cortisone concentrations and child-reported stress, suggesting hair cortisone as a new and easily applicable biomarker for future chronic stress research. Depending on the time period of interest, it should however be recommended to differentiate the type of biological matrix; i.e. saliva for shorter periods or hair for longer periods in the past, although future research should re-examine the maximum length of hair that can be used as a reliable estimate of cortisol exposure.

The hair matrix also showed its potential for nutritional epidemiological research: this thesis demonstrated that hair mineral concentrations could to a large extent be explained by food consumption frequencies. Further, reduced hair mineral levels were observed with poor metabolic health and increased stress in young girls. These findings altogether indicate that hair mineral analysis may, after further validation, be used as a complementary method to investigate dietary or more general health aspects of children. Particularly for long-term,

‘chronic’ research objectives, the hair matrix may offer opportunities, although research in this regard is still premature.

In conclusion, this thesis pleads for an increasing recognition of stress in young children and for further elucidation of the complex mechanisms and pathways involved in the adverse physical and mental health effects of stress in children. Extensive thinking is needed into the development of public health prevention strategies which require trans-disciplinary approaches: both children and adults involved in their daily care and education should be informed about the identification, consequences and possible management strategies of childhood stress, with a special attention for vulnerable groups and personalized approaches.

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SUMMARY

Over the last decade, research has acknowledged the impact of stress on children's life and health. Childhood stress has been defined as '*any intrusion into the child's normal physical or psychosocial life experiences that acutely or chronically unbalances the child's physiological or psychological equilibrium, threatens their security or safety, or distorts their physical or psychological growth or development*' (Sandberg S., 2007). In particular chronic stress may adversely affect the child's psychological and physical health, with psychosomatic complaints, affective or behavioural problems, depression and the development of obesity as associated health effects in children.

1 Importance of studying childhood stress – European framework

A first aim of this thesis was therefore to describe the prevalence and the psychosomatic, emotional outcomes of childhood stress in European pre- and primary-school children (4 to 11 years old). This objective was studied in the first follow-up survey (2009-2010) of the European IDEFICS project (Identification and prevention of Dietary- and lifestyle-induced health EFfects In Children and infantS), more specifically in the control regions of the participating countries (i.e. Belgium, Cyprus, Estonia, Germany, Hungary, Italy, Spain, Sweden). This study demonstrated that 1) certain adversities occur frequently (e.g. parental divorce), while others are more rare, 2) that a large percentage of children is shielded from stressors, while a small group of children is exposed to multiple, accumulating adversities, 3) that childhood adversities are associated and co-occur, resulting in potential cumulative childhood stress and that 4) the prevalence of childhood adversity is influenced by geographical location, age and sex (N=4637) (*Chapter 3.1*) (Vanaelst B. et al., 2012d). Further, childhood adversities were significantly related to the prevalence of psychosomatic and emotional symptoms in children, both quantitatively (i.e. an increasing number of adversities gradually amplified the risk for psychosomatic and emotional symptoms) and qualitatively (i.e. certain types of adversities were apparent risk factors for the occurrence of psychosomatic and emotional symptoms) (N=4066) (*Chapter 3.2*) (Vanaelst B. et al., 2012a). The first section of this thesis thus demonstrated the importance of recording and monitoring childhood stress in the future, given the widespread appearance of certain childhood stressors and their impact on the psychological health of young children.

2 Scalp hair as biological matrix for chronic stress assessments in children

To further monitor childhood stress, standardized, valid and reliable stress assessment methods are needed. Therefore, as part of this thesis, a literature review was performed to create an overview of commonly applied stress assessment methods in children, with an emphasis on epidemiological research (Vanaelst B. et al., 2012b). Based on this evaluation, the application of hair cortisol analysis appeared as a promising technique: evidence has accumulated on hair cortisol as a valid index of long-term systemic cortisol exposure and its potential as chronic stress biomarker. After all, a single hair sample has the unique characteristic to represent information over a prolonged period of months in the past (i.e., based on a general hair growth rate of 1 centimetre per month, a hair sample of, e.g., 6 cm reflects a 6 month's exposure time). However, hair cortisol analysis remained unexplored in childhood stress research.

The second objective of this thesis therefore relates to the practical utility of cortisol and cortisone (i.e. a metabolite of cortisol) measurements in hair samples as biological markers for chronic stress in elementary school children. This objective was studied in the ChiBS (Children's Body composition and Stress) project, which originated from the Belgian control arm of IDEFICS (i.e. the city Aalter), with a total number of 523 participants at baseline *(Chapter 2.2)* (Michels N. et al., 2012d. As this project was situated in the domain of *chronic* childhood stress, hair samples were exclusively taken from girls to ascertain the required hair length for analysis, i.e. 6 cm and thus a period of 6 months in the past. The results of this thesis, as presented below, thus only reflect observations in Flemish elementary school girls.

223 hair samples were obtained, after which physiological concentrations of cortisol and cortisone were measured in the most proximal 6 cm of the hair sample, using ultra performance liquid chromatography tandem mass-spectrometry. In a next step, the association between hair cortisol and cortisone, and child-reported stress estimates was investigated: while no association was observed for hair cortisol, hair cortisone concentrations were positively correlated with child-reported life events for the past 6 months (Coddington Life Events Scale) (N=165). This novel finding suggests the potential of hair cortisone as a new, additional or substituting stress biomarker for future stress research in children (*Chapter 3.3*) (Vanaelst B. et al., 2013a). Furthermore, inter-correlations of cortisol in different biological sample types (hair, serum, saliva) were investigated, and a triangulation method was

performed to examine which of the applied stress assessment methods could most accurately estimate childhood stress (*Chapter 3.4*) (Vanaelst B. et al., 2012c): salivary cortisol most accurately indicated 'true' childhood stress for periods up to 3 months in the past, whereas hair cortisol may be preferred above salivary measurements for more distant periods. This study aimed to encourage future childhood stress research by exploring the opportunities of cortisol and cortisone measurements in the hair matrix as stress biomarker in children. However, as this is a novel research area, further investigation into the metabolism and incorporation of cortisol and cortisone in hair and its response to stress is needed, as is research into potential confounding variables, standardised laboratory procedures and methodology. In this context, we recommend not to analyse segments up to 6 cm long, but to divide the hair samples into shorter fragments (e.g. 1 to 3 cm) to limit the effects of segmental loss and wash-out of hair hormone concentrations, and to consequently obtain an accurate reflection of HPA activity.

3 Scalp hair as bio-indicator for dietary habits in children and its association with metabolic health and stress

As literature suggests that stress and obesity may be associated with changes in the body's mineral status, the scope of this thesis was broadened to mineral analysis in scalp hair. As third objective of this thesis, the utility of hair mineral analysis as bio-indicator for the children's dietary habits was investigated. Each of the collected hair samples in the ChiBS project was split into two fractions, of which one was used for mineral analysis: the concentration of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), sodium (Na), phosphorus (P) and zinc (Zn) was quantitatively determined in the 6 cm hair segments using inductively coupled plasma mass spectrometry. Hair mineral reference values were calculated for our population of Flemish elementary school girls (N=218) and the relationship was examined with food consumption frequencies as a marker of the child's dietary pattern using RRR (reduced rank regression) (N=109): respectively 40, 50, 45, 46, 44 and 48% of variation in Ca, Cu, Fe, Mg, P and Zn in hair was explained by the consumption frequencies of certain foods. Although there is insufficient evidence to consider hair minerals as a direct representation of dietary habits, our findings indicate a certain influence of food consumption on hair mineral concentrations *(Chapter 3.5)* (Vanaelst B. et al., 2012e). Next, we examined the potential relationship between hair minerals, obesity and metabolic health which has been suggested in literature but has been unexplored in children *(Chapter 3.6)* (Vanaelst B. et al.,

2013b). An inverse correlation was observed between hair Ca, Mg, and Ca/P and the girls' metabolic score (N=151). Hair minerals may thus contribute to or result from a poor metabolic health in young girls, due to e.g. an unbalanced dietary intake or an altered mineral metabolism associated with metabolic conditions. In contrast to the hypothesis that overweight and obese children may be at risk for mineral deficiencies, we observed positive associations between certain minerals (Ca, Ca/Mg ratio and Ca/P ratio) and body composition parameters (BMI and body fat percentage) (N=218); a relationship which should be confirmed and further explored in larger, more heterogeneous populations. A last research question to be answered was the potential relationship between hair minerals and childhood stress estimates *(Chapter 3.7) (Vanaelst B. et al., 2013d)*. Independently from individual and behavioural factors, hair cortisone concentrations were inversely associated with concentrations of Ca, Mg, Zn, and the Ca/P ratio in hair (N=107), while child-reported stress (i.e. Coddington Life Events Scale) was not associated with hair minerals levels (N=140). This may point to an impact of the body's physiological stress response on hair minerals or mineral metabolism, although many details in this stress-mineral pathway are still unclear. In conclusion, this exploratory hair mineral research in children demonstrated that hair mineral analyses may, after further validation, be useful for future nutritional epidemiological studies investigating the body's mineral status or dietary habits over a long-term retrospectively.

4 Childhood stress and the association with overweight or obesity

Given the increasing evidence that chronic psychosocial stress may influence the development of obesity in children, a final aim of this study was to investigate the relationship between childhood stress and alterations in body composition of the child, taking into account the moderation or mediation effect of lifestyle factors *(Chapter 3.8)* (Vanaelst B. et al., 2013c). In contrast to the previous research objectives, this objective was studied in both boys and girls participating to the baseline ChiBS survey (no hair-measures were included into these analyses) (N=355). Linear regression analyses were used to study the cross-sectional relationship between psychosocial stress, stress-related lifestyle factors and body composition. We did not observe an association between body composition on the one hand and negative emotions, conduct or emotional problems and salivary cortisol on the other hand. However, negative life events (i.e. Coddington Life Events Scale) were positively and feelings of happiness were negatively associated with BMI z-score and WHtR (waist to height ratio). Peer problems and WHtR were positively associated in girls only. The lifestyle factors (high-

caloric snack consumption frequency, screen exposure time and sleep duration) were not observed to mediate the stress-body composition associations, although moderating effects were shown: the stress-body composition associations were strengthened by the most unhealthy lifestyle pattern (high snack frequency, high screen time, low sleep duration). This study pointed to another important health impact of psychosocial stress on children, i.e. the impact of stress on overall and central adiposity, and the impact of lifestyle behaviour as vulnerability factor in stress-induced adiposity. This important finding should be further investigated in interventional or longitudinal study designs.

5 In the future?

Although this thesis did not allow to study causality in the observed associations, this thesis is of importance because of its novel and exploratory research objectives which may promote several aspects of future childhood stress research. For example, future longitudinal study designs with larger and more heterogeneous study populations may expand the utility of hair cortisol/cortisone measurements as stress biomarker in children, or expand the knowledge of hair minerals as marker of dietary habits or other aspects of metabolism in children. Given the high prevalence of childhood stress and its diverse health effects, it may be recommended from a public health perspective to screen for the presence of psychosocial stress in young children and to incorporate education and stress management strategies in childhood stress - and obesity prevention programs.

SAMENVATTING

De laatste jaren kwamen stress en zijn impact op het leven en de gezondheid van kinderen steeds meer onder de aandacht. Sandberg S. (2007) definieerde stress bij kinderen als '*een inbreuk op de normale fysieke of psychosociale levenservaringen van het kind die acuut of chronisch het fysiologische of psychologische evenwicht van het kind verstoort, zijn veiligheid bedreigt, of zijn fysieke of psychologische groei en ontwikkeling uit balans brengt'*. Voornamelijk de chronische vorm van stress zou een ongunstige invloed hebben op de fysieke of psychologische gezondheid van kinderen, waarbij psychosomatische klachten, gedragsstoornissen, depressie en de ontwikkeling van obesitas reeds werden geassocieerd met stress bij kinderen.

1 Het belang van onderzoek naar stress bij kinderen – een Europees kader

Een eerste doelstelling van dit doctoraatsonderzoek was de prevalentie van stress bij Europese kinderen uit kleuter- en basisscholen (kinderen tussen 4 en 11 jaar) te beschrijven, alsook een beschrijving te geven van de psychosomatische en emotionele gevolgen van stress bij deze kinderen. Deze doelstelling werd onderzocht in de eerste opvolgfase (2009-2010) van het Europese IDEFICS project ('Identification and prevention of Dietary- and lifestyle-induced health EFfects In Children and infantS'); meer bepaald in de controle gebieden van de deelnemende landen België, Cyprus, Estland, Duitsland, Hongarije, Italië, Spanje en Zweden. Dit onderzoek toonde aan dat 1) bepaalde moeilijkheden/tegenslagen regelmatig voorkomen in het leven van kinderen (bv. scheiding van de ouders), terwijl andere eerder zeldzaam zijn, 2) dat een groot percentage van kinderen in een eerder 'beschermde' omgeving leeft (vrij van stressoren), terwijl een kleine groep kinderen wordt blootgesteld aan een complexe leefomgeving met meerdere, opeenstapelende tegenslagen/stressoren, 3) dat stressoren geassocieerd kunnen zijn en daardoor samen voorkomen, wat kan leiden tot potentiele 'cumulatieve stress' bij het kind en dat 4) the prevalentie van stress bij kinderen afhankelijk is van de geografische locatie, leeftijd en geslacht van het kind (N=4637) (*Chapter 3.1*) (Vanaelst B. et al., 2012d). Verder toonde dit onderzoek aan dat het ervaren van stressoren geassocieerd is met de prevalentie van psychosomatische en emotionele symptomen bij kinderen, zowel kwantitatief (d.w.z. dat met een toenemend aantal stressoren het risico op psychosomatische en emotionele symptomen graudeel stijgt) als kwalitatief (d.w.z. dat bepaalde types stressoren duidelijke risicofactoren zijn voor het voorkomen van psychosomatische en emotionele symptomen bij kinderen) (N=4066) (*Chapter 3.2*) (Vanaelst

B. et al., 2012a). Gezien het wijdverspreide voorkomen van bepaalde stressoren en hun impact op het psychologische welzijn van kinderen, toonde het eerste deel van dit doctoraatsonderzoek het belang aan van verdere opsporing en opvolging van stress bij kinderen.

2 Hoofdhaar als biologische matrix voor het meten van chronische stress bij kinderen

Om stress bij kinderen te monitoren, zijn gestandaardiseerde, valide en betrouwbare meetmethodes nodig. Daarom werd binnen dit onderzoek een grondig literatuuronderzoek uitgevoerd met als doel een overzicht te verkrijgen van gebruikelijke stress-meetmethoden bij kinderen, meer specifiek in het domein van epidemiologisch onderzoek (Vanaelst B. et al., 2012b). Uit dit literatuuronderzoek bleek de analyse van cortisol in haar een veelbelovende techniek te zijn: meer en meer studies tonen aan dat metingen van haar cortisol als een valide indicatie voor lange-termijn systemische cortisol blootstelling kan beschouwd worden, en dus ook als potentiële merker voor chronische stress. Aangezien hoofdhaar ongeveer 1 centimeter per maand groeit, kan één haarstaal informatie verschaffen over een lange periode in het verleden en dus over 'chronische' stress (bijv. een haarstaal van 6 centimeter lang reflecteert een periode van 6 maanden in het verleden). Echter, haar cortisol metingen zijn onbekend terrein binnen het onderzoekdomein van chronische stress bij kinderen.

De tweede doelstelling van dit doctoraatsonderzoek was daarom de praktische bruikbaarheid te onderzoeken van cortisol en cortisone (d.i. een metaboliet van cortisol) in hoofdhaar als biologische merker voor chronische stress bij lagere-school kinderen. Deze onderzoeksvraag werd bestudeerd in het 'ChiBS' project ('Children's Body composition and Stress'). De ChiBS studie heeft zijn oorsprong in de Belgische controle-arm van het IDEFICS project (nl. in de stad Aalter) met een totaal van 523 deelnemertjes bij de start van het onderzoek *(Chapter 2.2)* (Michels N. et al., 2012d). Omdat deze onderzoeksvraag zich richtte op c*hronische* stress bij kinderen, werden uitsluitend haarstalen afgenomen van meisjes om de vereiste haarlengte van 6 centimeter te kunnen bereiken (d.i. een periode van 6 maanden in het verleden). De resultaten die hieronder worden voorgesteld zijn dus uitsluitend observaties van Vlaamse lagere-school meisjes.

Van 223 lagere-school meisjes werd een haarstaal afgenomen waarvan in de meest proximale 6 centimeter van het haarstaal de fysiologische concentraties van cortisol en cortisone werden

geanalyseerd via een laboratoriummethode genaamd ‘ultra performantie vloeistof chromatografie tandem massa-spectrometrie’. In een volgende stap werd de associatie tussen haar cortisol en cortisone, en kind-gerapporteerde stress onderzocht: haar cortisone concentraties vertoonden een positieve correlatie met kind-gerapporteerde levensgebeurtenissen over de laatste 6 maanden (Coddington Life Events Scale) (N=165), terwijl deze associatie niet werd gevonden voor haar cortisol. Deze nieuwe bevinding suggereert dat haar cortisone concentraties als nieuwe, potentiële biomerker kan dienen voor toekomstig stressonderzoek bij kinderen (*Chapter 3.3*) (Vanaelst B. et al., 2013a). Vervolgens werden inter-correlaties van cortisol in verschillende biologische staaltypes onderzocht (nl. in hoofdhaar, serum en speeksel), en werd een driehoeksmeting-methode toegepast om te onderzoeken welke van de gebruikte stress-meetmethodes het meest accuraat stress bij kinderen kan schatten (*Chapter 3.4*) (Vanaelst B. et al., 2012c): speeksel cortisol duidde het meest accuraat ‘werkelijke’ stress bij kinderen aan voor perioden tot 3 maanden in het verleden, terwijl haar cortisol geprefereerd dient te worden boven speeksel cortisol voor periodes verder in het verleden.

Deze studie betrachtte toekomstig stressonderzoek bij kinderen te stimuleren door voor het eerst de mogelijkheden van cortisol en cortisone metingen in de haarmatrix te exploreren. Binnen dit nieuwe domein is echter verder onderzoek nodig naar het metabolisme en de incorporatie van cortisol en cortisone in hoofdhaar, alsook naar mogelijke ‘confounding’ variabelen, gestandaardiseerde laboratoriumprocedures en methodologie. Zo is het op methodologisch vlak bijvoorbeeld aan te raden in toekomstig onderzoek de afgenomen haarstalen te verdelen in kortere fragmenten van bijv. 1 tot 3 centimeter (i.p.v. volledige segmenten van 6 cm te analyseren) om zo de invloed van fragmentair verlies en ‘wash-out’ van haar-hormoonconcentraties te beperken, en bijgevolg een meer accurate reflectie van HPA activiteit te bekomen.

3 Hoofdhaar als bio-indicator voor de voedingsgewoonten van kinderen en de associatie met metabole gezondheid en stress

Aangezien wetenschappelijke literatuur aantoont dat stress en obesitas mogelijks geassocieerd zijn met veranderingen in de mineraalstatus van het lichaam, werden binnen dit doctoraatsonderzoek ook mineraal analyses in hoofdhaar uitgevoerd. De derde doelstelling van dit onderzoek was de bruikbaarheid van haar-mineraalanalyses te onderzoeken as bio-

indicator voor de voedingsgewoontes van kinderen. Elk van de afgenomen haarstalen in het ChiBS project werd verdeeld in twee fracties, waarvan één fractie werd gebruikt voor mineraalanalyse: de concentraties van calcium (Ca), koper (Cu), ijzer (Fe), magnesium (Mg), natrium (Na), fosfor (P) en zink (Zn) werden kwantitatief bepaald in 6 cm haarfragmenten via een laboratoriummethode genaamde 'inductively coupled plasma mass spectrometry'. Er werden referentiewaarden opgesteld voor deze haar-mineraalconcentraties voor onze populatie Vlaamse lagere-school meisjes (N=218) en via RRR (reduced rank regression) werd de relatie met voedselconsumptiefrequenties als merker voor het voedingspatroon van kinderen onderzocht (N=109): respectievelijk 40, 50, 45, 46, 44 en 48% van de variatie in Ca, Cu, Fe, Mg, P en Zn in haar werd verklaard door de consumptiefrequenties van bepaalde voedingsitems. Alhoewel er onvoldoende evidentie is om haar mineralen als directe uiting van voedingsgewoontes te beschouwen, duiden deze bevindingen toch op een bepaalde invloed van voedselconsumptie op haar-mineraalconcentraties *(Chapter 3.5)* (Vanaelst B. et al., 2012e). Nadien werd de relatie tussen haar mineralen, obesitas en metabole gezondheid onderzocht, dewelke reeds werd gesuggereerd in de wetenschappelijke literatuur maar nog niet werd onderzocht bij kinderen *(Chapter 3.6)* (Vanaelst B. et al., 2013b). Haar Ca, Mg en Ca/P vertoonden een inverse correlatie met de metabole score van de meisjes (N=151). Haar mineralen kunnen dus mogelijks bijdragen tot of resulteren uit een zwakke metabole gezondheid bij jonge meisjes; dit ten gevolge van bijv. een ongebalanceerd dieet of een gewijzigd mineraal metabolisme geassocieerd met metabole aandoeningen. In tegenstelling tot de hypothese dat kinderen met overgewicht en obesitas een verhoogd risico lopen op deficiënties voor bepaalde mineralen, stelden wij een positieve associatie vast tussen bepaalde mineralen (Ca, Ca/Mg ratio en Ca/P ratio) en lichaamssamenstelling (BMI en lichaamsvetpercentage) (N=218); een relatie die moet bevestigd en verder onderzocht worden in grotere en meer heterogene populaties. Een laatste onderzoeksvraag was de potentiële relatie tussen haar mineralen en stress bij kinderen *(Chapter 3.7).* Onafhankelijk van individuele– en gedragsfactoren, vertoonden haar cortisone concentraties een inverse associatie met concentraties van Ca, Mg, Zn, en de Ca/P ratio in haar (N=107), terwijl kind-gerapporteerde stress (nl. Coddington Life Events Scale) niet was geassocieerd met haar mineraal levels (N=140). Dit kan duiden op een invloed van de fysiologische stress-respons op haar-mineraalconcentraties of het mineraalmetabolisme, alhoewel vele details in dit stress-mineraal traject nog ongekend zijn. Samengevat, dit verkennende haar-mineraalonderzoek in kinderen demonstreerde dat haar-mineraalanalyses, na verdere validatie, zinvol kunnen zijn

voor toekomstig nutritioneel epidemiologisch onderzoek naar de lichaamsmineraalstatus of naar retrospectieve voedingsgewoontes.

4 Stress bij kinderen en de associatie met overgewicht of obesitas

Omdat er steeds meer evidentie is om aan te nemen dat chronische, psychosociale stress de ontwikkeling van obesitas bij kinderen in de hand kan werken, bestond de laatste doelstelling van dit onderzoek erin de relatie tussen stress bij kinderen en veranderingen in hun lichaamssamenstelling te bestuderen, met hierbij aandacht voor moderatie- of mediatie-effecten van levensstijlfactoren *(Chapter 3.8)* (Vanaelst B. et al., 2013c). In tegenstelling tot de vorige onderzoekobjectieven, werd deze onderzoekvraag bestudeerd zowel in jongens als meisjes die deelnamen aan de eerste onderzoekmodule van ChiBS (nl. metingen in 2010) (in deze analyses werden geen haar-metingen geïncludeerd) (N=355). Via lineaire regressie analyses werd de cross-sectionele relatie tussen psychosociale stress, stress-gerelateerde levensstijlfactoren en lichaamssamenstelling bestudeerd. Er werd geen associatie gevonden tussen lichaamssamenstelling enerzijds, en negatieve emoties, gedrags- of emotionele problemen en speeksel-cortisol anderzijds. Echter, negatieve levensgebeurtenissen (Coddington Life Events Scale) waren positief en gevoelens van geluk negatief geassocieerd met de BMI z-score en WHtR (waist-to-height ratio, middel tot lengte ratio). Problemen met leeftijdsgenoten en WHtR vertoonden enkel bij meisjes een positieve associatie. De levensstijlfactoren (hoge-calorische snack consumptie, scherm blootstellingstijd en slaapduur) medieerden de relatie tussen stress en lichaamssamenstelling niet, alhoewel moderatie-effecten werden geobserveerd: de stress-lichaamssamenstelling associaties werden versterkt door de meest ongezonde levensstijlpatronen (hoge snack consumptie, hoge scherm blootstellingstijd, korte slaapduur). Dit onderzoek wees op een andere belangrijke gezondheidsimpact van psychosociale stress bij kinderen, nl. de impact van stress op algemene en centrale adipositas, alsook op de invloed van levensstijl als kwetsbare factor in stress-geïnduceerde adipositas. Deze belangrijke bevinding dient verder onderzocht in interventionele of longitudinale studie-opzetten.

5 In de toekomst?

Alhoewel dit onderzoek niet toeliet causaliteit in de geobserveerde associaties te onderzoeken, ligt het belang van dit onderzoek in zijn nieuwe, exploratieve karakter dat verschillende aspecten van toekomstig stressonderzoek bij kinderen kan bevorderen. Zo kunnen

bijvoorbeeld toekomstige longitudinale of interventionele studies met grotere en meer heterogene studiepopulaties de bruikbaarheid van haar cortisol/cortisone metingen als stress biomerker voor kinderen uitbreiden, of de kennis expanderen omtrent haar mineralen als merker voor voedingsgewoontes of andere aspecten van het metabolisme bij kinderen. Gezien de hoge prevalentie van stress bij kinderen en zijn diverse effecten op de gezondheid, is het vanuit maatschappelijk gezondheidsperspectief aan te raden te screenen voor psychosociale stress bij jonge kinderen, alsook om educatie- en stressmanagement-strategieën te incorporeren in stress- en obesitas- preventieprogramma's voor kinderen.

ANNEXES

Annex 1: Photographical illustration of saliva sampling and instructions for collection

The purpose of this saliva collection is to measure stress by analyzing the hormone cortisol in saliva. Apart from the instructions with 2 checklists, you will also have 2 bags each containing 4 cotton swabs. With these swabs you can collect saliva from your child.

Time points for collection (the time points have to be followed strictly) during these 2 days:

On _____ and _____ (dates completed by the researcher) (if you want to chose another date, please contact the researcher on the phone number below!)

1. **Immediately after awakening, just after opening the eyes when the child is still in bed!**
2. **30 minutes after awakening**
3. **60 minutes after awakening**
4. **In the evening: at least one hour after dinner but between 7 and 8 PM**

Please pay attention to the following guidelines as it is of vital importance to standardize the sampling:

1) Be **punctual** in following the time points. In order to sample as quick as possible after awakening, leave the bag, a ballpoint and a watch beside your bed. Please, take a good look at the sampling steps the day before and familiarize yourself with the saliva tubes.
2) During **1 hour before sampling** your child should not:
   -**eat or drink** (except water)
   -**brush its teeth**
   During **2 hours before sampling** your child should not:
   -do intensive **physical activity**
   This means that your child should stay fasting until 1 hour after awakening and that teeth brushing should be postponed until then. Please, plan your morning routine by giving breakfast just before leaving to school or try to get up a bit earlier.
3) Pay attention to it that your child takes **as less medication as possible** on the sampling day and does **not consume caffeine** (coffee, cola, some energy drinks,…).
4) If you have more than 1 child who is sampling, be sure not to switch the samples.

Please, follow these steps for sampling:

1) Make sure you have some time free during the above-mentioned time points.
2) Take 1 bag per day and 1 tube per moment. On the tube the ID-number of your child is written with after the line a number between 1 and 8. These numbers should be used **chronological**. On day 1 you use tube 1 to 4, on day 2 you use tube 5 to 8. E.g. 1410000/1 immediately after awakening, 1410000/2 30 minutes after awakening,…
3) Open the tube and take the cotton swab. The small tube inside is for the analysis, please don't take it away.

4) Your child has to take **the whole cotton swab in the mouth** and move it around inside the mouth during 2 minutes, until the cotton roll is completely saturated with saliva. It is very important to collect as much saliva as possible! **Don't let your child chew or suck on it.**
5) Place the cotton roll back in the tube and **close the tube tightly**!

6) **Write the exact time point, the date and name of your child on the tube (with ballpoint). Don't forget to answer the questions in the checklist!**
7) Place the tube back in the bag.
8) Place the used tube immediately in the **refrigerator** (not on room temperature!).
9) Please bring the 8 tubes and the 2 checklists back to school on _____ (date completed by the researcher). The researchers will collect the tubes during the first morning session.

Please contact the researcher in the following situations:

- If you have <u>forgotten</u> to bring the tubes back to school on the instructed date.
- If you could not follow the guidelines on the instructed date (e.g. the child was sick, the time points could not be followed). We will suggest you <u>another day</u> so that we will be able to collect the tubes on the next day.
- If you have some problems or <u>questions</u>.

Thank you in advance for your helpful cooperation!

Annex 2: Photographical illustrations of hair sampling

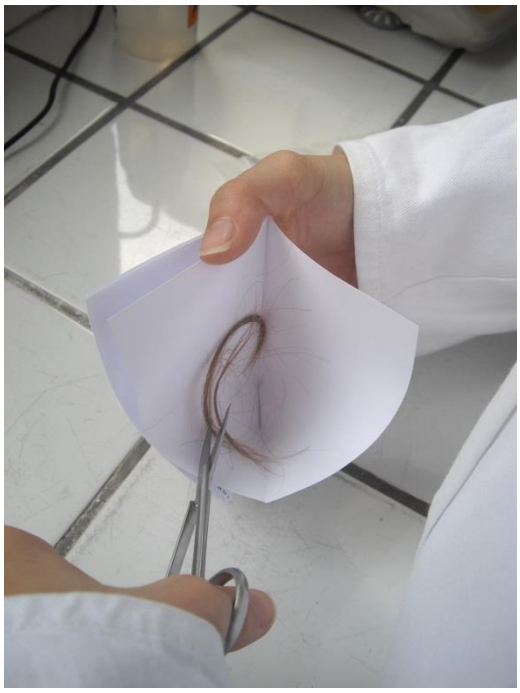
Required materials for hair sampling: scissor, comb, hairgrip, cord, paperclip, folded piece of paper and plastic zip-lock bag

Hair is separated at the back of the head with the hairgrip to expose the vertex posterior region of the head.


A strand of hair is separated, tied together using a cord at the proximal side of the hair strand and cut as close as possible to the scalp.


The strand of hair is fixed in a folded piece of paper using a paper clip at the side of the hair root (cord-side) without folding it. The hair sample is stored in a zip-lock bag with ID number.

Annex 3: Photographical illustrations of UPLC-MS/MS methodology



After the washing procedure, the most proximal 6 cm of the hair samples is finely minced using fine scissors. 50 mg of minced hair is weighed out in a tube.

Steroid extraction is performed by adding methanol to the minced hair sample and shaking the sample for 24 hours.

After centrifugation, the supernatant is transferred to a new glass tube. Methanol is added to the minced hair, vortexed, centrifuged, and the supernatant is compiled with the supernatant from previous centrifugation. This step is repeated twice. The supernatant is evaporated at 40°C under a constant stream of nitrogen till dryness of the samples.

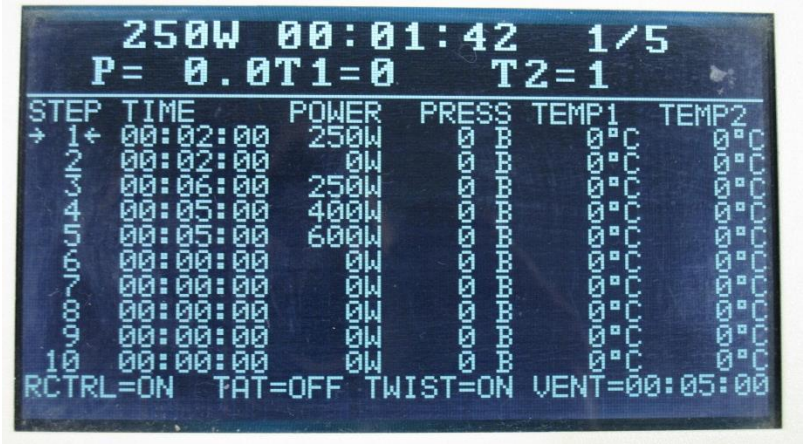
The sample is then prepared for injection into the UPLC-MS/MS system (Waters Acquity UPLC system – Quattro Premier XE tandem mass spectrometer).

Annex 4: Photographical illustrations of ICP-MS methodology

Approximately 0,1g of hair is weighed out in tube and subjected to a washing procedure in an ultrasonic bath.

Hair samples are dried till complete dryness in an oven and transferred to a Teflon tube.


After the washing procedure, the samples are digested by adding HNO_3 and H_2O_2, after which the Teflon tubes are closed and placed in the microwave.



The microwave digestion program is then started.

The digested hair samples are cooled, diluted and prepared for ICP-MS (Thermo Element XR).

DANKWOORD -
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ABOUT THE AUTHOR

Barbara Vanaelst was born in Oudenaarde on March 25th, 1986. In June 2004, she graduated from secondary school, i.e. Sint-Bernarduscollege (Oudenaarde), where she studied 'Latin and Sciences'. In September the same year, her university education 'Biomedical Sciences' was started at Ghent University. In June 2009, she obtained her MSc degree and shortly after, in September 2009, she started working as a PhD student at the Department of Public Health of the Faculty of Medicine and Health Sciences, Ghent University.

Under the supervision of Prof. Stefaan De Henauw and dr. Inge Huybrechts, she cooperated full-time in the fieldwork of the IDEFICS project during the academic years 2009-2010 and 2010-2011. She was involved in the communication and planning of the fieldwork in Flemish elementary schools, in the performance of anthropometrical measurements and physical fitness tests. In addition, she was responsible for the registration and storage of biological samples gathered in the IDEFICS project and for hair sampling. During this period, she further developed her own research project and a scholarship from the Research Foundation – Flanders (FWO) was obtained. Given the epidemiological nature of the PhD research, experts in the field of hair research were contacted for the analytical, biochemical part of the research: the analyses of hair cortisol, cortisone and minerals were outsourced to respectively the University of Strasbourg and the Department of Analytical Chemistry of Ghent University. During the PhD period, she combined her work with assisting bachelor and master thesis's, and attended several national and international congresses and symposia.

In April 2013, Barbara Vanaelst finalized her PhD research and started a new career at Bimetra Clinics, the Clinical Research Center of Ghent University Hospital.

Overview of first-author publications - A1

Vanaelst B, De Vriendt T, Huybrechts I, Rinaldi S, De Henauw S. Epidemiological approaches to measure childhood stress. *Paediatric and Perinatal Epidemiology (2012),* 26: 280-297.

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Overview of first-author publications - A2

Vanaelst B, Huybrechts I, De Bourdeaudhuij I, Bammann K, Hadjigeorgiou C, Eiben G, et al. Prevalence of negative life events and chronic adversities in European pre- and primary-school children: results from the IDEFICS study. *Archives of Public Health (2012),*26.

Michels N*, Vanaelst B*, Vyncke K, Sioen I, Huybrechts I, De Vriendt T, et al. Children's Body Composition and Stress - the ChiBS study: aims, design, methods and population characteristics. *Archives of Public Health (2012d),* 70: 17. * joint first authorship

Overview of first-author publications – national journals A3

Michels N*, Vanaelst B*, Vyncke K, Sioen I, Huybrechts I, De Vriendt T, De Henauw S. Relatie tussen stress en lichaamssamenstelling bij kinderen: design en eerste resultaten van de ChiBS studie. *Tijdschrift voor Voeding en Diëtiek (2012) 28: 8-11*. *joint first authorship