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Early stress, cortisol in hair and health among children in different psychosocial environments

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To Ida, Justus and Edith

“Reality leaves a lot to the imagination.”
— John Lennon



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ABSTRACT

Psychosocial circumstances during early life are increasingly recognized as crucial, not only for the growing individual but also for health throughout life. A possible mechanism could be physiologic dysregulation due to stress. Cortisol in hair is a new biomarker that allows assessment of long-term activity of the hypothalamic-pituitary-adrenal axis.

The objective of this thesis was to investigate the relationship between early stress, levels of cortisol in hair and health among children in different psychosocial environments.

The ABIS-study is a prospective population-based cohort study of every child born in southeast Sweden between Oct 1 1997 and Oct 1 1999 (N=21,700) in which approximately 17,000 families (79%) participated. The studies presented in Papers I, III and IV were based on ABIS data on children aged 1, 3, 5 and 8 years concerning stress related psychosocial variables as well as hair samples and diabetes related autoantibodies. Papers I and IV compared a subsample (n=2,448) from two different social environments. Paper III consisted of a subsample of 100 children as well as their mothers. Paper II covered 99 university students.

Paper I showed that the risk for diabetes-related autoantibodies, both against GADA and IA-2A (>95% cut off), was significantly higher ($p<0.0001$) among children from the blue-collar than from the white-collar city. This difference persisted still after adjustment for other previously documented risk factors. In paper II the method of measuring cortisol concentrations in hair was developed and mean cortisol levels were significantly related to serious life events ($p=0.045$) among the students. Paper III demonstrated that, in children from one to eight years of age, cortisol levels in hair decreased over time and correlated to each succeeding age, between years 1 and 3 ($r=0.30, p=0.002$), 3 and 5 ($r=0.39, p=0.001$), and 5 and 8 ($r=0.44, p=0.001$). Repeated measures gave a significant linear association over time ($p=0.001$). Maternal hair cortisol levels during the second and third trimester and child hair cortisol at year 1 and 3

was also significantly associated. Paper IV showed that children with prenatal psychosocial exposures had higher infant cortisol levels in hair ($B=0.40$, $p<0.0001$, adjusted for gender and size for gestational age) in a dose-response manner and were more often ($p\leq 0.05$) affected by 12 of the 14 most common childhood diagnoses with a general pattern of rising ORs.

In conclusion, the findings in this thesis showed that children born into an environment fraught with adverse psychosocial exposures seem to have an increased hypothalamic-pituitary-adrenal axis activity. It appears to be persistent throughout early childhood and affect health negatively, as evidenced through common childhood diseases and levels of autoantibodies. A widespread and dose response-like effect of adverse psychosocial circumstances was seen on the different outcomes studied throughout this thesis. This supports the model of physiologic dysregulation as a plausible pathway in how the duration and number of early detrimental exposures act as a trajectory to health disparities. Knowledge of these relationships could be valuable in selecting preventive measures, not least in primary care. Moreover, given the prolonged nature of exposure to a stressful social environment, the novel biomarker of cortisol in hair appears to be a useful aid in studies on how long-term stress affects health and may be particularly relevant when applied to research on children.

LIST OF PAPERS

This thesis is based on the original publications, which are referred to in the text by their Roman numerals I–IV.

Paper I. Karlén J, Faresjö T, Ludvigsson J. Could the social environment trigger the induction of diabetes related autoantibodies in young children? *Scand J Public Health* 2012;40:177–82.

Paper II. Karlén J, Ludvigsson J, Frostell A, Theodorsson E, Faresjö T. Cortisol in hair measured in young adults – a biomarker of major life stressors? *BMC Clin Pathol* 2011;11(1):12.

Paper III. Karlén J, Frostell A, Theodorsson E, Faresjö T, Ludvigsson J. Maternal influence on child HPA axis: a prospective study of cortisol levels in hair. *Pediatrics* 2013;132:1333-40.

Paper IV. Karlén J, Ludvigsson J, Hedmark M, Faresjö Å, Theodorsson E, Faresjö T. Impact of prenatal psychosocial exposures on hair cortisol levels and child health: cohort study. (*Submitted to Pediatrics, August 2014*)

ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
ABIS	all babies in southeast Sweden
ANOVA	analysis of variance
CRH	corticotropin-releasing hormone
ELISA	enzyme-linked immunosorbent assay
GADA	glutamic acid decarboxylase antibodies
HPA	hypothalamic-pituitary-adrenal
IA-2A	tyrosine phosphatase antibodies
ICD	international classification of diseases
OR	odds ratio
PSS	perceived stress scale
PTSD	posttraumatic stress disorder
RIA	radioimmunoassay
SAM	sympathetic-adrenomedullary
SES	socio-economic status
T1D	type 1 diabetes

INTRODUCTION

Psychosocial Stress

The evidence for stress as a cause of a range of diseases in modern industrial societies has grown stronger in recent decades.^{1,2} Psychosocial factors such as anxiety, social isolation, stressful life events and lack of control accumulate over life and increase the risk of many major public health diseases such as premature death, obesity, diabetes type 2, cardiovascular diseases and poor mental health.³⁻¹⁴ Increasing evidence is also emerging regarding other diseases, including for example, infections and autoimmune diseases.¹⁵⁻¹⁷

Children seem to be especially susceptible to psychosocial factors due to developmental dependence on the immediate psychosocial surroundings into which they are born.¹⁸ The impact can already be seen in childhood in terms of a wide range of ill health.¹⁹ According to the United Nations convention (1991) on the rights of the child, children have the right to 'the enjoyment of the highest attainable standard of health'. But the health of children is also important for other reasons. There is growing evidence that being born into environments fraught with adverse psychosocial circumstances also predisposes worse health later in life, thus early psychosocial stress acts as a health trajectory in to adulthood.^{12,20-22}

The actual pathways linking psychosocial environmental exposures to health disparities are difficult to uncover, but a number of plausible pathways have been suggested. They range from adverse health behaviours such as sedentary lifestyle or exposure to environmental insults, to physiological dysregulation of neuroendocrine and immune responses.²³⁻²⁵ Whether stressful exposure takes the form of individual experiences or an adverse social environment, it has an impact on the biopsychosocial self, physiologically affecting the individual in the same way as when their life is in danger. Similarly, a disturbed physiological regulation may affect the child, making it vulnerable

in the life just entered. This highlights the complexity of the highly integrated systems of stress.

Knowledge about the impact of early psychosocial stress and its physiologic entity can be useful as an aid in selecting preventive measures to support already vulnerable children and improving treatment approaches in health care. Studying the impact of early stress may therefore yield powerful tools in improving health for both children and future adults.

Concept of stress

In the twentieth century Walter Cannon introduced the term ‘homeostasis’ for the maintenance of physiological variables important for the survival of the organism. He believed that threats to this state of equilibrium awakens responses in the animal that he coined the *fight or flight* response. The body mobilizes to protect itself, e.g. blood is redirected to your muscles, the heart starts beating faster and blood pressure increases. Hans Selye, studying the pituitary–adrenocortical system developed this theory and defined the concept of ‘stress’ as the response of any demand placed upon the body. This concept was radical, because scientists previously believed that illnesses were caused by different pathogens. Later he introduced the term ‘stressor’, to disembrace the confusion caused by the term stress as the physiological response, not the actual force causing stress.² Hereafter the stress research field can be viewed as being divided in to three heuristic traditions: the biological, the psychological and the sociological tradition.

Biologically oriented research can be seen as a continuation of the work of Cannon and Selye, with the focus on physiological responses to different kinds of stimuli.²⁶ This is described in greater detail later on in this thesis.

The psychological tradition emphasizes the person’s own perception of threat to the well-being of the self, and thus represents an individual’s subjective conceptualization of stress. For example, Lazarus developed a theory of stress and coping (availability of cognitive and behavioural resources to deal with

demands), where he believed that none was measurable as a single factor but rather is a result of interaction between the self and the environment.²⁷

In the social approach, focus has been on the environmental component of stress and its characteristics, thus stress is seen as a stimulus. Early on the focus was on the concept of changes in life, so-called 'Life Events', measured through the Social Readjustment Rating Scale often used to assess vulnerability.²⁸ Another example is chronic strain, the persistent and demanding experiences of daily life.^{29,30} The group perspective is emphasized in social research on the macro level, e.g. a population perspective with place effects of the social environment on health.³¹⁻³⁴ Stress is suggested to be a possible mediator in the 'upstream' influences of more proximal determinants of health such as social inequity.^{20,35}

Stress is difficult to define. In the past 20 years stress research has developed rapidly, sub-specializing into widely different fields. This has created many different concepts of stress, which can be seen as a problem due to the inconsistent use of the term.³⁶ However, there has also been development towards a more integrative approach to stress since these different fields inevitably influence each other.³⁷ An example is the integration of sociology and psychology, often described with the umbrella term 'psychosocial' (Oxford English Dictionary's first brief definition: *...pertaining to the influence of social factors on an individual's mind or behaviour, and to the interrelation of behavioural and social factors*) in, for example, studies of the effect of Socio-Economic Status (SES) on health that partly could be attributed to every individual's subjective level of control (*Locus of control*).³⁸ Another evident example is that the tradition of neurobiology has evolved to be more integrated with various other disciplines such as psychology and sociology.³⁹ Although research has primarily focused on stress as something negative, early on there were thoughts of a distinction between positive, healthy stress and negative stress. Examples are Selye's stress/eustress and Lazarus's harm/challenge models.^{27,40}

Any single stress concept, be it social, psychological, physiological, environmental or genetic, is in the end a simplistic description of the complex whole that constitutes the human being. Therefore, the stress process is

probably best understood when focusing on the whole instead of on specific parts. In this context the cumulative approach is able to take into account the tendency of adverse factors to cluster into account.^{41,42} Another important aspect is the ability to uncover possible interactions or potentiating effects that are difficult to discern while studying single factors by themselves.⁴³⁻⁴⁷

Cumulative stress

The body's response to stress and consequently its ability to retain homeostasis is necessary for the viability, adaption and ultimately survival of the organism. But when mobilizing resources to deal with extreme, repeated or constant threat without sufficient periods of recovery it is thought that the stress system actually could cause harm upon the body.⁴⁸ Hence, time is an important factor when considering the harmful effect of stress on the individual.

Allostatic load, a term often used in the discussion of pathophysiological aspects of psychological stress, was coined in the 90s and further developed by McEwen.⁴⁹ It refers to the cumulative burden of the physiological responses of the organism, which the organism must undertake in order to adapt, ultimately exerting 'wear and tear' on the body with a potential pathological outcome.⁵⁰ In the social sciences the cumulative approach is usually applied as a summation index of dichotomized risk factors, e.g. in research concerning life course and the SES effect on health in adults as well as in children.^{1,19,51,52}

Early stress and health

It is accepted that psychosocial circumstances impact health in childhood, being associated with a wide range of ill health e.g. birth weight, neonatal mortality, stunted growth, injury, infections, obesity, mental health problems, asthma and immunological diseases.^{16,53-63} It is necessary, therefore to extend our knowledge on several aspects of children's stress and health. However, research into the effect of stress on children has lagged behind research into the effect on adults, and it is only during the last few decades that early childhood has been increasingly recognized as crucial for health throughout life.^{19,52,53,64-66} It is even suggested that conditions during foetal development affect health.^{67,68} Besides biological needs,⁶⁹ the close social systems (e.g. carers and family) can indirectly exert important psychosocial influence on the young child.¹⁸ Moreover, the integrative approach of the psychosocial environment may be especially important when studying the effects of stress on children, this due to their dependence on the close social systems throughout their development.⁴⁴

The actual biological pathways by which adverse psychosocial exposures lead to health disparities are very complex and difficult to define. However, as mentioned earlier, one plausible mechanism is physiologic dysregulation due to stress.⁷⁰⁻⁷³ Evidence suggests that early stress catalyses biological adaptations that change the way in which three highly integrated systems work, the *nervous*, the *endocrine*, and the *immune* systems, both individually and their interrelation.^{72,74-76} These are all integrated as a complex network that shares a common language of hormones, cytokines, receptors and neurotransmitters that facilitates communication across the network to maintain homeostasis. This network is thought to play a critical role in physical, cognitive, and emotional development by coordinating the body's response to stress in the environment. **See figure 1.**

As an example of this, the *stress hypothesis* concerning the induction of diabetes-related autoantibodies introduced by Johnny Ludvigsson 2006, suggests that psychological stress via cortisol and insulin resistance may induce beta-cell stress in an otherwise predisposed individual.⁷⁷ This could

trigger an autoimmune beta-cell destruction leading to Type 1 diabetes (T1D).^{16,78} Diabetes-related autoantibodies are markers of on-going beta-cell damage in the pancreas and appear before the onset of T1D. Higher concentrations of these autoantibodies, especially higher concentrations of more than one them, increase the risk of developing T1D and can consequently be used to identify subjects at risk of developing the disease.^{79,80} Another example is adverse life events or stress with a prolonged character, such as child abuse or neglect, which may lead children into risky health behaviours including smoking and alcoholism. This also appears to be associated to biological changes in the nervous system e.g. smaller cortex volume, the endocrine system, higher levels of cortisol in saliva and changes in immune functioning with elevated levels of the antibody IgA (immunoglobulin A).⁸¹⁻⁸³ These changes then possibly give rise to stress-related diseases in adulthood such as cardiovascular events and depression.^{8,53,84}

The use of the term *early childhood* is inconsistent in the literature. Most often it is referring to life from birth to about eight years, but according to international standards (WHO Commission on the Social Determinants of Health) it also includes the prenatal developmental period.^{85,86} In this thesis, early childhood represents the time period from birth to eight years, excluding the prenatal period. *Early life* as well as *early stress* is further used to describe both time periods.

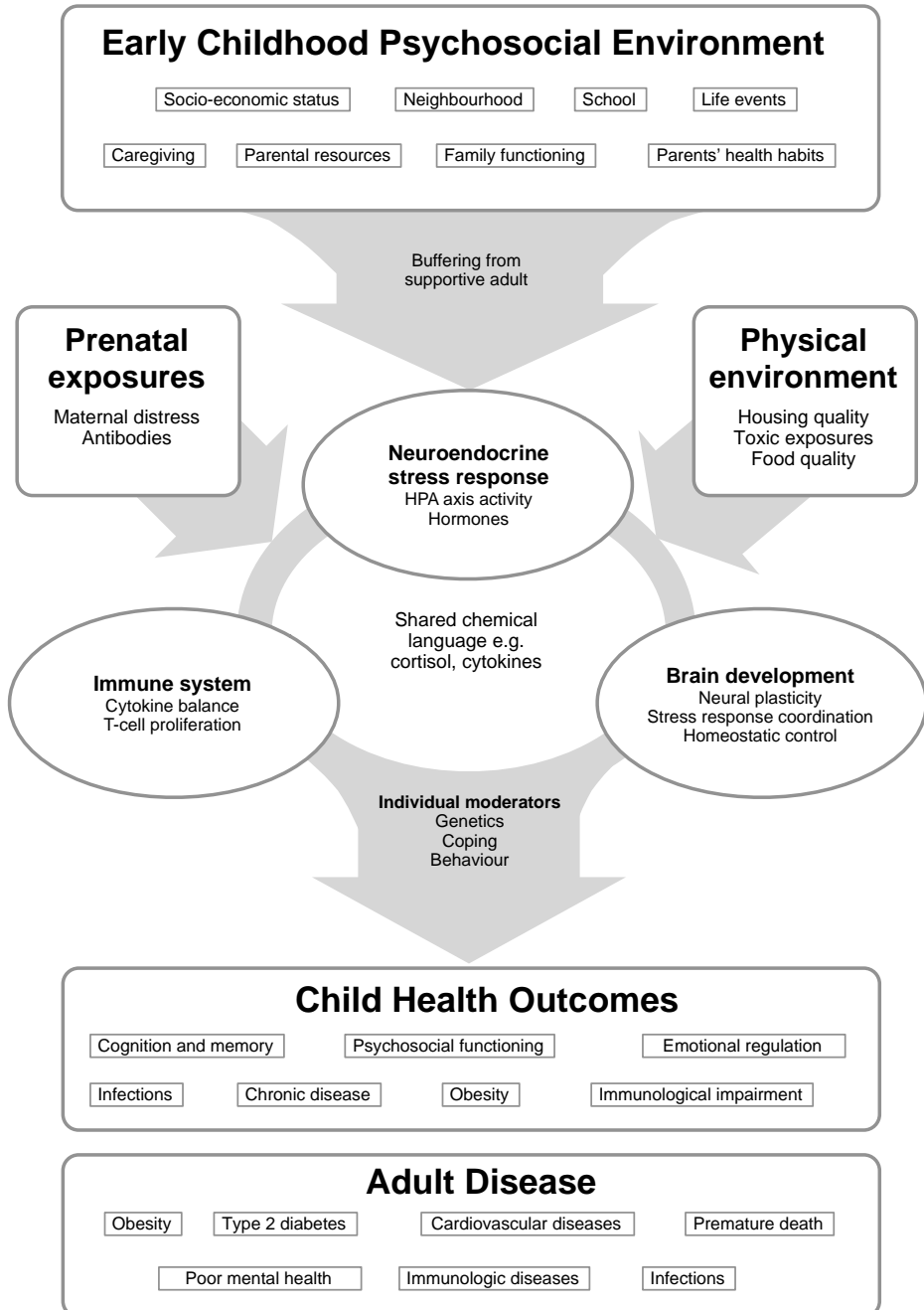


Figure 1. A model of early life experiences leading to individual health outcomes. (Inspired by figure, M Riis⁸⁵).

The Stress Response and HPA Axis

The neuroendocrine hypothalamic-pituitary-adrenal (HPA) axis is a well-studied pathway that together with the sympathetic-adrenomedullary (SAM) system is thought to be a main actor in physiological stress. Aside from these systems, other cytokines and hormones, such as angiotensin, prolactin and oxytocin, are released during the stress response.⁸⁷ In short, the HPA circuits' response to strain and stressors cause negative affective states, e.g. anxiety, that alter the production of corticotropin-releasing hormone (CRH) in the paraventricular nucleus of the hypothalamus. In response to levels of CRH, adrenocorticotrophic hormone (ACTH) is released from the pituitary gland out to the circulating blood, ultimately reaching the cortex of the adrenal gland, resulting in the synthesization of cortisol.⁸⁸ Cortisol regulates its own secretion through negative feedback inhibition by binding to its receptor on cells in the hypothalamus and pituitary gland, and has the effect of inhibiting secretion of tropic hormones. See **figure 2**.

This stress response is thought to be a plausible physiological mechanism through which stressors influence disease risk. For example, the HPA axis could be part of the pathophysiological mechanism between childhood abuse and later depression and anxiety.⁸⁹ Other examples are atrophy of parts of the brain due to chronic stress,³⁵ as well as a significant degree of cognitive impairment.⁹⁰ The latter two are examples of how interdependent these three systems are – psychosocial stress affects mental health, which leads to dysregulation of physiological systems, which in turn affects the brain and its mental ability.

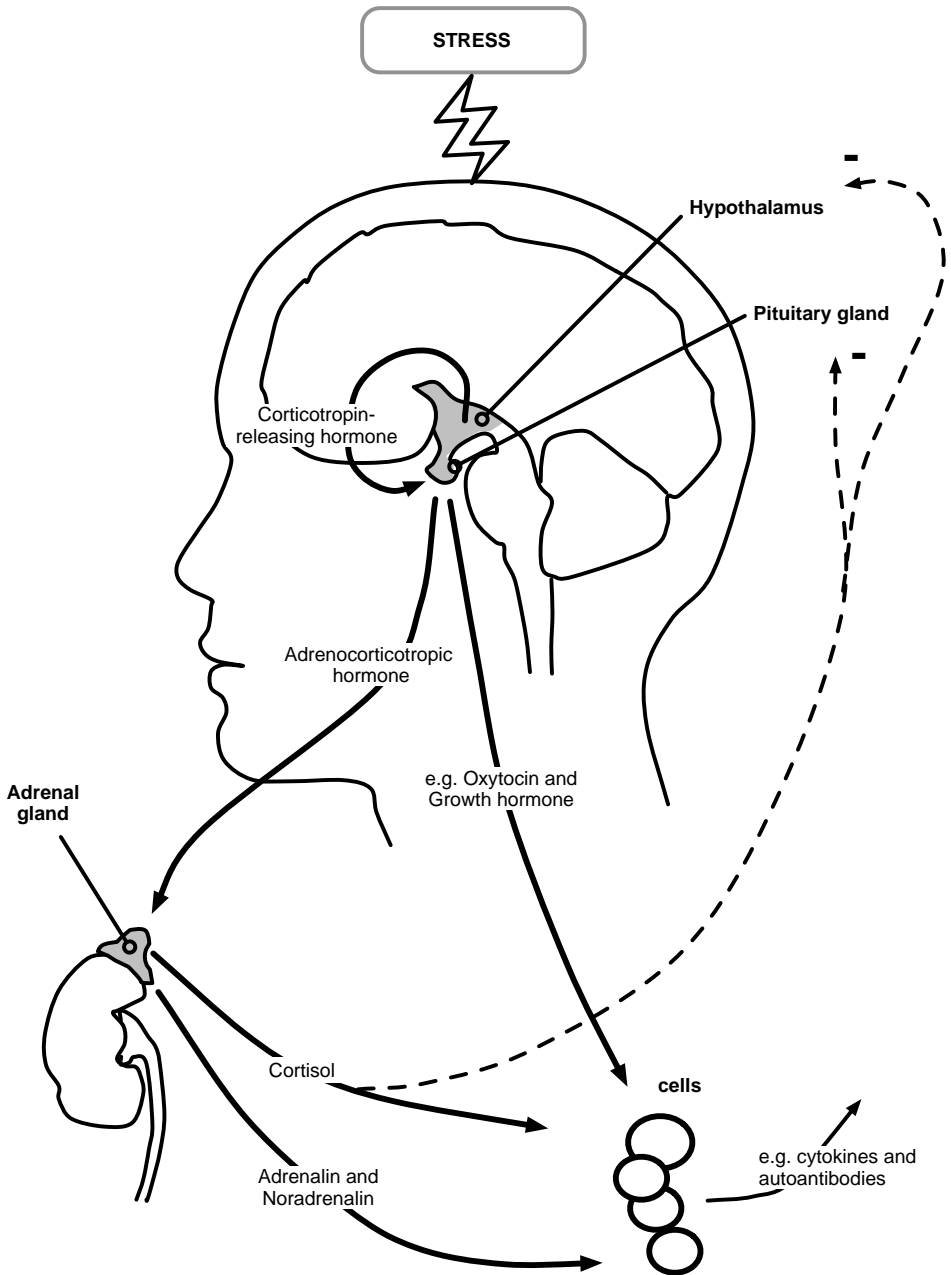


Figure 2. The stress response and function of the HPA axis.

Assessing HPA axis activity

The most extensively used method of measuring stress response is to assess the activity of the HPA axis through levels of cortisol concentrations in saliva.⁷¹ The method has certain advantages: it is non-invasive and consequently less stressful, cheap, and can be performed by non-medical personnel in many different environments. Its technical accessibility and non-invasiveness has made it popular and it is mostly used in psychological and neuroendocrine experimental studies, especially among studies in children. Studying behavioural and hormonal reactions to potentially stressful situations is common, for example temporal separation from the mother during attachment assessments.⁹¹

Unfortunately, current methods of measuring cortisol, including concentrations in saliva, blood or urine, only cover a spot time interval and cannot detect stress longitudinally or retrospectively. Typically, several measurements of cortisol are required from each individual in order to assess a representative cortisol concentration, with levels varying greatly in each individual depending on the circadian rhythm, among other things.^{92,93} Sauvé et al. also demonstrated this in their 2007 study by showing that cortisol in hair correlated with 24-hour urinary cortisol but not with serum or salivary cortisol, which only measure momentary levels of cortisol.⁹⁴ Also, since situational factors such as physical exercise in conjunction with sampling seem to affect cortisol levels,⁹⁵⁻⁹⁷ it is therefore reasonable that nervousness associated with sampling can affect cortisol levels in methods measuring momentary stress.

Child cortisol regulation

Since cortisol levels are important in the diagnosis of diseases related to the HPA axis, such as Cushing's and Addison's disease, there are some older studies based on rather crude blood reference values, especially in children.^{98,99} A rather large study of 670 children aged 1 day to 17 years showed that median serum cortisol levels were high in the first 15 days after birth and then steadily decreased until rising again at the onset of puberty, reaching a plateau at adolescence.¹⁰⁰ Moreover, cortisol studies in saliva indicate that the HPA axis is highly reactive and unstable at birth as well as correlating to the pubertal stage.¹⁰¹ Infants seem to be born without the pulsatile variation, the so-called circadian (diurnal) rhythm, where levels of cortisol are at their highest in the early morning.¹⁰² However, there are contradictory results as to when it begins. Some studies point toward the age of about one month while others see no circadian rhythm before nine months of age.^{103,104} Even when the circadian rhythm has started it seems to differ to that of adults,¹⁰¹ possibly because of variations according to sleep and feeding patterns.⁹³ Among neonates it has been shown that hospitalized infants have higher levels of cortisol in saliva compared to controls.¹⁰⁵ They also have higher cortisol levels as a function of the period of stay in the ventilator during neonatal intensive care.¹⁰⁶ Some evidence suggests that prenatal stress could shape the development of the HPA axis, often measured through short-term output of the stress hormone cortisol in saliva.¹⁰⁷

Cortisol in hair

The shortcomings of the present cortisol-measuring methods call for a better procedure in determining extended and long-term exposure to stress. Hair has been used as a substrate for measuring environmental agents, drugs or toxins for a number of years and quite recently has allowed the retrospective measurement of steroid hormones,^{108,109} including cortisol levels, in hair.¹¹⁰⁻¹¹² Cortisol is assumed to be incorporated into the hair after diffusion from blood plasma circulating in the capillaries that supply the hair roots as the hair grows.¹¹³ Furthermore, since hair grows approximately 1 centimetre per

month, it is also suggested that it could be used as a retrospective calendar of HPA activity during specific time periods preceding sample collection.¹¹³ Initial studies on primates showed that cortisol levels in their hair increased when exposed to the stress of relocation, and that the cortisol levels also increased in serum and saliva respectively.^{114,115} A few subsequent initial studies on humans indicate that the level of cortisol in hair may reflect the activity of the HPA axis.^{116,117} Furthermore, correlations could be seen between specific stressors and cortisol in hair in selected groups. For example, neonates were shown to have higher cortisol levels depending on the period of stay in neonatal intensive care.¹⁰⁶ For a group of patients with chronic pain, higher hair cortisol levels were found compared to a control group.¹¹⁸ In a proof-of-concept study by Kirshenbaum et al., cortisol levels increased in near-scalp hair segments of pregnant women, which corresponded to the well-known increase of blood cortisol levels in the third trimester of pregnancy.¹¹⁸⁻¹²²

The last couple of years have seen a rapid emergence of research concerning concentrations of cortisol in hair. So far it seems that there is a positive association between cortisol levels in hair and different kinds of stressors, such as life events, chronic pain, unemployment, shift work and parental income.¹²⁰⁻¹²³ So far, to our knowledge, no clinical standards or reference values have been presented, so the magnitude of the physiologically plausible cortisol in hair concentrations is unknown. The individual's perception of stress or self-reported stress-related measures is not necessarily equivalent to stress measured by biomarkers, and studies focusing on stress-related measures, such as Perceived Stress Scale (PSS), Hospital Anxiety and Depression scale, Trier Inventory for the Assessment of Chronic Stress, Beck Depression Inventory, have indeed found at best diverging associations.¹²³⁻¹²⁶ Furthermore, research focusing on mental illness has shown very inconsistent results, with studies reporting both high and low hair cortisol levels in association with, for example, depressed and patients diagnosed Post Traumatic Stress Disorder (PTSD).¹²⁷⁻¹²⁹ A few studies on cardiovascular-related problems have shown a relationship to obesity, diabetes type 2, level of heart failure as well as myocardial infarction.¹³⁰⁻¹³⁴

AIMS OF THE THESIS

The general aims of this thesis were to investigate the relationship between early stress, levels of cortisol in hair as a marker for HPA axis activity and health among children in different psychosocial environments.

The specific aim for each paper was:

Paper I. To compare the concentration of diabetes-related autoantibodies in one-year-old children from different social environments, two equally sized neighbouring but socio-economically different twin cities. This while taking other previously documented risk factors of autoimmunity into consideration.

Paper II. To investigate whether concentrations of cortisol measured in hair extracts correlate with perceived stress, serious life events and perceived health in young adults, as well as developing the method for measuring cortisol concentrations in hair.

Paper III. To explore hair cortisol concentrations prospectively in children from one to eight years of age and its possible association to the mother's cortisol levels during pregnancy. Furthermore, to investigate possible links between early stress exposures and child hair cortisol concentrations.

Paper IV. To examine whether adverse psychosocial circumstances, i.e. psychosocial vulnerability in the family during pregnancy, alter infant cortisol concentrations in hair. Furthermore, to investigate a possible relation to diagnose risk on children followed prospectively until the age of ten years.



METHOD

ABIS

All babies in southeast Sweden (ABIS) is a population-based prospective cohort study aimed to examine the aetiology of T1D and other immune-related diseases. The parents of all $N \approx 21,700$ babies in the southeast of Sweden born between 1 October 1997 to 1 October 1999 were asked to participate, **figure 3**. The participating families and their children have been followed prospectively with regular questionnaires and biological samples, such as hair, stool, breast milk, urine and blood samples, including cord blood. The parents of about 17,000 children (78%) initially gave their informed consent to participate. Data were collected by the nurses at 250 well-baby clinics in conjunction with the Swedish child check-ups. The current thesis was based on data provided at birth (2 weeks) and age 1, 3, 5 and 8. No reminders were used. At 8 years both the child and the parent answered a questionnaire that was sent home to the family. For an overview of the design of the ABIS-study data collection see **figure 4**. Follow-up rate at the age of 1 year was 67%. When comparing the ABIS population to available SCB-data (Statistics Sweden, 1999)¹³⁵ the sample was representative of Sweden concerning the parents' education. The proportion of parents born abroad in the study cohort was similar to the proportion of individuals born abroad in the region, and only slightly smaller than the proportion in Sweden as a total. Furthermore, the sample was representative of Sweden regarding the parents' education. There were no difference between the original ABIS-cohort or the follow-up samples concerning: foreign origin, education, and parental age.¹³⁶

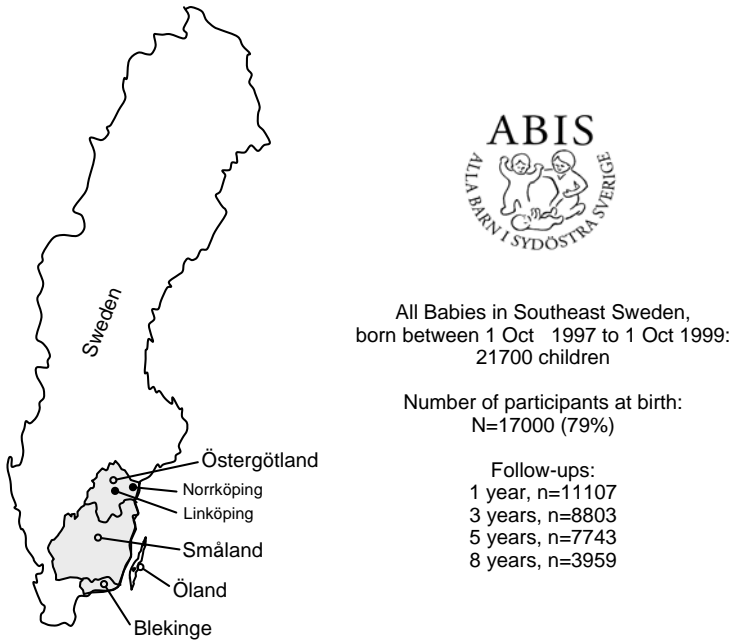


Figure 3. Map of southeast Sweden, showing the regions from where the sample for ABIS was drawn, including number of participants at the different follow-ups.

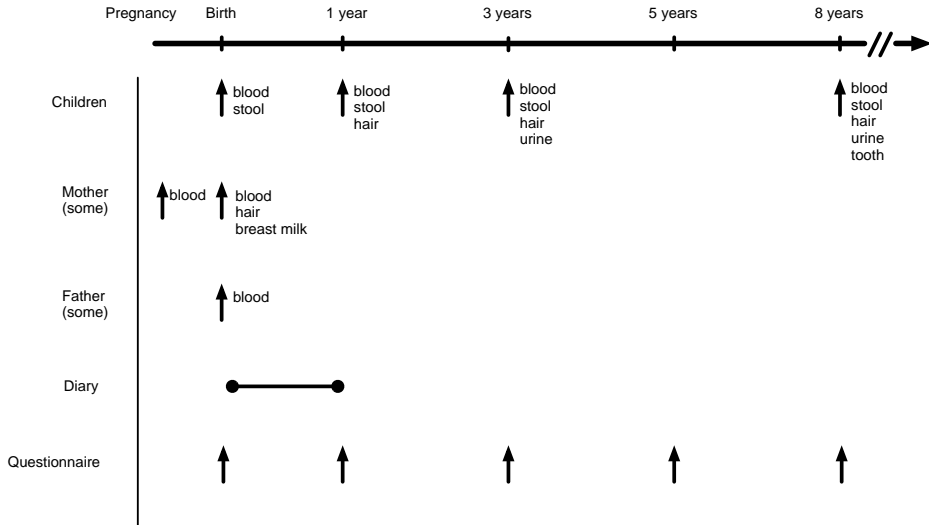


Figure 4. Design of the ABIS-study including data collection.

Twin Cities

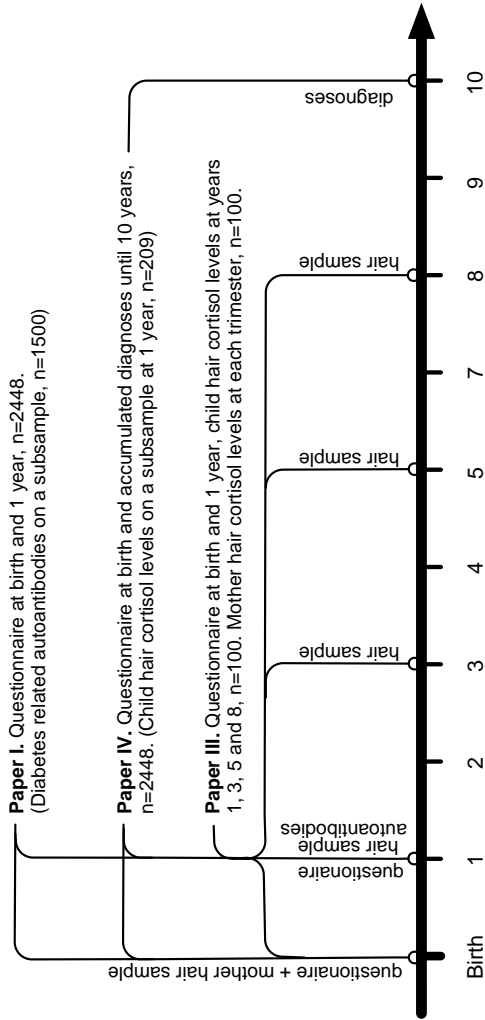
In this thesis, one of the underlying objectives was to focus on the stress and health of children in different social environments. On a structural community level the two Swedish cities Linköping and Norrköping in the county of Östergötland, are referred to as twin cities but they represent two different social environments. The cities are about equal in size, around 150,000 and 135,000 respectively, and geographically located within a distance of 40 kilometres. Today they could be considered as quite similar and comparable cities, but their social histories are very different as well as their present day public health.¹³⁷

Norrköping is referred to as a blue-collar city. It was industrialized as early as the seventeenth century and during the industrial revolution 200 years later became a centre for the textile industry, employing many women, whose work was low paid and monotonous. The city of Linköping is referred to as a white-collar city. It was historically centred on commerce, the Church, administration, and later also on higher education. Industrialization of Linköping started much later, in the early twentieth century, and mainly employed men within the metal industry. However, both cities underwent major structural changes from an industrial to an information society in the 1960–70s. In particular the blue-collar city was hard hit in the 1960s and 70s with factory shutdowns and mass lay-offs. The white-collar city managed this change better than the blue-collar one, most likely due to its tradition of, for example, administration and education to fall back on. These marked differences have been somewhat reduced during recent decades, but the cities may well be viewed as typical ‘white-collar’ and ‘blue-collar’ communities. Although the two cities are much alike today, there are some distinct differences in the populations.¹³⁸ The inhabitants of the blue-collar city have in general a lower education, are more often smokers, exercise less, are more overweight, have shorter life expectancy, and higher frequencies of cardiovascular disease, gastrointestinal disorders, but also mental illness, musculoskeletal diseases, and rheumatism etc.^{139,140} These differences seems to have remained stable over the years.¹³⁷

TwinABIS

In papers I and IV a subsample of the major ABIS-study was used. This so-called 'TwinABIS' constitutes a total of n=2448 children (from the city of Linköping n=1288 children, and from Norrköping n=1160 children). These cities were chosen because they represent two different social environments and because they are part of the same county council that is responsible for practically all health care with the same clinical practice guidelines. Furthermore, it is possible to study diagnoses of and health-care utilization by all the inhabitants in both primary care and hospital care in this county council, since an extensive regional Health Care Register is available

This subsample consists of every participating ABIS-child living in the urban parts of the neighbouring cities of Linköping and Norrköping. Thus it was based on the perception that there is a difference between the psychosocial environments of children living in the two cities. This difference is presumably not evident in the rural and countryside of the two communities. The number of omitted children due to this exclusion of children living in the rural parts of the two cities was n=129 (9%) in Linköping and n=20 (2%) in Norrköping. For an overview of the samples used in Papers I, III and IV (note, Paper III included data from the whole ABIS), see **figure 5**. For detailed information on each of these papers see headings below.



Paper I. Questionnaire at birth and 1 year, n=2448. (Diabetes related autoantibodies on a subsample, n=1500)

Paper IV. Questionnaire at birth and accumulated diagnoses until 10 years, n=2448. (Child hair cortisol levels on a subsample at 1 year, n=209)

Paper III. Questionnaire at birth and 1 year, child hair cortisol levels at years 1, 3, 5 and 8, n=100. Mother hair cortisol levels at each trimester, n=100.

Figure 5. The ABIS samples used in three of the papers in current thesis

Cortisol in Hair Analysis

Cortisol concentrations were expressed as pg/mg with a method developed in-house using a competitive radioimmunoassay (RIA) in methanol extracts of pulverised hair. An approximately 3 mm-thick hair and 3 cm-long sample was cut close to the scalp from the posterior vertex area of the head. The samples were minced and extracted in methanol as explained below. Hair samples were weighed on a Sartorius MC 210p microscale, in 2 mL QiaGen RB sample tubes. To each tube a 5 mm steel ball was added and batches of five tubes were put in each solid aluminium holder. The holders, including the samples and steel balls in the sample tubes, were frozen in liquid nitrogen for 2 minutes and rapidly minced in Retch TissueLyser II at 30 Hz for 2 min producing very fine hair powder. One mL pure methanol (Chromasolv, Sigma-Aldrich) was added to each tube and each tube fixed in a metal holder on an oblique (5 degrees from the horizontal) plate on a horizontal shaker (Edmund Bühler, type B1) at room temperature, making sure that the steel balls continuously moved back and forth for ten hours. The tubes were then centrifuged and 700 µL of the supernatant moved to another sample tube for lyophilization in SpeedVac Plus SC210A (Savant) using Edwards XDS 5 vacuum pump. The extracted hair samples were then measured for concentration of cortisol. First they were dissolved in 150 µL 0.1 mol/L phosphate buffer, pH 7.4 containing 0.02% bovine serum albumin (BSA) and 0.01% triton X-100, and concentrations of cortisol measured were analysed as described by Morelius et al.¹⁴¹

Ethical Considerations

Papers I, III and IV are based on data from the ABIS-study. At start-up, before the child was born, parents received oral and written information as well as being offered the opportunity to watch a video about the ABIS-study. The return of a completed questionnaire as well as biological samples was considered as an informed consent. At the time of the first questionnaire (when the child was just born) the parents also had the opportunity to give their informed consent for possible upcoming linkage in the future of data

from medical records and health-care registers. ABIS has been approved by the research ethics committee of the Faculty of Health Sciences at Linköping University, Sweden, at several times in conjunction with different sub-projects and at the Medical Faculty at Lund University, Sweden. Ethical considerations in the ABIS-project have thereafter been specifically studied in a dissertation from the university of Linköping.¹⁴² Data are stored without personal identification numbers and analyses are with due confidentiality. This thesis has a separate approval (2008-02-14, registration number M154-07) from the Regional Ethical Review Board at the Faculty of Health Sciences, Linköping University, Sweden.

Statistical Analyses

Data from questionnaires at the different follow-ups were optically scanned and manually checked for errors. Univariate analyses were executed as shown below. Multivariate regression analysis was used to control for other known associated factors and in Papers I and IV to estimate the ORs (95% CI). Independent variables found to be significant at the 5% level in the univariate analyses were included in the final multivariate analyses. For association of continued variables, Pearson's correlation coefficient was applied (Papers II, III, IV). Outliers in the measured cortisol concentrations were identified with Grubbs test and no statistics were calculated on mean values based on small groups (i.e. groups with fewer than ten individuals). Statistical analyses were made using the Statistical Package for the Social Sciences v. 16.0 to 20.0 software in the different papers (SPSS, Chicago, IL, USA).

In Paper I univariate analyses between exposed and non-exposed groups and differences in occurrence of glutamic acid decarboxylase (n=65; cut off 95th percentile positive reaction) were measured by χ^2 and, in the case of a few observations under 5), Fisher's exact test was used.

In Papers II and III analysis of variance (ANOVA) was used for univariate tests. Paper II concerned adults' levels of cortisol in hair with supposedly less biological variation than the children, and thus cortisol in hair values were not logarithmized prior to analyses and outliers were ruled out due to the possible

inclusion of measurement errors. Nevertheless, although data were not normally distributed, ANOVA was still subsequently used in all univariate analyses in paper II, including the variable *Serious Life Events*. This was done after consultation with two independent experienced statisticians who both recommended this method. According to them, this method is quite robust and it is even harder to get a statistically significant result with ANOVA than it is using non-parametric tests.¹⁴³ Also note that *Serious Life Events* is still statistically significant in the multivariate analysis.

In Papers III and IV the measured cortisol concentrations were log-transformed to a normal distribution before computing inferential statistics, since their distributions were skewed. Thereafter the geometric means were presented. Paper IV used a composite vulnerability scale to compare mean numbers of items against having a diagnosis or not, as well as being transformed in to a categorical variable when testing against binary diagnosis; in both cases independent samples t-test was used.

Paper I

Study sample

The TwinABIS sample in this study yielded n=1288 children from the white-collar twin city, and n=1160 children from the blue-collar twin city. Out of these children, 709 and 788 children, respectively, were tested for tyrosine phosphatase (IA-2A) and n=701 and n=708 children were tested for glutamic acid decarboxylase antibodies (GADA).

Autoantibodies

Autoantibody analyses were done in whole blood samples from randomly selected one-year-old children in the total ABIS cohort. n=5765 children were tested for autoantibodies to GADA and n=5635 children were tested for autoantibodies to IA-2A. The autoantibody assay has been described else-

where and validated in the Diabetes Autoantibody Standardization Program 2002 workshop with documented good sensitivity and specificity (specificity was 100% for IA-2A and 96 % for GADA assays and the sensitivity was 54% and 81%, respectively).^{144,145} In this report the cut-offs analysed were 95%.

Questionnaires

Data from questionnaires in the ABIS-study were also included in this report. The mother answered one questionnaire around two weeks after child delivery; she also answered yet another when the child was one year old. Both questionnaires concerned a large set of factors that could have a possible association to the development of T1D, such as family characteristics, dietary habits, lifestyle and psychosocial factors. Questions included in this analysis were explanatory factors documented as risk factors for increased GADA and IA-2A in earlier studies and factors concerning differences in socio-economic structure between the two cities.

Paper II

Study sample

Medical and nursing students in their second semester at the Faculty of Health Sciences at Linköping University were invited to voluntarily cut hair samples and to anonymously fill in a questionnaire. N=99 students decided to participate in the study. No exclusion criteria were applied, except for those students with crew cut or shaved hair, for which hair sampling was impossible.

Independent variables

The questionnaire covered the experience of: 1) *serious life events* during the last three months; this was assessed with the dichotomous (yes/no) question

during the last three months, have you experienced something that you would describe as a serious life event? (e.g. death of a close relative, serious illness or divorce.', with a 'yes' option to describe which kind of event; 2) *perceived health* during the last three months, assessed by a visual analogue scale coded as 0–10 (ranging from 'very poor' to 'excellent'); and 3) *perceived stress* during the last three months, assessed with the PSS which is a 14-item instrument assessed on 5-point Likert-scales (ranging from 'never' to 'very often').¹⁴⁶ The association between total PSS-score, as well as each PSS-item, and cortisol concentrations was statistically tested. Background factors were measured, including sex, age, and possible confounding factors, such as coloured/permed hair and regular use of prescribed pharmaceuticals the last three months.

Cortisol in hair

Hair samples with a length of 3 cm, cut closest to the scalp from the posterior vertex area of the head were analysed. Samples weighing 5 mg or more were needed for maintaining a total inter-assay coefficient of variation below 8% for hair extraction and measurement of cortisol by the radioimmunoassay. The intra-assay coefficient of variation for the radioimmunoassay itself was 7% at 10 nmol/L.

Paper III

Study sample

The extensive ABIS biobank was scrutinized and the first 100 children with enough hair stored to be able to measure cortisol in hair at the ages of 1, 3, 5 and 8 years of age as well as hair collected by their mothers at least covering the third trimester of the pregnancy were included.

Cortisol in hair

Hair strands for both children and their mothers were cut closely to the scalp from the top and back of the head and then deep-frozen. A minimum of 3 cm hair was cut reflecting exposures during the last three months. Samples of the mother's hair were cut after child delivery. If available, at least 9 cm hair was cut reflecting the 9 months period of pregnancy. These were thereafter cut into 3 cm pieces reflecting the pregnancy trimesters. The children's hair was cut in connection to the prospective data collection at the well-baby clinics at the age of 1 year, 3 years, 5 years and 8 years.

The lowest detectable concentration was 1 nmol/L and the within- and between assay coefficients of variation were 6.1% and 9.3%, respectively, at 10 nmol/L. Each of the multiple steps in the weighing, extraction and measurement contributes to the overall uncertainty, with sample mass as one of the important variables. Ten replicates of hair samples weighing 1, 2, 3, 4 and 5 mg were extracted and measured by the procedure described above and the coefficient of variation calculated, see **table 1** below. At least 5 mg of hair samples was subsequently used in the analysis.

Table 1. Coefficient of variation on cortisol concentrations in hair in relation to sample mass

Hair sample	Coefficient of variation
1 mg	46.2%
2 mg	16.1%
3 mg	12.4%
4 mg	13.6%
5 mg	7.9 %

Paper IV

Study sample

A subsample of $n=2447$ children, (TwinABIS) consisting of every participant living in the urban areas of the two cities of Linköping and Norrköping was chosen. In all, $n=571$ children were excluded due to incomplete data on the independent variable. The sample in this paper for subsequent analyses concerning vulnerability and International Classification of Diseases (ICD–10) diagnoses thus was $n=1876$ children ($n=926$ girls and $n=950$ boys). Concentration of cortisol in hair was analysed for a subsample of $n=209$ children ($n=103$ boys and $n=106$ girls) with sufficient hair samples collected at age one. This sample was stratified according to the distribution of the vulnerability scale and gave an oversampled 3+ category ($n=90$).

Psychosocial vulnerability scale

The mothers answered a questionnaire shortly after birth containing psychosocial factors or markers in areas such as socio-economic situation, social support, empowerment, living situation and sense of coherence. A ‘deficit accumulation approach’ employed in earlier studies was used to create a psychosocial vulnerability scale.⁴⁶ Variables in the existing database associated with detrimental impact on health as suggested in previous research were included. Some detailed answers were dichotomized to pinpoint the most vulnerable, e.g. schooling was simplified into having an education above elementary school or not (see below).

Eleven psychosocial items resulted in the final composite independent variable: the psychosocial vulnerability scale. They were: *father’s highest level of education elementary school; mother’s highest level of education elementary school; father unemployed or on sick leave the year before pregnancy; mother unemployed or on sick leave during pregnancy; living in an apartment, as opposed to an own house; single mother; parents born abroad; maternal serious life event during pregnancy* (‘Have you been exposed to something which you perceive as a serious life

event during your pregnancy?'); *maternal lack of support* ('Do you feel your surroundings give you the support you and your newborn child need?'); *mother not feeling safe* ('Do you feel safe and in the circumstances needed to give you and your newborn child a good start?'); *mother worried over the possibility of child falling ill with serious disease* ('How do you usually feel when you consider the possibility that your child could fall ill with a chronic or serious disease in the future?') For these, a stepwise 6-grade scale was applied, between not worried and very worried, with 4–6 being classified as worried.

Health outcome

ICD-diagnoses (International Classification of Diseases –10) were derived from the regional Health Care Register data of all health-care visits in primary as well as in hospital care within the public financed health care, which represent practically all health care in the region. Diagnoses from birth to February 2008 were obtained, and recorded on a one-per-case basis. Children were aged eight to ten at follow-up, hence describing the cumulative incidence from birth. This follow-up was almost 100%, since the dropout due to death or moving out the region was negligible. Closely related diagnoses were in some instances merged, e.g. codes J00 through to J06 were all registered in the category 'J00-J06: acute upper respiratory infections'. Categories Q, R and Z were omitted. There is no evidence of systematic misclassification in this health-care register. ¹⁴³

Cortisol in hair

Nurses at the well-baby clinics cut the children's hair from the posterior vertex area of the head at age one and the first 3 cm of outgrowth was analysed for cortisol concentrations using a competitive RIA in methanol extracts, as described earlier. Hair samples between 3–10 mg were required to maintain a total inter-assay coefficient of variation below 8% for hair extraction and measurement of cortisol by the radioimmunoassay. The intra-assay coefficient of variation for the radioimmunoassay itself was 7% at 10 nmol/L.

RESULTS

Paper I

Diabetes-related autoantibodies in the twin cities

The presence of the autoantibodies IA-2A and GADA (95% cut-off) among children aged one year in the two cities is presented in **figure 6**. The number of children with autoantibodies, IA-2A as well as GADA, were significantly higher ($p < 0.0001$) among children from the blue-collar twin city (8.9% and 7.1%) than among children from the white-collar twin city (1.0 and 1.3%). Children with both IA-2A and GADA autoantibodies were only found among the children from the blue-collar twin city (1.3% or $n=10$ children). This difference persisted still after adjustment for other previously documented risk factors. Since Paper 1 was published these results have been replicated at the age of three years, though notably with less differences between the twin cities. See **figure 7**.

Different factors with a possible association to GADA and IA-2A positivity (95% cut-off) are shown in **table 2** and **table 3** respectively. Some previously known factors were significantly associated to the autoantibody positivity in the univariate analysis. These factors remained independently and significantly associated in the multivariate analysis as well as the *twin cities* factor, that is, living in the blue-collar twin city compared to the white-collar city.

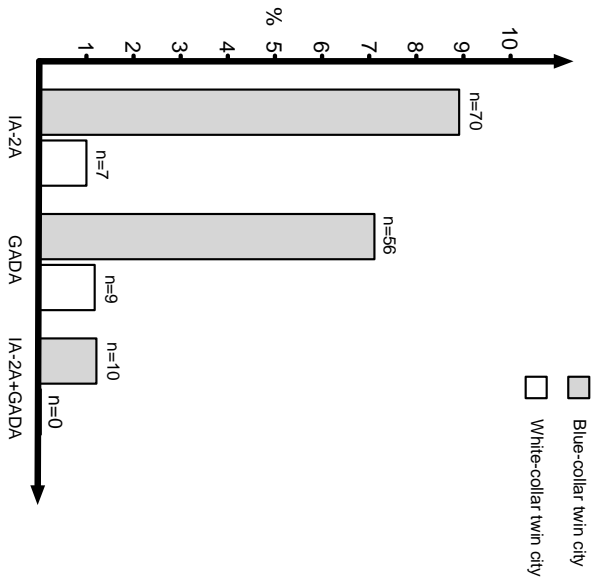


Figure 6. Distribution of children at 1 year of age in the blue-collar and the white-collar twin cities with the autoantibodies for tyrosine phosphatase (IA-2A) and glutamic acid decarboxylase (GADA) at 95% cut off.

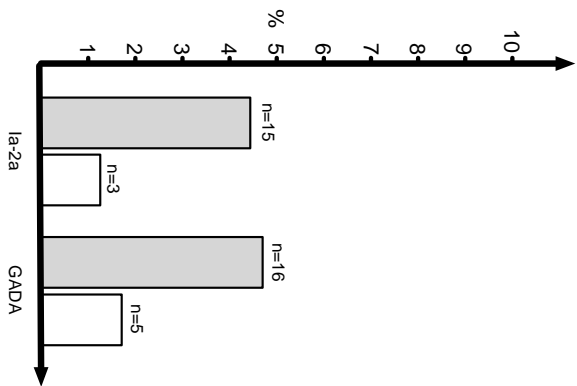


Figure 7. Distribution of children at 3 year of age in the blue-collar and the white-collar twin cities with the autoantibodies for tyrosine phosphatase (IA-2A) and glutamic acid decarboxylase (GADA) at 95% cut off.

Table 2. Bivariate and multiple logistic regression analysis of possible factors explaining the occurrence of GADA (n=65, cut 95th percentile positive reaction) for one-year-old children in different social environments, the twin cities.

	n	Bivariate analysis		Multiple logistic regression		
		OR	CI 95%	OR	CI 95%	p
Gender (male)	41	1.21	0.76-1.92			
High weight at birth	6	1.82	0.77-4.34			
High weight at 1 years of age	8	2.95	1.35-6.45	3.42	1.50-7.80	0.003
Born in the spring	23	1.12	0.67-1.88			
Mother below 30 years at child's birth	36	1.05	0.66-1.68			
Type 1 Diabetes in the family	10	1.33	0.67-2.63			
Coeliac disease in the family	11	3.10	1.58-6.08	3.24	1.36-7.73	0.008
Mother low education	6	1.25	0.53-2.94			
Father low education	11	1.84	0.95-3.58			
Mother born abroad	8	1.90	0.89-4.06			
Father born abroad	8	1.56	0.73-3.33			
Dairy products introduced before child was 4 months	28	0.74	0.46-1.19			
Breast feeding ends before 7 months	20	0.71	0.41-1.23			
Serious life event in the family before one 1 of age	1	0.46	0.06-3.38			
Living in the Blue-collar twin city	56	5.56	2.77-11.15	6.52	2.75-15.43	<0.0001

Table 3. Bivariate and multiple logistic regression analysis of possible factors explaining the occurrence of IA-2A (n=77, cut 95th percentile positive reaction) for one-year-old children in different social environments, the twin cities.

	n	Bivariate analysis		Multiple logistic regression		
		OR	CI 95%	OR	CI 95%	p
Gender (male)	48	1.11	0.73–1.69			
High weight at birth	7	1.82	0.82–4.09			
High weight at 1 years of age	7	1.93	0.85–4.36			
Born in the spring	36	1.52	0.98–2.37			
Mother below 30 years at child's birth	51	1.44	0.94–2.20			
Type 1 Diabetes in the family	9	0.91	0.45–1.84			
Coeliac disease in the family	7	1.42	0.64–3.15			
Mother low education	7	1.18	0.54–2.63			
Father low education	10	1.27	0.64–2.50			
Mother born abroad	8	1.50	0.70–3.18			
Father born abroad	9	1.37	0.67–2.80			
Dairy products introduced before child was 4 months	18	0.77	0.50–1.19			
Breast feeding ends before 7 months	42	1.20	0.76–1.92			
Serious life event in the family before one 1 of age	7	3.26	1.40–7.55	2.99	1.16–7.71	0.004
Living in the Blue-collar twin city	70	9.00	4.16–11.15	12.51	4.50–34.80	<0.0001

Paper II

Stress factors and cortisol in hair

Mean cortisol levels for the studied young adults were 19.93 pg/mg (s.d. 33.35), ranging from 1.45 to 212.03 pg/ mg. For males, the mean cortisol levels were 17.76 (s.d. 13.34) and for females 20.55 (s.d. 37.66), no difference between sexes in this respect ($p = 0.726$). Increased cortisol levels in hair were found among participants reporting that they, during the last three months, had experienced serious life events ($p = 0.045$). Also, participants reporting lower levels in the 14-item PSS had significantly higher levels of cortisol in their hair ($p=0.025$, $r=-0.061$) as seen in **table 4**. Cortisol levels were neither affected by coloured/permed hair, intake of prescribed pharmaceuticals nor self-reported health. Furthermore, in a follow-up question concerning pharmaceuticals – ‘If Yes, which kind?’ – no one reported the use of oral corticosteroids or other hormonal drugs of probable influence to cortisol levels. In a multiple regression model, indicators of perceived stress, perceived health and experience of serious life events were included. Only the indicator of experiences of serious life events in the last three months exhibited an independent and significant ($p=0.042$) explanation of increased levels of cortisol in hair. Four respondents had cortisol concentrations that were considerably higher than the average (692/750/1090 and 2027 pg/mg), and were considered as outliers. Despite the anonymous data collection, two of these four outliers could be reached by open announcement. Both of them reported serious psychological problems, anorexia nervosa and severe generalized anxiety disorder with panic attacks, respectively.

Table 4. Stress and health factors associated to cortisol levels in hair (pg/mg)

	n	mean	s.d.	Bivariate		Multivariate†	
				r*	pt	β	p
Background factors:							
Age (mean=22.1 s.d.=4.1)	95			-0.073	0.732		
Sex	Male	24	17.76	13.32			
	Female	71	20.53	37.66	0.726		
Coloured/permed hair	Yes	40	13.04	16.01			
	No	55	24.78	40.89	0.089		
Pharmaceuticals	Yes	28	22.85	44.86			
	No	67	18.58	27.19	0.570		
Stress and Health factors:							
Serious life event	Yes	20	32.98	56.90			
	No	75	16.32	22.53	0.045	0.212	0.041
Perceived stress (PSS)	Yes	95			-0.061	0.025	-0.078
	No	95			0.038	0.251	0.448

* Pearson correlation

† ANOVA, cortisol levels as dependent variable.

‡ Multivariate regression model: (df = 4) F = 3.43 p = 0.012, cortisol levels as dependent variable, background factors were not included in the regression model.

One single PSS item ('In the last three months, how often have you felt difficulties were piling up so high that you could not overcome them?') had a tendency towards exposure-response relationship, with increasing mean cortisol levels corresponding to higher levels of coping problems (*Never*=4.02, *Almost never*=9.75, *Sometimes*=14.79, *Fairly often*=21.86, *Very often*=32.91 pg/mg respectively), although confidence intervals overlapped between all but *Never* and *Sometimes*/*Fairly often*, see **figure 8**.

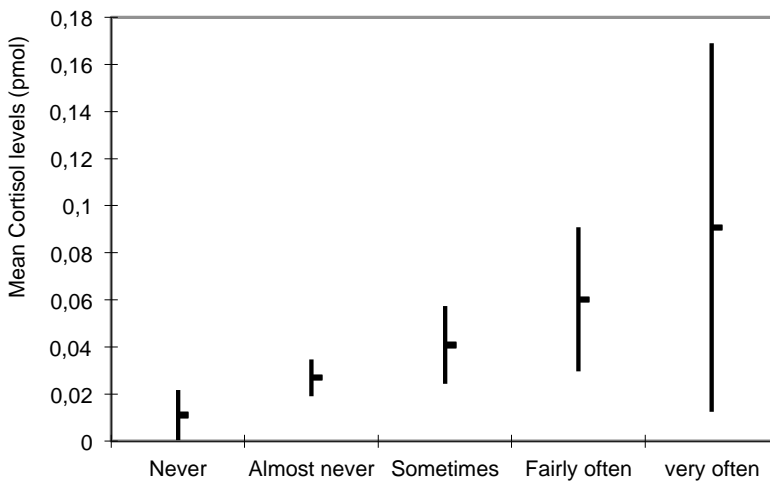


Figure 8. Mean cortisol levels (CI = 95%) and answers to the single PSS question 'In the last 3 months, how often have you felt difficulties were piling up so high that you could not overcome them?' (N=99).

Paper III

Child and mother hair cortisol

The prospective, non-logarithmized hair cortisol concentrations for the children at 1, 3, 5, and 8 years of age are shown in **figure 9**. At 1 year of age they ranged from 0.18 to 1667 pg/mg, at 3 from 0.87 to 983, at 5 from 0.36 to 1299, and at 8 from 0.34 to 402. Cortisol values for the children decreased over time, indicated by the geometric mean values, and they were significantly positively correlated between 1 and 3 years of age and between 3, 5, and 8 years. Repeated-measures ANOVAs were also used, and a significant linear association over time was observed ($\epsilon=0.807$, $p=0.001$). During pregnancy, the pregnant mother's cortisol concentrations increased over time and ranged during the first trimester from 0.62 to 50 pg/mg, during the second trimester from 0.33 to 263 pg/mg, and during the third trimester from 0.42 to 206 pg/mg. All trimesters were strongly correlated: first and second ($r=0.93$, $p=0.001$), second and third ($r=0.78$, $p=0.001$), and first and third ($r=0.74$, $p=0.001$). Maternal hair cortisol levels during the second ($n = 73$) and third trimester ($n=100$) correlated to child levels at years 1 and 3, whereas the first trimester ($n=40$) did not show significant correlations, see **table 5**.

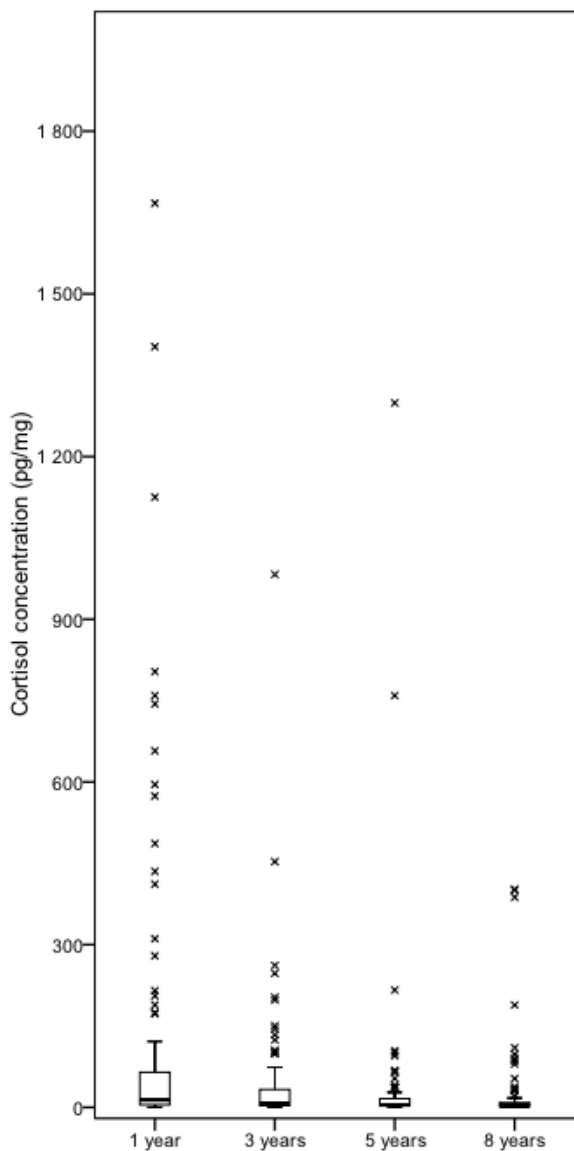


Figure 9. Boxplot of child (n=100) prospective hair cortisol concentrations (pg/mg) during early childhood. Box indicates IQR, asterisks indicate outliers (>1.5 IQR from Q2).

Table 5. Prospective childhood hair cortisol concentration (pg/mg) for 100 children at the ages 1, 3, 5 and 8 years and correlation to maternal hair cortisol concentration at the three trimesters of pregnancy.

	Cortisol geometric mean (95%CI)	1 year		3 years		5 years		8 years	
		r	p	r	p	r	p	r	p
Child, 1 year	19.87 (13.55–29.13)			0.30	0.002	0.12	0.905	0.12	0.220
Child, 3 years	11.30 (8.28–15.42)					0.39	<0.001	0.29	0.002
Child, 5 years	6.77 (5.08–9.03)							0.44	<0.001
Child, 8 years	4.99 (3.74–6.66)								
Mother third trimester (n=100)	2.62 (2.16–3.18)	0.23	0.024	0.27	0.007	0.04	0.667	0.16	0.109
Mother second trimester (n=73)	2.25 (1.75–2.90)	0.36	0.002	0.25	0.033	-0.01	0.966	0.10	0.409
Mother first trimester (n=40)	1.88 (1.33–2.65)	0.20	0.206	0.29	0.074	0.12	0.463	0.25	0.127

Cortisol in hair socio-demographic factors

Cortisol levels of children at one year of age and perinatal and socio-demographic factors with sample size ten or above were investigated. Gender (40 boys and 60 girls) had no effect on cortisol levels. Higher birth weight, inappropriate size for gestational age, residence type apartment, and living in an urban area were all significantly correlated to cortisol concentration in hair at a univariate level. Three of these, all but living in an urban area, were also statistically significant in the multivariate analyses. One child was prematurely born; excluding it from the analyses did not significantly change the results. One child whose mother reported a lack of social support during pregnancy had a cortisol concentration of 1667 pg/mg, and the only two children with single mothers had concentrations of 189 and 759 pg/mg (compared with the median for all children of 13.6 pg/mg at year 1). These cases were not overlapping. Of the two children with a single mother, the mother of the one with a concentration of 759 pg/mg was also young. Three children, who were attending a day-care centre before the age of one, also had high levels of 759 (the same child as above with single, young mother), 744, and 487 pg/mg. Furthermore, four mothers smoked during pregnancy, giving corresponding levels of 744 (also day-care centre before year 1), 311, 3.2, and 2.4 pg/mg among the children. Finally, six mothers reported serious life events, and their children had 86.5, 22.6, 8.0, 4.2, 2.4, and 0.8 pg/mg in raw hair cortisol concentrations.

Paper IV

There was no difference in logarithmized cortisol levels with respect to gender (boys=2.45 and girls=2.79 pg/mg) or weight. An association between vulnerability and lower birth weight and birth height for females was found. The reference group of female babies with no vulnerability in the family had a mean weight of 3570 g, those with 2 items had a mean weight of 3459 g ($p=0.023$), and those with 3+ items had a mean weight of 3413 g ($p=0.027$).

Children with 3+ items were also shorter at birth than the reference group, with a mean height of 49.8 cm versus 50.4 cm ($p=0.035$).

Psychosocial vulnerability and cortisol in hair

An association was found ($r=0.22$, $p=0.002$) between vulnerability and logarithmized cortisol concentrations in hair, as illustrated in **figure 10**. When adjusting for *gender* and *small for gestational age (SGA)*, vulnerability was still solely independently significant ($B=0.40$, $p<0.0001$). Vulnerability had a general tendency towards a dose response-like increase in cortisol concentrations; zero items ($n=33$) gave mean 1.90 pg/mg (95% CI=0.91–2.89); one item (46) gave 2.18 (2.17–4.16); three items (49) gave 2.76 (2.12–3.41); four items (24) gave 2.82 (1.99–3.65); five (10) gave 3.86 (2.46–5.26) and six items ($n=2$) gave mean 7.21 out of the values 6.88 and 7.54 pg/mg. Out of the 11 single dichotomous items, a significant difference in mean cortisol values was found for two variables: *mother unemployed or on sick leave during pregnancy* ($p=0.041$) and *living in an apartment compared to a house* ($p=0.031$), see **table 6**. Also, all but one of the items (*father's occupation*), exhibited higher mean cortisol levels in the exposed group, although non-significant.

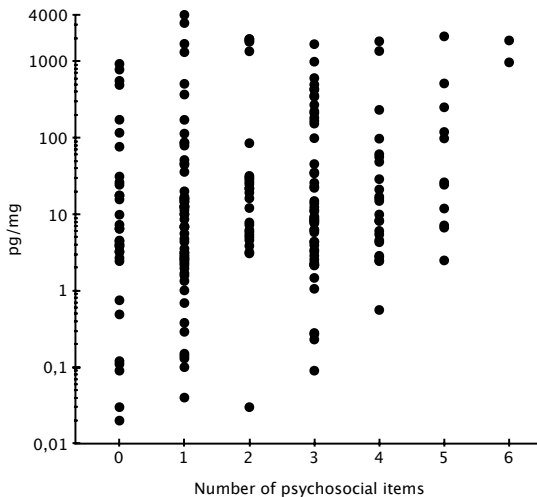


Figure 10. Prenatal psychosocial vulnerability and hair cortisol concentration at one year ($n=209$).

Table 6. Prenatal psychosocial vulnerability, single items and hair cortisol concentration (log pg/mg) at age one year.

Psychosocial exposure		n	Cortisol mean (95% CI)	p ^a
Vulnerability score (number of single items)		209	2.62 (2.28–2.96)	0.002 ^b
Single items				
Mother's marital status	In a relationship	191	2.43 (2.28–3.00)	0.733
	Single	18	2.64 (1.43–3.43)	
Mother's occupation	Working	162	2.43 (2.05–2.82)	0.041
	Unemployed/sick-leave	47	3.27 (2.54–4.00)	
Father's occupation	Working	193	2.62 (2.27–3.98)	0.941
	Unemployed/sick-leave	16	2.57 (1.39–3.77)	
Residence type	Villa	73	2.12 (1.50v2.73)	0.031
	Apartment	136	2.89 (2.49–3.29)	
Father's educational level	College or university	170	2.47 (2.08–2.86)	0.067
	High School/9-year	39	3.28 (2.57–3.99)	
Mother's educational level	College or university	182	2.55 (2.17–2.92)	0.257
	High School/9-year	27	3.13 (2.39–3.87)	
Foreign origin	None or one parent	191	2.54 (2.19–2.88)	0.096
	Both parents	18	3.55(2.19–4.91)	
Maternal serious life event	No	174	2.57 (2.20–2.95)	0.523
	Yes	35	2.88 (2.01–3.66)	
Maternal lack of support	No	208	2.60 (2.25–2.93)	-
	Yes	1	6.88 (-)	
Mother feeling safe ^c	Yes	204	2.59 (2.25–2.93)	-
	No	5	4.01 (2.08–6.93)	
Mother worried about child falling ill with serious disease	No	116	2.37 (2.92–2.82)	0.099
	Yes	93	2.94 (2.43–3.45)	

^a Mean cortisol in exposed group versus non-exposed group, independent samples t-test

^b Pearson correlation, $r=0.22$, $p=0.002$.

^c Question phrased, "Do you feel safe and in the circumstances needed to give you and your new-born child a good start?"

Psychosocial vulnerability and health outcome

The cumulative incidence was analysed for the 14 most common diagnoses, ranging from 0.81 (acute upper respiratory infections) to 0.09 (urticaria). Incidence rates below this were too low for reliable statistical analyses. Comparing the mean vulnerability in diagnosed versus undiagnosed groups, it was significantly higher for diagnosed children in 12 out of the 14 ICD-10 groupings. After adjusting for *gender* and *SGA*, ten diagnoses were still significant ($B=1.92$, $p<0.0001$), see **table 7**. Gender was also independently significant in some cases, among boys: acute upper respiratory infections ($p=0.002$); injury, poisoning and certain other consequences of external causes ($p<0.0001$) and asthma ($p=0.018$). Among girls, one diagnosis was more common, urinary tract infections ($p<0.0001$). Moreover, vulnerability correlated to the number of different diagnoses ($r=0.15$, $p<0.0001$). An increase in vulnerability was associated and graded to a corresponding rise in OR for most diagnoses, although they were only significant among those in the highest category of vulnerability, with a few exceptions. One exception to this general tendency was for urinary tract infections, where having some degree of vulnerability increased all of the ORs about twofold. The largest increases in ORs compared to the reference group, with a more than twofold increase in the 3+ category, were seen in the following diagnoses: viral infections of unspecified site, intestinal infectious disease and urticaria. Somewhat smaller but still significant ORs were seen in the two most common diagnoses: upper respiratory infections and otitis media.

Table 7. Prenatal psychosocial vulnerability and the risk for the 14 most common childhood diagnoses prospectively until age 10 years (n=1876).

Diagnoses (ICD-10)	Psychosocial items	n (%)	Diagnose risk		p ^a	p ^b
			OR	95% CI		
Acute upper respiratory infections (J00-J06)	0	346 (78)	ref	—	0.003	0.004
	1	603 (79)	1.06	0.80-1.41		
	2	368(83)	1.34	0.96-1.87		
	3+	196 (87)	1.83	1.17-2.86		
Otitis media (H65-H67)	0	288 (65)	ref	—	0.009	0.007
	1	496 (65)	1.00	0.79-1.28		
	2	298 (67)	1.09	0.83-1.44		
	3+	172 (76)	1.71	1.19-2.46		
Injury (S00-T98)	0	247 (56)	ref	—	0.048	0.053
	1	418 (55)	0.96	0.76-1.22		
	2	271 (61)	1.24	0.95-1.61		
	3+	138 (62)	1.24	0.90-1.76		
Viral infections of unspecified site (B34)	0	99 (22)	ref	—	< 0.0001	<0.0001
	1	210 (28)	1.32	1.01-1.74		
	2	138 (31)	1.56	1.16-2.11		
	3+	85 (38)	2.10	1.48-2.97		
Infections of the skin (L00-L08)	0	123 (28)	ref	—	0.618	—
	1	205 (27)	0.96	0.74-1.26		
	2	126 (28)	1.03	0.77-1.38		
	3+	66(29)	1.07	0.75-1.53		
Other acute lower respiratory infections (J20-J22)	0	81 (18)	ref	—	0.038	0.029
	1	133 (18)	0.95	0.70-1.28		
	2	92 (21)	1.17	0.84-1.62		
	3+	55 (24)	1.44	0.98-2.12		
Conjunctivitis (H10)	0	69 (16)	ref	—	0.012	0.020
	1	137 (18)	1.19	0.87-1.63		
	2	89 (20)	1.36	0.96-1.92		
	3+	52 (23)	1.62	0.98-2.12		
Dermatitis and eczema (L20-L30)	0	65 (15)	ref	—	0.001	0.032
	1	123(16)	1.12	0.81-1.55		
	2	82(18)	1.31	0.92-1.88		
	3+	57(25)	1.96	1.32-2.92		
Intestinal infectious diseases (A00-A09)	0	52 (12)	ref	—	< 0.0001	0.002
	1	102 (13)	1.16	1.18-2.92		
	2	74 (17)	1.50	1.34-3.51		
	3+	54 (24)	2.36	1.03-3.25		
Urinary tract infections (N30, N34, N39.0)	0	27 (6)	ref	—	0.013	0.003
	1	82 (11)	1.86	1.18-2.92		
	2	55 (12)	2.17	1.34-3.51		
	3+	24 (11)	1.83	1.03-3.25		
Viral infections characterised by skin and mucous lesion (B00-B09)	0	36 (8)	ref	—	0.021	0.061
	1	66 (9)	1.07	0.70-1.64		
	2	52 (12)	1.50	0.96-2.34		
	3+	28 (12)	1.60	0.95-2.70		
Pneumonia (J12-J18)	0	40 (9)	ref	—	0.160	—
	1	66 (9)	0.96	0.63-1.44		
	2	46 (10)	1.16	0.74-1.81		
	3+	27 (12)	1.37	0.82-2.29		
Asthma (J45)	0	32 (7)	ref	—	0.006	0.004
	1	63 (8)	1.16	0.77-1.80		
	2	48 (11)	1.55	0.97-2.48		
	3+	29 (13)	1.89	1.11-3.21		
Urticaria (L50)	0	29 (7)	ref	—	0.002	0.016
	1	64 (8)	1.31	0.83-2.06		
	2	47 (11)	1.69	1.04-2.73		
	3+	30 (13)	2.19	1.27-3.74		

^a Mean vulnerability in diagnosed versus undiagnosed groups, independent samples t-test.

^b Adjusted for *gender* and *size for gestational age*, binary regression.

Distribution in the twin cities

Additional data from this study but not included in the final submitted paper are presented below.

The distribution of vulnerability factors in the population and in the white- and blue-collar twin cities are listed in **table 8**. Four of the factors were significantly more prevalent in the blue-collar city: *mother unemployed or on sick leave during pregnancy* ($p=0.002$), *father unemployed or on sick leave the year before pregnancy* ($p=0.003$), *mother's highest level of education only elementary school* ($p=0.001$) and *mother worried over the possibility of child falling ill with serious disease* ($p=0.039$). The mean number of vulnerability factors in the family was 1.23 in the white-collar city and 1.40 in the blue-collar city ($p=0.001$). No single family had more than seven vulnerability factors, and the majority of families had none or only one factor.

The cumulative incidence of the 14 most common diagnoses in our sample during the ten-year follow-up in regard to sex and the twin cities is shown in **table 9**. Four diagnoses were more common among boys: *acute upper respiratory infections* ($p=0.007$); *injury, poisoning and certain other consequences of external causes* ($p=0.001$); *other acute lower respiratory infections* ($p=0.038$) and *asthma* ($p=0.011$). One diagnosis, *urinary tract infections* ($p=0.001$), was more common among girls. Five diagnoses had a significantly higher incidence in the blue-collar city: *acute upper respiratory infections* ($p=0.018$); *otitis media* ($p=0.001$); *viral infections of unspecified site* ($p=0.005$); *intestinal infectious diseases* ($p=0.003$) and *pneumonia* ($p=0.002$). None of the analysed diagnoses had a higher cumulative incidence in the white-collar city.

Table 8. Vulnerability factors in the family at birth for the TwinABIS sample (N=1876).

Vulnerability factor	Total		White collar city		Blue collar city		P
	n	%	n	%	n	%	
Living in an Apartment (as opposed to own house)	1008	53.7	544	54.3	464	53.1	0.602
Father's highest level of education – elementary school	143	7.6	67	6.7	76	8.7	0.102
Mother's highest level of education – elementary school	101	5.4	37	3.7	64	7.3	0.001
Father unemployed or on sick leave the year before pregnancy	65	3.5	23	2.3	42	4.8	0.003
Mother unemployed or on sick leave during pregnancy	210	11.2	91	9.1	119	13.6	0.002
Both parents born abroad	49	2.6	29	2.9	20	2.3	0.412
Mother living alone	32	1.7	14	1.4	18	2.1	0.269
Serious life event during pregnancy*	177	9.4	90	9.0	87	10.0	0.472
Mother not feeling supported†	13	0.7	6	0.6	7	0.8	0.599
Mother not feeling safe‡	11	0.6	7	0.7	4	0.5	0.495
Mother worried over the possibility of child falling ill with serious disease§	650	34.6	326	32.5	324	37.1	0.039

* Phrasing of survey question: "Have you been exposed to something which you perceive as a serious life event during your pregnancy?"

† Phrasing of survey question: "Do you feel your surroundings give you the support you and your newborn child need?"

‡ Phrasing of survey question: "Do you feel safe and in the circumstances needed to give you and your newborn child a good start?"

§ Phrasing of survey question: "How do you usually feel when you consider the possibility that your child could fall ill with a chronic or serious disease in the future?". Stepwise likert-type scale 1-6 between not worried and very worried. Mothers answering 4-6 were classified as worried.

Table 9. Cumulative incidence (percent affected) of the 14 most common childhood diagnoses, recorded from birth to the age of 8-10 years, n=1870^a

ICD-10 code(s)	Description	Total		Gender		p	Social environment		p
		%	%	Female %	Male %		White-collar %	Blue-collar %	
J00-J06	Acute upper respiratory infections	81	78	83	0.007	79	83	0.018	
H65-H67	Otitis media	67	65	69	0.069	64	71	0.001	
S00-T98	Injury, poisoning and certain other consequences of external causes	57	52	62	0.001	56	59	0.120	
B34	Viral infections of unspecified site	28	28	29	0.675	25	32	0.001	
L00-L08	Infections of the skin and subcutaneous tissue	28	26	29	0.269	29	26	0.140	
J20-J22	Other acute lower respiratory infections	19	17	21	0.038	19	20	0.654	
H10	Conjunctivitis	18	19	18	0.791	20	17	0.131	
L20-L30	Dermatitis and eczema	17	19	16	0.125	18	17	0.596	
A00-A09	Intestinal infectious diseases	15	16	14	0.453	13	18	0.003	
N30, N34, N39,0	Urinary tract infections	10	16	4	0.001	11	9	0.103	
B00-B09	Viral infections characterized by skin and mucous lesions	10	9	10	0.404	9	10	0.730	
J12-J18	Pneumonia	10	9	10	0.318	8	12	0.002	
J45	Asthma	9	7	11	0.011	9	9	0.889	
L50	Urticaria	9	10	9	0.460	8	10	0.057	

^a Internal dropout of 6 participants because of missing data.

DISCUSSION

Main Findings

This thesis aimed at examining the relationships between psychosocial stress in early life, child health and cortisol in hair as a marker for long-term HPA axis activity.

As shown in Paper I, there appears to be a difference in the risk of diabetes-related autoimmunity among children living in two different psychosocial environments. Those living in a blue-collar city had high levels of autoantibodies more often than those living in a white-collar city.

In Paper II, the method of measuring cortisol concentrations in hair was developed, which seems to have the potential of becoming a new indicator of long-term HPA axis activity, identifying possible stress exposures with an association to major life stressors.

Paper III shows that, in children from one to eight years of age, cortisol levels in hair are correlated over time, but also associated to maternal hair cortisol levels during pregnancy. This suggests a heritable trait or environmental calibration to maternal HPA axis activity.

Paper IV demonstrates that children born into families fraught with living under disadvantageous psychosocial exposures seem to have increased cortisol levels in hair and are more likely to be affected by diseases common in childhood in a dose–response-like manner.

Interpretation of Findings

Early psychosocial exposures and health

Papers I and IV cover potential health outcomes among children in different psychosocial environments.

As shown in Paper I, there appears to be a difference in the risk of diabetes-related autoimmunity among children living in two different social environments, even after adjusting for other known risk factors for T1D.^{79,147-149} An important strength in this study was the ability to control a number of other diabetes-related potential confounding factors that had already been studied in the ABIS material. These results have, after Paper I was published, been confirmed at the age of three years, notably though with slightly less difference between the cities, which supports the notion that the differences found did not arise by chance.

A high-titer of an autoantibody may indicate that an autoimmune process has begun, possibly leading to T1D, and is used to identify subjects at risk of developing the disease. The majority of the children who were positive for one of the diabetes-related autoantibodies at one or three years of age will never develop T1D. Consequently, the results in this study imply that psychosocial stress was only associated with the induction of diabetes-related autoimmunity. Nevertheless, these findings support the stress hypothesis concerning the induction of diabetes-related autoantibodies as a possible hypothesis among others, and are in line with earlier studies of stress as a possible aetiological factor of T1D.^{16,78}

Some earlier studies have indeed shown a correlation between psychosocial factors and the risk of T1D, although findings concerning the socio-economic gradient are too inconsistent to allow firm conclusions,^{80,144,150,151} and the difference in public health between the studied cities is marked.^{137,139} But what in the social environment could trigger the induction of autoantibodies? There are many different modulating factors that may covariate in the autoimmune process leading to T1D and the aetiology is unknown.⁷⁷ One could speculate

that a blue-collar social environment characterized by more widespread economical problems, lower education and higher unemployment rates gives a higher general level of stress in the community. Stress in the close family as a result of a more socially deprived environment could then possibly affect the children's immune system. Although it has been shown that stressful life events precede the development of beta-cell autoantibodies and this could also be valid on the community level. Could it be relevant to introduce a concept like 'community stress' that represents an accumulation of stressful events and negative psychosocial conditions that affects the population in a whole community? Similar mechanisms could contribute to the results in Paper I, possibly shedding light on the unspecified so-called 'black box', introduced by Susser, in disease aetiology between social exposures and autoantibody levels.³³ Although these results and the difference in levels of autoantibodies between children in the two cities are intriguing, the findings tell us little about the aetiology of T1D and should be seen mainly as hypothesis generating. However, beta-cell autoimmunity in itself indicates that something is wrong with the immune system, since it attacks and destroys own healthy tissues. If someone develops a larger quantity of antibodies than 95% of all healthy children, it is definitely an abnormal reaction and something has happened with the immune system of that child.

In Paper IV, prenatal detrimental psychosocial exposures resulted in a corresponding increase in the risk of being diagnosed with 12 of the 14 most common childhood diseases, with a general dose-response-like pattern of rising ORs, statistically significant for the most vulnerable children. These results were in line with earlier findings on early stress and poor health in childhood that suggest a relationship between early detrimental psychosocial factors and disease risks. Diagnoses are not a measure of actual disease; however, in Sweden, children with parents of low socio-economic status (SES) are less likely to visit a physician,¹⁵² and such bias could therefore have made the association even weaker in the present study than it actually is. Most of the childhood diagnoses were common infection diseases, as we did not have enough statistical power to analyse less common childhood diseases. Some possible explanations to this risk difference could, for example, be that the number of siblings and day-care use might expose vulnerable families to

infectious pathogens to a greater extent. Then again, earlier research on adults by, for example, Cohen show immune cells being unable to respond to hormonal control as well as showing a susceptibility to the common cold among adults suffering from psychosocial stress.^{153,154}

Psychosocial factors and cortisol in hair

Papers II, III and IV all suggest that the measurement of cumulated concentration of cortisol in hair is associated to earlier stressful psychosocial circumstances.

Paper II showed that higher hair cortisol concentrations were found among those reporting that they had experienced serious life events during the last three months, somewhat surprisingly as the Perceived Stress Scale was not significantly associated to cortisol levels. This could be a result of the weakness in the validity of PSS to a time period of three months as mentioned earlier in the method section. A general question is how well the concordance is for humans between perceived stress and the actual individual activity of the HPA axis. Furthermore, there were two other findings in this paper that support the association between psychosocial stressors and levels of cortisol. 1) Four outliers had extremely high cortisol levels and two of them could be reached through open announcement. Although single case findings, both reported severe psychological problems during the last three months, which raises the question whether this new biomarker also may reflect severe psychological illness. 2) Also, when testing every single PSS-item, the question 'In the last month, how often have you felt difficulties were piling up so high that you could not overcome them?' was significant ($p=0.004$) against higher levels of cortisol with a tendency towards exposure–response relationship (not included in final Paper II), arguing for an association to coping with everyday hassles, which in earlier studies has been shown to correlate more to stress as well as health status problems such as depression.¹⁵⁵

In Paper III, an interesting finding relating to the variables reflecting social exposures was that children living in a villa compared to an apartment had lower hair cortisol levels, possibly mirroring differences in the continuous stress exposure of the social environment.²⁰ Variables describing the mother's psychosocial circumstances are interesting as they tend to point in the expected direction according to the hypothesis of influence of psychosocial factors on stress in general. However, the number of mothers in the affected groups was small, so these findings should be interpreted with caution and only for generating hypotheses. One mother experiencing *lack of support* during pregnancy, and the two *single mothers* had children with very high hair cortisol levels at year 1 (notably not the same mothers). Also, being a very young mother was associated with higher cortisol levels of the child. This is also the case with the three children starting day care before the age of one, who all had higher cortisol levels than the mean. This finding is supported by studies on cortisol in saliva, where cortisol levels among children at day-care centres rose compared to those being at home, possibly reflecting the everyday social challenges in peer groups.¹⁵⁶ In the current study, educational levels of the parents were not related to hair cortisol levels, in agreement with what Vaghri et al. recently showed.¹⁵⁷ Nor were parental smoking, foreign origin of the parents or maternal experiences of serious life events during pregnancy associated to higher hair cortisol levels. Cortisol concentrations in hair for the children intercorrelated over ages 1, 3, 5 and 8. Children's cortisol levels at ages 1 and 3 were also correlated to maternal cortisol levels during pregnancy. This suggests that the physiological stress response has a very early set point, possibly genetic, which affects the levels of cortisol output later in life. Yet another explanation could be environmental calibration to maternal cortisol levels. A genetic background for the stress response is plausible but not thoroughly investigated.^{158,159} More recent theories suggest that epigenetics could be involved in modulating the transgenerational effects of stress.¹⁶⁰ One could hypothesize that this association could be part of the transmission gap in the attachment theory, assisting in the development of the child's own behavioural and physiological regulatory systems e.g. the stress response,¹⁶¹ perhaps explaining some of the strong association between parental attachment and infant attachment.^{162,163} Or it could be a reflection of the quality of the maternal

care, known to have a great impact on lifelong stress reactivity via 'programming' of the neuroendocrine stress response in rodent offspring.^{164,165} Furthermore, cortisol levels at year 1 had high variability, with very high values and a wider interquartile range compared to those at 3 years, narrowing down with every subsequent measure at 5 and 8 years. This supports the theory that the stress response system seems to mature over time and is consistent with previous studies on cortisol in saliva and serum.^{100,101}

As shown in Paper IV, added prenatal detrimental psychosocial vulnerability was associated to increased levels of cortisol in hair in a dose-response-like manner. When adjusting for gender and inappropriate SGA, vulnerability was still solely and independently significant. ($B=0.40$, $p<0.0001$) This has, to our knowledge, never been shown before; supporting the theory behind the vulnerability construct and the allostatic load hypothesis stating that the accumulation of adversities matters and that a dysregulation of the physiological stress system is one plausible mechanism in how psychosocial exposures affect health. Supporting these results is a study on patients with PTSD, where levels of cortisol in hair and the number of lifetime traumatic events seemed to have a dose-response relationship.¹²⁸ Even though the vulnerability construct could be developed further, including more validated variables at different levels of operation, as well as improving the method for measuring cortisol in hair, there is a probability that some of the individuals actually could have an attenuated stress response. There is recent evidence that exposure to intense stimuli could in some cases dampen HPA axis reactivity, and inconsistent reports of socio-economic status (SES).¹⁶⁶⁻¹⁶⁸ However, this has been shown among adults and again with methods measuring cortisol levels in saliva and blood, which makes comparison to cortisol in hair difficult. One could speculate that a developing child has a more reactive stress response compared to an adult (as shown in Paper III), due to being less mature and having experienced less stress. But then there are extreme cases, such as traumatized apathetic refugee children, that seem to have a blunted stress response.¹⁶⁹ Furthermore, two out of the eleven single psychosocial items were significantly correlated to elevated cortisol levels of the child (*mother unemployed or on sick leave during pregnancy* and *living in an apartment compared to a house*). In eight of the remaining nine items, mean cortisol levels were higher in

the exposed group, although not significantly so. These findings further support the possible association between psychosocial factors and heightened HPA axis activity, thus indicating that cortisol in hair mirrors the continuous stress load in the daily social environment.

The widespread positive correlation of the different psychosocial factors to cortisol in hair studied in this thesis, as well as the dose–response-like effect, suggest that there is an association between the accumulative nature of adverse psychosocial factors and increased HPA axis activity measured through hair cortisol concentrations, at least at group level. Supporting this is research published in recent years in this expanding field of interest. As an example, shift workers have a higher risk of cardiovascular disease,¹⁷⁰ and indeed, there seem to be elevated levels of cortisol in hair among shift workers.¹²⁰ Other studies have found association with, for example, unemployment and parental income.^{121,123} Psychosocial factors like SES are powerful predictors of adult cardiovascular morbidity¹⁷¹ – interestingly, some studies have found an association between cortisol levels in hair and obesity, diabetes type 2, level of heart failure as well myocardial infarction.¹³⁰⁻¹³⁴ Therefore, given the prolonged nature of the exposure to a stressful psychosocial environment and its detrimental effects on health, the novel biomarker of cortisol in hair could prove to be of major importance in this area of research.

In contrast, studies focusing on the individual's perception of stress or self-reported stress-related measures have mostly found none or diverging associations. The lack of covariance could possibly be explained by lag effects caused by different time exposures of the retrospective assessment, in Paper II illustrated by the fact that the perceived stress scale is validated only for the preceding four weeks while measuring cortisol levels representing the previous couple of months, as well as problems with recall bias.¹⁷² This could be part of the explanation as to why Papers II and III also showed diverging results concerning the variable *serious life events*, where there was a significant correlation in Paper II based on 99 studied students, but no such significant association was found when analysing the 100 pregnant mothers in Paper III. Although a small study, one interesting report is on the positive association of effort–reward imbalance among 39 female day-care teachers ($r = 0.33$, $p =$

0.006) to cortisol levels in hair, but no association with either the effort or the reward scale separately.¹⁷³ It seems that more research is needed to find or develop appropriate tools to assess perceived stress, protective factors, personality aspects and the psychological effects on the long-term assessment of the physiological stress response that cortisol in hair seems to constitute.

Study Limitations and Methodological Considerations

Design and analysis

Three out of four studies in this thesis were based on the ABIS study, with the general strength of a true prospective design and the relatively high initial participation rate (79%). The sample was representative of Sweden concerning the parents' education and origin at the time (Statistics Sweden, 1999), but it is uncertain whether it has changed over time. A strength that lies in the cohort design is that the maternal responses are unbiased concerning the different dependent variables, the mothers had no knowledge of the outcomes, such as autoantibodies, hair cortisol concentrations and later diagnoses for their child. Furthermore, the large sample size requires one to be cautious when interpreting the results, as small but significant differences may not always be clinically relevant. As such, although there is a very strong scientific advantage that lies within a prospective framework and high participant rate, it does not necessarily implicate that all correlations found are causal in nature and clinically significant. To be humble, correlations should mainly be considered as associations.

A general limitation for prospective studies is the tendency of diminishing participation rates over time. The dropout rates increase for each new follow-up. This is also evident in the ABIS study, although the follow-up rate of 67% (completed questionnaires) at 1 year must be considered acceptable. However, as shown in Paper IV, we have linked data of health-care utilization and ICD-diagnosis from a health-care register to fill up the prospective dropout gap.

There is some risk in prospective studies of possible bias due to social selection against the background of diminishing participating rates over time. The responses received might be the opinions of a motivated section of the sample, possibly giving an underrepresentation of individuals with lower SES. But it could also be that those with problems are more motivated to participate. In general, the least and most advantageous social groups tend not to participate in studies.¹⁷⁴

How to handle outliers is an intriguing question. If outliers reflect measurement errors they should be omitted from further analysis. However, the procedure in our laboratory is that if outliers are detected, their biological samples are replicated and analysed at two independent occasions to ensure no measurement error had occurred. The participants with extremely high levels of cortisol in hair i.e. outliers, were handled in two different ways in the papers, which calls for an explanation. In Paper II, where adults were tested for cortisol concentrations in hair, we found four outliers, which were excluded from the statistical analysis, but two of them were possible to follow-up by an interview. This follow-up with an interview revealed important case findings. In Papers III and IV, when cortisol levels in children were measured, we also found outliers. These were included in the statistical analysis after they were log-transformed. We found that the biological variability seemed to be greater in young children especially during their first year of life, which was also reported in earlier reports on cortisol levels in blood and saliva. Possibly the regulation of their cortisol response is not sufficiently calibrated at a very young age. The outliers of cortisol in hair included in Papers III and IV were not considered as measurement errors. Not including them would therefore have risked introducing selection bias into the novel sample of cortisol in hair, and were consequently included, although all values were log-transformed in the statistical analysis.

In Paper IV, only participants who had completed answers on each of the 11 items in the composite vulnerability scale were included. There is a possible underrepresentation of individuals in the higher categories of vulnerability i.e. the distribution is not so even. Possibly this is reflecting the actual situation, with the risk of being affected by many of these single vulnerability factors

being quite rare in the population. Paper II is not based on the ABIS material, but deals with students around 20 years of age. They do not represent the general population but rather a selective group of young students, which could be seen as a possible limitation of this study. However, one the purpose of Paper II was initially to test the novel biomarker cortisol in hair and its application together with questionnaires.

Psychosocial measures

The variables used to measure psychosocial exposures in Papers I, III and IV were restricted to the variables already collected in the ABIS questionnaires. These variables are obviously different in nature and represent a variety of psychological, social and environmental dimensions. One could argue that there is a limitation in the measures used as the parents answered the questions rather than the children. Investigating the role of early stress in the development of disease should, if possible, be measured within the same individual. However, besides the obvious ethical, scientific and practical problems of asking small children about their perceived stress, we believe that the parents' answers reflect quite well the psychosocial situation and social environment in which the children live their lives.¹⁸

The psychosocial variables used are all indicators of a child at psychosocial risk, but the interpretations of associations do not indicate a specific causal relationship. As an example in Paper I, what factors might have caused the difference in autoantibody distribution between children from the blue- and the white-collar city? The twin cities variable is a crude indicator, and a variety of methodological problems could be raised, for example the risk of ecological fallacy. Nevertheless, this variable has been shown in other studies to be a fairly good indicator of the social environment.^{137,138} Another example is the reports of experiences of unspecified serious life events that were measured with a dichotomous yes/no-question. The advantage is that it is a subjective measure where the mother decides whether the event was stressful or not. But, in reality, the perception of how stressful the impact of a life event actually was could differ between mother and child.

In Paper IV, a scale of psychosocial vulnerability was used. The calculation of this score was not based on any weighing of single variables; the presence of one single vulnerable variable gave simply one point on the score. Even though the items used were crude, the hypothesis was articulated a priori and the general direction of the outcome suggests that the results cannot be explained by chance. In using a composite scale, there is a risk of obscuring associations between individual items that could be seen as confounders. On the other hand, a composite scale could take interactions and potentiating effects into account.⁴⁶ We decided to only include participants with completed answers on each one of the 11 items in our composite vulnerability variable. This reduced the effective response rate to 57%. Looking at the whole TwinABIS sample of N=2447 and the 11 items making up the composite variable, individuals belonging to the vulnerable category in 9 of the items were more likely to have missing answers on other items than those in the not vulnerable category. Thus, our final sample of N=1876 doesn't mirror the true vulnerability status in the population. There is probably an under-representation of individuals in the higher categories of vulnerability and observed associations might have been even more pronounced if these children could have been included. In this study, the consistency of findings, the dose-response-like relationships to disease risk and cortisol levels, as well as no single item being seen as driving the associations, support the theoretical model of vulnerability – that the accumulation of adversities matters.

Paper II is not based on the ABIS questionnaires. The purpose of the independent measures was in this case to scan possible relations between perceived stress and health as well as serious life events with levels of cortisol in hair. The effects of serious life events on physical and mental health have a long research tradition from the early reports by Holmes and Rahe.²⁸ This was criticized on methodological grounds concerning the usefulness of generalized magnitude ratings of life events. Later this quite simple measurement, also used in this report, was more widely used.¹⁷⁵ The PSS used has been validated for a recall period of four weeks, but was used to assess three months of stress in the current study, which is a limitation. Perhaps it would have been better to collect PSS levels at three consecutive months to better reflect the same time interval as the measurement of cortisol levels in hair. Furthermore, there is

always a risk for recall biases for retrospective self-reports. Possibly, this is more conceivable for reports of perceived stress i.e. changes of emotions, behaviour etc.¹⁷⁶

Cortisol in hair

Cortisol in hair is a novel marker of HPA axis activity over time, and as such there is a set of natural features associated with the method. A straightforward restraint is that it is impossible to measure differences in short time interval reactions of an actual stress response to an outer stressor, for that purpose measurement of cortisol in, for example, saliva is preferable. Measurement of cortisol in hair, on the other hand, represents the cumulative concentration of cortisol measured over periods of months or weeks at least. Another obvious restraint is that not everyone has the amount of scalp hair required to measure cortisol levels, ruling out, for example, very thin-haired children as well as bald adults.

There are several possible confounders that should be considered when analysing cortisol in hair. External contamination through, for example, cortisone-containing creams could be a potential confounder. In our studies the ABIS question *During pregnancy, did you take any medicine? Cortisone (yes/no)* did not alter the results. In Paper II there was no correlation to dyed hair, supported by other studies.^{94,111,119} Bleached or permed hair seemingly shows no effect.¹³² There are also other characteristics that are insufficiently studied, e.g. the effect of gender, race and age.^{110,111,177,178} Other possible limitations of the method are variations in cortisol levels related to cultural differences, coping styles and personalities.^{179,180} Furthermore, we still do not fully understand in what way cortisol becomes incorporated into hair, there are even suggestions that the root of the hair strand could actually produce cortisol, even though the quantities seem to be very small.^{181,182} Contradicting this are a couple of studies on, for example, patients with hypogonadism in which hair testosterone levels follow the pattern of systemic levels and not of ACTH alterations that supposedly would have been the case if ACTH drives the follicular production.¹⁸³ Nevertheless, research so far shows that measuring

cortisol in hair seems to have a general robustness and stability that speak for its usefulness in further research.¹¹³ However, some caution is also warranted when interpreting individual cortisol concentrations, despite the fact that the extracts from hair reflect cortisol built into the hair over months. We cannot tell where in a cycle of periods of high- or low-stress exposures an individual is during the time period hair was collected for measuring cortisol concentrations. Cross-sectional measurements of cortisol in hair should therefore, for the time being, mainly be used for group comparisons.

Immunoassay analyses are commonly used for analysis of salivary cortisol as they are relatively easily conducted, are affordable and have become popular when measuring concentrations of cortisol in hair, including the current method. One difficulty is the use of different analysis methodologies by different laboratories. A critical examination of the different enzyme-linked immunosorbent assay (ELISA)-based methods used has begun, as a step towards standardization and uniform interpretation.¹⁸⁴ Another problem is that the methodology, regardless of the method used, still lacks direct validation research and sufficient methodological rigour.¹¹³

A particular strength of the current method in this thesis is the ability to reliably analyse cortisol concentrations in samples as minute as 3–5 mg of hair. In comparison, ELISA-based assays have methodologically refined over several years with samples ranging from 100 mg to today where at least 10 mg is required. Mass spectrometric identification is still regarded as the gold standard in forensic hair analysis,¹⁸⁵ but for this a sample size of about 25 mg is required (Robert Kronstrand 2013. Linköping University, IMH, Division of Drug Research. Personal communication).

General Conclusions

Children born into an environment fraught with adverse psychosocial exposures seem to have increased long-term HPA axis activity, which also appears to be persistent throughout early childhood. These children also seem to be more likely to be affected by common childhood diseases. This, together with the widespread and cumulative effect of adverse exposures seen on the different outcomes, support the model of physiologic dysregulation as a plausible pathway of how the duration and number of early detrimental psychosocial exposures act as a trajectory to health disparities. It also suggests that the multiplicity of early adversities is of importance and could be of help in selecting preventive methods at group level.

Moreover, the novel biomarker of cortisol in hair has the potential of becoming a new indicator for retrospective HPA axis activity over periods of months, possibly assessing the biological response to the broader context of an exposure to a stressful psychosocial environment. As such, it provides the possibility to further develop the method, and cortisol in hair could prove to be a very useful aid in the studies of effects of stress on health. It may be especially useful in research among children, with the obvious advantage of easy sample collection, small sample size, and being non-invasive, as well as being non-dependent on stress levels at sample collection.

Implications for Further Research

An important result in this thesis is the links between mother and child hair cortisol levels in Paper III. What causes this association? An obvious answer would be environmental calibration or genetic heritability. But there are also some reports on possible epigenetic mechanisms related to stress, e.g. evidence on increased HPA axis activity and epigenetic programming in the male offspring exposed to stress early in the prenatal period,¹⁸⁶ as well as in studies on mice where chronic stress in male mice results in altered miRNA content of a sperm in association with reduced HPA axis responsiveness in the pup.¹⁸⁷ Hence, it could be that life-course epidemiology should also include exposures from not only prenatal life, but also from previous generations.¹⁸⁸ Cortisol in hair as a biomarker of chronic stress could provide an invaluable opportunity for further investigation of the transgenerational mechanisms of epigenetics on the effects on physiological stress activity and stress-related health.

Randomized intervention trials aimed at reducing stress in early childhood by, for example, family intervention programmes at the well-baby clinics with follow-ups, could give leads concerning stress-related health outcomes. This kind of study could simultaneously be aimed at investigating whether the effect of early stress on cortisol regulation is reversible with an intervention, possibly improving the regulation of the biological systems in children.¹⁸⁹

A thorough investigation of children that have very high levels of cortisol in hair as well those with very low levels is warranted. This could provide leads in trying to understand which protective, salutogenic factors in the *stress-resilient child* (in Swedish 'maskrosbarn') promote a normal stress reaction and in the long run well-being and health. Furthermore, one could gain information on what kind of circumstances may cause a blunt stress response.

The psychosocial vulnerability scale used in this thesis is promising, but needs to be elaborated further, possibly leading to a standardized scale, and could, in clinical and preventive child health settings prove useful.

Immunoassay analyses of hair cortisol concentration need further validation in general, and current RIA method used in particular. This could be done, for example against mass spectrometry, but also against other sources of known levels of cortisol concentrations to affirm reflective cortisol secretion over extended periods of time. Moreover, other areas of interest could concern possible confounding influences of, for example, environmental exposures and use of pharmaceuticals.

Research possible within the ABIS-study

An outline of possible future research within the existing ABIS-study could be the following:

- Cortisol levels in hair and psychosocial vulnerability among children that have developed type 1 diabetes.
- General follows-ups on the TwinABIS subsample concerning psychosocial factors, cortisol levels and health outcome.
- Further development of the cortisol in hair method towards smaller hair samples so as to be able to measure even smaller already collected hair samples in the ABIS bio bank.
- Investigation of whether prenatal vulnerability has a stand-alone effect on HPA axis activity or whether it simply mirrors future vulnerability by examining psychosocial circumstances in the follow-ups.
- Case-control studies on specific sub-groups of children, e.g. those that at an early age had extreme levels of cortisol in hair or high vulnerability.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Den tidiga psykosociala miljön som barn växer upp i har visat sig vara viktig för hur hälsan utvecklas under barndomen men även långt upp i vuxen ålder. En möjlig förklarande biologisk mekanism mellan den psykosociala miljön och senare ohälsa kan vara att fysiologiska reaktionsmönster i kroppen, exempelvis det komplexa samspelet mellan hjärna, hormoner och immunsystem, förändras när man utsätts för tidig stress vilket påverkar kroppens förmåga att hantera sjukdomar. Människors stressnivåer mäts ofta genom att analysera nivåerna av stresshormonet kortisol i exempelvis saliv och blod, men dessa metoder mäter bara hur stressad individen är i själva mät-ögonblicket. Nu finns en ny metod där man kan mäta kortisol i människors hår som till skillnad mot tidigare metoder återspeglar stressnivåerna över längre perioder, som månader, och även bakåt i tiden, ungefär som årsringarna på ett träd.

Syftet med denna avhandling var att undersöka om det fanns några samband mellan tidig utsatthet för psykosocial stress och barnets senare hälsa, samt om detta påverkar barnets stressnivåer över längre tidsperioder under uppväxten, genom att mäta nivåer av kortisol i hår.

Delstudierna i den här avhandlingen bygger till stor del på ABIS-studien (alla barn i sydöstra Sverige), som är en stor långsiktig studie där man följer omkring 17 000 barn från födsel och framåt under uppväxten. En första studie visade att det fanns stora skillnader i förekomst av autoantikroppar relaterade till typ 1 diabetes mellan två socialt sett helt skilda städer, en gammal arbetarstad och en tjänstemannastad. Det skulle kunna tyda på att det fanns skillnader i stressnivåer för befolkningen i de båda städerna, man kanske skulle kunna benämna det som samhällsstress? Men vad är det som egentligen orsakar sådana skillnader? Vi utvecklade metoden att mäta kortisol i hår och testade den genom att göra en studie på studenter. Den visade att en del stressrelaterade faktorer och händelser som kunde uppfattas som stressfyllda

faktiskt var kopplade till nivåerna av kortisol i hår. Med denna upptäckt började vi sen undersöka om man kunde använda metoden på barn. Det visade sig att barnens nivåer av kortisol i hår verkar hänga ihop över hela den tidiga barndomen. Spädbarn med höga stressvärden verkade ha fortsatt höga värden under flera år framåt. Barnens nivåer var även kopplade till mammans kortisolnivåer, trots att de är helt olika biologiska varelser. Vi fann också att de barn som tidigt i livet var utsatta för olika typer av psykosocial stress hade en ökad risk att under uppväxten drabbas av nästan alla de vanliga sjukdomarna som barn drabbas av upp till 10 års ålder. Hos barn som tidigt var exponerade för olika former av psykosocial stress kunde vi också mäta högre nivåer av kortisol i hår, ökad nivå av stress gav motsvarande höjning av kortisolnivåer i hår.

De viktigaste fynden i den här avhandlingen stödjer alltså tidigare teorier om att barns fysiologiska stressrespons påverkas av tidig stress och att det påverkar barnets hälsa senare i livet. Den metod vi vidareutvecklat i våra studier som gäller att mäta kortisol i hår verkar vara en mycket användbar metod för att mäta stress i olika psykosociala miljöer, och passar kanske extra bra för forskning om stress på just barn.

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