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EARLY INDICATORS FOR ADVERSE DEVELOPMENT OF CARDIOVASCULAR, RENAL AND METABOLIC FUNCTION IN CHILDREN BORN WITH LOW BIRTH WEIGHT

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Karolinska Institutet

Stockholm 2018

Published by Karolinska Institutet.
Printed by E-Print AB 2018

ISBN 978-91-7831-179-8

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To Zsuzsanna and Nicolas

ABSTRACT

Prematurity affects more than 10% of infants worldwide and is the main reason for neonatal mortality. Improvements in neonatal care have led to higher survival rates into adulthood. Adverse events during organogenesis and development, intra-or extrauterine, can increase the risk for chronic disease later in life. Developmental origins of health and disease is the epidemiologic research field linking early life events to related clinical phenotypes.

In this thesis, we present 4 studies designed to follow up consequences of prematurity or low birth weight at term compared to term controls with normal birth weight in two different cohorts. The first cohort of children, studied at a mean age of 9.7 and again at 12.6 years (studies I-III), were born either very preterm (<32 weeks gestational age) or at term but small for gestational age. We studied kidney volume and function, the autonomous nervous system using heart rate variability and identified markers for insulin resistance. The second cohort of children, studied at a mean age of 7.7 years (study IV), were born extremely preterm (<28 weeks gestational age). We measured kidney volume and function and divided the group into those who developed and those who did not developed nephrocalcinosis during the neonatal period. We also studied blood pressure at the time of their visit, including 24-h ambulatory blood pressure measurements.

Kidney volume or function was not significantly different between the three groups in study I. In study IV we found that children born extremely premature had smaller kidneys then children born at term, in particular the right sided kidney volume was significantly smaller compared to controls. Preterm born girls had smaller kidneys than full-term born girls (controls) but preterm born boys were not different to controls. Among preterm born children without nephrocalcinosis girls, had smaller kidney volumes than boys. Kidney function was normal and not affected by kidney volume.

Paper II showed signs for insulin resistance in very preterm born children and children born small for gestational age. Preterm born children presented signs for hepatic insulin resistance while small for gestational age born children had a decreased peripheral insulin sensitivity.

Both, very preterm and full-term small for gestational age born children had a generalized depression of heart rate variability compared to controls indicating an impaired function of the autonomous nervous system (study III). Office blood pressure as well as 24-hour ambulatory blood pressure were in the normal range for children born very or extremely preterm as well as for children born small for gestational age at term. Circadian blood pressure regulation was adversely affected in 50% of children born extremely preterm illustrated by the absence of normal day-to-night dipping in 24-hour ambulatory blood pressure measurements (study IV).

In conclusion, children born preterm or full-term but small for gestational age showed several morphological or functional changes at early school age. The detected changes are indicating a possible development towards impaired kidney function, hypertension and the metabolic syndrome.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following publications and manuscript which will be referred to by their Roman numerals (I-IV)

I. Alexander Rakow, Stefan Johansson, Lena Legnevall, Robin Sevastik, Gianni Celci, Mikael Norman, Mireille Vanpée.
Renal volume and function in school-age children born preterm or small for gestational age.
Pediatric Nephrology 2008; 23:1309-1315

II. Anna Kistner, Alexander Rakow*, Lena Legnevall, Giovanna Marchini, Kerstin Brismar, Kerstin Hall, Mireille Vanpée.
(*First and second author have contributed equally.)
Differences in insulin resistance markers between children born small for gestational age or born preterm appropriate for gestational age.
Acta Paediatrica 2012; 101:1217-1224

III. Alexander Rakow, Miriam Katz-Salamon, Mats Ericson, Ann Edner, Mireille Vanpée.
Decreased heart rate variability in children born with low birth weight.
Pediatric Research 2013; 74:339-343

IV. Alexander Rakow, Åsa Laestadius, Ulrika Liliemark, Magnus Backheden, Lena Legnevall, Sylvie Kaiser, Mireille Vanpée.
Nephrocalcinosis in extremely preterm born infants as a risk factor for impaired renal size and function at early school age.
Manuscript

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LIST OF ABBREVIATIONS

ABPM	Ambulatory Blood Pressure Monitoring
AGA	Appropriate for Gestational Age
ANS	Autonomic Nervous System
ASD	Autism Spectrum Disease
BMI	Body Mass Index
BPD	Bronchopulmonary Dysplasia
BSA	Body Surface Area
CAPA	Caucasian Asian Pediatric Adult
DM	Diabetes Mellitus
DOHaD	Developmental Origins of Health and Disease
EPT	Extremely Preterm
EUGR	Extrauterine Growth Restriction
GA	Gestational Age
eGFR	Estimated Glomerular Filtration Rate
HbA1c	Hemoglobin A1c
HOMA-IR	Homoeostasis Model Assessment Insulin Resistance
HRV	Heart Rate Variability
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein
IR	Insulin Resistance
IUGR	Intrauterine Growth Restriction
LBW	Low Birth Weight
MetS	Metabolic Syndrome
RDS	Respiratory Distress Syndrome
SGA	Small for Gestational Age
VLBW	Very Low Birth Weight
WBISI	Whole Body Insulin Sensitivity Index

1 INTRODUCTION

An association between an environmental event very early in life (before or around birth) and an increment in disease susceptibility at a certain point or period later in life is the main paradigm of developmental origins of health and disease (DOHaD)[1].The DOHaD research field is a rather young area of research. Initiated by British and Scandinavian researchers already in the 1930's but then pioneered and brought to scientific acknowledgement by David Barker in the 1980's[2-4]. DOHaD started with the discovery of an association between undernutrition during fetal development (maternal undernutrition) and the development of cardiovascular diseases and diabetes later in life[2,5,6]. But since than DOHaD research has expanded and found associations between adverse events occurring during a sensitive developmental phase early in life or before birth and outcomes or disease like asthma and allergy, immune and autoimmune diseases, cancer, depression, psychiatric, neurodevelopmental disorders and of course obesity, diabetes, hypertension and cardiovascular diseases[7-11]. The mechanisms behind the environmental effects on development and the clinical phenotype are largely explained by epigenetics which describes molecular mechanisms affecting gene expression patterns without alterations in DNA base sequence[6]. Epigenetic modification can happen via different mechanisms but the best studied so far are DNA methylation and histone modification[12].

Most of the research in this field was initially focusing on children born with low birth weight (LBW) in general assuming that the vulnerable period for development is limited to the fetal live. That was also reflected by the terminology and definition at that time where the research field was called "fetal" programming and later "fetal" origins of adult disease until first 2002 the official name DOHaD was established by the main society. It was than accepted that the concept of DOHaD includes a huge span of environmental factors acting during different phases of developmental and potentially increasing the risk for later chronic diseases[1,6]. For the above reasons not much emphasis was put on the differentiation of the etiology of LBW, whether prematurity or IUGR being the cause. A distinction between these two different perinatal exposures seems reasonable as they display different etiologies. However, so far it appears that most of the long-term consequences are remarkably similar despite the different perinatal conditions (IUGR vs prematurity). The latency between the environmental adverse event and the possible subsequent manifestation of sequels is obviously very long which may complicate the appreciation of the relationship and delays diagnostics but also preventive and therapeutic measures. Development of early biomarkers as well as uncovering of differences in pathomechanisms and improvement of understanding of common trajectories for later disease risk is essential.

With the research presented in this thesis we try to contribute to more detailed and specific clinically relevant evidence for this high-risk group and possibly improve knowledge and thereby open up for better and earlier prevention and management.

2 BACKGROUND

2.1 PREMATURITY

Gestational age describes the duration of gestation starting from the first day of the last menstruation and ending at birth and is expressed in completed weeks and days. By definition all infants being delivered prior to 37 weeks of gestation are premature. Prematurity can be sub divided into late preterm (32-36+6) very preterm (28-31+6) and extremely preterm (EPT, below 28 weeks of gestation). Postterm is defined as delivery after 42 weeks of gestation[13]. Worldwide the rate of prematurity is around 15 million infants per year with the lowest rates in northern Europe (~5%) and the highest in sub-Saharan Africa (Malawi, 18%). Around 60% of all prematurity occurs in Asia and sub-Saharan Africa[14]. A combination of decline in stillbirth paralleled by lowering the threshold for preterm cesarean section delivery as well as an increase in late preterm birth has led to a rise in preterm birth in high-income countries[15-17]. According to the recent numbers of the Swedish National Quality register and the Swedish Board of Health and Welfare (SNQ, Socialstyrelsen) rates are 5.6% for infants born prior to completion of 37 weeks of gestation, 1% for very preterm born (< 32weeks GA) and 0.3-0.4% for extremely preterm born children (< 28 weeks of GA) for the 120 000 children born per year[18,19].

2.1.1 Etiology of prematurity

Despite a huge variety of accepted risk factors for spontaneous prematurity, no clear cause can be identified in the majority of cases[20]. Infection and inflammation, social stress and race and genetics can be used as the main pillars to explain spontaneous preterm birth[21]. Race, maternal education and maternal age as well as smoking during pregnancy or severe overweight or underweight increase the risk for preterm birth or low birth weight significantly[22]. Infections have been suspected as a cause for preterm birth for a long time and even though they are in most cases not clinically apparent they may contribute to as much as 25% of prematurity[23,24]. Maternal history of preterm delivery is a strong risk factor for recurrent preterm delivery which makes it likely that genetic factors or at least gene-environmental factors contribute to the timing of birth[25]. Also the mothers own prematurity increases the odds for delivering preterm[26]. Common maternal reasons for preterm delivery are preeclampsia, fetal distress and severe intrauterine growth retardation leading to medical induced preterm delivery. The variety in pathophysiological causes for prematurity results in each preterm born baby carrying his or her own individual profile of comorbidity.

2.1.2 Survival and Mortality

As the thresholds for viability were lowered over the last decades and the survival rates of extreme preterm born infants are improving the contribution of prematurity to mortality rates in children below five years consequently increased. Prematurity accounts for 35% of death among newborns and is globally with 17.9% the leading cause of deaths in children under the age of five years[27]. There is a great variance in survival and mortality rates not only between but also within countries. Infrastructural differences, attitudes towards decision making, quality of

care and socioeconomic circumstances may all lead to variations in mortality rates[28,29]. Child mortality in Sweden is among the lowest in the world (<5years: 2.9/1000)[30]. Also neonatal mortality in Sweden is rather low when compared internationally (SE: 1.5/1000; USA: 3.7/1000 ; UK: 2.6/1000) [18,30,31].

2.1.3 Short and long-term morbidity of prematurity

2.1.3.1 Respiratory distress syndrome

Inversely related to gestational age with the most premature being mainly affected is the respiratory distress syndrome (RDS). Immature lungs with a deficiency of endogenous surfactant lead to alveolar instability and collapse[32]. This is the main reason for invasive or non-invasive respiratory support in extremely preterm infants. Preventive and rescue strategies include antenatal steroids, exogenous surfactant application and continuous positive airway pressure.[33,34].

2.1.3.2 Bronchopulmonary dysplasia

BPD is a complication in postnatal lung development mainly due to extreme prematurity, baro- and volutrauma following mechanical ventilation and inflammation. It is defined by the need for supplemental oxygen after 36 weeks of postmenstrual age and can be further divided into mild, moderate and severe where the latter is defined by inspired oxygen > 30% or need for respiratory support [35,36]. The incidence of BPD increases with decreasing gestational age at birth. Higher survival rates of more extreme preterm born infants have led to an increase of what sometimes is called the "new BPD" [37]. in a recent survey the prevalence of BPD for infants born before 32 weeks of gestation in Sweden was 6%[38]. Mortality numbers for children suffering from severe BPD are between 10-20% during the first year of life. Pulmonary long-term complications are reactive airway diseases and increased risk for severe pneumonia and bronchiolitis. Neurodevelopmental delay and growth failure are commonly seen in children suffering from severe BPD[39,40].

2.1.3.3 Germinal matrix and intraventricular hemorrhage

The extreme premature infant is especially vulnerable to sudden changes in pressure and blood flow to the brain which may lead to bleedings often originating from a highly vascularized and cellular active area called the germinal matrix. These bleedings may extend into the ventricle system and potentially even into the parenchyma. Post hemorrhagic ventricle dilatation and hydrocephalus are potential complications. Severity of germinal matrix and intraventricular hemorrhage is most often graded by the Papile classification[41]. The prevalence of severe IVH (grade III+IV) is currently 3.4% in the Stockholm area for infants born before 32 weeks of gestation[38]. Neuro developmental outcome is strongly associated with intraventricular hemorrhage (IVH)[42,43].

2.1.3.4 Periventricular leukomalacia /White matter injury

Ischemia, hypoxia and inflammation to the brain are mainly affecting the periventricular white matter, predominantly in preterm newborns, resulting in persistent lesions which often are responsible for later cognitive, motor or sensory impairments[44,45].

2.1.3.5 Persistent ductus arteriosus

The ductus arteriosus is an essential vascular communication in utero. Postnatally the duct closes itself but remains often open in preterm infants. Shunt direction, volume and duration of patency determine the relevance of the persistent ductus arteriosus (PDA). Significant left-to-right shunting leads to pulmonary edema as well as to reduced systemic perfusion[46,47]. Opinions about when and if a PDA should be treated are varying significantly among institutions and clinicians. Treatment options can be pharmacological or surgical and interventional using transcatheter occlusion-technique[48].

2.1.3.6 Necrotizing enterocolitis

Necrotizing enterocolitis (NEC) is a multifactorial severe gastrointestinal complication, almost exclusive in preterm, resulting in severe inflammation and tissue necrosis with the risk for perforation of the gastrointestinal tract. The pathomechanisms involved are decreased intestinal blood flow, mucosal injury and bacterial invasion but the complete etiology is still poorly understood. In case of severe NEC (1.3% in Stockholm) extensive surgery might be needed potentially leading to a short bowel syndrome, a rare but significant complication of NEC with an incidence of around 20%[38,49]. The mortality from NEC is high (20-30%) particularly for those in need for surgery[50].

2.1.3.7 Retinopathy of prematurity

Retinopathy of prematurity (ROP) is the consequence of disturbed neurovascular development of the retina due to short gestational age and birth into a hyperoxic environment combined with inflammation. The abnormal neovascularization may lead to fibrosis, and ultimately retinal detachment resulting in blindness[51].

2.1.3.8 Neurodevelopmental delay

Neurodevelopmental outcome is closely related to the spectrum of complications described above but also to prematurity itself[50,52]. The EXPRESS study group reported some kind of disability in 58% of extreme preterm born children in Sweden where severity of disability is inversely proportional to increasing gestational age[53]. Between 5-10 % of extremely preterm born infants, discharged alive from the neonatal care, developed cerebral palsy[53].

2.1.3.9 Autism spectrum disorder

Autism spectrum disorders (ASD) can be described as a lifelong developmental disability mainly affecting social interaction, communication and often shows repetitive behavior. There is growing evidence that ASD is overrepresented in extreme preterm born children. In the EPT

born children it is suspected that ASD may be the consequence of abnormal brain development [54,55].

2.2 INTRA UTERINE GROWTH RETARDATION

Intra uterine growth restriction (IUGR) describes a process where fetal growth fails to achieve the genetically determined growth potential of the individual due to one or multiple pathological factors[56]. The incidence of IUGR lies between 5-15% of all pregnancies in Europe and in the United States but can be as high as 55% in low income countries[57].

2.3 SMALL FOR GESTATIONAL AGE

A newborn who weighs less than 2 standard deviations (SD) below the mean value for gestational age for a given population is described as small for gestational age (SGA). Alternatively, the World Health Organization (WHO) has chosen birthweight below the 10th percentile for identifying SGA[58,59]. Among children born preterm roughly 30% are also SGA[38]. Not all children born SGA have suffered from IUGR. The majority of children born SGA are constitutionally small and absolute healthy[60]. However, in this thesis and in studies I-III we mainly use the term SGA when referring to IUGR related LBW.

2.4 RENAL DEVELOPMENT

The development of the kidney is a complex process divided into three different phases where the first two phases (pronephros and mesonephros) are transient but leave their remains which are not only crucial for the further development of the kidneys but also play a role in the development of the genitals. The formation of the metanephros defines the final phase of renal development and starts at around 5 weeks of gestation and forms the permanent kidney[61]. The metanephros consists of the uretic bud epithelium as well as mesenchymal metanephric tissue components. The uretic bud epithelium differentiates into collecting duct and pelvis while the mesenchymal tissue develops into the proximal and distal loop as well as the glomerulus[62]. Nephrogenesis is a process of branching morphogenesis defining the final number of nephrons. In humans nephrogenesis is believed to continue up to 36 weeks of gestation leaving each individual with a finite nephron number endowment between 210 000 to 2.7 million nephrons[63] (figure 2). After that period kidney growth is mainly due to tubular growth and hypertrophy and if further nephrons are formed the number of abnormal glomeruli among those is high [64-68]. However, glomeruli and tubular function continues to develop during the first months of life in term and also preterm born children which makes them particularly vulnerable to nephrotoxic drugs, severe infections or impaired perfusion[69,70].

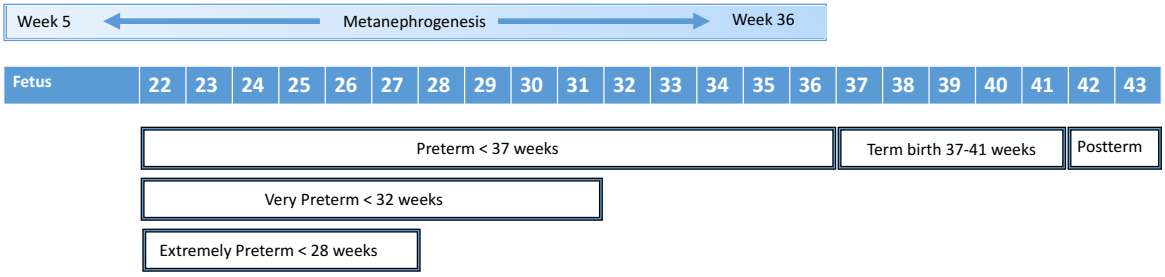


Figure 1. *Duration of nephrogenesis in relation to gestational age (Modified after Tucker and Singh et al.[13,71])*

2.5 KIDNEYS ROLE IN REGULATION OF ARTERIAL BLOOD PRESSURE

The kidneys have a role in regulating body fluid homeostasis and thereby a long-lasting effect on blood pressure. Pressure diuresis and pressure natriuresis as well as the renin- angiotensin-aldosteron system (RAAS) are slow but also fast acting mechanisms for blood pressure control. Reduced excretion capacity for sodium and water due to reduced filtration surface as seen in patients with nephron deficit can lead to hypertension[65,72-74]. Nephron number is reduced following low protein diet throughout pregnancy, utero placental insufficiency or preterm birth[67,75]. But apart from the "nephron number-Brenner hypothesis" other renal mechanisms can increase the risk for the development of hypertension.

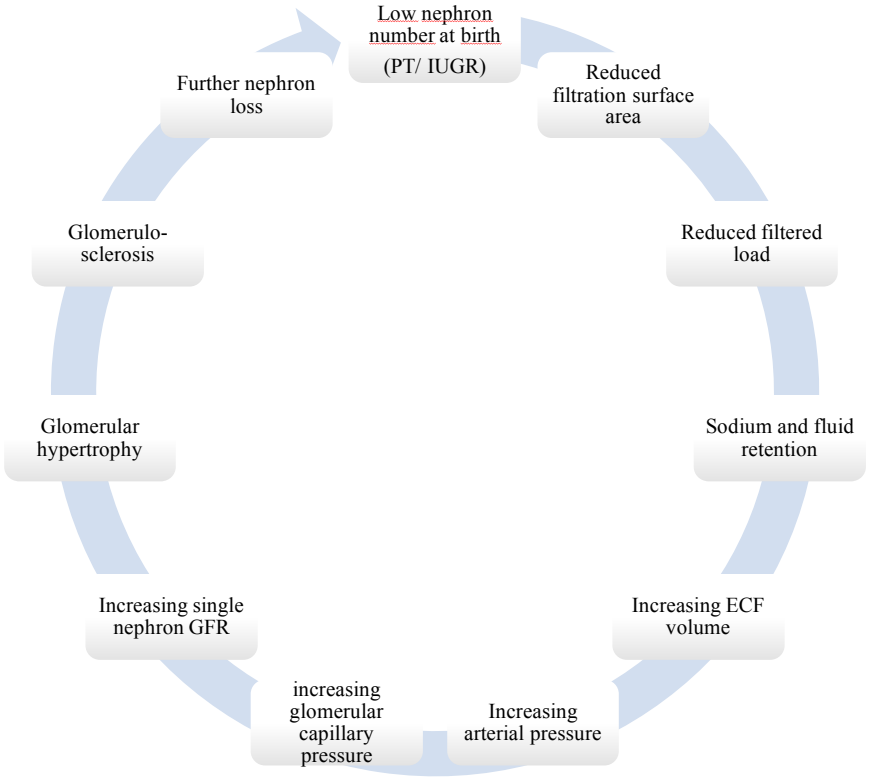


Figure 2. *Congenital nephron deficit and development of hypertension and renal diseases (adapted from Brenner et al.[72])*

Overactivity of the RAAS reflected by higher levels for angiotensin II, angiotensin converting enzyme and aldosterone have been described for children with low birth weight[76,77]. Vascular tone and small artery resistance regulations by the sympathetic nervous system are other possible contributing factors to renal hypertension and glomerular damage[78]. However, in the studies presented we focus on kidney volume which can be regarded as a proxy for nephron number[67] and can be measured with ultrasound technique[79-81]. Subjects with low nephron number are having a 70% increased risk for kidney disease[82] and due to the vicious cycle, described by Brenner et al., (Figure 2) a higher risk for the development of cardiovascular morbidities[65,72,73,83].

2.6 ELEVATED CHILDHOOD BLOOD PRESSURE

Hypertension affects about 20% of all adults and is the main risk factor for cardiovascular morbidity worldwide[84,85]. Unlike for the adult patient blood pressure has to be adjusted for age, sex and height in children. In children blood pressure below the 90th percentile for systolic and diastolic pressure is classified as "normal" whereas above the 90th but below the 95th percentile blood pressure is classified as "elevated normal" blood pressure or pre-hypertension and above the 95th percentile it is classified as hypertension[86,87]. Currently the prevalence of established hypertension in children is about 4% and for pre-hypertension it is 10%[88-90].It has been shown that childhood hypertension tracks into adulthood and is associated with an increased risk for atherosclerotic lesions leading to increased cardiovascular morbidity[91-97]. A raise of blood pressure in a young adult by only 5mmHg will increase the risk to die by stroke later in life with 34%[98].

2.7 NEPHROCALCINOSIS

Nephrocalcinosis (NC) is defined as the abnormal deposition of mainly calcium oxalate crystals into the renal parenchyma[99]. With an incidence varying between 7 and 41% among extremely preterm born infants (up to 64% in a single center[100]) NC is a rather common complication in this high risk group[101-104]. Immaturity of the kidneys with impaired excretory capacity and induced hypercalciuria by commonly used drugs during the neonatal period are suggested etiological explanations[105-107]. NC is reliably diagnosed with ultrasound technique[108,109]. Whether NC has an aggravating effect on kidney function in preterm born infants with possible reduced nephron number is currently under debate[104,110-113].

2.8 METABOLIC SYNDROME

The metabolic syndrome (MetS) can be described as a cluster of pathologies potentially leading to cardiovascular diseases like atherosclerosis, hypertension myocardial infraction as well as to diabetes. By definition the MetS is present if some (at least 3) or all of the following symptoms are established: Insulin resistance, hyperinsulinemia, central obesity, high blood pressure, dyslipidemia (high triglycerides, low HDL, high LDL), procoagulant state (high plasma fibrinogen and plasminogen activator inhibitor 1), vascular abnormalities (increased albumin excretion in urine, endothelial dysfunction) and hyperuricemia[114,115]. According to a recent survey more than a third of all US adults met the criteria for the metabolic syndrome[116].For non-diabetic adult Europeans the prevalence is around 15%[117]. Among children the rise of childhood

obesity had accompanied a significant increase in MetS as well. A study in obese children from five European countries using different definitions for the MetS showed a prevalence range between 16.4 to 35.7 % depending on the criteria used[118].

2.9 INSULIN RESISTANCE

Insulin resistance (IR) is defined as the reduced biological capability of insulin to inhibit hepatic glucose production and facilitate glucose disposal[119]. IR is thought to be the central pathomechanism towards the MetS[120]. Hyperglycemia, obesity followed by inflammation and increasing free fatty acids (FFA) along with dyslipidemia and impaired endothelial function are steps ultimately resulting in the MetS.[121,122]. Hyperinsulinemia (indicative of insulin resistance) can also lead to hypertension by increasing plasma volume through promoting sodium reabsorption, increasing sympathetic activity as well as the proliferation of vascular smooth muscle cells[123-125]. IR can be either peripheral (or whole body) or central (hepatic). The pathomechanism for peripheral IR is impaired glucose uptake and consumption in muscle and fat tissue while hepatic or central IR results in unrestricted glucose production in the liver[126,127].

2.10 ROLE OF INSULIN-LIKE GROWTH FACTOR-1AND ITS BINDING PROTEINS

Insulin-like growth factor-1 (IGF-1) is an amino acid single chain polypeptide with high similarity to insulin which is involved in growth, metabolism, differentiation and also angiogenesis[128,129]. IGF-1 is mainly produced by the liver due to growth hormone stimulation and applies its action via paracrine, autocrine and endocrine pathways[130]. Of the endocrine IGF-1 approximately 95-99% is bound to binding proteins (IGFBPs) of which IGFBP-3 is the main binding protein regulating the action of IGF-1. IGFBP-1 is regarded as an important marker for glucose homeostasis[131]. Low levels of IGFBP-1 correlate with increased risk for the metabolic syndrome and type-2 diabetes mellitus(T2DM)[132,133]. Very recent research even suggests that IGFBP-1 has regenerative effect on beta cells and thereby reduces the risk for T2DM[134,135]

2.11 NERVOUS CONTROL OF PERFUSION AND BLOOD PRESSURE

The autonomic nervous system (ANS) is a central regulator collecting afferent and efferent neurons that link and synchronize the central nervous system with visceral effectors[136]. The neural control of the circulatory system depends on the interaction of the two major components of the ANS, the sympathetic and parasympathetic arms of the ANS. The sympathetic branch of the ANS controls vascular tone, heart and kidneys and the adrenal medulla via barosensitive, thermosensitive and glucosensitive efferent neurons. The parasympathetic branch decreases heart frequency and contractility via fibers in the vagus nerves distributed mainly to the atria[137-139]. The barosensitive efferents of the sympathetic nervous system are responsible for short term blood pressure fluctuations by affecting resistance arterioles or noradrenalin release but they have also a long-term effect on blood pressure regulation by influencing renin secretion, renal tubular sodium reabsorption and renal blood flow [140,141].

2.12 HEART RATE VARIABILITY

ANS function can be assessed non-invasively by measuring heart rate variability (HRV). HRV represents the variation of time between consecutive heartbeats controlled by the two branches of the ANS and reflects the heart's ability to respond to different stimuli and circumstances. HRV has initially been use for observation and detection of fetuses in distress and led to the development of the cardiotocograph (CTG)[142].The measurement of HRV can be used as a tool for examining cardiovascular autonomic control but is also giving information about activity related to respiration and thermoregulation. Abnormalities in HRV can be regarded as a proxy for impaired ANS function and thereby a predictor for cardiac and metabolic dysfunction in patients with preceded cardiac diseases but even among individuals free from cardio-vascular disease[143-146]. The measurement and illustration of HRV can be done in different ways. Most frequently time domain and frequency domain measurements are used[147].

3 AIMS

The overall objective for this thesis is to identify early markers for an increased cardiovascular, renal and metabolic risk for children born with LBW.

- To investigate whether school children born with LBW have already impaired kidney volume and function (I+IV).
- To investigate whether nephrocalcinosis is associated with long term kidney health in extremely pretem born infants (IV).
- To evaluate early markers for insulin resistance (II).
- To investigate whether the autonomous nervous system is affected by prematurity or LBW (III).
- To investigate whether prematurity or LBW at term are equally associated with outcomes investigated in study I-III.
- To investigate if the above described suspected alterations have an effect on blood pressure (IV).

4 SUBJECTS AND METHODS

4.1 STUDY POPULATION STUDIES I-III

The retrospective follow-up studies I-III consists of children born between 1990-1993 who all belong to the same cohort. A total of 390 children born at the Karolinska University Hospital, Stockholm fulfilled the inclusion criteria and were eligible for the follow-up. Inclusion criteria were preterm birth before 32 weeks of GA, and birth at term but with a birth weight below the -2 SD according to the Swedish reference data for fetal growth[148]. Exclusion criteria were chromosomal anomalies, congenital infections, congenital anomalies of the urogenital tract or other life threatening congenital anomalies. Healthy children born at term with normal birth weight were selected from the delivery room records (Controls). One hundred thirty-three families were lost to follow up due to changed address. Of the remaining 257 contacted families 105 accepted to participate in the study.

The participating children were divided by their perinatal exposures. Thirty-nine children were born very preterm (<32 gestational weeks, Preterm), 29 children were born at term but were small for gestational age (SGA) and 37 children were born at term with appropriate weight for gestational age (Control). Children from the Preterm and SGA group were cared for at the neonatal intensive care unit (NICU) or maternity ward at the Karolinska University Hospital Stockholm, Sweden respectively. Children from the control group were healthy term born children matched for gender and date of birth, selected from the delivery room records from the same institution. Children not participating in the study were not different in their maternal, perinatal and neonatal characteristics. The children were initially investigated at an average age of 9.7 years and again at an average age of 12.6 years (Study I). Information about maternal anthropometrics, hypertension, diabetes, gestational diabetes, smoking habits, duration of lactation and any medical treatment or other significant disease during or before pregnancy were collected. Perinatal and neonatal information included antenatal steroids, mode of delivery, Apgar scores, birth anthropometrics and relevant information towards neonatal complications and severity of illness were collected.

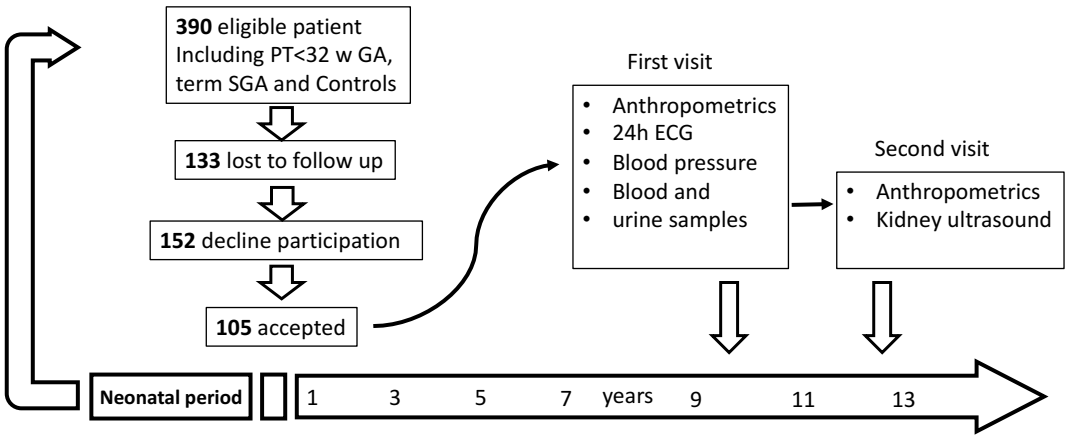


Figure 3. *Recruitment and study design (retrospective cohort study)*

Table 1. *Neonatal Characteristics for cohort 1 for the three different studies (I-III) for the three different groups: Children born preterm (<32 weeks of gestation) (Preterm), children born at term but with birth weight<-2 SD for gestational age (SGA), and children born at term with normal birthweight (Control).*

	Preterm			SGA			Control		
Study	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**
N	39	21	31	29	26	27	37	30	28
GA (w)	26.6* (2.0)	26.4* (1.7)	26.7* (2.1)	39.3 (1.4)	39.3 (1.2)	39.3 (1.4)	39.6 (1.0)	39.6 (1.0)	39.6 (1.0)
BW (g)	954* (203)	987.6* (217.7)	965* (202)	2436* (331)	2,467* (276.3)	2,441* (334)	3,485 (502)	3,557 (504)	3,503 (515)
BL (cm)	35.5* (2.9)	35.6* (3.4)	35.4* (2.9)	46.9* (2.4)	46.7* (2.1)	46.8* (2.3)	50.0 (2.2)	50.2 (2.2)	49.7 (2.2)
Girls (%)	56	42	48.4	55	57	59.3	65	63	64.3

*Values presented as means with (SD). Statistics were done with one-way Anova for comparison of all three groups. * denotes significant difference in comparison to control (P values <0.05). N: numbers, GA: Gestational age, w: Weeks, BW: Birth weight, g: grams, BL: Birth length.*

4.1.1 Methods Studies I-III

All anthropometric measurements were performed by the same research nurse. Length, weight, head circumference, sitting height and abdominal circumference were measured. Children were wearing light indoor clothing for weight measurements. A wall mounted stadiometer was used for height measurements. Abdominal circumference was measured midway between the lower rib margin and the iliac crest using a normal measuring tape (only in 43 subjects (Preterm :7, SGA: 20, Control:16). Sex specific body mass index (BMI, kg/m^2) was calculated. Body surface area was estimated by using the equation from Haycock[149].

4.1.1.1 Blood pressure recordings

Office. blood pressure measurements were performed at a resting state after at least one-hour acclimatization to the environment. At least 3 measurements were taken using an automated , non-invasive oscillometric technique (Dinamap™, Criticon Inc., Tampa, Florida, USA)[150] with appropriate cuff size for age and size around the upper right arm. An average out of the three measurements was calculated for systolic, diastolic and mean arterial pressure.

4.1.1.2 Blood and urine sampling

Blood samples were obtained from 84 of the children (28 Preterm, 23 SGA and 33 Control) after placing a topical anesthetic cream containing 2.5% lidocaine and 2.5% prilocaine

(EMLA®; AstraZeneca, Sodertälje, Sweden). All blood and urine samples were analyzed at the Department of Clinical Pharmacology and Division of Clinical Chemistry at the Karolinska University Hospital Laboratory, Stockholm, Sweden.

Blood and urine samples collected for study I were serum creatinine analyzed using the Jaffe method (Beckman Coulters instruments LX, Fullerton California, USA), serum cystatin-C (Dade Behring Nephelometer II (BNII)) urine albumin, immunoglobulin and alpha-1 microglobulin using immunonephelometry method as well as urinary N-acetylglucosamine (U-NAG) analyzed with colorimetric method (Cobas Mira, Hoffmann-La Roche AG, Basel, Switzerland). The estimated glomerular filtration rate in ml/min/1.73m^2 BSA (eGFR) was calculated with the Schwartz formula using serum creatinine [151].

Blood samples for study II were blood glucose, measured by photometry (HemoCue AB, Ängelholm, Sweden), serum-insulin measured by electrochemiluminescence immunoassay (Roche Diagnostics GmbH, Mannheim, Germany), HbA1c measured by cation exchange chromatography (MonoS column) with high-performance liquid chromatography (Roche Diagnostics, Basel, Switzerland), IGF-I and its binding protein (IGFBP-I) were measured with in-house radioimmunoassay's (RIAs). Serum amyloid protein A (SAA) was measured by nephelometric technology (BN ProSPEC system, Siemens Healthcare, Erlangen Germany). High-sensitive C-reactive protein was measured by turbidimetry, infrared immunoassay rate method. Cholesterol and triglycerides were measured by enzymatic method and low density and high-density lipids (LDL, HDL) were measured by homogenous method (DXC800 (2020) Beckman Coulter Inc., Brea CA, USA). Apolipopreotein A1 and B were measured by turbidimetry (Beckman AB, Synchron LX, Beckman Coulter Inc., Diamond diagnostics, Holliston, MA, USA).

4.1.1.3 Oral glucose tolerance test

Following a 10-12 hour overnight fast a standardized oral glucose tolerance test (OGTT) was performed in all children giving 1.75g/kg body weight glucose (Orangedax; Custom laboratories, Baltimore, MD, USA) up to a maximum of 75g[152] per individual. The above described blood samples were taken at 0 min as base line followed by blood samples for blood glucose, insulin and IGFBP-1 taken at 30 and 120 min from an intravenous catheter with heparin lock flush injection system (heplock, Baxter; One Baxter Parkway Deerfield, IL, USA).

To estimate insulin resistance we used the homeostasis model assessment of insulin resistance (HOMA-IR= fasting plasma insulin x fasting plasma glucose/22.5)[153] and the whole body insulin sensitivity index (WBISI= 10 000/square root of {(fasting glucose x fasting insulin) x (mean glucose x mean insulin during OGTT at 0,30,120min)}[154].

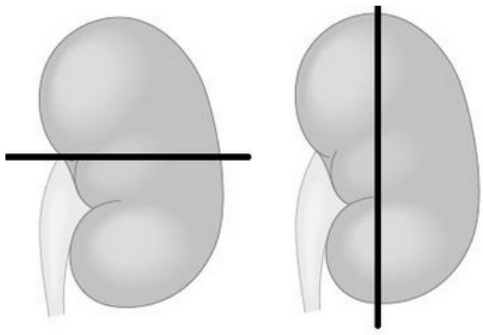
4.1.1.4 Heart rate variability (HRV), Study III

A twenty-four-hour Holter electrocardiography (ECG) using an ambulatory recorder unit (Braemer DL700; Braemer, Burnsville, MN, USA) was obtained from 86 children. The Holter ECG system (Danica Holter Replay Unit; Danica Biomedical, Borlänge , Sweden) automatically analyzed cardiac conduction and rhythm disturbances as well as distinguished normal from non-normal QRS complexes. The values for consecutive RR intervals as well as

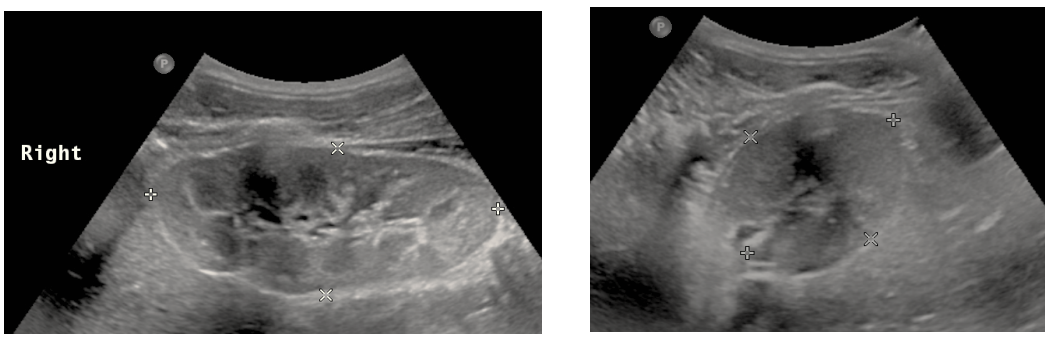
their corresponding classification code were exported to an ASCII text file. For the frequency domain measurements of HRV, 5-minute data episodes were analyzes by custom-made software[155]. Gaps in the time series due to non-normal RR intervals were filled with values calculated by linear interpolation between the adjacent normal RR interval. The software controlled for misclassified drop beats deviating more than three SD from the mean normal RR interval of each epoch. A minimum of 50% of the recordings had to be of acceptable accuracy in order to be included[147]. The frequency domain of the time series of RR intervals was analyzed with auto-regression method. Four different frequency bands of the total power spectrum were defined as by the standards of measurements[147]. Total power (Tot Pow, 0.0033-0.4 Hz (ms2)); very low frequency power (VLF Pow, 0.0033-0.04 Hz (ms2)); low frequency power (LF Pow, 0.04-0.15 Hz (ms2)); and high frequency power (HF pow, 0.15-0.40 Hz (ms2)) bands. For the time domain measurements, the mean of the SD of all normal RR intervals for all 5 min segments of each ECG recording (SDNN index) was calculated.

4.1.1.5 Kidney ultrasound, Study I

Kidney volume was measured in 86 of the children by using ultrasound technique. All measurements were performed by the same investigator using an Acuson 128xp system (Acuson, Mountain View, California, USA) with a 3.5-5MHZ linear transducer. Children were examined while in prone position lying on a pillow to compensate for lumbar lordosis if needed. Multiple images were taken from left and right kidney in longitudinal and transvers projections and the average from the largest measurements for length, width and thickness were entered in the formula for an ellipsoid (Figure 4 a, b) (volume= length x thickness x width x 0,523)[156].



a.)



b.)

Figure 4. a.: *Illustration of ultrasound projections, b.: longitudinal and transversal projections of the right kidney of a 9 year old, male Control child.*

4.1.2 Statistical analysis

Data in all three studies are presented as numbers, percentage, mean values with 1 SD or confidence interval, or medians with quartiles and minimum and maximum values as indicated. For study I statistical analysis was computed with STATA software package, version 9, using the analysis of variance (ANOVA) command presenting regression coefficients for the specified model. Reported p-values from the ANOVA command were the p-values from the regression coefficients for the preterm and term SGA categories. We used ANOVA to analyze group difference in kidney function and volume. We considered group differences in renal volume and function of 10% as important. Therefore, we calculated the sample size to 30 in each group, with 80% power and a significance level of 0.05. For kidney volume comparison, we adjusted for gender, age, BSA and BMI. We used regression analyses to study possible associations between kidney volume and function and perinatal factors. Simple logistic regression was used for continuous variables and the chi-square test for binary variables. Variables with $P<0.20$ in simple regression were entered in stepwise forward regression. Those variables were: gestational age at birth, birth weight, gender, Apgar Score (10min), birth weight z-score, maternal hypertension during pregnancy, delivery mode, pre-and postnatal steroids, PDA and duration of continuous positive pressure support. For study II analysis were performed using Statistica StatSoft version 10.IGF-1 and IGFBP-1 were not normally distributed and therefore log-transformed. Comparison between the groups were analyzed with ANOVA test followed by post hoc Fischer's test for comparison between the separate groups. Pearson's chi-square test was used for comparison of categorical data or percentages. Linear regression was performed using ponderal index (PI), weight and length SDS, mothers and father's height, BMI, the percentage of IGFBP-1 change during OGTT (t0-t120), the percentage change of insulin at 30 min, glucose and log transformed blood values. Analysis of covariance (ANCOVA) was used with BMI as a covariance variable and post hoc test was performed by planned comparison and Bonferroni correction. For study III analyses were computed in JMP software package, version 8.0.1 (SAS Institute, Cary, NY) using nonparametric Kruskal-Wallis test. Stepwise multiple regressions were performed to identify variables influencing HRV (gender, age at visit, BMI, maternal hypertension, maternal diabetes and prenatal steroid administration). The independent variables with F-to-enter=4 were entered into logistic analysis model. For all studies a p-value of <0.05 was defined as significant.

4.1.3 Informed consent and ethics

The parents received oral and written information about the study protocol and the purpose of the study. All parents gave informed and signed consent prior to inclusion. The studies were approved by the Karolinska Institutet research ethics committee (I-III, Dnr 97-186).

Table 2. *Characteristics at first visit for the three different studies (I-III) for the three different groups: Children born preterm (PT), small for gestational age (SGA) and with normal birth weight at term (control).*

	Preterm			SGA			Control		
Study	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**
N	39	21	31	29	26	27	37	30	28
Age, (years)	9.6* (0.3)	9.5* (0.3)	9.6* (0.3)	9.8 (0.3)	9.8 (0.3)	9.8 (03)	9.8 (0.2)	9.8 (0.2)	9.8 (0.2)
Girls, n (%)	22 (56)	9 (42)	15 (48.4)	16 (55)	15 (57)	16 (59.3)	24 (65)	19 (63)	18 (64.3)
Bodyweight, (kg)	32* (7.2)	29* (6.8)	31.8* (7.1)	32* (7.3)	30* (5.5)	31.8* (7.3)	38 (9.0)	36 (8.1)	37.9 (8.1)
Height, (cm)	134* (6.6)	137* (7.5)	134* (6.8)	137* (7.1)	136* (5.9)	137* (7.3)	142 (7.6)	143 (7.7)	142.6 (7.5)
Body mass index (kg/m²)	17.7 (3.0)	16.4 (2.4)	17.6 (2.9)	16.8* (2.5)	15.9* (1.9)	16.8* (2.5)	18.5 (3.2)	17.3 (2.7	18.6 (2.8)

*Values presented as means with (SD) or percentage. Statistics were done with one-way Anova for comparison of all three groups. * denotes significant difference in comparison to control (P values <0.05).*

Table 3. *Characteristics at second visit in children born preterm (PT), small for gestational age (SGA) and with normal birth weight at term (control) for study I.*

	Preterm (n=33)	**SGA (n=24)**	**Control (n=29)**
Age, mean (SD), years	12.9 (0.3)	12.0 (0.3)*	12.7 (0.2)
Females, n (%)	17 (51)	12 (50)	18 (62)
Bodyweight, mean (SD), kg	47.5 (10.4)*	43.9 (10.6)*	52.2 (12.8)
Height, mean (SD) cm	153.9 (9.4)*	152.9 (9.5)*	159.2 (8.9)
Body mass index (BMI), mean (SD) kg/m^2	20.1 (3.2)	18.3 (3.0)	20.6 (3.7)
Body surface area (BSA), mean (SD) m^2	1.42 (0.19)	1.35 (0.2)*	1.51 (0.22)

*Values presented as means with (SD)or percentage. Statistics were done with one-way Anova for comparison of all three groups. * denotes significant difference in comparison to control (P values <0.05).*

4.2 STUDY POPULATION STUDY IV

A total of 213 infants born and cared for at the Karolinska University Hospital between 2008-2011 with a gestational age below 28 weeks were eligible for inclusion in the study. Of those 213 children 105 had a renal ultrasound investigation during their neonatal period performed by a pediatric radiologist to evaluate for nephrocalcinosis (NC) as by the clinical protocol. In 37 cases the investigation result and/or the images were lost to follow up. Of the remaining 68 investigated children 34 had been diagnosed with NC (NC+) where the other half showed no signs for NC (NC-). Of the initial 213 children 38 children died. For the group of 68 children with a kidney ultrasound one child from the NC+ and 3 children form NC- group died (Figure 5.). Information on all relevant data reflecting on nephrotoxic substances as aminoglycosides, vancomycin, loop diuretics, thiazide diuretics, NSAID like Ibuprofen for treatment of–PDA, antenatal and postnatal steroids were collected during the chart review process. Duration of treatment was considered. Severity of illness as well as course of complication during the neonatal period was estimated by collecting data on Apgar Score, IUGR, RDS, BPD, acute kidney injury (AKI) defined and staged by the KDIGO guidelines, PDA receiving treatment, sepsis episodes (clinical and/or culture verified), NEC Bell stage II or more, surgical interventions, ROP grade III or higher (and or any plus disease) and IVH or parenchymal hemorrhage[157-159]. SGA was defined as a birth weight < mean −2 standard deviations (SDs) according to Swedish reference data for normal fetal growth[148]. There was no significant difference between the two groups of EPT born children with regards to the above listed treatments or complications. Three children from the NC+ group 5 children from the NC- group and none from the control group were SGA. None of the participating children had kidney or urinary tract malformations, congenital metabolic disorders, congenital abnormalities, genetic disorders or a positive family history for hyperoxaluria, cystinuria or any type of renal tubular acidosis.

At school age families to the surviving 64 children with available ultrasound results from their neonatal period were contacted. In total 41/64 families agreed to participate and 20 children in the NC+ group and 21 children in the NC- group could be investigated (Figure 5). All renal ultrasound images from the neonatal period were reviewed by a single senior pediatric radiologist. Diagnosis was confirmed in 39 out of 41 cases. In each group (NC+/NC-) one patient was misdiagnosed and moved to the opposite group. The degree of nephrocalcinosis was sub classified into mild, moderate and severe during the review process. Of the 20 children 12 had mild, 5 had moderate and 3 had severe NC. The 23/64 non-participants were not different to the study population with regards to gestational age, birth anthropometrics or severity of illness.

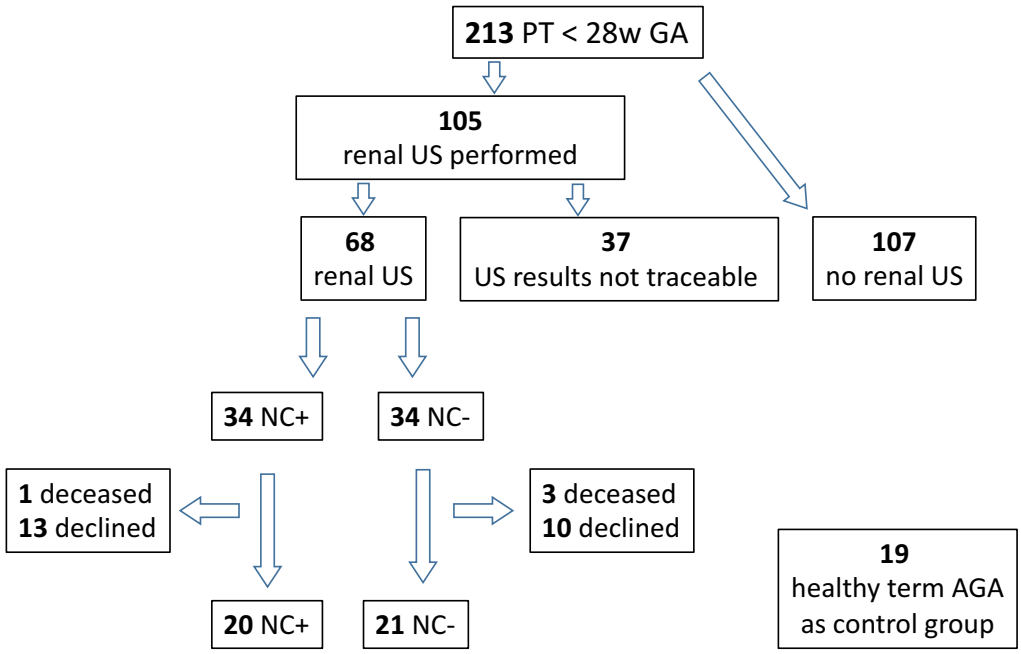


Figure 5. *Recruitment flow chart for study IV*

Table 4. *Neonatal characteristics for the three groups: Extremely preterm infants born <28 weeks gestational age (EPT) with nephrocalcinos (NC+) or without nephrocalcinosis (NC-) during the neonatal period and full-term controls (Control*

	EPT NC+ (n = 20)	**EPT NC- (n = 21)**	**Control (n=19)**	**P, ANOVA / NC+ vs NC-**
Males n (%)	9 (45)	13 (61.9)	10 (52.6)	0.55 / 0.27
Gestational age, mean (SD) in weeks	25.5 (1.2)	25.9 (1.3)	39.7 (1.6)	<0.0001 / 0.22
Birth weight, mean (SD) in g	755 (124)	841 (202)	3586 (477)	<0.0001 / 0.10
Birth weight, SDS, (SD)	-0.93 (0.78)	-0.87 (1.22)	0.19 (0.93)	0.0012 / 0.85
Birth length, mean (SD) in cm	32.4 (1.8)	33.6 (2.6)	50.4 (1.9)	<0.0001 / 0.08
Head circumference, mean (SD) in cm	23.3 (1.3)	24.0 (1.8)	34.6 (1.4)	<0.0001 / 0.12
SGA, n (%)	3 (15%)	5 (24%)	0 (0)	0.028 / 0.47
Apgar score at **5** min, mean (SD)	6.7 (2.9)	7.4 (2.1)	10 (0.0)	0.0007 / 0.36
Apgar score at **10** min, mean (SD)	8.4 (1.7)	8.7 (1.9)	10 (0.0)	0.022 / 0.59

Values are presented as numbers and percent (n, (%)). Statistics were done with one-way Anova for comparison of all three groups followed by post-hoc students t-test for continuous variables and Pearson's Chi-square test for categorical data when compared NC+ with NC-. P values <0.05 were considered significant.

4.2.1 Methods Study IV

Basic anthropometric measurements were identical to those for study I-III. In addition, lean body mass in kg was measured using DEXA technique by subtracting the percentage body fat from the total body weight. We were able to use this information as this study is part of a larger project were body composition measurements are of importance. DEXA results were available for 12, 16 and 17 children from the NC+, NC- and control group respectively.

4.2.1.1 Blood pressure recordings

Office blood pressure was measured using oscillometric technique (Dinamap Carescape V100, GE Healthcare, Illinois, USA) following standardized recommendations[87]. We also monitored 24-hour ambulatory blood pressure (ABPM) using a SPACELABS 90217A device (SpaceLabs Medical Inc., Redmond, Washington, USA) in children born preterm but not in controls where normative reference data were used instead[86]. Day-to-night BP decline was calculated by the equation (sleep $BP_{systolic}$-awake $BP_{systolic}$/awake $BP_{systolic}$) x100. "Extreme dipper" was defined as BP decline more than 20%, normal decline from day to night BP was defined as 10-20%, decline less than 10% was defined as "non-dipper" and an increase in sleep BP was defined as "riser"[160].

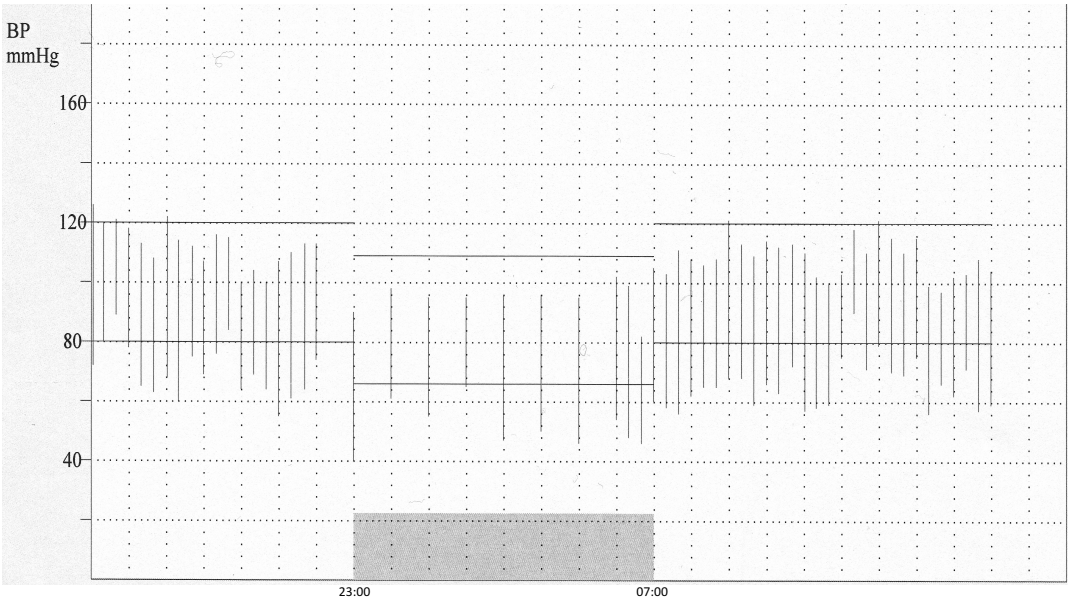


Figure 6. *24hour ambulatory blood pressure monitoring (ABPM) from a patient (NC+) illustrating normal (>10%) day-to-night decrease of systolic and diastolic blood pressure.*

4.2.1.2 Blood and urine sampling

Blood samples were collected in 54 patients (17 NC+, 20 NC-, 17 controls). Blood samples were taken after placing a topical anesthetic cream containing 2.5% lidocaine and 2.5% prilocaine (EMLA; Astra Zeneca, Södertälje, Sweden). Urine sample were collected in 58 patients (19 NC+, 20 NC -, 19 controls). Blood samples were investigated for plasma sodium, potassium using potentiometry with ion selective electrodes (Cobas 8000, Cobas C ISE2, Roche, Basel Switzerland), calcium, phosphate, alkaline phosphatase, creatinine and urea

using photometric technique and cystatine-C using immunochemical and turbodimetric method (Cobas 8000 Cobas CC 701 Roche, Basel Switzerland). Urine samples were investigated for sodium and potassium measured using potentiometry with ion selective electrodes (Cobas 8000, Cobas C ISE2, Roche, Basel Switzerland). Phosphate, calcium and magnesium were measured using photometric technique (Cobas 8000 Cobas CC 701 Roche, Basel Switzerland) and albumin was measured using immunochemical and turbodimetric method (Cobas 8000 Cobas CC 701 Roche, Basel Switzerland). Protein HC and immunoglobulin G was measured by immunochemical and nephelometric method (BN Pro Spec, Siemens Healthcare, Erlangen, Germany). For the estimation of GFR we used the simplified Schwartz formula as well as the cystatin-C based CAPA formula described elsewere[151,161].

4.2.1.3 Kidney ultrasound

Kidney volume in study IV was measured in the same way as in study I. Again all measurements were performed by the same investigator. A newer, different ultrasound system was used (Philips EPIQ 7G with SW1.5.2 software (Philips Ultrasound, Inc. 22100 Bothell Everett Hwy Bothell, WA 98021-8431, USA) and a 2-9 MHZ frequency (Phillips C9-2) curved transducer was used. All investigations for the neonatal period were performed by different specialists in pediatric radiology using a Siemens S 2000 system with a 6C2 curved transducer (Siemens, Erlangen, Germany). As mentioned above, all renal ultrasound images from the neonatal period were reviewed by a single senior consultant in pediatric radiology in 2018.

Table 5. *Characteristics at visit for the three groups: Extremely preterm infants born <28 weeks gestational age (EPT) with nephrocalcinos (NC+) or without nephrocalcinosis (NC-) during the neonatal period and full-term controls.*

	PT NC+ (n = 20)	**PT NC- (n = 21)**	**Control (n = 19)**	**ANOVA/ NC+ vs NC-**
Age at visit, mean (SD) in years	7.8 (1.0)	7.4 (1.1)	8.1 (1.2)	0.14/0.2
Body weight, mean (SD) in g	22.5 (5.8)	22.3 (5.4)	26.7 (4.0)	0.02 /0.9
Body weight SDS (SD)	-1.26 (1.5)	-0.87(1.3)	-0.02 (0.7)	0.01 /0.4
Body height, mean (SD) in cm	120 (7.6)	121 (8.3)	129 (8.6)	0.0005 /0.5
Body Height SDS (SD)	-1.2 (1.2)	-0.5 (0.9)	0.2 (0.7)	0.0001/0.03
Head circumference, mean (SD) in cm	51.1 (1.6)	51.8 (2.2)	53.4 (1.6)	0.0009/0.2
Waist circumference, mean (SD) in cm	58.0 (7.4)	55.5 (5.5)	57.7 (3.3)	0.3/0.2
Waist-to-height ratio, mean (SD)	0.48 (0.05)	0.45 (0.03)	0.44 (0.02)	0.005/0.04
BMI, mean (SD) in kg/m^2	15.5 (2.4)	14.9 (1.8)	15.7 (1.2)	0.4/0.4
BMI SDS (SD)	-0.7 (1.4)	-0.9 (1.6)	-0.2 (0.8)	0.26/0.6
BSA, mean (SD) in m^2	0.86 (0.13)	0.86 (0.12)	0.98 (0.11)	0.003/0.9
Lean body mass (12/16/17) in kg	15.8 (2.7)	15.6 (3.0)	19.6 (3.5)	0.001/0.8

Values are presented as numbers and percent (n (%)). Statistics were done with one-way Anova for comparison of all three groups followed by post-hoc students t-test for continuous variables when compared NC+ with NC-. P values <0.05 were considered significant.

4.2.2 Statistical analysis

For descriptive statistics, continuous variables were presented with mean and standard deviation (SD). Continuous variables, approximately normally distributed were analyzed with respect to the three defined groups, using Analysis of variance (ANOVA). In order to adjust for continuous prognostic variables covariance analysis was used (ANCOVA). Stepwise regression analysis was used to examine the impact of a set of prognostic variables. Non-normal continuous variables were analyzed with Kruskal Wallis test. Dichotomous variables were analyzed with cross tables and Pearson's Chi-square test. As an additional analyses the kidney volume was analyzed with a mixed effects model including right and left volume in the same analysis. A hierarchical model with the child as the main unit was set up, taking into consideration the correlation between right and left side, with the unstructured covariance structure unstructured (UN). In all statistical analyses the relevant assumptions were checked. A p value <0.05 was considered significant.

4.2.3 Informed consent and ethics

The parents received oral and written information about the study protocol and the purpose of the study. All parents gave informed and signed consent prior to inclusion. The study was approved by the Karolinska Institutet research ethics committee (IV, Dnr 2016/1258-31).

5 RESULTS

5.1 STUDY I

Renal volume and function in school-age children born preterm or small for gestational age.

Anthropometrics

At first visit weight, height and BSA were significant lower in PT and SGA group in comparison to controls. BMI was lower in SGA group but not for the PT group. At second visit weight and height were still lower among children in the PT and SGA group than in controls but BMI was lower in SGA and not in the PT group. BSA was lower in the SGA group at second visit (Table 2,3). At first visit age in the PT group was lower in comparison to the controls and SGA group ($p<0,05$). At second visit due to the slightly different composition of study individuals out of the same groups, age was significant lower in the SGA group but not in the PT group in comparison to controls (Table 3).

Blood pressure

Systolic and diastolic blood pressure measured at rest, orthostatic test and following a mathematical stress test was not different between the three groups. The detailed results for blood pressure measurements reflecting on the cohort investigated for study I-III are published by our research group but are not part of this thesis[162].

Kidney volume

Unadjusted kidney volume was lower in the group of children born preterm in comparison to controls (means (SD) 162ml (31) vs 182ml (47), p=0,035). The difference between the two groups remained significant when adjusting for gender, age and BMI (p=0,046).

Children born at term but small for gestational age had also a lower total kidney volume than controls but the difference did not reach significance (163ml (26) vs 182ml (47), p= 0,073). After adjustment for BSA, age and gender the difference between PT and controls diminished (20 ml to 7,5 ml) and was no longer significant. The difference in total kidney volume between SGA and controls remained insignificant after adjustments. Adjusted left and right kidney volume separately were not significant different among the groups.

Correlation analysis showed that birth weight correlated to total renal volume (r=0,23, p=0,03) but not to gestational age (r=0.33, p=0.06).For the preterm group birth length correlated to total kidney volume (r=0,36, p=0,4).

Kidney function

Estimation of renal function with serum creatinine using the Schwartz formula was possible in 84 subjects (PT=28, SGA=23, controls=33). There was no significant difference in eGFR between the three groups. Serum concentrations for creatinine and cystatin C did not differ between the three groups. The urinary protein excretion calculated as ratio to urinary creatinine were not different between the groups (Table 7).

Table 6. *Unadjusted kidney volume in healthy children born preterm (PT), small for gestational age (SGA) and with normal birth weight at term (Control). Values are given in means (SD)*

	PT (n= 33)	**SGA (n= 24)**	**Control (n=29)**
Total kidney volume (ml)	162* (31)	163 (26)	182 (47)
Right kidney volume (ml)	82.4	78.4	91.1
Left kidney volume (ml)	79.5	84.8	90.4

*Results from one-way ANOVA ,*denotes significant difference (p< 0.05) compared to controls*

Table 7. *Kidney function parameters, urinary protein excretion expressed as ratios to urinary creatinine in healthy children born preterm (PT), small for gestational age (SGA) and with normal birth weight at term (Control). All values given as means (SD)*

	PT (n=28)	**SGA (n=23)**	**Control (n=33)**
Serum- **creatinine,** mmol/l	77.1 (1.8)	77.9 (1.9)	81.6 (1.6)
Serume **cystatin-C**, mg/l	0.91 (0.03)	0.88 (0.03)	0.83 (0.03)
eGFR ml/min/1.73m^2 body surface area	89.6 (13.5)	88.8 (12.3)	88.3 (10.7)
	(n=12)	(n=22)	(n=16)
Urine **albumin** mg/mmol	0.6 (0.4)	0.8 (0.7)	0.7 (1.0)
Urine **IgG** mg/mmol	0.2 (0.1)	0.3 (0.2)	0.4 (0.4)
Urine **A1M** mg/mmol	0.7 (0.7)	0.6 (0.6)	0.6 (0.7)
Urine **NAG** nkat/mmol	4.7 (3.4)	3.2 (1.5)	3.7 (3.5)

Results from one-way ANOVA. Urine IgG: urine immunoglobulin, Urine A1M: urin-alpha-1-microglobulin, Urine NAG: urine-N-acetylglucosamin

5.2 STUDY II

Differences in insulin resistance markers between children born small for gestational age or born preterm appropriate for gestational age.

We investigated 84 children from the same cohort as in study I but individuals participating in this study are not completely identical to those investigated in study I.

Anthropometrics

Children born preterm were younger at study time than children born SGA or control (Table 2). Both groups of children with low birth weight (preterm and SGA group) were significantly lighter and shorter and standard deviation score (SDS) for weight and height were also significant lower in comparison to controls ($p \leq 0.02$ for all)). Head circumference at time of investigation was still significantly lower in children born preterm or SGA ($p= 0.004$, <0.001 respectively) and BMI only in term children born SGA versus control. Among the SGA children only girls had significantly lower BMI but not boys ($p= 0.001$ vs 0.89). Children born preterm had no different BMI than controls which remained unchanged for boys and girls. BMI SDS was only significant lower for the SGA group but not for preterm ($p= 0.003$; 0.22 respectively) in comparison to controls.

Insulin resistance

Results for the OGTT showed adequate and normal values for all three groups without any significant difference between the groups. HBA1c levels were normal for all groups with no significant difference between the groups ($p=0,13$). Unadjusted insulin resistance indexes HOMA-IR and WIBSI as well as baseline values for insulin, IGF-1 and its binding protein were not different between the groups of children with different perinatal exposure (Table 8). After adjustment for BMI HOMA-IR was significantly higher and WIBSI significantly lower in the group of children born SGA compared to controls. Fasting and stimulated Insulin levels at 30 minutes in the SGA group were significantly higher in comparison with controls A difference not seen for preterm born children (Table 9).

During the OGTT the decrease of IGFBP-1, following glucose stimulation, was significantly slower for the children born preterm compared to controls and children born SGA at term ($p= 0.045$; 0.007 respectively).

Inflammatory markers, hsCRP and SAA, were not different among the children of the three groups ($p= 0.3$, $p=0.46$ respectively). The lipoproteins, cholesterol, HDL and LDL and their ratios (cholesterol/HDL, LDL/HDL) as well as triglycerides, Apo A1 and Apo B did not differ significantly between the Preterm, SGA and control group.

Table 8. *Unadjusted results for OGTT at fasting level (t0), at 30 min (t30) and at 120 min (t120) for the group of preterm born children (PT), term born but SGA (SGA)and term with adequate birth weight (Control).*

	PT (n=21)	**SGA (n=26)**	**Control (n=30)**	**P-value**
HbA1c, %, t0	4.6 (4.5-4.7	4.4 (4.3-4.5)	4.4 (4.3-4.5)	NS
B-glucose, mmol/L t0	4.4 (4.1–4.6)	4.4* (4.2–4.7)	4.1 (3.9–4.3)	NS SGA vs C: 0.042
B-glucose, mmol/L t30	8.0* (7.4–8.7)	7.8 (7.2–8.3)	7.2 (6.7–7.7)	NS PT vs C: 0.039
B-glucose, mmol/L t120	6.0 (5.5–6.4)	5.8 (5.4–6.3)	5.8 (5.5–6.2)	NS
B-glucose increase, t0–t30	3.7 (3.0–4.3)	3.3 (2.7–3.9)	3.1 (2.5–3.6)	NS
S-Insulin mU/L t0	4.3 (3.3–5.5)	5.3 (4.3–6.7)	4.8 (3.9–5.9)	NS
S-Insulin mU/L t30	49 (37–63)	52 (40–66)	41 (32–52)	NS
S-Insulin mU/L t120	27 (20–36)	30 (23–40)	29 (22–38)	NS
Insulin ratio, t30/t0	11.4 (9 -14.4)	9.7 (7.6–12.4)	8.6 (6.9–10.6)	NS
S-IGFBP-1 lg/L t0	45 (34–60)	43 (35–54)	44 (36–54)	NS
S-IGFBP-1 lg/L t30	46 (34–64)	42 (34–52)	40 (32–50)	NS
S-IGFBP-1 lg/L t120	23 (16–31)	18 (14–23)	19 (15–25)	NS
% IGFBP-1, t120/t0	52 (47–58)	42 (37–47)	45 (40–49)	0.023 PT vs C: 0.045 PT vs SGA 0.007
S-IGF-1 lg/L t0	229 (194–270)	198 (170–231)	207 (180–238)	NS
HOMA–IR	0.82 (0.61–1.11)	1.05 (0.86–1.28)	0.87 (0.68–1.13)	NS
WBISI	2445 (2074–2815)	2115 (1903–2326)	2386 (2090–2683)	NS

p-values according to ANOVA followed by post-hoc Fisher's test. Values are in mean (95% CI)(geometric means for hormone values and HOMA-IR) or %.

Table 9. *Results for OGTT at fasting level (t0), at 30 min (t30) and at 120 min (t120) adjusted for BMI for the group of preterm born children (PT), term born but SGA (SGA)and term with adequate birth weight (Control).*

	PT (n=21)	**SGA (n=26)**	**Control (n=30)**	**P-value ANCOVA PT vs C SGA vs C PT vs SGA**
S-Insulin, mU/L t0	4.3 (3.5–5.3)	6.0 (4.9–7.4)	4.3 (3.5–5.1)	0.025 1.0 0.029 0.045
S-Insulin, mU/L t30	49 (39–62)	58 (46–73)	37 (30–46)	0.020 0.16 0.012 0.62
S-IGFBP-1, lg/L, t0	45 (37–54)	38 (31–46)	50 (42–59)	0.11 0.8 0.076 0.46
HOMA–IR	0.83 (0.66–1.05)	1.19 (0.96–1.48)	0.78 (0.64–0.95)	0.016 1.0 0.013 0.051
WBISI	2429 (2156-2702)	1971 (1713-2229)	2526 (2282-2770)	0.008 1.0 0.007 0.035

p-values are according to ANCOVA test with BMI as co-variance variable, followed by post-hoc planned comparison and Bonferroni correlation. Values are in mean (95% CI) (geometric means for hormone values and HOMA-IR). BMI: body mass index, IGFBP-1 Insulin like growth factor binding protein-1, HOMA-IR: Homeostasis model assessment-insulin resistance, WBISI: Whole-body insulin sensitivity index.

5.3 STUDY III

Decreased heart rate variability in children born with low birth weight

Maternal data

Prevalence of maternal smoking, gestational diabetes, gestational hypertension or history of hypertension were not different between the three groups.

Anthropometrics at visit

At visit, children born preterm were younger than children born SGA or control AGA (Table 2). Children from the preterm and SGA group were significantly lighter and shorter than controls. BMI was only lower in the SGA group compared to controls (Table 2).

Heart rate variability

Frequency domain parameters for the groups born preterm and SGA were significantly lower in comparison to healthy term born children (controls) at visit (Table). The ratio between low frequency and high frequency power (LF/HF ratio) was not different between the three groups. Time domain parameters as Mean RR and SDNN were also significant lower for preterm and SGA born children.

Table 10. *Results for heart rate variability parameters in time and frequency domains for children born preterm (PT), born at term but small for gestational age (SGA) and children born at term with normal birth weight (Controls). Values are given as means and 95% confidence intervals.*

	Preterm (n=31)	**SGA (n=27)**	**Control (n= 28)**	**P value**
Tot Pow (ms^2)	4.405* (3.514–5.297)	3.965* (3.030–4.901)	6.344 (5.109–7.579)	0.008
HF Pow (ms^2)	1.815* (1.299–2.331)	1.363* (994–1.732)	2.934 (2.099–3.769)	0.01
VLF Pow (ms^2)	1.160* (980–1.340)	1.203* (884–1.522)	1.535 (1.298–1.771)	0.02
LF Pow (ms^2)	1.429* (1.132–1.744)	1.399* (1.036–1.738)	1.875 (1.509–2.074)	0.04
LF/HF ratio	1.4 (1.2–1.6)	1.6 (1.4–1.8)	1.4 (1.1–1.7)	0.09
Mean RR (ms)	701* (670–732)	684* (648–720)	739 (711–767)	0.02
SDNN (ms)	74* (67–81)	71* (63–79)	89 (80–98)	0.006

Denotes $p < 0.05$ in comparison to controls according to Kruskal-Wallis test. Tot Pow: Total power, HF Pow: high frequency power, VLF Pow: very low frequency power, LF Pow: low frequency power, LF/HF ratio: low frequency/high frequency ratio, Mean RR: mean of R wave to R wave variation, SDNN: the mean of the SD of all normal RR intervals for all 5-min segments.

5.4 STUDY IV

Nephrocalcinosis in extremely preterm born infants as a risk factor for impaired renal size and function at early school age.

Maternal data

Prevalence of maternal smoking, gestational diabetes, gestational hypertension or history of hypertension were not different between the three groups.

Anthropometrics at visit

Children in the control group were insignificantly older than both groups of preterm born children but significantly heaver, taller and with a larger head circumference. However, BMI and waist circumference was not different between the three groups. Mean waist circumference and waist-to-height ratio was highest in the EPT NC+ group (Table 11).

Table 11. *Characteristics at visit for the three groups: Extremely preterm infants born <28 weeks gestational age (EPT) with nephrocalcinosis (NC+) or without nephrocalcinosis (NC-) during the neonatal period and full-term controls.*

	EPT + NC (n = 20)	**EPT –NC (n = 21)**	**Control (n = 19)**	**P, ANOVA / NC+ vs NC-**
Age, mean (SD) in years	7.8 (1.0)	7.4 (1.1)	8.1 (1.2)	0.14 / 0.2
Body weight, mean (SD) in g	22.5 (5.8)	22.3 (5.4)	26.7 (4.0)	0.016 / 0.89
Body weight SDS	-1.26 (1.5)	-0.87(1.3)	-0.02 (0.7)	0.01 / 0.39
Body height, mean (SD) in cm	120 (7.6)	121 (8.3)	129 (8.6)	0.0005 / 0.5
Body Height SDS	-1.2 (1.2)	-0.5 (0.9)	0.2 (0.7)	0.0001/ 0.03
Head circumference, mean (SD) in cm	51.1 (1.6)	51.8 (2.2)	53.4 (1.6)	0.0009 / 0.25
Waist circumference, mean (SD) in cm	58.0 (7.4)	55.5 (5.5)	57.7 (3.3)	0.34 / 0.24
Waist-to-height ratio, mean (SD)	0.48 (0.05)	0.45 (0.03)	0.44 (0.02)	0.005 / 0.04
BMI, mean (SD) in kg/m^2	15.5 (2.4)	14.9 (1.8)	15.7 (1.2)	0.44 / 0.45
BMI SDS	-0.7 (1.4)	-0.9 (1.6)	-0.2 (0.8)	0.26 / 0.65
BSA, mean (SD) in m^2	0.86 (0.13)	0.86 (0.12)	0.98 (0.11)	0.003 / 0.9

Values are presented as means and standard deviation (SD). Statistics were done with one-way Anova for comparison of all three groups followed by post-hoc students t-test for continuous variables when compared NC+ with NC-. P values <0.05 were considered significant

Kidney volume

All kidney volumes were adjusted for BSA following linear regression analysis. Total kidney volume in girls was significantly lower for both preterm groups in comparison to controls (Table 12). In boys, no difference could be seen between preterm groups and controls. Using the mixed effects model analysis where BSA, gender and each kidney side were included, the direct comparison between boys and girls showed a significant lower kidney volume for girls in both preterm groups in comparison to the control group (p=0.0048; p=0.016 respectively). Both gender taken together total kidney volume between preterm and controls reached only borderline significance (p=0.056). Right sided kidney volume was significantly lower in NC+ and NC- group separately and together compared to controls. Left kidney size was not different between the three groups. Right sided kidney length was significantly shorter for NC+ and NC- group when compared to controls. Left sided kidney length was not different between the three groups. In a mixed effect model including right and left volume for all groups we found a trend towards lower right sided kidney volume for all groups including controls (p=0.055).

An alternative way to adjust for body size and volume has recently been described and has been used in comparable studies. A simple quote was used to adjust kidney volumes for BSA by dividing measured volume by BSA (KV/BSA)[163]. When using this adjustment, the NC+ group had significantly lower total kidney volume than controls but not NC- group of preterm born children (Table 13). Both preterm groups taken together had significant lower kidney volume in comparison to controls (Table 13). When looked at side specific kidney volumes by using (KV/BSA) right sided kidney volume was significantly lower for NC+ preterm born children while for the left side the NC- group had lower kidney volume (Table 13). When we directly compared the NC+ group of preterm born children with NC-group no significant difference could be detected regardless of the method used (Table 13).

We calculated predicted left and right renal volume for each individual using the formula described by Dinkel[156]. When we compared measured kidney volume with predicted kidney volume the most children < 75% of predicted came from the NC+ group and the right side was mainly affected (Table 14). Also when calculating the percentage of total kidney volume in relation to predicted volume for each group, the NC+ group reached the lowest percentage (NC+:85.2%, NC- 90.2% Control: 97.2 of predicted, p= 0.029 (Kruskal-Wallis test)) (Table 14).

Table12. *Results for kidney volume adjusted for BSA and kidney length adjusted for height at visit comparing the different groups: Extremely preterm infants born <28 weeks gestational age with nephrocalcinosis (NC+) or without nephrocalcinosis during the neonatal period (NC-) and full- term controls (Control).*

	Estimated difference (CI 95%)	**P-value**
Total Kidney volume		
NC+ vs NC-	-2.97 (-13.09 +7.14)	0.55
NC+ vs Control	-11.12 (-22.57 +0.31)	0.056
NC- vs Control	-8.15 (-19.4 +3.06)	0.15
(NC+ NC -) vs Control	-9.64 (-19.79 +0.51)	0.062
Right Kidney volume		
NC+ vs NC-	-4.72 (-10.31 +0.86)	0.09
NC+ vs Control	-8.7 (-14.9 -2.5)	0.0068
NC- vs Control	-3.97 (-10.09 +2.13)	0.19
(NC+ NC -) vs Control	-6.34 (-11.83 -0.85)	0.024
Left Kidney Volume		
NC+ vs NC-	2.23 (-5.25 +9.72)	0.55
NC+ vs Control	-5.16 (-13.47 +3.1)	0.21
NC- vs Control	-7.4 (-15.59 +0.79)	0.07
(NC+ NC -) vs Control	-6.28 (-13.63 +1.07)	0.09
Total Kidney volume Girls		
NC+ vs NC-	7.66 (-6.93 + 22.25)	0.28
NC+ vs Control	-14.76 (-29.04 -0.49)	0.04
NC- vs Control	-22.42 (-38.76 -6.08)	0.009
(NC+ NC -) vs Control	-18.59 (-32.09 -5.1)	0.009
Total Kidney volume Boys		
NC+ vs NC-	-6.83 (-21.88 +8.22)	0.36
NC+ vs Control	-6.7 (-25.29 +11.89)	0.46
NC- vs Control	0.12 (-15.63 +15.94)	0.98
(NC+ NC -) vs Control	-3.28 (-18-81 +12.24)	0.66
Right Kidney length		
NC+ vs NC-	-0.22 (-0.55 +0.1)	0.17
NC+ vs Control	-0.64 (-1.02 -0.26)	0.001
NC- vs Control	-0.42 (-0.78 -0.06)	0.023
(NC+ NC -) vs Control	-0.53 (-0.86 -0.20)	0.002
Left Kidney Length		
NC+ vs NC-	0.22 (-0.19 +0.64)	0.29
NC+ vs Control	-0.03 (-0.52 +0.44)	0.88
NC- vs Control	-0.25 (-0.72 +0.21)	0.27
(NC+ NC -) vs Control	-0.14 (-0.57 +0.27)	0.49

Results from the ANCOVA analysis models and planned comparisons.

Table 13. *Results for Kidney volumes using BSA related KV (KV/BSA) for the different groups: Extremely preterm infants born <28 weeks gestational age with nephrocalcinosis (NC+) or without nephrocalcinosis during the neonatal period (NC-) and full-term controls (Control).*

	Estimated difference (CI 95%)	P-value
Total Kidney volume (KV/BSA)		
NC+ vs NC-	-4.21 (-15.29 +6.86)	0.44
NC+ vs Control	-14.02 (-25.37 -2.66)	0.016
NC- vs Control	-9.8 (-20.88 +1.27)	0.08
(NC+ NC -) vs Control	-11.8 (-21.51 -2.09)	0.018
Right Kidney volume (KV/BSA)		
NC+ vs NC-	-5.04 (- 11.0 +0.93)	0.09
NC+ vs Control	-9.21 (-15.33 -3.08)	0.003
NC- vs Control	-4.16 (-10.22 +1.89)	0.17
(NC+ NC -) vs Control	-6.62 (-12.01 -1.23)	0.016
Left Kidney Volume (KV/BSA)		
NC+ vs NC-	-2.4 (-10.9 +5.96)	0.55
NC+ vs Control	-6.99 (-15.64 +1.64)	0.11
NC- vs Control	-9.47 (- 18.01 -0.92)	0.03
(NC+ NC -) vs Control	-8.26 (-15.7 -0.82)	0.03

Results from the ANCOVA analysis models and planned comparisons

Table 14. *Results for kidney volume in numbers and percentage from predicted volume for the different groups: Extremely preterm infants born <28 weeks gestational age with nephrocalcinosis (NC+) or without nephrocalcinosis during the neonatal period (NC-) and ful- term controls (Control).*

	EPT NC+	EPT NC-	Control	P-value
n **<75%** of predicted **total** kidney volume	4	3	1	0.21
n **<75%** of predicted **right** kidney volume	9	4	1	0.01
n **<75%** of predicted **left** kidney volume	4	5	1	0.25
Percent of **predicted total kidney** volume (SD)	85.2% (11.6)	90.2% (16.5)	97.2 % (12.2)	0.029

Differences between number of patients with <75% of predicted kidney volume are calculated with Pearson's Chi-square test. Differences in percentage are analyzed using Kruskal-Wallis test.

Kidney Function

The estimated glomerular filtration rate (eGFR) by using the cystatin-C based CAPA formula showed no difference between preterm born children with or without NC (p= 0.11). Both preterm groups taken together had significantly lower but normal eGFR compared to controls (p=0.036). Plasma creatinine levels were higher in the control group (p=0.003) but still normal while cystatin-C values were insignificantly lower in the control group (p=0.22).

Using the simplified Schwartz formula showed insignificantly higher eGFR values for the preterm group (PT means 113 ml/min/1,73m^2 (SD 19.9), Controls 104.6 ml/min/1,73m^2 (SD 17.7)). Plasma electrolytes sodium, potassium, phosphate and calcium as well as urea and alkaline phosphatase were not different between the three groups. Urinary proteins and electrolytes as well as ratios (albumin/creatinine, magnesium/creatinine, calcium /creatinine, phosphate/creatinine) were in the normal range for all groups. Urinary phosphate excretion was higher in preterm born children without NC (p=0.015). NC+ preterm born children had a trend towards higher calcium/creatinine ratio but not reaching significance (p=0.08).

Table 15. *Kidney function results for extremely preterm infants born <28 weeks gestational age with nephrocalcinosis (NC+) or without nephrocalcinosis during the neonatal period (NC-) and full-term controls (Control).*

	Estimated difference (CI 95%)	**P-value**
eGFR Cystatin-C		
NC+ vs NC-	-7.05 (-16.85 +2.74)	0.15
NC+ vs Control	-6.29 (-17.01 +4.42)	0.24
NC- vs Control	-13.35 (-23.7 -3.0)	0.012*
(NC+ NC -) vs Control	-10.11 (-0.69 -19.5)	0.035*
eGFR Creatinine		
NC+ vs NC-	-1.92 (-14.7 +10.84)	0.76
NC+ vs Control	-9.8 (-23.25 +3.6)	0.14
NC- vs Control	-7.89 (-20.66 +4.87)	0.22
(NC+ NC -) vs Control	8.75 (+20.07 -2.5)	0.12
Albumine/Creatinine		
NC+ vs NC-	-0.08 (-1.93 +1.77)	0.93
NC+ vs Control	-1.03 (-2.84 +0.76)	0.25
NC- vs Control	-0.95 (-2.73 +0.81)	0.28
(NC+ NC -) vs Control	+0.9 (+0.51 -2.5)	0.19

Results from the ANCOVA analysis models and planned comparisons

Blood pressure

Office blood pressure, systolic, diastolic and mean were not different between the three groups. Four out of a total of 41 preterm born children had office systolic blood pressure above the 90th percentile. Three of these 4 children also had diastolic blood pressure above the 90th percentile. All other children had normal blood pressure below the 90th percentile.

Ambulatory blood pressure measurements were analyzed in 34 preterm born children (NC+:14; NC-: 20). In the remaining 7 patients 24h-BP measurements were either not possible or had to be discarded because of insufficient quality. Day-to-night decline below 10% was absent in 9 of 14 patients (64.3%) in the NC+ group and in 8 of 20 (40%) of NC- children. Both preterm born groups combined, 50% of the children did nor reduce their blood pressure by 10% from day to night. The mean reduction of systolic blood pressure from day to night for the NC+ group was -5.8% (SD 2,9) and -5.4% (SD 3.3) for the NC- group.

Table 16. *Results for office blood pressure SDS for children born extremely preterm with nephrocalcinosis (EPT NC+) and children born extremely preterm without nephrocalcinosis (EPT NC-) and healthy children born at term with normal birth weight (control). Presented as means (SD)*

	EPT NC+ (n= 20)	**EPT NC- (n=21)**	**Control (n=19)**	**P-value**
Office BP, systolic SDS	O.18 (0.94)	-0.07 (0.72)	-0.005 (0.72)	0.58
Office BP, diastolic SDS	0.12 (0.79)	0.02 (0.51)	0.07 (0.57)	0.87

Results from one-way ANOVA

Table 17. *Results for ABPM for children born extremely preterm with nephrocalcinosis (EPT NC+) and children born extremely preterm without nephrocalcinosis (EPT NC-) and healthy children born at term with normal birth weight (control) (presented as numbers for percentiles) for the three groups*

	EPT NC+ (n=14)	**EPT NC- (n=20)**	**P -value**
24h ABPM systolic percentile			
<50th	9	14	0.7
50-75th	5	3	0.2
75-90th	0	3	0.1
> 90th	0	0	
24h ABPM diastolic percentile			
<50th	13	17	0.5
50-75th	1	2	0.7
75-90th	0	1	0.4
> 90th	0	0	
Day-to-night decrease < 10%			
Patients, n (%)	9 (64.3%)	8 (40%)	0.16

Differences between NC+ and NC- group analyzed with Pearson's Chi-square test, the normal percentiles are taken from reference [87]

6 DISCUSSION

The presented studies were designed to identify morphological and functional alterations in school children allowing associations between prematurity or SGA and the pathological development towards cardiovascular, renal and metabolic diseases.

Kidney volume

Using different calculations and adjustment, we found evidence and trends for smaller kidneys in school aged children born preterm but no significant difference for SGA born children compared to controls (study IV+I respectively). Our findings are in line with the few comparable previous studies showing an effect of prematurity on kidney volume or size in this age group [112,164-166]. Others showed a relation between birth weight and kidney volume during the neonatal period and in infancy or later in young adulthood[167-169]. Several post mortem studies have confirmed these findings[66,67,170].

Kidneys were smaller in study IV when children, born extremely preterm had a mean age of 7.7 years but the difference in kidney volume was more discrete in study I where children were almost five years older. This could either indicate that study I was not powered well enough to prove the difference or that the selection of exclusively extreme preterm (< 28w GA) born children in study IV compared to the less premature born children (<32w GA) in study I explained the difference. It is also possible that the kidneys have caught up on growth between 7.7 and 12.5 years of age, potentially by hypertrophy with however the same number of nephrons. It has previously been shown that kidneys in children or adults born premature or SGA have fewer but larger glomeruli due to hyperperfusion of the remaining nephrons followed by compensatory enlargement of the filtration surface for the individual glomerulus leading to glomeruli hypertrophy [72,73,167,170,171]. As the glomeruli constitute a large amount of the kidney volume hypertrophy consequently leads to larger kidney volume[172].

Since we wanted to be able to discriminate between LBW due to prematurity or due to IUGR in the first cohort and even though the mean KV for the SGA group was almost as low as for the PT-group the difference to the control group was not significant. Schmidt et al. followed infants and toddlers and found significantly smaller kidney volumes for children born SGA not even showing a catch-up growth during the 18 months follow up in their study[173]. Much earlier Hinchliffe et al. could show in an autopsy study that IUGR stillbirth had significant reduction in nephron number compared to non-IUGR infants[174]. It is possible that our study was underpowered to prove a significant difference between SGA and control kidney volume.

Looking at gender differences we found that EPT born girls had significantly smaller kidney volumes in comparison to girls in the control group but not boys. A finding which was not present in study I. There is conflicting evidence with regards to gender difference for kidney volume measured with ultrasound. Dinkel et al. could not see a difference between healthy boys and girls born at term while Schmidt et al. in a large longitudinal cohort study in 717 healthy children found that boys had significant larger kidney volumes than girls at the ages 0, 3 and 18 month using ultrasound technique[156,175]. A Korean study investigated kidney volume in children using scintigraphy (technicium-99m dimercaptosuccinic acid) could not find a difference between boys and girls[176]. However, in a post mortem histological study

Neuengarten et al. showed that kidney weight was greater in men but no difference in glomerular number between the sexes was observed. They explained the larger kidneys with greater BSA in men[177]. Also earlier Nyengaard et al. showed a difference in gender with girls being disadvantaged in kidney size[79]. Nevertheless, even if kidney volumes are in general lower in girls compared to boys we showed that kidney volume in preterm born girls, adjusted for BSA, are significantly lower than in girls born at term with normal birth weight. This finding suggests that girls are more vulnerable than boys. Keijzer-Veen et al. investigated 51 preterm bon young adults at the age of 20 years and could also show smaller kidneys in females born premature in comparison to preterm born males[178].

Besides the gender difference there was also a difference between right and left kidney volume in study IV. Mean values for right sided kidney volume was lower than the left sided kidney volume. In a mixed effects model the difference between right and left kidney reached almost significance for all three groups (p=0.055) implicating that the right kidney is independently smaller. These findings are in concurrence with most of the previous studies on this subject but often describing discrete and not always significant differences [156,175,176,178-181]. However, we could not detect any difference between the right and left side in our first study (Study I). Contradicting to our results are the findings from a recent Dutch study who found in young adults born preterm and SGA (<32 weeks of gestation and < -2 SDS) in about 60% the right kidney to be larger[178]. More recently a large study using three-dimensional ultrasound technique confirmed our results and detected significantly smaller kidneys on the right side in their population of healthy children between 1 and 12 years of age [182]. This laterality in reduced kidney volume might appear trivial but could be of importance when it comes to organ donation as more often the left kidney is donated because of easier access and vascular surgery [183]. This means that the smaller, right kidney remains with the donor which might not be a problem as long as there is no reduced nephron endowment primarily.

In study IV we tested the hypothesis that NC developed in extreme preterm born infants during their neonatal period can be used as a surrogate for prolonged kidney injury and severity of illness and investigated whether it is related to kidney volume. Especially in extreme preterm children where there is incomplete nephrogenesis, postnatally acquired kidney injury (AKI) is of great importance for the individual's future renal health and can be regarded as a "second hit" to the potentially deficient and more vulnerable EPT infants kidneys[184,185]. On direct comparison of the two groups (NC+ and NC-) we could not see any significant difference with regards to kidney volume. Nevertheless, comparing the two groups to controls revealed that the NC+ EPT group was more affected than the NC- group of EPT born children (Total KV, Right KV, Right KL, Total BSA/KV, Right BSA/KV) (Tables 11,12,13). Kist-van Holthe measured kidney length in children born preterm below 32 weeks of gestation with and without NC but could not detect a difference between the two either[112]. Another study, following 107 preterm children (63 with PT NC+ and 44 PT NC- Controls) to the age of 24 months found reduced kidney length for the PT NC+ up to 12 months in both kidneys and up to 24 months on the right sided kidney only in PT NC+ in comparison to the controls (PT NC-) [104]. Some clinicians propose that the routine ultrasound investigation for the presence of NC in EPT infants is unnecessary as they regard NC as transient, self-resolving, benign and no consequences will arise from the positive finding[186]. However, there

is some evidence that NC has an effect on future kidney function and further the knowledge of NC might be relevant to the responsible clinician and possibly avoid or limit the use of nephrotoxic drugs as well as initiate adequate follow up for the patient[104,112].

To briefly summarize the finding for kidney volume, we found smaller kidneys in children born preterm, mainly in girls and mainly on the right side and we could not detect a significant effect of NC on kidney volume.

Kidney function

We studied kidney function by using the simplified creatinine based Schwartz formula to estimate GFR in study I and in addition the cystatin-C based CAPA formula for study IV. When using the Schwartz formula for study I no difference in GFR could be detected among the three groups. In study IV four children in the preterm groups and one child from the control group had eGFR below 85 ml/min/1.73m^2 ,which can be defined as mild renal insufficiency[187]. When we were using the cystatin-C based CAPA formula eGFR was normal in all groups. However, there was a significantly lower but still normal eGFR measured with the cystatin-c based formula in the group of children born prematurely in comparison to the control group (p= 0.036). Cystatin-C has been regarded to be superior to creatinine as a filtration marker for GFR assessment because of its independency to body mass[188]. It has also been suggested to combine creatinine and cystatin-C in a composite equation to estimate GFR[189,190] but mainly in adults and a clear improvement in accuracy has not yet been described for children[191]. When using the Schwartz formula we saw a lower (but normal) eGFR in the Control group compared to both preterm groups most likely due to higher muscle mass in controls and therefore elevated plasma creatinine levels.

As kidney volume seem to be smaller in children born preterm than in controls born at term and kidney volume can be regarded a proxy for nephron number one would speculate that kidney function should be impaired when nephron numbers are low[192]. However, it has been suggested that the reduced total filtration surface is compensated by single glomerular hyperfiltration in kidneys with fewer nephrons [73]. We might have investigated this high-risk group of ex preterm or SGA children too early to detected kidney function impairments but soon enough to detect lower kidney volume. Keijzer-Veen showed that kidney function was impaired in preterm born SGA children at 19 years of age[193]. Contradicting to our findings are the results from Rodriquez-Soriano et al. who could not see any difference in kidney length and volume between preterm and control but a diminished GFR in school age children[194]. Kwinta et al. was showing both, reduced kidney volume as well as increased cystatin-C levels[164]. However, cystatin-C levels were perfectly normal in Kwinta's study in the preterm group and no eGFR was calculated. A more recent study from the same group followed up on a cohort of EPT born children at the age of 7 and 11 years and compared them to children born at term with birth weight > 2500g. Their results are in line with our results showing smaller kidneys, higher but normal cystatin-C values and lower but normal GFR in the EPT group[165].

In study IV we identified NC as a potential marker or cause for impaired function but we could not detect any difference between preterm born children with or without NC with regards to eGFR (Table14). Kist van Holthe showed a higher number of children in the NC+ group with mild chronic renal insufficiency but these results were not significantly different

to the children born preterm without NC[112]. Another more recent study could not detect an effect of NC on GFR in preterm born children[104]. Others have tried to correlate neonatal AKI to worsened renal function at mean age of 6.6 years but could not detect any significant difference in GFR or microalbuminuria at this age[184].

Reflecting on the above discussed evidence, it appears that most studies show trends and tendencies towards impaired function but no clear pathology is evident in this age group yet. In none of the studies focusing on kidney function we were able to detect significant changes or differences for urine proteins or electrolytes or their ratios. In study IV we were expecting slightly abnormal electrolyte ratios but as no residual NC was detectable on ultrasound investigation it was not surprising that urinary electrolytes were in the normal range and no significant difference between the groups was found. Preterm children with NC had higher ratio for urinary calcium/creatinine but not reaching significance and still in the normal range ($p=0.08$). Only urinary phosphate was significantly higher in children born preterm without NC ($p=0.05$). The phosphate/creatine ratio was highest in children born preterm without NC compared to controls ($p=0.015$). Kist-van Holthe et al. investigated tubular function in preterm born children with and without NC but could not detect any evidence for impaired proximal or distal tubular function either[112].

Blood pressure

Blood pressure measurements were performed in both cohorts but ABPM only for study IV. The results of the blood pressure measurements from the first cohort are published separately and are not part of this thesis. However, the results showed normal BP in all subjects and no significant difference between the groups[162]. In study IV 4/41 preterm born children and one child out of the control group had office systolic blood pressure values above the 90th which is defined as "high-normal BP" or pre-hypertension[86]. Whether this was real "high-normal BP" or more likely to be a "white coat effect" was initially uncertain[87]. Only one child with elevated office BP had abnormal BP in the ABPM. The ABPM results were normal for all other preterm born and control children according to age, gender and height reference percentiles in study IV[87]. These results are in line with a recent large cohort study showing that children born extremely preterm have slightly higher but still normal office blood pressure at the age of six years [195]. A different study followed very preterm born young adults at the age of 20 years and found higher day- time BP in comparison to normal controls[196].

In study IV 16/41 (39%) patients from the preterm groups had neurodevelopmental disorders of the autism spectrum disease (ASD) and/or ADHD type which reduced the compliance for the ABPM. In seven out of those 16 patients the parents either indicated directly that the ABPM would not be tolerated or we had to discard the measurements afterwards because of insufficient readings and poor quality.

The results for the 34 successful ABPM showed that 50% of the EPT born children did not decrease their blood pressure from day-to-night in an appropriate way indicating abnormal circadian blood pressure regulation. These findings are of great interest as the phenomenon of "non-dipping" has previously mainly been described for children or adults with established pathologies like primary hypertension, DM, renal pathology (autosomal dominant polycystic kidney disease, ADPKD) and overweight or obesity[197-199]. In adults, non-dipping has been associated with worse cardiovascular outcomes, microvascular complications and can be

regarded as an indicator for the development of hypertension[200,201].Few studies have described insufficient day-to-night decline of BP in preterm born children. A study in 41 preterm born children (26-36 weeks of gestation) at the age of 7 years found in comparison to 27 healthy controls insufficient night dipping in 73% compared to 41% in the control group[202]. It is rather unclear why the prevalence of non-dipping was so high among healthy control children in this study. Another recent and slightly larger study investigate 78 preterm born children (27 weeks mean GA) and compared them to 38 healthy term control children at the mean age of 6.7 years but could only find 16.7% versus 5.2% non-dippers[164]. We were unfortunately unable to perform ABPM in our control group and used published reference data for comparison[87]. However, there is scare information about the prevalence of non-dippers in healthy term born children. There is also uncertainty with regards to what an expected night time decline in BP should be in children and therefore adult criteria had been used so far. A slightly older study investigated 61 health children and 40 patients with renal diseases between the age of 3 to 6 years with ABPM and found that the healthy children had a mean systolic day-to night dip of 8-10% from day systolic blood pressure[203]. All the children investigated in our study were above 6 years of age (mean 7.7years). We therefore expected a dip of 10% to be appropriate for our patient group.

Circadian blood pressure dysregulations in particular a reduced BP drop from day to night had been associated with nocturia or nocturnal enuresis in adults and children[204,205]. This symptom was prevalent in 6/17 (35%) of our non-dipping patients, also in 14 (34%) of all preterm born children but in none of the controls. In the absence of hypertension, pressure diuresis and pressure natriuresis can't explain nocturnal enuresis in these patients. An immaturity or dysfunction of the ANS has been speculated to play a role in those patients where other common problems like decreased bladder capacity, bladder over activity, increased nocturnal urine production or the inability to wake up cannot explain the condition [206-208].

Circadian fluctuations of blood pressure including day-to-night decline are regulated by the ANS and the hypothalamo-pituary-adrenal axis[209]. In study III we were able to show that the overall activity of the ANS measured by HRV is significantly diminished in children born preterm or SGA. Even though the findings of non-dipping and impaired ANS function measured by HRV are not established in the same cohort we felt it was appropriate to create a connection between these results.

Heart rate variability and the autonomic nervous system

As a reminder, HRV is a measure for the ANS function, illustrating heart-brain interactions and ANS dynamics. In the context of the above discussed phenomena of non-dipping and circadian regulation of blood pressure these activities would be reflected by the very-low or ultra-low frequency spectrum (VLF and ULF) in HRV measurements. The "Low-frequency band" confusingly also called "mid-frequency band" is primarily reflecting baroreceptor activity at rest. Higher frequencies (HF) reflect parasympathetic or vagal activity often also called the "respiratory frequency band" as it reflects much of the variations due to the respiratory cycle. The above described finding of "non-dipping" can be related to the findings in study III showing low HRV for PT and SGA. The corresponding frequency band for circadian regulation (VLF) is significantly lower in PT and SGA groups compared to

controls. However, a direct association between low VLF power and "non-dipping" in ABPM remains speculative as we do not investigate the same patients. It would have been of interest to have both 24-hour Holter ECG and ABPM measurements in the first cohort to be able to see if HRV is most affected in the group of children with an insufficient decline in day-to-night blood pressure. Nevertheless, even though the VLF power band has traditionally "only" been associated with thermoregulation, circadian regulation, hormonal factors including renin angiotensin system, it has a stronger association to mortality than LF and HF components of power spectrum[144,210,211]. There is evidence that disturbances of the ANS measured by HRV are visible in children with neurodevelopmental disturbances and autism spectrum disease (ASD) in different age groups[212-215]. In study IV the prevalence of neurodevelopmental disorders including ASD was high among children born preterm (16/41, 39%). However, the HRV results are reflecting on cohort I where the prevalence of neurodevelopmental disorders was much lower (6/58, 10%). We could not detect an imbalance of the two branches of the ANS on HRV measurements. The so called "autonomic balance hypothesis" claims that the parasympathetic and sympathetic systems competitively regulate sinoatrial node firing where the increase of one leads to a decrease of the other. It seems that the relationship between PNS and SNS is however nonlinear and probably more complex[216]. In view of the non-dipping phenomena in our group of preterm born children the LF/HF ratio is not of great interest. As mentioned before circadian dysregulations would mainly be reflected in the VLF band or even ultra-low frequency (ULF) power bands. Apart from the abnormal circadian regulation leading to the non-dipping phenomenon it is difficult to interpret the results from study III. Unlike for the adult population there is limited HRV reference data available stratified by age and gender and corrected for confounding factors like BMI and blood pressure in children [147,217,218]. Some researchers have speculated that a sympathetic over activity might be responsible for later development of hypertension and cardiovascular complications while others have stated a vagal or parasympathetic withdrawal as the pathological mechanism[219-223]. It is likely that an early environmental effect, whether intrauterine or extrauterine, alters the development of the ANS which mainly occurs during the third trimester of gestation. A very recent study investigating extremely preterm born infants without destructive brain injury at term equivalent and compared HRV parameters to normal term controls showed a general depression of HRV in the preterm born group[224].

Insulin resistance and the metabolic syndrome

Autonomic dysfunction has been observed in patients with type I diabetes mellitus (T1DM) and or insulin resistance but also in subjects with overweight and obesity[225,226]. Cho et al. investigated 125 girls aged 8-18 with T1DM and found also a suppression of total, LF and HF power and no difference in the LF:HF ratio[225]. In study II we focus on insulin resistance markers in the same cohort investigated for HRV. We found discreet signs for an altered glucose handling in children born preterm and those born at term with SGA[227]. Insulin resistance is regarded as a precursor to DM and the MetS and also linked to hypertension[115,228]. A recent study from Taiwan found that changes in the ANS are detectable prior to the onset of insulin resistance and the initiation of MetS[229]. That is a rather relevant finding for our study as we can detect altered autonomic function while the markers for insulin resistance are still subtle in the group of children born preterm or at term with SGA. After adjustments for BMI the group of term SGA children had significantly higher HOMA-

IR and lower WBISI values in comparison to controls while children born preterm had a diminished decrease of IGFBP-1 during OGTT.

IGFBP-1 has been shown to be a strong predictor for the development of diabetes and can be used as a marker for hepatic insulin sensitivity in a test setting using oral or intravenous glucose load [230,231]. This has already been proven to be valid for children as well[232]. Non-obese children born preterm in our cohort showed an attenuated fall of IGFBP-1 following glucose provocation which we interpreted as a discrete but significant sign. For the SGA group we observed a more peripheral IR which was expressed by higher HOMA-IR, lower WBISI and a higher basal insulin during OGTT. This has been shown earlier by Dallar et al. but in contrast to our SGA group their SGA children had significantly higher BMI than the corresponding controls[233]. Both our findings are indicating a different glucose handling resulting in signs for decreased peripheral insulin sensitivity for the SGA born children and a more hepatic insulin resistance type for the preterm born children. It seems that the different perinatal exposures like postnatal extrauterine stress and antenatal intrauterine stress have different consequences on glucose metabolism. Slightly opposing the above statement, a Finnish research group had shown that children born preterm share almost the same risk as children born with low birth weight following IUGR for reduced insulin sensitivity[234,235] indicating that the effect of slow fetal growth and short gestation are somehow comparable. The same group postulated that the combination of prematurity and SGA doesn't lead to "more" insulin resistance. They also suggested that possibly the pre-and postnatal pathomechanisms for the SGA and PT respectively, including stress, malnutrition and hypoxia, are almost equally leading to the same effect of insulin resistance[235,236]. A more recent and much larger study by Wang et al. showed that plasma insulin levels were inversely related to gestational age and thereafter tracking into early childhood indicating impaired insulin homeostasis and possible development of insulin resistance. They also speculate that the pathomechanisms by which children born preterm or SGA exhibit signs for insulin resistance resemble each other[237]. However, none of the studies distinguished between the different types of IR. It seems worrying that these early signs for IR, although discrete, are visible in both our groups with pathological perinatal exposure (PT and SGA).

Insulin resistance is thought to be the central pathomechanism towards the MetS[120]. It is among the aims of this thesis to try to identify markers or components of the MetS for our cohorts as the consequences are of utmost importance to these patients. A recent study from Denmark followed very preterm born children (<32weeks GA) at the age of 6 years who received different post discharge nutritional regimes (breast feeding, formula or fortified breast milk) for signs of the MetS. The research group could only see relatively week trends and association but no clear evidence for the presence of MetS in patients following a certain growth pattern due to formula feeding [238]. Others claim to have detected high blood pressure, high triglycerides, low HDL and elevated fasting glucose levels suggesting reduced insulin sensitivity in VLBW infants born preterm or SGA at the age of 2 years[239,240]. It is for obvious reasons difficult to find all aspects of the MetS at such young age. We were able to detect discrete signs for insulin resistance, an affected autonomous nervous system, abnormal circadian regulation and smaller kidneys in our target groups with risk factor prematurity or SGA. These findings are indicators towards components of the metabolic syndrome. It is of great importance to validate and follow up on these indicators in order to enable appropriate

timing for intervention and preventive measures. Interventions may lose effectiveness when applied too late.

Much of the research in the DOHaD field has been targeting children or adults born with LBW not differentiating between the etiologies for reduced birth weight. This led to the view that mainly the fetal period is most vulnerable rather than appreciating that the plastic phase of development extends throughout postnatal life in particular for those born preterm. Adverse events or development in utero is mainly characterized by malnourishment and impaired perfusion and maternal conditions while postnatal adversity might be more characterized by stress, pain, suboptimal nutrition, invasive infections and toxic substances in form of drugs. We therefore designed our first cohort in a way allowing us to differentiate between LBW due to prematurity or due to IUGR. With regards to kidney volume and function as well as HRV we could not see a significant difference between PT and SGA groups and it seems that intrauterine and extrauterine adverse events and development were leading to comparable effects. Only when we were studying markers for insulin resistance we detected slightly different clinical phenotypes where preterm born children showed a hepatic type of insulin resistance while children born SGA showed peripheral insulin resistance. However, it seems that there are only very few clinical outcomes related to either intrauterine or extrauterine adverse events and that in general the pathomechanismsn paving the path towards cardiovascular or metabolic sequels are not so different[236,237,241-243].

6.1 METHODOLOGICAL CONSIDERATIONS

All four studies presented here are observational, **retrospective cohort studies**. Out of a population of newborns born during a certain time period we retrospectively choose those who were born preterm and those who were born term but SGA (Study I-III) and defined these conditions as the "risk factor" or "exposure" for an "outcome". We defined outcome as kidney volume, and function, insulin resistance and HRV parameters at school age (Study I-III). For study IV we added NC as a risk factor to prematurity and had kidney volume, function and blood pressure as outcome factors.

Both selected cohorts are well defined and representative for the population to which we intended to generalize the results to. All of the individuals for study I-III, those with the exposure and controls, have been recruited from a single hospital where all the patient data was stored in similar data systems and all patients had been treated according to the same clinical guidelines. This can be regarded as an augmentation in **internal validity**. However, **external validity** is limited as we have to accept that we do not know how applicable our findings are to the risks and effects for children born and cared for at other hospitals following different protocols.

The retrospective design of a cohort study saves time and money in comparison to a prospective set up. But equivalent to the "drop outs" in a prospective cohort study the retrospective study suffers from the "lost to follow up "problem. The total numbers for both our cohorts were rather small. The selection of subjects with an exposure (in our case prematurity (all) or SGA (study I-III)) was limited to the children born with this exposure during a certain time period at our hospital (1990-1993, 2008-2011). The retrospective design

allows for **selection bias**. It is possible that among those children who were lost to follow up or those who declined to participate were children who were sicker or families felt that their child already had too many doctors' visits or would not be willing or able to comply to the study protocol. However, we analyzed the perinatal and neonatal characteristics of those non-participants and couldn't find a significant difference to the participants. All controls for the first cohort were matched for date of birth and gender. For the second cohort matching was difficult and we therefore also accepted children volunteering for controls who were slightly older (Table 10).

We have an obvious selection bias in study IV with regards to NC. During the neonatal period, it was to the attending consultant's discretion to decide whether an infant should have an ultrasound examination of the kidney to check for NC. It is likely that those infants who had been chosen appeared sicker or at higher risk to the clinicians. This also may explain the rather high numbers of NC in our cohort (50%) amongst those investigated.

The outcomes defined in our studies are supposed to be mainly affected by the exposure. We tried to restrict the effects of third factors and minimized **confounding bias** by applying inclusion and exclusion criteria as well as adjusting for confounding factors in our analysis. The outcome kidney volume and function as well as HRV parameters can be regarded as mainly affected by the exposure, namely prematurity and SGA at term. But there is of course the chance that these outcomes might have been effected by events during infancy and childhood which we haven't adjusted for. Also, the complex conditions and the complicated neonatal course for extreme preterm born children almost invites for interactions or **effect modifications** we could not control for. For example, do we not know if certain medication or treatment strategies during the neonatal period had a modifying effect on the ANS leading to the depressed activity we measured with HRV. Or if a certain diet under a certain period after discharge had an effect on kidney growth or glucose metabolism.

The additional "exposure" or "risk factor" NC was particularly vulnerable for effect modification as there is no absolute certainty about the etiology and pathogenesis of NC. However, we screened for hereditary of rare stone promoting diseases and we also reviewed all images and results from the neonatal period at the time of visit. It is also uncertain if a child screened negative for NC didn't develop signs for NC one or two weeks later. Multiple investigations would have minimized this problem. We didn't put too much emphasis on the grading of NC for the same reasons as mild NC at screening day could develop to sever NC some time later or may have been resolved. Except for the ultrasound investigation for study I where the investigator did only know the perinatal exposure of the participants after the examination there was no **blinding** in our studies.

A further weakness for study I was the fact that we investigate kidney function and kidney volume at different times with a few years in-between. It would have been beneficial to repeat blood sampling at the second visit when ultrasound investigations of the kidney and anthropometrics were performed.

We also did no distinguish between different ethnicities in our study as numbers were low. It is known from earlier research that anthropometrics are different between children from

different ethnic background and therefore also linked to organ size and volume which is again related to function[192].

Finally, to mention is that we use findings in study III (ANS/HRV) to explain finding in study IV (non-dipping). The studies were performed in different cohorts where the exposure is not entirely identical as preterm in cohort one were slightly more mature than in cohort two. However, we felt that it was appropriate to connect these findings.

Thanks to the British statistician and epidemiologist Sir Austin Bradford Hill there is a guidance using 9 criteria to evaluate hypothesized relationships. We tested one of our "effects" kidney volume, using these criteria[244,245].

The cause precedes the effect (Temporality).

Yes, prematurity or SGA at term preceded the effect of low kidney volume.

The association should be plausible/the result should be biological sensible (Plausibility/Coherence).

It seems plausible and biological sensible that IUGR or extrauterine growth restriction (EUGR) may have an effect on kidney growth and size later in life.

There should be consistent results from a number of studies (Consistency).

There are multiple studies available on this topic.

The association between the cause and the effect should be strong (Strength).

We see an association but the strength is debatable.

There should be a dose depended relationship with the effect (Biological gradient).

Yes, the association is stronger the more preterm the children were born (Study I vs IV).

The exposure causes only one disease (Specificity).

We can't state this for our studies. First prematurity doesn't automatically lead to a diseases and secondly prematurity has multiple and different effects.

Intervention or cessation of exposure should eliminate the effect (Experiment).

Yes, elimination of prematurity or IUGR should eliminate the fact that children later in life can have smaller kidneys. However, kidneys could be smaller because of other reasons for example acquired kidney diseases or by ethnicity.

If one causal agent is known and accepted the likelihood for a second agent to be causal is less (Analogy).

In our case, there is no accepted cause for having smaller kidneys as an ex-preterm born child.

It is important to remind that the studies presented in this thesis are neither designed nor powered to assess **causality**. We are describing **associations**.

6.2 ETHICAL CONSIDERATIONS

The whole field of DOHaD illustrates an ethical dilemma. For generations parents and patients have believed that once the neonatal period is overcome with reasonable few scars and acceptable development that the chapter can be closed. DOHaD research has shown that just by being born too early or being born with significantly lower birth weight than adequate for gestational age increases the risk for the development of a variety of serious conditions partly addressed in this thesis. This fact illustrates one of the greatest ethical challenges. Recruiting families required informing them about why we regarded this research important and why we wanted to include their child. By informing them about DOHaD research we certainly increased the anxiety level for the family again even though their child might be perfectly well of. Most parents were completely unaware about the increased risk for renal, metabolic or cardiovascular problems like hypertension or insulin resistance and the risk for DM to their children. We initiated more follow up for a few participants as we found some of their results worrying and worth checking. In most of these cases the control investigations were fine but we worried the families anyhow. The fear we created will stay. But more important, the awareness will eventually lead to closer follow up and maybe to a healthier lifestyle. At the day of the investigations we always repeated the reasoning behind this research project and we always highlighted the importance of lifestyle modifications where needed and the enormous risk of overweight and lack of physical activity. However, we also mentioned that other factors as for example genetic factors have a huge impact on the development of health and disease and that not all adverse development should or could be explained by the perinatal exposure.

For a few mothers and/or fathers it was difficult to come back to the place where some of them experienced a lot of pain and suffering. Others on the contrary were excited to meet doctors and/or nurses they still remembered from their neonatal period. Some families could use the meeting to ask and discuss problems which they had the feeling they couldn't discuss with their community nurses or doctors.

The examinations included in our studies were apart from line insertion and blood sampling not very invasive. An experienced nurse or doctor was responsible for line insertion after local anesthesia. The 24-hour Holter ECG as well as the 24-hour blood pressure measurements were leading to some discomfort but were overall well tolerated.

Apart from the ethical impact of this type of research on the patients and their families it raises a fundamental challenge to the field of neonatology. DOHaD research has an impact on the perspectives for this particular group of patients in terms of outcomes and quality of life. It may challenge the field of neonatology with its high speed technical advances and its strive to stretch the borders of viability if long term outcomes are worrying and difficult to improve.

Also daily practice and management might be associated with ethical competing risks for example when decision on nutritional strategies have to be made where one could wish for early catch up growth to benefit brain development but maybe to the expense of increasing risk for cardiovascular diseases and diabetes in early adulthood or the other way around[246].

Without the DOHaD research neonatologist around the world would most likely have a much more limited view on the outcomes of their patients.

7 CONCLUSION

Main findings

- Kidney volume is significantly lower in extremly preterm born children but not in very preterm or SGA born children at early school age.
- Kidney function is normal in all children born preterm or SGA at school age
- Nephrocalcinosis has no significant effect on renal size or function in children born extremely preterm at school age.
- Office and 24-hour ambulatory blood pressure measurements are normal among extremly preterm born children at school age.
- Circadian blood pressure regulation is affected in extremely preterm born children indicating a pre-hypertensive state.
- Overall HRV is significantly depressed in children born very preterm or SGA compared to controls at school age indicating dysfunction of the ANS.
- Signs for insulin resistance are present in children born preterm or SGA at school age.
- Preterm born children and children born SGA show comparable effects for HRV and kidney function but differ in typ of insulin resistance markers.

Main message

Children born very preterm or full-term with LBW have measurable morphological and or functional changes already at school age predicting a higher risk for adverse development of the cardiovascular, renal and metabolic systems.

8 FUTURE PERSPECTIVES

Despite the wide spread accessibility of medical information via social media and the internet patients, parents and families know very little about the possible long-term consequences of prematurity or IUGR addressed by DOHaD research[247]. This is unfortunately also true for medical professionals. It seems unprofessional if not unethical that we spend an enormous amount of work, resources and money on rescuing extremely preterm infants and then neither prepare the patients and parents nor the health care professionals involved in this patient category with knowledge and tools to limit the potential risk for development of chronic diseases.

So far, the prevention of prematurity hasn't been very successful. Antenatal and neonatal care need to be modified in a way that organogenesis and development is as little affected as possible. Aspects of knowledge gained from DOHaD research need to be incorporated into neonatal practice. Applying the results from this thesis into daily clinical practice could for example implicate that more attention should be given to avoid nephrotoxic drugs and drugs with the potential to negatively influence development, to avoid pain and excessive stress, to balance nutritional goals for post discharge and prevent excessive catch up and to advise and inform parents and care takers early about the detrimental effect of childhood overweight and about the need for a healthy life style. Again, preventions and interventions may lose effectiveness when applied too late.

Professional follow up needs to start in early childhood and include the regular control of blood pressure, renal function and metabolic status.

More studies are needed trying to specify the risk groups and discover pathomechanisms which would enable treatment as well as evaluating preventive measures. Ideally these studies should include more subjects but the value of rather small trials should not be underestimated.

Study IV is part of a larger project where we apart from the nephrological and cardiovascular aspect also investigate bone metabolism, growth and nutrition. We will continuously include children from the same cohort of extreme preterm born children. We will gather more data on kidney volume, function and blood pressure from this cohort and are hoping to publish this data soon.

9 SVENSK SAMMANFATTNING

Omkring 10 % av alla barn i världen föds för tidigt, före 37 graviditetsveckor. För tidig födsel är huvudanledningen till neonatal dödlighet.

Förbättringar inom neonatalvården har lett till att allt fler barn överlever till vuxenålder men en påverkad organutveckling kan leda till en ökad risk för kroniska sjukdomar senare i livet. ”Developmental Origins of Health and Disease” är ett nytt forskningsområde om hur händelser tidigt i livet kan få effekter senare i livet.

Denna avhandling omfattar fyra studier om konsekvenser av för tidig födelse eller av låg födelsevikt hos barn födda i fullgången tid.

Den första kohorten bestod av barn undersökta vid 9 och 12 års ålder, och som var födda för tidigt (före 32 veckor), födda i fullgången tid med mycket låg födelsevikt (SGA) eller födda i fullgången tid med normal födelsevikt (delarbete I-III). Förutom mätningar av njurvolym/-funktion och markörer för insulinresistens, bedömdes det autonoma nervsystemet med hjälp av hjärtfrekvens-variabilitet.

Den andra kohorten utgjordes av barn som föddes extremt för tidigt (före 28 veckor) mellan 2008 och 2011 och var i genomsnitt 7 år gamla vid undersökningen (delarbete IV). Som i delarbete I undersöktes njurvolym/-funktion. Gruppen av extremt för tidigt födda barn delades upp beroende på om de hade haft njurförkalkningar under neonatalperioden (nefrokalcinos) och friska fullgångna barn var kontrollgrupp. Vi undersökte också 24 timmars blodtryck i denna kohort.

I skolåldern var njurvolymen lägre bland barn födda för tidigt men skillnaden var enbart signifikant i studie IV. Den lägre njurvolymen hade dock ingen mätbar effekt på njurfunktionen. Flickor verkade vara mer drabbade av minskad njurvolym än pojkar. Den högre njuren var mindre än den vänstra hos extremt för tidigt födda barn men samma mönster sågs i alla grupper, även kontrollgruppen (ej signifikant).

I delarbete II hittade vi diskreta men signifikanta tecken på insulinresistens bland barn födda för tidigt och bland barn var födda fullgångna med låg födelsevikt (SGA). De två grupperna skilde sig dock: barn födda för tidigt hade en insulinresistens på central nivå (levern) medan barn som föddes SGA hade en mer perifer typ av insulinresistens (som muskelvävnad).

Båda grupperna, barn födda för tidigt och barn födda fullgångna med låg födelsevikt (SGA), hade en tydligt minskad hjärtevariabilitet som kan tolkas som en avvikande aktivitet i det autonoma nervsystemet (studie III). Vid 24 timmars blodtrycksmätning uppvisade drygt hälften inte den normala sänkningen av blodtryck från dag till natt (studie IV). Blodtrycksvärdena var i övrigt inte annorlunda mellan grupperna och befann sig huvudsakligen i normalområden.

Sammanfattningsvis uppvisar skolbarn med låg födelsevikt (för tidigt födda eller fullgångna med låg födelsevikt) strukturella och funktionella organförändringar som kan vara indikatorer för högt blodtryck, diabetes mellitus typ 2 och njurpåverkan senare i livet.

10 ACKNOWLEDGEMENTS

I am very grateful for all the support, collaboration and contribution I have received over the years which enabled me to complete this thesis but I would like to express my special gratitude to the following people:

Mireille Vanpée, my friend and main supervisor. Thank you for including me into your projects in the first place and then to encourage me to finish them. It has been a long journey and even when it was difficult sometimes I wouldn't have wanted anybody else as my main supervisor. You even kept me going when I hoped I had escaped. And thank you for being able to keep things separate.

Baldvin Jonsson, my co-supervisor and mentor. Thank you for all your guidance and clever advices and sometimes very practical help.

Mikael Norman, co-supervisor and co-author. Thank you for all your support, encouragement and aspiring guidance.

Gianni Celsi, my co-supervisor. We haven't met much but the few times we did were giving and helpful. Thank you for that.

Miriam Katz-Salamon, co supervisor, co-author and fantastic, inspiring and extremely knowledgeable person without whom study III never would have been possible.

Robert Lindvall, my official external mentor. Most of the mentoring happened during nights when we were on call. Thank you for sharing your enthusiasm for research.

Stefan Johansson, my friend, colleague and co-author. Thank you for all your help and advice and real hard work but even more important for the good times we had and will have together. I hope someday we will share a "LEXUS HEADQUARTER" again.

Anna Kistner, co-author. Thank you for your contagious attention to details.

Åsa Laestadius, co-author and research companion and friend. Thank you for all the important help you gave me but also for your expertise and knowledge. The best parts are still to come.

Ulrika Liliemark, co-author and hardworking researcher. Thank you for the enormous work you did. I really hope we will continue working together for a long time.

Boubou Hallberg, Head of department. Thank you for your support, encouragement and generosity. You are constantly surprising me, please continue to do so.

Björn Westrup, colleague and companion throughout the good years at Danderyd. Thank you for all the encouragement, good advices and the space you gave me.

Eva Berggren Broström, my highly respected former chief of service for all your smart advices and the possibilities and trust you gave me.

Hugo Lagercrantz, my very first boss (in Sweden), thank you for taking the risk in letting "The Germans" in but also for your support throughout, even when I was at the other side of the channel.

Lars, Kajsa and Morten for backing me up when I had to hide and for being reliable and very nice colleagues.

Urban Fläring, Peter Radell, Jonas Berner, Peter Larsson and Tova Hamrin Hannegård for opening doors and keeping them open, for inspiration and stimulation even in research and for just being extremely nice colleagues.

Lena Legnevall the one and only research nurse. Thank you for your extraordinary flexibility and the simplicity with which you made it all happen. You are the personalized 24/7.

Gordana Printz, for the wonderful job you did in recruiting all these children.

Denise, for always taking care of me and taking care of all these little things without nothing bigger would evolve.

Anna Sandberg, for your patience and friendliness and all your help with the exciting field of administration.

Kristina Jonsson, **Jessica Schiöt** and **Siri Lilliesköld** for your contagious optimism and constant smiles.

Anna Gudmundsdottir and **Ewa Henckel** fellow PhD students, for all the pep-talk and for sharing the PhD experience.

My son **Nicolas**, for the "unlimited mileage" you made in retrieving blood pressure monitors from families for me.

My wife **Zsuzsanna,** for coping with me throughout these last few months. Next summer will be "with" holiday, szeretlek.

Also to **all my colleagues** at the neonatal department in particular all my junior colleagues for creating a climate which makes work so much more enjoyable.

Most of all I would like to express my sincere gratitude to all the parents and children participating in our studies.

Funding:

The research studies presented in this thesis have been generously supported by grants from: Karolinska University Hospital ALF project, The Swedish Order of Freemasons Foundation for Children's Welfare, Samaritan Foundation, Sällskapet Barnavård and Lilla Barnets Fond.

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