Department of Medical Epidemiology and Biostatistics
Karolinska Institutet, Stockholm, Sweden

# HEALTH CONSEQUENCES OF ADVERSE FETAL GROWTH – STUDIES IN TWINS

Sara Öberg


Stockholm 2011

The picture “Två bröder ser på stjärnor” on the front page is reproduced with kind permission from the artist Helena Bergenrud (www.bergenrud.com)

All previously published papers were reproduced with permission from the publishers indicated in the list of publications

Published by Karolinska Institutet
Printed by Larserics Digital Print AB

© Sara Öberg, 2011
ISBN 978-91-7457-371-8

The real voyage of discovery consists not in seeking new lands
but in having new eyes

-Marcel Proust

# ABSTRACT

Findings of associations between birth weight and adult health outcomes have been taken to indicate that fetal growth – and fetal nutrition in particular – may program future health and disease in the developing individual. However, the study of prenatal exposures in humans is challenging, and most of the evidence to support fetal programming thus rests on proxy measures, such as birth weight and birth weight for gestational age. These measures are not only crude indicators for fetal growth and fetal nutrition, but also influenced by many other factors, some of which may be involved in disease development (common causes).

The aim of this thesis has been to make use of the unique features of twins to increase the understanding of potential health consequences of adverse fetal growth. Birth weight differences within twin pairs target a specific measure of fetal growth, and within-twinpair comparison further allows for evaluation of the influence of fetal growth independent of factors shared by the twins in a twin pair. Some of these factors (e.g. early socioeconomic environment and genetic factors) have been proposed to potentially confound associations between birth weight and adult disease. Twins as a group are further exposed to a more adverse fetal environment than singletons. By comparing adult morbidity and mortality in twins to singletons from twin families, the potential influence of twinning on later health could be evaluated, independent of twin family factors.

In a cohort of bi-ethnic adolescent twins in Georgia (US) we could confirm that the previously reported inverse linear association between birth weight and blood pressure was present also in African Americans, and independent of familial factors. The findings support a role for fetal programming in African Americans that warrants further tracking of the association into adulthood.

Associations between birth weight and adult outcomes were further studied in a cohort of like-sexed twins of the Swedish Twin Register, born 1926 to 1958. First the previously reported positive association between birth weight and breast cancer was investigated in over 11 000 female twins. A linear association (from 2,500 grams and upward) was noted for the log-rate of breast cancer diagnosed before the age of 50 among unrelated twins as well as within twin pairs, indicating that the intrauterine experience may play a role in the development of early onset breast cancer. Next we performed conditional logistic regression on all twin pairs discordant for cardiovascular disease (N=1942), finding birth weight inversely associated with coronary heart disease and stroke within dizygotic (DZ) but not within monozygotic (MZ) twin pairs. Understanding which factors are shared within MZ but not DZ twin pairs could help shed some light on the underlying mechanisms to the association between birth weight and cardiovascular disease.

Lastly, we compared cumulative risks of cardiovascular disease, overall cancer and death in twins, singletons from twin families as well as from the population (identified in the Multi-Generation Register and born between 1932-1958). For all three outcomes twins appeared similar to singletons of twin families, and these in turn were not found any different from singletons of non-twin families overall. The findings indicate that the unique experience of twinning does not influence risk of adult morbidity or mortality compared to the general population.

# LIST OF PUBLICATIONS

I. Oberg S, Ge D, Cnattingius S, Svensson A, Treiber F.A, Snieder H, Iliadou A. Ethnic Differences in the Association of Birth Weight and Blood Pressure - The Georgia Cardiovascular Twin Study
*Am J Hypertens*. 2007 Dec; 20(12):1235-41.
© Nature Publishing Group

II. Oberg S, Cnattingius S, Sandin S, Lichtenstein P, Iliadou A. Birth Weight – Breast Cancer Revisited: Is the Association Confounded by Familial Factors?
*Cancer Epidemiol Biomarkers Prev*. 2009 Sep;18(9):2447-52.
© American Association for Cancer Research

III. Oberg S, Cnattingius S, Sandin S, Lichtenstein P, Iliadou A.N. Birth weight predicts risk of CVD within DZ but not MZ twin pairs: a large population-based co-twin-control study
*Circulation.* 2011 (in press)
© American Heart Association

IV. Oberg S, Cnattingius S, Sandin S, Lichtenstein P, Morley R, Iliadou A.N. Twinship influence on morbidity and mortality across the lifespan
(*Manuscript*)

# TABLE OF CONTENTS

**ABSTRACT** ..... **1**

**LIST OF PUBLICATIONS** ..... **2**

**TABLE OF CONTENTS** ..... **3**

**LIST OF ABBREVIATIONS** ..... **5**

**1 INTRODUCTION** ..... **6**

**2 BACKGROUND** ..... **8**

**2.1 FETAL GROWTH** ..... **8**

2.1.1 FETAL GROWTH REGULATION ..... 8

2.1.2 MONITORING AND MEASUREMENT ..... 9

2.1.3 PATTERNS OF FETAL GROWTH ..... 10

**2.2 HEALTH CONSEQUENCES OF ADVERSE FETAL GROWTH** ..... **11**

2.2.1 DEVELOPMENTAL ORIGINS OF ADULT HEALTH AND DISEASE ..... 11

2.2.2 CARDIOVASCULAR DISEASE ..... 12

2.2.3 BLOOD PRESSURE ..... 14

2.2.4 BREAST CANCER ..... 15

2.2.5 POTENTIAL MECHANISMS ..... 16

**2.3 CAUSAL INFERENCE IN OBSERVATIONAL STUDIES** ..... **20**

2.3.1 COMMON THREATS TO CAUSAL INFERENCE ..... 20

**2.4 TWINS** ..... **25**

2.4.1 BIOLOGY ..... 25

2.4.2 FETAL GROWTH IN TWINS ..... 26

2.4.3 THE USE OF TWINS IN DOHAD ..... 28

**3 AIMS** ..... **30**

**4 MATERIALS & METHODS** ..... **31**

**4.1 SETTINGS** ..... **31**

**4.2 DATA SOURCES** ..... **31**

4.2.1 TWIN COHORTS ..... 31

4.2.2 NATIONAL REGISTERS AND LINKAGE ..... 34

**4.3 STATISTICAL METHODS FOR TWIN DATA** ..... **36**

4.3.1 METHODS TO HANDLE NON-INDEPENDENT OBSERVATIONS ..... 36

4.3.2 METHODS TO COMPARE TWINS IN A TWIN PAIR ..... 37

**4.4 STUDY DESIGNS & METHODS** ..... **40**

4.4.1 PAPER I ..... 40

4.4.2 PAPER II ..... 41

4.4.3 PAPER III ..... 43

4.4.4 PAPER IV ..... 44

**5 RESULTS** ..... **47**
**5.1 BIRTH WEIGHT & BLOOD PRESSURE IN BIETHNIC TWINS (I)** ..... **47**
**5.2 BIRTH WEIGHT & BREAST CANCER IN FEMALE TWINS (II)** ..... **48**
**5.3 BIRTH WEIGHT & CARDIOVASCULAR DISEASE IN TWINS (III)** ..... **49**
**5.4 MORBIDITY & MORTALITY IN TWINS AND SINGLETONS (IV)** ..... **50**
**6 DISCUSSION** ..... **51**
**6.1 TWINS AND DOHAD** ..... **52**
6.1.1 EXTERNAL VALIDITY OF TWIN STUDIES ..... 52
6.1.2 INTERNAL VALIDITY OF TWIN STUDIES ..... 53
**6.2 SPECIFIC METHODOLOGICAL CONSIDERATIONS** ..... **54**
**6.3 FINDINGS & IMPLICATIONS** ..... **57**
**6.4 OVERALL CONCLUSIONS** ..... **62**
**7 ACKNOWLEDGEMENTS** ..... **64**
**8 REFERENCES** ..... **66**

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AA | African American |
| BMI | Body mass index |
| CHD | Coronary heart disease |
| CI | Confidence interval |
| CVD | Cardiovascular disease |
| DC | Dichorionic |
| DOHaD | Developmental Origins of Health and Disease |
| DZ | Dizygotic (fraternal) |
| EA | European American |
| e.g. | For example |
| GCTS | The Georgia Cardiovascular Twin Study |
| HR | Hazard ratio |
| i.e. | That is |
| IGF | Insulin like growth factor |
| MC | Monochorionic |
| MCMA | Monochorionic monoamniotic |
| MZ | Monozygotic (identical) |
| MZMC | Monozygotic monochorionic |
| NIDDM | Non-insulin dependent diabetes |
| OR | Odds ratio |
| SALT | Screening Across the Lifespan Twin study |
| SD | Standard deviation |
| SGA | Small for gestational age |
| STR | The Swedish Twin Register |
| resp. | Respectively |
| vs. | Versus |

# 1 INTRODUCTION

The time we spend in utero is undoubtedly *the* most important developmental time period of our lives; we go from one cell to a viable individual in roughly 38 weeks! Potential disturbances of growth and maturation in the perinatal period may seriously affect the future functioning of the developing individual. If responsible triggers and insults could be correctly identified, carefully weighted interventions could have great impact on the life and health of the individual. However, if a targeted agent is not causal, the intervention may be ineffective, costly and potentially even harmful. In the special case of pregnancy the importance of establishing causality is further emphasized, as interventions may affect both mother and fetus.

The last two decades have seen the emergence of a research field dedicated to the Developmental Origins of Health and Disease (DOHaD). Following the first findings of an inverse association between birth weight and mortality from coronary heart disease, birth weight has been found inversely associated with both cardiovascular disease and its risk factors, including hypertension and non-insulin dependent diabetes (NIDDM) [1-4]. Considering birth weight a proxy for fetal growth and fetal nutrition, it has been suggested that critical insults to fetal nutrition may program later disease [5]. With most focus on the role of fetal and maternal nutrition, this has paved way for sophisticated evolutionary theories of developmental plasticity, whereby the fetus adapts in preparation for the outside world in response to signals via the nutritional supply line from the mother [6]. While this field of research has moved on to consider such signals (prenatal exposures) that may not even influence birth weight or fetal growth, the underlying mechanisms of the original findings remain to be explained. Birth weight is a product of gestational age and fetal growth, and is thus a crude proxy for fetal growth, which in turn is an even more crude proxy for fetal nutrition. The factors that influence birth weight and fetal growth may also be involved in cardiovascular disease development, e.g. socioeconomic and genetic factors. It is thus possible that the reported associations could be partly or entirely explained by the presence of shared common causes (confounding). It is also possible that suboptimal fetal growth results in morphological and structural changes in the developing fetus that will ultimately influence later disease risk.

The perhaps most studied association within this field is that between birth weight and blood pressure [7-9]. Drawing on this well-established inverse association, it has been proposed that the higher blood pressure levels seen in African Americans (AAs) compared to European Americans (EAs) could be explained by fetal programming, as AAs also experience higher incidence of both low birth weight and small for gestational age, compared to EAs [10, 11].

At the other end of the spectrum, excessive fetal growth and high birth weight may not necessarily benefit future health. In 1990 Trichopoulos proposed that breast cancer may originate in utero [12], speculating that intrauterine exposure

to pregnancy estrogens could increase breast cancer risk in female offspring, both by increasing the amount of cells at risk and the likelihood for malignant transformation [13]. Considering birth weight a proxy for hormone exposure in utero [14, 15], the positive association between birth weight and risk of breast cancer [16-19] has been put forward as supporting evidence of the hypothesis. It is also possible that birth weight and breast cancer share common causes, which confound the association.

Twin siblings share early environment, and a varying degree of genetic factors. As a result, birth weight differences within twin pairs will reflect fetal growth that is independent of gestational age, maternal and other factors shared by the twins. Within monozygotic (MZ) twin pairs birth weight reflects fetal growth that is independent also of genetic factors. Thus, evaluating the effects of birth weight within twin pairs may help gain more insights about the early life influence on adult disease. Previous investigations of disease-discordant twin pairs from the Swedish Twin Registry have shown attenuation of birth weight associations within MZ twin siblings for NIDDM [20] but not for hypertension [21].

Findings in twins are commonly questioned concerning their representativeness of the general population. This is of particular concern with respect to perinatal conditions where differences in prenatal environment between twins and singletons need to be considered. Due to spatial and nutritional constraints, twins generally have shorter gestation as well as lower birth weights for gestational age, compared to singletons. Yet, twins do not appear to be different from the general population with respect to cardiovascular mortality in adulthood [22, 23]. At the same time, there are many reports of associations between birth weight and adult diseases in twin samples similar to those widely reported in singleton populations [20, 21, 24]. It has been suggested that the *general growth constraint* of twinning (due to sharing of space and supply line) may not influence adult disease risk, whereas the factors that make twins experience different growth (compared to unrelated twins and/or the co-twin) could [25]. For cancer overall, twins do not appear to differ from the general population. Reports from studies of specific types of cancer indicate that twins may experience lower risk of some types of cancers [26-28] and higher risk of others [26, 28]. Previous twin-singleton comparisons could also be confounded if twins as a group were to have a slightly different genetic or socioeconomic background than the general population [29].

The main aim of this thesis has been to take advantage of the unique features of twins to increase the understanding of how early life exposure may influence later health. In the first three studies previously well-described associations between birth weight and later health outcomes are re-assessed within twin pairs in order to evaluate the influence of fetal growth that is independent of factors shared by twins in a twin pair. In the final study, the health consequences (in terms of adult morbidity and mortality) of the twin experience, whether being a twin or a member of a twin family, are investigated.

# 2 BACKGROUND

## 2.1 FETAL GROWTH

By tradition, a pregnancy dates from the first day of the last menstrual period [30]. Because of the variability of the menstrual cycle, both within and between women, this will be between 1 to 3 weeks before the ovulation that enables fertilization takes place [31]. It follows that the gestational age will overestimate that of the fetus by approximately 2 weeks. A normal pregnancy lasts 281-282 gestational days [32]. Stages of growth and development are commonly described in relation to the three trimesters of pregnancy, referring to the first 14 weeks (1st trimester), weeks 14-28 (2nd trimester) and weeks 28-40 (3rd trimester).

Essentially all vital structures and organs are formed already during the first 8 weeks of life, in what is called the embryonic period. For the remainder of the pregnancy, the fetal period, focus is on growth, development and maturation.

Fetal growth is a carefully weighted process, in which the needs of the fetus must be balanced against the capacity of the mother. To secure survival of the offspring the fetus has to be allowed to grow and mature, but not more than it can also be delivered without complication. According to the parent-offspring conflict, the mother may also have an invested interest to protect her future reproductive capacity by not putting all effort into one offspring [33]. Along these lines, parental imprinting of genes has inspired the idea of an evolutionary genetic struggle between paternal and maternal genes, where paternal genes would seek to optimize growth whereas the maternal would balance or counter this [34].

### 2.1.1 FETAL GROWTH REGULATION

The premises for fetal growth are thus a fetus with a certain growth potential, and a space and supply line to enable it. In other words, the full realization of the fetal genetic growth potential will depend on the physical constraints (maternal physiognomy) and availability of nutrients and oxygen (the supply line from the mother). Thus, the mother influences the potential for growth both by providing genes (to the fetus) and intrauterine environment (which in turn will be a result of both her genes and environment). Maternal genetic factors have been found to account for approximately 20%, and fetal genetic factors for 30 %, of the normal variation in birth weight [35] and the liability for small for gestational age [36].

Since the mother is the sole supply provider, her own consumption of nutrients as well as other substances (such as caffeine, alcohol and tobacco) may affect fetal growth. Other impairing maternal conditions, such as hypoxia and systemic disease, may also influence the availability of nutrients and oxygen to the fetus. Some of the maternal exposures may further exercise an influence by impairing the function of the placenta, which is the main regulator of the supply line. In addition to enabling and maintaining the critical exchange of nutrients and

oxygen, the placenta serves as a barrier (e.g. for maternal glucocorticoids) as well as a communicator between the mother and fetus. The placenta is also an important producer of hormones (e.g. estrogens, progesterone, human placental lactogen and leptin) and local growth factors (e.g. insulin-like, fibroblast, and vascular endothelial growth factors) which may regulate fetal growth by influencing the growth and function of the placenta [37].

A number of factors influencing fetal growth can be expected to do so through one or several of these pathways [38]. Height and weight of the mother could for example be linked to fetal growth through the (genetic) growth potential and also the maternal milieu. Among the most consistent predictors of fetal growth are socioeconomic status (SES), ethnicity and fetal sex. The influence of SES is likely mediated by maternal factors such as diet, smoking, alcohol, body constitution, etc. While the same applies to some of the observed ethnic influence as well, it may not account for all of the discrepancy between Americans of African and European descent [38]. Lastly, while an influence of sex on fetal growth is well known, the mechanisms and patterns are not well understood. Male embryos appear to experience more rapid cell division and end up, on average, larger than girls of the same gestational age at birth. Still, they are at higher risk of adverse perinatal outcome, and accumulating evidence suggests that boys are more vulnerable to various influences throughout gestation [39].

### 2.1.2 MONITORING AND MEASUREMENT

Growth is a dynamic property, and as such needs to be evaluated between at least two points in time. Rough estimates of fetal growth could be obtained from monitoring maternal weight gain and measurements of symphysis-fundus height throughout pregnancy [40]. In the last decades ultrasound examination has made biometry of the fetus possible, but in clinical practice repeated monitoring is generally only performed on indication (high-risk pregnancies) [41] .

Anthropometric measures (weight, length and head circumference) are routinely recorded at birth. As with any cross-sectional anthropometric measure, these will be a result of the cumulative growth and age of the individual. It is thus common for fetal growth to be evaluated from curves based on birth weight for gestational age. Importantly, for any given gestational age, the weight prediction of such a curve will be based on those who were born at that age. Such revealed weight curves may not be representative of the intrauterine population, and may underestimate fetal growth in preterm born infants [42].

Of the three most commonly recorded anthropometric measures at birth, weight is the most readily available as well as the most reliably measured [43]. As an approximation of growth, some claim birth weight may be more indicative of environmental influences in later gestation compared to length and head circumference, possibly due to “sparing” of head and length [35, 44].

### 2.1.3 PATTERNS OF FETAL GROWTH

Based on the sigmoid shape of standard intrauterine growth curves, fetal growth is often described to be approximately linear, with a noticeable decline in the rate of growth toward term [45]. The decline has further been attributed to a gradual depletion of the nutritional influx (either from mother, placenta or both) [46]. Others claim that the plateau seen in growth curves towards term may be a result of inaccurate pregnancy dating, placing birth weights of earlier gestation at term (due to overestimation by the last menstrual period method) [47]. Curves based on ultrasonographically estimated fetal weights in uncomplicated term pregnancies indicate a fairly constant rate of growth until term [42].

It is also commonly assumed that the overall linear growth should be the result of a sequential timing of the body dimensional growth such that length growth is favored in the 2nd trimester and weight in the 3rd [48]. The data to support this rests on cross-sectional measures of live-born at various gestational ages [49, 50] . More recent longitudinal measurements using ultrasound have failed to confirm the proposed timing of growth in length versus weight [51, 52].

Intrauterine growth restriction is defined as a reduction in the expected pattern of fetal growth (i.e. failure to reach the growth potential). Assessment thus requires repeated measures; nevertheless in most practice evaluation is made cross-sectionally, by relating size to gestational age. Based on the distribution in the sample or a reference population, a cut-off either at 2 standard deviations (SD) below the mean or below the 10th percentile is used to define small for gestational age (SGA). Measures that describe the tail of the normal distribution may not successfully discriminate between those who are constitutionally small and those that are pathologically small. In an attempt to address this, some advocate the use of customized growth curves, in which various predictors of growth may be taken into account (e.g. sex, ethnicity, maternal stature, parity etc.) [47]. Others caution against inappropriate adjustment for factors associated with pathological growth restriction, as in the case of ethnic (AA vs. EA) differences in fetal growth [53, 54].

Intrauterine growth restriction is commonly assumed to result in two different phenotypes with respect to body proportionality (at birth). These have further been attributed different etiologies on account of timing and type of insult. The "timing hypothesis" stipulates that early and/or chronic insults (e.g. congenital malformations, maternal smoking and low socioeconomic status) will result in symmetric growth restriction. Conversely, asymmetric growth restriction would be the consequence of a faltering supply line later in pregnancy, resulting in short (if in in 2nd trimester) or long and thin neonates (if in 3rd trimester) [48]. Building on the notion of a sequential fetal growth pattern, the timing hypothesis has had great impact both in the theoretical and the practical world of perinatology and obstetrics. The theory has also been used in epidemiological practice as a means of identifying potential critical periods of prenatal insults, not least with respect to the potential influences on adult disease [55].

Independent of the more recent longitudinal findings failing to support the sequential growth pattern on which the timing hypothesis rests, several others have questioned the use of proportionality as a means to infer etiology of the insult (whether in time or character) [56]. With respect to growth restriction, proportionality has rather been proposed to reflect a continuum, with progressive disproportionality as severity of growth restriction increases [44]. The weak support for the existence of proportionality phenotypes in the normal population should further discourage their use in attempts to identify stages of development at which undernutrition would lead to permanent, negative health outcomes [57].

## 2.2 HEALTH CONSEQUENCES OF ADVERSE FETAL GROWTH

Adverse fetal growth may put the fetus at risk of complications both inside and outside the womb. Severe growth restriction may lead to intrauterine death, and growth restricted newborns are at increased risk of perinatal morbidity and mortality. Babies that are large on the other hand are at increased risk of delivery complications, with negative consequences for both infant and mother. In this thesis however, the focus is on the potential long-term consequences of adverse fetal growth, which have attracted much research attention in the last decades.

### 2.2.1 DEVELOPMENTAL ORIGINS OF ADULT HEALTH AND DISEASE

In 1986, drawing from their findings in an ecological study geographically linking previous infant mortality to later coronary heart disease mortality, David Barker and Clive Osmond proposed that early life deprivation could predispose coronary heart disease [58]. This was by no means a novel idea, but rather built on findings by Kermack, Rose and Forsdahl who in the 1930s, 1960s and 1970s respectively had noted that the early environment appeared to be of importance for later health [59-61]. What made Barker & Osmond's paper seminal was the emphasis made on the influence of nutrition in prenatal and early postnatal life, from which the hypothesis of a fetal origin of adult disease was developed. This hypothesis was further elaborated in a number of papers (and books) and by 1995 refined to state "that coronary heart disease is associated with specific patterns of disproportionate fetal growth that result from fetal undernutrition in middle to late gestation" [5]. By then the Southampton group had linked individual anthropometric measures at birth, and birth weight in particular, to coronary heart disease mortality, blood pressure and NIDDM [1, 62, 63].

With the launch of the fetal origins hypothesis, the interest in early nutrition came to almost exclusively target the prenatal period, sparking an avalanche of observational studies linking size at birth to a number of outcomes later in life. Other than cardiovascular and metabolic outcomes, size at birth has for example been shown to predict psychiatric disorders and asthma [64, 65]. Parallel to this process, clinical researchers were becoming increasingly concerned with the long-term effects of postnatal nutrition, leading them to invoke the concept of "programming", whereby a stimulus or insult at a critical period will have lasting consequences [66].

To explain their findings of an inverse association between birth weight and glucose intolerance and NIDDM, Hales and Barker further hypothesized about a "thrifty phenotype". According to this, a fetus subjected to scarce resources may develop insulin resistance in order to limit growth and thereby increase the chance of survival [67]. The mismatch that occurs if such a phenotype enters a world of plenty would then lead to the development of metabolic disorders.

The idea of early programming as an adaptive response has recently been adopted and further refined by evolutionary theorists. Through a process known as plasticity, it has been suggested that the fetus adjusts its phenotype according to predictions about the future, made possible by nutritional or hormonal signaling from the mother [68]. The fact that these mechanisms persist, and that the environments we live in today are so different from those within which they evolved, may further contribute to explain the increasing burden of metabolic and cardiovascular disease [69].

In an effort to unify the past and ongoing research of the field and to signify the "paradigm shift" in the perception of early life influence, the term Developmental Origins of Adult Health and Disease (DOHaD) was proposed in 2004 [69]. This field now covers a broad range of research including experimental, evolutionary, clinical, and laboratory research, and life-course epidemiology.

To note, the increasing focus and reliance on animal models (in which the maternal diet can be manipulated) within the DOHaD framework has led to the recognition that birth weight (and presumably also fetal growth) may not necessarily be on the causal pathway between such insults that trigger adaptive responses and their consequences [70]. However, other than natural experiments of intrauterine malnutrition during the Dutch famine and Leningrad siege [71, 72], studies in humans have been restricted to the use of proxies for fetal growth, predominantly size at birth. While these measures are poor proxies for fetal nutrition (or other triggers of an adaptive response), their association to later health outcomes appears to be robust (replicated in various populations) and also strikingly consistent across the entire range of normal variation (of birth weight).

### 2.2.2 CARDIOVASCULAR DISEASE

In the past century there has been an epidemiological transition with cancer and cardiovascular disease (CVD) replacing infectious disease and malnutrition as the main causes of death in most high-income countries [73]. At the turn of the millennium CVD was the leading cause of death worldwide [74]. Although mortality from CVD is declining in the developed world as a result of improved diagnosis, treatment and prevention, coronary heart disease and stroke are still the 1st and 3rd most common causes of death [75].

Identification of risk profiles for CVD has been one of the most important public health contributions of epidemiology, improving not only prevention but also diagnosis and treatment. At the time Barker launched his hypothesis the main life-

style and other modifiable risk factors (e.g. raised blood pressure, blood glucose and lipids, obesity, tobacco smoking, and physical inactivity) had been established, and genetic association studies had not yet been well developed. It is perhaps then not so surprising that the promise of an entirely new framework with potentially modifiable precursors was met with much interest and enthusiasm.

Following Barker and colleagues' first findings of inverse associations between birth weight and mortality from coronary heart disease [1], a number of studies have linked weight at birth to cardiovascular disease overall, hypertension, coronary heart disease and stroke . Overall, it appears as though the association is (inverse) linear, with a decrease in birth weight associated with an increase in the risk of CVD across the entire range of normal birth weights.

There have also been several attempts to use other anthropometric measures at birth, alone or in relation to birth weight (as with ponderal index, where birth weight is divided by the birth length cubed) in order to target distinct temporal patterns of undernutrition (building on the notion that timing of growth restriction results in a particular body shape) [55]. Maternal pelvic size and placental weight have also been used to speculate about the roles of maternal nutrition and placental function in fetal programming [76]. Only a minority of studies have had the ability to adjust for gestational age, or potential confounders such as maternal smoking or socioeconomic status. On the other hand, most studies have adjusted for measures of body size later in life (e.g. weight in infancy or BMI in adulthood). Interestingly this does not only appear to generally strengthen the association at hand, but in many studies in fact be necessary to achieve statistical significance. A potential explanation, as well as cause for caution concerning this practice, is presented in the causal inference section (page 22).

#### 2.2.2.1 CORONARY HEART DISEASE

In a systematic review and meta-analysis from 2007, Huxley et al. evaluated 18 published and 2 unpublished studies, and found that all had reported an inverse association between birth weight and risk of coronary heart disease [2]. There further appeared to be little heterogeneity between the results of the studies. Another more recent meta-analysis reviewed the association between birth weight and cardiovascular disease mortality [77]. Out of 14 studies included, 11 had reported of an inverse association. Neither of these reviews found any indication of difference in effect between men and women. They also shared the conclusion that many of the included studies had had limited ability to adjust for gestational age and potential confounders.

#### 2.2.2.2 STROKE

Low birth weight has been shown to predict stroke in both men and women [76, 78-81]. Some indications of a more pronounced association for hemorrhagic stroke [78, 79] have not been confirmed in larger studies [80, 81]. The number of hemorrhagic cases also tends to be small, leading to imprecise estimates.

#### 2.2.2.3 HYPERTENSION

Upon their first finding of an association between weight at birth and blood pressure in middle-aged men and women, the Southampton group concluded that prevention of hypertension may depend on improving maternal health and nutrition [82]. While a few later studies have evaluated hypertension as main outcome [3, 21, 83], the overwhelming majority of studies that followed have assessed the association with blood pressure continuously. As a result it was concluded a decade later that the inverse association between birth weight and blood pressure appeared robust, albeit modest [7]. To reconcile the small effects of birth weight on blood pressure with its proposed large effect on the risk of hypertension, it was later suggested that slow fetal growth initiates a self-perpetuating process that in turn leads to hypertension in adulthood [84].

### 2.2.3 BLOOD PRESSURE

As already indicated, the best-studied outcome with respect to early life influence on later health is, by far, blood pressure. This may perhaps in part be a result of the discovery that the association could be studied in children, facilitating follow-up and search for birth data. A joint effort of two reviews of the literature up until the year of 2000 found 80 studies that had explored the association between birth weight and blood pressure in children and adults [85]. The majority had reported that blood pressure fell with increasing birth weight, with an effect size of -2 mmHg/kg (pooled). A closer look at the 55 studies that had reported regression coefficients indicated that this effect may have been overestimated due to publication bias (favoring of small studies with large effects), failure to account for random error, inappropriate (current body size) and inadequate adjustments for potential confounding [7].

#### 2.2.3.1 AGE

Among the first to explore the association between birth weight and blood pressure in children, Whincup *et al*. noted that an inverse association was present already in 5-7-year olds, however only statistically significant with adjustment for current size [86]. With exception of the newly born, it appears as though the inverse association may be apparent across the lifespan, and potentially amplify with age [9, 87]. The association also appears to temporarily attenuate throughout adolescence [85, 88], which has been suggested to be a result of perturbed tracking of blood pressure during the adolescent growth phase [89, 90] possibly due to puberty-related hormonal changes [88]. The general indications are thus that the association appears already in early childhood, attenuates with puberty, and then manifests and potentially even amplifies throughout adulthood.

#### 2.2.3.2 ETHNICITY

In the US it has been long recognized that African Americans have higher blood pressure levels than European Americans, and the difference appears to manifest already in childhood and adolescence [91]. Since AAs are also at increased risk of being born small compared to EAs of the same gestational age [92], it has been proposed that the ethnic difference in blood pressure originates in utero [10, 11]. However, the association motivating such a claim has been established in predominantly white populations. Two previous smaller studies of AA adults have found no correlation between size at birth and blood pressure [93, 94]. In children, findings have been inconsistent, with some indications of a positive association in young children [95, 96]. Longitudinal tracking of a bi-ethnic sample however has shown an inverse association in both AAs and EAs, amplifying with age (into adulthood) [97].

### 2.2.4 BREAST CANCER

Parallel to the developing theory of a fetal origin for cardiovascular disease, it was hypothesized that breast cancer may originate in utero [12]. In 1990, Trichopoulos speculated that prenatal estrogen exposure would create a "fertile soil" for breast cancer disease development, and that diet during pregnancy could be a potential determinant of pregnancy estrogen levels [12]. The hypothesis was inspired by observations that exposure to prenatal ionizing radiation increase risks of childhood leukemia [98], and that daughters of mothers using diethylstilbestrol (DES) during pregnancy were at increased risk of vaginal adenocarcinoma [99]. It was thus speculated that risk factors of cancer could operate already in the fetal period. An increasing body of evidence had already supported a link between estrogen and breast cancer (via reproductive factors indicative of endogenous estrogen exposure, such as parity, age at menarche, first birth and menopause [100, 101] and exogenous exposure in the form of hormone replacement therapies [102]). Trichopoulos further hypothesized that prenatal estrogen exposure would increase the number of susceptible stem cells in the mammary gland, in which cancer may later develop [103].

A number of observational studies have reported positive associations between breast cancer and potential indicators of prenatal estrogen exposure, such as maternal age [104, 105], preeclampsia [106] and twinning [106, 107]. Trichopoulos' initial proposal that twins may be at lower risk due to shorter gestation was modified with the suggestions of higher maternal estrogen levels in twin pregnancies, and dizygotic in particular [108, 109]. Evidence to support an increased risk in twins has however been inconclusive. Overall twins do not appear to be at different risk of breast cancer compared to singletons [17], and a modest risk increase in dizygotic twins remains speculative [17].

Birth weight is the most studied indicator of prenatal estrogen exposure to date. While birth weight is positively correlated with maternal estrogen [110], available data to date do not support any correlation with fetal estrogen concentrations [111]. The original hypothesis' focus on estrogens has nevertheless been

generalized to include other hormones [112], with particular interest in the role of insulin-like growth factor I and II (IGF-I and IGF-II). These peptides have been implicated in breast carcinogenesis [113, 114], and are also believed to be major modulators of both fetal and placental growth [115, 116].

In recent years several reviews and a large re-analysis of individual level data including over 22,000 cases have all confirmed a positive association between birth weight and risk of breast cancer, in particular when diagnosed before menopause [16-19]. While the majority of studies report of a linear association, some have noted an increased risk of breast cancer also in the lower end of the birth weight range [105, 117]. Birth length has also been reported a predictor of breast cancer, independent of birth weight [19].

### 2.2.5 POTENTIAL MECHANISMS

Crude measures of fetal growth (predominantly birth weight) have been shown to predict a number of health outcomes later in life, with inverse associations to blood pressure, risks of CVD and NIDDM being the most consistently reported. While these findings have inspired to well-developed and widely accepted theories about early life influence on health and disease, the underlying mechanisms to the associations are yet to be unraveled.

#### 2.2.5.1 SUBOPTIMAL GROWTH – SUBOPTIMAL FUNCTION

Considering not only the degree of development and growth in the prenatal period, but also the vital features and structures of nearly every organ, it is quite astonishing that organ malformation or malfunctioning is so rare. In this light it also seems quite plausible that disruptions that interrupt or impair these processes may result in structural and functional long-term effects. With respect to cardiovascular disease development, the vascular tree will be of importance and impaired endothelial function and arterial stiffness are risk factors for both hypertension and stroke [118]. Another often mentioned plausible candidate to suffer structural changes if deprived during development is the kidney. A few studies in humans have found a linear association between birth weight and nephron number [119, 120]. Reduced nephron number has in turn, on account of animal models, been hypothesized to lead to increased filtration pressure (glomerular hyper filtration) and, ultimately, hypertension [121]. Another strong candidate proposed to play a role both in the occurrence and/or perpetuation of growth restriction and the pathogenesis of disease is glucocorticoid exposure. It has been speculated that prenatal glucocorticoid exposure could reset the hypothalamic-pituitary axis and the following endocrine changes could contribute to the progression of disease [15]. Structural changes in vital organs, such as muscle, liver, and pancreatic tissue, may further lead to impaired metabolic control, that could contribute to disease development later in life [122-124].

While there are a number of studies to support structural changes in animals, evidence from humans is scarce. In humans, most studies into the potential mechanisms have involved linking birth weight to known or hypothesized antecedents of cardiovascular disease (to evaluate a potential causal pathway). Birth weight can for example predict endothelial function in adulthood [125] and low birth weight has been linked to sympathetic nerve activity [126], beta-cell function [127], and low grade inflammation [128], all of which have been shown to play a part in cardiovascular disease development.

#### 2.2.5.2 DEVELOPMENTAL PLASTICITY AND EPIGENETICS

While structural changes may be plausible in the case of fetal growth restriction, they may be more difficult to reconcile with the consistent observations of effects across the entire range of birth weight, and not just in the supposedly growth restricted [6]. Building on the theory of a mismatch between the early and later nutritional environment, the adaptive predictive response was proposed as a unifying explanation to observed associations [68]. Through this, nutritional stimuli would work as clues about the outside world according to which the fetus could tune its metabolism in expectation of the future (to increase the chance of survival in the long term, rather than the short). However, if the predictions fail (deprived intrauterine environment does not equal deprived outside world) the mismatch of the altered homeostatic capacity and the environment could lead to negative health outcomes. The interest for the theory of developmental plasticity to explain the impact of early life on later health and disease has surged with the advent of epigenetics, offering a promising mechanism through which it could act.

Developmental plasticity is described as a regulated phenomenon by which one genotype can give rise to a range of different phenotypes. While environmental stimuli may directly affect gene expression, such effects are often transient and reversible, and may thus not readily explain the long-term changes proposed to act in programming. Chemical modification of the DNA or chromatin – i.e. epigenetic modulation – on the other hand, can lead to a permanent switch into "on" or "off" mode. Environmentally induced epigenetic changes thus offer a sophisticated and plausible mechanism for how environmental influences could produce the proposed adaptive programming of various phenotypes [68].

Epigenetic modulation of the genome plays an integral part in the regulation of gene expression throughout development. By silencing specific genes it initiates and perpetuates cell differentiation, and is also the mechanism behind imprinting (asymmetrical expression of alleles depending on their parent-of-origin) and X-chromosome inactivation in females. Importantly, epigenetic change can occur in response to extracellular signals, e.g. endocrine and nutrient. Commonly, epigenetic modulation is defined as heritable changes in gene expression not caused by altered DNA sequence [129]. This stems from the observation that all epigenetic mechanisms are mitotically heritable (maintaining e.g. cell-specific differentiation). Although imprinted genes appear to have developed mechanisms to convey epigenetic information across generations, the extent to which such transmission is possible for other epigenetic changes is not known [129].

With respect to DOHaD, an increasing number of animal models indicate that nutrition (and other environmental stimuli) during early development may influence the establishment and/or maturation of epigenetic mechanisms, ultimately leading to permanent changes in gene expression [130]. The role of epigenetics in cardiovascular disease development remains to be elucidated. The strongest link between CVD and epigenetics to date appears to be through homocysteine, as hyper-homocysteinemia is associated with increased risk of CVD and can also impair the one-carbon metabolism, of importance for DNA-methylation (the most widely known epigenetic mechanism) [131]. There are also some indications of links between epigenetic changes and early vascular atherogenesis [132, 133]. However, as a recent review of epigenetics and CVD concluded "prenatal epigenetic contributions to adult CVD risk in humans are often inferred, but difficult to confirm in observational studies" [134].

While most of the mechanisms (e.g. altered function of the kidney, vascular tree and pancreas) proposed to bridge fetal growth restriction with adult disease are also implicated in developmental plasticity, the key discriminating factor is that these are then not the result of structural damage due to impaired fetal growth, but rather secondary to epigenetic changes in gene expression [6].

#### 2.2.5.3 BREAST CANCER AND EPIGENETICS

Epigenetics have also been proposed to affect virtually every step in cancer progression [135]. 10-15% of women with non-familial breast cancer have for example been found to have a hypermethylated BRCA1 gene [135]. Interestingly, loss or relaxation of imprinting of the IGF2 gene resulting in bi-allelic expression has been linked to cancer of the breast [113, 114] as well as to fetal growth stimulation [116]. However, due to the cross-sectional nature of studies showing epigenetic changes in tumor cells, it is not possible to establish whether these are causes or effects of the carcinogenous process [130].

#### 2.2.5.4 CONFOUNDING

Another mechanism by which size at birth would appear associated with later health outcomes is if they share common causes. In such a case, the association could be partly or entirely due to confounding.

In the early ecological studies both Forsdahl and Barker discussed the possibilities that the associations seen may be the result of other factors independently related to infant mortality and cardiovascular disease mortality, notably maternal smoking and the socioeconomic environment [58, 61]. With respect to the findings of individual birth weight and coronary heart disease mortality, it was also speculated that the environment in which low birth weights are more common may also negatively influence health in adulthood [62].

Socioeconomic environment is a predictor of birth weight and fetal growth [38] and it was recently shown that the risk of CVD was inversely related to (fathers) social class at the time of birth [136]. Maternal smoking during pregnancy also influences birth weight (by a negative effect on both fetal growth and length of gestation) [137] and children of mothers who smoked during pregnancy have been found to have higher blood pressure than those of mothers who did not smoke [138]. Although maternal smoking has been proposed to explain the connection between birth weight and blood pressure [139], this has not been supported by studies evaluating the influence of maternal smoking during pregnancy on the association [140, 141]. Several studies have also attempted to account for the potentially confounding influence of the socioeconomic environment at birth on the association between birth weight and adult outcomes. Overall, results from these studies indicate that adjusting for various measures of social class and/or level of education has quite modest effects on the association [2]. Importantly, measured variables of SES such as education, occupation and income, cannot be expected to capture the full range of mechanisms by which social factor may influence health.

Breast cancer has been reported to be associated with SES both at the individual and community-level, and women of higher education in particular appear to be at increased risk of breast cancer [142]. Family socioeconomic status is a strong predictor of educational attainment (also in adoptees) [143, 144]. Thus, an affluent environment in early life (and childhood) may not only be more likely to produce full grown (well-nourished) newborns, but also women that become well-educated and possibly more likely to develop breast cancer.

Genetic factors are important determinants of birth weight and fetal growth restriction [35, 36], and of the outcomes these have been found associated with, e.g. blood pressure, coronary heart disease, NIDDM, and breast cancer [145-148]. If some of these genetic factors were to be involved in both fetal growth regulation and disease development, the influence of such common causes could explain all or parts of the observed association between birth weight and disease.

The genetic perspective has been put forward in the “fetal insulin hypothesis”, proposing a common genetic background for fetal growth and insulin resistance [149]. Indeed, allelic variations in genes involved in insulin regulation and action (INS, IGF1, IRS-I and ADCY5) have been found associated with low birth weight and glucose intolerance and NIDDM [150, 151] and myocardial infarction [77, 152, 153]. Most recently, common polymorphisms in the promotor region of PONI (encoding Paraoxonase-I which protects low-density lipoproteins from oxidative modification), previously linked to oxidative stress and risk of CVD [154], were found to be determinants of occurrence of small for gestational age [155].

## 2.3 CAUSAL INFERENCE IN OBSERVATIONAL STUDIES

Epidemiology involves the description and explanation of human variability (generally with respect to health). Although it may not always be apparent (even to the researchers themselves), most epidemiological studies are concerned with causality. Several definitions of epidemiology, such as that of the WHO, include "the application of this study to the control of diseases and other health problems" [156]. With an ultimate goal of successful (and safe) prevention of disease, correct identification of causal relationships should be vital.

In observational studies causation can never be proved. In epidemiology it has traditionally been inferred from observed associations, sometimes with the guidance of a set of criteria for causality [157]. Coincidental with the growing interest for early life influence on health, there has however been a development toward a formal theory for causal inference. Counterfactual reasoning and the use of directed acyclic graphs in particular are now gradually making their way into epidemiological practice.

For a given individual, the causal effect is defined as a comparison between the outcome under exposure and that under no exposure, ceteris paribus. At the population level this translates into comparing the average outcome had everyone been exposed to that had everyone not been exposed. In contrast to this counterfactual definition of a causal effect, epidemiological measures of association come from comparing the actually exposed individuals to the actually unexposed (since we can observe only one counterfactual scenario per individual). This explains the well-known dictum that association is not causation. For association to be causation, the average outcome under no exposure in those that were actually exposed would have to be the same as the average outcome in the actually non-exposed. Such exchangeability (with respect to counterfactual outcomes) between the exposed and unexposed can be assumed if the exposure is assigned completely at random. Due to ethical and/or practical reasons however, many research questions do not lend themselves to randomized experiments and what is left is the possibility to observe the consequences of "natural" exposure assignment. The great challenge then involves identification of and proper dealing with sources of non-exchangeability between the naturally exposed and unexposed individuals.

### 2.3.1 COMMON THREATS TO CAUSAL INFERENCE

Validity concerns how closely we measure what we intend to measure. The most common threats to validity are bias and random variability. Since an infinitely large population rarely can be studied, sampling will introduce random variability. This affects the precision of estimation; the more random variability, the less precision. The resulting *random error* can be reduced by increasing the sample size (thereby increasing the precision). The presence or consequence of bias, on the other hand, cannot be mitigated by increased sample size. The *systematic errors* that result from bias need to be addressed by proper study design, data collection, analysis and interpretation.

#### 2.3.1.1 SYSTEMATIC ERRORS

Sources of bias can be described, explained and classified using directed acyclic graphs (DAGs). Construction of causal diagrams using DAGs may also help clarify causal questions and identify how/if they can be tested (the initiated reader may choose to turn to the work by Judea Pearl for a formal introduction) [158, 159]. Variables in a DAG are linked by directed arrows, which represent a direct causal effect from one variable to another. The graph is acyclic: by following the direction of the arrows one can never end up where one started (in other words there can be no arrows from effects to causes). A *causal* DAG should include all variables, measured and unmeasured, which are common causes of any pair of variables in the DAG [159, 160]. Importantly, this implies that in a causal DAG there will be information in missing variables, as well as in missing arrows. This also explains why construction of causal diagrams will require subject-matter knowledge [161]. DAGs come attached with a set of rules on how to decide whether two variables are independent or associated (d-separation). Generally, these are evaluated on the basis of marginal versus conditional independence, or association. The act of conditioning on a variable in a DAG is represented graphically by enclosing it in a box/square.

##### 2.3.1.1.1 Confounding

We say there is confounding when the exposure and outcome share common causes. If two variables share a common cause, this will induce a marginal association between the two, even though neither of them causes the other; an association due to common causes is referred to as "spurious".

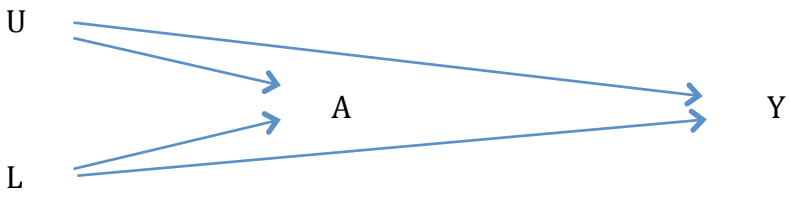



Figure 1. DAG representing confounding:
An association between birth weight or fetal growth (A) and CVD (Y) is due to shared socioeconomic factors (L) and/or shared genetic factors (U) [1]

A confounder is any variable that can be used to block the spurious association produced by the common cause. Such blocking can be achieved by methods such as standardization or stratification (most commonly conditioning on the confounder by including it in a regression model). It should be noted that there are situations in which the standard method (stratification) may not be appropriate, as in the case of e.g. time-varying exposure and/or confounding.

[1] No arrow from A to Y in this DAG implies that there is no causal effect of A on Y, and any association between the two is the result of shared common causes. The DAG merely serves to illustrate the structure of the bias; in real life we would probably have preferred to include an arrow from A to Y as well, since we do not know if e.g. fetal growth impairment may cause CVD.

2.3.1.1.2 Selection bias

Selection bias arises from the act of conditioning on a common effect of both the exposure and the outcome, or a common effect of a cause of the exposure and a cause of the outcome, or a combination of the two.

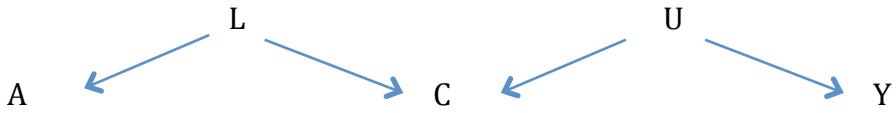



Figure 2. DAG illustrating selection bias:
An association between birth weight (A) and blood pressure (Y) is introduced by conditioning on participation in the study (C) if this is an effect of a cause of A, e.g., socioeconomic status (L), and of a cause of Y, e.g. health consciousness/healthy behavior (U)

Selection bias can arise as subjects are selected into a study (e.g. volunteer bias or inappropriate control selection), into a particular analysis (missing data bias), or when certain subjects are censored before the outcome can be observed (differential loss to follow-up). Ideally, this type of bias should be handled in the design phase of a study (e.g. by proper selection of controls, data collection etc.), whereas a potential analysis solution would be to block the path that was opened by conditioning on C, which in the example above could be made by conditioning on L. Similar to the case of handling confounding however, there are situations in which stratification may not serve to remove bias. The use of DAGs may help identify when other methods would be more appropriate (e.g. when the variable we wish to condition on to block the path is itself an effect of A).

Variables that are effects of more than one cause (i.e. have more than one arrow pointing to them) are called colliders, and the bias is therefore sometimes also referred to as collider-stratification bias. A special case of this type of bias arises with the act of conditioning on a variable in the causal pathway, when there are also unmeasured common causes of the intermediate and the outcome.

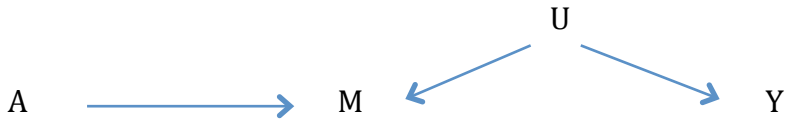



Figure 3. DAG illustrating "collider-stratification" bias:
The association between fetal growth (A) and blood pressure (Y) is due to conditioning on the intermediate adult BMI (M) which is an effect of A and a cause of Y but also shares common cause physical activity (U) with Y [1]

Conditioning on a variable in the causal pathway is often cautioned against but less so with a motivation. Other than the concern for spurious associations [162], the relevance or plausibility of such practice could perhaps be questioned in some situations (e.g. when holding the intermediate constant makes little sense).

#### 2.3.1.1.3 Information bias

Information bias occurs when the exposure and/or outcome cannot be measured perfectly. The resulting misclassification can be expressed in terms of sensitivity and specificity. The *sensitivity* describes the proportion of correctly classified among those truly affected (by e.g. exposure or disease) and the *specificity* refers to the proportion correctly identified among the non-affected (correspondingly the unexposed or unhealthy). The degree of information bias will depend on the sensitivity and specificity of the classification method, and the prevalence of the state of interest (exposure or outcome) [163]. If the misclassification of exposure is random with respect to the outcome, or vice versa, the misclassification is traditionally referred to as non-differential.

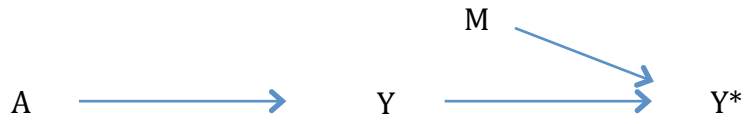


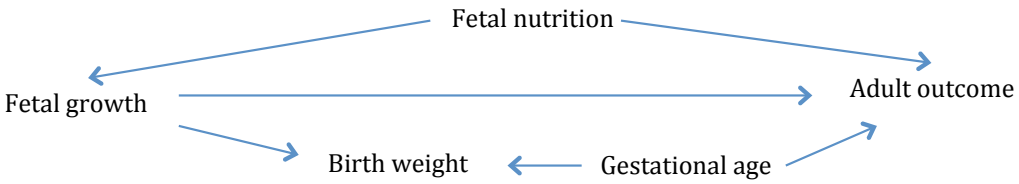

Figure 4. DAG illustrating information bias (non-differential misclassification): The association between fetal growth (A) and a diagnosis of CVD in the Inpatient Register (Y*) is expected to underestimate a potential causal effect of A on CVD (Y) if Y* is not perfectly correlated with Y (M can be a vector of reasons for receiving disease status in the register, e.g. whether the onset of disease warrants hospital care)

Non-differential misclassification of a dichotomous trait (as with CVD in the example above) tends to lead to an attenuation of the true effect. In the case of differential misclassification, i.e. misclassification that differs systematically between the groups contrasted (diseased/non-diseased in the case of exposure misclassification or exposed/unexposed in the case of outcome misclassification), the bias can go in any direction (and sometimes it may be hard to predict which).

### 2.3.1.2 EXTERNAL VALIDITY

The closer a study measures what it intends to measure (whether a causal effect or association) the higher the internal validity. Thus the degree of random and systematic error in a study determines its internal validity. As the "internal" implies, this pertains only to the sample studied. If the study subjects are representative of the source population, and/or other populations, the findings may be generalized to these populations as well. Such generalizability, or representativeness, is referred to as external validity.

In a sense, the studies of this thesis were motivated by the "association is not causation" axiom, and the reservation that associations that have not only inspired extensive research and advanced theories, but potentially also the promotion of preventive measures, may not represent the proposed causal effect. First, and most important, none of the studies directly measure the exposure believed to be causal (fetal and/or maternal nutrition). The proxies used are poor and at times only speculative (such as with the proportionality phenotypes used to target types and timing of insults). Also, it is unclear whether they are in the causal pathway between fetal nutrition and outcome (as could be the case with fetal growth), or associated with the outcome because they share the exposure as a common cause (likely the case with birth weight, as illustrated in the DAG below).




In addition, the indicators used to proxy the true exposure have many other determinants, several of which may also be involved in disease development (represented by gestational age in the DAG above, and familial factors in the DAG illustrating confounding on page 21). Previous studies have had limited ability to address such potential confounding from common causes of the exposure and the outcome. What the majority of studies appear to have done however, is to adjust for potential colliders such as adult BMI, which is likely to share common causes with the outcomes of interest (e.g. healthy lifestyle). Infants that are born small have a tendency to remain small (due to e.g. genetic factors). Thus if only large adults are compared, those who were smaller at birth will be more likely to have a less healthy lifestyle, introducing a spurious association between birth weight and outcome (similar to the DAG example of collider-stratification bias on page 22).

By studying twins, this thesis-work has attempted to address some of these issues. The use of twins allows for a more specific measure of fetal growth, so that several of the problems with misclassification in previous studies may be avoided. Also, because this measure is independent of several of the causes potentially shared with the outcome, associations cannot be due to these. Aiming to increase our understanding of the role of fetal growth, these investigations might also provide some clues about the underlying mechanisms to previously reported associations. Nevertheless, while the present studies address some important shortcomings of previous studies they are not exempt from problems or error themselves. Some of the known or possible issues and their potential influence on the findings and conclusions will be further discussed under methodological considerations in the discussion section (pages 52-56).

## 2.4 TWINS

The phenomenon of twinning has been an inspiration to many myths and stories, from Castor & Pollux of Greek mythology to Viola & Sebastian in Shakespeare's Twelfth night. With sir Francis Galton noting (in 1875) that the study of twins may allow "to weigh in just scales the effects of Nature and Nurture" [164], the unique features of twins have also intrigued and inspired scientists.

### 2.4.1 BIOLOGY

Twinning occurs as a result of the implantation (and maintenance) of two embryos. This can essentially happen spontaneously in two different ways; either through the separate fertilization of two eggs, or through the early division of one fertilized egg (zygote) into two viable embryos. Roughly one-third of all twins stem from the same zygote and are thus called monozygotic (MZ or identical twins) whereas the remaining two-thirds are dizygotic (DZ or fraternal twins; stemming from two separate zygotes)[165].

The rate of twinning varies greatly across different geographic regions and between ethnic groups (from 8-16/1000 maternities in the Nordic countries to up to 1/20 in Nigeria) [166, 167]. The general perception appears to be that MZ twinning rates are relatively constant across both time and populations, so that differences in overall twinning rates roughly reflect the variability in DZ twinning [165]. With no identified risk factors MZ twinning is generally considered a random event, whereas DZ twinning has been shown to increase with maternal age, parity, and height [168]. It has also been proposed that diet could influence DZ twinning rates [169].

Dizygotic twins stem from two separately fertilized eggs and will therefore be genetically related like any full siblings. Originating from the same zygote, monozygotic twins, on the other hand, are expected to share the same genetic setup. However, all MZ twin pairs may not be strictly genetically identical due to rare post-zygotic genomic changes, such as chromosomal abnormalities and point mutations [170, 171]. There is also a recent report that copy variation profiles may differ between MZ co-twins [172], but the extent and consequences of this for phenotypic variation requires further study.

Although the genetic material is expected to be overall very similar within MZ twin pairs, epigenetic modulation of the DNA (potentially influencing gene expression) may differ. Permanent epigenetic silencing, such as the X-chromosome inactivation in females, may differ in MZ twin pairs depending on the timing of division and the commitment to inactivation [173]. Epigenetic modulation of the genome can also occur throughout the life course, possibly in response to environmental factors [174], and could be responsible for phenotypic differences within monozygotic twin pairs [175]. According to a recent study, epigenetic markers appear to be overall very similar in young MZ twins but significantly different in elderly MZ twin pairs [176].

In utero, twins will need to share both space and supply line from the mother, and the premises for this sharing will mainly depend on placentation. Normally, an embryo is enclosed in two membranes; the inner *amnion* forming the amniotic sac around the embryo, and the outer *chorion*, which forms the connection to the maternal circulation via the placenta. Embryos that are separate will develop their own membranes and are thus able to form individual placentas. This is the case for roughly all of DZ and ~30% of MZ twins. Approximately half of these dichorionic placentas will fuse together throughout the course of pregnancy [177]. If in MZ twins the division occurs after the chorion has formed (>3 days after fertilization) there will only be one chorion, and thus one placenta, to share. Monochorionic placentas are more prone to vascular anastomoses than fused dichorionic placentas [177] . Also, peripheral chord insertion (into the membranes) tends to be more common in monochorionic placentas [178]. In rare cases the division can occur even after the amnion has formed, at which the twins share both amnion and chorion. 1-2% of all MZ pregnancies turn out to be monochorionic-monoamniotic (MCMA), and the potential for their chords to twist around each other put these twins at greater risk of complications [165].

### 2.4.2 FETAL GROWTH IN TWINS

The prenatal growth of twins is commonly described to be similar to that of singletons up until the 3rd trimester when a downregulation of growth occurs [179]. According to ultrasound measurements this decrease in the twin growth velocity begins around week 32 [180, 181]. The primary process for the slowed growth in multiple pregnancies is believed to be a response to the environment when the size of the fetuses combined exceeds a certain threshold [182]. Others have claimed that twins may downregulate their growth already in the first half of gestation, and by setting a lower growth trajectory this early would be better protected against undernutrition later in gestation [183].

Twins also experience shorter gestations than singletons (3 weeks on average). The lower birth weights seen in twins compared to singletons may thus be a result of growth restriction or shorter gestation, or both.

In comparison to singletons, the unique constraints on twins due to their sharing of space and supply line is likely to overshadow most other influences on growth. The variation in growth between (unrelated) twins however, is likely to be influenced by the same determinants described for singletons, i.e. the individual growth potential and the supply line supporting it. In a twin cohort we would therefore expect differences in fetal genes, maternal factors and placental function to influence the variation in fetal growth. In twins, zygosity and/or chorionicity can predict fetal growth, which probably mainly is a reflection of the importance of placentation, and in particular the location of the chord insertion (which if peripheral risks impairing the critical exchange between mother and fetus) [184]. A peripheral chord insertion appears even further unfavorable in the case of shared or fused placentas [184].

#### 2.4.2.1 TWIN GROWTH DISCORDANCE

Twins in a twin pair may also experience different growth. Discordance in the availability of nutrients and oxygen may cause restriction of the growth of one twin relative to the other. In DZ twin pairs a difference in growth could also stem from differences in the genetic growth potential. It has been speculated that lower levels of discordance (the difference in birth weight being <25% of the weight of the heavier twin) reflect natural differences between twin siblings, whereas more severe discordance would rather be the result of growth restriction in response to an exhausted intrauterine environment [185]. It has further been proposed that twin growth discordance may be an adaptation to the limited space, aimed at promoting maturity by increasing the length of gestation (as discordant twins appear to be delivered later than concordant twins of the same combined weight) [186].

Allocation of nutrients and oxygen is mainly a result of placentation. In dichorionic twins, whether placentas are separate or fused, allocation may vary according to differences in the location of the chord insertion as well as the function of the individual placenta. The location of the chord insertion appears even more important in monochorionic twins who share one placenta. The monochorionic placenta is also more richly vascularized with anastomoses than a fused placenta. Plenty of vascular anastomoses may protect against unilateral shunting of blood from one twin to the other (as occurs in the twin to twin transfusion syndrome, TTTS) and, similarly, compensate for an unfavorable placental function in one twin to mitigate growth discordance [187]. Conversely, a paucity of vascular anastomoses could increase the risk of TTTS and severe growth discordancy in monochorionic twins [188]. Irrespective of this, birth weight discordance in MC has been shown to be the result of unequal sharing of the placenta and/or chord insertion, and that these in turn are not associated with the vascular architecture (anastomoses) of the placenta [189, 190].

To conclude, other than the potential influence from the difference in genetic resemblance between DZ and MZ, the main determinants of growth discordance appear to be the same in both monochorionic and dichorionic twins, namely placental function and chord insertion [189, 190].

### 2.4.3 THE USE OF TWINS IN DOHAD

In the classical twin study, the degree of twin pair similarity in MZ and DZ is used to estimate the relative contributions of genes and environment to phenotypic variation. The similarities of twins may also offer unique opportunities to assess associations that are independent of all factors shared by the twins in a twin pair.

When a comparison is made between the twins in a twin pair all factors they have in common are held constant, thus any differences between them cannot be due to the factors that they share. All twins share early environment and a varying degree of genetic factors, which together are commonly referred to as familial factors. Birth weight differences within twin pairs will thus be independent of e.g. gestational age, maternal factors such as constitution and behaviors, early socioeconomic environment, etc. In monozygotic twin pairs a difference in birth weight can further (generally) not be attributed to any genetic influence. As previously described, growth discordance in twin pairs (which can be measured by the discordance in birth weights) is a result of the twins' unequal share of the supply line - and in DZ twins also fetal genetic factors. The availability of the supply line, in turn, is a reflection of fetal nutrition.

Thus, by targeting the influence of fetal growth that is independent of familial factors (and genetic factors in MZ), assessments of the association between birth weight and later health outcome within twin pairs may help provide some clues about the potential early life (fetal) influence on health and disease.

---

Twins have been used to explore DOHaD associations previously, in several different ways. First, the adverse intrauterine experience of twins compared to singletons has been proposed as a test for the fetal origins hypothesis [22, 23]. Reports of no difference in CVD mortality between twins and the general population however sparked a debate about the role of fetal programming in twins. Some argue that growth in twins is inherently different from that in singletons. For example, twins would be under much stronger influence of determinants (such as maternal constraint) that may not be importantly linked to later disease development [183]. The same authors also propose that twins may downregulate their growth early so as to avoid negative consequences of fetal growth restriction. Still, twin growth appears similar to that of singletons up until at least the third trimester (which is the originally proposed critical window for fetal programming) [180, 181]. No differences between twins and the general population have nevertheless been taken as an indication that the general adverse environment and growth constraint of twin pregnancies (compared to singletons) may not be pathological [25]. It has also been pointed out that twin-singleton comparisons may be confounded if twins as a group were to have a slightly different genetic or socioeconomic background than the general population [29].

The contrast between DZ and MZ twins has also been used to indicate a varying degree of intrauterine exposure (using zygosity as a proxy for placentation). By this logic, differences in glucose metabolism between MZ and DZ have been proposed to be due to a more adverse fetal experience in MZ compared to DZ (due to a greater degree of placental sharing among MZMC) [191]. Conversely, the presence of two hormone-producing placentas has been proposed to put DZ at greater risk of breast cancer (compared to singletons or MZ) [17].

Several studies have also used within-twinpair comparisons in the DOHaD field to explore the role of shared environment and genetic factors. The reasoning is such that if an association remains holding shared factors constant (i.e. within-twinpairs), such factors (e.g. maternal and socioeconomic factors) are not likely to be influential for the original association. Conversely, if an association changes within-twinpairs compared to between unrelated twins, the shared factors may play a role in the appearance of the original association. Commonly, this role has been attributed that of a common cause, so that inference about the influence of shared factors has been expressed in terms of potential confounding of the studied association. These types of inferences are also commonly based on a two-step approach, in which a "crude" association of birth weight and outcome (ignoring twin pair structure) is first contrasted to the within-twinpair association in all twins, and then in DZ and MZ twins separately. If the within-twinpair association is different from the "crude" (between unrelated twins), this is taken as an indication that the original association could be confounded by familial factors. Differing within-twinpair associations between DZ and MZ is sometimes further attributed to a genetic influence (based on the assumption that the only factors shared within MZ twin pairs and not DZ twin pairs, are genetic).

As with DOHaD studies in singletons, the majority of within-twinpair comparisons have assessed the association between birth weight and blood pressure. A meta-analysis of the first 10 studies reported of great heterogeneity between these, and also that all estimates had been adjusted for current BMI [192]. More recently, a large study from the STR has found an association between birth weight and hypertension within both DZ and MZ pairs, with and without adjustment for adult factors [21]. In the same cohort no association between birth weight and NIDDM within MZ has been interpreted as supporting a common genetic etiology for low birth weight and NIDDM (in contrast with findings in smaller twin samples of birth weight being associated with glucose metabolism within MZ [193, 194]) [20]. Attenuation of the association between birth weight and acute myocardial infarction in a small co-twin-control study in twins of the STR has further been taken as an indication of familial confounding of the original DOHaD association [24]. The only previous study of the association between birth weight and breast cancer in like-sexed twins is also a small co-twin-control from the STR. In this study, indications were of increasing risk of breast cancer with increasing birth weight within twins, but estimates were not statistically significant [195].

# 3 AIMS

- To study the potential influence of ethnicity and familial factors on the association between birth weight and blood pressure in adolescence

  (PAPER I)

  — Is there any support for the claim that ethnic differences in blood pressure originate in utero?

- To investigate whether the reported positive association between birth weight and risk of breast cancer is present also within twin pairs

  (PAPER II)

  — Does fetal growth that is independent of familial factors predict risk of breast cancer in like-sexed twins?

- To study the potential influence of within-twinpair fetal growth on the risk of cardiovascular disease in adulthood

  (PAPER III)

  — Is there an association between birth weight and cardiovascular disease independent of factors shared within twin pairs?

- To study the potential influence of twinning on adult mortality and morbidity from cardiovascular disease and cancer

  (PAPER IV)

  — Are twins different from singletons with respect to morbidity and mortality in adulthood? If so, is this a result of the unique experience of being a twin, or of belonging to a twin family (or both)?

# 4 MATERIALS & METHODS

## 4.1 SETTINGS

Observational studies of twins are generally based on data collected in volunteer twin cohorts or population-based twin registers. This thesis has predominantly been based on data from the Swedish Twin Register, but has also made use of data from a volunteer cohort of bi-ethnic adolescent twins in the US. In addition, the Swedish Multi-Generation Register has been used to identify twins and their singleton siblings, as well as random samples of singletons from the population.

## 4.2 DATA SOURCES

### 4.2.1 TWIN COHORTS

#### 4.2.1.1 THE GEORGIA CARDIOVASCULAR TWIN STUDY

The Georgia Cardiovascular Twin Study (GCTS) is a longitudinal study of >500 African American (AA) and European American (EA) twin pairs. The cohort was established to study changes in influence of genetic and environmental factors on development of bio-behavioral risk factors for cardiovascular disease in youth.

Twins were recruited from schools in Augusta, Georgia and 308 EA and 223 AA pairs attended a baseline visit between 1997 and 2000. During their scheduled follow-up visit between 2001 and 2004, an additional 53 EA and 51 AA pairs were recruited. All subjects were healthy (as assessed by parental reports about previous medical history, and not using any hypertensive drug) when entering the study. The Institutional Review Board of the Medical College of Georgia has approved the GCTS and written consent has been obtained from all subjects (or parents, if subject was aged <18 years). [196]

##### 4.2.1.1.1 Data collection

Ethnic designation was determined from self-reports. Subjects were classified as either EA or AA if 1) both parents reported being of European or African ancestry respectively; 2) parents and child were born and raised in the United States; and 3) parents considered themselves and their child to be EA or AA respectively (and not of Hispanic, Native American, or Asian descent). Self-identification of ethnic designation has been strongly advocated when used for human categorization in biomedical and genetic research [197].

Zygosity was determined using 5 standard microsatellite markers (TPOX, THO1, FGA, F13A01, FES/FPS) on DNA collected through mucosal swabs. The likelihood of MZ using Bayes Theorem (Q = 1.8) for five concordant markers for EA and AA was 99.0% and 99.2%, respectively [198].

All physical tests were performed in a laboratory setting, and the procedures used were the same during baseline recruitment and follow-up. Blood pressures were examined using a Dinamap Vital Signs Monitor (Model 1864 SX, Criticon, Inc., Tampa, FL) after 11, 13, and 15 min in supine position (subjects were instructed to relax on a hospital bed). An average of the three measurements was calculated and used as the resting value.

#### 4.2.1.1.2 Birth records

Information on birth weight was self-reported by the twins' mother (N = 810; 54% EA and 46% AA). In a subset of twins, birth weight information was also available from birth register data of the state of Georgia (N = 617; 53% EA and 47% AA). In the 399 subjects with both types of information available, self-reports were found to be in good agreement with registers (r = 0.92, $P = 0.001$).

Other self-reported information included family social status, maternal age at birth and maternal smoking habits at the time of the interview (yes/no).

### 4.2.1.2 THE SWEDISH TWIN REGISTER

The Swedish Twin Register (STR) was established in the late 1950s to study the health effects of smoking, with ability to adjust for genetic liability for disease. The register consists of four consecutive birth cohorts, spanning over more than a century (1886 to 2001) and including over 170 000 twins [199]. Since the cohorts were compiled at different time points, slightly different methods of ascertainment and data collection have been used (multiple births were for example identified in national parish records in the first cohort, and in national birth registrations in the following). In the two first cohorts, both twins of the twin pair had to be alive and traceable at the time of compilation. Although population-based, it follows that the register does not include *all* twins born in Sweden between 1886 and 2001. Moreover, most studies in twins rely on information that requires the twins to have participated in one or more rounds of data collection (e.g. mail-, telephone- or web-questionnaires). The STR is hosted at the Department of Medical Epidemiology and Biostatistics at Karolinska Insitutet.

The twins under study in this thesis were born between 1926 and 1958. This 2nd cohort of the STR was compiled in 1970, and thus includes all identified as twin pairs, where both twins were alive and traceable in 1971 (N=58 836).

#### 4.2.1.2.1 Data collection

During 1972-1973 a paper-based questionnaire was mailed out to all alive and traceable like-sexed twins born 1926-1958 (N= 38 955). Over 14,000 pairs responded (overall response rate 81%) to questions about twin similarity, anthropometric measures (height and weight), health related behaviors (smoking, drinking and dietary habits) and health symptoms (cardiovascular and respiratory symptoms including allergies) [200].

During 1998-2002 the Screening Across the Lifespan Twin Study was performed in all living twins born 1958 or earlier. Twins were contacted for an extensive telephone interview, including questions about twin similarity, birth order, health-related behaviors and indicators of health status (incl. prescription drug use). In those of the 1926-1958 cohort who were still alive and traceable the response rate was 74%. [199]

#### 4.2.1.2.2 Zygosity determination

Questions used to determine zygosity in the questionnaire (Q73) and telephone interview (SALT) are shown below. For Q73, pair response agreement classified twins as either monozygotic or dizygotic, whereas non-agreement or missing response(s) resulted in undetermined zygosity. This method has been reported to correctly classify zygosity in over 95% of Swedish twins (estimated from the twins that could be classified; excluding unclassified twins from the denominator) [201]. In SALT, twins were asked an additional question about twin similarity, and DNA analysis of a subsample of 199 twins found this method for zygosity determination to be 99% accurate [199].

| Questions about twin similarity | Q73 | SALT |
|---|---|---|
| 1. During childhood, were you and your twin partner as like as ''two peas in a pod'' or not more alike than siblings in general? (direct translation from Swedish actually "as like as two berries") | * | * |
| 2. How often did strangers have difficulty in distinguishing between you and your twin partner when you were children | | * |

The STR presently uses the best available information of zygosity, based on the following algorithm:
1. DNA analysis
2. Agreement in pair response to Q73
3. Agreement in pair response to SALT if undetermined in Q73
4. Agreement in singleton response in SALT (2 questions)

#### 4.2.1.2.3 Birth records

In the beginning of the new millennium, a large effort was made to locate the birth records of all twins born 1926-1958. Records of births were collected from all over Sweden, including hospital records for hospital deliveries and midwives records for home deliveries. The records include maternal information such as age, marital status, occupation, parity, last menstrual period, and potential disease(s) during pregnancy. The time of birth and type of delivery have been registered, together with anthropometric measures of the offspring at birth and birth order of twins. If the twins were baptized at birth, their Christian names were attached to their birth order in the report to the official birth registration.

Anthropometric measures at birth include birth weight (measured with scales in grams), birth length (measured with measuring tape with the baby lying on its back with legs stretched and the head against a vertical headboard) and head circumference (measured with measuring tape around the scull above the ears).

The establishment and data collection of this cohort of the STR has been approved by the Swedish Data Inspection Board (DNR 3083-74; 7254-95).

### 4.2.2 NATIONAL REGISTERS AND LINKAGE

Residents in Sweden are assigned a civic registration number, which includes the complete birthdate (yyyy-mm-dd) and four administrative numbers (-xxxx) [202]. Through this unique identifier, individuals can be tracked and linked to national registries of health and demographic information. National registers of health are kept by the National Board of Health and Welfare, whereas registers containing demographic information and censuses are kept by Statistics Sweden.

#### 4.2.2.1 THE INPATIENT REGISTER

Data in the Inpatient Register exist from 1964, when 6 counties in Sweden started recording their hospital admissions. Coverage was up to 85% by 1983, and the register includes all public admissions in Sweden from 1987 and onwards. Information includes dates of admission and discharge and up to eight discharge diagnoses, coded according to the International Classification of Disease (ICD). The register also includes information about potential procedures performed during the hospital stay. Validation of the recorded main diagnoses (combining translation error, coding error and diagnosing error) has shown an overall misclassification of 12% at the three-digit level of the ICD code, and 4% at the ICD-chapter level. [203]

#### 4.2.2.2 THE CANCER REGISTER

The Cancer Register holds information on all primary incident cancers in Sweden since 1958 [204]. Reporting is mandatory for physicians as well as pathologists and cytologists separately, ensuring all cancers found in autopsy, histologic, and/or cytological specimens are included and are classified according to the ICD. 99 % of the registered cancers have been morphologically verified. In 2006 the registry roughly covered 95% of all cancers reported in the Cause of Death Register, the deficit mainly represented by lung, pancreatic, and prostate cancers in patients above the age of 75 [204]. Validation studies restricted to females have found the completeness of the registry to be 99% [205, 206].

#### 4.2.2.3 THE CAUSE OF DEATH REGISTER

National records of cause of death have been computerized since 1952, and are considered reliable from 1961 and onwards. Reports of death and filing of cause of death certificates are mandatory, so that the record completely covers all deaths occurring in Sweden. Information includes date of death, underlying cause of death and up to 10 contributing diagnoses classified according to ICD. There is also information of potential autopsy. [207]

#### 4.2.2.4 THE MULTI-GENERATION REGISTER

The Multi-Generation Register links all Swedish residents to their parents, thereby allowing for identification of full and half-siblings, as well as more complex family structures. The register was created in the early 1990s and includes all individuals who have been registered in Sweden since 1961. Of these, all individuals aged 15 years or younger at the time the personal registration number was introduced (in 1947) were included in the register together with information on their parents. Index individuals thus include those born from 1932 and onwards, who were alive and residing in Sweden sometime after 1961. [208]

Completeness of parental information depends on birth year, with a greater extent of missing data on one or both of the parents in the oldest cohorts (more commonly the father, as paternity is established either by marital connection at the time of birth, or "by acknowledgement" if not married). Non-biological or adoptive relations are flagged. From the structure of the register it follows that an individual can only appear once as an index person, but may be recorded several times as parent (to new index individuals).

#### 4.2.2.5 THE POPULATION AND HOUSING CENSUSES

Population censuses have been conducted in Sweden since 1860, and housing censuses since 1945. Censuses are performed every decade through postal enquiries, and for the twins under study in this thesis, data was available from the 1970, 1980 and 1990 censuses. The censuses include socioeconomic indices such as income, occupation and education. There is also information on type, size and quality of housing, etc. Demographic information of migration (emigration -1961 and immigration -1969) is further available in the register of the total population. [209]

## 4.3 STATISTICAL METHODS FOR TWIN DATA

In this thesis, we use the unique features of twins to gain more insight about the role of the fetal environment for health and disease development later in life. While the degree of similarity in twins tends to be the (direct or indirect) target in most studies of twins, it is also a feature that requires special care in the methods used for statistical inference. Twins in a twin pair are likely to be more correlated than any two random individuals, and twin data thus violates an assumption most commonly used in statistical inference, namely that of independent observations.

### 4.3.1 METHODS TO HANDLE NON-INDEPENDENT OBSERVATIONS

Assuming that observations are independent when they are in fact positively correlated can lead to an overestimation of the statistical information of the data. As a consequence, the precision of statistical predictions may become overstated; reflected by too narrow confidence intervals and too low *P*-values [210]. It is generally recognized that for most standard regression techniques, the estimation of the (fix) parameters will be consistent irrespective of potential dependencies in the data, provided that the model for the mean has been correctly specified [211]. Some of the methods to account for non-dependence in data are therefore solely aimed at improving the estimation of the variance (standard errors). The efficiency of the parameter estimation could however be improved by making full use of the information of the data (including the dependencies). Methods that improve both parameter and variance estimation at the same time either require the specification of a full probability model or use a quasi-likelihood approach. In the methods used in this thesis (described below) the size of the correlations are not of interest by their own, but rather treated as nuisance factors that need to be accounted for.

#### 4.3.1.1 BOOTSTRAPPING

In the bootstrap method, the sampling variability of the parameter of interest is estimated from an empirical distribution of parameter estimates obtained in subsamples of the data [212]. These are created through a series of resamplings of the observed data, where study subjects are drawn with replacement to achieve samples with the same size each time. In paper IV of this thesis, the bootstrap method was used to estimate the confidence intervals of the standardized cumulative risk functions. This approach also accounts for the dependencies in the data brought on by the comparison of members from the same family. While implementation of the bootstrap method is simple in theory, it may become quite demanding computationally.

#### 4.3.1.2 ROBUST COVARIANCE ESTIMATOR

Also known as the empirical covariance matrix estimator, the sandwich estimator, or the Huber/White estimator, the robust covariance estimator has been developed to provide consistent estimates of variance in the presence of model

misspecification (particularly when the assumption of homoscedasticity or independence of the error terms is violated) [213, 214]. Essentially this is achieved by including an empirical estimate of the covariance of the error terms in the variance estimation of the parameter. The sandwich variance estimators tend to have larger variance than variance estimates obtained by methods that improve the estimation of parameters and variance simultaneously. In paper II of this thesis Cox proportional hazard regression is used to study the association between birth weight and the instantaneous rate (hazard) of breast cancer. To account for the dependence within twin pairs a robust estimator proposed for Cox regression [215, 216] was used.

#### 4.3.1.3 GENERALIZED ESTIMATING EQUATIONS

The generalized estimating equations (GEEs) are a quasi-likelihood approach to estimation which does not require any specification of the distribution of the error terms [217]. The specification of a working correlation matrix for the covariance increases the efficiency of the parameter estimation, leading to reduced variance compared to a method solely aimed at improving the variance estimation [211]. Also, if the robust sandwich method is used for the estimation of variance, this will be consistently estimated even under misspecification of the working correlation matrix. GEEs can be implemented in all generalized linear models [218], and were used to assess the association between birth weight and blood pressure in paper I.

### 4.3.2 METHODS TO COMPARE TWINS IN A TWIN PAIR

The objective of using twins in this thesis has been to enable within-twinpair comparisons. To achieve this in turn, two different methods have been used.

#### 4.3.2.1 BETWEEN AND WITHIN DESIGN

The clustering of correlated observations may not always be treated merely as a nuisance to statistical inference. Cluster information may provide an opportunity to investigate the relative contributions of between-cluster and within-cluster effects to that of the individual (which when estimated alone will be a weighted average of the between- and within-cluster effects) [219].

While some use this type of separation to remove bias of the individual level exposure effect (from confounding by the cluster level mean exposure), others may be interested to evaluate the influence of the contextual effect (e.g. a neighborhood cluster) on individual response.

In the between – within model, the individual exposure is partitioned into a between-cluster and a within-cluster component. Applied to twins, these will correspond to the twin pair mean and the individual deviance from that mean. Formally, the exposure $x_{ij}$ of the i$^{th}$ twin of the j$^{th}$ twin pair is replaced by the twin pair mean $\bar{x}_j$ and the individual deviance from the twin pair mean $x_{ij} - \bar{x}_j$.

A regression model of the independent outcome $Y_{ij}$ could thus look like:

$$link\ (E(Y_{ij})) = \beta_0 + \beta_1\ \bar{x}_j\ +\ \beta_2\ (x_{ij} - \bar{x}_j)$$

Conceptually, the between regression coefficient measures the effect of exposure between twin pairs, whereas the within regression coefficient measures the effect within twin pairs. The between and within components are orthogonal i.e. statistically independent of each other. If estimation of either effect is not dependent on the simultaneous estimation of the other, it follows that centering the individual predictor around it's cluster mean is what achieves the within-cluster comparison. This is further equivalent to including the cluster mean ($\bar{x}_j$) in a model with the individual predictor ($x_{ij}$) [220].

The formulation of the model above makes the assumption that the effect of the individual deviance from the mean is linearly related to the mean outcome modeled. The method can thus be used in the generalized linear regression setting as long as care is taken that the effect of the individual deviance from the twin pair mean exposure on the mean outcome modeled is linear (e.g. the log-odds or log-rate). The partitioning into between and within component for continuous predictors is less flexible for dichotomous exposures in twins (where the deviation from the mean is restricted to -0.5 or 0.5 in discordant pairs and 0 in concordant pairs). In such instances it has been recommended to instead perform within-pair comparisons of discordant pairs [221].

When the individual deviance from the twin pair mean (within-component) is used to obtain within-twinpair comparison, all twins with information about exposure for their co-twin can be used for the effect estimation. The ability to study all twins in a cohort contrasts with the co-twin-control method used in study III (and described next), which only includes disease-discordant twins.

The individual deviance in birth weight from the twin pair mean birth weight can be seen as an estimate of fetal growth that is independent of all factors shared by the twins in a twin pair. In this thesis we evaluate its association with systolic blood pressure (paper I) and log-hazard of breast cancer (paper II).

#### 4.3.2.2 CO-TWIN-CONTROL METHOD

Another way of achieving within-twinpair comparison is by matching twins to their co-twins. Similar to a matched cohort design, twin pairs discordant for the exposure can be followed with respect to outcome [222]. Alternatively, a matched case-control design can be applied in which cases are matched to their disease-free co-twins.

In the case-control design, the study population is selected on the basis of disease-status. Cases (diseased) and controls (non-diseased) can be sampled from a theoretical study base, or from a well-defined cohort. In both cases the purpose of the controls is to represent the exposure status of the underlying study base/cohort. In a matched case control study *n* number of disease-free controls are matched to each case with respect to certain characteristics (matching factors). In the co-twin-control method, the disease-free control is the co-twin of the case, thus matched on all factors shared by the twins of the twin pair. Control-selection (evaluation of disease-status) could be made at the end of a fixed follow-up time (as in a traditional case-control study), or at the time of case ascertainment, when a co-twin is matched if found to be disease-free (incidence-density sampling). The latter method is used in paper III to sample disease-discordant twin pairs from the underlying cohort of twins in the STR.

As previously mentioned, this co-twin-control method only includes disease discordant twins. In addition, only twin pairs that are also discordant with respect to the exposure will contribute to the estimation of effect.

In any matched case-control design, the parameter estimates will be independent of the matching factors if the analyses are made conditional on the matching factors. In the co-twin control design, matching takes place at the twin-pair level, and estimates of effect will thus be independent of all factors that are shared by the twins in a twin pair. In the 1:1 matched sample produced by twin pair matching, conditional logistic regression will estimate the effect (change in log-odds of the outcome) of the difference between the two observations in the strata [223]. Thus, a continuous measure of birth weight will estimate the linear effect of the within-twinpair difference in birth weight.

## 4.4 STUDY DESIGNS & METHODS

### 4.4.1 PAPER I

To evaluate the influence of ethnicity and familial factors on the birth weight – blood pressure association, baseline measurements for all individuals in the Georgia Cardiovascular Twin Study (N=1270, 635 pairs) were considered. Due to missing birth weight information (N=243), the final study population consisted of 1027 individuals. Of these, 562 were EA (279 pairs and 4 singletons), and 465 were AA (232 pairs and one singleton).

EAs were more frequent among those with missing information on birth weight (64% versus 54%), as were MZs (56% compared with 48%). After adjustment for sex and ethnicity, missing cases were not different from the study population in any of the characteristics investigated (age, height, weight, systolic and diastolic blood pressure, maternal smoking and parental education).

The association between birth weight and blood pressure (systolic and diastolic) was evaluated through linear regression using GEEs to account for the dependence within twin pairs (SAS GENMOD procedure [224]). Birth weight and individual deviance from the twin pair mean birth weight were used to estimate separate cohort and within effects respectively. Potential differences in effect (on the absolute scale) between groups were tested by the introduction of interaction terms between the main effect and ethnicity, and both cohort and within associations were also evaluated in ethnic groups separately.

In the cohort analyses, potential confounding factors under consideration included sex, age, maternal age at birth and maternal smoking, as well as the highest level of education of the parents. Unfortunately there was no information on gestational age. However, the individual deviance in birth weight from the twin pair mean is independent of all factors shared by the twins in a twin pair, including gestational age. In this study we also performed all analyses with and without adjustment for BMI at baseline. The problems with adjusting for potential colliders - such as adult anthropometric measures in studies of fetal growth and later disease - are further illustrated and discussed in the causal inference section (pages 22 & 24).

All statistical analyses were performed in SAS software version 9.1.

### 4.4.2 PAPER II

In order to study the association between birth weight and breast cancer, all like-sexed female twins of the 1926-1958 birth cohort for which zygosity was known (N=16 895) were selected. Of these, 16 604 were alive and at-risk (no previous breast cancer diagnosis) at the start of follow-up in 1973. Birth records were available in 79% of these, and further restrictions due to missing birth weight data (n=39) and the requirement of correct birth identification of each twin within a twin pair (n=1185) resulted in a final study population of 11 923 twins (of which 5859 were intact twin pairs).

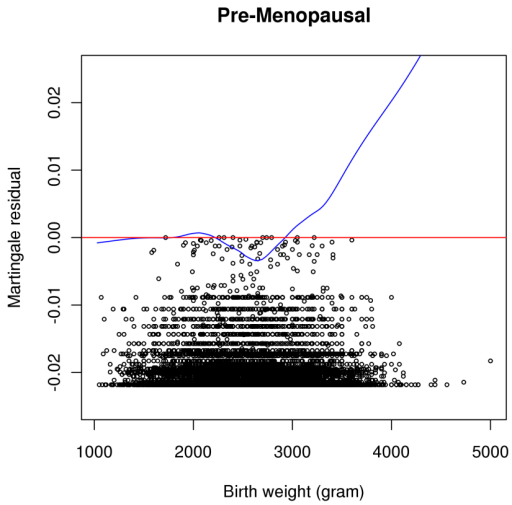
Gestational age was estimated from the first day of the last menstrual period (self-reported by the mother) and the twins' birth date. Socioeconomic status at birth was derived from the highest self-reported occupation of the parents. From the 1972-1973 questionnaire self-reported height (cm), weight (kg) and smoking status (yes/no) was also available.

A diagnosis of breast cancer was defined as a registration of either ICD code 171 ($7^{th}$ revision) and/or C50 ($10^{th}$ revision) in the Cancer Register. Time at-risk was defined from the start of 1973 (when zygosity was first established) until incident disease, death or end of follow-up in Dec 31 2006.

The association between birth weight and breast cancer was evaluated through Cox proportional hazards regression in the SAS PHREG procedure, using robust standard errors to account for the dependence between twins in a twin pair [215]. Attained age was used as underlying time scale and baseline hazards allowed to vary with year of birth. When Schoenfeld residuals from a model with birth weight predicting the log-hazard of breast cancer were plotted against age, an indication of non-proportional hazards was revealed. This was further confirmed by a statistically significant difference in effect between women over the age of 50 compared to women aged 50 or younger. As a consequence we performed all analyses in these two groups of women separately.

Further evaluation of the nature of the relationship between birth weight and the log-hazard of breast cancer was conducted by plotting Martingale residuals against birth weight. As shown in figure 1 on the next page, for women aged 50 or under, these indicated a positive linear relationship between birth weight and log-hazard of breast cancer from 2,500 grams and upward. For women above the age of 50 there were weak indications of an opposite pattern (results not shown).

Figure 1. Martingale residuals from a model predicting the log-hazard of breast cancer plotted against birth weight in grams.




For both cohort and within-twinpair analyses other covariates considered included other anthropometric measures at birth (birth length and head circumference), individual socioeconomic status (highest level of attained education in 1970) and smoking status in 1973. For cohort analyses zygosity, gestational age and socioeconomic status at birth were also considered.

All statistical analyses were performed in SAS software version 9.2.

### 4.4.3 PAPER III

In the study of birth weight and cardiovascular disease, all like-sexed twins born between 1926 and 1958 (N=37 194) were considered. Birth records were available in N=26 204 (81%) of those with known zygosity (N=32 539). Further requirements of birth order ascertainment (twins either having to have been baptized at birth or answered in agreement to questions about birth order in the telephone interview) limited the study population to N=23 689 twins. In this sample, the mean birth weight discordance between like-sexed twin siblings was 350 grams (range 0-2250 grams) in DZ and 304 grams (range 0-2420 grams) in MZ twins.

Twins were followed in national registries of hospital admissions, causes of death and population statistics (concerning migration). Time at-risk was defined from the beginning of 1973 (when zygosity was first established) until incident disease, migration, death or end of follow-up in Dec 31 2006. Cerebrovascular and coronary heart diseases (CHD) were combined for a relatively strict definition of overall CVD. CHD and stroke were also considered separately, and the latter further categorized according to subtype (ischemic, hemorrhagic or not otherwise specified). Due to the potentially fatal outcome of these diseases, the few cases identified through the Cause of Death Register only were also included. The diagnose codes included from each revision of the ICD are presented below, along with the total number of cases identified (only in Cause of Death register).

| | Cardiovascular | Coronary heart | | Stroke | |
|---|---|---|---|---|---|
| | disease | disease | Hemorrhagic | Ischemic | Not specified |
| | | | ICD CODES INCLUDED | | |
| 8th revision | 410-414; 430-438 | 410-414 | 430-431 | 432-434 | 436 |
| 9th revision | 410-414; 430-438 | 410-414 | 430-432 | 433-434 | 436 |
| 10th revision | I20-I25; I60-I69; G45 | I20-I25 | I60-I62 | I63 | I64 |
| | | | STUDY SAMPLES | | |
| Twins at risk in 1973 | 23,455 | 23,467 | | 23,461 | |
| Total number of cases | 2,548 (204) | 1,740 (182) | 240 (27) | 513 (6) | 74 (6) |
| Pairs discordant for disease | 1,942 (172) | 1,334 (151) | 210 (26) | 414 (5) | 62 (6) |

Co-twin-control selection was performed for each of the three main outcomes. Eligible pairs were those where both twins were at risk of the disease until the date of case diagnosis. The final study samples amounted to 1942 pairs discordant for CVD (1194 male and 748 female), 1334 pairs discordant for CHD (884 male and 450 female), and 686 pairs discordant for stroke (388 male and 298 female).

Conditional logistic regression was used in the SAS LOGISTIC procedure [225] to evaluate the association between birth weight and CVD within (matched) twin pairs. Information on adult characteristics not shared by the twins of the pair (e.g. height, weight, smoking and socioeconomic status) were considered and discarded on the basis of their likely roles in causal diagrams.

All statistical analyses were performed in SAS software version 9.2.

### 4.4.4 PAPER IV

To investigate a potential influence of twinship on adult morbidity and mortality, three cohorts with varying degree of exposure to twinning were compared.

| Group | Feature | Twin exposure | |
|---|---|---|---|
| | | Co-twin | Twin family |
| Cohort 1 | Twins (identified through MGR) | YES | YES |
| Cohort 2 | Singleton full siblings of twins in cohort 1 | NO | YES |
| Cohort 3 | Singletons of other families than cohort 1 | NO | NO |

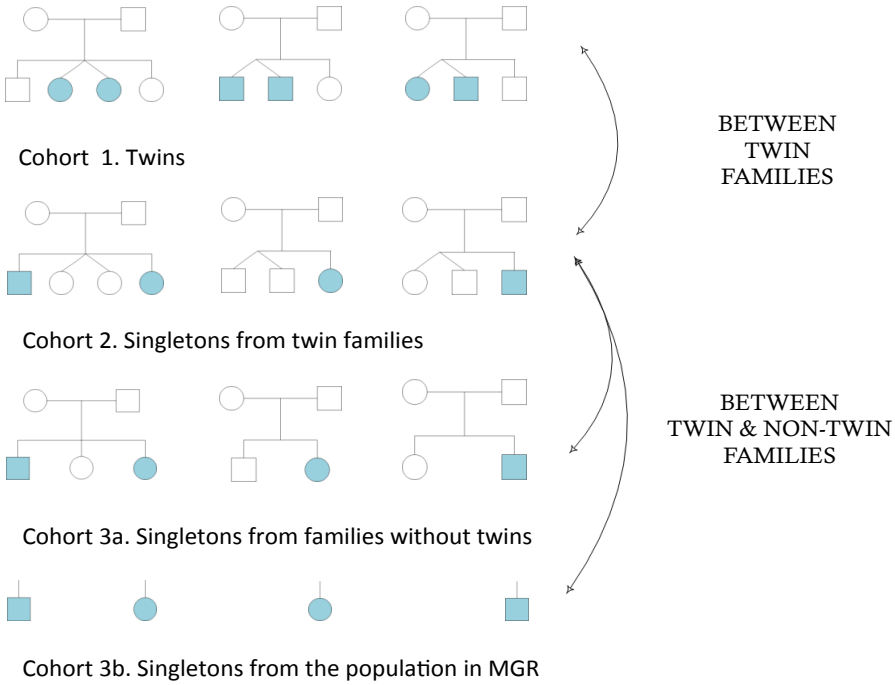
Overall morbidity and mortality of twins was compared to that of their singleton siblings. By accounting for factors shared by twin families, these comparisons aimed to capture the effect of the unique experience of being a twin on morbidity and mortality (independent of potential twin family influence on health). Further comparison of singleton from twin families and non-twin families, was aimed at providing some insight into whether belonging to a twin family (rather than being a twin) influences health (the comparisons are illustrated in figure 2).

Families with and without twins were identified in the Multi-Generation Register (MGR). To identify twins, any pair of individuals born to the same mother within 3 days of each other was considered eligible. This method identified N=24 578 twin pairs born between 1932 and 1958, 771 of which were born within 1 to 3 days of each other. The twin pairs represented a total of 24 150 families, some families containing two (n=408) or three (n=10) twin pairs. The twin cohort identified through the MGR is illustrated in Figure 1 (cohort 1) and comprised of 7631 like-sexed male, 7757 like-sexed female and 9190 opposite-sexed pairs.

In 18 098 of the twin families, one or more singleton full sibling was identified (Figure 1, cohort 2). The number of singleton full-siblings in these families ranged from 1-13 (averaging at 1.6 per twin family). Overall N=38 364 singletons were found in twin families, but to allow for comparison across birth cohorts only those born between 1932 and 1958 were considered (cohort 2, N=35 227).

Lastly, two random samples from the general population were considered for the "unexposed" comparison group. First, the aim was to ensure that the comparison group was truly unexposed to twinning (no twins in the family). Therefore, for each individual in cohort 2, up to 10 individuals were retrieved from the MGR with same birth year, sex and family constellation (cohort 3a, figure 2). For some unique or rare sibling constellations the number of eligible individuals in the MGR was not enough, and as a consequence the average number of matches in cohort 3a was 4.5 (range 0-10). Second, to allow for a comparison group to represent the population, all individuals of the MGR who had not already been included in cohorts 1 or 2 were eligible for selection to the second sample. From this population up to 10 individuals from the MGR were selected, matched to the singletons in cohort 2 with respect to birth year and sex (cohort 3b, figure 2).

Figure 2. Study populations and matching design for paper IV




The main outcomes considered were cardiovascular disease, overall cancer, and death, according to the diagnostic codes of the International Classification of Disease (revisions 7-10). To capture such cardiovascular diseases that predominantly occur later in life, CVD was defined as coronary heart disease (including angina pectoris) or cerebrovascular disease (ICD-7 codes 330-334, 420-422; ICD-8 and ICD-9 codes 410-414, 430-438; and ICD-10 codes G45, I20-25, I60-69). The diagnoses used to identify overall cancers were ICD-7 codes 140-206 and ICD-10 codes C00-C97. Information of migration to or from Sweden was available from the Register of the Total Population.

All individuals were followed with respect to incident case of cardiovascular disease, cancer and death, respectively. Due to the inclusion criteria of the MGR, the earliest start of follow-up was in 1961, when individuals were between 3 and 29 years old. For each outcome, time at-risk extended up until incident event, emigration from Sweden, death or end of follow-up on Dec 31 2007.

Table 1 presents the characteristics of each of the cohorts under study, after exclusion of individuals with a record of migration before 1961. There was a higher representation of younger birth cohorts among the twins compared to the singletons from twin families, and the samples matched to them. The birth cohort and sex distributions of the latter (cohort 3a and b) did not perfectly follow that of cohort 2 as a result of incomplete matching and exclusion of migrators before 1961. Information on family size (number of full siblings identified in the MGR) was available for cohorts 1, 2 and 3a (Table 1). Larger family sizes tended to be overrepresented in cohort 2 (twin families with singletons) compared with cohort 1 (all twin families) and cohort 3a (families with only singletons). In cohort 3a the number of matches per individual in cohort 2 decreased as family size increased.

**Table 1. Characterstics of the study populations**

| | | Twins | Singletons | | |
|---|---|---|---|---|---|
| | | Cohort 1 | Cohort 2 | Cohort 3a | Cohort 3b |
| GENDER, N (%) | Male | 23 718 (50) | 17 530 (51) | 76 718 (51) | 130 042 (51) |
| | Female | 23 878 (50) | 16 604 (49) | 74 070 (49) | 126 362 (49) |
| | Total | 47 596 | 34 134 | 150 788 | 256 404 |
| BIRTH COHORT, N (%) | 1932-1939 | 9043 (19) | 8462 (25) | 35 503 (24) | 56 584 (22) |
| | 1940-1949 | 21 171 (44) | 17 211 (50) | 76 422 (51) | 133 283 (52) |
| | 1950-1958 | 17 382 (37) | 8461 (25) | 38 863 (26) | 66 537 (26) |
| FAMILY SIZE, N (%) | Missing | 0 (0) | 0 (0) | 0 (0) | N/A |
| | 2 siblings | 11 501 (24) | 0 (0) | 69 612 (46) | N/A |
| | 3 siblings | 15 121 (32) | 7033 (21) | 73 780 (49) | N/A |
| | 4 siblings | 10 140 (21) | 8940 (26) | 6831 (5) | N/A |
| | 5 siblings | 5236 (11) | 6644 (20) | 448 (0.3) | N/A |
| | >5 siblings | 5598 (12) | 11 517 (34) | 117 (0.1) | N/A |
| AGE, Mean (Median) | At entry (yrs) | 13 (13) | 15 (16) | 15 (15) | 15 (15) |
| | At exit (yrs) | 58 (59) | 60 (61) | 60 (61) | 60 (61) |

The three main contrast samples under evaluation were; twins and singleton siblings of twins (cohort 1 vs. cohort 2); singletons from twin families and non-twin families (cohort 2 vs. cohort 3a or b); and twins and singletons from the population (cohort 1 vs. cohort 3b).

Survival functions were estimated through Cox proportional hazards regression (SAS PHREG procedure [215]), with age as underlying time scale and allowing non-proportional hazards for the two groups being contrasted. To account for the noted systematic differences in distribution of predictors of the outcome (e.g. birth year, sex and family size) between the groups, the survival functions were standardized to the distribution of the covariates in the contrast sample under study. In the traditional method of standardization, the predicted outcome under a set of covariates (the confounders) in the exposed versus the unexposed is weighted according to the joint distribution of those covariates in a reference sample (e.g. the combined sample of exposed and unexposed). The method thus relies on a correct model specification for the outcome given the covariates. In order to evaluate the influence of potential misspecification of this model, a standardization method that only requires model specifications for the exposure was also used (inverse probability weighting), and the results compared. All standardized survival estimates were converted into cumulative risks of failure (f = 1 - s) and plotted against age. Bootstrapping methods were used to obtain estimates of statistical precision (95% confidence intervals) for the risk differences between groups.

All statistical analyses were performed in SAS software version 9.22.

# 5 RESULTS

## 5.1 BIRTH WEIGHT & BLOOD PRESSURE IN BIETHNIC TWINS (I)

Baseline characteristics of the twins of the GCTS are shown in Table 2 below.

**Table 2. Characteristics of the GCTS, ethnic and gender groups separate**

| | BOYS | | | GIRLS | | |
|---|---|---|---|---|---|---|
| | **African American** | **European American** | | **African American** | **European American** | |
| | Mean (SD) | Mean (SD) | *P* | Mean (SD) | Mean (SD) | *P* |
| Age (years) | 14.1 (2.66) | 14.4 (2.61) | 0.36 | 14.6 (2.90) | 14.7 (2.85) | 0.54 |
| SBP (mmHg) | 113 (11) | 110 (9.3) | <0.01 | 110 (10) | 106 (8.5) | <0.01 |
| DBP (mmHg) | 59 (5.8) | 56 (5.8) | <0.01 | 61 (7.0) | 58 (5.4) | <0.01 |
| Height (m) | 163 (14) | 163 (14) | 0.86 | 160 (7.5) | 158 (9.6) | 0.12 |
| Weight (kg) | 59 (19) | 58 (20) | 0.54 | 59 (17) | 54 (16) | <0.01 |
| Birth weight (g) | 2562 (633) | 2565 (601) | 0.72 | 2306 (508) | 2508 (518) | <0.01 |

NOTE: Mean and standard deviation (SD)

*P*-values from test of mean difference between ethnic groups (boys and girls separate)

AAs had significantly higher blood pressure levels than EAs (both boys and girls). It was however only girls that differed in body size; AA girls being smaller at birth and bigger (higher BMI) at baseline compared to EA girls (Table 2).

Diastolic blood pressure was not associated to birth weight in either ethnic group. In the cohort combined a 1 kg increase in birth weight was associated with a mean drop in systolic blood pressure by 1.4 (-2.6 to -0.20) mmHg. Stratified analysis showed a statistically significant inverse association in AAs and a weak non-significant inverse association in EAs. Although the within-twinpair comparisons were hampered by decreased precision, indications were of a remaining inverse association in AAs but not in EAs (in which it appeared reversed) (Table 3).

**Table 3. Mean change in SBP for 1kg increase in birth weight, cohort and within**

| | **African Americans** | **European Americans** |
|---|---|---|
| | Adjusted β (95% CI) | Adjusted β (95% CI) |
| Cohort ALL | -2.3 (-4.4 to -0.35) | -0.50 (-1.9 to 0.91) |
| Within ALL | -3.0 (-6.4 to 0.35) | 2.4 (-0.24 to 4.9) |
| Within DZ | -3.0 (-7.4 to 1.5) | 2.8 (-0.76 to 6.4) |
| Within MZ | -3.5 (-8.0 to 1.1) | 2.0 (-1.5 to 5.4) |

NOTE: Beta-estimate β and 95 % confidence interval (95% CI)

Precision was further decreased with stratification according to zygosity, but indications were of an inverse association within AAs in both zygotic groups.

## 5.2 BIRTH WEIGHT & BREAST CANCER IN FEMALE TWINS (II)

In the cohort of 11 923 women, 590 developed breast cancer during follow-up.

High birth weight (≥3000 grams) was associated with an increased rate of early (≤50 years) but not late (>50 years) breast cancer. Birth length and head circumference were not statistically significant associated with rate of breast cancer, whereas adult height was positively associated with breast cancer diagnosed early. Maternal age, parity, individual BMI and smoking status were not statistically significantly associated with rate of breast cancer in either age group.

Table 4 presents the hazard ratios of breast cancer diagnosed at 50 years or earlier, and after the age of 50 according to birth weight categories. Adjusting for other anthropometric measures at birth did not change the overall findings of an increased rate of early breast cancer in the highest birth weight category, and no association between birth weight and breast cancer diagnosed after the age of 50. With indications of a slightly increased rate also in the lower birth weight category, linearity could not be assumed.

**Table 4. Hazard ratios of breast cancer according to birth weight (g) in female twins below and above the age of 50**

| | | ≤50 years | | >50 years | |
|---|---|---|---|---|---|
| | | Adjusted for GA | Fully adjusted | Adjusted for GA | Fully adjusted |
| $N_{cases}$ / $N_{tot}$ | | 225 / 11 363 | 219 / 10 995 | 333 / 9776 | 315 / 9447 |
| Birth weight, g | <2,500 | 1.27 (0.89 to 1.79) | 1.37 (0.93-2.01) | 1.02 (0.79 to 1.31) | 0.96 (0.68-1.34) |
| | 2,500-2,999 | 1.00 | 1.00 | 1.00 | 1.00 |
| | ≥3,000 | 1.63 (1.14 to 2.32) | 1.58 (1.03-2.42) | 0.87 (0.64 to 1.18) | 0.80 (0.57-1.12) |

NOTE: Hazard ratios and 95% confidence intervals

Fully adjusted incl. gestational age (GA), zygosity, birth length and head circumference (categorized)

Plotting of Martingale residuals confirmed a positive linear association between birth weight and log-hazard of early (≤50 years) breast cancer from 2,500g and upward. As a consequence, the effect of birth weight continuous and individual deviance from twin pair mean birth weight on the log-hazard of early diagnosed breast cancer were evaluated in twins weighting 2,500g or more (Table 5).

**Table 5. Hazard ratios of breast cancer according to 500g increase in birthweight in twins up to the age of 50, ≥2,500g**

| **≤50yrs** (birth weight ≥2,500g) | Cohort | Within ALL | Within DZ | Within MZ |
|---|---|---|---|---|
| $N_{cases}$ / $N_{tot}$ | 132 / 6886 | 132 / 6886 | 95 / 4428 | 37 / 2458 |
| Crude HR (95% CI) | 1.53 (1.21-1.93) | 1.46 (1.00-2.13) | 1.53 (1.00-2.34) | 1.21 (0.56-2.62) |
| $N_{cases}$ / $N_{tot}$ | 128 / 6636 | 128 / 6636 | 91 / 4242 | 37 / 2394 |
| Adjusted HR (95% CI) | 1.54 (1.13-2.11) | 1.53 (1.00-2.35) | 1.56 (0.94-2.59) | 1.38 (0.64-2.95) |

NOTE: Hazard ratio HR and 95% confidence interval (95% CI)

Adjusted for birth length and head circumference (in cohort both crude and adjusted also include gestational age)

The effect estimates were similar in the cohort and within twin pairs overall. While a slight attenuation could be noted in MZ, the sample was less than half of the DZs and the confidence intervals in fact completely overlapped/included those of the DZ estimates.

## 5.3 BIRTH WEIGHT & CARDIOVASCULAR DISEASE IN TWINS (III)

Table 6 shows the within-twinpair estimates of birth weight and CVD, CHD, and stroke in like-sexed dizygotic twins and monozygotic twins respectively.

Overall, there was an inverse association between birth weight and CVD within DZ [*P* for linear trend=0.005] but not MZ [*P* for linear trend=0.82] twin pairs (Table 5). The pattern was consistent for both coronary heart disease and stroke. With further stratification according to subtypes of stroke, interpretation was slightly hampered by reduced statistical power. Nevertheless, for ischemic stroke the same pattern of an inverse linear association was obvious within DZ but not within MZ twin pairs. For hemorrhagic stroke, estimates were imprecise for both DZ and MZ, precluding interpretation of potential effects in either group.

**Table 6. Within-pair association between birth weight and cardiovascular disease in dizygotic and monozygotic twin pairs**

| | Cardiovascular disease | Coronary heart disease | Stroke | |
|---|---|---|---|---|
| | | | Ischemic | Hemmorrhagic |
| | OR (95% CI) | OR (95% CI) | OR (95% CI) | OR (95% CI) |
| **Dizygotic twin pairs** (N) | 2248 | 1706 | 546 | 228 |
| Birth weight, g <2,500 | 1.49 (1.12-1.96) | 1.37 (0.97-1.91) | 2.04 (1.06-3.90) | 1.59 (0.66-3.80) |
| 2,500-2,999 | 1.26 (1.01-1.56) | 1.27 (0.98-1.64) | 1.02 (0.61-1.70) | 1.57 (0.79-3.12) |
| ≥3,000 | 1.00 | 1.00 | 1.00 | 1.00 |
| per 1000 gram increase | 0.73 (0.57-0.92) | 0.74 (0.56-0.98) | 0.49 (0.28-0.88) | 0.51 (0.23-1.13) |
| **Monozygotic twin pairs** (N) | 1436 | 962 | 282 | 192 |
| Birth weight, g <2,500 | 1.01 (0.66-1.54) | 0.85 (0.51-1.42) | 0.49 (0.18-1.39) | 1.56 (0.44-5.51) |
| 2,500-2,999 | 0.91 (0.64-1.31) | 0.79 (0.51-1.23) | 0.50 (0.20-1.26) | 1.89 (0.75-4.78) |
| ≥3,000 | 1.00 | 1.00 | 1.00 | 1.00 |
| per 1000 gram increase | 0.93 (0.65-1.32) | 1.10 (0.73-1.68) | 0.98 (0.41-2.33) | 0.74 (0.25-2.24) |

NOTE: Odds ratio OR and 95% confidence interval (95% CI)

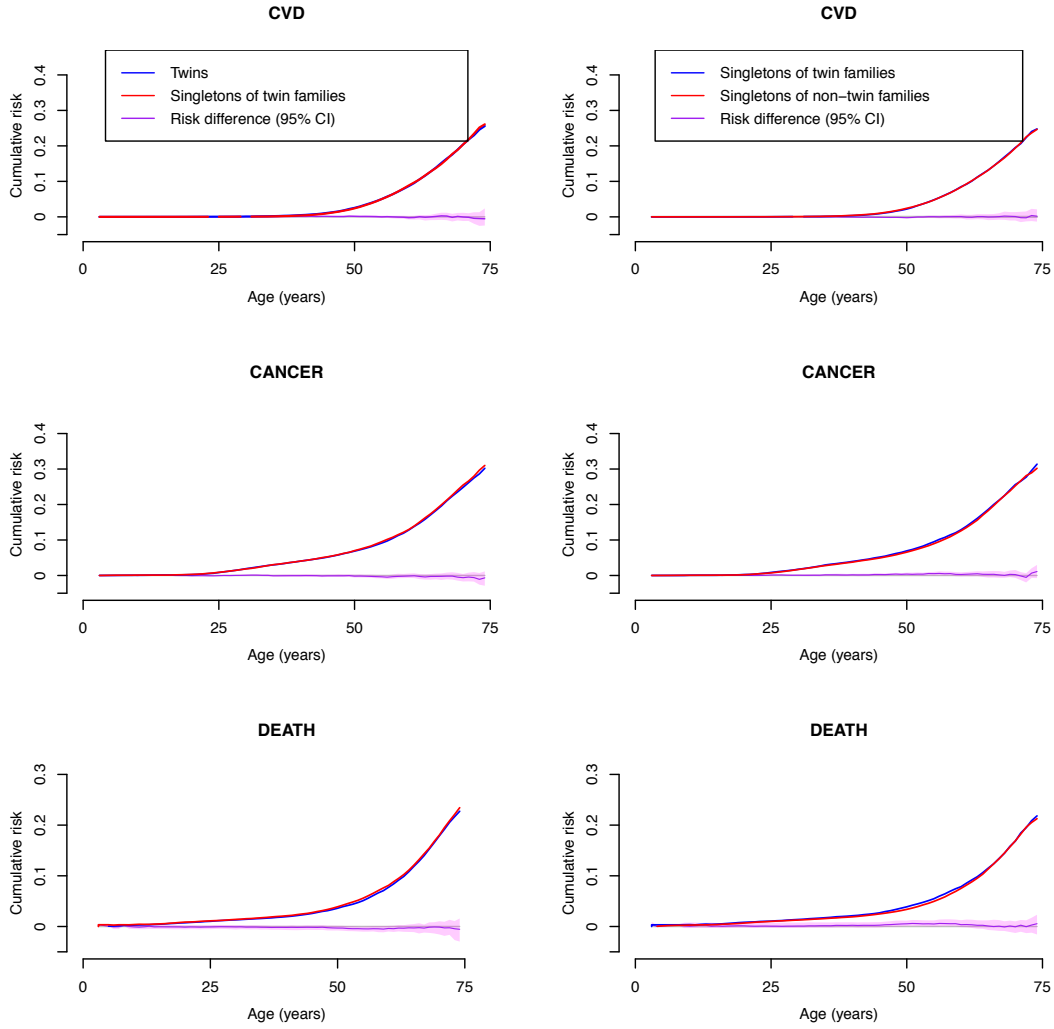
The difference in the within-twinpair associations between DZ and MZ pairs was further tested by the introduction of an interaction term between birth weight and zygosity in a model including all twin pairs. Global Wald test of overall significance of this interaction term (with birth weight categorized to allow for potential non-linearity of the within pair effect in MZ) yielded *P*-values of 0.27 for CVD; 0.18 for CHD; and 0.06 for ischemic stroke.

The differences between DZ and MZ in the estimate for the lowest birth weight category compared to the reference were 1.47 (0.96 to 2.24) for CVD; 1.61 (0.97 to 2.66) for CHD; and 4.13 (1.45 to 11.8) for ischemic stroke.

## 5.4 MORBIDITY & MORTALITY IN TWINS AND SINGLETONS (IV)

Figure 3 below shows cumulative risks of cardiovascular disease, overall cancer and all-cause death respectively, contrasting twins and singletons of twin families in the left column, and singletons of twin and non-twin families in the right column. In both, the underlying survival functions have been standardized to the sex, birth cohort and family size distributions of the contrasted cohorts combined.

**Figure 3. Cumulative risks of CVD, cancer and all-cause death**




Overall, the morbidity and mortality of twins appeared to be similar to that of singletons of twin families, which in turn did not appear to differ from singletons from non-twin families. The indication that there then should not be any differences between twins and singletons of the population was further confirmed by comparing all twins to a random sample of the population of singletons included in the MGR (i.e. no appreciable differences between cohorts 1 and 3b for either of the outcomes was found, results not shown).

Reassuringly the method used for standardization (whether based on models for the outcome, as for the results shown here, or models of the exposure) had negligible influence on the results overall.

# 6 DISCUSSION

The interest for the importance of early life exposures with respect to health and disease development has grown exponentially during the last decades. Spurred by the early reports of associations between birth weight and cardiovascular disease mortality, extensive studies have found birth weight a predictor of a wide variety of disorders including asthma, psychiatric disorders, hypertension, non-insulin dependent diabetes, some types of cancer, and cardiovascular disease. From the early theories of maternal nutrition and the "paradigm shift" to an evolutionary perspective and developmental plasticity [69], the true underlying mechanisms to the associations have proved difficult to unravel. In humans, several attempts have been made to link birth weight to indicators of potential pathways of pathogenesis, such as hormonal exposures [110], endothelial function [125], sympathetic nerve activity [126], beta-cell function [127], and low-grade inflammation [128]. Exploring the effects of causes or components of birth weight in turn has turned out to be more complicated, at least in humans. Instead, animal experiments have provided important insights, however most such studies involve manipulation of the maternal diet, which may or may not influence fetal growth and/or birth weight [70]. As a consequence, it has even been argued that programming mechanisms may be independent of birth weight (and fetal growth), suggesting the study of birth weight inappropriate in the first place [70]. However, apart from natural experiments, like the Leningrad siege [71] and the Dutch famine [72], findings to support fetal programming of adult disease in humans rest mainly on associations with birth weight or other proxies of fetal growth, and the underlying reasons for their association with later health outcomes remain unresolved.

In this thesis twins are used in an attempt to increase our understanding of the potential health consequences of adverse fetal growth. By comparing fetal growth within twin pairs, a specific measure of fetal growth is targeted. Since all factors shared by the twins in a twin pair are held constant (e.g. early socioeconomic environment and common genetic factors), these cannot influence the within-twinpair associations. Twins as a group are further exposed to a more adverse fetal environment than singletons. By comparing adult morbidity and mortality in twins to singletons from twin families, the influence of twinning on later health will be independent of factors shared by twin families. The comparison serves as an instrument to evaluate whether twins are different from singletons with respect to large common disease outcomes. Potential differences throughout the life course may have implications for the extent to which findings in twins can be generalized to singletons or the population at large.

## 6.1 TWINS AND DOHAD

The validity of using twins to evaluate DOHaD related hypotheses has been questioned. The predominant reservations appear to concern whether findings in twins can be considered representative of singleton populations.

### 6.1.1 EXTERNAL VALIDITY OF TWIN STUDIES

Generalizability of twin studies to the general population is commonly questioned. A potential threat to representativeness could be if twins were to be different from the general (predominantly singleton) population with respect to the phenotypes under study. With the advent of fetal programming theory, it was suggested that the growth constraint that twins experience compared to singletons, would put twins at a different risk of some adult diseases. When twins were compared to the general population with respect to cardiovascular mortality in adulthood, however, no differences were found [22, 23]. The findings have further been confirmed by our comparison of twins and singletons from twin families concerning all-cause mortality and adult morbidity in cardiovascular disease and cancer (paper IV).

In response to these findings, it has been argued that twins may not be useful in exploring fetal origins of adult disease, because the regulation of growth in twins may be inherently different from that in singletons [183]. Twins have also been suggested to physiologically downregulate their growth early in gestation, and as a consequence become less sensitive to environmental insults throughout gestation [183]. Others have speculated that the splitting into two embryos from one zygote may lead to a delay of the embryonic development in MZ, which in turn could result in less sensitivity to insults compared to a more rapidly developing fetus [165]. With respect to developmental plasticity, it has also been suggested that epigenetic change (in response to maternal nutrition) may occur already in the preimplantation embryo, and that the environmental influence that causes discordant growth in twins would then occur too late to induce any systemic epigenetic change [130].

In contrast to these suggestions of a greater resilience in twins against fetal programming effects, the associations seen between birth weight and adult diseases in singletons appear to be present also in twin samples [20, 21, 24]. To reconcile these findings with those of no differences between singletons and twins with respect to adult outcomes, it has been suggested that the general growth constraint of twinning (due to sharing of space and supply line) does not influence adult disease risks, whereas factors that make twins experience different growth (from other twins and potentially also from their co-twin) could [25]. Several of these factors would be fetal growth determinants common also for singletons, such as gestational length, maternal, socioeconomic, and genetic factors.

### 6.1.2 INTERNAL VALIDITY OF TWIN STUDIES

The main challenge of studying fetal exposures in humans concerns the procurement of valid measures for the exposures of interest. Indicators of the fetal experience are commonly evaluated at birth, with measures of size for example taken to indicate growth in utero. The foundation of DOHaD theories relies on such proxy measures of fetal growth, which in turn appears to have been taken to indicate fetal nutrition. As discussed in the background, both proxy measures (such as birth weight) and fetal growth are determined by many other factors than fetal nutrition. Unrelated individuals may differ in these measures due to differences in gestational age, maternal constitution, environment and behavior, genetic differences, etc. Other than the potential for misclassification when using proxies, there is concern that some of the factors influencing these may also be involved in disease development, introducing a spurious association.

In twins, growth discordance is mainly a result of unequal share of the supply line (in DZ twins also a difference in fetal genetic factors). The availability of the supply line in turn, is a reflection of individual fetal nutrition. In addition, the birth weight discordance within twin pairs is commonly relatively large and develops predominantly in the third trimester, which by the original fetal origins hypothesis was stipulated to be the most critical period for insults.

Moreover, when comparisons are made within twin pairs, all factors shared by the pair are held constant. Birth weight differences within twin pairs will thus be independent of factors such as gestational age, maternal factors (e.g. constitution and behaviors), the early socioeconomic environment, etc. In monozygotic twin pairs, a difference in birth weight can further not be attributed to genetic influence.

The unique features of twins have also been used to evaluate the role of shared environment and common genes on associations between birth weight and later outcome, by comparing within-twinpair associations to the crude (i.e. between twins). The validity of such practice relies on assumptions that are not often made explicit. The perhaps most important pertains to the influence of other sources of bias. While going from crude to within comparison serves to remove the (potential confounding) influence of familial factors, this action could potentially also change the influence of other biases. Such a concern may arise, when, for instance, the sample used to obtain the within-twinpair estimate is much reduced (selected) compared to the between-twins comparison. Potential unmeasured individual confounding could also influence the between and within-pair association differently [226].

Additional speculation about the specific role of genetic factors has further come from contrasting DZ and MZ. The assumption that MZ are more alike than DZ purely as a result of their genetic resemblance may however be put into question. While the common reservation that MZ pairs may be treated differently (and therefore become more similar) does not quite apply to the intrauterine environment, the plausibility of an equal environment in utero may still need

scrutiny. A difference in the shared intrauterine environment for DZ and MZ would mainly be attributed to the fraction of monochorionic MZ twins, which have a greater potential for inter-fetal exchange (through vascular anastomoses in the placenta they share). With respect to twin growth discordance, however, other than the difference in genetic resemblance, the main determinants appear to be the same in monochorionic and dichorionic twins, namely unequal sharing of the supply line due to placental and chord function.

By allowing for a measure of fetal growth that is independent of gestational age, maternal factors and (to a varying degree) genetic factors, within-twin pair comparisons of birth weight can address several of the shortcomings of previous studies. Like all observational studies, however, internal validity will also depend on the potential influence of other sources of bias. Below follows a discussion of the methodological considerations specific to the papers in this thesis.

## 6.2 SPECIFIC METHODOLOGICAL CONSIDERATIONS

The study subjects in this thesis are all members of cohorts sampled from some theoretical source population. Individuals that meet a set of inclusion criteria (e.g. birth year, inclusion in a register, participation in data collection, etc.) are selected and followed prospectively, usually for a fixed period of time. When follow-up extends for a long time, as when subjects are followed in registers of health (studies II-IV), another type of selection occurs as subjects may be censored due to migration or death from other causes. Both types of selection (to and from) may have consequences for validity.

Other than fulfilling the features that define the source population (such as being twins of AA or EA descent and attending public schools in Georgia) the twins of the GCTS were characterized by their willingness to participate in a study. Volunteerism can be associated with health consciousness. In twin volunteer cohorts females and monozygotic twin pairs are often overrepresented, and this was noted also in the GCTS. Any greater influence from potential unmeasured common causes of volunteering and blood pressure (e.g. health consciousness), and birth weight (e.g. socioeconomic status, as illustrated in the DAG on page 22), is considered unlikely, and not least with respect to within-twinpair birth weight. We see no reason why the within-twinpair comparison of the GCTS twins would further not be representative for the source population.

For the STR cohort used in this thesis, and the matched cohorts of twins and singletons from the MGR, selection depends on inclusion criteria of the registers. Because the registers were established several years after the study subjects were born, the members of the cohorts will be survivors up to a certain age. In the cohorts sampled from the MGR, individuals had to be alive from 3 up to 29 years. In the STR both twins of the pair had to be alive in 1971, and in addition, zygosity status had to be determined through data collection participation. Over 80% response rate in the 1972-1973 questionnaire should however mitigate concerns about strong volunteer selection.

The potential influence of survival selection bias was of particular interest in the contrasting of twins and singleton morbidity and mortality "across the lifespan". Twins are at increased risk of perinatal and infant mortality [227], and early selection could potentially make twins a more resilient group (on average) compared to singleton survivors. In the comparison of survivors one would then expect a lower risk of mortality (and potentially also lower risk of other negative health outcomes) in the resilient group (twins) compared to singletons. The fact that we did not see any such tendencies could either indicate that such survival bias does not exist, that it has little influence, or that its expected effect has been outweighed by the presence of another negative health influence unique to twins.

In cohort studies, exposure status is established before the outcome occurs. This prospective design ensures that the exposure assessment is not influenced by the outcome. If the exposure is assessed in retrospect once the outcome has occurred, the accuracy with which exposure is reported may differ between the diseased and the non-diseased (a differential misclassification referred to as recall bias). In the Georgia cohort, birth weight information was self-reported by the mothers at the twins' first visit. Since our outcome blood pressure was also measured at baseline, this self-reported exposure assessment was not prospective. At the time of recall, it was nevertheless unlikely that mothers were aware of their healthy children's blood pressure.

Moreover, birth weight information was also retrieved from official birth records and used when available, so that self-reports were used in 40% of the sample. In the twins that had both types of data, the self-reports were found to be in good agreement with birth records (r=0.92, $P<0.01$). A difference in the degree of misclassification (as a result of using self-reports) could potentially contribute to the difference seen between AAs and EAs. Still, there was no greater difference in the distribution of self-reports, or their agreement with birth records, between ethnic groups.

In within-twinpair comparison of exposures at birth in particular, the potential influence from cross-misclassification also needs to be considered. Assigning a twin the characteristics of its co-twin (and vice versa) will distort any true influence of that characteristic, and conclusions from within-twinpair comparisons may be biased as a result. In the GCTS we had little ability to evaluate potential existence and influence of cross-misclassification, other than indirect indications from the high correlation between self-reports and birth records. In the STR cohort, however, efforts were made to minimize cross-misclassification, by requiring twins to either have been baptized at birth or to have accurately reported their birth order (in agreement with the recorded birth order).

Outcome classification was, in the three Swedish studies, based on diagnose identification in national registers of health. This practice assumes that incident events occur in Sweden, and are correctly diagnosed and recorded to the register(s). In this thesis the cardiovascular disease definition was restricted to acute events, in part to target the main adult CVDs, but also because these outcomes are likely to be caught in either hospital or cause of death records (high sensitivity). For breast cancer, there was a concern that cancer cases may be missed due to the limited ability to account for migration. In the study population considered at risk, individuals could have migrated (from Sweden and back) during the follow-up. If a woman was diagnosed (and treated) abroad, her cancer status could be delayed or missed in the Swedish Cancer Register. The likelihood of this happening in the estimated small number of migrators was, however, judged to be low. Later linkage with migration data has also allowed verification of no discernible outcome misclassification bias.

The presence of collider-stratification bias may, as previously discussed, be of concern for the topics under study here. In the field of early origins of later disease there are a number of things happening from the time of exposure until the onset of disease that may or may not be appropriate to account for. The careful reader may notice that the present work is not exempt from potentially inappropriate practice. In the first study, our aim was to explore whether there was any support for the claim that ethnic differences originate in utero. For this to hold, it was argued that fetal growth would have to be associated with blood pressure independent of familial factors in African Americans. Before exploring this through within-twinpair comparison, we assessed whether the effect of ethnicity on blood pressure remained after adjustment for birth weight. Ethnicity is a strong predictor of birth weight, and we do suspect that there can be unmeasured common causes of birth weight and blood pressure (e.g. socioeconomic and genetic factors). Since conditioning on birth weight thus may have introduced a spurious association between ethnicity and blood pressure, the claim that birth weight cannot "explain" ethnic differences in blood pressure may not be valid.

In studies of associations between birth weight and later health outcomes, the influence from potential confounding is of particular concern. The cohort analyses in this thesis share, and illustrate, several of the limitations of previous studies in unrelated individuals. In the GCTS there was, for example, no ability to account for gestational age, which may be an independent determinant of blood pressure. In addition, information on maternal morbidities and parental socioeconomic status was incomplete both in the GCTS and the STR. The purpose of the within-twinpair comparisons, however, has been to address several of these shortcomings. While the associations obtained from within-twinpair comparison cannot be confounded by the factors shared by the twin pair, they are still susceptible to potential confounding from factors that are not shared by the twins of a twin pair. In these studies of prenatal growth, influence from individual common causes of the exposure and later outcome may be of a less concern.

## 6.3 FINDINGS & IMPLICATIONS

In paper I, the aim was to investigate the grounds for the claim that ethnic differences in blood pressure originate in utero. For this to hold, we would expect there to be an association between birth weight and blood pressure, independent of familial factors, in African Americans. While abundant evidence exist to support such an association predominantly in white populations of all ages [85], investigations in AAs are still scarce. Only one of the few studies assessing the association in AA children or young adults has reported of an inverse association in AAs similar to that seen in EAs, amplifying with age [97]. Another study tracking children into adolescence found an inverse association only in EAs [88]. In these and most other studies, the ability to evaluate confounding by common causes of birth weight and blood pressure was limited.

As expected, mean blood pressure levels were found higher in AAs than in EAs in our sample of adolescent twins. However, only the girls were also significantly different with respect to mean birth weight (AA girls weighted less than EA girls). More importantly, an inverse association between birth weight and systolic blood pressure was present, and found to be independent of familial factors in AA, but not in EA twins.

The inconsistency of results from previous bi-ethnic samples could be due to small sample sizes, different age distributions when measuring blood pressure, and inadequate control for confounding factors. Particularly the variation with age may be of importance in this case, as a general attenuation of the association of birth weight and blood pressure during adolescence has been noted and attributed to changes during puberty [89]. Potential ethnic differences, in for instance sexual maturation, could thus in part account for disparities in the birth weight – blood pressure association. Catch-up growth, and especially centile crossing catch-up in childhood by babies small at birth, is often discussed as an important component in the pathway towards future morbidity [228]. Without knowledge of the early postnatal growth trajectories in this cohort, we cannot exclude that ethnic differences also with respect to catch-up growth could explain some of our findings.

Ethnic differences in mean blood pressure levels found in this sample were not consistently matched by corresponding differences in birth weight. However, the finding of an inverse association between birth weight and systolic blood pressure in AAs (independent of familial factors) suggests that the generally less favorable birth weight outcome in AAs may be of consequence for future blood pressure. The findings support further tracking of the birth weight association to blood pressure into adulthood in this ethnic group.

In like-sexed Swedish twins of known zygosity, high birth weight was found to confer an increased risk of breast cancer diagnosed before or at the age of 50. The positive linear association seen in twins above 2,500 g also appeared to be independent of familial factors (shared early environment and common genes).

The findings are in agreement with the results from recent reviews and meta-analyses [16-19] which all confirm a modest increased risk of breast cancer with increasing birth weight, particularly in women diagnosed before or at menopause. In a large re-analysis of individual-level data, birth length was found to be a stronger predictor of breast cancer risk than birth weight [19]. Birth length had no effect on the risk of breast cancer in our study, which could potentially be a result of imprecise measurement (birth length generally being more prone to measurement error).

Finding a positive association between birth weight and risk of early onset breast cancer also within twin pairs indicates a role for the individual prenatal experience with respect to fetal nutrition and growth. The association between birth weight and breast cancer has been proposed to reflect uterine hormonal exposure, which could both stimulate fetal growth and have carcinogenic effects on the breast [12]. Our findings have no straightforward translation to inference concerning potential hormone exposure pathways. Little is known of fetal hormonal exposure in twins, both in relation to the mother and the co-twin. While pregnancy is associated with high estrogen levels in the mother [229], the correlations to the levels in the fetal cord blood seem moderate [230]. Furthermore, the levels of estrogen in the fetal circulation throughout pregnancy are still largely unknown. Maternal estrogen levels are higher in twin compared with singleton pregnancies [109], but to which extent prenatal hormonal exposures are shared within twin siblings is also unknown.

There is increasing interest for the potential role of epigenetics in DOHaD [130]. Epigenetic modulation may (due to differing gene expression) lead to phenotypic differences within monozygotic twin pairs [175]. Fetal growth may in part be epigenetically determined through imprinting, with most imprinted genes also being expressed in the placenta [231]. The imprinted IGF2 gene has been proposed to control both placental supply and fetal demand for nutrients [115], and loss of imprinting and bi-allelic expression of the IGF2 allele has been linked to both fetal growth [115, 116] and breast cancer development [113, 114]. With respect to our findings, any individually acquired epigenetic changes during prenatal life (possibly in response to individual environmental stimuli) would appear independent of familial factors.

These findings indicate that the intrauterine experience may be of importance for the development of early onset breast cancer. In the continued efforts to understand how fetal factors influence breast cancer etiology, these findings support further investigation of prenatal exposures or events that are independent of familial factors.

Including also the like-sexed men of the 1926-1958 STR cohort, we could further show that birth weight was inversely associated with risk of CVD within dizygotic, but not within monozygotic twin pairs. Similar to previous findings in singletons, associations were seen for both coronary heart disease and ischemic stroke, but only within dizygotic twin pairs. The findings indicate that understanding what factors are shared within monozygotic but not within dizygotic twin pairs could provide insights to the mechanisms behind birth weight – CVD association.

Factors that are shared within MZ twin pairs but not DZ pairs are discussed in detail in the background (twins; pages 24-27). Vascular anastomoses in the placentas of MZMC twins allow for a unique sharing between these twins compared to MZDC and DZ. Within-twinpair estimates of growth will be independent of such factors, so that if these were to be the causal antecedents of fetal programming, we would not expect to see a within-twinpair association in MZMC. It is however unclear what these factors would be. The other fundamental difference between MZ and DZ with respect to within-twinpair sharing concerns the degree of shared genetic factors. Since the within-twinpair association is independent of genetic factors within MZ but not DZ twin pairs, our findings are compatible with there being a common genetic background for fetal growth and cardiovascular disease. In such a case an intergenerational association between the two phenotypes could be expected, and has been confirmed in some studies (offspring birth weight and parental/grandparental CVD mortality) [232, 233].

Another potential and speculative explanation to the lack of association within MZ twins would be if epigenetic changes in response to maternal nutrition only occur in the preimplantation embryo [130]. Epigenetic changes occurring at this early stage would be independent of the processes that later produce within-twinpair growth discordance. However, under this scenario we see no principal reason why this would not also apply to DZ twins who also share early environment, and we would thus not expect to find an association within any type of twin pairs.

It has further been proposed that the underlying genotype may modify the programming effects of early life exposures [234]. If susceptibility to early life exposures depends on underlying genotype, only susceptible individuals would contribute to the observed associations. This would also be the case in twins, where only pairs in which the exposed twin was also genetically susceptible would contribute to a within pair association. It follows that for this scenario to be compatible with the present findings, there would have to be a systematic difference in distribution of genetic susceptibility between DZ and MZ twins; notably MZ twins would have to be significantly less genetically susceptible to programming in utero compared to DZ twins.

Our findings indicate that understanding which factors are shared within MZ but not DZ twin pairs, may help identify the underlying mechanisms to the association between birth weight and cardiovascular disease.

Past the first years of life, we found no indication that twins would have any survival disadvantage (or advantage) compared to singletons. Overall, twins did not appear to fare worse than singletons from twin families with respect to risk of common diseases such as cancer and CVD, or with respect to all-cause mortality. Nor did individuals from twin families appear to be at any different risk of morbidity and mortality compared to individuals from non-twin families.

These findings confirm those of previous studies of CVD and all-cause mortality in Swedish and Danish twins [22, 23, 235]. The lack of difference in risk between twins and the general population has been taken as an indication that the general growth constraint due to twinning (sharing space and supply line) is different from the type of growth impairment (experienced by both twins and singletons) that appears to be associated with diseases later in life [25]. If the apparent association between birth weight and CVD were to be the result of common genetic factors (which would be in line with the results of our assessment of the association in disease-discordant twins) there would be no reason to suspect any difference in CVD risk between twins and singletons. The present method to compare twins to singletons of twin families should further mitigate any concern for potential confounding from twin family factors (e.g. socioeconomic or genetic factors shared by twin families at large). Still, the unique twinning experience in this setting does not discriminate between the pre- and postnatal environment.

With respect to overall cancer risk the situation may be less straightforward. Overall, our findings do not support any effect of twinning on overall cancer risk. This is in line with previous findings from comparisons of twins and the population in Sweden [26, 28], whereas in Norway twins have been found at decreased risk of cancer overall compared to the general population [27]. Cancer is a heterogeneous group of diseases, and it is possible that increased risks for some type(s) are washed out by decreased risks for other(s), when considered all together. Decreased risks of skin and colorectal cancer have been noted in both Norwegian and Swedish twins [26, 27], whereas the Swedish studies also found young twins at increased risk of testicular cancer [26, 28] and breast cancer [28]. Since these studies compared the experience of twins to rates in the general population, it should be interesting to evaluate risks of subtypes of cancer using the present study design – with ability to make comparisons independent of twin family factors. Hence, even though the present findings of no effect of twinning on overall risk of cancer may present some comfort with respect to representativeness, we believe that further research is required, as twinning (and potentially also zygosity) may influence certain specific cancer diseases.

Finding that the unique experience of twinning does not appear to put twins at any different risk of common complex diseases compared to singletons should be reassuring for the use of twins in epidemiological research. The ability to generalize findings in twins is of great importance for all types of twin studies. Particularly with respect to cardiovascular disease and overall mortality – which have been associated with an adverse intrauterine experience – the present findings are in line with previous claims that the twinning experience per se may not put twins at greater risk of common adverse health outcomes.

The time we spend in utero is undoubtedly the most important developmental period of our lives. Experiences in this critical time may influence the future functioning, and thereby health, of the developing individual. The emergence and explosion of the DOHaD research field has been instrumental in increasing the awareness and interest for the importance of early life and development. Although extensive research has produced sophisticated theories and plausible potential mechanisms, the complete pathways through which the fetal environment may influence future disease development in humans remain to be unraveled, and even more so the specific (and potentially modifiable) insults or triggers.

In this thesis the aim has been to target a more specific measure of intrauterine exposure, obtained by the difference in growth between twins in a twin pair. While providing some clues about the role of fetal growth in DOHaD, the findings also illustrate the complexity of the field, and of attributing various health outcomes to one unifying hypothesis. Similarly, the associations seen between birth weight and later disease are not likely to share one comprehensive explanation, but rather reflect a varying degree of interplay between influence from genetic factors, the fetal environment and possibly also the postnatal period.

The major challenge in this field of research clearly concerns the ability to study specific intrauterine exposures. While animal models can contribute with information about the consequences of specific prenatal insults by allowing experimental manipulation, the extent to which such findings apply to the generally much slower fetal development in humans is inconspicuous.

While we believe within-twinpair comparisons can provide valuable clues about the role of fetal growth in "fetal programming", they come with their own challenges and potential limitations. Among the things that will require further study is the evaluation of potential threats to generalizability from twin studies concerning the role of the fetal environment for adult disease. This includes a better understanding of the nature of twin sharing of e.g. hormonal factors in utero and the influence of inter-fetal sharing via vascular anastomoses (in MZMC). Potential systematic differences in genetic factors and/or patterns of epigenetic modulation in twins (DZ vs. MZ) also need further investigation. Naturally, understanding if and to what extent environmentally induced epigenetic changes could also be inherited should be of great consequence. The potential presence of, and influence from, individual common causes of exposure and outcome in within-twinpair comparisons will also require further study. Lastly, many previous within-twinpair comparisons have been hampered by limited power (so also papers I & II of this thesis). Stratification beyond that into zygotic groups reduces the precision with which associations are estimated. Thus, even with high validity large samples will be required for inference from within-twinpair comparisons.

## 6.4 OVERALL CONCLUSIONS

- The previously well-established inverse association between birth weight and blood pressure appears to be present also in African American adolescent twins, independent of familial factors.

- Increasing birth weight (from 2,500g and upward) in female twins is associated with an increased risk of breast cancer diagnosed at 50 years or earlier, and the association appears independent of familial factors.

- Birth weight is inversely associated with risk of cardiovascular disease within dizygotic but not monozygotic twin pairs. In twins, there is no support for an association between birth weight and cardiovascular disease in the absence of genetic variation.

- Twins do not fare worse than their singleton siblings with respect to cumulative risks of cardiovascular disease, overall cancer or mortality. Furthermore, twin family membership does not appear to influence the risk of either of these outcomes. Overall, twins do not appear different from the general population with respect to adult morbidity and mortality.

The studies in this thesis have been generously supported by:

The Swedish Council for Working Life and Social Research
(No. 2004-0174, 2004-1654, 2007-0231)
The Swedish Research Council
(No. K2006-71X-14676-04-2, K2007-70P-20518-01-4 & K2008-54X-20638-01-3)
The Swedish Cancer Society (No. 4594-B01-01XAC and 4594-B04-04XAB)
The European Union funded Network of Excellence Lifespan (No. FP6036894)
The National Institutes of Health (No. HL56622)

# 7 ACKNOWLEDGEMENTS

**Anastasia Nyman Iliadou**, my main-advisor: For your endless enthusiastic, wise, and incredibly generous academic support. If possible even more for always looking out for me, and your unique ability to keep me grounded while encouraging me to fly!

**Sven Cnattingius**, my co-advisor: For introducing me to the world of perinatal epidemiology and sharing your vast expertise as well as connections in the field. Consulting with you has always left me confident and excited about what we do!

**Paul Lichtenstein**, my co-advisor: For your critical appraisal and most valuable input. Also for providing guidance and support in times when I've needed it the most!

**Sven Sandin**, co-author, SAS genius and statistical counsel: For always meting my endless questions and hang-ups with patience, interest, enthusiasm, and of course, sound advice.

**Ulf Rydberg**, my mentor: For first raising my interest for the importance of the prenatal period (way back in the early days of medical school)! For remaining an adamant supporter, academic role model, wise and caring mentor, and most of all a good friend.

I am naturally also grateful for the generous contributions of my co-authors **Harold Snieder, Dongliang Ge, Frank Treiber, Anna Svensson** and **Ruth Morley**.

To my Epi-gang; a collection of brilliant, dedicated, and spirited gastronomes: **Christin Bexelius, Thomas Frisell, Arvid Sjölander & Anna Svensson.** This wouldn't have been half as fun, challenging, or exciting without you.. Academia and I need you!

For enabling a stable and stimulating work environment, the current and former heads of department **Henrik Grönberg**, **Nancy Pedersen,** and **Hans-Olov Adami** (without whose vision and leadership the department would not be, nor would probably my connection to Boston, and for your enthusiastic and generous support of that I'm forever grateful).

For always listening and supporting, and sometimes even joining the students' initiatives to improve postgraduate education / conditions at the Department, the former study directors **Kamila Czene** and **Paul Dickman**

Much of the research at MEB would not be possible without the supportive infrastructure of IT, data management and administrative personnel. I would never have pulled off all the stuff that needs to be done outside of the actual research without the three lovely ladies **Gunilla Sonnebring, Katarina Ekenäs** and **Camilla Ahlqvist**. In some of the more practical aspects of research I greatly appreciate the assistance from **Rozita Broumandi, Ann-Marie Nyberg** and **Gunnar Pettersson**

For setting excellent standards, my predecessors in Perinatal Epidemiology: **Lena George, Niklas Bergvall, Stefan Johansson** and **Susanne Buchmayer**

To all the former and current PhD-student colleagues, for keeping the spirit, working toward student improvements and most of all for your good company

To hosts, collaborators and friends at Melbourne University: **Drs. Ruth Morley, John Carlin, Terrence Dwyer, Elya Wood, Katrina Scurrah, Lyle Gurrin,** and **John Hopper**

For enabling incredible opportunities to expand my academic training and experience outside of MEB, I'm most grateful to the **NIHCHD/CIHR** for their Summer Institute of Reproductive and Perinatal Epidemiology, the **Swedish Council for Working Life and Social Research** for allowing my visit at the Murdoch Childrens Research Institute in Melbourne, and to the **Fulbright Commission** for supporting my master studies in the US

To all my **HSPH peeps** for contributing to the best year ever, and for continuing to be the most amazing circle of friends, y'all know who you are!

To the *really* old friends that never falter; **Elinor** & **Charlotte** for your longstanding, honest and always sound love, **Adam** & **Kajsa** for your warm and wise friendship, and **Marie** & **Carl** for your support and great company (whether over Grange or Rock Band!)

To **David, Maja** & **Michaela**, for proving there can be rocks outside of family

And last, only because you matter the most, **to my family**, without whom I would not be

# 8 REFERENCES


1. Barker, D.J., et al., *Weight in infancy and death from ischaemic heart disease.* Lancet, 1989. **2**(8663): p. 577-80.
2. Huxley, R., et al., *Is birth weight a risk factor for ischemic heart disease in later life?* Am J Clin Nutr, 2007. **85**(5): p. 1244-50.
3. Curhan, G.C., et al., *Birth weight and adult hypertension, diabetes mellitus, and obesity in US men.* Circulation, 1996. **94**(12): p. 3246-50.
4. Whincup, P.H., et al., *Birth weight and risk of type 2 diabetes: a systematic review.* Jama, 2008. **300**(24): p. 2886-97.
5. Barker, D.J., *Fetal origins of coronary heart disease.* Bmj, 1995. **311**(6998): p. 171-4.
6. Gluckman, P.D., M.A. Hanson, and C. Pinal, *The developmental origins of adult disease.* Maternal & Child Nutrition, 2005. **1**(3): p. 130-141.
7. Huxley, R., A. Neil, and R. Collins, *Unravelling the fetal origins hypothesis: is there really an inverse association between birthweight and subsequent blood pressure?* Lancet, 2002. **360**(9334): p. 659-65.
8. Leon, D.A. and I. Koupilova, *Birthweight, blood pressure and hypertension: epidemiological studies.*, in *Fetal origins of Cardiovascular and Lung Disease*, D.J. Barker, Editor 2000, National Institutes of Health: Bethseda, Md.
9. Law, C.M., et al., *Initiation of hypertension in utero and its amplification throughout life.* Bmj, 1993. **306**(6869): p. 24-7.
10. Lopes, A.A. and F.K. Port, *The low birth weight hypothesis as a plausible explanation for the black/white differences in hypertension, non-insulin-dependent diabetes, and end-stage renal disease.* Am J Kidney Dis, 1995. **25**(2): p. 350-6.
11. Forrester, T., *Historic and early life origins of hypertension in Africans.* J Nutr, 2004. **134**(1): p. 211-6.
12. Trichopoulos, D., *Hypothesis: does breast cancer originate in utero?* Lancet, 1990. **335**(8695): p. 939-40.
13. Anbazhagan, R. and B.A. Gusterson, *Prenatal factors may influence predisposition to breast cancer.* Eur J Cancer, 1994. **30A**(1): p. 1-3.
14. Petridou, E., et al., *Tobacco smoking, pregnancy estrogens, and birth weight.* Epidemiology, 1990. **1**(3): p. 247-50.
15. Fowden, A.L. and A.J. Forhead, *Endocrine mechanisms of intrauterine programming.* Reproduction, 2004. **127**(5): p. 515-26.
16. Michels, K.B. and F. Xue, *Role of birthweight in the etiology of breast cancer.* Int J Cancer, 2006.
17. Xue, F. and K.B. Michels, *Intrauterine factors and risk of breast cancer: a systematic review and meta-analysis of current evidence.* Lancet Oncol, 2007. **8**(12): p. 1088-100.
18. Park, S.K., et al., *Intrauterine environments and breast cancer risk: meta-analysis and systematic review.* Breast Cancer Res, 2008. **10**(1): p. R8.
19. Silva Idos, S., B. De Stavola, and V. McCormack, *Birth size and breast cancer risk: re-analysis of individual participant data from 32 studies.* PLoS Med, 2008. **5**(9): p. e193.
20. Johansson, S., et al., *The association between low birth weight and type 2 diabetes: contribution of genetic factors.* Epidemiology, 2008. **19**(5): p. 659-65.
21. Bergvall, N., et al., *Genetic and shared environmental factors do not confound the association between birth weight and hypertension: a study among Swedish twins.* Circulation, 2007. **115**(23): p. 2931-8.
22. Christensen, K., et al., *Cardiovascular mortality in twins and the fetal origins hypothesis.* Twin Res, 2001. **4**(5): p. 344-9.

23. Vagero, D. and D. Leon, *Ischaemic heart disease and low birth weight: a test of the fetal-origins hypothesis from the Swedish Twin Registry.* Lancet, 1994. **343**(8892): p. 260-3.
24. Hubinette, A., et al., *Birthweight, early environment, and genetics: a study of twins discordant for acute myocardial infarction.* Lancet, 2001. **357**(9273): p. 1997-2001.
25. Morley, R., *Fetal origins of adult disease.* Semin Fetal Neonatal Med, 2006. **11**(2): p. 73-8.
26. Hemminki, K. and B. Chen, *Are twins at risk of cancer: results from the Swedish family-cancer database.* Twin Res Hum Genet, 2005. **8**(5): p. 509-14.
27. Iversen, T., S. Tretli, and E. Kringlen, *An epidemiological study of cancer in adult twins born in Norway 1905-1945.* Br J Cancer, 2001. **84**(11): p. 1463-5.
28. Braun, M.M., et al., *Effect of twinship on incidence of cancer of the testis, breast, and other sites (Sweden).* Cancer Causes Control, 1995. **6**(6): p. 519-24.
29. Posthuma, D., et al., *Twin-singleton differences in intelligence?* Twin Res, 2000. **3**(2): p. 83-7.
30. Baskett, T.F. and F. Nagele, *Naegele's rule: a reappraisal.* Bjog, 2000. **107**(11): p. 1433-5.
31. Fehring, R.J., M. Schneider, and K. Raviele, *Variability in the Phases of the Menstrual Cycle.* Journal of Obstetric, Gynecologic, & Neonatal Nursing, 2006. **35**(3): p. 376-384.
32. Bergsjo, P., et al., *Duration of human singleton pregnancy. A population-based study.* Acta Obstet Gynecol Scand, 1990. **69**(3): p. 197-207.
33. Trivers, R.L., *Parent-offspring conflict.* Am Zool, 1974. **14**: p. 249-64.
34. Moore, T. and D. Haig, *Genomic imprinting in mammalian development: a parental tug-of-war.* Trends Genet, 1991. **7**(2): p. 45-9.
35. Lunde, A., et al., *Genetic and environmental influences on birth weight, birth length, head circumference, and gestational age by use of population-based parent-offspring data.* Am J Epidemiol, 2007. **165**(7): p. 734-41.
36. Svensson, A.C., et al., *Familial aggregation of small-for-gestational-age births: the importance of fetal genetic effects.* Am J Obstet Gynecol, 2006. **194**(2): p. 475-9.
37. Murphy, V.E., et al., *Endocrine Regulation of Human Fetal Growth: The Role of the Mother, Placenta, and Fetus.* Endocr Rev, 2006. **27**(2): p. 141-169.
38. Kramer, M.S., *Intrauterine growth and gestational duration determinants.* Pediatrics, 1987. **80**(4): p. 502-11.
39. Lampl, M., et al., *Sex differences in fetal growth responses to maternal height and weight.* American Journal of Human Biology, 2010. **22**(4): p. 431-443.
40. Hakansson, A., et al., *A new symphysis-fundus height growth chart based on a well defined female population with ultrasound-dated singleton pregnancies.* Acta Obstet Gynecol Scand, 1995. **74**(9): p. 682-6.
41. Brodszki, J. and K. Marsal, *Management of pregnancies with suspected intrauterine growth retardation in Sweden. Results of a questionnaire.* Acta Obstet Gynecol Scand, 2000. **79**(9): p. 723-8.
42. Marsal, K., et al., *Intrauterine growth curves based on ultrasonically estimated foetal weights.* Acta Paediatr, 1996. **85**(7): p. 843-8.
43. Wilcox, A.J., *On the importance--and the unimportance--of birthweight.* Int J Epidemiol, 2001. **30**(6): p. 1233-41.
44. Kramer, M.S., et al., *Body proportionality and head and length 'sparing' in growth-retarded neonates: a critical reappraisal.* Pediatrics, 1989. **84**(4): p. 717-23.
45. Luke, B., *Nutritional influences on fetal growth.* Clin Obstet Gynecol, 1994. **37**(3): p. 538-49.
46. Gruenwald, P., *Growth of the human fetus. I. Normal growth and its variation.* Am J Obstet Gynecol, 1966. **94**(8): p. 1112-9.
47. Gardosi, J., *New definition of small for gestational age based on fetal growth potential.* Horm Res, 2006. **65 Suppl 3**: p. 15-8.

48. Villar, J. and J.M. Belizan, *The timing factor in the pathophysiology of the intrauterine growth retardation syndrome.* Obstet Gynecol Surv, 1982. **37**(8): p. 499-506.
49. Tanner, J.M., *Fetus into Man*1978, Boston: Harvard University Press.
50. Gruenwald, P., *Intrauterine growth*, in *Perinatal Physiology*, U. Stave, Editor 1978, Plenum: New York.
51. Vik, T., et al., *Prenatal growth in symmetric and asymmetric small-for-gestational-age infants.* Early Hum Dev, 1997. **48**(1-2): p. 167-76.
52. Lampl, M. and P. Jeanty, *Timing is everything: a reconsideration of fetal growth velocity patterns identifies the importance of individual and sex differences.* Am J Hum Biol, 2003. **15**(5): p. 667-80.
53. Kramer, M.S., et al., *US Black vs White disparities in foetal growth: physiological or pathological?* Int J Epidemiol, 2006. **35**(5): p. 1187-95.
54. Joseph, K.S., et al., *Customized birth weight for gestational age standards: Perinatal mortality patterns are consistent with separate standards for males and females but not for blacks and whites.* BMC Pregnancy Childbirth, 2005. **5**(1): p. 3.
55. Barker, D.J., et al., *The relation of small head circumference and thinness at birth to death from cardiovascular disease in adult life.* Bmj, 1993. **306**(6875): p. 422-6.
56. Bryan, S.M. and P.C. Hindmarsh, *Normal and abnormal fetal growth.* Horm Res, 2006. **65 Suppl 3**: p. 19-27.
57. Hindmarsh, P.C., et al., *Intrauterine growth and its relationship to size and shape at birth.* Pediatr Res, 2002. **52**(2): p. 263-8.
58. Barker, D.J. and C. Osmond, *Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales.* Lancet, 1986. **1**(8489): p. 1077-81.
59. Kermack, W.O., A.G. McKendrick, and P.L. McKinlay, *Death-rates in Great Britain and Sweden. Some general regularities and their significance.* Int J Epidemiol, 2001. **30**(4): p. 678-83.
60. Rose, G., *Familial Patterns in Ischaemic Heart Disease.* Br J Prev Soc Med, 1964. **18**: p. 75-80.
61. Forsdahl, A., *Are poor living conditions in childhood and adolescence an important risk factor for arteriosclerotic heart disease?* Br J Prev Soc Med, 1977. **31**(2): p. 91-5.
62. Barker, D.J., et al., *Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease.* Bmj, 1989. **298**(6673): p. 564-7.
63. Hales, C.N., et al., *Fetal and infant growth and impaired glucose tolerance at age 64.* Bmj, 1991. **303**(6809): p. 1019-22.
64. Abel, K.M., et al., *Birth Weight, Schizophrenia, and Adult Mental Disorder: Is Risk Confined to the Smallest Babies?* Arch Gen Psychiatry, 2010. **67**(9): p. 923-930.
65. Shaheen, S.O., et al., *Birth weight, body mass index and asthma in young adults.* Thorax, 1999. **54**(5): p. 396-402.
66. Lucas, A., *Programming by early nutrition in man.* Ciba Found Symp, 1991. **156**: p. 38-50; discussion 50-5.
67. Hales, C.N. and D.J. Barker, *Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis.* Diabetologia, 1992. **35**(7): p. 595-601.
68. Gluckman, P.D., M.A. Hanson, and H.G. Spencer, *Predictive adaptive responses and human evolution.* Trends Ecol Evol, 2005. **20**(10): p. 527-33.
69. Gluckman, P.D. and M.A. Hanson, *Developmental origins of disease paradigm: a mechanistic and evolutionary perspective.* Pediatr Res, 2004. **56**(3): p. 311-7.
70. Hoet, J.J. and M.A. Hanson, *Intrauterine nutrition: its importance during critical periods for cardiovascular and endocrine development.* J Physiol, 1999. **514 ( Pt 3)**: p. 617-27.
71. Stanner, S.A., et al., *Does malnutrition in utero determine diabetes and coronary heart disease in adulthood? Results from the Leningrad siege study, a cross sectional study.* Bmj, 1997. **315**(7119): p. 1342-8.

72. Roseboom, T.J., et al., *Coronary heart disease after prenatal exposure to the Dutch famine, 1944-45.* Heart, 2000. **84**(6): p. 595-8.
73. Omran, A.R., *EPIDEMIOLOGIC TRANSITION - THEORY OF EPIDEMIOLOGY OF POPULATION CHANGE.* Milbank Memorial Fund Quarterly, 1971. **49**(4): p. 509-&.
74. Gaziano, T.A., *Cardiovascular Disease in the Developing World and Its Cost-Effective Management.* Circulation, 2005. **112**(23): p. 3547-3553.
75. WHO. *The Atlas of Heart Diseases and Stroke*. 2004 [cited 2011 May 1]; Available from: http://www.who.int/cardiovascular_diseases/resources/atlas/en/.
76. Martyn, C.N., D.J. Barker, and C. Osmond, *Mothers' pelvic size, fetal growth, and death from stroke and coronary heart disease in men in the UK.* Lancet, 1996. **348**(9037): p. 1264-8.
77. Risnes, K.R., et al., *Birthweight and mortality in adulthood: a systematic review and meta-analysis.* Int J Epidemiol, 2011.
78. Eriksson, J.G., et al., *Early growth, adult income, and risk of stroke.* Stroke, 2000. **31**(4): p. 869-74.
79. Hypponen, E., et al., *Prenatal growth and risk of occlusive and haemorrhagic stroke in Swedish men and women born 1915-29: historical cohort study.* Bmj, 2001. **323**(7320): p. 1033-4.
80. Lawlor, D.A., et al., *Birth weight is inversely associated with incident coronary heart disease and stroke among individuals born in the 1950s: findings from the Aberdeen Children of the 1950s prospective cohort study.* Circulation, 2005. **112**(10): p. 1414-8.
81. Rich-Edwards, J.W., et al., *Longitudinal study of birth weight and adult body mass index in predicting risk of coronary heart disease and stroke in women.* Bmj, 2005. **330**(7500): p. 1115.
82. Barker, D.J., et al., *Fetal and placental size and risk of hypertension in adult life.* Bmj, 1990. **301**(6746): p. 259-62.
83. Koupil, I., D.A. Leon, and L. Byberg, *Birth weight, hypertension and /`white coat/' hypertension: size at birth in relation to office and 24-h ambulatory blood pressure.* J Hum Hypertens, 2005. **19**(8): p. 635-642.
84. Yliharsila, H., et al., *Self-Perpetuating Effects of Birth Size on Blood Pressure Levels in Elderly People.* Hypertension, 2003. **41**(3): p. 446-450.
85. Huxley, R.R., A.W. Shiell, and C.M. Law, *The role of size at birth and postnatal catch-up growth in determining systolic blood pressure: a systematic review of the literature.* J Hypertens, 2000. **18**(7): p. 815-31.
86. Whincup, P.H., D.G. Cook, and A.G. Shaper, *Early influences on blood pressure: a study of children aged 5-7 years.* Bmj, 1989. **299**(6699): p. 587-91.
87. Davies, A.A., et al., *Association between birth weight and blood pressure is robust, amplifies with age, and may be underestimated.* Hypertension, 2006. **48**(3): p. 431-6.
88. Li, C., et al., *Birth weight, puberty, and systolic blood pressure in children and adolescents: a longitudinal analysis.* J Hum Hypertens, 2006. **20**(6): p. 444-50.
89. Barker, D.J. and C.M. Law, *Birth weight and blood pressure in adolescence. Studies may be misleading.* Bmj, 1994. **308**(6944): p. 1634.
90. Lever, A.F. and S.B. Harrap, *Essential hypertension: a disorder of growth with origins in childhood?* J Hypertens, 1992. **10**(2): p. 101-20.
91. Dekkers, J.C., et al., *Moderators of blood pressure development from childhood to adulthood: a 10-year longitudinal study.* J Pediatr, 2002. **141**(6): p. 770-9.
92. Salihu, H.M., L. Fitzpatrick, and M.H. Aliyu, *Racial disparity in fetal growth inhibition among singletons and multiples.* Am J Obstet Gynecol, 2005. **193**(2): p. 467-74.
93. Falkner, B., S. Hulman, and H. Kushner, *Birth weight versus childhood growth as determinants of adult blood pressure.* Hypertension, 1998. **31**(1): p. 145-50.

94. Hughson, M.D., et al., *Hypertension, glomerular number, and birth weight in African Americans and white subjects in the southeastern United States.* Kidney Int, 2006. **69**(4): p. 671-8.
95. Hemachandra, A.H., M.A. Klebanoff, and S.L. Furth, *Racial disparities in the association between birth weight in the term infant and blood pressure at age 7 years: results from the collaborative perinatal project.* J Am Soc Nephrol, 2006. **17**(9): p. 2576-81.
96. Rostand, S.G., S.P. Cliver, and R.L. Goldenberg, *Racial disparities in the association of foetal growth retardation to childhood blood pressure.* Nephrol Dial Transplant, 2005. **20**(8): p. 1592-7.
97. Chen, W., S.R. Srinivasan, and G.S. Berenson, *Amplification of the association between birthweight and blood pressure with age: the Bogalusa Heart Study.* J Hypertens, 2010. **28**(10): p. 2046-52.
98. Macmahon, B., *Prenatal x-ray exposure and childhood cancer.* J Natl Cancer Inst, 1962. **28**: p. 1173-91.
99. Greenwald, P., et al., *Vaginal cancer after maternal treatment with synthetic estrogens.* N Engl J Med, 1971. **285**(7): p. 390-2.
100. Kelsey, J.L., M.D. Gammon, and E.M. John, *Reproductive factors and breast cancer.* Epidemiol Rev, 1993. **15**(1): p. 36-47.
101. Travis, R.C. and T.J. Key, *Oestrogen exposure and breast cancer risk.* Breast Cancer Res, 2003. **5**(5): p. 239-47.
102. Bergkvist, L., et al., *The risk of breast cancer after estrogen and estrogen-progestin replacement.* N Engl J Med, 1989. **321**(5): p. 293-7.
103. Trichopoulos, D. and R.D. Lipman, *Mammary gland mass and breast cancer risk.* Epidemiology, 1992. **3**(6): p. 523-6.
104. Rothman, K.J., et al., *Maternal age and birth rank of women with breast cancer.* J Natl Cancer Inst, 1980. **65**(4): p. 719-22.
105. Innes, K., T. Byers, and M. Schymura, *Birth characteristics and subsequent risk for breast cancer in very young women.* Am J Epidemiol, 2000. **152**(12): p. 1121-8.
106. Ekbom, A., et al., *Evidence of prenatal influences on breast cancer risk.* Lancet, 1992. **340**(8826): p. 1015-8.
107. Cerhan, J.R., et al., *Twinship and risk of postmenopausal breast cancer.* J Natl Cancer Inst, 2000. **92**(3): p. 261-5.
108. Murphy, M. and J. Doherty, *Letter: Does breast cancer originate in utero?* Lancet, 1990. **335**(8705): p. 1604.
109. Kappel, B., et al., *Human placental lactogen and dU-estrogen levels in normal twin pregnancies.* Acta Genet Med Gemellol (Roma), 1985. **34**(1-2): p. 59-65.
110. Kaijser, M., et al., *Maternal pregnancy estriol levels in relation to anamnestic and fetal anthropometric data.* Epidemiology, 2000. **11**(3): p. 315-9.
111. Troisi, R., N. Potischman, and R.N. Hoover, *Exploring the underlying hormonal mechanisms of prenatal risk factors for breast cancer: a review and commentary.* Cancer Epidemiol Biomarkers Prev, 2007. **16**(9): p. 1700-12.
112. Trichopoulos, D., *Intrauterine environment, mammary gland mass and breast cancer risk.* Breast Cancer Res, 2003. **5**(1): p. 42-4.
113. Wu, H.K., et al., *Relaxation of imprinting of human insulin-like growth factor II gene, IGF2, in sporadic breast carcinomas.* Biochem Biophys Res Commun, 1997. **235**(1): p. 123-9.
114. van Roozendaal, C.E., et al., *Loss of imprinting of IGF2 and not H19 in breast cancer, adjacent normal tissue and derived fibroblast cultures.* FEBS Lett, 1998. **437**(1-2): p. 107-11.
115. Constancia, M., et al., *Placental-specific IGF-II is a major modulator of placental and fetal growth.* Nature, 2002. **417**(6892): p. 945-8.
116. Fowden, A.L., *The insulin-like growth factors and feto-placental growth.* Placenta, 2003. **24**(8-9): p. 803-12.

117. Mellemkjaer, L., et al., *Birth weight and risk of early-onset breast cancer (Denmark).* Cancer Causes Control, 2003. **14**(1): p. 61-4.
118. Brawley, L., L. Poston, and M.A. Hanson, *Mechanisms underlying the programming of small artery dysfunction: review of the model using low protein diet in pregnancy in the rat.* Arch Physiol Biochem, 2003. **111**(1): p. 23-35.
119. Hughson, M., et al., *Glomerular number and size in autopsy kidneys: the relationship to birth weight.* Kidney Int, 2003. **63**(6): p. 2113-22.
120. Hoy, W.E., et al., *Nephron number, hypertension, renal disease, and renal failure.* J Am Soc Nephrol, 2005. **16**(9): p. 2557-64.
121. Zandi-Nejad, K., V.A. Luyckx, and B.M. Brenner, *Adult hypertension and kidney disease: the role of fetal programming.* Hypertension, 2006. **47**(3): p. 502-8.
122. Desai, M., et al., *Organ-selective growth in the offspring of protein-restricted mothers.* Br J Nutr, 1996. **76**(4): p. 591-603.
123. Petrik, J., et al., *A low protein diet alters the balance of islet cell replication and apoptosis in the fetal and neonatal rat and is associated with a reduced pancreatic expression of insulin-like growth factor-II.* Endocrinology, 1999. **140**(10): p. 4861-73.
124. Poulsen, P., et al., *Low birth weight and zygosity status is associated with defective muscle glycogen and glycogen synthase regulation in elderly twins.* Diabetes, 2007. **56**(11): p. 2710-4.
125. Leeson, C.P., et al., *Impact of low birth weight and cardiovascular risk factors on endothelial function in early adult life.* Circulation, 2001. **103**(9): p. 1264-8.
126. Boguszewski, M.C., et al., *Low birth size and final height predict high sympathetic nerve activity in adulthood.* J Hypertens, 2004. **22**(6): p. 1157-63.
127. Li, C., M.S. Johnson, and M.I. Goran, *Effects of low birth weight on insulin resistance syndrome in caucasian and African-American children.* Diabetes Care, 2001. **24**(12): p. 2035-42.
128. Tzoulaki, I., et al., *Size at birth, weight gain over the life course, and low-grade inflammation in young adulthood: northern Finland 1966 Birth Cohort study.* Eur Heart J, 2008. **29**(8): p. 1049-56.
129. Riggs, A.D., R.A. Martienssen, and V.E. Russo, *Introduction*, in *Epigenetic Mechanisms of Gene Regulation*, V.E. Russo, R.A. Martienssen, and A.D. Riggs, Editors. 1996, Cold Spring Harbor Lab. Press: Plainview, NY. p. 1-4.
130. Waterland, R.A. and K.B. Michels, *Epigenetic epidemiology of the developmental origins hypothesis.* Annu Rev Nutr, 2007. **27**: p. 363-88.
131. Castro, R., et al., *Homocysteine metabolism, hyperhomocysteinaemia and vascular disease: an overview.* J Inherit Metab Dis, 2006. **29**(1): p. 3-20.
132. Ying, A.K., et al., *Methylation of the estrogen receptor-alpha gene promoter is selectively increased in proliferating human aortic smooth muscle cells.* Cardiovasc Res, 2000. **46**(1): p. 172-9.
133. Lund, G., et al., *DNA methylation polymorphisms precede any histological sign of atherosclerosis in mice lacking apolipoprotein E.* J Biol Chem, 2004. **279**(28): p. 29147-54.
134. Ordovas, J.M. and C.E. Smith, *Epigenetics and cardiovascular disease.* Nat Rev Cardiol, 2010. **7**(9): p. 510-9.
135. Jones, P.A. and S.B. Baylin, *The fundamental role of epigenetic events in cancer.* Nat Rev Genet, 2002. **3**(6): p. 415-28.
136. Lawlor, D.A., et al., *Family socioeconomic position at birth and future cardiovascular disease risk: findings from the Aberdeen Children of the 1950s cohort study.* Am J Public Health, 2006. **96**(7): p. 1271-7.
137. Cnattingius, S., *The epidemiology of smoking during pregnancy: smoking prevalence, maternal characteristics, and pregnancy outcomes.* Nicotine Tob Res, 2004. **6 Suppl 2**: p. S125-40.

138. Lawlor, D.A., et al., *Associations of parental, birth, and early life characteristics with systolic blood pressure at 5 years of age: findings from the Mater-University study of pregnancy and its outcomes.* Circulation, 2004. **110**(16): p. 2417-23.
139. Brion, M.J., et al., *Similar associations of parental prenatal smoking suggest child blood pressure is not influenced by intrauterine effects.* Hypertension, 2007. **49**(6): p. 1422-8.
140. Leon, D.A., et al., *Fetal, developmental, and parental influences on childhood systolic blood pressure in 600 sib pairs: the Uppsala Family study.* Circulation, 2005. **112**(22): p. 3478-85.
141. Blake, K.V., et al., *Maternal cigarette smoking during pregnancy, low birth weight and subsequent blood pressure in early childhood.* Early Hum Dev, 2000. **57**(2): p. 137-47.
142. Webster, T.F., et al., *Community- and individual-level socioeconomic status and breast cancer risk: multilevel modeling on Cape Cod, Massachusetts.* Environ Health Perspect, 2008. **116**(8): p. 1125-9.
143. Maughan, B., S. Collishaw, and A. Pickles, *School achievement and adult qualifications among adoptees: a longitudinal study.* J Child Psychol Psychiatry, 1998. **39**(5): p. 669-85.
144. Entwisle, D.R., K.L. Alexander, and L. Steffel Olson, *First Grade and Educational Attainment by Age 22: A New Story.* American Journal of Sociology, 2005. **110**(5): p. 1458-1502.
145. Snieder, H., G.A. Harshfield, and F.A. Treiber, *Heritability of blood pressure and hemodynamics in African- and European-American youth.* Hypertension, 2003. **41**(6): p. 1196-201.
146. Marenberg, M.E., et al., *Genetic susceptibility to death from coronary heart disease in a study of twins.* N Engl J Med, 1994. **330**(15): p. 1041-6.
147. Poulsen, P., et al., *Heritability of type II (non-insulin-dependent) diabetes mellitus and abnormal glucose tolerance--a population-based twin study.* Diabetologia, 1999. **42**(2): p. 139-45.
148. Lichtenstein, P., et al., *Environmental and heritable factors in the causation of cancer--analyses of cohorts of twins from Sweden, Denmark, and Finland.* N Engl J Med, 2000. **343**(2): p. 78-85.
149. Hattersley, A.T. and J.E. Tooke, *The fetal insulin hypothesis: an alternative explanation of the association of low birthweight with diabetes and vascular disease.* Lancet, 1999. **353**(9166): p. 1789-92.
150. Tamemoto, H., et al., *Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1.* Nature, 1994. **372**(6502): p. 182-6.
151. Almind, K., et al., *Aminoacid polymorphisms of insulin receptor substrate-1 in non-insulin-dependent diabetes mellitus.* Lancet, 1993. **342**(8875): p. 828-32.
152. Lindsay, R.S., et al., *The insulin gene variable number tandem repeat class I/III polymorphism is in linkage disequilibrium with birth weight but not Type 2 diabetes in the Pima population.* Diabetes, 2003. **52**(1): p. 187-93.
153. Vaessen, N., et al., *Association between genetic variation in the gene for insulin-like growth factor-I and low birthweight.* Lancet, 2002. **359**(9311): p. 1036-7.
154. Bhattacharyya, T., et al., *Relationship of paraoxonase 1 (PON1) gene polymorphisms and functional activity with systemic oxidative stress and cardiovascular risk.* Jama, 2008. **299**(11): p. 1265-76.
155. Infante-Rivard, C., *Genetic Association Between Single Nucleotide Polymorphisms in the Paraoxonase 1 (PON1) Gene and Small-for-Gestational-Age Birth in Related and Unrelated Subjects.* Am J Epidemiol.
156. WHO. *Health topics: Epidemiology*. 2011 [cited 2011 May 7]; Available from: http://www.who.int/topics/epidemiology/en/.
157. Rothman, K.J. and S. Greenland, *Causation and causal inference in epidemiology.* Am J Public Health, 2005. **95 Suppl 1**: p. S144-50.

158. Pearl, J., *Causal diagrams for empirical research.* Biometrika, 1995. **82**(4): p. 669-688.
159. Pearl, J., *Causality: Models, Reasoning and Inference*2000, Cambridge: Cambridge University Press.
160. Spirtes, P., C. Glymor, and R. Scheines, *Causation, Prediction, and Search*. 2nd ed2000, Cambridge, MA: The MIT Press.
161. Robins, J.M., *Data, design, and background knowledge in etiologic inference.* Epidemiology, 2001. **12**(3): p. 313-20.
162. Cole, S.R. and M.A. Hernan, *Fallibility in estimating direct effects.* Int J Epidemiol, 2002. **31**(1): p. 163-5.
163. Szklo, M. and J. Nieto, *Information bias*, in *Epidemiology: beyond the basics*, M. Szklo and J. Nieto, Editors. 2007, Jones & Barlett Learning.
164. Galton, F., *The history of twins, as a criterion of the relative powers of nature and nurture.* J Anthropol Inst, 1875. **12**: p. 566-76.
165. Hall, J.G., *Twinning.* Lancet, 2003. **362**(9385): p. 735-43.
166. Kapiro, J. and R. Marttila, *Demographic trends in Nordic countries*, in *Multiple pregnancy - epidemiology, gestation and perinatal outcome*, I.K. Blickstein, L.G, Editor 2005, Informa UK Ltd.: London, UK.
167. Nylander, P.P., *The twinning incidence of Nigeria.* Acta Genet Med Gemellol (Roma), 1979. **28**(4): p. 261-3.
168. Campbell, D., *Epidemiology of Twinning.* Current Obstetrics & Gynaecology, 1998. **8**: p. 126-134.
169. Steinman, G., *Can the chance of having twins be modified by diet?* Lancet, 2006. **367**(9521): p. 1461-2.
170. Machin, G.A., *Some causes of genotypic and phenotypic discordance in monozygotic twin pairs.* Am J Med Genet, 1996. **61**(3): p. 216-28.
171. Gringras, P. and W. Chen, *Mechanisms for differences in monozygous twins.* Early Hum Dev, 2001. **64**(2): p. 105-17.
172. Bruder, C.E., et al., *Phenotypically concordant and discordant monozygotic twins display different DNA copy-number-variation profiles.* Am J Hum Genet, 2008. **82**(3): p. 763-71.
173. Monteiro, J., et al., *Commitment to X inactivation precedes the twinning event in monochorionic MZ twins.* Am J Hum Genet, 1998. **63**(2): p. 339-46.
174. Bjornsson, H.T., M.D. Fallin, and A.P. Feinberg, *An integrated epigenetic and genetic approach to common human disease.* Trends Genet, 2004. **20**(8): p. 350-8.
175. Petronis, A., *Epigenetics and twins: three variations on the theme.* Trends Genet, 2006. **22**(7): p. 347-50.
176. Fraga, M.F., et al., *Epigenetic differences arise during the lifetime of monozygotic twins.* Proc Natl Acad Sci U S A, 2005. **102**(30): p. 10604-9.
177. Fox, H. and N.J. Sebire, *Pathology of the placenta*. Third ed2007, Philadelphia, USA: Saunders Elsevier.
178. Bleker, O.P., W. Breur, and B.L. Huidekoper, *A study of birth weight, placental weight and mortality of twins as compared to singletons.* Br J Obstet Gynaecol, 1979. **86**(2): p. 111-8.
179. Alexander, G.R., et al., *What are the fetal growth patterns of singletons, twins, and triplets in the United States?* Clin Obstet Gynecol, 1998. **41**(1): p. 114-25.
180. Grumbach, K., et al., *Twin and singleton growth patterns compared using US.* Radiology, 1986. **158**(1): p. 237-41.
181. Smith, A.P., et al., *A prospective longitudinal study of growth velocity in twin pregnancy.* Ultrasound Obstet Gynecol, 2001. **18**(5): p. 485-7.
182. McKeown, T. and R.G. Record, *Observations on foetal growth in multiple pregnancy in man.* J Endocrinol, 1952. **8**(4): p. 386-401.
183. Phillips, D.I., M.J. Davies, and J.S. Robinson, *Fetal growth and the fetal origins hypothesis in twins--problems and perspectives.* Twin Res, 2001. **4**(5): p. 327-31.

184. Loos, R.J., et al., *Determinants of birthweight and intrauterine growth in liveborn twins.* Paediatr Perinat Epidemiol, 2005. **19 Suppl 1**: p. 15-22.
185. Blickstein, I. and R.B. Kalish, *Birthweight discordance in multiple pregnancy.* Twin Res, 2003. **6**(6): p. 526-31.
186. Blickstein, I. and R.D. Goldman, *Intertwin birth weight discordance as a potential adaptive measure to promote gestational age.* J Reprod Med, 2003. **48**(6): p. 449-54.
187. Lewi, L., et al., *Placental sharing, birthweight discordance, and vascular anastomoses in monochorionic diamniotic twin placentas.* Am J Obstet Gynecol, 2007. **197**(6): p. 587 e1-8.
188. Bajoria, R., *Vascular anatomy of monochorionic placenta in relation to discordant growth and amniotic fluid volume.* Hum Reprod, 1998. **13**(10): p. 2933-40.
189. Fick, A.L., et al., *Unequal placental sharing and birth weight discordance in monochorionic diamniotic twins.* Am J Obstet Gynecol, 2006. **195**(1): p. 178-83.
190. De Paepe, M.E., et al., *Placental characteristics of selective birth weight discordance in diamniotic-monochorionic twin gestations.* Placenta. **31**(5): p. 380-6.
191. Poulsen, P. and A. Vaag, *Glucose and insulin metabolism in twins: influence of zygosity and birth weight.* Twin Res, 2001. **4**(5): p. 350-5.
192. McNeill, G., C. Tuya, and W.C. Smith, *The role of genetic and environmental factors in the association between birthweight and blood pressure: evidence from meta-analysis of twin studies.* Int J Epidemiol, 2004. **33**(5): p. 995-1001.
193. Poulsen, P., et al., *Low birth weight is associated with NIDDM in discordant monozygotic and dizygotic twin pairs.* Diabetologia, 1997. **40**(4): p. 439-46.
194. Poulsen, P., et al., *Age-dependent impact of zygosity and birth weight on insulin secretion and insulin action in twins.* Diabetologia, 2002. **45**(12): p. 1649-57.
195. Hubinette, A., et al., *Birth characteristics and breast cancer risk: a study among like-sexed twins.* Int J Cancer, 2001. **91**(2): p. 248-51.
196. Snieder, H., et al., *Beta2-adrenergic receptor gene and resting hemodynamics in European and African American youth.* Am J Hypertens, 2002. **15**(11): p. 973-9.
197. Risch, N., et al., *Categorization of humans in biomedical research: genes, race and disease.* Genome Biol, 2002. **3**(7): p. comment2007.
198. Jackson, R.W., et al., *Determination of twin zygosity: a comparison of DNA with various questionnaire indices.* Twin Res, 2001. **4**(1): p. 12-8.
199. Lichtenstein, P., et al., *The Swedish Twin Registry: a unique resource for clinical, epidemiological and genetic studies.* J Intern Med, 2002. **252**(3): p. 184-205.
200. Medlund, P., et al., *A new Swedish twin registry containing environmental and medical base line data from about 14,000 same-sexed pairs born 1926-58.* Acta Med Scand Suppl, 1976. **600**: p. 1-111.
201. Cederlof, R., et al., *Studies on similarity diagnosis in twins with the aid of mailed questionnaires.* Acta Genet Stat Med, 1961. **11**: p. 338-62.
202. Ludvigsson, J.F., et al., *The Swedish personal identity number: possibilities and pitfalls in healthcare and medical research.* Eur J Epidemiol, 2009. **24**(11): p. 659-67.
203. Socialstyrelsen, *Inpatient diseases in Sweden 1987-2007 [in Swedish]*, 2008, National Board of Health and Welfare: Stockholm.
204. Socialstyrelsen, *Cancer incidence in Sweden (in Swedish)* 2008, National Board of Health and Welfare: Stockholm.
205. Helgesson, O., et al., *Malignant disease observed in a cohort of women. A validation of Swedish Cancer Registry data.* Scand J Soc Med, 1994. **22**(1): p. 46-9.
206. Garne, J.P., K. Aspegren, and T. Moller, *Validity of breast cancer registration from one hospital into the Swedish National Cancer Registry 1971-1991.* Acta Oncol, 1995. **34**(2): p. 153-6.
207. Socialstyrelsen, *Causes of Death 2006 [in Swedish]*, 2008, National Board of Health and Welfare: Stockholm.

208. Statistics_Sweden, *Multi-Generation Register. A description of contents and quality*, 2005: Örebro.
209. Statistics_Sweden, *SCB-data för forskning [swedish]*, 2006.
210. Zeger, S.L. and K.Y. Liang, *An overview of methods for the analysis of longitudinal data.* Stat Med, 1992. **11**(14-15): p. 1825-39.
211. Carlin, J.B., et al., *Analysis of binary outcomes in longitudinal studies using weighted estimating equations and discrete-time survival methods: prevalence and incidence of smoking in an adolescent cohort.* Stat Med, 1999. **18**(19): p. 2655-79.
212. Efron, B. and R.J. Tibshirani, *An Introduction to the Bootstrap* 1993, New York, NY, USA: Chapman and Hall.
213. Huber, P., *The behaviour of maximum likelihood estimated under non-standard conditions*, in *Fifth Berkeley Symposium of Mathematical Statistics and Probability*1967, University of California Press: Berkeley, CA.
214. White, H., *Maximum likelihood estimation of misspecified models.* Econometrica, 1980. **50**: p. 1/25.
215. SAS_Institute_Inc., *The PHREG Procedure (Book Excerpt)*, in *SAS/STAT® 9.2 User's Guide*2008, SAS Institute Inc.: Cary, NC, USA. p. 4722-4515.
216. Lin, D.Y. and L.J. Wei, *The Robust Inference for the Proportional Hazards Model.* Journal of the American Statistical Association, 1989. **84**: p. 1074-1078.
217. Zeger, S.L., K.Y. Liang, and P.S. Albert, *Models for longitudinal data: a generalized estimating equation approach.* Biometrics, 1988. **44**(4): p. 1049-60.
218. Fitzmaurice, G.M., N.M. Laird, and J.H. Ware, *Applied Longitudinal Analysis, 2nd Edition*2011, Hoboken, NJ, USA: John Wiley & Sons.
219. Neuhaus, J.M. and J.D. Kalbfleisch, *Between- and within-cluster covariate effects in the analysis of clustered data.* Biometrics, 1998. **54**(2): p. 638-45.
220. Begg, M.D. and M.K. Parides, *Separation of individual-level and cluster-level covariate effects in regression analysis of correlated data.* Stat Med, 2003. **22**(16): p. 2591-602.
221. Carlin, J.B., et al., *Regression models for twin studies: a critical review.* Int J Epidemiol, 2005. **34**(5): p. 1089-99.
222. Hrubec, Z. and C.D. Robinette, *The study of human twins in medical research.* N Engl J Med, 1984. **310**(7): p. 435-41.
223. Hosmer, D.W. and S. Lemeshow, *Applied Logistic Regression*. Wiley series in probability and mathematical statistics. Applied probability and statistics section, ed. Wiley1989: John Wiley & Sons, Inc.
224. SAS_Institute_Inc., *The GENMOD Procedure (Book Excerpt)*, in *SAS/STAT® 9.2 User's Guide*2008, SAS Institute Inc.: Cary, NC, USA.
225. SAS_Institute_Inc., *The LOGISTIC Procedure (Book Excerpt)*, in *SAS/STAT® 9.2 User's Guide*2008, SAS Institute Inc.: Cary, NC, USA. p. 4255-4474.
226. Kaufman, J.S. and M.M. Glymour, *Splitting the differences: problems in using twin controls to study the effects of BMI on mortality.* Epidemiology, 2011. **22**(1): p. 104-6; discussion 107-8.
227. Rydhstroem, H. and F. Heraib, *Gestational duration, and fetal and infant mortality for twins vs singletons.* Twin Res, 2001. **4**(4): p. 227-31.
228. Lucas, A., M.S. Fewtrell, and T.J. Cole, *Fetal origins of adult disease-the hypothesis revisited.* Bmj, 1999. **319**(7204): p. 245-9.
229. Lindberg, B.S., E.D. Johansson, and B.A. Nilsson, *Plasma levels of nonconjugated oestrone, oestradiol-17beta and oestriol during uncomplicated pregnancy.* Acta Obstet Gynecol Scand Suppl, 1974. **32**(0): p. 21-36.
230. Troisi, R., et al., *Correlation of serum hormone concentrations in maternal and umbilical cord samples.* Cancer Epidemiol Biomarkers Prev, 2003. **12**(5): p. 452-6.
231. Reik, W. and J. Walter, *Genomic imprinting: parental influence on the genome.* Nat Rev Genet, 2001. **2**(1): p. 21-32.

232. Davey Smith, G., et al., *Offspring birth weight and parental mortality: prospective observational study and meta-analysis.* Am J Epidemiol, 2007. **166**(2): p. 160-9.
233. Manor, O. and I. Koupil, *Birth weight of infants and mortality in their parents and grandparents: the Uppsala Birth Cohort Study.* Int J Epidemiol, 2010.
234. Pennell, C.E., et al., *Approaches to evaluate gene-environment interactions underlying the developmental origins of adult health and disease*, in *Early life origins of human health and disease*, J.P. Newnham and M.G. Ross, Editors. 2009, Karger: Basel. p. 205-217.
235. Christensen, K., et al., *Mortality among twins after age 6: fetal origins hypothesis versus twin method.* Bmj, 1995. **310**(6977): p. 432-6.