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# Spontaneous abortion: Risk factors and measurement of exposures

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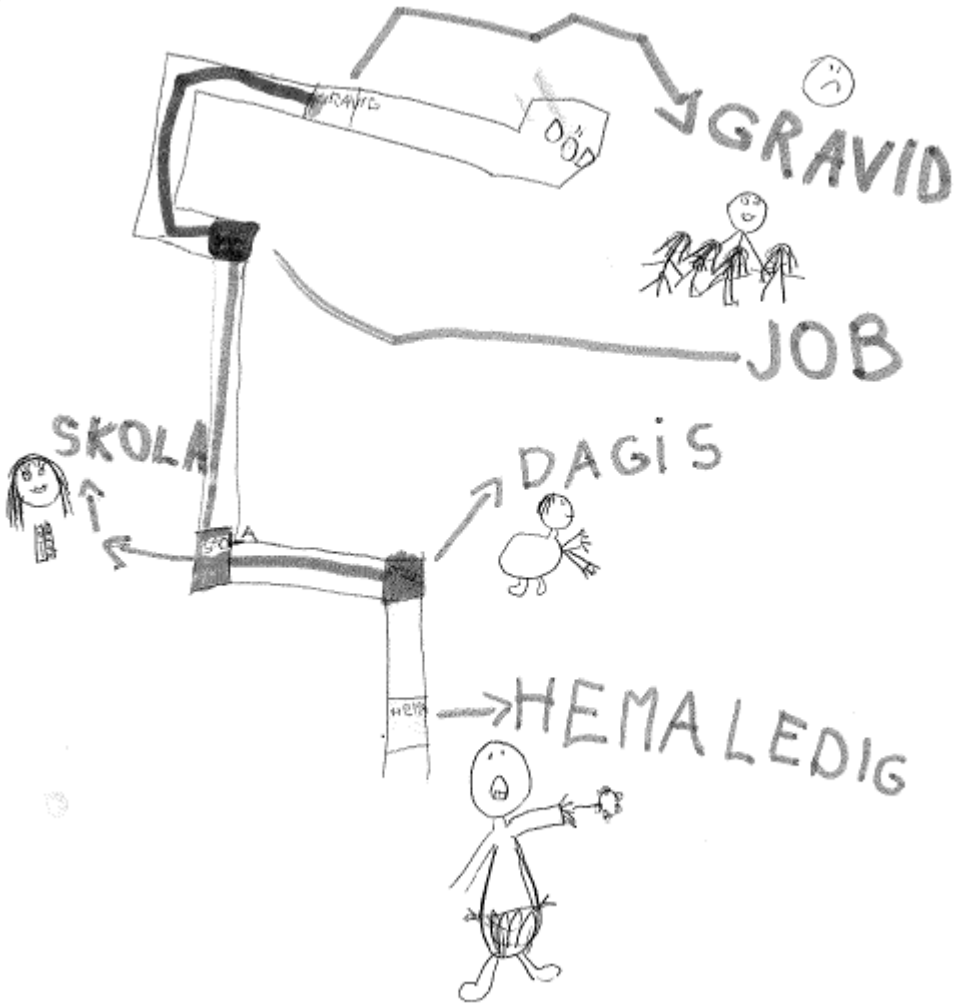


Klara Allenmark 2002
En kvinnas livslinje [Life cycle of a woman]
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# ABSTRACT

The overall objectives of this thesis were to increase the understanding of the etiology of spontaneous abortion and repeated spontaneous abortion, and to evaluate possibilities of exposure assessment during pregnancy.

Data from a Swedish population-based case-control study of spontaneous abortion, performed in Uppsala County 1996-98, formed the base for the three first studies of the thesis. The final study population included 562 cases with a spontaneous abortion in gestational week 6-12 and 1,028 pregnant controls frequency-matched to the cases according to week of gestation. Exposure information was assessed through in-person interviews using structured questionnaires, and through plasma measurements of folate and cotinine levels. Intrauterine tissue was sent for chromosomal analyses among cases with incomplete spontaneous abortion. Conditional logistic regression models were used to calculate odds ratios to estimate the relative risk of spontaneous abortion.

Women with folate levels <5 nmo/l were at 60% increased risk of spontaneous abortion as compared with women with folate levels 5.0-8.9 nmol/l. High maternal folate levels were associated with a non-significant trend towards a protective effect. Folate deficiency was predominantly associated with abnormal fetal karyotype abortions, however we could not show a differential effect of folate between normal and abnormal fetal karyotype abortions. The use of a folic acid supplement, reported by <5% of all women, was not associated with a statistically significant increased risk of spontaneous abortion.

The association between sociodemographic and anthropometric factors, obstetric history, and life style factors, with respect to risks of repeated spontaneous abortion, were explored among 108 cases with two or more consecutive first trimester spontaneous abortions and 583 controls with at least two pregnancies. High and low maternal age, and previous spontaneous abortions were found to be major risk factors. Smokers were at increased risk of repeated spontaneous abortion. Among nonsmoking women with high caffeine intake there was an increased risk, whereas there was no such association among smokers. Folate deficiency was not associated with increased risk of repeated spontaneous abortion.

Measurement of plasma cotinine was used to assess the dose received from exposure to environmental tobacco smoke (ETS) and active smoking. The prevalence of ETS exposure (cotinine 0.1-15 ng/ml) was 24% among women with spontaneous abortion and 19% among women with a normal pregnancy in the first trimester. Nonsmoking pregnant women exposed to ETS faced a 70% increased risk of spontaneous abortion as compared with unexposed women. Active smoking was associated with a more than two-fold increased risk of spontaneous abortion. ETS exposure was associated with normal, abnormal, and unknown fetal karyotype abortions.

In a prospective follow-up of 953 women from early pregnancy until delivery, maternal self-reported information on active smoking, smoking cessation during pregnancy, and ETS exposure during pregnancy, was validated using cotinine measurements as gold standard. Sensitivity, specificity, positive and negative predictive values, and likelihood ratios were calculated as measures of accuracy in gestational weeks 6-12 and 31-34. The validity of self-reported active smoking in early and late pregnancy was reasonably high. Among women reporting smoking cessation before the first interview and between the first and second interview, 13% and 25% misreported active smoking, respectively. Self-reported information on ETS exposure misclassified most women as unexposed throughout the pregnancy.



*Key words:* Case-control study, caffeine, cotinine, environmental tobacco smoke, epidemiology, fetal karyotype, folate, maternal smoking, pregnancy, repeated spontaneous abortion, risk assessment, smoking cessation, spontaneous abortion, validation study.

# CONTENTS

# LIST OF PAPERS

The thesis is based upon the following papers, which will be referred to by their Roman numerals:

I. George L, Mills JL, Johansson ALV, Nordmark A, Olander B, Granath F, and Cnattingius S**.**
Plasma folate levels and risk of spontaneous abortion.
*JAMA* 2002; 288:1867-1873.


II. George L, Granath F, Johansson ALV, Olander B, and Cnattingius S.
Risks of repeated miscarriage.
*Paediatric and Perinatal Epidemiology* 2006; 20:119-126.


III. George L, Granath F, Johansson ALV, Annerén G, and Cnattingius S.
Environmental tobacco smoke and risk of spontaneous abortion.
*Epidemiology* 2006; 17:500-505**.**


IV. George L, Granath F, Johansson ALV, and Cnattingius S.
Self-reported nicotine exposure and plasma levels of cotinine in early and late pregnancy.
In press, *Acta Obstetricia et Gynecologica Scandinavica.*

# ABBREVIATIONS

| | |
|---|---|
| CI | confidence interval |
| e.g. | for example (*exempli gratia*) |
| ETS | environmental tobacco smoke |
| hCG | human chorinic gonadotropin |
| i.e. | that is (*id est*) |
| LR | likelihood ratio |
| MTHFR | metylenetetrahydrofolate reductase |
| NPV | negative predictive value |
| NTD | neural tube defect |
| OR | odds ratio |
| PPV | positive predictive value |
| SD | standard deviation |
| vs | versus |

# 1. INTRODUCTION

Spontaneous abortion is the most common adverse pregnancy outcome. The total rate of spontaneous abortions, including subclinical abortions, has been estimated to 31%. Recurrent spontaneous abortion, defined as three or more consecutive spontaneous abortions, affects approximately 1% of all women.

Both folate deficiency and folic acid supplements have been reported to increase the risk of spontaneous abortion. Thus, the results are inconclusive and actual measurements of folate levels have not been available in all studies. Studies on environmental tobacco smoke and spontaneous abortion are limited to a few studies of self-reported exposure. In fact, most studies of active and passive smoking and risk of adverse pregnancy outcomes are based on maternal self-reported exposure information, which may underestimate the risk compared with biochemical exposure assessments. Measurement of cotinine, a metabolite of nicotine, can be used to measure the dose received from active smoking and from exposure to environmental tobacco smoke.

Using data from a large Swedish case-control study of spontaneous abortion, the aims of this thesis were to investigate maternal folate and cotinine levels in relation to risk of spontaneous abortion, and to explore risk factors for repeated spontaneous abortion (i.e. at least two consecutive spontaneous abortions), with special reference to potentially preventable risk factors. We also aimed to validate self-reported data of maternal nicotine exposures in a prospective follow-up of a cohort of pregnant women.

# 2. BACKGROUND

In this chapter definitions, incidence, and etiologic mechanisms for spontaneous abortion and recurrent spontaneous abortion will be described. Also known risk factors for spontaneous abortion and recurrent spontaneous abortion, with an emphasis on the main risk factors of importance in the studies included in this thesis, are presented.

## SPONTANEOUS ABORTION

### Definitions, incidence and mechanisms

In 1970, the World Health Organization (WHO) defined spontaneous abortion, or miscarriage, as an involuntary loss of an intrauterine pregnancy before 28 completed weeks of gestation in which the fetus was dead when expelled (Kline 1984). In many countries, Sweden included, this is still the legal definition. As the survival of premature infants born before 28 weeks of gestation has increased, the upper cut-off limit for spontaneous abortion has been reduced. Today, spontaneous abortion is usually defined as an involuntary loss of an intrauterine pregnancy before fetal viability, with an upper limit of 20 completed weeks of gestation (USDHHS 2006). Spontaneous abortions may be subdivided into unrecognised and recognised spontaneous abortions (Weinberg 1998, Nguyen 2005), and into first and second trimester spontaneous abortions. An *unrecognised (subclinical) spontaneous abortion* occurs after the conception but before the woman is aware she is pregnant, and is detected by measurement of human chorionic gonadotropin (hCG) levels in blood or urine (biochemical pregnancy). A *recognised (clinical) spontaneous abortion* occurs after the woman realises she is pregnant. The recognition of a pregnancy is influenced by individual, social and economical circumstances, and occurs around the time of the first missed menstrual period, or later.

Underreported and unrecognised spontaneous abortions, and differences in definitions lead to diverse estimated rates of spontaneous abortion across studies. The risk of a clinically recognised spontaneous abortion has generally been estimated to 9-15% (Wilcox 1988, Regan 1989, Goldhaber 1991, Nybo Andersen 2000a, Gindler 2001). Studies also including unrecognised losses yield higher frequencies. However, since there is no marker of conception, it is not possible to capture all losses, especially not those close to conception. Between 1983 and 1985, Wilcox and colleagues (1988) studied risk of early spontaneous abortion among 221 American women who aimed to become pregnant. Daily urine samples were collected for a total of 707 menstrual cycles for biochemical diagnosis of pregnancy. The incidence of unrecognised hCG-detected spontaneous abortion was 22%, and when including recognised spontaneous abortions the total incidence was 31%.

Risk of spontaneous abortion changes over the course of pregnancy, and is highest during the first trimester (Wilcox 1988, Goldhaber 1991), when approximately 80% of spontaneous abortions occur. Cumulative weekly risk of subsequent fetal loss was calculated in a life-table analysis in a Danish cohort of 27,432 pregnancies (Nybo Andersen 2000b). The risk of fetal loss (including spontaneous abortion, hydatiform moles, ectopic pregnancies and stillbirths) peaked in the first trimester, and thereafter it levelled off (Figure 1).

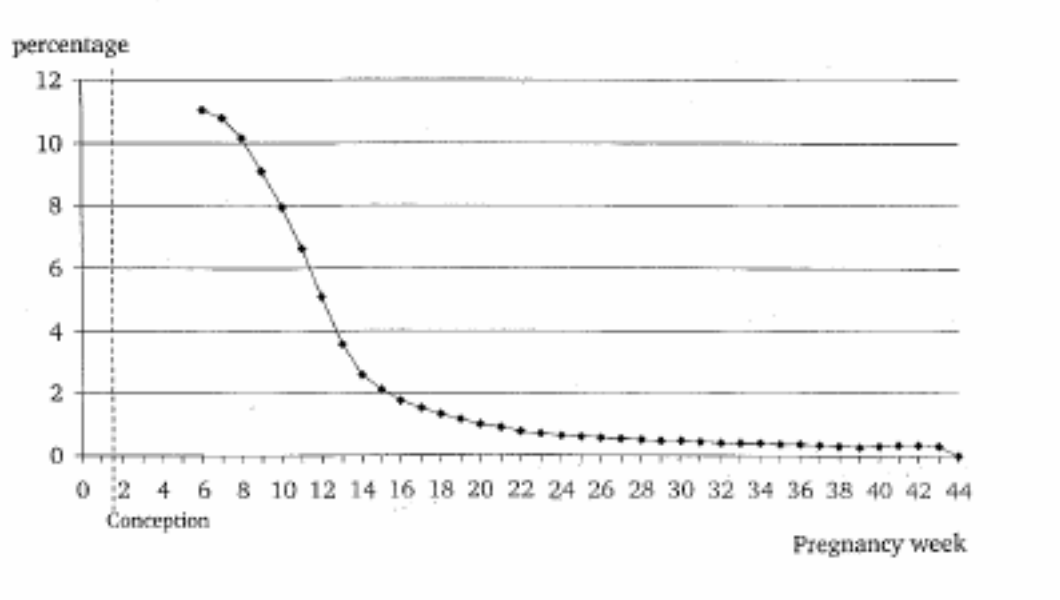


Figure 1. Risk of subsequent fetal loss given fetal survival to various pregnancy weeks (Source: Fetal Death Epidemiological Studies, PhD-thesis, Anne-Marie Nybo Andersen, University of Copenhagen, 2000b).

The etiology of spontaneous abortion is heterogeneous and not quite understood. Spontaneous abortions may occur as a result of genetic factors (chromosomal abnormalities, mutant genes), environmental toxins (drugs, lead, ionizing radiation), infectious agents (viruses, bacteria), uterine abnormalities (malformations, fibroids, cervical insufficiency, post operative changes), and other maternal or paternal factors (chronic disease). Various causes may act in different weeks of gestation. Early spontaneous abortions are more likely to be caused by chromosomal abnormalities, whereas other factors such as cervical incompetence predominantly causes second trimester losses. Most chromosomal abnormalities are lethal and spontaneously aborted in early pregnancy. The frequency of aberrations among lost pregnancies is inversely related with advancing week of gestation, and has been estimated to 35% in clinically recognised spontaneous abortions, 4% among stillborn infants, and 0.4% among live born infants (Kline 1984, Hassold 2001). Transmissible parental balanced translocations are associated with fetal chromosomal aberrations; however, the single most important risk factor for chromosomal abnormalities is, as will be discussed below, high maternal age. Except for ionizing radiation (Hook 1984), relatively little is known about other aneuploidy inducing agents (Hassold 2001).

There has been no evidence of changed trends of incidence of spontaneous abortion over calendar time. In 1984, Kline and Stein reviewed 15 earlier studies (from 1956-1976) and found probabilities of recognised spontaneous abortion (until weeks 20-28), from 6 to 20% (Kline 1984). Crude population-based risks depend on the age composition of the population. Wilcox and colleagues compared two cohorts of pregnant women from the 1930s and 1960s, and found no differences in age-specific risks of spontaneous abortion over time (Wilcox 1981). Goldhaber et al (1991) compared life-table risks of spontaneous abortion (weeks 5-27) among three Californian cohorts of pregnant women (1959-1966, 1974-1977, and 1981-1982), and estimated risks to 14.8%, 14.3%, and 12.6% respectively. The authors concluded that a biased selection of women at high risk for miscarriage may explain the higher incidence in the older cohorts. Further, improved diagnostic tools (introduction of routine ultrasound examinations, and sensitive urine-based hCG-tests) may lead to identification of more early spontaneous abortions that previously would have passed unrecognised. Altered therapeutic routines (surgical vs. medical vs. expectant management) may also distort the distribution of recorded spontaneous abortions in, at least, in-patient registers. A Danish prospective register-

based study included information on 1221,546 pregnancy outcomes, and it was estimated that 80% of all recognised spontaneous abortions were hospitalised (Nybo Andersen 2000a). The reported over-all incidence of spontaneous abortion (until week 28) was 9.3% in 1978-82, 11.1% in 1983-1987, and 12.5% in 1988-92.

## Risk factors

The most consistently reported risk factors for spontaneous abortion are high maternal age and a history of previous spontaneous abortions. Below, the epidemiologic credibility of most well known risk factors for spontaneous abortion will briefly be described. The main exposures of importance in the studies included in this thesis, that is folate and tobacco smoke, will be presented more thoroughly.

### *Maternal age and previous reproductive history*

Most descriptive and analytic studies have reported an increased occurrence of spontaneous abortion as the maternal age rises over 30 years (Kline 1984, Alberman 1987), and the risk increases sharply after the age of 35 (Hook 1984). The increased risk of spontaneous abortion with advancing maternal age applies both for fetuses with abnormal and normal karyotype (Kline 1984, Alberman 1987). The maternal age effect has been found for almost all trisomies, and may be explained by ovarian aging which changes the rate of meiotic errors in the oocyte (Hassold 2001).

Nybo Andersen and colleagues (2000a) investigated risk of spontaneous abortion in relation to maternal age and previous reproductive history. In this register-based study, including 101,851 spontaneous abortions, the age-specific risk of spontaneous abortion was 9% at 20-24 years, and 75% above 44 years. A similar age-related increased risk of spontaneous abortion was observed across all strata of parity, previous spontaneous abortions, and calendar period. Also, the risk of spontaneous abortion increased with a history of previous spontaneous abortion in all age categories regardless of parity. For example, among nulliparous women aged 20-25 years, the risk of spontaneous abortion was 12% after one, 23% after two, and 45% after three previous spontaneous abortions, and for parous women of the same age the corresponding risk estimates were 12%, 18% and 35%. A history of previous spontaneous abortions has been associated with increased risk in several other studies (Regan 1989, Coste 1991, Reagan 1991, Parazzini 1997, Ogasawara 2000). Thus, it can be concluded that advanced maternal age and previous spontaneous abortions are independent risk factors for a spontaneous abortion.

### *Caffeine*

The case-control study that formed the base for the studies included in this thesis was designed to investigate the relation between caffeine intake during pregnancy and spontaneous abortion (Cnattingius 2000). In that study there was a significant interaction between caffeine ingestion and smoking with regard to the risk of spontaneous abortion: caffeine ingestion increased the risk of spontaneous abortion among nonsmokers but not among smokers. It was found that nonsmoking women with a daily caffeine intake of 500 mg or more faced a doubled increase in risk of spontaneous abortion (OR 2.2, 95% CI 1.3-3.8), and the risk was predominantly confined to normal karyotype abortions. High caffeine consumption among smokers was not associated with increased risk. Findings in other epidemiologic studies investigating the association between caffeine ingestion and spontaneous abortion have been inconsistent, however all studies did not take nausea into

adequate consideration (Fenster 1991, Kline 1991, Armstrong 1992, Dlugosz 1992, Mills 1993, Parazzini 1998, Klebanoff 1999, Bech 2005).

### *Folate*

In 1931, the British physician Lucy Wills found that megaloblastic anemia during pregnancy was corrected with yeast extract (Hoffbrand 2001). Folic acid was given its name in 1941, when it was isolated for the first time from spinach by Mitchell and colleagues (Folium [Latin] = leaf) (Hoffbrand 2001). Natural occuring folates constitute of a group of compounds (mostly pteroylpolyglutamates), which are synthesized by microorganisms and plants (Snow 1999, Eichholzer 2006). The synthetic form, folic acid (pteroylmonoglutamic acid), is used in vitamin supplements and fortified foods (Eichholzer 2006). Folate is a general term, and usually refers to both natural folates and folic acid. Folate is a water-soluble B-vitamin and humans are entirely dependent on exogenous sources for their folate supply. The major food sources of folate include leafy green vegetables (e.g. spinach), legumes (e.g. beans and peas), citrus fruits, yeast, liver, and kidney.

Pregnancy is associated with a physiologic reduction in folate levels from the second trimester (Shojania 1984, Tamura 2006). Possible responsible mechanisms for the decline are increased fetal and placental demands, increased urinary clearance, and a physiologic increase in plasma volume (Bailey 2000, Tamura 2006). The recommended daily dietary allowance of folates is 0.4 mg for non-pregnant adults and, due to increased demands, 0.6 mg during pregnancy (Bailey 2000, Hultdin 2003). Folate deficiency is defined as a plasma folate level below 5 nmol/l in Sweden (Hultdin 2003). Folate deficiency during pregnancy has been associated with young age, low parity, low education, low income, and low socioeconomic status (Larroque 1992, Scholl 2000). Smoking has a negative effect on folate status (Larroque 1992, Bailey 2000). While beer has a high folate content, alcohol abuse is associated with chronic folate depletion (Bailey 2000). Antifolate drugs include antiepileptic medications, antibiotics (trimethoprim, sulfasalazine), metotrexate and pyrimethamine.

#### *Measurement of exposure*

Self-reported dietary intake of folate is not easily assessed without adequate conversion tables for dietary content of folates (Bailey 1990, Mikkelsen 2006). There is no consensus for the laboratory assessment of folate status (Klee 2000). A measurement of plasma or serum folate levels reflects folate status during the last days, and is an indicator of folate levels available for fetal replicating cells (Scholl 2000, Tamura 2006). Red cell folate levels reflect the long-term (2-3 months before the analysis) folate status (Bailey 2000), but is a less sensitive indicator for early stages of folate deficiency (Hultdin 2003). Although the red cell folate level is less influenced by dietary fluctuations, this measurement has analytical limitations with large across-laboratory variations (Klee 2000). An elevated homocysteine level is another indicator of folate or vitamine B12 deficiency (Christensen 1995, Hultdin 2003), and marker of dysfunctional folate metabolism (Nelen 2000a). However, homocysteine levels may not reflect folate status during pregnancy, possible due to hemodilution and other physiologic changes in pregnancy (Bonnette 1998).

#### *Folate and neural tube defects*

Neural tube defects (NTDs) are malformations of the spinal cord or the meninges. In two large intervention studies (MRC Vitamin Study Research Group 1991, Czeizel 1992) periconceptional use of folic acid had a strong protective effect against occurrence and

recurrence of NTDs. Based on this evidence, folic acid has been recommended for millions of women of childbearing age worldwide since 1992 to prevent these malformations (Cornel 1997). However, the official policies differ between countries. In many countries all women who could become pregnant are advised to use a supplement containing 400 μg of folic acid per day. In other countries, e.g. the U.S., Canada, Costa Rica, Chile, and Brazil, mandatory folic acid food fortification has been introduced (Tamura 2006). The birth prevalence of NTDs after food fortification has declined with 19% in the US (Honein 2001), and 78% in Newfoundland (taking induced terminations into account) (Liu 2004). Concern of adverse effects of folic acid on the general population, such as masking the diagnosis of a vitamin B12-deficiency among elderly (Eichholzer 2006), has been arguments against fortification. There has also been controversy on reported increased occurrence of spontaneous abortion (discussed below) and multiple births among folic acid supplement takers (Ericson 2001, Li 2003, Czeizel 2004). The Swedish Council on Technology Assessment in Health Care (SBU) is currently investigating benefits and risks of a possible folic acid food fortification program in Sweden, and a report is expected in the fall of 2006 (www.sbu.se).

*Folate and spontaneous abortion*

Studies examining the relationship between folate deficiency and spontaneous abortion have come to inconsistent results. Folate deficiency was assessed with serum or plasma folate measurements (Streiff 1967, Pietrzik 1992, Neiger 1993, Ronnenberg 2002, de Weerd 2003), red cell folate measurements (Hibbard 1975, Neela 1997, de Weerd 2003), or with measurements of forminoglutamic acid (figlu) excretionin in the urine after histidine loading (Friedman 1977). Some studies (Hibbard 1975, Friedman 1977, Pietrzik 1992, Neiger 1993, Ronnenberg 2002) but not all (Streiff 1967, Neela 1997, de Weerd 2003), found that folate deficiency was a risk factor for spontaneous abortion. These conflicting results could be due to small sample sizes (number of included cases was 34 to 115), a highly selected population, or lack of control for potential confounders such as age, smoking and alcohol consumption. Moreover, folate levels change during pregnancy (Bailey 2000), and many studies have included non-pregnant women as controls.

Conversely, it has also been suggested that folic acid supplementation may increase the rate of, or shift the timing of spontaneous abortion. In 1997, data from one of the large randomised trials on folic acid supplementation and NTD occurrence (Czeizel 1992) was analysed and the authors reported that periconceptional use of folic acid was associated with a significant increased risk (RR 1.16, 95% CI 1.01-1.3) of spontaneous abortion.(Hook 1997). This finding was supported a few years later (Windham 2000) when data from a cohort of pregnant women (Windham 1997) was analysed. Compared with non-use, use of folic acid or multivitamins was associated with increased risk (RR 1.14, 95% CI 0.96-1.35) for miscarriage. Researchers from the other large randomised trial on folic acid and NTD recurrence (MRC Vitamin Study Research Group 1991) contradicted these findings (Wald 1997), and reported a relative risk for miscarriage of 0.98 (95% CI 0.64-1.49), when analysis was restricted to women only taking folic acid and compared with women taking no vitamins (Wald 2001). More recently, a large population-based cohort study of 23,806 mostly primiparous Chinese women (Gindler 2001), concluded that miscarriage rate did not differ between women with and without periconceptional use of folic acid (9.6% vs. 9.3%; RR 1.03, 95% CI 0.89-1.20). Gestational age at miscarriage was not influenced by use of folic acid supplementation.

***Homocysteine***

Vitamine B12 acts as a cofactor for normal function of folate, and B12 in cooperation with folate lowers plasma homocysteine levels (Christensen 1995, Eichholzer 2006). Folate or vitamin B12 deficiency causes hyperhomocysteinemia. Elevated homocysteine levels can also be related to genetic defects with polymorphisms of metabolising enzymes, for instance mutations in the metylenetetrahydrofolate reductase (MTHFR) gene (Klee 2000). Beside hyperhomocysteinemia, persons with these common polymorphisms may have a functional folate deficiency and need higher levels of folate for normal function (Hultdin 2003). Hyperhomocysteinemia has been associated with arterial and venous tromboembolic disease (Welch 1998), and with some pregnancy related outcomes such as neural tube defects, placental abruption, and preeclampsia (Nelen 2000b). It is believed that the biochemical consequences of hyperhomocysteinemia can be modified by folate and vitamine B12 supplementation (Zetterberg 2004).

Elevated homocysteine levels have been associated with spontaneous abortion (Gris 2003), and recurrent spontaneous abortion (Steegers-Theunissen 1992, Wouters 1993, Nelen 2000a, Nelen 2000b). The relation between homozygosity for the MTHFR mutation and recurrent spontaneous abortion has been inconclusive (Nelen 1997, Kutteh 1999, Nelen 2000b). In a more recent meta-analysis on trombophilias and recurrent miscarriage (Krabbendam 2005), strict criteria were applied and 11 valid studies out of 69 were selected for comparison. The authors reported hyperhomocystemia as a significant risk factor for recurrent spontaneous abortion, but no association with the MTHFR mutation.

***Tobacco smoke***

Sweden is one of the few countries in the world where female inhabitants smoke more than males, and the smoking prevalence was 18.0% among women and 13.9% among men in 2005 (Statistics Sweden 2005). Despite a declining trend, smoking is not uncommon among pregnant Swedish women. In 2002, 21% of pregnant women were smokers three months before conception and 11% at registration to antenatal care (National Board of Health and Welfare 2004). Many smoking women are probably smokers at the time of conception, and quit when they learn of their pregnancy in early gestation. In some groups, smoking is more prevalent: for instance 37% among 20-24 year old women, and 55% of younger women smoked three months before conception in 2002 (National Board of Health and Welfare 2004). The smoking prevalence during pregnancy is influenced by socioeconomic factors. High education both has a protective effect on smoking initiation and a positive effect on smoking cessation.

In Sweden and many other industrialized countries in the Western world, the number of smokers during pregnancy has declined over the last two decades. However, in former East Germany, smoking prevalence among young women increased from 27% to 47% between 1993 and 1997 (Cnattingius 2004). In 1983, when smoking information was first included in the Swedish Medical Birth Register, 31% reported smoking in early pregnancy. This frequency has then successively dropped: to 26% in 1993, 20% in 1997, and 12% in 2001 (Cnattingius 2006, in press). In developing countries, smoking is still uncommon among women whereas the prevalence is high among men. However, there is concern that smoking prevalence among women in developing countries will increase, as it has in developed countries.

There is strong evidence that smoking during pregnancy is causally related to fetal growth restriction, and possible also with many other adverse pregnancy outcomes such as placental

abruption, premature birth, stillbirth and sudden infant death syndrome (USDHHS 2004). Since smoking is amenable to intervention, great efforts have been made in Swedish antenatal care settings to reduce smoking during pregnancy. According to the Swedish Medical Birth Register, approximately 50% of smokers quit in early pregnancy. Studies from other parts of the world have reported smoking cessation during pregnancy in a very wide range from 11 to 65 % (Solomon 2004, USDHHS 2004). There is a great social pressure on pregnant smokers to abstain from smoking, and it has been proposed that some women who are not able to quit will try to conceal actual smoking habits. Inadequate recall on smoking information may then be an issue of concern.

*Environmental tobacco smoke*

Environmental tobacco smoke (ETS), or passive smoke, or second hand smoke, is the tobacco smoke produced by active smokers that spreads and pollutes the surrounding air. ETS constitutes of two components; mainstream tobacco smoke that is exhaled by the smoker, and sidestream tobacco smoke that is emitted directly from the burning cigarette between puffs (Dockery 1997). Sidestream tobacco smoke contributes the most to ETS, and contains higher concentrations of many toxic compounds than mainstream smoke, possibly because temperatures are lower when sidestream smoke is produced (Cal/EPA 2006). The prevalence of ETS exposure in populations of pregnant women has ranged from 5% to 36% (Haddow 1988, Eskenazi 1995, Rebagliato 1995, Peacock 1998) in studies where cotinine measurements were obtained to define ETS exposure in nonsmoking women.

Environmental exposure to tobacco smoke in nonsmokers during pregnancy has been associated with risk of restricted fetal growth (in the range of 10-100 gram), sudden infant death syndrome and possible preterm birth (Windham 1999b, Cal/EPA 2006, USDHHS 2006) As a consequence of the growing evidence that nonsmokers exposed to ETS face risk of developing several of the diseases as active smokers (Dockery 1997), many countries have prohibited smoking in public areas and workplaces to restrict the general population's exposure to ETS. One of the first states with such legislation was California, where smoking in all public places was banned in 1998. In Sweden, smoking was prohibited in restaurants and bars on June 1, 2005.

*Measurement of exposure*

Active smoking and ETS exposure can be assessed by self-reported exposure information or biomarker measurements. A measurement of a biological marker of exposure is thought to be an indicator of uptake of toxic constituents of tobacco smoke as a whole (Dockery 1997). Because tobacco smoke and ETS is a mixture of compounds it cannot be measured directly. The magnitude of an individual's dose received from active smoking is dependent on number of smoked cigarettes, smoking intensity, type of cigarette, and filter. The magnitude of an individual's dose received of ETS is determined by the air concentration of tobacco smoke constituents and the time the person spends in the location (Cal/EPA 2006). The air concentration in turn, is conditional on number of smoked cigarettes, smoking pattern, distance to smokers, room size, furnishing and ventilation. The measured level of the biomarker will, for smokers and nonsmokers, be influenced by individual factors such as differences in nicotine metabolism and elimination, and analytical methods (Rebagliato 2002).

Biomarkers that are specific to tobacco smoke exposure and that can detect ETS exposure among nonsmokers, are nicotine, cotinine, and tobacco specific nitrosamines (NNAL and

NNAL-glucuronide) (Cal/EPA 2006). The most widely used biomarker in research studies is cotinine, the major metabolite of nicotine (Jarvis 1984, Benowitz 1996), which can be measured in blood, urine, saliva and hair. Measurement of cotinine has been shown to be a valid summary measure of the dose received from active smoking, and from ETS among nonsmokers during pregnancy (Haddow 1987, Peacock 1998). Analytical methods for cotinine assays include radioimmune assay and gas chromatography. Radioimmune assay is sensitive (1-2 ng/ml) but non-specific, and gas chromatography is both sensitive (0.1-0.2 ng/ml) and highly specific (Benowitz 1996). Other nicotine containing products, including oral snuff and nicotine replacement therapy, also cause detectable cotinine levels (Dempsey 2001). The half-life of cotinine in blood is approximately 17 hours for non-pregnant women (Benowitz 1996), and close to 9 hours among women between 16 and 40 weeks of gestation (Dempsey 2002). Thus, plasma cotinine is an indicator of the exposure over the last days preceding measurement. Cotinine levels are stable over time in blood, and a random cotinine measurement has been considered to be a reasonable indicator of daily ETS exposure (Benowitz 1996). Hair cotinine measurements may reflect exposure over the last months; additional knowledge is needed regarding the influence on assessments from differences in hair growth rate, and pigmentation for example (USDHHS 2006). Presently, there are no biomarkers for long-term exposure, which has to be assessed with self-reported exposure information. There is no uniform cut-off level of cotinine in blood to distinguish active smokers from nonsmokers. Previous research and validation studies of pregnant women have used the following limits of plasma cotinine: 17.5 ng/ml (Lindqvist 2002), 15.0 ng/ (Peacock 1998), 14 ng/ml (Rebagliato 1995), 10 ng/ml (Eskenazi 1995, Klebanoff 2001, DeLorenze 2002, Kharrazi 2004), and 5 mg/ml (Parna 2005).

Most studies on maternal smoking or ETS exposure and adverse pregnancy outcomes, including those from the Swedish Medical Birth Register, are based on self-reported exposure information. The accuracy of self-reported information on active tobacco smoke and ETS exposure can be validated using cotinine measurements as the reference (Jarvis 1984, Haddow 1987, Benowitz 1996). Studies evaluating self-reported information on maternal active smoking have shown that 1%-73% of reported nonsmokers have cotinine levels indicating active smoking. Although most validation studies have found maternal self-reported smoking information reliable with less than 10% underreporting of active smoking (Walsh 1996, Klebanoff 1998, Peacock 1998, Klebanoff 2001, Owen 2001, Lindqvist 2002, Hanke 2004, Pickett 2005), higher rates of underreporting of smoking (20-35%) have generally been observed in smoking cessation trails (Boyd 1998, Greaves 2001, Britton 2004). Part of the divergence may also be attributed to different cut-off levels of cotinine, variations in study settings and sample size, diverse interview situations, and type of used questionnaire. It may not be possible to generalize findings between populations with different prevalence of smoking, which would imply a need for validation studies in different settings of pregnant women.

Rates on smoking cessation during pregnancy have also usually been estimates based on self-reported information. Solomon and colleagues (2004) reviewed the literature on spontaneous smoking cessation in early pregnancy. They reported results from one Australian and three US studies, where 9-21% of women who stated that they had quit smoking, had cotinine measurements indicating smoking. The accuracy of maternal self-reported ETS exposure information has been verified with cotinine measurements in a limited number of studies, which have found poor (O'Connor 1995) to moderate (Rebagliato 1995, DeLorenze 2002, Kaufman 2002) correlations.

*Active smoking and spontaneous abortion*

Maternal smoking has been associated with spontaneous abortion in most (Kline 1977, Armstrong 1992, Dominguez-Rojas 1994, Kline 1995, Chatenoud 1998, Ness 1999, Windham 1999a, Winter 2002), but not all (Wilcox 1990, Ahlborg 1991, Windham 1992, Rasch 2003, Wisborg 2003) studies. The risk estimates have been moderately increased ranging from 1.2-3.4 (Cnattingius 2004, USDHHS 2004). A dose-response relationship was observed in two large investigations (Kline 1981, Armstrong 1992). Not many studies have investigated the smoking associated risk of spontaneous abortion by fetal karyotype. In the case-control comparison by Kline and colleagues (1995), smokers as compared to nonsmokers were at increased risk of normal karyotype spontaneous abortion (OR 1.5, 95% CI 1.2-2.0), and trisomic spontaneous abortion (OR 1.2, 95% CI 0.8-1.9). Studies on maternal smoking and spontaneous abortion have generally been based on self-reported exposure information, which may have underestimated the risk in comparison with biochemical measures of exposure. Ness and colleagues (1999) found that maternal smoking assessed with cotinine measurements in urine was more strongly (OR 1.8, 95% CI 1.3-2.6) related to spontaneous abortion than was self-reported (OR 1.4, 95% CI 1.0-1.9) smoking information.

*Environmental tobacco smoke and spontaneous abortion*

Previous studies of ETS and risk of spontaneous abortion are limited to a few studies of self-reported exposure to ETS or paternal smoke, and the results have been inconsistent. In a PubMed literature search, the following studies were found, of which three report a positive (Ahlborg 1991, Windham 1992, Venners 2004) and two a negative (Chatenoud 1998, Windham 1999a) association with ETS exposure and spontaneous abortion. No studies were found on the association between cotinine measurements of ETS and risk of spontaneous abortion. However, an association with elevated serum cotinine levels in the second trimester and fetal loss after gestational week 20 has been observed in a cohort study of pregnant nonsmokers (Kharrazi 2004).

In the following section, studies relating ETS exposure and spontaneous abortion will be reviewed. In the most recent study, Venners and colleagues (2004) collected daily urine for hCG assay to detect subclinical and clinical spontaneous abortions in a cohort of 526 married nonsmoking Chinese textile workers, who aimed to become pregnant. ETS exposure was assessed through self-reported information of spousal smoking. Compared with women with a nonsmoking husband, the adjusted OR for spontaneous abortion was 1.04 (95% CI 0.67-1.63) for women with a husband smoking <20 cigarettes daily and 1.81 (1.00-3.29) for women with a husband smoking ≥20 cigarettes daily. Windham and colleagues (1999) examined ETS exposure and risk of spontaneous abortion in a cohort of 4,209 nonsmoking Californian women from prenatal care where 9.6% of the women experienced a spontaneous abortion between registration in the first trimester until week 20 of gestation. ETS exposure was assessed through self-reported information on paternal smoking and average numbers of hours of exposure at home and at work. The investigators found no association with ETS exposure with risk of spontaneous abortion (OR 1.01, 95% CI 0.80-1.27), or with spousal smoking (OR 0.97, 95% CI 0.41-2.3). In an Italian case-control study of 782 cases with first trimester miscarriage and 1,543 control women after a normal delivery (Chatenoud 1998), no association with current (OR 0.8, 95% CI 0.7-1.0) or former spousal smoking was found. In an earlier case-control study by Windham et al (1992), the risk of spontaneous abortion was investigated among 491 nonsmoking case women and 1,044 controls after a normal delivery. Self-reported ETS exposure of ≥1 hour per day was associated with spontaneous abortion (OR 1.6, 95% CI 1.2-2.1), and paternal smoking was not significantly associated (OR 1.4, 95% CI 0.7-2.6). Ahlborg and Bodin (1991) assessed ETS exposure through self-administered

questionnaires among 4,687 pregnant women recruited from prenatal care centres in Sweden between 1980 and 1983. ETS exposure at home (defined as living with a person who smoked inside the home) was not associated, while ETS exposure at work (defined as spending most of the time at work in rooms where other persons were smoking) was associated with first trimester spontaneous abortion (RR 2.16, 95% CI 1.23-2.16).

Above cited studies were all based on self-reported exposure information, which may not properly account for all possible exposures at home, work, and in public places, and may thus misclassify some exposed women as unexposed (Benowitz 1999, DeLorenze 2002). Spousal smoking were used as a proxy of ETS exposure in some of the studies (Windham 1992, Chatenoud 1998, Windham 1999a, Venners 2004). However, women may also be exposed to other sources of ETS exposure, and women living with a smoking partner may not necessarily be exposed to ETS. The quality of self-reported exposure assessment may account for much of the inconsistent results among previous studies on ETS exposure and spontaneous abortion. Effects of ETS exposure should be investigated among nonsmokers, which were done in all but one study (Chatenoud 1998).

***Maternal disesases***

Women with uncontrolled diabetes mellitus, as measured with glucose and glycosylated haemoglobin levels, were at increased risk for spontaneous abortion (Mills 1988), whereas diabetic women with good metabolic control were at the same risk as nondiabetic women. Presence of maternal hemostasis-related autoantibodies, such as antiphospholipid antibodies, has been associated with first trimester spontaneous abortion (Gris 2003). An association with infections has been reported. Parazzini and collegues (1997) found an association with pelvic inflammatory disease and spontaneous abortion (OR 5.1, 95% CI 1.0-26.2). Fever during pregnancy has been associated with fetal loss in one investigation (Kline 1985), but not in a more recent study (Andersen 2002).

***Drugs and alcohol***

Prenatal exposure to diethylstilbestrol (Stillman 1982) has been reprted to be associated with miscarriage. An increased risk of spontaneous abortion with the use of nonsteroidal anti-inflammatory drugs and aspirin has been reported (Nielsen 2001, Li 2003), and contradicted just recently (Keim 2006). Ness and colleagues (1999) found that maternal use of cocaine measured in hair analysis increased the risk for spontaneous abortion independent of smoking status. In two studies investigating the relation between marijuana use and spontaneous abortion (Wilcox 1990, Ness 1999), no association was found. Alcohol has been associated with increased risk of spontaneous abortion in some investigations (Kline 1981, Armstrong 1992, Kesmodel 2002, Rasch 2003), but not in others (Wilcox 1990, Dlugosz 1992, Ness 1999). A methodological limitation may be maternal under-reporting of alcohol consumption (Ernhart 1988, Verkerk 1992). Animal studies have pointed out that high blood alcohol levels can directly trigger spontaneous abortion (Abel 1997).

***Other risk factors***

Other factors reported to be associated with risk of early spontaneous abortion are high paternal age (de la Rochebrochard 2002, Nybo Andersen 2004), psychologic stress (Coste 1991, Neugebauer 1996), irradiation (Kline 1984), maternal lead exposure (Kline 1984, Hertz-Picciotto 2000), and paternal exposure to lead or mercury (Anttila 1995). Occupational exposures, such as work in daycare nursery has been proposed as a risk factor (Gothe 1992).

Nurses working in anesthesiology have been suspected to be at risk, but this has not been confirmed in other studies (Ericson 1985, Eger 1991).

## RECURRENT SPONTANEOUS ABORTION

### Definitions, incidence and mechanisms

Recurrent spontaneous abortion is usually defined as three consecutive spontaneous abortions (Stirrat 1990, Christiansen 2006). Recurrent spontaneous abortion has been reported to affect 1% of all women (Dhont 2003, Rai 2006). This is a greater frequency than expected by chance. Anticipating that 15% of all recognised pregnancies are spontaneously aborted, it can be estimated that 0.3% ($0.15^3$/100) would have been aborted consecutively three times by chance (Warburton 1987). It is believed that among women who experience recurrent spontaneous abortion there is a persistent factor between pregnancies that causes fetal loss repeatedly. Possible causes can be thought of as either congenital (genetic factors or anomalies of the reproductive tract), or acquired causes (trauma, infective agents, tumours, hormonal disturbances, immune disorders, chronic diseases, or environmental toxins). The etiology of recurrent spontaneous abortion may be multifactorial (Christiansen 2006), and even with no treatment at least 60% of patients will carry next pregnancy to term (Reagan 1991). Recurrent spontaneous abortions have been associated with the loss of a fetus with normal karyotype to a greater extent as compared with sporadic spontaneous abortions (Warburton 1987, Coste 1991, Ogasawara 2000).

### Risk factors

Recurrent spontaneous abortion is a heterogeneous disorder, and there have been inconsistencies in the definition of the diagnosis across research studies. Some studies have included women with two consecutive spontaneous abortions, and different upper cut-off limits of gestation have been used. Several factors involved in human reproduction have been proposed as risk factors including genetic factors (chromosomal abnormalities or balanced translocations of either parent), uterine factors (malformations, trauma, tumours), endocrine factors (uncontrolled diabetes mellitus, insulin resistance, untreated thyroid disease, hyperprolactinaemia, luteal phase deficiency, hCG deficiency, hypersecretion of luteinising hormone, polycystic ovary syndrome, premature ovarian failure), immune factors (antiphospholipid syndrome, alloimmune factors), trombophilic defects (activated protein C resistance, Factor V Leiden mutation, defect proteine C or S, defect antitrombin III, hyperhomocysteinemia, protrombin gene mutation), infectious agents (bacterial vaginosis, chlamydia tracomatis, human papillomaviruses, toxoplasmosis, rubella, cytomegalovirus, herpes, listeria), environmental factors (heavy metals, organic solvents, smoking, caffeine, alcohol, drugs, hyperthermia, pesticides), and psychological factors (trauma, stress) (Stirrat 1990, Reagan 1991, Bulletti 1996, Cramer 2000, Gardella 2000, Li 2002, Dhont 2003, Christiansen 2005, Arredondo 2006, Kutteh 2006, Rai 2006).

Below, the epidemiologic evidence for the risk factors investigated in this thesis, namely smoking, caffeine intake, and folate deficiency will be summarized.

***Smoking, caffeine and folate deficinency***

Smoking and caffeine intake and risk of recurrent spontaneous abortion have been examined in a limited number of studies. Modest, not statistically significant smoking-associated increased risks have been reported (Parazzini 1990, Finan 2002), whereas other studies have been unable to find an association (Strobino 1986, Zusterzeel 2000, Nelen 2000a). Among three studies that reported on caffeine intake and risk of recurrent spontaneous abortion, unadjusted increased risks were found in two studies (Parazzini 1990, Nelen 2000a), and no association could be found in a Dutch study (Zusterzeel 2000).

An association with folate deficiency and recurrent spontaneous abortion has been observed in some (Hibbard 1964, Pietrzik 1992, Wouters 1993, Nelen 2000a), but not all epidemiological studies (Sutterlin 1997). Hyperhomocysteinemia and mutations in the metylenetetrahydrofolate reductase (MTHFR) gene are associated with folate deficiency, and the relation with recurrent spontaneous abortion was described in a previous section of this chapter.

Many studies of risk factors for recurrent spontaneous abortion have methodological shortcomings, such as lack of power (due to limited sample size) and insufficient control for potential confounding factors. Another main drawback of above cited observational studies is that almost all have a skewed selection of controls, e.g. non-pregnant women, term pregnant women, or women with less number of previous pregnancies than the cases (Hibbard 1964, Parazzini 1990, Pietrzik 1992, Wouters 1993, Sutterlin 1997, Zusterzeel 2000, Nelen 2000a).

# 3. AIMS

The overall objectives of this thesis were to increase the understanding of the etiology of spontaneous abortion and repeated spontaneous abortions, and to evaluate some possibilities of exposure assessment during pregnancy.

The specific aims of the studies included in this thesis were:

- To study the association between plasma folate levels and risk of spontaneous abortion (Paper I).

- To investigate risk factors for repeated spontaneous abortion with a focus on the possible modifiable exposures smoking, caffeine intake and folate deficiency (Paper II).

- To study the risk of spontaneous abortion related to passive and active smoking, as defined by plasma cotinine levels (Paper III).

- To assess the validity of maternal self-reported data on active smoking and self-reported exposure to environmental tobacco smoke, using plasma cotinine measurements as gold standard (Paper IV).

# 4. MATERIAL AND METHODS

## SETTING

A population-based, matched case-control study of spontaneous abortion was conducted in Uppsala County, Sweden (Cnattingius 2000), and formed the base for Papers I-III. The source population consisted of pregnant women living in Uppsala County during the study period (1996-1998). Women recruited in early pregnancy were not only used as controls in the case-control study of spontaneous abortion, but were also followed as a prospective cohort until delivery and formed the base for Paper IV. Oral informed consent was obtained from all the women, and the ethics committee of the medical faculty at Uppsala University approved the studies before the start of data collection.

## STUDY SUBJECTS

From January 1996 to June 1998, cases with spontaneous abortion were identified at the Department of Obstetrics and Gynecology of Uppsala University Hospital, which was the only place in Uppsala County for the care of women with spontaneous abortion during the time period. Inclusion criteria were a gestational age of 6 to 12 completed weeks, and a confirmed pregnancy by a positive urine pregnancy test (human chorionic gonadotropin test). The diagnosis of spontaneous abortion was based on clinical history, examination, and endovaginal ultrasound scanning. Of 652 eligible cases identified, 562 agreed to participate (86% participation rate). Surgical curettage was performed in all women with incomplete spontaneous abortion, and obtained intrauterine tissue was sent for histological examination.

Pregnant women seeking prenatal care in Uppsala County were asked to participate as controls, as well as to participate in a prospective cohort study during pregnancy until delivery. From January 1996 through December 1998 1,037 women were asked to participate, and 953 agreed (participation rate 92%). Inclusion criteria were that the women were Swedish speaking, living and seeking prenatal care in Uppsala County and pregnant at 6 to 12 weeks when entering the study. Since these women served as controls in the case-control study of spontaneous abortion they were frequency-matched to the cases by week of gestation. All potential controls were examined with endovaginal ultrasound before the interview to verify the viability of the fetus. If a non-viable intrauterine pregnancy was detected, the woman was recruited as a case patient. If a woman recruited as a control had a spontaneous abortion before 13 completed weeks of gestation, she was also included as a case and subjected to a new interview.

In order to select controls from the whole source population that generated the cases, controls were also sampled among women who planned to have an induced abortion. In Uppsala County there were approximately 3 induced abortions for every 10 completed pregnancies. In total, 310 women who would terminate their pregnancies were asked to participate and 274 agreed (88% participation rate). Of these, 75 women were randomly selected and added to the control group. The number was estimated according to the gestational age distribution of induced abortions in Uppsala County during the study period. In total 1,028 controls were included in the case-control study of spontaneous abortion (953 prenatal care patients and 75 pregnant women who planned to have an induced abortion).

Gestational age was defined as number of completed weeks of gestation. In the first interview (Papers I-IV) gestational age was calculated from the first day of the last menstrual period for both case and control women. In the second interview (Paper IV) we used the gestational age assessed in routine ultrasound screening (15-18 weeks of gestation).

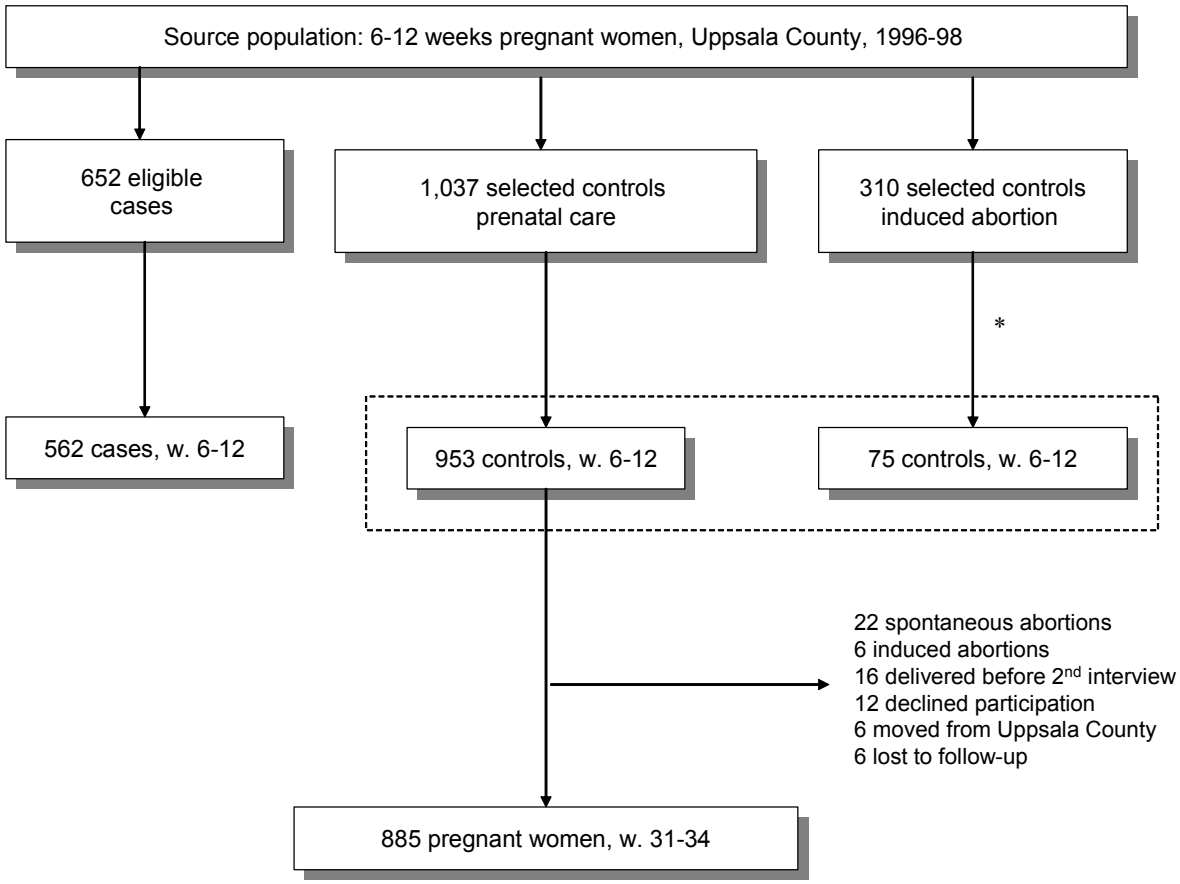


Figure 2. Flow-chart of study subjects.
* 75 controls were randomly selected among 274 women with induced abortions who agreed to participate.

*Paper I*
To investigate the association between maternal folate levels and spontaneous abortion, we included 468 cases and 921 matched pregnant controls with information on plasma folate levels.

*Paper II*
All women included in the study had been pregnant at least twice. We defined cases as women with two or more consecutive first trimester spontaneous abortions. Controls were defined as women with at least two pregnancies, of which the last was required to be a normal intrauterine pregnancy confirmed by ultrasound examination. Controls were selected from women seeking prenatal care. From the case-control study of spontaneous abortion we used data on 108 cases and 583 controls to study risk factors for repeated spontaneous abortion.

*Paper III*
In this paper, we studied the association between active and passive smoking (defined by cotinine levels) and spontaneous abortion. We excluded 23 cases and 58 controls who used oral snuff or nicotine replacement therapy in order to avoid misclassification of exposure due

to elevated cotinine levels (Dempsey 2001). The definition of nonsmokers was set to nonsmoking during pregnancy; therefore 22 cases and 78 controls with cotinine ≤15 ng/ml who reported smoking during pregnancy were excluded. In all, 463 cases and 864 controls from the case-control study of spontaneous abortion were included in the analyses.

*Paper IV*
A cohort of pregnant women was followed prospectively from early pregnancy until delivery and data were used to validate self-reports of passive and active smoking during pregnancy. Of the 953 women recruited in early pregnancy, 68 did not complete the interview in late pregnancy: 22 miscarried, 6 electively terminated their pregnancy, 16 delivered before the second interview, 12 women opted to withdraw from the study, 6 moved outside Uppsala County, and 6 were lost to follow up. Another 52 women had missing cotinine measurements in early or late pregnancy. We also excluded 48 women who used oral snuff or nicotine replacement therapy in order to avoid misclassification of exposure due to elevated cotinine levels (Dempsey 2001). In all, we included 785 women in the validation study.

## DATA COLLECTION

In-person interviews were performed with all women between 6-12 weeks of gestation, and for the women who continued their pregnancy also at 31-34 weeks. A structured questionnaire was used to reduce bias, since the interviewers could not be blinded to as to case-control status. Three specially trained research midwives conducted the interviews with the cases and the controls recruited among women receiving prenatal care. Two physicians conducted interviews with the control subjects who would undergo induced abortions. Ninety percent of the cases were interviewed within two weeks after the diagnosis of spontaneous abortion, and the other ten percent were interviewed within seven weeks. All pregnant women were interviewed within six days after their last completed week of gestation used in matching. Women were asked about possible risk factors for spontaneous abortion, including sociodemographic, anthropometric, and life-style factors, obstetric and medical history. Use of prescription or non-prescription drugs was asked for on a weekly basis. Women reported presence and severity of the pregnancy symptoms nausea, vomiting, and fatigue on a week-by-week basis.

Women were asked to provide blood samples, which were obtained from the cases at the time of miscarriage diagnosis and from the controls at the time they were interviewed. There were virtually no differences in baseline maternal characteristics and exposures between the cases and the controls who agreed to participate in the study and the cases and the controls from whom blood samples were available. Pregnant women also provided blood samples in gestational weeks 31-34. Blood samples were kept frozen at -80°C until assayed. Plasma blood samples were analyzed for cotinine and folate concentrations.

### Folic acid

*Self-reported exposure*
Self-reported use of folic acid-containing supplements was recorded on a weekly basis. In accordance to the recommended dose to reduce the risk of a neural tube defect (Locksmith 1998)**,** we defined daily folic acid supplementation as intake of at least 400 μg per day, beginning at a minimum four weeks before conception (i.e., two weeks before the last menstrual period) and continuing daily through the last completed week of pregnancy.

*Folate measurement*

Folate analyses were performed with an immunoassay analyzer (AxSYM, Abbot Laboratories, Abbot Park, Ill), using ion capture reaction technology. The analysis is quantified by measuring the amount of unoccupied folate specific binding sites bound to matrix using a conjugate of pteroic acid (a folate analog) and alkaline phosphatase as the signal-generating molecule, and a substrate, 4-methylumbelliferyl phosphate. Folate measurements were missing for 94 (17%) of the cases, 101 (11%) of the controls from prenatal care, and from 6 (8 %) of the controls with induced abortions. Plasma folate levels were categorized before data analyses. Low plasma folate level was defined as 4.9 nmol/l or lower, which corresponded to the cut-off for recommending folic acid supplementation (Fex 1997). There were no standard definition of high plasma folate levels in Sweden, and folate levels were categorized numerically into 5.0-8.9 nmol/l (reference group), 9.0-13.9 nmol/l, and 14.0 nmol/l or more.

## Caffeine

*Self-reported exposure*

Women were asked to report intake of various caffeine sources during each week of pregnancy, starting four weeks before the last menstrual period and ending in the most recently completed week of gestation. Sources of caffeine included coffee (brewed, boiled, instant, and decaffeinated), tea (loose tea, tea bags, and herbal tea), cocoa, chocolate, soft drinks, and caffeine-containing medications. Respondents were offered four cup-sizes from which to choose (1.0 dl, 1.5 dl, 2.0 dl, and 3.0 dl). The women estimated weekly consumption of soft drinks in centiliters. We estimated the intake of caffeine using the following conversion factors shown in Table 1 (Barone 1996).

Table 1. Caffeine content of different products.

| Product | | Volume or weight | Caffeine content (mg) |
|---|---|---|---|
| Coffee | brewed | 150 ml | 115 |
| | boiled | | 90 |
| | instant | | 60 |
| Tea | loose tea / tea bag | 150 ml | 39 |
| | herbal | | 0 |
| Soft drinks | (cola) | 150 ml | 15 |
| Cocoa | | 150 ml | 4 |
| Chocolate bar | | 1 g | 0.3 |
| Drug product | | per tablet* | 15-200 |

*depending on type of drug product

The mean daily caffeine intake during pregnancy was calculated from the time of estimated conception through the most recently completed week of gestation.

## Nicotine

*Self-reported exposure*

In early pregnancy (6-12 weeks of gestation), women were asked about all nicotine exposures, including exposure to environmental tobacco smoke (ETS). This information was collected on a weekly basis, starting four weeks before last menstrual period until the most recently completed week of gestation. Each woman was asked in detail about previous and current smoking habits. Information was collected on active cigarette smoking (number of cigarettes smoked per day), use of oral snuff and nicotine replacement therapy (transdermal patches and chewing gum). The women were asked to report ETS exposure with the question: *"Have you been exposed to passive smoke (indoors with other person smoking)?"* Information on ETS exposure was recorded as daily exposed (numbers of hours per day), non-daily exposed, and nonexposed. Smoking status of the partner was also recorded.

In late pregnancy (31-34 weeks of gestation), information on nicotine exposures was asked on a biweekly basis from the time of the first interview until the last completed week of gestation. Nicotine exposure information was categorized in the same way as in the first interview.

*Cotinine measurement*

Plasma cotinine was measured by gas chromatography with use of N-ethylnorcotinine as an internal standard (detection limit 0.1 ng/ml) (Olsson 1991). At 6-12 weeks of gestation, information on plasma cotinine measurements were missing for 54 (10%) of the cases, 23 (2%) of the controls from prenatal care, and 5 (7 %) of the controls with induced abortions. At 31-34 weeks of gestation, cotinine measurements were missing for 34 (4%) of the women.

*Definition of smoking status*

In Papers I and II, smokers were defined as women with a plasma cotinine level higher than 15 ng/ml and nonsmokers as women with cotinine ≤15 ng/ml (Peacock 1998). Self-reported daily smoking during all weeks of pregnancy was allowed to over-ride missing cotinine measurements for classification of smoking status.

In Papers III and IV, the following definition was used: active smokers were defined as women with a plasma cotinine of more than 15.0 ng/ml; women were classified as ETS-exposed if they had a plasma cotinine concentration from 0.1 to 15.0 ng/ml, and as nonexposed to tobacco smoke if plasma cotinine levels were below 0.1 ng/ml (Peacock 1998).

## Fetal karyotype

Fetal karyotype analysis was possible if chorionic villi were identified in intrauterine tissue obtained by dilatation and curettage. The success rate of karyotyping when villi were identified was 88% (258 out of 293 cases). Cytogenetic analysis was performed using direct preparation, and the chromosomes were banded with Giemsa stain. Eleven cells in metaphase were routinely analyzed, and karyotyping was considered unsuccessful if fewer than three cells in metaphase were obtained.

## STATISTICAL METHODS

### Papers I-III

The controls were frequency-matched to the cases by gestational week. Data in Papers I-III were analysed as matched case-control studies with the use of conditional logistic regression models using SAS PROC PHREG (SAS Institute Inc 1999). Odds ratios (ORs) with 95% confidence intervals (CIs) were used to estimate the relative risk. The overall effect of the main exposure was tested by using a Wald $\chi^2$ test. The test considers all strata in determining significance and not just pair-wise comparisons with the reference group. Since the study was frequency-matched, all controls were considered in the sub-analyses of risks of spontaneous abortion according to fetal karyotype and gestational age. Modification of the effect of the main study variable (folate, cotinine) was evaluated as deviance from multiplicative effects.

In *Paper I v*ariables were included in the multivariable analyses (maternal age, cigarette smoking, average caffeine intake during pregnancy, previous spontaneous abortions, education, parity, country of birth, body mass index, change of eating habits, and the pregnancy symptoms nausea, vomiting and fatigue) if they were judged à priori to be potential confounders, and if they were associated with risk of spontaneous abortion, with low or high folate levels, or with both ($p<0.05$). The interaction between fetal karyotype and folate levels was assessed in a case-case comparison by a $\chi^2$ test. Interaction analyses between folate levels and maternal age, smoking, caffeine intake, and gestational age were assessed by a likelihood ratio test.

In *Paper II, v*ariables were included in the multivariable analyses if they were judged à priori to be associated with risk of spontaneous abortion or if they were univariably associated with risk of repeated spontaneous abortion. In the final model we included maternal age, obstetric history before the two index pregnancies, induced abortions, myoma, time to conceive, marital status, smoking status, caffeine intake, alcohol intake during pregnancy, and plasma folate levels. The obstetric history before the two index pregnancies was categorized as: only index pregnancies (i.e., two pregnancies); index pregnancies and no previous miscarriage; index pregnancies and at least one previous miscarriage.

In the original case-control study of spontaneous abortion (Cnattingius 2000), there was a significant interaction between caffeine ingestion and smoking with regard to the risk of miscarriage: caffeine ingestion increased the risk of miscarriage among nonsmokers but not among smokers. We therefore stratified the analyses of caffeine ingestion and risk of repeated spontaneous abortion according to smoking status. The interaction was tested by introducing an interaction term between caffeine intake and smoking in the model and assessed by a likelihood ratio test.

In *Paper III, v*ariables were included in the multivariable analyses if they were judged à priori to be potential confounders. We included the following covariates: maternal age, average caffeine intake during pregnancy, education, country of birth, previous spontaneous abortions, parity, and the pregnancy symptoms change of eating habits, nausea, vomiting and fiatigue.

To investigate whether nausea modified the ETS-associated risks, we performed interaction analyses between cotinine and nausea with regard to risk of spontaneous abortion. The interaction was tested by introducing an interaction term between nausea and cotinine in the logistic model and was assessed by a likelihood ratio test. In the first model, nausea was defined as the highest level reported during any week of pregnancy (also used in our main analyses), and in the second model, nausea was defined as the nausea level reported at the last

completed week of gestation (in order to time the history of nausea more closely to cotinine measurements). We also assessed the interaction between fetal karyotype and ETS exposure status in a case-only comparison by a $\chi^2$ test.

### Paper IV

The validity of maternal self-reports of smoking, smoking cessation during pregnancy, and ETS exposure during pregnancy was estimated using cotinine measurements as the gold standard. As measures of accuracy, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated in early and late pregnancy. Ninety-five percent confidence intervals (CIs) were computed by the exact method based on the binomial distribution. We also calculated the likelihood ratio (LR) of a positive and of a negative test.

When evaluating the accuracy of self-reported smoking information, cotinine values were dichotomized into >15 ng/ml (gold standard smoker) and ≤15 ng/ml (gold standard nonsmoker) (Peacock 1998). Self-reported information of smoking during the last completed week of gestation was dichotomized into daily smoking or non-daily smoking. The measures of accuracy were defined as follows (Table 2).

Table 2. Validity measures in determining accuracy of self-reported smoking compared with cotinine measurements.

| Test | Gold standard Cotinine (ng/ml) | | Total |
|---|---|---|---|
| Self-report | >15 | ≤15 | |
| Smoker | a | b | a+b |
| Nonsmoker | c | d | c+d |
| Total | a+c | b+d | |

Sensitivity=a/(a+c), Specificity=d/(b+d)
Positive predictive value=PPV=a/(a+b)
Negative predictive value=NPV=d/(c+d)
Likelihood ratio of a positive test=[a/(a+c)]/[b/(b+d)]
=sensitivity/(1-specificity)
Likelihood ratio of a negative test=[c/(a+c)]/[d/(b+d)]
=(1-sensitivity)/specificity

The *sensitivity* was the proportion of gold standard smokers who reported that they were smokers. The *specificity* was the proportion of gold standard nonsmokers who reported that they were nonsmokers. The *positive predicted value* was the proportion of women who reported that they were smokers who had cotinine levels >15 ng/ml. The *negative predicted value* was the proportion of women who reported that they were nonsmokers who had cotinine levels ≤15 ng/ml. The *likelihood ratio of a positive test* was the ratio of the probability of reporting smoking among gold standard smokers to the probability of reporting smoking among gold standard nonsmokers. The *likelihood ratio of a negative test* was the ratio of the probability of reporting nonsmoking among gold standard smokers to the probability of reporting nonsmoking among gold standard nonsmokers (Chien 2001).

Self-reported smoking cessation during pregnancy was validated among smokers. First we validated self-reported smoking in early pregnancy among women who stated they were smokers at time of conception (n=122). Secondly, we validated self-reported smoking in late pregnancy among women who stated they were smokers in early pregnancy (n=60).

Self-reported ETS exposure was validated among nonsmokers. The definition of a nonsmoker was a woman who reported nonsmoking the week preceding the interview and who had a cotinine level ≤15 ng/ml. When evaluating the accuracy of self-reported ETS exposure, cotinine measurements were dichotomized into ETS-exposed (0.1-15.0 ng/ml) and nonexposed (<0.1 ng/ml). Self-reports of ETS exposure during the last completed week of gestation were dichotomized into daily (≥1 hours/day) and non-daily exposure. We calculated the same measures of accuracy as for active smoking.

# 5. RESULTS

First in this chapter, general results from the case-control study of spontaneous abortion will be presented. Thereafter, the most important results in Paper I through IV will be reviewed.

## General results

Compared to the controls from prenatal care, the women with spontaneous abortion were older (≥35 years), were more likely to be born outside the Nordic countries (Sweden, Norway, Denmark, Finland, or Iceland), and to have had more previous births and spontaneous abortions (Table 3). The controls with induced abortions were younger than the controls from prenatal care, and had lower education, were more often living alone, and were more often nulliparas.

Table 3. Selected characteristics for cases and controls.

| Characteristic | Cases (n=562) No. (%) | Controls from prenatal care (n=953) No. (%) | Controls with induced abortions (n=274) No. (%) |
|---|---|---|---|
| Age (years) | | | |
| ≤24 | 69 (12) | 173 (18) | 113 (41) |
| 25-29 | 160 (28) | 374 (39) | 62 (23) |
| 30-34 | 175 (31) | 317 (33) | 54 (20) |
| ≥35 | 158 (28) | 89 (9) | 45 (16) |
| Country of birth | | | |
| Nordic | 507 (90) | 908 (95) | 257 (94) |
| Non-nordic | 55 (10) | 45 (5) | 17 (6) |
| Education | | | |
| <12 years | 184 (33) | 334 (35) | 117 (43) |
| 12-13 years | 92 (16) | 191 (20) | 66 (24) |
| Graduate school (1-3 years) | 155 (28) | 200 (21) | 56 (20) |
| Graduate school (>3 years) | 131 (23) | 228 (24) | 35 (13) |
| Marital status* | | | |
| Cohabitating | 532 (95) | 933 (98) | 115 (42) |
| Single | 27 (5) | 17 (2) | 159 (58) |
| Parity | | | |
| 0 | 229 (41) | 429 (45) | 148 (54) |
| 1-2 | 280 (50) | 487 (51) | 93 (34) |
| ≥3 | 53 (9) | 37 (4) | 33 (12) |
| Previous spontaneous abortions | | | |
| 0 | 393 (70) | 775 (81) | 242 (88) |
| 1 | 107 (19) | 148 (16) | 25 (9) |
| ≥2 | 62 (11) | 30 (3) | 7 (3) |

*Information was missing for 3 cases and 3 prenatal care controls.

*Chromosomal analysis*

We were able to determine the fetal karyotype in 46% (258 out of 562) of the cases with spontaneous abortion (Table 4). We found that 101 fetuses had normal karyotype, 157 fetuses had abnormal karyotype, and the fetal karyotype was unknown in 304 cases. The most common chromosomal aberration was trisomy 16 (in 37 cases), which is in accordance with

previous reports (Hook 1984, Hassold 2001). The success rate of karyotyping increased by week of gestation. In gestational weeks 6-8, only 25% of fetal karyotypes could be determined, whereas in weeks 9-10 and 11-12, the success rates were 51% and 57%, respectively.

Table 4. Results from chromosomal analyses of spontaneous abortions (number of subjects).

| Fetal karyotype | Type of change | Total | XX | XY |
|---|---|---|---|---|
| Normal (n=101) | | 101 | 43 | 58 |
| Abnormal (n=157) | Trisomy | 97 | 41 | 56 |
| | Double trisomy | 7 | 2 | 5 |
| | Sex chromosome changes | 30 | | |
| | 45,X | | 16 | |
| | 47,XXX | | 4 | |
| | 47,XXY | | | 9 |
| | 47,XYY | | | 1 |
| | Triploidy | 16 | | |
| | Tetraploidy | 6 | | |
| | Other* | 1 | | 1 |
| Unknown (n=304) | | 304 | - | - |

* 46,XY,14q+

In Papers I and III, the power to study spontaneous abortion by fetal karyotype was limited due to low numbers of known karyotypes included. We performed interaction analyses based on a case-only comparison stratified on fetal karyotype. These analyses could not show a differential effect of folate associated risk patterns between normal and abnormal fetal karyotype abortions (p=0.38), or ETS-associated risk patterns between normal and abnormal fetal karyotype abortions (p=0.94).

## Maternal folate levels (Paper I)

Folate deficiency (plasma folate ≤4.9 nmol/l) was present among 17% of cases and 14% of controls. Compared with women with plasma folate levels between 5.0-8.9 nmol/l, women with folate deficiency were at increased risk of spontaneous abortion in the univariable analysis (OR 1.31, 95% CI 0.95-1.80, Table 5). In the multivariable analysis, low folate levels were associated with a fifty percent increase in risk of spontaneous abortion (OR 1.47, 95% CI 1.01-2.14). The increase in risk was mainly due to elimination of confounding by maternal age; when we only adjusted for age in the model the corresponding OR was 1.45 (95% CI 1.04-2.01). Among women with higher folate levels there was a non-significant trend towards a protective effect. When we stratified the analyses by gestational age (6-8, 9-10, and 11-12 weeks of gestation), low folate levels were associated with non-significant increased risks of spontaneous abortion (ORs 1.4-1.6).

Table 5. Crude and adjusted odds ratios and 95% confidence intervals (CI) for the risk of spontaneous abortion associated with folate levels.

| Plasma folate (nmol/l) | Odds ratio (95% CI) | Odds ratio (95% CI)* |
|---|---|---|
| ≤4.9 | 1.31 (0.95-1.80) | 1.47 (1.01-2.14) |
| 5.0-8.9 | 1.00 (referent) | 1.00 (referent) |
| 9.0-13.9 | 1.00 (0.73-1.33) | 0.84 (0.59-1.20) |
| ≥14.0 | 1.07 (0.74-1.55) | 0.74 (0.47-1.16) |
| *P*-value† | 0.39 | 0.04 |

*Adjusted for maternal age, cigarette smoking, average caffeine intake, previous spontaneous abortions, education, parity, country of birth, body mass index, change of eating habits, and the pregnancy symptoms nausea, vomiting and fatigue

† Wald test of the overall effect (test of general heterogeneity)

During the study period there was no recommendation in Sweden for pregnant women to use folic acid supplements. In all, 4.7% (30 cases and 35 controls) of the women reported daily intake of a folic acid supplement. Women (cases and controls) taking folic acid supplementation had higher average folate levels (mean 18.2 nmol/l [SD 8.1 nmol/l]) compared to women not taking a supplement (8.2 nmol/l [4.6 nmol/l]). Among supplement takers, none had folate deficiency (Table 6). The adjusted odds ratio for spontaneous abortion among women taking a folic acid supplement compared with those not taking a supplement was 1.3 (95% CI 0.7-2.4). Women taking folic acid supplementation experienced spontaneous abortion earlier than women not taking supplements.

Table 6. Plasma folate levels among folic acid supplement takers and non-takers.

| | Plasma folate (nmol/l) | | | | |
|---|---|---|---|---|---|
| Folic acid supplementation* | ≤4.9 No. (%) | 5.0-8.9 No. (%) | 9.0-13.9 No. (%) | ≥14.0 No. (%) | Total No. |
| No | 206 (16) | 766 (58) | 245 (18) | 107 (8) | 1324 |
| Yes | 0 | 6 (9) | 17 (26) | 42 (65) | 65 |

*Defined as daily intake of ≥400 µg folic acid from 4 weeks before conception until last completed week of gestation.

### Risk factors for repeated spontaneous abortion (Paper II)

In concordance with previous studies (Nybo Andersen 2000a, Dhont 2003) we found that high maternal age and a history of previous spontaneous abortions were strong risk factors for repeated spontaneous abortion (OR 2.9 and 4.8, respectively).

Women with high daily caffeine intake (≥300 mg caffeine) and smokers were at increased risk of repeated spontaneous abortion (Table 7). In stratified analyses we found that the caffeine associated risk was present only among nonsmokers (daily exposure to ≥300mg caffeine: OR 2.7 [95% CI 1.1-6.2] for nonsmokers vs. OR 0.4 [95% CI 0.05-4.1] for smokers). However, the interaction between caffeine and smoking was not statistically significant in this study (p=0.30). Women with a history of at least one preceding miscarriage prior to the two index pregnancies, women reporting prolonged time to conceive, and women with a history of myoma, faced a more than four-fold increased risk of repeated spontaneous abortion respectively.

Table 7. Adjusted odds ratios and 95% confidence intervals (CI) for the risk of repeated spontaneous abortion.

| Characteristic | Odds ratio (95% CI) * | *P*-value† |
|---|---|---|
| Caffeine intake (mg/day) | | |
| 0-99 | 1.0 (referent) | |
| 100-299 | 1.6 (0.7-3.3) | |
| ≥300 | 1.8 (0.8-3.9) | 0.31 |
| Smoking | | |
| No | 1.0 (referent) | |
| Yes | 2.1 (1.1-4.1) | 0.02 |
| Plasma folate (nmol/l) | | |
| ≤4.9 | 0.8 (0.4-1.9) | |
| 5.0-8.9 | 1.0 (referent) | |
| 9.0-13.9 | 2.3 (1.1-4.6) | |
| ≥14.0 | 2.2 (1.0-4.9) | 0.04 |

*Adjusted for the other covariates in the table, maternal age, obstetric history, induced abortions, myoma, time to conceive, marital status, and alcohol intake during pregnancy. 87 cases and 526 controls were included in the multivariable model.

† Wald test of the overall effect (test of general heterogeneity)

Analyses of folate-associated risks were hampered by limited power due to small sample size and missing data. Information on folate levels was missing for 19% of the cases (21 out of 108) and 9% of the controls (52 out of 583). Folate deficiency was not associated with risk of repeated spontaneous abortion. Contrary to the à priori hypothesis, we found that higher folate levels (9-13.9 and ≥14.0 nmol/l) as compared with the reference category were associated with increased risks (Table 7). Daily folic acid supplementation was reported by 11% of the cases and 4% of the controls. The use of a folic acid supplement was associated with risk of repeated spontaneous abortion (crude OR 3.1), and correlated with high folate levels. In an analysis restricted to non-supplement takers women with moderately high folate levels (9.0-13.9 nmol/l), were still at increased risk, whereas women with high levels were not at increased risk.

## Passive and active smoking (Paper III)

Cotinine levels had a bimodal distribution among women with detectable levels: for active smokers cotinine levels were distributed well above the cut-off (15 ng/ml) and for nonsmokers below 10 ng/ml. The distribution of plasma cotinine levels (median, range, mean, SD) was virtually identical among ETS-exposed cases and controls (Table 8). Among smokers, the median and mean cotinine values were higher among cases than controls.

Table 8. Distribution of cotinine levels among cases and controls according to exposure status.

| | Cotinine (ng/ml) | |
|---|---|---|
| | Cases | Controls |
| | Median [90% central range] | Median [90% central range] |
| ETS-exposed | 3.3 [1.2-7.5] | 3.4 [0.8-8.9] |
| Active smokers | 137.5 [20.2-327.9] | 98.5 [22.4-291.9] |
| | Mean (SD)* | Mean (SD)* |
| ETS-exposed | 3.8 (2.1) | 3.8 (2.2) |
| Active smokers | 152.7 (104.0) | 118.7 (98.5) |

*SD=standard deviation

Women with spontaneous abortion were more likely to be ETS-exposed (cotinine levels 0.1-15 ng/ml) than the control women (24% vs. 19%, Table 9). There was a stepwise increase in risk of spontaneous abortion with increasing cotinine category. We found that nonsmoking ETS-exposed pregnant women faced a more than 60% increased risk of spontaneous abortion compared with nonexposed women in the crude analysis (OR 1.65, 95% CI 1.22-2.22). After adjustment for potential confounding factors this risk remained essentially unchanged (Table 9). When the analysis was restricted to life-long nonsmokers, the risk for ETS-exposed women increased to 1.90 (95% CI 1.26-2.86). Active smokers (cotinine >15 ng/ml) faced a more than two-fold increased risk of spontaneous abortion compared with nonexposed women.

Table 9. Odds ratios (OR) and 95% confidence intervals (CI) for the risk of spontaneous abortion associated with cotinine levels.

| Cotinine (ng/ml) | Cases (n=463) No. (%) | Controls (n=864) No. (%) | Crude OR* (95% CI) | Adjusted OR*† (95% CI) |
|---|---|---|---|---|
| <0.1 | 262 (56.6) | 597 (69.1) | 1.00 (referent) | 1.00 (referent) |
| 0.1-≤15 | 111 (24.0) | 161 (18.6) | 1.65 (1.22-2.22) | 1.67 (1.17-2.38) |
| >15 | 90 (19.4) | 106 (12.3) | 2.05 (1.48-2.84) | 2.11 (1.36-3.27) |
| *P*-value‡ | | | <0.001 | <0.001 |

* Crude and adjusted analyses were based on 419 cases and 792 controls with complete covariate information

† Adjusted for age, country of birth, education, marital status, shift work, parity, previous miscarriages, average caffeine intake during pregnancy, folate levels, change of eating habits, and pregnancy symptoms (nausea, vomiting, and fatigue)

‡ Wald test of the overall effect (test of general heterogeneity)

We also stratified the analyses according to paternal smoking status. Among nonsmoking women who reported having a smoking partner (76 cases and 81 controls), the adjusted OR of spontaneous abortion among ETS-exposed (as defined by plasma cotinine) compared with nonexposed women was 1.47 (95% CI 0.39-5.61). Among women with a nonsmoking partner (379 cases and 723 controls), the corresponding OR was 1.51 (95% CI 1.02-2.24).

### Validation of self-reports (Paper IV)

In early pregnancy (6-12 weeks of gestation), 53 out of 66 cotinine indicated smokers reported active smoking (sensitivity 80%), and 712 out of 719 cotinine indicated nonsmokers reported nonsmoking (specificity 99%, Table 10). Among 60 women who reported active smoking, 53 had cotinine levels >15 ng/ml (PPV 88%), and among 725 women who reported nonsmoking, 712 had cotinine levels ≤15 ng/ml (NPV 98%). Thus, only 2% of self-reported nonsmokers underreported active smoking. In late pregnancy (31-34 weeks of gestation) we obtained similar results.

Table 10. Self-reported smoking in early pregnancy compared with cotinine measurements (number of subjects).

| Daily smoking | Cotinine (ng/ml) | | Total | Likelihood ratio |
|---|---|---|---|---|
| | >15 | ≤15 | | |
| Yes | 53 | 7 | 60 | 82.5 |
| No | 13 | 712 | 725 | 0.2 |
| Total | 66 | 719 | 785 | |

Sensitivity 80% (95% CI 69-89), specificity 99% (95% CI 98-99.6), positive predictive value (PPV) 88% (95% CI 77-95), negative predictive value (NPV) 98% (95% CI 97-99).

Thus, the validity of self-reported smoking was reasonably high in this cohort. However, when we separately studied the validity of self-reports among women who reported smoking cessation, the specificity and negative predictive values decreased. For example, among self-reported quitters between conception and the first interview, specificity was 89% and NPV 87%. This means that 13% (8 out of 62) misreported active smoking. Among self-reported quitters between the first and second interview, specificity was 86% and NPV 75%, thus 25% (4 out of 16) misreported active smoking in late pregnancy.

According to cotinine measurements 22% (n=159) of nonsmoking women were exposed to environmental tobacco smoke (ETS) in early pregnancy (Table 11). In late pregnancy 8% (n=55) were ETS-exposed. The validity of self-reported ETS exposure in early pregnancy was poor. Among 159 cotinine-indicated ETS-exposed women, only 9 reported daily exposure (sensitivity 6%), while among 32 women who reported daily ETS exposure, only 9 were exposed according to cotinine measurements (PPV 28%). The likelihood ratio (LR) of a positive test was low (i.e., the ratio between the probability of reporting daily ETS exposure among cotinine-indicated ETS-exposed women [9/159] to the probability of reporting daily ETS exposure among cotinine-indicated nonexposed women [23/553]). The LR of a negative test was 1.0, thus the probability for reporting none-exposure was equal among cotinine-indicated ETS-exposed and unexposed women. Thus, self-reported ETS exposure misclassified most women as unexposed. Similar results were obtained in late pregnancy.

Table 11. Self-reported ETS exposure in early pregnancy compared with cotinine measurements among nonsmokers (number of subjects).

| Daily ETS exposure* | Cotinine (ng/ml) | | Total | Likelihood ratio |
|---|---|---|---|---|
| | 0.1-15 | <0.1 | | |
| Yes | 9 | 23 | 32 | 1.4 |
| No | 150 | 530 | 680 | 1.0 |
| Total | 159 | 553 | 712 | |

Sensitivity 6% (95% CI 3-10), specificity 96% (95% CI 94-97), positive predictive value 28% (95% CI 14-47), negative predictive value 78% (95% CI 75-81).
*Daily ETS exposure was classified as ≥1 hour/day, and non-daily exposure as <1 hour/day.

## Smoking cessation during pregnancy (Paper IV)

We lacked information on cotinine levels at the time of conception. When we investigated self-reported smoking information we found that 16% (122 out of 785) of the women in this cohort reported that they were smokers at the time of conception. According to cotinine measurements 50% of those smokers had quit smoking before 6-12 weeks of gestation and 7% had quit in late pregnancy (after 6-12 weeks but before 31-34 weeks of gestation). Thus, the entire smoking cessation rate during pregnancy in this cohort was 57%.

# 6. GENERAL DISCUSSION

## METHODOLOGICAL CONSIDERATIONS

### Study design

In this thesis, we aimed to study different possible preventable exposures, and to what extent those risk factors were associated with risk of spontaneous abortion and recurrent spontaneous abortion, respectively. In the choice of the most suitable study design there are many factors to consider. A randomised clinical trial is often considered the gold standard, however, some exposures such as tobacco smoke and folate deficiency are not ethically or clinically possible to allocate in a randomised trial. The case-control design is suitable for the efficient study of several risk factors and the association with the outcome. The case-control study used in this thesis was originally designed to study the caffeine related risk of spontaneous abortion. In this study, several exposures were carefully assessed, including plasma measurements of cotinine concentrations, sociodemographic and anthropometric factors, obstetric history, life style factors, and other potential confounding factors. Since plasma samples were stored, we also had the opportunity to later analyze plasma folate concentrations. Thus, this study was appropriate to use for the fulfilment of our aims.

Frequency-matching with regard to duration of gestation was conducted to ensure that the distribution of gestational age would be the same among women with spontaneous abortion as among the control women. This design enabled a more efficient control of gestational age in the analysis. The matching factor gestational week was chosen since it was originally regarded as a potential confounder of the association between caffeine intake and spontaneous abortion. Regarding the hypothesis under study in Papers I-III, the matching was beneficial. Pregnancy is associated with a physiologic reduction in folate levels (Tamura 2006), and with increased nicotine and cotinine metabolism from the second trimester (Dempsey 2002). Since blood samples among controls were drawn in closely matched gestational age to the cases, we could avoid systematic differences in exposure assessment between cases and cntrols.

The proper definition of the diagnosis spontaneous abortion may vary, as mentioned previously. Different diagnostic criteria across studies may affect the obtained results, and make comparisons between studies more complex. In the case-control study, the upper boundary of gestational age was set to 12 completed weeks. The efficiency of case recruitment was improved by our choice to study first trimester spontaneous abortions, because this is when a majority of the abortions occur. The lower boundary of gestational age was set to six completed weeks, since many pregnancies are not recognised before that week. This increased the efficiency in recruitment of subjects, and enhanced the possibilities to determine fetal karyotypes.

In general, studies of spontaneous abortions will, to some extent, suffer from incomplete case ascertainment since very early losses close to conception, including those before implantation, will not be recognised (Wilcox 1988). Because we do not know all fetuses at risk for spontaneous abortion, the true incidence of spontaneous abortion cannot be calculated. In this thesis, association between exposure and spontaneous abortion was studied in a time-window of 6-12 weeks gestation, when most pregnancies are recognised. It cannot be excluded, however, that observed associations may result both from factors that increase the probability of fetal death, and from factors that influence the timing of fetal death. If the exposure under

study was related to a larger proportion of the losses to occur in the time-window of study, the risk estimates would be biased.

In Paper IV, a prospective design was used to follow a cohort of pregnant women from early pregnancy until delivery. This enabled the collection of detailed self-reported information on all possible nicotine exposures, changes in smoking behaviour, and repeated assessment of cotinine levels over the course of pregnancy.

## Validity

A study has a high internal validity if bias, confounding, and chance are unlikely to explain the associations (MacMahon 1996). This section will describe the process of selecting the cases and controls, and the assessment of information on the exposures of interest. Possible sources of bias in the studies of this thesis, and how it could have distorted the obtained results, will be considered.

### *Selection of subjects to compare*

Selection bias can be avoided when controls are a representative sample of the source population that generated the study cases (Wacholder 1992). In the present case-control study, subjects were selected with a population-based approach from a geographically and temporally defined population of pregnant women. We attempted to identify all cases of 6-12 weeks spontaneous abortions. Since the true incidence of early fetal loss is not known, we can only estimate the success of case ascertainment. There is an inherent possibility of case self-selection in the case-control design of early fetal loss. We recruited cases from women seeking medical care, and we do not know if all women with spontaneous abortion seek medical care. At least, we are not aware of any factors related to the exposures under study that would influence the utilization of medical services. Our data can only relate to the spontaneous abortions that are both recognised and reported.

Non-attendance to prenatal care would be a potential source of selection bias of control women, if it was related to the exposure of interest. We cannot rule out that pregnant women not attending prenatal care settings may differ from those attending with respect to lifestyle-related factors, for example smoking. In Sweden, health care coverage is nationwide and free of cost, and the attendance of pregnant women to prenatal care is high. Between 1995 and 1996, 95% registered before their 15th week of gestation (Darj 2002). Furthermore, some cases with spontaneous abortion were generated from women that would have chosen to terminate their pregnancy, provided it had continued. Women with induced abortions may differ from women continuing their pregnancies in terms of factors associated with risk of spontaneous abortion such as age, smoking and possibly other lifestyle habits. To avoid a potential selection bias of the controls, we therefore also sampled controls among women who planned to have an induced abortion.

In Paper II, power limitations due to small sample size forced us to study women with at least two, instead of three, consecutive spontaneous abortions. Also many previous studies of this outcome have included women with only two miscarriages (Christiansen 2006), probably since this facilitates case ascertainment. The fact that women with two instead of three consecutive spontaneous abortions were studied, could have lead to attenuation of estimated exposure risks due to random misclassification of the outcome. However, in a crude analysis of our data, the distribution of risk factors was similar among women with three and two consecutive spontaneous abortions. Controls in Paper II were restricted to women with at least

two pregnancies, of which the last was required to be a normal intrauterine pregnancy. This design aimed to select controls and cases from the same study base. All controls were at risk to develop the outcome. The potential for selection bias was reduced, whereas many observational studies have a skewed selection of controls, e.g. non-pregnant women, term pregnant women, or women with less number of previous pregnancies than the cases.

In Paper IV, a cohort of pregnant women was followed prospectively from early pregnancy until delivery. The drop out of non-participants, pregnancy losses before the second interview, women with missing exposure information, and the exclusion of users of other nicotine containing products resulted in a selection of subjects to compare with regard to the smoking prevalence. For instance, in early pregnancy 12% of the 953 women in the cohort were active smokers, and in late pregnancy, the smoking prevalence was 7% among the remaining 785 women in the cohort. When looking in early pregnancy among the same 785 women, the smoking prevalence was 8%. Thus, we have selected a healthier cohort, with lower smoking prevalence than the general population for the validation study. This fact does not threat the internal validity of the results; however the generalizability of the findings to other populations may be limited.

### *Information on outcome*

In this work, strict diagnostic criteria were applied to avoid misclassification of women with a normal pregnancy and women with a spontaneous abortion. All pregnancies were confirmed by a positive hCG-test, and an endovaginal ultrasound scanning was performed to determine fetal viability.

### *Information on exposure*

All women were interviewed face-to-face by trained interviewers using structured questionnaires close to the time of the miscarriage in cases, or the corresponding gestational week for controls, which limited errors in exposure assessment. Because we were able to measure folate and cotinine levels rather than having to rely on self-reported information, the potential for recall bias was avoided.

Misclassification of environmental tobacco smoke exposure was considered in Papers III and IV. Although cotinine measurement is a well accepted biomarker of ETS exposure (Benowitz 1999), it may also be subjected to exposure misclassification. Most importantly, misclassification by active smokers has to be ruled out. Misclassification of an active smoker as ETS-exposed would lead to biased estimates. To limit the possibility of having any recent quitters, or occasional smokers in our "ETS-group" we excluded women who reported smoking during pregnancy. Users of oral snuff and nicotine replacement therapies were also excluded to avoid exposure misclassification (Dempsey 2001). However, there is still a possibility that women smoking cigarettes on an intermittent basis might be misclassified as exposed to ETS in our data.

One limitation with the case-control design is that information on the supposed cause can only be retained after the person has been identified with the disease. Since death of the fetus may occur days or weeks before vaginal bleeding and clinical diagnosis (Mills 1999), this study design limited our possibility to infer a temporal relationship between exposure and outcome. Plasma cotinine is a precise indicator of exposure over recent past (Dempsey 2002), and among nonsmokers it reflects environmental exposure to tobacco smoke during the last days. Plasma folate reflects folate status during the days or weeks preceding assessment (Shojania

1984, Scholl 2000). Howerver, a single cotinine or folate measurement is an imperfect marker of the exposure over the etiologic period of interest. We did not have information about the time when each blood sample was collected. In Paper III, systematic differences between cases and controls may have influenced levels of plasma cotinine. In controls, blood was collected during office hours (i.e. from 8 am to 4 pm). Even though most women with pregnancy bleedings first contacted the Department of Obstetrics and Gynecology by phone, and thereafter were scheduled for a visit during office hours, blood samples were, among cases, also collected in evenings and sometimes at night. If recency or amount of ETS exposure differed between cases and controls, we would have expected differences in prevalence of ETS exposure, as well as differences in the distribution of cotinine values among these classified as being exposed to ETS. However, the distribution of cotinine values (median, range, mean, SD) among cases and controls exposed to ETS were virtually identical, indicating that timing and dose of exposure for cases and controls were similar. Thus, possible misclassification of ETS exposure based on a single cotinine measurement should be non-differential between cases and controls.

In Paper II, information on maternal characteristics and exposures were only collected at the last pregnancy. We preferred, because of validity concerns, not to retrospectively assess exposure information from the preceding pregnancy. There is a possibility of differential reporting of exposures (recall bias) between cases and controls, which may be larger than in the study of a single spontaneous abortion. By matching the cases and controls on gestational week and interviewing controls in early pregnancy and cases strictly after the miscarriage, we tried to limit the risk of recall bias.

### *Confounding and effect modification*

Confounding leads to biased estimates, however, if confounding is known and measured, it can be controlled for in the analysis. Information on many potential confounding factors was identified in the studies of this thesis. In Paper I, the crude estimated association for folate deficiency-spontaneous abortion was biased toward the null-value (i.e. negative confounding), because low maternal age was positively associated with folate deficiency, and also negatively associated with spontaneous abortion. After adjustment, the increase in risk was mainly due to elimination of confounding by maternal age. Controlling for other possible confounding factors in the analyses had only a marginal effect on the folate-spontaneous abortion association.

In Paper II, confounding by indication may be an alternative explanation for the observed association with high folate levels and repeated spontaneous abortion. For example, women who experience reproductive problems may become more health conscious, and eat more folate rich food. In fact, we found that a history of previous spontaneous abortions was associated with taking folic acid supplementation. We tried to account for this supposed bias in the analyses with restriction of the sample to non-supplement users, and then observed that the dose-response relationship between plasma folate levels and risk of repeated miscarriage was less clear, and the overall association was non-significant.

In Paper III, we were able to adjust for a wide range of potential confounding factors, which had little effect on the association between ETS exposure and risk of spontaneous abortion relationship. In order to take confounding by nausea into account, we adjusted for pregnancy symptoms in the analyses. Nausea did not explain the association between ETS exposure and spontaneous abortion. Nevertheless, the relation between environmental exposures (such as tobacco smoke), symptoms of pregnancy (such as nausea), and fetal viability is complex.

Women with a viable pregnancy experience more nausea and it has been proposed that this in turn may cause women to avoid various environmental exposures (Stein 1991, Savitz 2002). However, in interaction analyses between cotinine and nausea with regard to risk of spontaneous abortion, we found no evidence of an effect modification by nausea.

Since there was an interaction between caffeine ingestion and smoking with regard to the risk of miscarriage (Cnattingius 2000), a modification of the effect of ETS by caffeine was investigated in Paper III. In this investigation there appeared to be no effect-modification by caffeine intake among women exposed to ETS, although this conclusion may be hampered by limited power. Instead, our data showed that the significant interaction effect between cotinine and caffeine was mainly driven by active smoking.

Unmeasured confounding can never be excluded in an observational study; however, a hidden confounder has to be very strong to change the effect estimate very much. In Papers I and III, we lacked information on alcohol exposure among the controls drawn from the pool of women with induced abortions. The remainder of cases and controls reported very low levels of alcohol consumption during pregnancy, however, we cannot rule out maternal under-reporting of alcohol intake (Ernhart 1988).

### Chance

Although chance can never be excluded as an explanation of the observed findings, with the use of statistical methods the role of chance as an explanation can be estimated (Hennekens 1987). In this thesis, the influence of random errors or chance was estimated through calculations of p-values, and confidence intervals around the estimates. The confidence level was set to 95 percent. The large sample size in Papers I and III resulted in a reduction of random errors and increased precision of the effect estimates, and allowed efficient control of potential confounding factors.

Power calculations were performed with an α-level of 0.05, and a ß-level of 0.20 (80% power). In Papers I and III, with prevalence of exposure (among the controls) ranging from 5-50%, we could detect odds ratios ranging from 1.9 to 1.4. However, the power for subgroup analyses of fetal karyotype (Papers I and III), and of folate supplementation (Paper I) was limited due to small numbers. In paper II, with prevalence of exposure ranging from 5-50%, we could detect odds ratios ranging from 2.9 to 1.8. Thus, in Paper II we had low power to detect even relatively high relative risks.

## FINDINGS AND IMPLICATIONS

### Folate (Paper I)

Our aim was to investigate if folate deficiency was a risk factor of spontaneous abortion. Since there was no food fortification in Sweden during the study period and also a low rate of supplement use, the choice of study population and time period was optimal for this objective. We observed that women with folate deficiency were at increased risk of spontaneous abortion. Many previous observational studies on folate status and early fetal loss have methodological drawbacks including small sample size, lack of control for potential confounders, or inclusion of non-pregnant women as controls. However, our finding was in coherence with most (Hibbard 1975, Friedman 1977, Pietrzik 1992, Neiger 1993, Ray 1999, Ronnenberg 2002), but not all (Streiff 1967, Neela 1997, de Weerd 2003) investigations. Our findings could not support the concern that high folate levels increased the risk of spontaneous abortion (Hook 1997, Windham 2000). Instead, there was a trend towards a protective effect among women with high folate levels. However, it must be noted that women living in countries with folic acid food fortification have higher average folate levels than what was considered high levels in the present study.

The mechanisms by which low folate could cause spontaneous abortion, and the mechanisms of the preventive effect of folic acid on NTDs remain unknown. Folate is essential in single-carbon transfer reactions (Mattson 2003), and used in the body in a large number of biochemical reactions including metabolism of amino acids, DNA methylation, and synthesis of nucleic acids (Christensen 1995, Hultdin 2003). Folate stimulates the hematopoetic system, and folic acid is used in the treatment and prevention of folate deficiencies and megaloblastic anemia. Folate deficiency has been tentatively associated with abruptio placentae and preeclampsia (Ray 1999), and early vascular effects related to folate deficiency might also increase the risk of spontaneous abortion. Low folate increases the incidence of NTDs, and fetuses affected with NTDs are more commonly aborted spontaneously (Byrne 1986). Byrne and colleagues (1986) estimated that the incidence of NTD was ten times higher in miscarriages than in term births. Women who give birth to infants affected by NTD have more often a history of previous miscarriages compared with women who gave birth to infants with other birth defects (48% vs. 20%)(Carmi 1994). However, NTD is a rare condition, and can only explain a small part of the association between low folate levels and spontaneous abortions. It is known that folate is fundamental in DNA synthesis and regulation. DNA methylation is important in regulation of gene expression and differentiation during development (Christensen 1995). Thus, it has been suggested that the rapidly developing cells in the embryo may be damaged by lack of adequate folate (Scott 1995). Failure to produce sufficient DNA and to regulate DNA function may lead to spontaneous abortion among both normal and abnormal fetuses.

Analyses stratified by fetal karyotype were based on small numbers. We found that folate deficiency was primarily confined to spontaneous abortions with abnormal karyotype. Most chromosomally abnormal conceptuses are spontaneously aborted, and we found no differences in the severity of fetal karyotype abnormality (lethal vs. non-lethal karyotype) by plasma folate levels. It is possible that low folate status causes a larger proportion of these losses to occur in the 6-12 week period. However, we also performed a test for interaction between karyotype and folate to test whether the distribution of folate levels differed between the groups of normal and abnormal karyotype. This test was non-significant, and we cannot claim a differential effect of folate status by fetal karyotype in this data. Although our study

has far better data than previous studies of folate effects on chromosomal status and timing of losses, future studies will be required to resolve these issues.

There has been controversy on the folic acid-miscarriage association, where some investigators claimed a harmful association with periconceptional supplement use, but later investigations have found no such association (Hook 1997, Wald 1997, Windham 2000, Gindler 2001, Wald 2001, Lumley 2005). The risk of miscarriage associated with periconceptional folic acid supplementation could be estimated with a limited precision in this study due to low numbers. The low rate of periconceptional folic acid supplementation during the study period was due to a lack of recommendations for Swedish women to take supplementation during pregnancy. In Paper I, no adverse effects of folate supplementation were detected in terms of any statistically significant increased risk in spontaneous abortion.

To summarize; the observed association with folate deficiency and spontaneous abortion is consistent with most previous evidence, and there is plausible biologic basis to explain the association. The existing evidence is not sufficient to infer a causal relationship between low folate and miscarriage. For example, we were unable to ascertain the temporal relationship between exposure and outcome. The finding also needs to be confirmed in prospective studies within other populations with adequate range of exposure. The combined observations in this work lend further support to the existing strong evidence that women of childbearing age should be advised to take a folic acid supplement during pregnancy to prevent birth defects.

### Repeated spontaneous abortion (Paper II)

Well known risk factors for recurrent spontaneous abortion, such as high maternal age and previous spontaneous abortions, could be confirmed in this study. Many of our analyses in this paper relied on small numbers, producing statistically imprecise estimates that should be interpreted with caution. Our à priori hypothesis that folate deficiency was related to repeated spontaneous abortion could not be confirmed in this work. On the contrary, high folate levels were associated with repeated miscarriage. As was discussed in the previous section, the observed association with folate levels and repeated spontaneous abortion may, however, not be valid due to confounding by indication. Chance, as an alternative explanation, can not be excluded with reasonable confidence.

In this study, we also aimed to explore factors that would be possible to eliminate from the woman's environment and the relationship with recurrent spontaneous abortion. We found that maternal smoking and high caffeine intake were associated with increased risk of repeated spontaneous abortion. However, due to an interaction between these two variables, the main effect of smoking could not be assessed, and the effect of caffeine intake was only observed among nonsmokers. There is plausible biologic evidence for fetal toxicity of tobacco smoke (USDHHS 2004), and it seems likely that a persisting environmental exposure between pregnancies may cause repeated fetal loss. Findings in previous observational studies of repeated miscarriage are inconsistent, and may be flawed by systematic errors such as a skewed selection of controls, e.g. non-pregnant women, term pregnant women, or women with less number of previous pregnancies than the cases (Hibbard 1964, Parazzini 1990, Wouters 1993, Sutterlin 1997, Zusterzeel 2000, Nelen 2000a).

We also found that a history of myoma was associated with risk of repeated spontaneous abortion. This association might not be causal, and may be explained by systematic differences between case and control women. Women with previous abortions are more likely to have been closely investigated with previous gynecological examinations, ultrasound

scannings, and surgical curettage, with the possibility of detection of myoma. There is also a possibility of differential reporting of myoma (recall bias) between cases and controls.

Risk estimates for women below 25 years of age were slightly increased in the univariable analysis, but increased markedly in the multivariable analysis. In fact, we observed a u-formed risk of repeated spontaneous abortion with maternal age in the adjusted analysis. In a Danish study (Nybo Andersen 2000a), the risk of miscarriage according to maternal age followed a J-shaped curve; however the risk among the youngest women disappeared after adjustment for induced abortions. In our study, the age-related risks remained also after adjusting for induced abortions. This association should be interpreted cautiously; it may reflect residual effects of unmeasured maternal characteristics associated with risk of spontaneous abortion and with low maternal age, such as socioeconomic and life-style factors.

Given the limitations of this study, the findings suggest that all women with recurrent spontaneous abortion should be encouraged to stop smoking and probably also to reduce caffeine consumption. Environmental factors may be overlooked in the very extensive investigation that is generally offered couples with this diagnosis. However, both these exposures are largely a matter of personal choice, and if motivated, both factors may be possible to eliminate from the environment.

### Maternal smoking (Papers III and IV)

Our finding that active smoking was associated with increased risk of spontaneous abortion confirmed most but not all previous investigations (Cnattingius 2004, USDHHS 2004). It has been reported that cotinine measurements better predict risk of low birth weight (Peacock 1998), and more strongly relate to risk of spontaneous abortion (Ness 1999), than maternal self-reported smoking information. In the validation study, we found that maternal self-reported information on active smoking could be considered reasonably valid in early and late pregnancy. Still, a fraction of pregnant smokers in clinical settings will be undetected with self-reported exposure information, and may not receive appropriate interventions. In research settings, the use of self-reported smoking information may lead to underestimated smoking-related risks, and inconsistent results between studies.

In the cohort of pregnant women, we found that the smoking cessation rate during pregnancy was 57%, according to cotinine measurements. Almost all women who quit smoking did so before the first interview in week 6-12 of pregnancy. These findings are reassuring and also in concordance with the self-reported information in the Swedish Medical Birth Register. Although based on small numbers in this work, it is worth noting that self-reported information on smoking cessation during pregnancy was unreliable, especially if the quitting was late in gestation. Previous studies with biochemical verification of smoking cessation, have observed an over-reporting of quitting (Solomon 2004). This implies that smoking cessation interventions should be provided throughout the pregnancy to all women who report smoking around time of conception, and that self-reports alone in this group are insufficient to assess smoking status.

A more active approach worth considering is routine biochemical assessment of tobacco smoke exposure in prenatal care settings (McClure 2004). Prenatal tobacco smoke exposure is still not negligible, and the evidence of causal adverse perinatal effects is strong (USDHHS 2004). With this strategy, smoking interventions during pregnancy can be targeted where needed, and biomarker feedback may also increase smoking cessation rates during pregnancy.

### Environmental tobacco smoke (Papers III and IV)

This study is presumably the first using cotinine measurements to assess ETS exposure in relation to risk of spontaneous abortion. We observed an increased risk of spontaneous abortion among ETS-exposed nonsmoking pregnant women. There is a lack of evidence regarding effects of ETS on early fetal loss (USDHHS 2006). Previous studies have assessed ETS with self-reported exposure information, or with spousal smoking as a proxy measure (Ahlborg 1991, Windham 1992, Chatenoud 1998, Windham 1999a, Venners 2004). It is plausible that exposure misclassification accounts for much of the inconsistent results among those studies.

We found that self-reported information on ETS exposure was subject to a large degree of misclassification. In fact, most women who were ETS-exposed according to cotinine values classified themselves as unexposed to ETS. Self-reported information of ETS exposure during pregnancy has only been validated in a limited number of studies (O'Connor 1995, Rebagliato 1995, DeLorenze 2002, Kaufman 2002), which have found low to moderate correlations. A misclassification of exposure status that is random among cases and controls, may lead to estimates biased towards the null value and failure to find a relationship that truly exists.

The prevalence of ETS exposure among nonsmoking women was surprisingly high in this work (24% of cases, and 19% of women with a normal pregnancy). These rates were, however, in agreement with other populations of pregnant women where ETS exposure was assessed with cotinine measurements (Haddow 1988, Eskenazi 1995, Rebagliato 1995, Peacock 1998). It can be anticipated that the rate of exposure is lower today in Sweden, after the legislation in 2005, when smoking in all public places was banned in restaurants and bars. However, over-all smoking prevalence was close to 16% in Sweden 2005 (Statistics Sweden 2005), and there is evidence that ETS exposure can also occur outdoors (Cal/EPA 2006).

In the present study, we were not able to disentangle information on where the nonsmoking women were exposed to tobacco smoke. Assessment of self-reported exposure information was based on number of hours exposed daily, but did not separate possible sources of exposures, i.e., ETS exposure at work, at home and in public places. Thus, limitations in the used questionnaire probably accounted for some of this inability. Spousal smoking explained only a part of the exposure to ETS. Not all women with smoking partners were ETS-exposed, and many women with a nonsmoking partner were ETS-exposed. Thus, ETS-related risk estimates for spontaneous abortion were equal among women with a smoking partner as among those with a nonsmoking partner. This finding indicates that the exposure occurred outside the home. It also rules out the possibility that the risk was related solely to active paternal smoking.

There are plausible underlying biologic mechanisms for the association between tobacco smoke and spontaneous abortion. Tobacco smoke is classified as carcinogenic to humans (Group I) by the International Agency for Research on Cancer (IARC), and it contains millions of components (e.g., nicotine, carbon monoxide, and cyanide) that are potentially toxic for the developing fetus. Nicotine is the main addictive compound of tobacco smoke, and its metabolite, cotinine, has been detected in fetal tissue in 7$^{th}$ week of gestation among smokers and nonsmokers (Jauniaux 1999). Meconium analyses of new-born infants (Ostrea 1994) found that cotinine concentrations were similar among infants whose mothers were light active smokers and infants whose mothers were exposed to ETS (34.7 ng/ml vs. 31.6 ng/ml).

Sidestream tobacco smoke contains many of the same toxic constituents as mainstream tobacco smoke. Possibly because temperatures are lower when sidestream smoke is produced, it contains even higher concentrations of many compounds than mainstream smoke (Cal/EPA 2006). The biologic mechanisms underlying an association between ETS and spontaneous abortion may involve pathways similar to those for active smoking. Nicotine has strong vasoconstrictive effects leading to reduced placental blood flow. Carbonmonoxide binds to hemoglobin, causing maternal and fetal hypoxia (USDHHS 2001), which may interfere with the development of the growing conceptus and induce fetal demise. There have been comparatively few animal studies that have investigated the relation between ETS and mechanisms for spontaneous abortions. In a study of sea urchins (Longo 1970), there was a correlation between the degree of polyspermic eggs and nicotine concentration. Previous studies have mainly hypothesized that tobacco smoke is associated with spontaneous abortions of normal fetal karyotype (Kline 1995). Still, it is plausible that tobacco smoke also could influence, or promote abnormal conceptuses to be spontaneously aborted early in gestation through similar mechanisms. It has also been speculated if constituents of tobacco smoke could even cause changes in the chromosomes of germ cells. The proportion of diploid oocytes is highly associated with the number of cigarettes smoked per day (DeMarini 2004), and direct evidence of tobacco-associated intrauterine mutagenesis (structural chromosomal abnormalities in amniocytes) has recently been reported (de la Chica 2005). Another mechanistic consideration is the potential direct adverse effects of paternal smoking. It has been hypothesized that spousal smoking may increase the risk of miscarriage through direct effects of active smoking on sperm (Venners 2004), and smoking has been reported to induce aneuploidy in sperm for certain chromosomes (DeMarini 2004).

We observed that ETS exposure was associated with both normal, abnormal, and unknown fetal karyotype abortions. This is plausible since early tobacco-related changes, i.e. vascular and hypoxic changes, may affect all conceptuses to be spontaneously aborted. However, the power to study ETS and risk of spontaneous abortion by fetal kryotype was again limited, because our analyses included only 75 cases with normal and 120 cases with abnormal karyotypes.

In conclusion, the finding that ETS exposure was associated with risk of early fetal loss is biologically plausible and coherent with previous evidence. The case-control study was well powered, and carefully conducted to limit systematic errors. Exposure was assessed with a well-accepted biomarker. However, the possibility to determine the time sequence between exposure and outcome was limited, which warrants further studies. Even if the strength of the association was modest, the prevalence of ETS exposure among nonsmoking pregnant women was high, which may have substantial public health consequences. Since self-reports was an imprecise measure of ETS exposure in this as well as in previous studies, forthcoming exposure assessment may have to rely on biomarker measurements. The studies of this thesis, add to the evidence that efforts should be continued to limit ETS exposure, with the promotion of smoke-free homes, work places and public areas.

## FUTURE CHALLENGES

Despite the numerous investigations that have been undertaken to elucidate the etiology of spontaneous abortion, surprisingly few established risk factors exist. The etiology is heterogeneous, and more than one factor may interact in causation of fetal loss. Chromosomal abnormalities are often claimed as the cause for a miscarriage; however, the etiology behind aneuploidy is even more elusive. It has often been stated that the etiology is not important, since most women who experience a spontaneous abortion will, with no treatment, carry next pregnancy to term. On the other hand, studies of developmental toxicants can be regarded as studies of mortality. An intervention that would reduce the incidence of fetal loss with only a fraction would render enormously large health and economic benefits on a population level.

Given the existing methodological challenges in the studies of determinants of early reproduction, the first important obstacle to overcome would be to find a biomarker of human conception. If a biomarker of conception was available, also early losses would be captured, the timing of death could be determined, and the correct incidence of spontaneous abortion could be estimated. Then it would be possible to disentangle etiologic from prognostic factors, and to investigate if etiology differs between recognised and unrecognised spontaneous abortions.

More specific, findings in the studies presented in this thesis have rendered some future questions to be answered:

- Does the risk of spontaneous abortion related to smoking, environmental tobacco smoke, and low plasma folate vary by
    - gestational age?
    - fetal karyotype?
- What is the prevalence of ETS exposure today among pregnant women after the changed legislation?
- How can we identify the main sources of ETS exposure?
- How can we reduce exposure to ETS?

# 7. CONCLUSIONS

From the studies of which this thesis consists, the following conclusions could be drawn:

- Low plasma folate levels were associated with an increased risk of spontaneous abortion.
- Smoking and possibly high caffeine intake during pregnancy, were associated with increased risk of repeated spontaneous abortion.
- Folate deficiency was not an observed risk factor for repeated spontaneous abortion.
- Almost one out of five nonsmoking pregnant women was exposed to environmental tobacco smoke according to cotinine measurements.
- Nonsmoking pregnant women exposed to environmental tobacco smoke were at increased risk of spontaneous abortion.
- Active smoking was associated with increased risk of spontaneous abortion.
- Self-reported smoking information throughout pregnancy had a reasonably high validity.
- Self-reported information on smoking cessation during pregnancy had a limited validity.
- Self-reported information on environmental tobacco smoke exposure was inaccurate throughout the pregnancy, and misclassified most women as unexposed.

# 8. SVENSK SAMMANFATTNING (Swedish Summary)

## Bakgrund

Missfall drabbar 15-30% av alla graviditeter. Habituella missfall (minst tre upprepade missfall i följd) drabbar 1% av alla kvinnor. Sambandet mellan folsyrabrist och missfall har varit oklart, och tidigare studier har stora metodologiska brister. Tillskott av folsyra under graviditet minskar risken för ryggmärgsbråck hos det väntade barnet. Sedan 1998 berikas därför livsmedel med folsyra i bland annat USA. I Sverige utreds frågan om folsyraberikning av Statens beredning för medicinsk utvärdering (SBU), vilka förväntas publicera en rapport under hösten 2006.

I Sverige röker i dag fler kvinnor än män. Trots att prevalensen av rökning minskat, rökte fortfarande 12 % under tidig graviditet 2001 enligt data från Medicinska Födelseregistret. Orsakssambandet mellan rökning under graviditet och intruterin tillväxthämning är starkt och väldokumenterat. Mindre allmänt känt är att även exponering för passiv rökning ökar risken för låg födelsevikt, för tidig födsel och plötslig spädbarnsdöd. Endast några få tidigare studier har studerat passiv rökning och risk för missfall, och resultaten talar för ett möjligt samband. De flesta studier av rökning och passiv rökning och risk för graviditetskomplikationer baseras dock på självrapporterad exponeringsinformation. Validiteten av självrapporterad aktiv och framförallt passiv rökning under graviditet har ifrågasatts. Med hjälp av en biomarkör i blod, cotinin, kan exponering av både aktiv och passiv rökning under graviditet mätas.

## Syfte

Syftet med denna avhandling var att undersöka potentiellt förändringsbara riskfaktorers betydelse för uppkomst av missfall och upprepade missfall, samt att undersöka möjligheter för exponeringsmätning under graviditet. Specifik målsättning var att undersöka sambandet mellan folsyranivåer i tidig graviditet och risk för missfall (delarbete I), att undersöka riskfaktorer för upprepade (≥2) missfall (delarbete II), att undersöka sambandet mellan passiv rökning (definierat av cotininnivåer) och risk för missfall (delarbete III), och att validera självrapporterad information av aktiv och passiv rökning, samt rökstopp under graviditet (delarbete IV).

## Material och metod

Delarbete I, II och III baseras på en svensk populationsbaserad fall-kontrollstudie över tidiga missfall. Studien genomfördes i Uppsala län 1996-98. I studien inkluderades 562 kvinnor med missfall i graviditetsvecka 6-12. Kontrollgruppen utgjordes av 1028 kvinnor med en konstaterad normal graviditet. Kontrollerna var frekvensmatchade till fallen med avseende på graviditetslängd. Alla kvinnor genomgick personliga intervjuer enligt ett strukturerat formulär. Vid intervjutillfället togs även blodprov för analys av folsyra- och cotininnivåer. Kromosomanalys av tidiga missfall visade 101 foster med normal och 157 foster med abnorm kromosomuppsättning (samt 304 missfall med okänd karyotyp). Med multipel logistisk regression beräknades relativ risk för missfall och upprepade missfall i form av odds ratio (OR), med 95% konfidensintervall (CI), justerade för potentiella confounders. Delarbete IV baseras på den gravida kontrollgruppen (953 kvinnor), som intervjuades och lämnade blodprov i graviditetsvecka 31-34. Självrapporterad information om aktiv rökning, rökstopp under graviditeten, och exponering för passiv rökning jämfördes med cotinin uppmätt i

plasma som referens, i tidig (vecka 6-12) och sen graviditet (vecka 31-34). Sensitivitet, specificitet, positivt och negativt prediktivt värde, och likelihood ratio beräknades som mått på tillförlitligheten av självrapporterad exponeringsinformation.

### Delarbete I

I denna studie inkluderades 468 kvinnor med missfall och 921 gravida kvinnor i kontrollgruppen med folsyranivåer analyserat i blodplasma. Vi fann att kvinnor med låga folsyranivåer (<5 nmol/l) löpte en nära femtio procent högre risk för missfall (OR 1.47, 95% CI 1.01-2.14) jämfört med kvinnor med normala folsyranivåer (5.0-8.9 nmol/l). Kvinnor med högre folsyranivåer (9.0-13.9 samt ≥14.0 nmol/l) löpte ingen ökad risk för missfall (OR 0.84, 95% CI 0.59-1.20 respektive OR 0.74, 95% CI 0.47-1.16) jämfört med kvinnor med normala folsyranivåer. Endast knappt 5% av kvinnorna hade tagit vitamintillskott med folsyra under graviditeten, och nästan alla dessa kvinnor hade höga folsyranivåer (>9 nmol/l). Vi analyserade även missfallsrisk med avseende på fostrets kromosomuppsättning, och fann att folsyrabrist var associerat med ökade risker för foster med abnorm och okänd kromosomuppsättning. Analyserna av foster med normal kromosomuppsättning inkluderade endast 83 kvinnor, och inga statistiskt säkerställda risker kunde identifieras för denna grupp.

### Delarbete II

Totalt identifierades 108 kvinnor med minst två konsekutiva missfall i första trimestern. Kontrollgruppen utgjordes av 583 kvinnor med minst två graviditeter, där den senaste graviditeten var konstaterat viabel med ultraljud. Jämfört med referensgruppen (25-29 år), fann vi att risken för upprepade missfall var mer än fördubblad för kvinnor över 34 år (OR 2.9, 95% CI 1.4-5.8) och för kvinnor yngre än 25 år (OR 2.8, 95% CI 1.1-6.8). Kvinnor med anamnes på minst ett tidigare missfall, förlängd tid till konception (>1år), eller myom hade en mer än fyrfaldigt ökad risk för upprepade missfall. Rökare hade en fördubblad risk för missfall (OR 2.1, 95% CI 1.1-4.1) jämfört med ickerökare. Ickerökande kvinnor med högt koffeinintag hade en ökad risk för upprepade missfall, en association som inte fanns hos rökande kvinnor. Folsyrabrist var inte associerat med ökade risker för upprepade missfall i denna studie.

### Delarbete III

I denna fall-kontrollstudie inkluderades 463 kvinnor med tidigt missfall och 864 gravida kvinnor i kontrollgruppen. Exponeringsstatus indelades i tre kategorier baserade på cotininnivåer i plasma; oexponerad (cotinin <0.1 ng/ml), passiv rökare (0.1-15 ng/ml), och aktiv rökare (>15 ng/ml). Vi fann att en betydande andel av de gravida ickerökande kvinnorna var exponerade för passiv rökning (19 % i kontrollpopulationen och 24 % av kvinnorna med missfall). Jämfört med oexponerade var missfallsrisken ökad med drygt 60% för passiva rökare (OR 1.67, 95% CI 1.17-2.38), och risken var dubblerad för aktiva rökare (OR 2.11, 95% CI 1.36-3.27). Även om passiv rökning ökade riskerna för missfall med normal, abnorm och okänd kromosomuppsättning, kunde inga statistiskt säkerställda skillnader av cotininets effekt mellan grupperna påvisas. Enligt våra efterforskningar är detta den första studien av passiv rökning relaterat till missfall där exponering mätts med cotinin. Givet den höga prevalensen av passiv rökning och att missfall är graviditetens vanligaste komplikation, kan effekterna av passiv rökning på tidiga missfall på en populationsnivå vara avsevärda.

### Delarbete IV

I denna prospektiva studie fann vi att självrapporterad information av aktiv rökning var tillförlitlig i tidig och sen graviditet, med en relativt hög sensitivitet och specificitet. Däremot ökade felklassificeringen självrapporterad information bland kvinnor som slutat röka under graviditeten. Av kvinnor som rapporterade att de slutat röka innan första intervjun rökte 13% enligt cotininmätnningen, och av kvinnor som rapporterade rökstopp mellan första och andra intervjun rökte 25% enligt cotininmätningen. Vidare fann vi att 22% av ickerökande gravida kvinnor var utsatta för passiv rökning i tidig graviditet (cotinin 0.1-15 ng/ml). Validiteten av självrapporterad information av exponering för passiv rökning var däremot dålig i både tidig och sen graviditet, med låg sensitivitet och lågt positivt prediktivt värde. Självrapporterad exponeringsinformation felklassificerade de flesta kvinnor som oexponerade. Resultat från denna studie talar för att framtida studier bör överväga att använda biomarkörer för att estimera exponering av passiv rökning.

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