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PERINATAL SNUS EXPOSURE AND CARDIOVASCULAR FUNCTION IN THE CHILD

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To My Family

ABSTRACT

Maternal use of smoking tobacco during pregnancy is one of the most important preventable risk factors during pregnancy. Maternal smoking is associated with alterations in autonomic cardiac control and long-term cardiovascular effects on blood pressure and arterial wall properties. Earlier studies have had difficulties in determining the specific contributions of prenatal and postnatal exposures, respectively, as well as distinguishing acute from chronic effects, as these children are frequently exposed to smoking both before birth and during childhood. The specific role of nicotine is also unclear as smoking contains many toxic combustion agents in addition to nicotine. While cigarette smoking is decreasing globally, other forms of tobacco- and nicotine-containing products are gaining in popularity and in Sweden and Norway, use of smokeless tobacco (*Swedish Snus*) is increasing among women.

Snus delivers high doses of nicotine to the fetus so we hypothesized that prenatal snus exposure had long term cardiovascular associations with increased blood pressure, arterial wall stiffness, intima media thickness and altered autonomic cardiac control in the offspring. We also aimed to investigate the levels of nicotine and metabolites in the breastmilk of snus-using mothers.

We included women from a larger national cohort with women recruited during early pregnancy during 2006–2011, residing in Stockholm, Östersund and Umeå. The pregnant women were grouped, based on their tobacco use at inclusion, into snus users, smokers or tobacco-free controls. Dual users were excluded. At infant age one to two months, we tested the infant's heart rate variability and also the infant's urine and mother's breastmilk for nicotine and metabolites. At child age 5-6 years, we tested heart rate variability, blood pressure, carotid intima media thickness and calculated arterial stiffness based on pulsatile changes in pressure and diameters.

Heart rate variability showed a higher low frequency/high frequency (LF/HF) ratio, indicating a lower vagal activity, in infants with snus exposure *in utero* compared with tobacco-free controls and the ratio was similar to that seen in smoke-exposed children. Breastmilk from snus users showed high levels of nicotine and cotinine. In addition, nicotine was still detected in breastmilk after more than 12 hours of abstention. The snus-exposed 5–6- year-old children showed higher systolic blood pressure, higher LF/HF ratio and stiffer arterial walls than tobacco-free controls. There was no significant difference in carotid intima media thickness between snus-exposed children and controls.

In conclusion, several long-lasting associations with prenatal snus exposure were discovered, indicating a prenatal programming of the cardiovascular function. Pregnant women should be recommended to abstain from all tobacco- and nicotine-containing products during the entire pregnancy.

LIST OF SCIENTIFIC PAPERS

I. **Nordenstam F**, Lundell B, Cohen G, Tessma MK, Raaschou P, Wickstrom R. Prenatal exposure to snus alters heart rate variability in the infant. *Nicotine Tob Res*. 2017;19(7):797-803.

II. **Nordenstam F**, Lundell B, Edstedt Bonamy AK, Raaschou P, Wickstrom R. Snus users had high levels of nicotine, cotinine and 3-hydroxycotinine in their breastmilk, and the clearance was slower than in smoking mothers. *Acta Paediatr*. 2018.

III. **Nordenstam F**, Norman M, Wickstrom R. Blood pressure and heart rate variability in preschool children exposed to smokeless tobacco in fetal life Submitted for publication 2019.

IV. **Nordenstam F**, Norman M, Caidahl K, Wickstrom R. Arterial stiffness and carotid intima media thickness in children exposed to smokeless tobacco in fetal life. Manuscript 2019.

CONTENTS

1 Introduction 1
2 Background 2
2.1 History of tobacco 2
2.2 Prevalence of tobacco use during pregnancy 3
2.2.1 Smoking during pregnancy 3
2.2.2 Smokeless tobacco including snus during pregnancy 4
2.2.3 E-cigarettes 4
2.3 Snus – oral moist snuff 5
2.3.1 Constituents of Swedish snus 5
2.3.2 The history of snus 5
2.3.3 Snus prevalence 6
2.3.4 "The Swedish experience" 7
2.4 Prenatal snus exposure and perinatal outcome 9
2.5 Sudden infant death Syndrome 11
2.6 Long-term effects of maternal tobacco use 12
2.7 Nicotine 13
2.7.1 Nicotine pharmacokinetics and metabolism 13
2.7.2 Nicotinic effects 15
2.7.3 Effects of nicotine during fetal development 16
2.8 Autonomic nervous system and cardiovascular regulation 17
2.8.1 ANS and cardiovascular regulation 17
2.8.2 Indices of autonomic nervous system activity and heart rate variability 19
2.8.3 Prenatal programming and DOHaD 21
3 Aims 22
4 Methodological considerations 23
4.1 Ethical considerations 23
4.2 Study design and setting 23
4.2.1 Women and infants included in the HRV and breastmilk studies 24
4.2.2 Preschool children included in the HRV, BP and carotid artery studies 26
4.3 Cohort size and power 27
4.4 Tobacco use self-reported through questionnaires 27
4.5 Heart rate variability (HRV) 28
4.6 Oscillometric blood pressure 29
4.7 Vascular assessment with B-mode ultrasound 29
4.8 Statistical methods 31
5 Results and Discussion 33
5.1 Main findings 33
5.2 Tobacco alters autonomic cardiac regulation with decreased parasympathetic activity 33

5.2.1 Even early prenatal exposure matters – there are no safe periods..... 35
5.3 High levels of nicotine and metabolites are found in breastmilk from snus- using mothers 37
5.4 A higher systolic blood pressure is seen following prenatal snus exposure..... 38
5.5 Stiffer arterial walls are also seen following snus exposure 39
5.6 Interactions between the autonomic nervous system and Blood pressure and arterial wall stiffness 41
5.7 Boys may be more vulnerable to snus exposure 43
5.8 Future aspects..... 45
5.9 clinical considerations and prevention 46
5.10 Conclusion 47
6 Populärvetenskaplig sammanfattning 48
7 Acknowledgements 51
8 References..... 53

LIST OF ABBREVIATIONS

ANS	Autonomic nervous system
BMI	Body mass index
BP	Blood pressure
cIMT	Carotid intima media thickness
CNS	Central nervous system
DBP	Diastolic blood pressure
DOHaD	Developmental Origin of Health and Disease
ECG	Electrocardiogram
EU	European Union
HRV	Heart rate variability
LF/HF	Low frequency/high frequency ratio
MBR	Medical birth register
nAChR	Nicotinic acetylcholine receptor
NO	Nitric oxide
NRT	Nicotine replacement therapy
PAH	Polycyclic aromatic hydrocarbons
PNS	Parasympathetic nervous system
RAAS	Renin-angiotensin-aldosterone system
RSA	Respiratory sinus arrhythmia
SBP	Systolic blood pressure
SIDS	Sudden infant death syndrome
SNS	Sympathetic nervous system
SUDI	Sudden unexpected death of infancy
TPR	Total peripheral resistance
TSNA	Tobacco specific nitrosamines

1 INTRODUCTION

Pregnancy is an overwhelming and emotional time. Exciting and yet a time full of anxiety and worries. There are recommendations for all kinds of things: restrictions of food, medications and drugs. At best, the recommendations are explicit, scientific and easy to follow, but unfortunately this is not always the case. How do you, as a snus-using mother, know what to do? How do you as a professional health worker, know how to advise a mother with a tobacco habit?

When we started to plan this study snus was gaining in popularity and was promoted as a means for smoke cessation and harm reduction. Snus is a moist tobacco product for oral use, without any of the combustion toxins found in cigarette smoke, although it contains high doses of nicotine. Today, snus and other nicotine containing products are considered to be less harmful alternatives to smoking and a way out of a smoking habit. When discussing harm reduction by snus, the complex situation of being pregnant and the need to care for the baby's health have been neglected. For a pregnant woman, snus is probably a better alternative than smoking, but is that also true for her baby?

Cigarette smoking is a modifiable and independent risk factor for cardiovascular disease and, during pregnancy, one of the most important preventable risk factors for adverse birth outcomes. Although cigarette smoking during pregnancy is decreasing globally and is now at around 1.7%, there are substantial regional differences (1). In the United States and European countries, the prevalence of smoking during pregnancy ranges from 5–38%, with Sweden at the lower end of spectrum (1). The Swedish tobacco tradition includes snus; although mostly used by men, snus is gaining in popularity among women of a childbearing age. The prevalence of snus use in pregnancy is around 1.3%, with as many as 4.7% of women reporting snus use 3 months before pregnancy (2). Among the thousands of different toxins found in cigarette smoke, nicotine is regarded as the main culprit of the adverse effects seen in pregnancy. Thus, the safety of using nicotine products and even nicotine replacement therapy (NRT) in pregnancy is highly debatable and randomized controlled studies have not provided any clear answers, due mainly to lack of compliance and problems with study design (3, 4).

By studying snus use during pregnancy, we could differentiate between the prenatal effects caused by nicotine and those caused by the combustion products in cigarette smoke. Furthermore, and more importantly, we avoided the confounding factor of exposure to second hand smoke during childhood. The interpretation of the results from studies of long-term effects of maternal smoking have been complicated by exposure to second hand smoke, leading to difficulties in separating prenatal effects from postnatal, as well as acute effects from chronic ones.

The rationale for the four studies in this thesis was to explore prenatal and perinatal exposure to snus as regards short- and long-term association with cardiovascular function in the offspring.

2 BACKGROUND

2.1 HISTORY OF TOBACCO

The tobacco plant *Nicotiana tabacum*, native to Peru and Ecuador, has been cultivated since 5000 B.C. The practice of smoking tobacco appears to have arisen from snuffing, i.e., inhaling through the nose, and the most ancient tobacco-related artifacts found are snuffing devices. The first Europeans smoking tobacco were the crew on Christopher Columbus' ship in 1492 after reaching Cuba. However, tobacco was not just smoked or snuffed, it was chewed, drunk, eaten, smeared over the body and put in the eyes. It was used to keep fleas and bugs away, for medical reasons and for religious and social purposes. It was cultivated and spread all over America and Europe. Royalty snuffed tobacco for the perceived medical effects; ironically, it was even believed to cure and prevent cancer (5).

Manufactured cigarettes were first marketed in England in 1850 and during the First World War the habit spread dramatically; use doubled in the United States during the first half of the 20th Century. The first epidemiological reports about the association of lung cancer and smoking were published in 1950 (6), but it was not until 1964, when the Surgeon General's report on "Smoking and Health" described the hazardous effects of smoking, that these became common knowledge. However, the report had little impact on smoking rates (7).

Picture 1. Dried tobacco leaves*. Source: Swedish Match.*

The estimated prevalence of smoking has decreased in the last 30 years and there has been a shift in the main locations of use, from high-income countries to developing countries, where the smoking prevalence remains high. The difference in prevalence between men and women is narrowing, with an increase in smoking among young women (8). Around 80% of the 1.1 billion smokers in the world live in low- or middle-income countries (9). Out of the 7 million

tobacco-related deaths per year, 890,000 are deaths because of exposure to second hand smoke and 28% of these are children (9).

2.2 PREVALENCE OF TOBACCO USE DURING PREGNANCY

2.2.1 Smoking during pregnancy

In a systematic review and meta-analysis of 295 reports published in 1985–2016, the estimated global prevalence of smoking during pregnancy was low, around 1.7% (1). However, there were large regional differences, with the highest figures of estimated prevalence in Ireland (38%), Uruguay and Bulgaria (29%) (1), see Figure 1.

The proportion of women who were daily smokers and continued smoking during pregnancy was 53% globally, ranging from 30% in European regions (including Russia) to 79% in the Western Pacific region (including China and Australia) (1). These numbers highlight that although the prevalence is decreasing overall, there are still countries where smoking during pregnancy is prevalent.

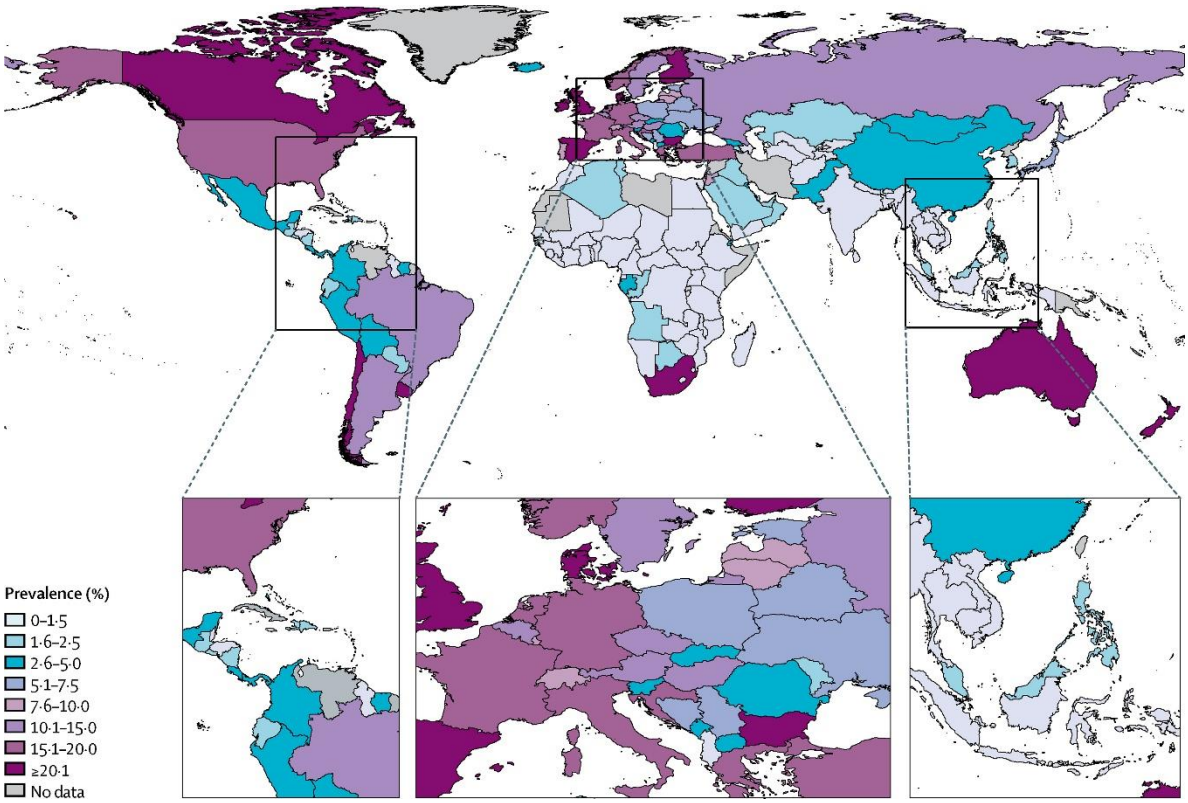


Figure 1. National prevalence of smoking during pregnancy*. Lange et al. Regional, national, and global prevalence of smoking during pregnancy in the general population: A systematic review and meta-analysis. Lancet 2018. Open publish under a creative common license. https://creativecommons.org/licenses/by-nc-nd/4.0/ and https://doi.org/10.1016/S2214-109X(18)30223-7.*

2.2.2 Smokeless tobacco including snus during pregnancy

Smokeless tobacco is prevalent and gaining popularity; it is often less expensive than cigarettes and considered safer. In many low- and middle-income countries, it is part of a long cultural tradition and used by both women and men. An estimated 300 million people use smokeless tobacco in different forms, most frequently in India and Bangladesh, but also for example among the Inuits in Canada and Alaska. The tobacco can be chewed, sucked, dissolved in the mouth, applied to the gums as a paste or ground into the gums. In Scandinavia and the United States, the most common type is an oral moist snuff (snus) which is put under the lip. There is also dry powder snuff that can be inhaled nasally. To avoid misunderstanding the word snus is used in this thesis when referring to the Swedish type of oral moist snuff described in detail below.

The worldwide prevalence of smokeless tobacco use among pregnant women is not known, regional studies show a wide range: 33% in Orissa, India, 17% in Mumbai, India, 14% in Alaska, and 7% in Soweto, South Africa (10, 11). Smokeless tobacco is rare in high income countries, except Scandinavia where the use of snus is increasing, especially among women in Sweden and Norway. Although the highest prevalence of 18–20% is seen among Swedish men, the Swedish National Board of Health reported in 2017 that the use among women had almost doubled in ten years and 4.7% of women reported snus use 3 months before pregnancy. The estimated prevalence of snus during pregnancy is 1.3% in Sweden, 3.4% in Norway and < 1% in the United States (12, 13).

2.2.3 E-cigarettes

E-cigarettes and water pipes (hookahs) are gaining in popularity especially among young people. Other forms of nicotine products are evolving on the market, like tobacco-free sachets, nasal sprays, lozenges, gums and patches and the line between replacement products and new addictive products is far from obvious. Cigarette smokers may find these products helpful as harm reduction but there is a substantial influx of new users to these nicotine products and the tobacco industry is enthusiastic in finding new products and new customers (14). In e-cigarettes, a fluid containing flavors and the preferred amount of nicotine is electronically heated and the vapor is inhaled. High school students in Sweden have reported occasional use of e-cigarettes (vaping) in 45% of males and 37% of females (15) and reports from the United States show an increased risk among adolescents to move from e-cigarettes to traditional tobacco cigarettes (16). A study from the United States published in 2017 showed that at least as many pregnant women used e-cigarettes as tobacco cigarettes and the women viewed e-cigarettes as safer than tobacco cigarettes (17).

2.3 SNUS – ORAL MOIST SNUFF

2.3.1 Constituents of Swedish snus

Snus is a moist tobacco product for oral use, packaged in small pouches or in a loose powder to put under the lip. All the women in the studies included in the thesis used snus pouches. Swedish snus contains pulverized tobacco, water, sodium carbonate, sodium chloride, moisturizer and flavoring(18). Compared with American snuff and other smokeless tobacco, Swedish snus contains significantly lower levels of toxic substances such as tobacco-specific nitrosamines (TSNA), polycyclic aldehydes (PAH) and heavy metals (18). The major snus company in Sweden (Swedish Match) has its own quality controller, Gothiatek, which reviews the manufacturing, packaging and storage. Gothiatek is responsible for checking that levels of nitrate, TSNA, PAH, aflatoxins and heavy metals are below the recommendations of the Swedish National Food Agency.

Picture 2. Swedish snus in pouches*. Photo: Niclas Grunewald*

Swedish snus delivers low doses of harmful chemicals and delivers high doses of nicotine. The amount of nicotine in snus varies from 3 to -12 grams per pouch, but the perceived strength depends on other factors, such as the acidity and humidity of the snus. Cigarette smoke contains around 4,000 different agents, many of which are toxic, including nicotine, carbon monoxide and TSNA. By burning the tobacco, carcinogenic agents like PAH and formaldehydes are created. The tobacco in cigarettes is acidic and must be inhaled into the lungs to be absorbed, while the less acidic tobacco in pipes and cigars can be absorbed more easily by the oral mucosa.

2.3.2 The history of snus

Swedes have been growing tobacco since the 17th Century and it was initially mostly used by the in high society. During the 18th Century, tobacco was cultivated in more than 70 Swedish towns. When dry snuff was replaced by moist snus, it gained popularity also among ordinary people and workers and the high society started to smoke cigars instead. During 1915–1961, the snus industry was subject to a monopoly in Sweden and the income was initially used for

military needs. After World War I, snus consumption reached an all-time high with a yearly consumption of 1.2 kg per person, after which snus use declined in favor of cigarettes. In 1970, snus began to be sold in portions (small pouches) as a less messy alternative to loose snus and slowly gained in popularity again. Since 1992, the European Union (EU) has banned snus and when Sweden joined the EU in 1995, a national exception was made. In 2002, the snus ban was legally challenged, unsuccessfully, in the European Court of Justice.

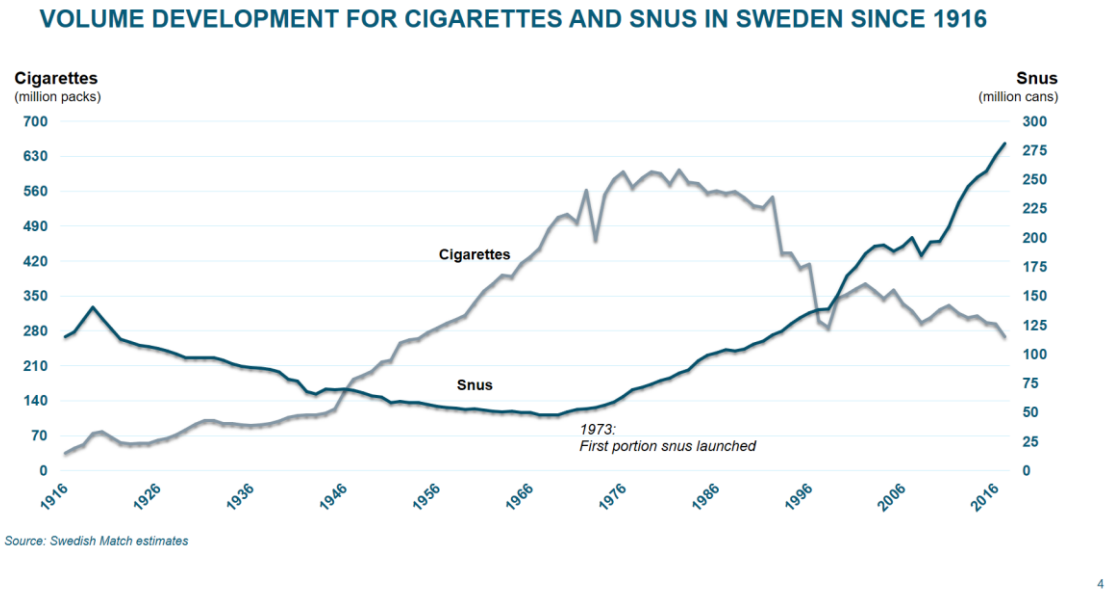


Figure 2. Volumes of cigarettes and snus sold in Sweden 1916–2016*. Published with permission from Swedish Match.*

2.3.3 Snus prevalence

The use of cigarettes has decreased in Sweden in the last 30 years, while snus use has increased. Consequently, the total tobacco use in Sweden is at the same high level as in many other European countries, around 20% (19).

Snus use among women has increased in Sweden and, in 2016, 4.7% of women reported using snus 12 weeks before pregnancy, an increase from 2.4% in 2007, according to the Medical Birth Registry (MBR) (2). There are large regional differences in Sweden, with the counties of Norrbotten and Västerbotten reporting 17% of women using snus 12 weeks before pregnancy. The age group reporting the highest snus use was women 25-29 years of age. The reported number of women using snus during pregnancy is around 1.3%, again with large regional differences. For example, in the small town of Lycksele (8,000 citizens) as many as 23% of the women used snus during pregnancy (2).

The information in MBR is based on self-reports, with prospectively collected data minimizing the risk of recall bias. However, given the increasing knowledge of detrimental effects of tobacco use during pregnancy there is a risk of underreporting. Gunnerbeck et al. showed in the original cohort study, named the SNUS study, that self-reporting was valid, but that many of the snus users were misclassified as non-users in the MBR, especially in late pregnancy (20). MBR has collected data about snus use during pregnancy since 2000, but there is no information about snus dose and the question about tobacco use could be misinterpreted to refer to cigarette use. In addition, there are regional differences in the reporting of tobacco, with a high percentage of non-response in some counties. A study from the United States concluded that health care staff neglected to screen for smokeless tobacco and other nicotine containing-products and that the knowledge of adverse pregnancy outcomes was limited (21).

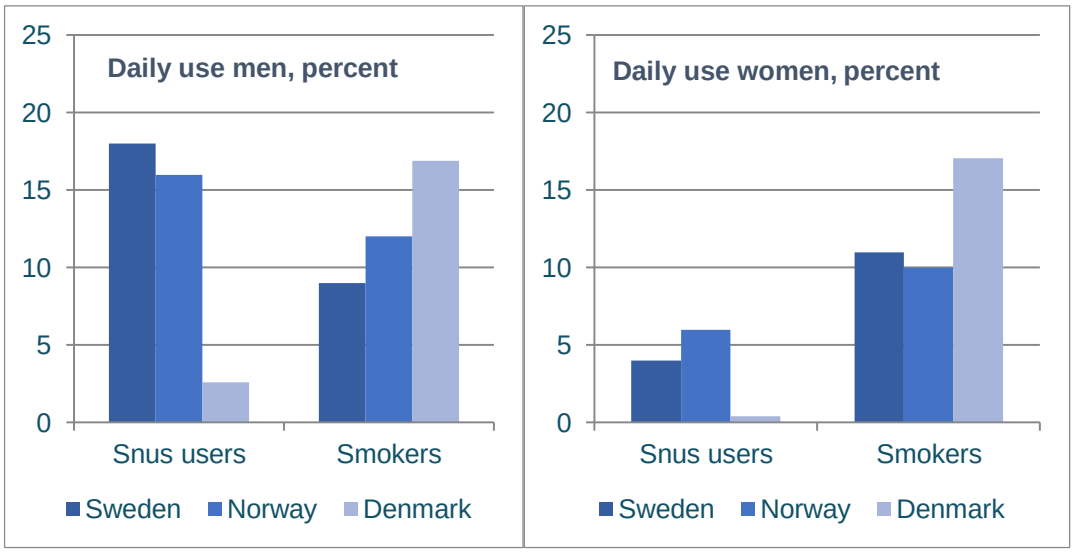


Figure 3. Prevalence snus use and smoking in Scandinavia in 2017-2018*. More women than men smoke in Sweden. Sources: Public Health Agency of Sweden (Folkhälsomyndigheten), Statistics Norway (Statistisk sentralbyrå ,SSB), Danish Health Authority (Sundhetsstyrelsen) adapted by Swedish Match.*

2.3.4 "The Swedish experience"

The tobacco-related mortality is lower in Sweden than in other European countries, although the total tobacco consumption is similar. This phenomena has been called the "Swedish Experience". This is claimed to reflect that "Swedes don´t smoke as much as others because they use snus" and is used to promote snus as an effective method of harm reduction (19), see Figure 4. The discussion is ongoing among politicians, public health experts and researchers, and there is no easy answer as to whether snus is an effective way out of a smoking addiction. Snus is an addictive tobacco product with suggested associations to some forms of cancer, although the overall risk of cancer is lower than for smoking cigarettes (22,

23). There is no evidence that snus is associated with increased risk of hypertension, myocardial infarction or stroke (24). However, the outcome after myocardial infarction is worse when snus use is continued afterwards, due to increased risk of arrhythmias (25). There are studies describing an association between snus use and metabolic changes such as insulin resistance and increased risk of diabetes type 2 (26), although the latter finding is challenged by other reports (27, 28).

Other forms of smokeless tobacco (SLT) have been associated with an increased risk of cancer in the airways and upper digestive canal and also an increased risk of stroke (29). A large review of worldwide studies of deaths caused by SLT report that South East Asia is a region with a substantial risk of death caused by SLT, due to cancer, ischemic heart disease and stroke (30). The regional differences in risk may be explained by various types of SLT used, with variations in content and route of absorption (30).

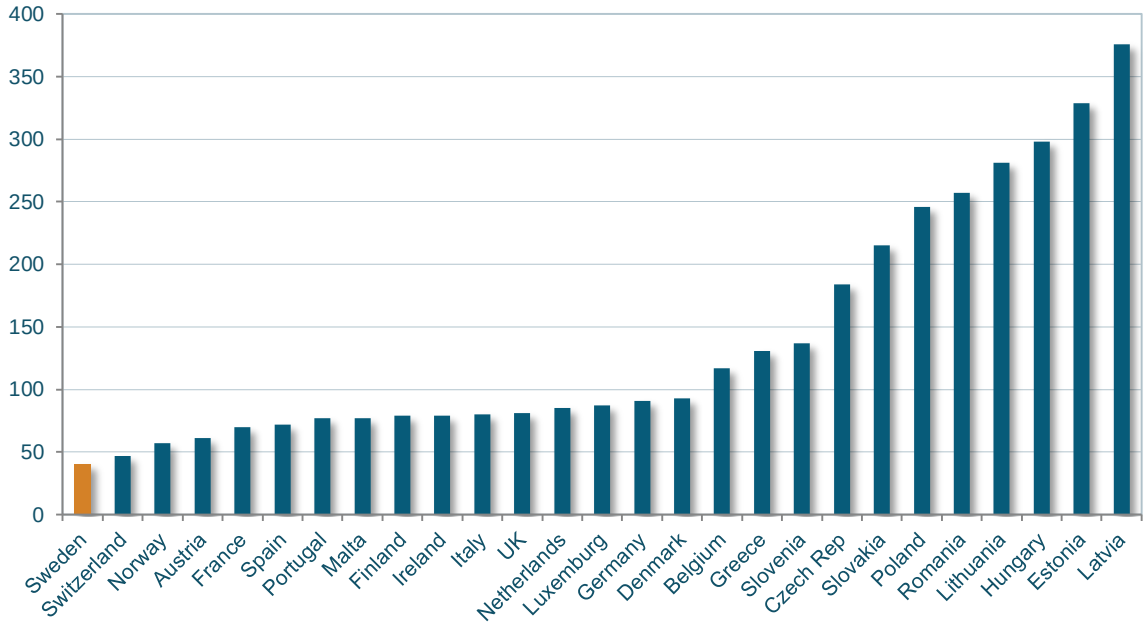


Figure 4. The proportions of cardiovascular mortality attributable to tobacco in European males in death rates /100 000 men. *Source: Swedish Match. Adapted from World Health Organization (WHO): Mortality Attributable to tobacco, 2012 (19).*

There is little direct evidence of Swedish snus or plain nicotine use in healthy adults contributing to poorer health (31, 32). However, the evidence from animal research and in vitro settings is very strong of nicotinic volatile actions and supports the notion of nicotine as an addictive toxin leading to an increased risk of cardiovascular disease as well as cancer (33, 34).

The risks associated with snus use during pregnancy are discussed below.

2.4 PRENATAL SNUS EXPOSURE AND PERINATAL OUTCOME

Maternal smoking during pregnancy is associated with increased risk of spontaneous abortion, ectopic pregnancies, placenta previa, placental abruption and placental dysfunction (35). The placental problems are partially responsible for the increased risk of stillbirth and preterm birth seen in smokers. The increased risk of preterm birth in combination with growth restriction is partially responsible for the increased risk of stillbirth and neonatal death (36). Maternal snus use is also associated with preterm birth (37-39). On the other hand, snus is not associated with placenta previa or abruption and, importantly, the impact on fetal growth is not as strong as in maternal smoking (11, 40), see Table 1.

There is also an interesting difference in association with preeclampsia, with smoking having a protective effect and reduced risk of preeclampsia, which snus use does not (38, 41). Taken together, this implies that there are combustion products explaining the differences between smoking and snus use. Carbon monoxide is suggested to be involved in the protective mechanism for preeclampsia and is probably also involved in the placenta problems and fetal growth restriction (41, 42).

The association between cigarette smoking and the risk of malformations is not very strong, but some studies have demonstrated associations with oral cleft, clubfoot, anal atresia, gastroschisis, heart defects (atrial septal defects, pulmonary stenosis), pyloric stenosis, craniosynostosis, limb reduction defects, hernia and undescended testes (43, 44). There is only one study of the association with snus and malformations, indicating increased risk of oral cleft (45). No studies are available of possible associations between other forms of smokeless tobacco and congenital malformations in humans.

Many of the risks mentioned above are dose-dependent and if smoking or snus use ceases during early pregnancy, several of the risks are reduced or eliminated (43, 46).

Increased risk of:	**Smoking:**	**Snus:**	**References smoking:** **(43, 44, 47-52)**	**References snus:** **(37-40, 45, 46, 53)**
Stillbirth			(Marufu, Ahankari, Coleman, & Lewis, 2015; Pineles, Hsu, Park, & Samet, 2016)	(Baba, Wikstrom, Stephansson, & Cnattingius, 2014; Wikstrom, Cnattingius, & Stephansson, 2010)
Preterm birth			(Ion & Bernal, 2015; Ko et al., 2014; Wallace, Aland, Blatt, Moore, & DeFranco, 2017)	(Dahlin, Gunnerbeck, Wikstrom, Cnattingius, & Edstedt Bonamy, 2016; England et al., 2003; Wikstrom, Cnattingius, Galanti, Kieler, & Stephansson, 2010)
Small for gestational age, low birth weight			(Ko et al., 2014; Pereira, Da Mata, Figueiredo, de Andrade, & Pereira, 2017)	(Baba, Wikstrom, Stephansson, & Cnattingius, 2013; England et al., 2003)
Oral cleft			(Hackshaw, Rodeck, & Boniface, 2011; Leite, Albieri, Kjaer, & Jensen, 2014(48, 54-56))	(Gunnerbeck et al., 2014)
Neonatal death			(Dietz et al., 2010; Pineles et al., 2016)	(Baba et al., 2014)
Sudden infant death syndrome		?	(Chong, Yip, & Karlberg, 2004; Dietz et al., 2010; Haglund & Cnattingius, 1990)	

Table 1. Prenatal smoking or snus exposure and neonatal outcome. *The broad arrows indicate a strong correlation while the thin arrows indicate a weaker correlation.*

2.5 SUDDEN INFANT DEATH SYNDROME

Sudden infant death syndrome (SIDS) is defined as the sudden death of an infant under one year of age that remains unexplained after autopsy, investigation of the scene of death and review of the medical history (57). SIDS is a subcategory of sudden unexpected death of infancy (SUDI), which encompasses any sudden or unexpected death occurring during infancy, whether explained or unexplained. SUDI includes causes of death such as asphyxia, suffocation, infection, metabolic disease or ion channel disease.

The mounting of extrinsic and intrinsic risk factors during a critical developmental time period increases the risk for SIDS. Intrinsic factors suggested are male sex, prenatal smoke exposure, preterm birth and genetic polymorphism. Extrinsic factors are sleeping position, soft bedding and bed sharing. The "theory of the triple risk" for SIDS describes the complex relationship between an infant's pathological vulnerability and an unsafe sleeping environment in combination with a critical developmental period (58). After the "back to sleep" (supine sleeping) campaign in 1992, maternal smoking became the most important risk factor, with an almost threefold increased risk in babies to smoking mothers before the campaign, jumping to a fivefold risk increase afterward (59).

Since the supine sleeping campaign, the number of SIDS events has decreased. Still, it is the leading cause of death among infants from 1 month up to 1 year in the United States (around 0.49/1,000 births) and in Sweden (around 0.15/1,000 births) (60, 61).

The exact mechanism of how maternal smoking increases the risk of SIDS is not yet understood. It has been suggested that nicotine binds to endogenous nicotinic acetylcholine receptors expressed in the fetal brain from gestational weeks 4–5. When these receptors are inappropriately stimulated by nicotine, the processes of cell survival, neurite outgrowth, synapse formation and transmitter release are affected (62-64). Many theories include some common mechanisms involving serotonergic pathways in the brainstem leading to disturbances in arousal and autonomic and cardiorespiratory control (56, 65, 66). Findings in brainstems of SIDS victims include delayed maturation of synapses in respiratory centers, delayed neural maturation and decreased serotonin (5-HT) receptors consistent with abnormalities in autonomic regulation (60, 67). In addition, a reduced lung capacity following prenatal nicotine exposure resulting in chronic hypoxia has been described (60, 68). Nearly two-thirds of SIDS victims showed structural evidence of pre-existing, chronic low-grade asphyxia and biochemical markers of asphyxia at autopsy (68, 69).

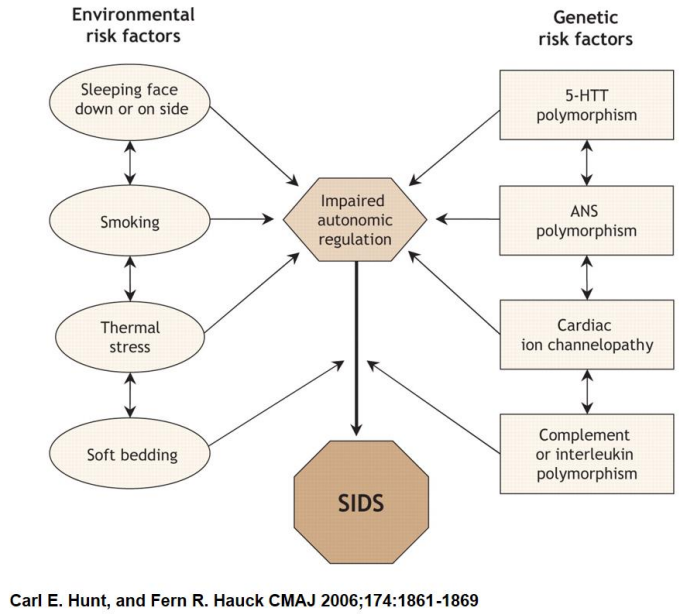


Figure 5. Schematic summary of potential interactions between environmental and genetic risk factors for SIDS. *Printed with kind permission from the authors and CMAJ. Hunt et Hauck 2006* (66).

Maternal smoking is associated with disturbed autonomic cardiac control, impaired arousal and neonatal apneas in the infant. Nicotine is suggested as the main toxin mediating these effects, this is supported by a vast number of animal studies. Nevertheless, snus has not been associated with SIDS, although a Swedish study of snus-exposed infants showed an association with apnea diagnosis (70).

2.6 LONG-TERM EFFECTS OF MATERNAL TOBACCO USE

Parental smoking and exposure to second hand smoking have a causal relationship with asthma in a child (71). Other respiratory problems such as bronchiolitis, pneumonia and coughing are also described (72, 73). Several studies suggest an association between exposure to smoking *in utero* and asthma during childhood (74, 75). However, most of these studies struggle with the confounding factor of second hand smoke exposure during childhood, as most parents who smoke during pregnancy continue to do so during the upbringing of the child.

Overweight and higher BMI (body mass index) in the offspring of smoking mothers have been reported (76, 77). Neurobehavioral problems, such as attention deficit disorder and poor school performance, are also reported following parental smoking (78, 79).

Increased thickness of the carotid intima media, an early sign of an atherosclerotic process, has been found in adolescents and young adults with a history of parental smoking (80).

Lastly, parental smoking has also been associated with acute lymphoblastic leukemia in childhood and brain tumors diagnosed before 2 years of age (81, 82). Maternal snus and its possible long-term associations have not been studied before.

2.7 NICOTINE

2.7.1 Nicotine pharmacokinetics and metabolism

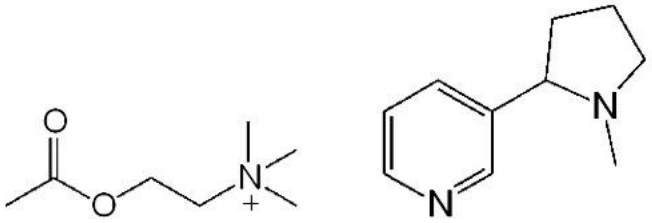


***Figure 6. Acetylcholine** (left) **and nicotine** (right).*

Nicotine occurs naturally in tobacco leaves and was used as an effective pesticide until banned by the EU in 2009. Nicotine makes up about 1.5–3% of dry tobacco. An average cigarette contains 10–14 mg nicotine and around 1–2 mg is absorbed when smoking depending on the puffing of the smoker (83). Nicotine is a weak base with a pH of around 8 and its absorption through biological membranes is pH-dependent. The smoke from a cigarette is acidic and nicotine is ionized and not easily absorbed by oral mucosa. The smoke from pipes and cigars is more alkaline and therefore more easily absorbed in the mouth. When cigarette smoke reaches the alveoli in the lungs it is absorbed and reaches the circulation quickly. After a puff from a cigarette nicotine reaches the brain in 10–20 seconds, faster than via an intravenous injection. The nicotine concentration in the blood increases rapidly and reaches a peak within ten minutes. The nicotine concentration can be manipulated by the smoker on a puff-to-puff basis and that is why a smoker can extract more or less nicotine from the same nicotine-containing cigarette (84).

A pouch of snus contains 4–12 mg of nicotine and the absorbed nicotine dose depends on the pH, the amount of moisture, the time in contact with mucosa and how the pouch is moved around in the mouth. Estimated extraction of the nicotine content ranges between 30 and 60% (85). The absorption from oral moist tobacco is slower than from cigarettes; the peak occurs within 30 minutes, but the serum concentration remains at a high level for longer (86), see Figure 7. Absorption of nicotine from snus occurs mainly through the oral mucosa; nicotine swallowed is poorly absorbed in the stomach.

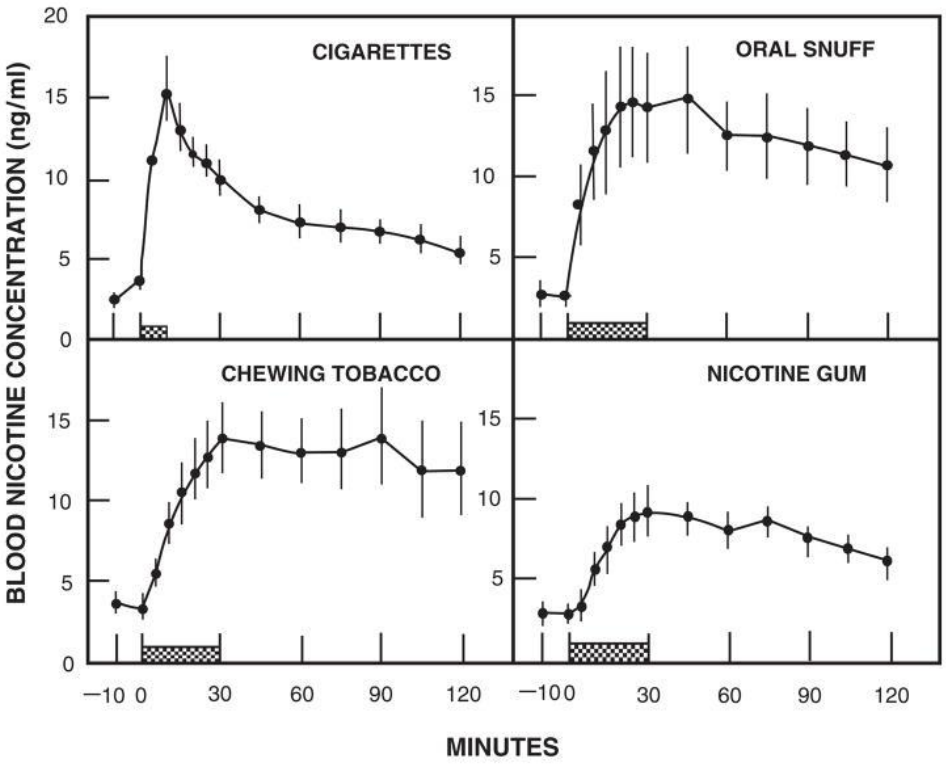


Figure 7. Blood nicotine concentrations during and after cigarette smoking for 9 minutes, oral snuff 2.5 g, chewing tobacco 7.9 g, and nicotine gum 4 mg. *Reprinted from Benowitz et al. 1988. With kind permission from the author and American Society for Clinical Pharmacology and Therapeutics.*

Nicotine is easily absorbed through the skin, which is a well-known problem in tobacco harvesting. Green tobacco sickness occurs when in contact with the moist tobacco leaves, with symptoms of nicotine poisoning, such as nausea, vomiting and headaches. The transdermal absorption mechanism is used for nicotine patches and although the absorption is high, it takes one hour before nicotine is found in blood and there is no peak. This slow administration without a peak allows the central nervous system (CNS) to adapt and the risk of addiction is nil (87).

Nicotine is metabolized in the liver by the cytochrome P450 system. There are findings suggesting a potential metabolism of nicotine and cotinine in other tissues than the liver, for example the brain and fetal lungs, but their contribution to the metabolism is considered small. The most important metabolite is cotinine, almost 80% of nicotine is metabolized to cotinine in humans and approximately 10% of the nicotine is excreted in the urine. Cotinine is also metabolized in the liver by the CYP2A6 enzyme, with trans-3-hydroxycotinine (OH-cot) being the major cotinine metabolite found in urine. The ratio between OH-cot and cotinine is used as an estimate of the metabolic rate of the CYP2A6 enzyme and nicotine metabolism (84). The large differences found in nicotine metabolism both intra- and inter-individually are explained by genetic factors, race, gender, age, pregnancy and additives like menthol flavoring. The half-life of nicotine is around 2 hours, whereas it is 16 hours for

cotinine. The long half-life of cotinine has made it commonly used as a biomarker for estimating nicotine exposure (84).

Women have a higher metabolism of both nicotine and cotinine than men and the metabolism is even higher during pregnancy (88). A higher metabolic rate increases the need for more nicotine, leading to a greater challenge for a woman to quit nicotine during pregnancy. Another aspect of the pregnancy-induced metabolism is that lower levels of biomarkers (nicotine and metabolites) in urine or serum does not necessarily mean lower consumption in pregnancy (88). A higher rate of metabolism is also seen in women using oral contraceptives than in non-pregnant women not taking hormones (89).

The metabolism of nicotine in neonates is slower, with a half-life 3–4 times longer than in adults. Interestingly, the half-life of cotinine seems to be similar in neonates and adults. This discrepancy in metabolism is suggested to be a consequence of different enzymatic activity or tissue distribution (90).

Nicotine easily passes through the placenta into fetal circulation, where around 20–30% of the blood is shunted via the ductus venosus bypassing the liver and directed to the brain via fetal shunts (91). Nicotine is suggested to accumulate in amniotic fluid. Luck et al found 50% higher concentrations of nicotine in amniotic fluid compared with in maternal serum, but the extent of absorption through the skin of the fetus is unknown (92). The exact pathways of absorption and metabolism in the fetus are thus not well-described. Animal models may not be optimal for testing nicotine metabolism during pregnancy as their metabolism may differ from humans (93). Serum concentrations in fetal cord blood were 15% higher than maternal concentrations measured in the third trimester (92). In conclusion, the fetus is exposed to equal or higher levels of nicotine as compared with the mother.

Nicotine in serum and breastmilk reaches a steady state within minutes. Nicotine is also accumulated in breastmilk with a ratio of 2.9, while cotinine is found in breastmilk with a ratio of 0.8 (94). The nicotine-containing breastmilk is swallowed by the infant; the absorption in the stomach is presumably low, but data is insufficient. The urinary cotinine levels found in breastfeeding infants of smoking mothers were 10 times higher than levels from bottle-fed infants of smoking mothers (95). According to Luck et al. the half-life of nicotine in breastmilk was almost 100 minutes, while the half-life in serum was 80 minutes and cotinine remained at the same levels in breastmilk over the course of 4 hours (94).

2.7.2 Nicotinic effects

The acute physiological effects of nicotine include cardiovascular responses with an increase in heart rate, cardiac contractility, respiratory rate, muscular blood flow and blood pressure. The systemic and cutaneous vessels contract and urine production diminishes. The platelets are activated and the immune system is suppressed. The effects on the CNS involve enhanced concentration and alertness and a suppression of appetite (86). These effects are described by snus users as a “pleasant feeling of being relaxed and more alert at the same time.”

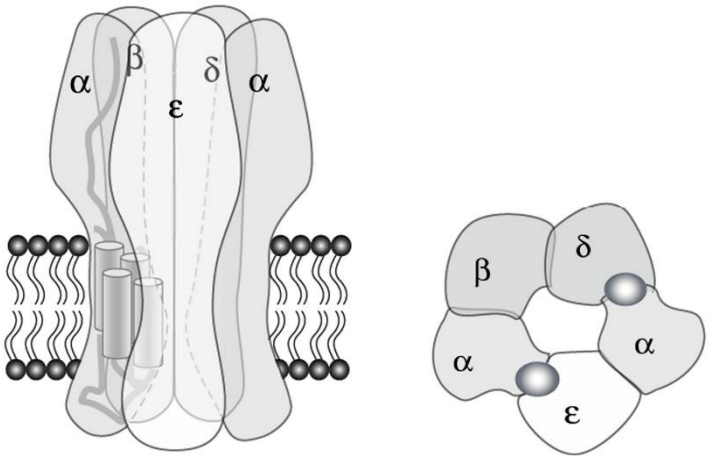


Figure 8. Nicotinic acetylcholine receptor with nicotine and acetylcholine binding sites marked on the α-subunits. *Adapted from Hurst et al. 2012, reprinted with kind permission from Elsevier.*

Nicotine binds to endogenous nicotinic acetylcholine receptors (nAChR) present in the brain, autonomic nervous system and neuromuscular junctions, and also in non-neural tissues such as endothelial cells, airway epithelial cells, inflammatory cells and keratinocytes (96). The receptor is composed of five subunits, which may vary but include at least two α-subunits on which the nicotine binding site is located. Nicotine binds to the outside of the receptor and opens the central canal, mediating an influx of sodium and calcium and an efflux of potassium (97).

There is also a ligand-gated receptor in non-neural cells with signaling through phosphorylation. The effects of these different types of nAChRs vary, where the cholinergic system in the CNS involves cognitive function, memory, attention and emotional processing. In the autonomic nervous system nAChRs are found in the brainstem and preganglionic connections involved in regulation of respiration, heart rate and contractility, and blood pressure. The non-neural cholinergic system is critical in controlling cell proliferation, differentiation, migration and apoptosis (98).

2.7.3 Effects of nicotine during fetal development

The varying and complex actions of nicotine are even more intriguing and somewhat different during vulnerable developmental periods such as fetal life (98). The nAChRs are present in the brain of fetus as early as gestational week 4-5 (63). Acetylcholine is important for the maturation of the brain and nicotine acts as a potent neuro teratogen when interacting with the trophic function of the cholinergic systems (99). Nicotine binding to nAChRs affects other signaling systems as well, such as the serotonergic and dopaminergic systems (96).

Neural cell replication and differentiation, apoptosis and migration are all important developmental steps in the formation of the nervous system and they may all be altered by nicotine exposure with long-term consequences (99, 100). Prenatal nicotine exposure is also suggested to affect the cardiovascular system, lung volume and size, endocrine and immunological system and cause epigenetic changes (98).

The full extent and precise mechanisms of prenatal nicotine exposure are not fully understood, although animal research and studies of children of smoking mothers have provided substantial insights. The timing and the dose may have importance and the effects are further complicated by adjustments with an initial upregulation with more nAChRs that eventually leads to a functional downregulation (101, 102). Not only nicotine may be of importance in prenatal exposure; the metabolite cotinine can also bind to nAChRs, with slightly different effects from nicotine. The significance of cotinine binding is not known (103).

2.8 AUTONOMIC NERVOUS SYSTEM AND CARDIOVASCULAR REGULATION

The autonomic nervous system (ANS) influences the function of almost all tissues in the body, by innervation of smooth muscle cells, cardiac muscle and pacemaker cells, exocrine and endocrine glands, lymphatic tissue, liver cells and white and brown fat. Without any conscious effort required, it regulates many physiological processes, including blood flow, blood pressure, heart rate, airway resistance, body temperature, metabolism, fluid balance, immune system, inflammatory processes, sexual function and pupil diameter (104, 105).

The two anatomically and functionally different branches of the ANS, the parasympathetic and the sympathetic nervous systems, can function antagonistically, synergistically or independently. We often think of the sympathetic nervous system (SNS) as a “fight and flight” reaction but actually there is ongoing sympathetic activity even in resting conditions. Similarly, the parasympathetic nervous system (PNS) is not only active in “rest and digest” situations.

2.8.1 ANS and cardiovascular regulation

In the heart, the SNS and PNS have mainly counteracting effects, controlling heart rate, AV node conduction and contractility. The effects of the SNS are mediated via adrenergic receptors. The dominant β_1 adrenergic receptors are expressed in the sinoatrial node (SA node), AV node and on atrial and ventricular cardiomyocytes. The activation of β_1 receptors increases heart rate, atrial and ventricular contractility and AV node conduction velocity. In addition, activation of this receptor also induces renin release by the kidneys. β_2 adrenergic receptors are mainly expressed in vascular smooth muscle, skeletal muscle and in the coronary circulation and elicit vasodilatation with increased blood flow to the liver, heart and

muscles. These non-innervated receptors are stimulated by circulating epinephrine. The adrenergic α_1 and α_2 receptors are expressed in vascular smooth muscle and elicit vasoconstriction (104, 105).

The main neurotransmitter for the PNS is acetylcholine in both preganglionic and postganglionic neurons. Acetylcholine is excitatory, mediating its effects via nAChRs in ganglion synapses and in skeletal muscle cells. In addition to the nAChRs there are two kinds of muscarinic AChRs in the cardiovascular system. The M_2 type is expressed in nodal and atrial tissue and also in the ventricles. Acetylcholinic activation of these receptors decreases heart rate and reduces AV node velocity and may also reduce contractility in the atria. The M_3 receptor is mainly expressed in the vascular endothelium and when activated stimulates nitric oxide (NO) production, resulting in vasodilatation.

Cardiovascular function is also influenced by numerous endocrine hormones. The adrenal gland releases norepinephrine, epinephrine and dopamine in response to sympathetic activation. The renin-angiotensin-aldosterone system (RAAS) is important for blood pressure control and renin is released by the kidney after sympathetic stimuli and activates the RAAS.

Antidiuretic hormone is released by the posterior pituitary gland and stimulates water retention by the kidneys. There are also hormones released by the heart: atrial natriuretic peptide (ANP) from the atria and brain natriuretic peptide (BNP) from the ventricles, which both respond to increased stretch of the heart and inhibit catecholamine (104, 105).

The most important reflex involved in autonomic cardiovascular function is the baroreceptor loop. Baroreceptors in the aortic arch and carotid bodies respond to stretching and the afferent signal is conducted to the CNS and vital centers in the medulla oblongata that interact with the endocrine response, respiratory control and arousal. In a simplified description (see Figure 9), a fall in blood pressure is sensed by the baroreceptors and the signal is connected to efferent sympathetic fibers in the medulla, stimulating the heart to raise the heart rate and contractility to increase cardiac output. At the same time, the SNS stimulates the smooth muscle cells in vessels to contract and increase resistance, with additional help from catecholamines released from the adrenal medulla and stimulation of the RAAS.

All these actions lead to an immediate response with increased blood pressure. This system works the other way as well, if blood pressure increases, the PNS will decrease the cardiac output. These systems work together in fine tuning of blood pressure homeostasis in daily life. In hypertension, the baroreceptors will adapt to the new situation and the baroreceptor loop will only react to deviations from the hypertensive situation (104, 105). For a further discussion of the ANS and blood pressure, see Results and Discussion.

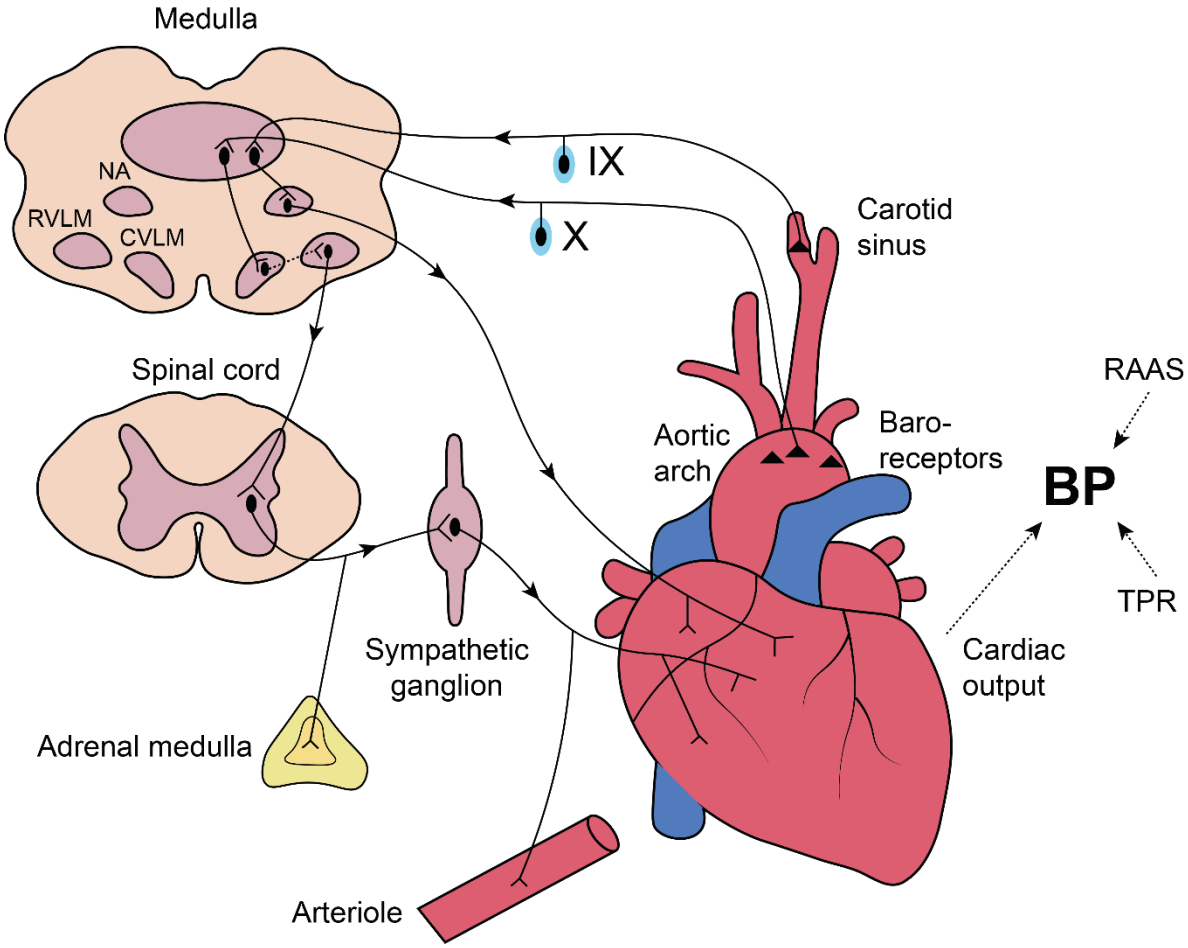
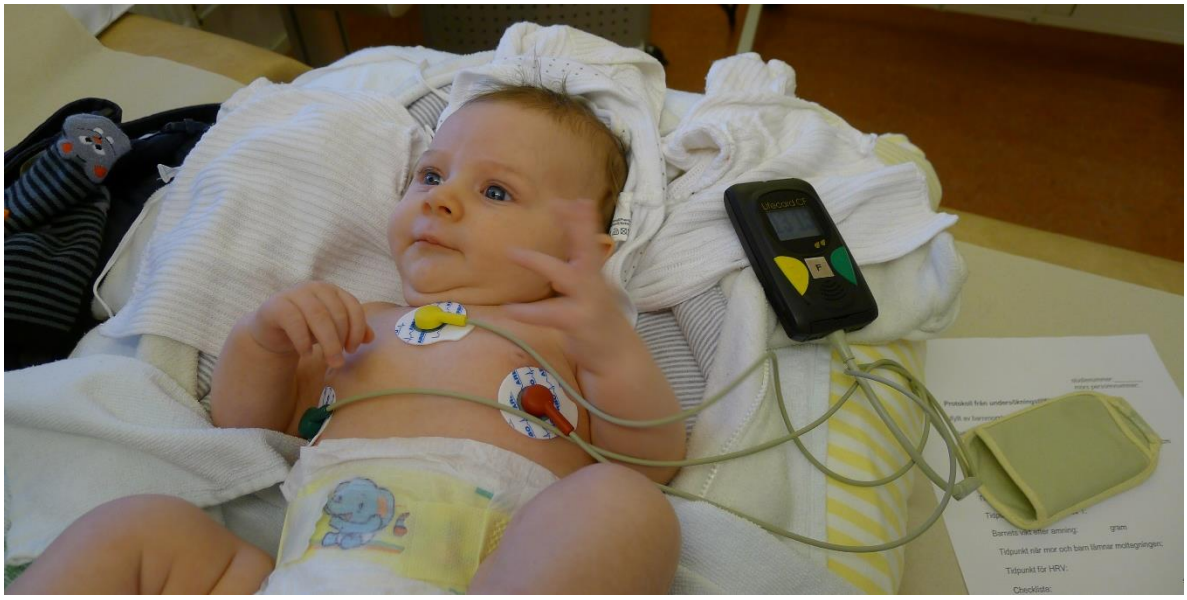


Figure 9. Schematic picture of the baroreceptor loop. *The baroreceptor signal from the aorta and carotid sinus is conducted via vagal (X) and glossopharyngeal (IX) nerves and coupled with centers in the medulla (NA: nucleus ambiguous. RVLM, CVLM: rostral and caudal ventrolateral medulla). Parasympathetic and sympathetic nerve input to the heart increases or decreases cardiac output and interacts with the renin-angiotensin-aldosterone system (RAAS) and total peripheral resistance (TPR) to modify blood pressure (BP). Picture modified from Kaur et al 2016 (106).*

2.8.2 Indices of autonomic nervous system activity and heart rate variability

Some of the commonly used assessment methods of ANS function are based on triggering of the cardiovascular reflexes through altering blood pressure and baroreflex responses. These interventions include the Valsalva maneuver, carotid massage and tilt-test and mainly assess the sympathetic response. A noninvasive approach that provides information on both the PNS and the SNS is spectral analysis of beat-to-beat fluctuations in heart rate, which can be used in both healthy and sick individuals of all ages.



Picture 3. ECG (electrocardiogram) recording in a participant (non-tobacco-exposed control) from Study 1. *The ECG recording was used for a HRV analysis. Published with kind permission from the parents. Photo: Felicia Nordenstam*

The heart rate is regulated by the PNS in resting conditions on a beat-to-beat basis; the signaling from the PNS acts directly on the next heartbeat and there are constant small fluctuations in the pace between every heartbeat. These small fluctuations appear at different wavelengths and are used in heart rate variability (HRV) analysis, where the most well-known fluctuations are connected to breathing and called respiratory sinus arrhythmia (RSA). The RSA appears within the high frequency (HF) range and changes in the HF spectrum band are considered to reflect changes of parasympathetic activity. The activity in the low frequency (LF) band is suggested to reflect a combination of sympathetic and parasympathetic activity on blood pressure control, body temperature, circadian rhythms and the renin-angiotensin-aldosterone system.

The ratio of LF/HF is often used as an estimate of the balance between the sympathetic and parasympathetic system. This interpretation needs to be used carefully and combined with information on the activity in the LF and HF bands, respectively. A high LF/HF ratio can be indicative of either a high sympathetic activity, a low parasympathetic activity or a combination of both. The very low frequency (VLF) band is even less understood, but activity in this band is unaffected by sympathetic blockade and is suggested to correspond to an intrinsic activity of the heart (107). In 1996, Malik et al. defined standards of HRV measurements in a Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology; this document is still the gold standard for HRV (107). The details of HRV analysis and the technical aspects are not discussed here, for further reading, see *Paper I* and the Task Force 1996 (107).

HRV analysis is a frequently used method in studies for testing autonomic function, stress and as a prognostic tool for cardiac conditions. In adult patients with early stages of chronic heart failure (CHF), a significant increase in LF and decrease in HF has been reported, corresponding to an altered autonomic balance with less vagal influence. The lack of vagal protection is considered to explain the increased risks of arrhythmia and sudden death and is used as a negative prognostic sign (108). Severe heart failure patients show reduced activity also in LF areas and in very advanced stages of CHF the heart behaves in a similar way to the cardiac denervation seen in patients with recently transplanted hearts (109). The re-innervation of a transplanted heart after 1–2 years is usually of sympathetic origin, but respiratory rhythmic changes have been observed. Altered HRV has been described in diabetes, hypertension and as a predictor of risk for sudden death (107). Children with uni-ventricular hearts and Fontan surgery have been shown to have lower HF and the HRV could predict future risk of arrhythmias (110).

Studies in preterm babies showed decreased HRV and preterm babies improved their HRV after massage with a more pronounced effect in males (111). Preterm birth is suggested to delay the maturation of the PNS, which continues during infancy (112-114). Altered HRV with lower vagal activity and elevated sympathetic activity in preterm infants of smoking mothers has been reported (115).

2.8.3 Prenatal programming and DOHaD

The idea of cardiovascular disease in the adult originating from fetal malnutrition sprang from Barker's observation in 1986 of the similar geographical distribution between infant mortality in the early 1900s and cardiac ischemic deaths 60 years later (116). The Barker hypothesis described that malnutrition in fetal life with a low birth weight correlated with blood pressure and arterial compliance in adult life (117).

Preeclampsia, placental dysfunction and maternal smoking are other risk factors associated with poor fetal growth and low birth weight. Exposure to a suboptimal environment *in utero* may affect the fetus during critical stages of development and thus cause a long-term effect on organs and function, a process known as prenatal programming. This concept has developed beyond malnutrition and now also includes overweight and obesity during pregnancy.

During the last years, an even wider concept has been introduced, called Developmental Origins of Health and Disease (DOHaD). The DOHaD concept includes factors affecting the fetus or child during development and is not confined to intrauterine events. The underlying mechanisms are still obscure, but involvement of glucocorticoids, sex hormones, RAAS, ANS, oxidative stress and inflammation have been suggested (118). Altogether, early developmental influences affecting future health are increasingly considered to be explained by epigenetics (118, 119).

3 AIMS

The general aims of this thesis were to explore long-term cardiovascular function in children with exposure to snus in fetal life by assessing autonomic cardiac regulation, blood pressure and arterial wall properties. We also aimed to investigate the concentration of nicotine and metabolites in the breastmilk from snus using mothers.

The specific aims were:

- To study heart rate variability (HRV) in infants with prenatal exposure to snus or cigarette smoke. ***The hypothesis*** was that both snus and smoke exposed infants had an altered HRV compared to tobacco-free controls. *Paper I.*
- To estimate nicotine, cotinine and OH-cotinine concentrations in breastmilk from snus using and smoking mothers before and after tobacco use. ***The hypothesis*** was that snus users had as high or higher concentrations of nicotine and metabolites in breastmilk compared to smokers. *Paper II.*
- To study blood pressure and heart rate variability in 5-6 years old children exposed to snus in fetal life. ***The hypothesis*** was that snus exposed children had higher blood pressure and altered heart rate variability compared to tobacco-free controls. *Paper III.*
- To study carotid artery dimensions, carotid intima media thickness and arterial stiffness in 5-6 years old children with prenatal exposure to snus in fetal life. ***The hypothesis*** was that snus exposed children had thicker intima media, smaller arterial dimensions and stiffer arterial walls compared to tobacco-free controls. *Paper IV*.

4 METHODOLOGICAL CONSIDERATIONS

The methods used in the studies are described in the articles and will not all be discussed in this chapter. Rather, the focus will be on the general outline of the studies, reasons for choosing certain methods and the limitations and strengths of the methods.

4.1 ETHICAL CONSIDERATIONS

Ethical approvals from the local ethical committee in Stockholm were obtained for all studies before they commenced. Written informed consent was collected from all parents. Children above 5 years of age were informed and asked directly. All studies were performed in accordance with the World Medical Association Declaration of Helsinki.

All tobacco-using women were offered tobacco cessation advice during pregnancy via the antenatal clinic without any connection to the study. None of the researchers involved in the studies have any connections to the tobacco industry.

4.2 STUDY DESIGN AND SETTING

Three of the studies in this thesis are of prospective observational design (cohort studies) and one is a case-control study. The participants originated from a larger national study of prenatal smokeless tobacco exposure (SNUS). The women in the original SNUS cohort were included when first visiting an antenatal clinic early in pregnancy. Recruitment took place 2006–2011 in several parts of Sweden and a total of 474 women were included.

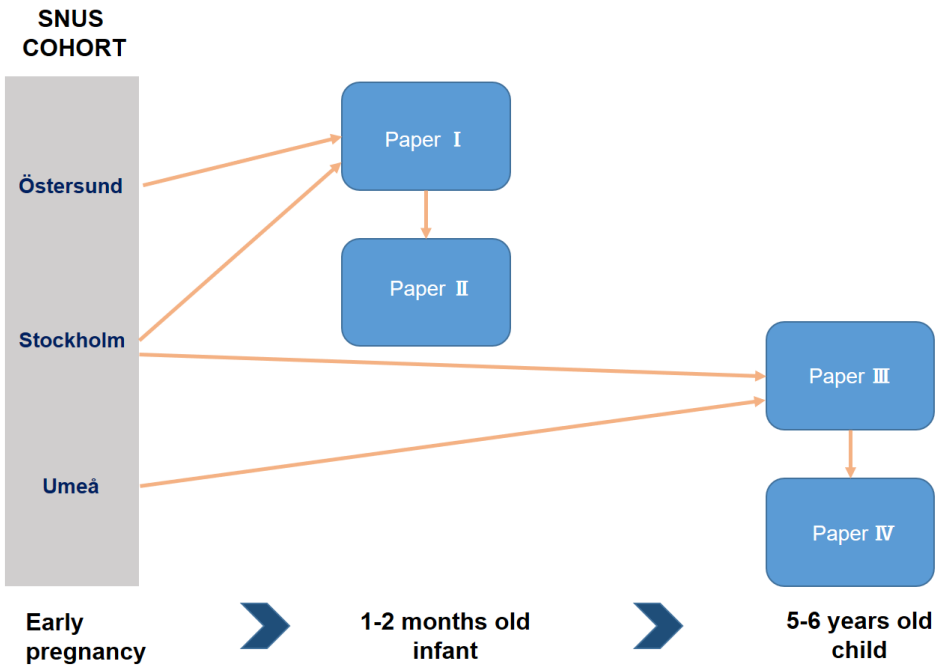


Figure 10. Flow chart and time line for the four studies included in the thesis.

Based on their tobacco use when first visiting the antenatal clinic, they were classified as either snus users, cigarette smokers or tobacco-free controls. No dual users or users of any other nicotine source than cigarettes or snus were included. During pregnancy, the women answered questionnaires about tobacco habits and perinatal data were collected by the Swedish Medical Birth Register.

4.2.1 Women and infants included in the HRV and breastmilk studies

Women in the counties of Stockholm and Östersund were informed in early pregnancy about the possibility of being contacted after delivery with an invitation to a study of their 1–2-month-old child (Studies 1 and 2). Families with a healthy newborn were contacted and informed about the study and new informed consent was collected from the mother. The appointment was scheduled at the pediatric ward when the baby was 6–8 (± 2) weeks.

All families contacted accepted the invitation to participate in Study 1 and those who were breastfeeding at the time of the appointment were invited to participate in Study 2.

The optimal timing of testing was set to 6–8 weeks after birth, as breastfeeding and daily routines and physiological adaptations after birth were expected to be settled at that time. We also expected any physiological influence of tobacco withdrawal after birth or any other impact on the autonomic nervous system from the "stress of being born" to have faded. Furthermore, as the risk for sudden infant death syndrome (SIDS) sharply increases at one month of age, we assumed that the alterations in autonomic control that are potentially associated with an increased risk of SIDS could be detected at that age (58, 66).

A potential problem with the timing was that the autonomic nervous system (ANS) is not fully matured at birth, especially not the parasympathetic part. There are suggestions of a developmental autonomic shift around the age of one to two months; thus we may have introduced a bias of different maturing in the ANS (120). However, when performing sub-group analysis with age as the variable, we could not see any association with age in weeks and HRV.

The women were divided into three groups based on their tobacco habits at inclusion early in pregnancy around gestational weeks 7–10. The pattern of use during pregnancy varied, with some women managing to quit during early pregnancy or decrease their daily dose. In contrast, some women increased their use during pregnancy. Thus, the tobacco exposure of the fetus could differ over time. There were no dual users or other nicotine containing products used. Furthermore, the amount of nicotine to which an infant is exposed depends on the route of exposure. For example, snus-using mothers who chose not to breastfeed their infants did not further expose their infants to any nicotine after birth. In contrast, smoking mothers who did not breastfeed still exposed their infants to nicotine via second hand smoke.

The breastfeeding women were asked to abstain from tobacco use for 12 hours before the appointment in the pediatric ward early in the morning. However, this turned out to be challenging for many of the women, especially the snus users, and hence the period of

abstention varied between 30 minutes and 12.5 hours. In addition to introducing a variable time of abstention not part of the study design, this also underlines the highly addictive properties of nicotine.

A concern with the testing of breastmilk is that samples collected for a couple of minutes from one breast might not have a representative concentration. The foremilk is more aqueous with lower fat content than the hind milk and the content of weak bases as nicotine might be lower than in the rest of the milk. To make sure to measure a true concentration of the breastmilk it would be preferable to empty the breast and then take a sample from the whole amount of milk. Further, an approach with repeated samples over a time period of 30 minutes could have provided more information about the highest levels of concentrations.

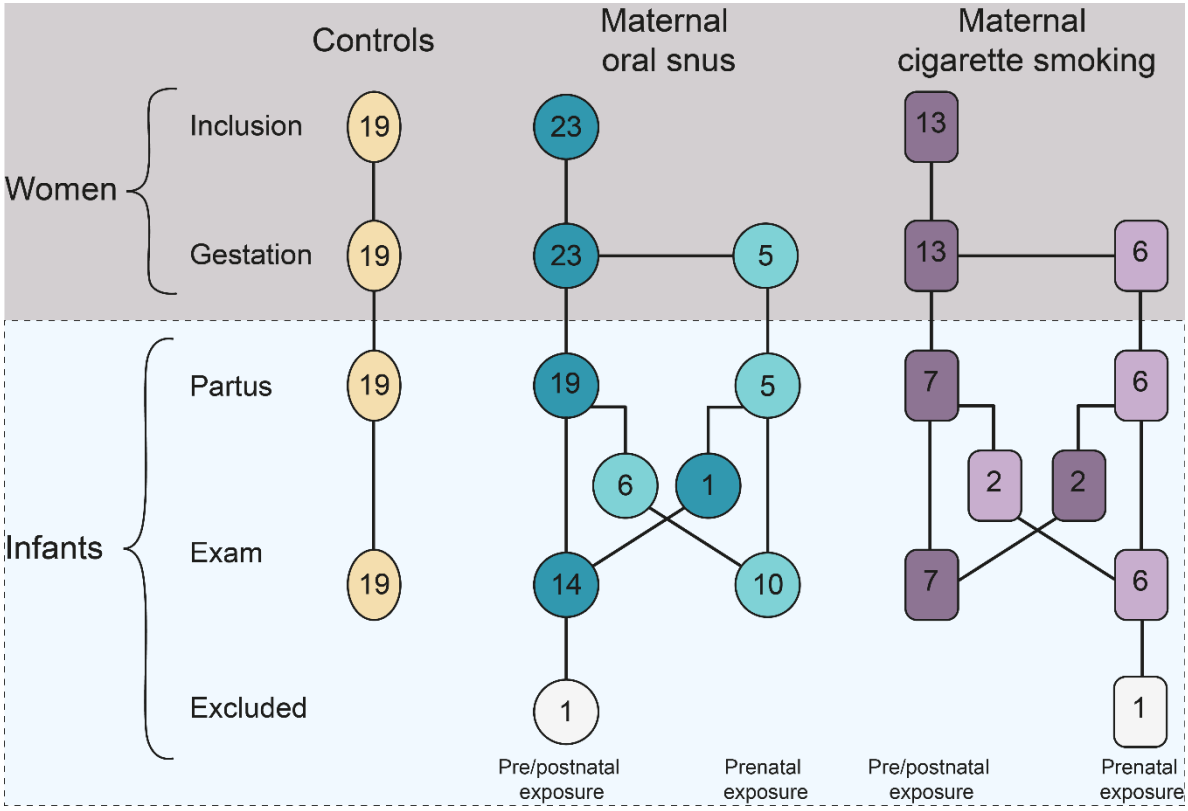


Figure 11. Flowchart for inclusion for Study 1*. Time line from inclusion at top, to examination at bottom. At inclusion the pregnant women were categorized based on their use of snus or cigarettes. After inclusion, some tobacco-using women quit during pregnancy (lighter blue for snus and lighter purple for smokers in the figure). After birth, the exposure of the infants in the snus group, depended on whether they were breastfed or not. The complex crossing after birth displays that one snus user started using snus again and two smokers started smoking after delivery. In the snus group there was one duplex pregnancy.*

4.2.2 Preschool children included in the HRV, BP and carotid artery studies

In Studies 3 and 4, women from the original SNUS cohort were again recruited, this time only women residing in the counties of Umeå or Stockholm. Women with a healthy 5–6-year-old child were invited to participate. We chose the county of Umeå as had many women included in the SNUS cohort, and there were identical ultrasound machines at the two centers. The inclusion criteria were strict in order to exclude as many confounding factors as possible. Exclusion criteria were any of the following: preterm birth, low birth weight, multiple birth, malformations or chronic health issues or exposure to second hand smoke during childhood, see Figure 12.

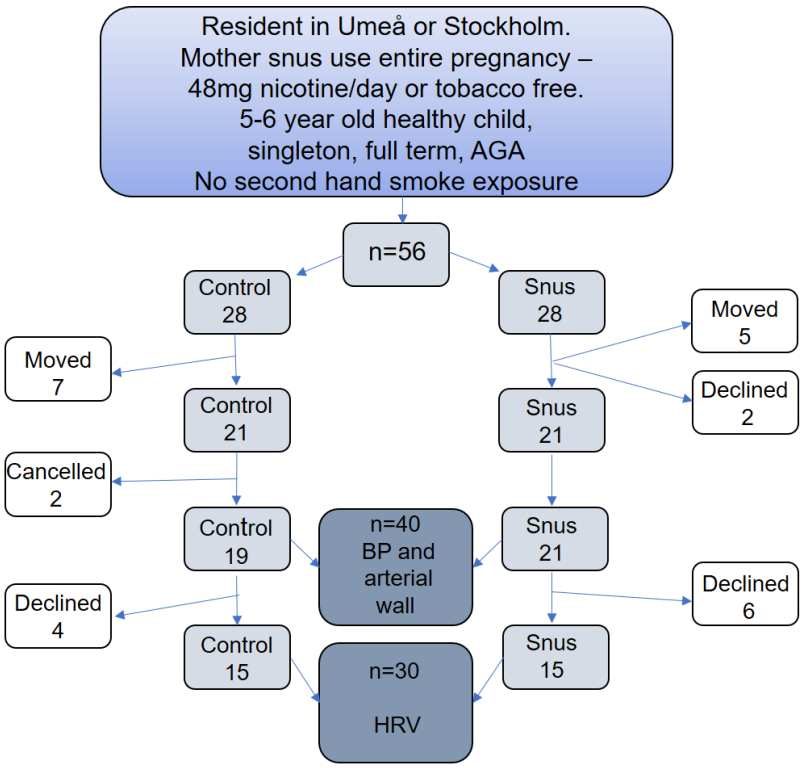


Figure 12. Flowchart Study 3 (BP and HRV) and Study 4 (arterial wall properties). *There were 42 children included in the studies, 40 children in the BP and arterial wall analysis and 30 children in the HRV analysis.*

We chose the age of 5–6 years so the child would be able to collaborate in achieving a high quality ultrasound examination of the carotid artery and because of the availability of reference data for that age group. All of the appointments were scheduled in the daytime and most in late afternoon. The time limit for each appointment was 60 minutes and included measuring weight, height, BP, ultrasound examination of the carotid artery and the heart, setting up the 24-hour ECG, giving information about the study and completion of a questionnaire by the parent during the ultrasound examination.

4.3 COHORT SIZE AND POWER

The cohort size for Study 1 was based on an earlier study on heart rate variability in infants (121). To attain 80 % power with a 5% significance level and expecting a mean difference in LF/HF of 1.15 with a standard deviation of 0.95, we estimated that 54 participants would be sufficient. For Studies 3 and 4, we estimated that 40 subjects would be sufficient, assuming the same power and significance levels and a mean difference in systolic blood pressure of 4 mmHg with a standard deviation of 6 and a difference in strain of 4% with a SD of 5 (122, 123).

However, when trying to do subgroup analysis the power was not sufficient. This proved to be an overall problem in all four studies. There were several interesting research questions based on subgroup analysis that we had not anticipated when planning the studies and where the data did not have enough power to enable any conclusions. For example, with more participants we could have explored differences between boys and girls further. In addition, in the breastmilk analysis we could see a tendency towards higher nicotine and cotinine concentrations in snus users compared with smokers, but it did not reach statistical significance, possibly representing a Type II error (β error). All four studies had limited numbers of participants and therefore limited possibilities for further stratified analysis.

4.4 TOBACCO USE SELF-REPORTED THROUGH QUESTIONNAIRES

The women answered questionnaires about their tobacco use at three separate occasions during gestation: at the antenatal booking in early pregnancy, in late pregnancy and immediately after birth. The participants in Studies 1 and 2 also answered a questionnaire concerning postnatal use at the study appointment 1–2 months after birth. The women reported daily use of tobacco for each gestational week, including the name/brand of the tobacco product, any other source of nicotine and exposure to second hand smoke (hours/day). The snus dose was reported as number of pouches per day and categorized as follows: not daily, 1–2, 3–4, 5–6 or ≥ 7 pouches per day. The cigarette dose was reported as number of cigarettes per day and categorized as follows: not daily, 1–5, 6–10 or > 10 cigarettes per day. The women were informed that their urine might be tested for cotinine in late pregnancy and that both breastmilk and infant urine would be tested at the appointment 1–2 months after birth.

When analyzing the data, the cut-offs for the dose categories for snus use were found to be set too low. There were women using up to 20 pouches per day and the high dose cut-off at > 7 pouches per day might have blunted the dose-response testing. Errors like this can be identified and corrected by early sub-analysis during enrollment.

4.5 HEART RATE VARIABILITY (HRV)

There are several non-invasive methods for testing autonomic cardiovascular regulation, e.g. the tilt-test, cardiovascular reflex tests (Valsalva maneuver and controlled breathing) and HRV. The cardiovascular reflex tests require cooperation from the subject and are therefore not applicable in infants. The tilt-test is used in testing infants, but is time-consuming and needs a special set-up that is not easily moved between different locations. HRV has become the most popular method used for autonomic cardiac assessment, even more so now than when we initially planned the studies. HRV is simple to perform in infants with the recording done at home after setting up the recorder at a hospital and does not require any adjustments in day-to-day life.

HRV is widely used and the number of published scientific papers using the method is impressive. In 1996, HRV guidelines were published by the European Society of Cardiology and the North American Society of Pacing Electrophysiology (107), yet there are considerable variations in how HRV is performed. The inconsistency is even larger when performed in pediatric populations, which makes it highly important to describe the method in detail and use a careful approach when comparing results from different studies.

In our studies we preferred a long-time HRV because short-time HRV (2-10 minutes) has too many confounding factors and a substantial risk of not detecting changes in the low frequencies. We focused on frequency domains (please see Introduction for further description), which are often used clinically in the pediatric population. After discussing and consulting with technical engineers with expertise in HRV, we decided to analyze a two-hour segment during night time sleep. In those two hours, we expected to have periods of both active sleep and quiet sleep and an elimination of major interferences such as noise, light and movement.

Two hours is in the range of a long-time HRV and therefore we used Fast Fourier Transformation (FFT) analysis with the time epoch set to 5 minutes and window processing using Bartlett. When analyzing short-time HRV, auto regression is used and window processing is not necessary. All 24 hours of recording were carefully edited manually, all beats corrected and artifacts rejected with the nicotine status blinded for the trained analyst. The selected two-hour segment was recorded between 22 pm and 6 am, artifact-free and with a low heart rate indicating sleep. The heart rate changes during this two-hour period indicated different states of sleep, although this is not a validated method for sleep state evaluation (124).

The respiratory peak is found in the high frequency band. The breathing frequency of an infant is high, around 40 breaths per minute and may even reach up to 60 breaths per minute. According to the Task Force, the upper limit for the HF band should be set at 0.5 Hz (based on HRV in adults), which leads to a potential risk of missing the respiratory peak in the infant (125). To make sure that we covered even high respiratory rates, we did additional analyses with the cut-off at 0.8 Hz. Surprisingly, doing so did not add any additional data or change the results, and we therefore reported our data using the Task Force limits of the HF band. In our HRV analysis, the respiratory peak shown as respiratory sinus arrhythmia (RSA) was not as dominant as expected in any of the infants. The explanation for this is not clear, either the RSA is not very dominant at 1–2 months of age or we failed to detect it. Infants have a

different breathing pattern with elements of periodical breathing that may explain the lack of a prominent RSA as seen in older children and adults. This theory is supported by other studies of infants who were also deficient in RSA in the HF band (113). Interestingly, in a study from 1989, Schechtman et al. reported that the RSA diminishes during the first month after birth to slowly increase again after 1–2 months (120).

In planning the studies of the older children the same design was used and a two-hour segment of ECG during night time sleep was analyzed. The preschool children showed a typical respiratory peak as expected at a frequency of around 0.26 Hz.

4.6 OSCILLOMETRIC BLOOD PRESSURE

The gold standard for noninvasive blood pressure measurement is by sphygmomanometry of the brachial artery. Many of the reference tables with sex, age and height corrected values are based on sphygmanometric measurements. These may differ from the measurements of oscillometry devices, which are the most common in clinical practice nowadays. The guide lines from the American Academy of Pediatrics and update on the 2004 "Fourth report on the Diagnosis, Evaluation and Treatment of High Blood pressure in Children and adolescents" recommend using a validated oscillometric device, and to use a sphygmanometer to verify any suspicion of hypertension (126, 127). Oscillometric devices are easy to use and accepted for research use. We used Dinamap (GE Health Care, Fairfield, Connecticut, USA) for blood pressure measurement and the reference tables from the Fourth report for sex-, age- and height-corrected values presented in centiles.

We followed a strict protocol with systolic blood pressure (SBP) and diastolic blood pressure (DBP) measured after 15 minutes of calm adaptation to the examining room and with the child sitting down resting. Three consecutive measurements in the right arm with at least one minute between the measurements were obtained by a trained nurse who was blinded to the category of the child. The BP was only tested at this single appointment and could have been influenced by other factors such as anxiety, including "white coat hypertension." Although we do not know anything about the BP over time, we found a difference at the appointments between the snus-exposed and the controls. Other studies imply that an anxiety reaction or white coat hypertension also might matter in the long run (128).

4.7 VASCULAR ASSESSMENT WITH B-MODE ULTRASOUND

The ultrasound examination of the 5–6 year-old children included a B-mode ultrasound of the right carotid artery and a full echocardiographic examination of the heart, to exclude any unknown cardiac abnormalities. All the examined children had normal results. All ultrasounds were performed by the same trained sonographer (Felicia Nordenstam) who was blinded to the group category.

In both centers a Philips iE33 ultrasound machine with a linear high-frequency vascular probe (15 Hz) and simultaneous ECG recording was used for the vascular assessments. Loops of 3–5 heart cycles were stored digitally on CDs for later calculations. The examination of the carotid intima media thickness (cIMT) was performed in accordance with the AEPC recommendations with the child in supine position, with the neck slightly extended. The artery was identified in short axis view and then examined in long axis view with the carotid bulb identified; the region of interest was the intima media in the far wall for a segment of 10 mm, proximal to the carotid bulb.

Three recordings of the common carotid artery were saved and used for later analysis. The cIMT was measured in end diastole (129) identified through ECG and the mean of three separate measurements was calculated with a max, mean and SD of cIMT for each participant using the semi-automated software Arterial Health Package from Siemens. Many reports emphasize the importance of using special software for these calculations. Considering that the layers measured in children were 380–420 µm and that the axial resolution in a high frequency probe was approximately 0.3–0.5 mm, manual measurements raise questions about validity of results.

The carotid dimensions were measured from leading edge to leading edge of the adventitia in systole and late diastole based on the ECG recording in long axis view. The carotid artery strain was calculated as the relative pulsatile diameter change (delta D) using the formula:

Peak systolic diameter (ESD) – end-diastolic diameter (EDD) / EDD X 100

The stiffness index (beta) for the carotid artery was calculated using the formula:

ln (SBP/DBP) / (ESD-EDD/EDD)(123, 130)

One limitation with the carotid examination was that only the right artery was examined. This decision was taken as we examined 5-year-old children and wanted them to be comfortable and at ease during the examination. Studies of the carotid artery in children do not describe any differences in strain, stiffness or cIMT (131-133) between the right and left carotid artery, although examination of both arteries are recommended for evaluation of cIMT (134). There is one study in adults reporting different impact of ageing on cIMT, where the left carotid artery displays changes earlier than the right (135). Some studies of cIMT and association with cardiovascular risk factors show differences between right and left cIMT(136, 137). These studies do not provide any explanation for the incongruences and the findings are described in general terms. The theoretical background for the diversity is different anatomical origins for the vessels and different hemodynamic impact due to the different anatomies. Furthermore, fetal circulation provides a potential difference in blood flow and in oxygen and nutrient content of the circulation in carotid arteries is accentuated during periods of circulatory redistribution (“brain sparing”) when blood from the ductus arteriosus might contribute to the circulation of the brain via retrograde circulation in the isthmus. Whether

there are any important differences in cIMT, strain or stiffness between the two carotid arteries needs further exploration.

4.8 STATISTICAL METHODS

The statistical methods used in the studies are described in detail in each paper. Descriptive statistics were presented as means and standard deviations and medians and ranges or interquartile ranges for numerical variables, or as frequencies for categorical variables. Differences between groups were examined using the chi-squared test or Fischer's exact test for categorical variables. Student's T-test and the Mann-Whitney U-test were used for comparing two groups and one-way ANOVA for comparing three groups with continuous variables. Further analysis with Tukey's post hoc pairwise comparison was made if the overall ANOVA test was significant. Correlations were tested with Pearson's correlation or Spearman's correlation.

Multiple linear regression was employed to control for potential confounders. The dependent variable and relevant clinical and demographic explanatory variables were tested with a systematic approach. In a simple linear regression model the relationship between the predictor variables and outcome was examined and thereafter the variables whose univariate test had a P-value <0.1 were considered candidates for further testing along with clinical relevant variables. The variables identified were entered into a multivariate model.

In the first paper the effect size was calculated with partial η^2 (eta squared). When presented with a difference between groups, a reader wants to know whether it is large or small. The P-value provides information on whether a finding is significant and not caused by chance; it does not include information on if the finding or difference is of importance. This consideration is even more important when using large cohorts, where a very large cohort can show a significant difference even if the difference is very small and may not be of clinical importance. An oversized cohort was not a problem in our studies, though some of our outcomes were more difficult to interpret than others. For example, it is easy for most readers to realize that a mean difference of 5.4 mmHg in SBP is quite large. On the other hand, the LF/HF ratio is much harder to grasp, making it difficult to understand if the mean difference of 1.16 is small or large. In ANOVA, analysis of partial η^2 is an appropriate method for estimating effect size and our result was 0.22; values above 0.14 are considered large (138).

5 RESULTS AND DISCUSSION

The most important results and conclusions from the papers included in the thesis are presented and discussed in this section. For complete results for all studies, see the published papers and manuscripts.

5.1 MAIN FINDINGS

The rationale for the studies in this thesis was to explore prenatal and perinatal exposure to smokeless tobacco (Swedish snus) and subsequent cardiovascular function in the infant/child. We explored autonomic cardiac regulation in 1–2-months-old infants and also estimated exposure by analyzing nicotine and metabolites in infants' urine and mothers' breastmilk. Snus-exposed infants were compared to tobacco-free controls of same age; infants of smoking mothers were also tested.

Furthermore, a group of 5–6-year-old children with snus exposure in fetal life, and no additional tobacco exposure after weaning from breastfeeding, had their blood pressure, autonomic cardiac regulation and characteristics of the carotid artery evaluated.

Several long-lasting associations with prenatal snus exposure were discovered indicating a prenatal programming of cardiovascular function. *These findings included an altered autonomic cardiac regulation, higher systolic blood pressure and stiffer arterial walls following prenatal snus exposure compared with in tobacco-free controls.*

5.2 TOBACCO ALTERS AUTONOMIC CARDIAC REGULATION WITH DECREASED PARASYMPATHETIC ACTIVITY

The autonomic cardiac regulation was explored through spectral analysis of HRV and we found a higher LF/HF ratio in infants at one to two months of age following snus exposure compared with tobacco-free controls *(Paper I).* The higher LF/HF ratio was mainly explained by a lower power in the high frequency domain, indicating decreased parasympathetic activity. The LF/HF ratio did not differ between snus- and smoke-exposed infants, which suggests that nicotine, the common constituent in tobacco, mediates the impact on autonomic regulation regardless of absorption route.

Other studies of infants with smoking mothers report similar findings with a higher LF/HF during sleep in infants aged 6–16 weeks (139) and abnormal heart rate response to tilt-tests in infants aged one to three weeks and three months (140). This has been considered a sign of autonomic instability. However, interpretation is complicated by the fact that reference values for HRV variables in children are scarce, mainly due to differences between studies in methodology, age and sex. A study of 201 children from 3 days to 14 years with a long time HRV showed coherence with our HRV data in infants (LF/HF ratio 2.15) and 5–6 year-old

children (LF/HF ratio 0.7) (141). It is not possible from to draw any conclusions from these studies about what should be categorized as risk values.

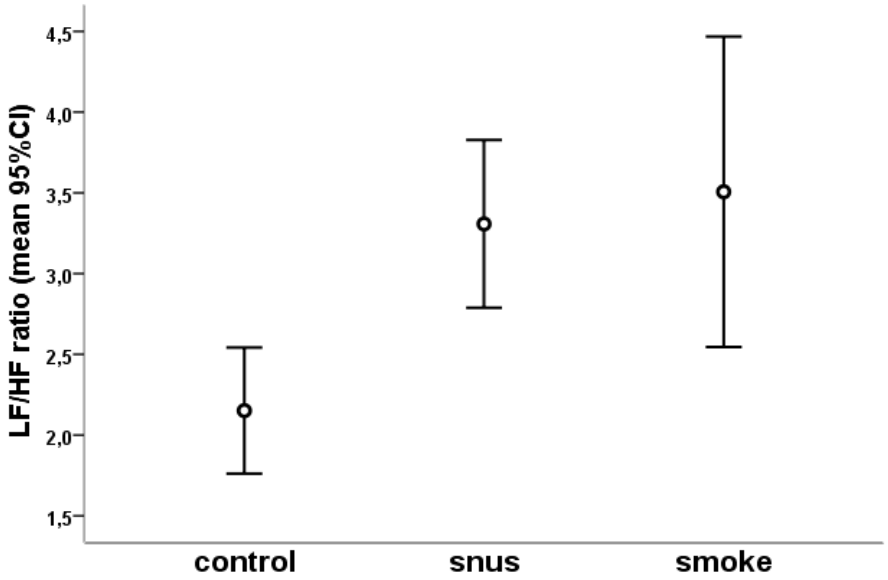


Figure 13. LF/HF ratio in controls (n = 19) and snus (n = 23) and smoke-exposed (n = 12) infants*. Results from Study 1*.

Although not fully understood, autonomic dysregulation is considered to be a crucial component in the multifactorial mechanisms underlying SIDS (142). Studies of infants who experienced apparent life threatening events (ALTE) or later succumbed to SIDS revealed changes in HRV indicating an immature or altered parasympathetic tone (143-146). Factors influencing autonomic control may thus have the potential to increase the risk for SIDS, and parental smoking is the major risk factor for SIDS following the sleeping campaigns (56, 57, 147, 148). This raises the question of whether other tobacco forms or nicotine containing products are safe. Snus use during pregnancy is associated with increased risk of intrauterine death and apneas although increased risk of SIDS has not been described (53, 70).

Among the several thousands of toxins in cigarette smoke, nicotine is the major neurotoxic substance. It binds to endogenous neuronal nicotinic acetylcholine receptors in the fetal brain and brainstem (63, 149-151), with consequences such as impaired arousability (65, 152), altered parasympathetic control (149, 153) and impaired autonomic cardiac control (154, 155). All these pathophysiological effects may have implications for the risk of SIDS.

During fetal life and in the newborn the sympathetic drive tends to dominate. As the ANS matures the parasympathetic influences increase and modulate the autonomic balance with increasing age (156, 157). Our findings suggested a decreased parasympathetic activity and we postulated that there was either a delay in the ANS maturation due to nicotine exposure or

a long-term alteration of the ANS. At the time of publication, there were several studies supporting a delay in maturation, but a long-term effect from nicotine could not be ruled out. A study by Cohen et al. suggested long term effects on autonomic control up to one year of age, although the children in that study were continuously exposed to second hand smoke (140). Our results, showing altered autonomic activity in 5–6 year-old children with perinatal snus exposure (Study 3), support the theory that it is a long-term effect that may have implications beyond the risk period of SIDS. There are also more recent studies suggesting long-term effects on autonomic activity in adults who were born preterm (158).

5.2.1 Even early prenatal exposure matters – there are no safe periods

There were no differences found between the snus and smoke groups and we therefore merged the two tobacco groups to study the timing of exposure. Infants were divided into three new groups: prenatal early exposure, prenatal continuous exposure and prenatal plus postnatal exposure. Eight women managed to quit during the first or second trimester and were classified as "prenatal –early." Seven women did not expose their infants to any tobacco after birth, they were either smoking women who quit or snus users who did not breastfeed. The group of infants exposed in fetal life but not after birth were classified as "prenatal – continuous." The group with both pre- and postnatal exposure included 20 children with an active ongoing exposure at the time of testing, classified as "prenatal plus postnatal."

All three groups with tobacco exposure had higher LF/HF than the control group. Even the prenatal -early group, which had no exposure for the last 4–9 months, had a higher LF/HF ratio compared with controls. See Figure 14.

To our knowledge, a lasting effect from nicotine exposure limited to early gestational exposure only has not been described in humans before and indicates a prenatal programming with a long-term effect on autonomic control. Most animal studies have included both prenatal and postnatal exposure. This is in many ways similar to the situation for infants of smoking mothers, where children will be exposed also postnatally via second hand smoke. Studies of maternal smoking and snus use has suggested that terminating exposure in pregnancy may reverse negative effects on birth weight, risk of stillbirth or preterm birth and other risks for the infant (40, 43, 46, 159). Our findings indicate that early exposure is also of importance. This leads to the conclusion that there may be no time periods that are "safe" regarding nicotine exposure and consequently that tobacco cessation programs need to be implemented before conception.

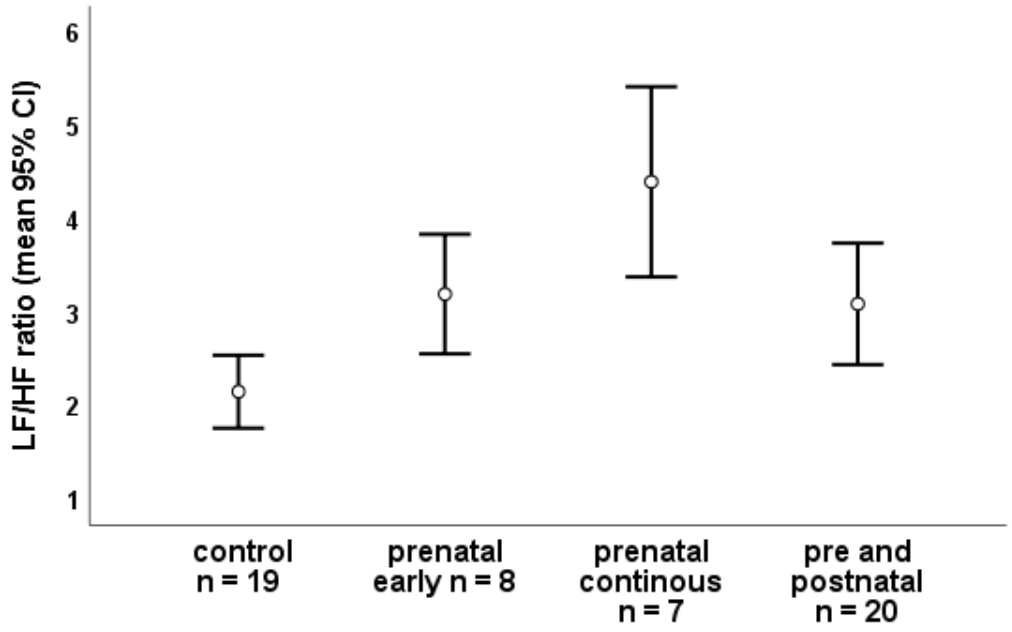


Figure 14. Tobacco exposure (smoking and snus) in different periods in fetal life and after birth and the association with HRV. *Results from Study 1.*

The prenatal -continuous group had an even higher LF/HF ratio than the prenatal -early group. This suggests either a dose-response effect, where this group have been exposed to a higher total nicotine dose than the prenatal -early group, or an impact of exposure during sensitive developmental periods in fetal life. Mid-gestation has been suggested as a vulnerable period with a rapid change in the profile of nicotine receptor subtypes in the brainstem (160). A recent study in rats tested exposure to second hand smoke and neurotoxicity during three periods of gestation, reporting a small effect from premating exposure, an intermediate effect from early gestation and a large effect from late gestation (161), a pattern similar to our findings.

A more puzzling finding was that the group with previous and acute exposure (prenatal plus postnatal) had a significantly lower LF/HF than the prenatal continuous group, albeit still higher than the controls and on a level comparable to that of the prenatal -early group. One possible explanation may be that a signaling system accustomed to high nicotine levels resets and adapts to a new situation when depleted of nicotine. Another possible explanation is that breastfeeding has a positive impact on the LF/HF ratio, either *per se* or via a breastmilk constituent. Breastfeeding protects against SIDS and the effect is stronger if the baby is exclusively breastfed (162). There are studies supporting a theory of different autonomic function in breastfed infants compared with formula-fed, but the results are inconclusive (163, 164). These findings should be explored further and corroborated, as they may point to mechanisms of intervention that could be of importance.

5.3 HIGH LEVELS OF NICOTINE AND METABOLITES ARE FOUND IN BREASTMILK FROM SNUS- USING MOTHERS

Breastmilk from breastfeeding women and urine from infants were analyzed for nicotine and its metabolites cotinine and OH-cotinine. At the time of testing (~ 6 weeks after birth), 19 out of 24 snus- exposed infants and 11 out of 13 smoke- exposed infants were breastfed, as were all the non- exposed infants *(Paper I).* Breastfeeding is generally recommended even for mothers using tobacco- or nicotine- containing products, as the beneficial effects are considered to outweigh the potential risks. Nevertheless, breastfeeding is less common among smoking mothers than tobacco-free mothers with multifactorial explanations including concerns of nicotine passing to breastmilk, decreased milk production and a higher incidence of colic (165-167). Our finding that snus- using mothers breastfed to a lower extent may have similar reasons, but this was not explored in the present studies.

We found high concentrations of nicotine and its metabolites in breastmilk from snus users with the highest nicotine levels around 30 minutes after snus use *(Paper II).* The highest detected concentrations of nicotine (137 ng/mL) and cotinine (958 ng/mL) were found in breastmilk from snus users, but the overall median concentrations did not differ from in smoking mothers. Furthermore, while breastmilk samples from smoking mothers were all nicotine-free after four hours of abstention, samples from snus- using mothers had detectable levels up to 12 hours of tobacco abstention. A plausible explanation for this is the plateau of high nicotine concentrations seen in snus users, described earlier, and that accumulated nicotine in other tissues continues to contribute to the concentrations found in breastmilk. The balance between serum and breastmilk may shift back and forth, with levels of nicotine remaining high for a longer time.

Trying to time breastfeeding before taking pouch of snus is probably better than the opposite, but these findings indicate that levels will remain high. Thus, cutting down doses or quitting will be far more effective in reducing the nicotine content in breastmilk.

The urine of the infants contained high concentrations of cotinine and OH-cotinine in both tobacco groups without any differences between the groups. The metabolites found in the infants' urine are likely a result of both the infants' own metabolisms and direct exposure to metabolites via the breastmilk. Previously, cotinine and OH-cotinine have been considered to lack physiological effects. However, recent studies in rats have suggested that the physiological effects of cotinine seem to counteract those of nicotine, where cotinine lowers heart rate and BP and weakens adrenergic vasoconstriction (168, 169). If that is true also in humans, the lower response in LF/HF seen in infants with postnatal exposure may be mediated by acute cotinine exposure. Analyzing cotinine and OH-cotinine may thus be of importance in future studies, in order to understand the findings and draw clinical conclusions.

5.4 A HIGHER SYSTOLIC BLOOD PRESSURE IS SEEN FOLLOWING PRENATAL SNUS EXPOSURE

Children aged 5–6 years who were exposed to snus in fetal life had a higher SBP than tobacco-free controls *(Paper III).* The mean difference in SBP was larger than in other studies of parental smoking and BP and remained high after the SBP was corrected for age, sex and height (126, 127). See Figure 15. There were two snus- exposed children with an elevated SBP (>90th centile) and none in the tobacco-free group. Since blood pressure development during the lifespan follows a trajectory, a minor change in blood pressure as a child might become larger later in life (170-172). At a population level, a minor change may have significant importance for the prevalence of hypertension and cardiovascular disease. DBP did not differ between the groups, although there was a trend towards higher DBP in the snus- exposed children.

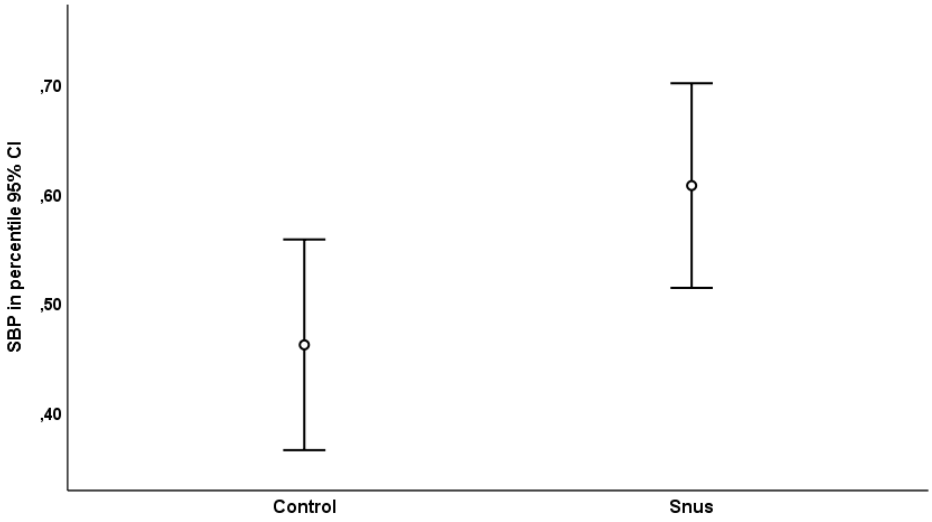


Figure 15. SBP in snus exposed (n = 21) and control children (n = 19). *The SBP is age-, sex- and height- adjusted and presented in percentiles. Results from Study 3.*

Although no significant difference was found in weight or BMI, there was a trend towards higher BMI in snus- exposed children. Overweight, high BMI and obesity have been described in children exposed to smoking (76, 77). Simonetti et al. reported some of the most important factors for higher SBP in children including overweight, preterm birth or low birth weight and maternal smoking (173). We found a correlation between SBP and weight for both snus-exposed children and tobacco-free controls. See Figure 16.

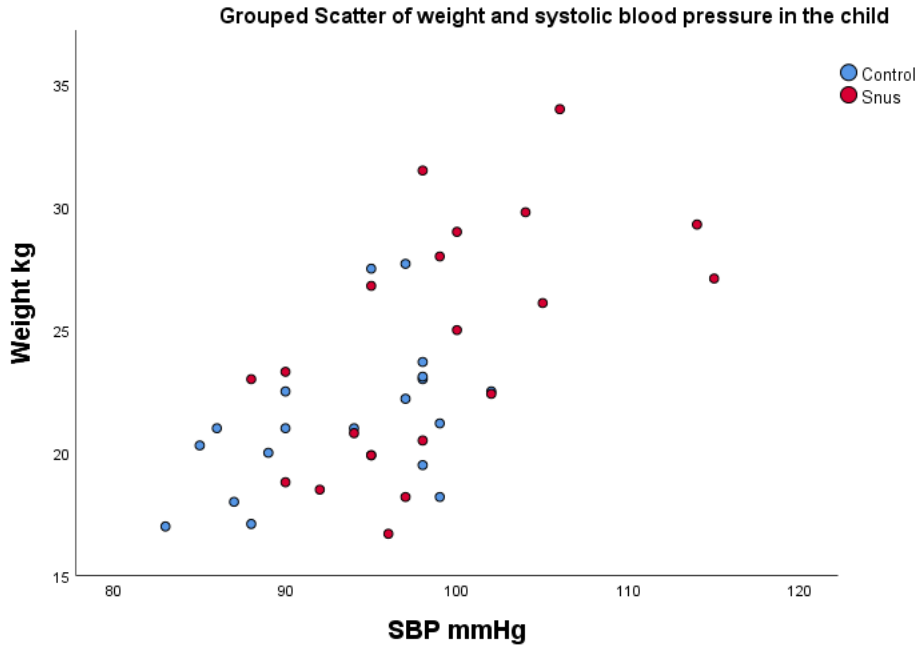


Figure 16. The correlation between weight and SBP. *Results from Study 3.*

Acute exposure to nicotine raises the blood pressure, but in chronic tobacco users the blood pressure control seems to be more complicated. Some studies have even reported lower BP in adult smokers than in controls (174). Maternal smoking during pregnancy has been associated with higher BP in exposed children, considered to be a long-lasting effect from prenatal nicotine exposure. However, these studies struggle with the confounding factor of continued exposure to second hand smoke during childhood, which complicates interpretation (77, 175-178). In exploring snus exposure during pregnancy and breastfeeding we were able to eliminate both the influence of combustion toxins from cigarette smoke and that of second hand smoke exposure during childhood. Our results reveal a long-lasting association between perinatal nicotine exposure and a higher SBP in the offspring as a result of prenatal programming.

5.5 STIFFER ARTERIAL WALLS ARE ALSO SEEN FOLLOWING SNUS EXPOSURE

In addition to a having a SBP, the snus-exposed children displayed stiffer arterial walls and lower strain than the tobacco-free children *(Paper IV).* Arterial stiffness describes the loss of elasticity of an artery with an incapability to respond to pressure changes and is influenced by several parameters, such as hypertension, smoking, obesity and duration of breastfeeding (179-183). Both acute and chronic changes with increased arterial stiffness have been reported in adults smoking (180), using nicotine-containing electronic cigarettes or nicotine tablets (184-186).

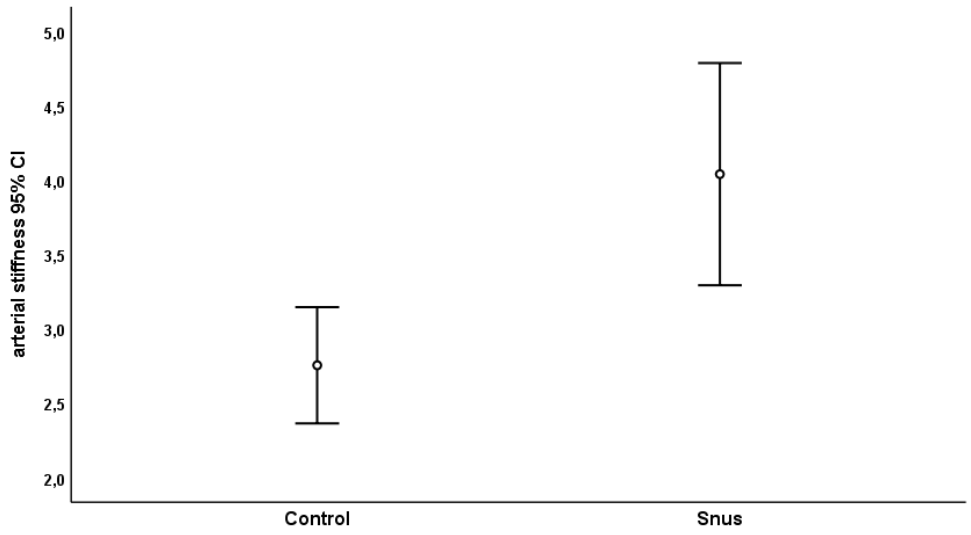


Figure 17. Carotid arterial stiffness in tobacco-free (n = 19)and snus-exposed children (n = 21). *Result from Study 4.*

Our measurements of stiffness index are congruent with reports from 5–10- year- old control children (131, 187). The stiffness index is calculated from pulsatile changes in blood pressure and diameter of the common carotid artery. The systolic carotid diameters were similar in snus-exposed and control children, but the diastolic carotid diameters were larger in snus-exposed children. Although this finding is not easily explained, one plausible explanation could be higher peripheral vascular resistance in the snus- exposed individuals. Consequently, a higher DBP would be expected. However, there was no significant difference in DBP, though the snus- exposed children had slightly higher values.

The common carotid artery is a large elastic vessel with a thick developed tunica media with elastic fibers arranged in concentric layers. The compliance of the wall is dependent on the ratio between elastin and collagen proteins. Furthermore, factors like glycation proteins crosslinking with collagen and extracellular proteins are involved in complex processes that may reduce vessel compliance (188). Ageing processes, inflammation, hypertension and shear stress favor collagen production and undermine elastin synthesis (188). Prenatal exposure to nicotine disturbs the elastin production in pulmonary arteries of monkeys, resulting in stiffer vessels and increased pulmonary hypertension (189).

In our study (*Paper IV)* there was no difference in cIMT between the snus- exposed and the tobacco-free controls. The connection between cigarette smoking and atherosclerosis is strong and thicker cIMT is an early sign of atherosclerotic disease (190, 191). The pathophysiological process of atherosclerosis is promoted by cigarette smoke through contribution of inflammation, insulin resistance, dyslipidemia, thrombosis and endothelial dysregulation.(192, 193) Nicotine is at least partly responsible for these changes and in addition, nicotine affects autonomic regulation with raised blood pressure as a consequence.

There are numerous studies in animals suggesting that arterial wall changes are associated with exposure to nicotine (194-197). Nevertheless, a study of adult snus users could not show any changes in cIMT (198). Pathological changes of the arterial wall characteristics, especially early in life, do not necessarily imply a beginning atherosclerotic process. Arterial stiffness due to prenatal nicotine exposure may be an entity of its own, disparate from ageing vessels in adult smokers.

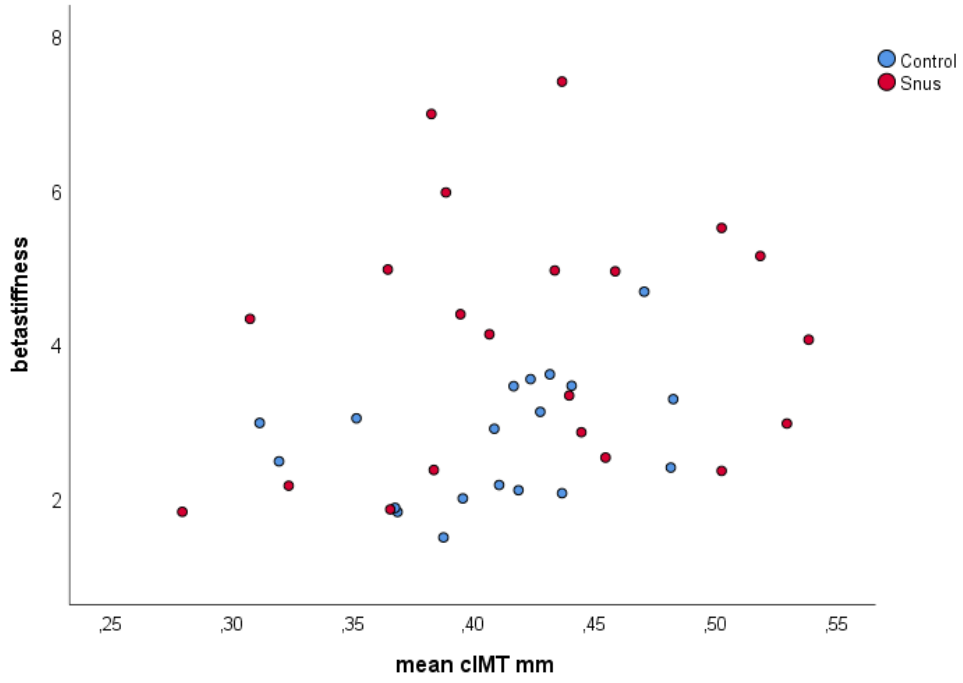


Figure 18. Arterial stiffness and cIMT (mm) in snus- exposed and control children. *There is no correlation between arterial stiffness and cIMT. Results from Study 4.*

The scarce reports about maternal smoking and intima media wall changes in children are incongruent. In newborns, altered umbilical artery walls (199) and aortic walls (200) have been described, and in 5-year-old children exposed to cigarette smoke cIMT was increased (132). On the other hand, a report of 8-year-old children with both prenatal and second hand exposure during childhood did not show any changes in cIMT (201).

5.6 INTERACTIONS BETWEEN THE AUTONOMIC NERVOUS SYSTEM AND BLOOD PRESSURE AND ARTERIAL WALL STIFFNESS

The autonomic cardiac regulation in the 5–6 year-old children was also altered, suggesting a long-term prenatal programming from nicotine exposure and not just a delayed maturation in the autonomic cardiac regulation *(Paper III).* The mechanism behind prenatal nicotine exposure and altered cardiac control and blood pressure regulation is not fully understood, but involves numerous reflexes and connecting systems.

The baroreceptor reflex loop and the renin-angiotensin-aldosterone system (RAAS) are involved in autonomic regulation of the blood pressure (202-204). We could see a correlation between LF/HF and the systolic blood pressures above the 60th centile in snus-exposed children, confirming a connection, although there was no correlation when including blood pressures and LF/HF from all snus-exposed children. An explanation could be that the strongest association with nicotine exposure and autonomic control is found in this higher range of SBP. Alternatively, the correlation could be explained by some outliers. The cutoff at the 60th centile was chosen as it exceeded the 95% CI of the controls and the median of the snus users was the 60th centile.

Nicotine exerts a wide range of effects on the autonomic nervous system through binding to nAChR in the brain, autonomic ganglia, adrenal medulla and neuromuscular junction, and affects other neurotransmitter systems via both pre- and postsynaptic effects (96, 97). Furthermore, nicotine may also interfere with ANS and BP control via structural changes leading to physiological effects. Indeed, smaller kidneys have been described in children exposed to maternal smoking during pregnancy and a potential subsequent dysfunction in the RAAS (205, 206).

The composition and characteristics of the arterial walls are of importance in blood pressure control, where a stiff and thick arterial wall increases resistance and may increase pressure. On the other hand, a high BP promotes shear stress on the arterial wall and the wall becomes thicker and stiffer in response to the increased pressure (207), see Figure 19.

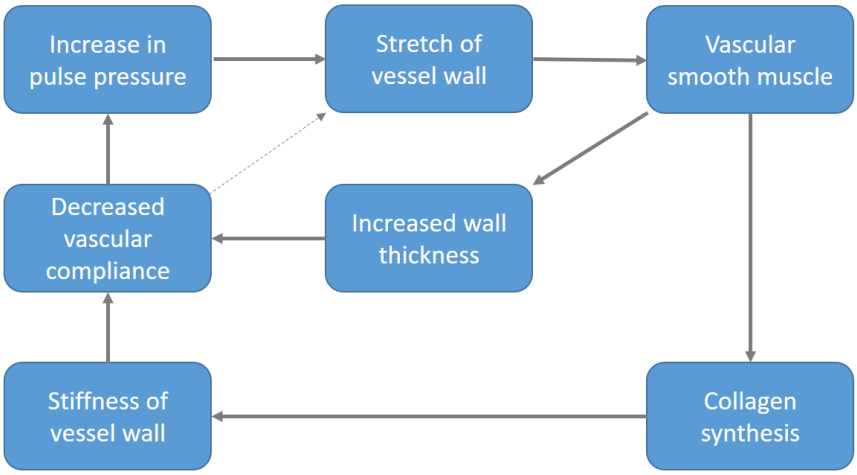


Figure 19. The response to increased pressure on the arterial wall with increased wall thickness and stiffness. *Source: Adapted from Martyn et Greenwald, The Lancet 1997. Published with kind permission from Elsevier.*

Arterial wall changes have been described in both neonates and older children following maternal smoking during pregnancy (199, 208). Prenatal nicotine exposure in monkeys also leads to thicker walls, increased collagen in adventitia and lowers the elastin compound in pulmonary arteries (189). Other animal studies show vascular dysfunction, endothelial dysfunction, increased response in SBP to angiotensin II and impaired perivascular adipose tissue with effects on arterial contraction following prenatal nicotine exposure (194, 203, 209, 210). The opposite connection between the arterial wall and ANS is not as clear and reports are rare, but endothelial function and NO seem to modulate the activity of sympathetic fibers in the arterial wall (211).

Nicotinic involvement in the process of arterial wall changes is evident but the process is truly complex and the crosstalk between arterial wall function, blood pressure and autonomic control is intriguing (212, 213); they are linked, interconnected and they are all susceptible to nicotinic actions.

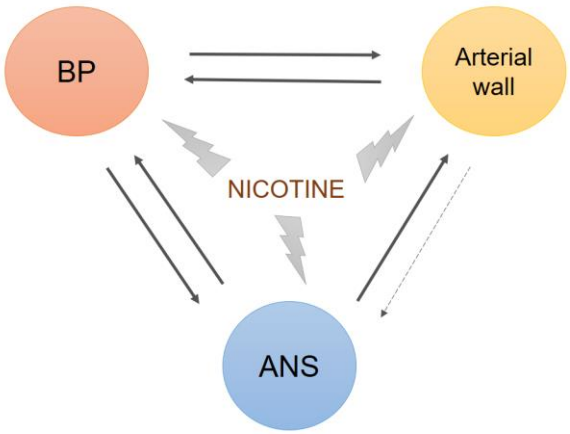


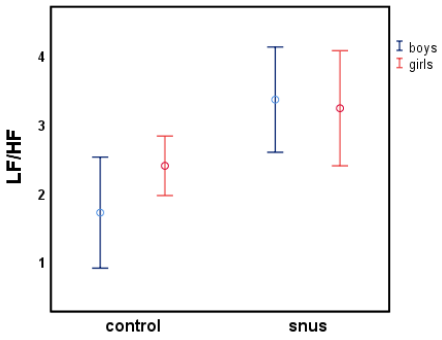
Figure 20. The crosstalk between autonomic nervous system (ANS), blood pressure (BP) and arterial wall, under the influence of nicotine.

5.7 BOYS MAY BE MORE VULNERABLE TO SNUS EXPOSURE

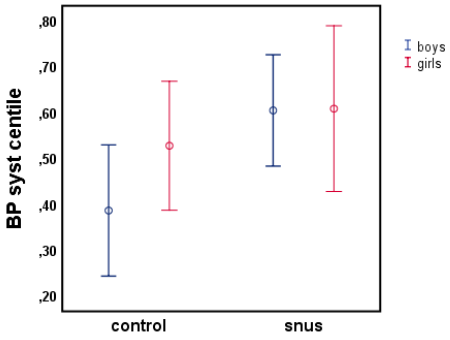
It is well-known that boys are more likely to be affected by prenatal stressors: being born preterm, having poorer neonatal outcome and an having increased risk of SIDS (214). Although our cohorts were too small to allow stratified analysis of sex differences, we could see a trend where boys seemed more affected by nicotine exposure. In HRV analysis, the difference between controls and tobacco-exposed infants was larger in boys than in girls and a similar trend was seen in SBP in the 5–6-year-old children. Arterial strain and stiffness did not show any tendencies towards sex differences at all. However, the cIMT measurements

exposed a significant difference between boys and girls in the snus-exposed group which was more accentuated than in the control children.

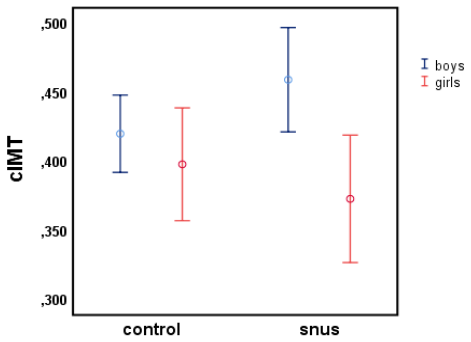
Although it is not possible to draw any conclusions from these tendencies, there are animal studies that support the theory of different responses in males following prenatal nicotine exposure. Such sex differences to prenatal nicotine exposure have been demonstrated in HRV (215), arterial contractility (209) and a stronger response in male rats BP after angiotensin II stimulation (203). In humans, maternal smoking decreased RSA more in boys than in girls, which is interesting given that boys have higher risk of SIDS than girls. Studies of BP or cIMT in children following maternal smoking have not considered or reported any sex differences (132, 173, 175, 216). There are studies reporting sex differences in arterial wall composition in normal control children but the data are incongruent (133, 217).



A



B



C

Figure 21. Are boys more affected by nicotine?

A: Heart rate variability infants (Study 1)

B: Systolic blood pressure (Study 3)

C: Carotid intima media thickness (Study 4)

Altogether, these findings are merely indications of boys being more vulnerable to nicotine exposure and need to be further elaborated. It is important to recognize possibilities of sex differences when planning studies also in children, which requires a meticulous study design and the performance of subgroup analyses.

5.8 FUTURE ASPECTS

This thesis has added important data increasing the understanding of prenatal tobacco exposure during vulnerable developmental periods and the associations with risk factors for future cardiovascular disease. However, it has also raised questions that need further studies.

The main aim of this project was to study whether it is beneficial for the child if the mother uses snus instead of cigarettes and we believe that this thesis and other studies have answered that question. It is clearly not safe for the child to be exposed to snus during fetal life and there appear to be no safe periods or safe doses. It is therefore unclear what added data future studies of tobacco use during pregnancy could offer, beyond corroborating its harmful effects. Instead, future studies should focus on effective ways to help women quit tobacco and nicotine during pregnancy. This is not a new research field and many studies have addressed this issue, but effective measures remain to be defined, illustrating also the enormous addictive potential of nicotine use. Finding ways to help women quit their nicotine use during pregnancy has the potential to significantly improve child health and effects continuing into adulthood. It should thus be a highly prioritized research question.

Although the harmful effects of nicotine exposure during gestation may be regarded as established, the present studies have indicated some specific areas that may be worthwhile to explore from a mechanistic point of view.

Firstly, the effects and mechanisms involved in the adaptation of neuronal signaling systems to nicotine exposure remain unclear. This is illustrated by the complex response following nicotine depletion after a period of nicotine exposure during development. The underlying mechanisms behind this paradoxical response should be explored on a basic scientific level, including animal studies and cellular models.

Secondly, the beneficial effects from breastfeeding need further exploration. The positive effects may be due to important constituents in breastmilk, i.e., anti-inflammatory and immune- stimulating factors – or maybe breastfeeding reflects a mother in good health. Perhaps, being unable to breastfeed is a manifestation of the detrimental effects of tobacco on both the mother and the baby.

Thirdly, the physiological role of the metabolite cotinine needs to be further investigated as this will be of great importance for the understanding of different physiological effects in an *in vivo* setting.

Finally, the indications of important sex differences between girls and boys in several of the studies in this thesis need to be confirmed in studies sufficiently powered to perform analyses thereof. The underlying mechanism(s) for such differences is unclear, but our findings clearly point to the importance of future studies including testing of sex differences in children exposed to tobacco and nicotine.

5.9 CLINICAL CONSIDERATIONS AND PREVENTION

Tobacco and nicotine products are not safe during pregnancy and should be avoided. Although there are indications of some associations between dose and negative outcome, there is no dose that is considered safe.

We have showed that even early exposure matters and that there are no safe periods. Tobacco cessation program should be offered in Youth Health Guidance Centers and antenatal centers, before, during and after a pregnancy. Preferably, cessation should be achieved before conception (218), a recommendation supported by our findings of effects from early exposure. The support and abstention of the partner is important for successful tobacco cessation in the mother-to-be and the partner should be included in a cessation program. Advice and recommendations should be specific, scientific and easy to follow for both the tobacco user and the health care professionals. When it comes to recommendations for the group that really struggles with total abstention, the health care professionals find it challenging to advise on NRT and other nicotine products (219).

According to the American Academy of Pediatrics Policy Statements "Clinical practice policy to protect children from tobacco, nicotine and tobacco smoke" from 2015 there is strong evidence for not recommending e-cigarettes as a cessation aid (220), it is actually associated with decreased rates of stopping smoking cigarettes in adolescence (220).

The tobacco industry needs to be regulated through laws on advertising, labelling of products and introducing new tobacco or nicotine products. Restrictions of visibility and product layout in stores are effective actions. There is a need for regulations on new nicotine products and how they are presented and sold beside NRT products, as this may be confusing and misleading for a customer.

More extensive laws regulating exposure to second hand smoke and limiting cigarette smoking in public areas are needed to make sure that children are protected from second hand smoke. The nicotine containing liquid used for e-cigarettes is highly toxic and many products candy or fruits flavors; there have been severe cases of poisoning in small children accidentally ingesting liquid nicotine (221, 222).

Furthermore, preventive actions are needed to stop the next generation from developing a nicotine or tobacco addiction. One method could be to regulate smoking in movies and TV programs that may be seen by children. The American Academy of Pediatrics and the 2014 General Surgeon's report have addressed "movie smoking" as a problem and suggested that restricting smoking in movies could have a significant impact (220).

Secondary prevention may also be considered. Babies to mothers who have used tobacco during pregnancy could be offered an examination and, if necessary, prescription of apnea alarms. A clinical examination of tobacco-exposed children in preschool age with blood

pressure check-up may be considered. Furthermore, if risk factors add up with prenatal nicotine exposure, increased systolic blood pressure, overweight, hereditary of hypertension and overweight, an intervention in early childhood might prevent future cardiovascular disease.

5.10 CONCLUSION

Maternal snus use during pregnancy showed long-lasting associations with altered autonomic cardiac control, increased systolic blood pressure and stiff arterial walls in the offspring, suggesting a prenatal programming from nicotine exposure. These alterations in cardiovascular function may follow a trajectory in to adult life with increased risk of future cardiovascular disease. Identifying early signs of cardiovascular risk factors during childhood may be of importance for preventive actions.

Nicotine exposure even early in pregnancy matter and there are no safe periods during embryonic development and no safe level of nicotine exposure established. Women of childbearing age are therefore recommended to abstain from tobacco and nicotine in all forms during pregnancy and breastfeeding.

6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Användningen av snus ökar i Sverige och Norge, även bland kvinnor i fertil ålder. Enligt Socialstyrelsen uppger 4,7% av kvinnor att de snusat tolv veckor före graviditeten. Under graviditeten uppger 1,3% av svenska kvinnor att de snusar.

Snusare slipper de giftiga förbränningsprodukter som bildas i cigarettrök, dock innehåller snus höga doser av nikotin. Nikotinet passerar lätt över till fostret. Fostervattnet kan till och med innehålla högre nivåer av nikotin än vad man finner i mammans blod. Effekterna av exponering av nikotin under fosterlivet är inte helt klarlagda. Man har beskrivit ökad risk för fosterdöd, för tidig födsel, lägre födelsevikt, gomspalt och även ökad risk för död i nyföddhetsperioden. De liknar de risker som har beskrivits vid rökning under graviditet, även om barn till rökare har en mer uttalad risk för tillväxthämning. Den flerfaldigt ökade risken för plötslig spädbarnsdöd som föreligger vid rökning har inte kunnat visas hos barn till snusare.

Vi ville undersöka om barn som exponerats för snus under fosterlivet uppvisade några effekter på hjärta och kärlsystem. Rökning under graviditet har rapporterats ge förändrad autonom reglering av hjärtrytmen, förhöjt blodtryck och stelare och förtjockade kärl. Denna hjärt- och kärlpåverkan tros bero på nikotin-exponernig under fosterlivet. Men tolkningen har försvårats av att cigarettrök innehåller tusentals andra potentiellt giftiga substanser. Dessutom har de flesta undersökningar gjorts på barn som även har varit utsatta för cigarettrök från föräldrarna under uppväxten. Genom att studera barn till snusare kunde vi utesluta exponering från andra giftiga substanser än nikotin och även utesluta effekter från fortsatt exponering under uppväxten.

I vår första studie undersökte vi barn vid en till två månaders ålder vars mödrar snusat under en del av, alternativt hela, graviditeten. Barnens autonoma reglering av hjärtrytmen undersöktes med EKG och analys av hjärtfrekvensvariabilitet jämfördes I dessa hänseenden med barn till rökare och helt nikotin- och tobaksfria barn. Barn till både rökare och snusare uppvisade förändrad hjärtfrekvensvariabilitet jämfört med kontrollbarnen och denna förändring liknade den man tidigare har sett hos barn med ökad risk för plötslig spädbarnsdöd. Lite överraskande visade även de barn vars hade mödrar slutat med tobak tidigt under graviditeten en förändrad hjärtfrekvensvariabilitet. Vi drog slutsatsen att det inte finns någon säker period för tobak under fostertiden.

Vi undersökte också nivåerna av nikotin och dess nedbrytningsprodukter i bröstmjölk hos snusande och rökande mammor. Nivåerna av nikotin var minst lika höga i bröstmjölk från snusare som I bröstmjölk från rökare. Dock var alla bröstmjölksprover från rökare fria från nikotin fyra timmar efter rökning, medan vi hos snusarna fortfarande kunde påvisa nikotin mer än 12 timmar efter senaste snusdos.

I studierna tre och fyra undersökte vi 5-6 åringar som hade varit exponerade för snus under hela fosterlivet och under amning. Dessa barn uppvisade högre systoliskt blodtryck jämfört med icke-exponerade barn och de hade också en förändrad hjärtfrekvensvariabilitet, trots att det hade gått så lång tid sen exponeringen upphört. Vi tolkar detta som att nikotin under fosterlivet hade gett upphov till långvariga effekter som kan ha betydelse för framtida hjärt- och kärlhälsa hos dessa individer.

I studie fyra undersökte vi barnens halskärl och tittade på kärlets eftergivlighet och kärlets tjocklek, även kallat "intima media" tjocklek. Vi fann att barn till snusare hade stelare kärl jämfört med kontrollerna men ingen skillnad i kärltjocklek. Denna kärlstelhet kan vara ett förstadium till åderförkalkning och kan medföra en ökad risk för hjärtkärlsjukdomar senare under vuxenlivet.

Sammanfattningsvis fann vi förändringar som kan innebära ökad risk för plötslig spädbarnsdöd och risk för utveckling av framtida hjärt- kärl sjukdomar. Vi tolkar det som att nikotinexponering under fosterlivet ger långvariga och potentiellt bestående förändringar som kan innebära en ökad risk för de exponerade individernas hälsa under vuxenlivet. Vår rekommendation är att avstå från alla former av tobak och nikotinprodukter under graviditeten. Även snusande kvinnor bör rekommenderas att amma sina barn, men om möjligt avstå från nikotin eller åtminstone minska doserna.

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