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Original investigation

Prenatal Exposure to Snus Alters Heart Rate Variability in the Infant

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Abstract

Introduction: Maternal use of smoked tobacco during pregnancy causes significant morbidity and mortality in the human infant including alterations in autonomic control with increased risk of sudden infant death syndrome. We hypothesized that maternal snus (smokeless tobacco) use during pregnancy affects autonomic cardiac regulation in the infant, as measured by heart rate variability (HRV) and the low frequency and high frequency ratio (LF/HF ratio).

Methods: A prospective observational study of 56 infants of women who used snus (n = 23) or cigarettes (n = 13) during pregnancy versus tobacco- and nicotine-free controls (n = 19). The nicotine dose was estimated by questionnaires at 4 timepoints pre- and post-natally. The infants' urine cotinine concentration and HRV during 2 hours of sleep were studied 1–2 months after birth.

Results: LF/HF ratio was higher in snus (mean 3.31; 95% CI 2.78–3.83) and smoke (3.51;2.54–4.47) compared to controls (2.15; 1.76–2.54, p = .002). Early prenatal nicotine exposure "without" any further exposure increased the LF/HF ratio (3.19; 2.55–3.84, p = .02). Continuous prenatal nicotine exposure "without" postnatal exposure was also associated with a residual increase in LF/HF ratio (4.40; 3.38–5.42, p < .001). There was no difference between infants exposed to smokeless versus smoked tobacco, suggesting a common constituent (nicotine) altering autonomic cardiac regulation.

Conclusion: Infants to mothers who used snus during pregnancy showed lower vagal activity with an increased LF/HF ratio compared to controls, and similar to infants of smokers. Even early prenatal exposure to snus has a lasting impact on autonomic cardiac regulation suggesting a fetal "re-programing" of the developing autonomic nervous system.

Implications: The results indicate that smokeless tobacco (Swedish snus) affects the developing autonomic nervous system during gestation. Even if exposure is interrupted during the first or second trimester, effects in autonomic cardiac regulation are seen in the 1–2 month-old infant. This underlines the importance of abstaining from all types of tobacco use during the whole pregnancy. Our findings may also have more general relevance to other routes by which nicotine can be delivered to a fetus and newborn.

Introduction

Tobacco use by pregnant women is a global public health hazard with both short- and long-term effects on the fetus and child. Exposure to tobacco and nicotine by pregnant women differs across cultures and between continents, and includes smoking, use of chewing tobacco and snus as well as use of nicotine replacement products such as patches, gums, and e-cigarettes. The use of tobacco alternatives to cigarettes and a combination of different nicotine sources are increasing amongst youth in the United States and may produce a different pattern of nicotine addiction in the near future.[1] In the industrialized world, the use of oral, smokeless tobacco during pregnancy is rare, except in Sweden and Norway, where oral moist snuff (Swedish snus) use amongst pregnant women is not uncommon.[2,3] While 1.3% of pregnant women use snus in Sweden overall, there are regional differences, with more than 5% reported in some northern parts (Swedish National Board of Health and Welfare 2014).[4]

The detrimental effects of maternal smoking during pregnancy and postpartum (eg, increased rates of preterm birth, intrauterine growth retardation and sudden infant death syndrome [SIDS]) are well known.[5,6] Although cigarettes contain many toxins, nicotine is the proposed linkage between maternal smoking and increased risk of SIDS.[7] Nicotine passes through the placenta and exposes the fetus to nicotine levels comparable to or even higher than those in the pregnant woman herself.[8] Swedish snus is pulverized or powdered tobacco that contains water, sodium carbonate, sodium chloride, moisturizer, flavoring, and nicotine.[9] It is placed behind the upper lip as a loose powder or sealed in small pouches. Although snus delivers lower concentrations of some harmful chemicals, for example, nitrosamines and metals, than other tobacco products, it does deliver high doses of nicotine.[9,10]

Studies from Swedish health registers show that adverse neonatal outcomes such as preterm birth,[11] stillbirth[12] and neonatal apneas[13] are associated with prenatal exposure to snus. Lower birth weight[14,15] has also been described, although the risk of being born small for gestational age (SGA) is not as pronounced as for active maternal smoking during pregnancy.[3,15,16] Animal studies indicate that nicotine is, by and large, partly responsible for these adverse effects,[17] but discrepancies between the adverse effects of smoked tobacco versus snus on neonates suggest that nicotine may not be the only culprit.[15,18] In humans, comparative data on the effects of prenatal nicotine exposure via routes other than smoking are extremely limited.[17,19] This is an important and troublesome knowledge gap, particularly because nicotine replacement therapy (NRT) is approved by some authorities as a smoking cessation strategy in pregnancy, despite concerns about its lack of safety or efficacy.[20]

In this study we have made use of the prevalence of snus usage amongst pregnant women in Sweden to investigate the likely effect of prenatal and postnatal exposure to nicotine on infant HRV. HRV is a functional marker of autonomic cardiorespiratory control. Small fluctuations in the time interval between heartbeats (the interval between the R peaks of QRS complexes; R-R interval) of an ECG recording are measured and expressed as discrete bands across a frequency spectrum. The high frequency (HF) component is generally thought to represent efferent vagal activity, whilst low frequency (LF) power is more complex, modulated by both vagal and sympathetic influences. The LF/HF ratio is considered to be an indirect index of overall sympatho-vagal balance.[21] Altered cardiac autonomic regulation has long been thought to be a "marker" of increased risk for SIDS in infants of smoker mothers.[22–24] We therefore studied HRV of 4–10 week-old infants to investigate whether maternal use of smokeless tobacco (snus) during pregnancy is also associated with altered cardiac autonomic regulation.

Methods

Study Design

This prospective observational study is a part of a larger Swedish project studying the effects on the health of the infant of perinatal smokeless tobacco (Swedish snus) exposure. Healthy pregnant women (n = 55) were included prospectively at their first antenatal appointment. All women included in the study thereafter completed standardized questionnaires at the time of inclusion, again at gestational week 32, after delivery and at the time their infants were tested at 1–2 months after birth. Upon inclusion, women were grouped into snus users (n = 23), smokers (n = 13) and nicotine-free controls (n = 19). Dual users were excluded from the analysis.

To investigate the effects of "prenatal exposure without postnatal exposure," the tobacco-exposed groups were merged into groups by time of exposure: the first comprising infants with only early prenatal exposure (until gestational week 6–26); and the second comprising those infants exposed continuously during the entire period of gestation. Children whose mothers did not use tobacco postnatally, or who were formula-fed by snus-using mothers, were labelled as not postnatally exposed.

In addition, infants with "prenatal and (ongoing) postnatal exposure" were compared to nicotine-free controls and the prenatal exposure only group. The smoke group was exposed postnatally by smoke from the mother and by breast milk if breast fed. Second-hand smoke was considered prevalent if the mother was exposed to passive smoking.

At 4–10 weeks after delivery, mother and infant presented to the nursery at their hospital. A urine sample from the infants was collected for cotinine analysis. A portable ECG recorder (LifeCard CF, Spacelabs Healthcare Limited, Hertford, UK), connected with three channel electrodes, was used to record a digital 24-hour ECG (sampling frequency 1024 Hz). All recordings were made at home.

Questionnaires

Questions included medications and health status of the mother and infant as well as the type and pattern of tobacco use. The questionnaires also covered mothers' exposure to second-hand smoking (hours per day) and maternal use of alcohol and drugs. The reported average daily snus intake numbers (0–1, 2–3, 4–5, 6–7, >7 portions) were converted to mean nicotine dose (mg/day) taking into account the nicotine content of different brands. Regular snus brands contain ~8 mg/portion whereas "mini" portions contain ~4 mg/portion. Cigarettes were defined accordingly (<1, 1–5, 6–10, 11–15, 16–20, >20) with an estimated nicotine dose of 2 mg/cigarette.[25,26] Daily doses were estimated for each week during pregnancy and after the baby was born. Doses were defined as high if >56 mg/day for snus or >40 mg/day for smokers.[27] The different ways of absorption and individual differences in smoking a cigarette or in the amount of time used for each snus portion makes it difficult to directly compare the estimated nicotine doses between the two tobacco groups.[25,26]

Cotinine Analysis

Following preparation, urine samples were analyzed by mass spectrometry (UHPLC-MS/MS in MRM mode). Measuring range was from 5–1000 ng/mL for all analyses. For urine analysis of cotinine, precision at 7 and 700 ng/mL was 8.3 and 4.3 CV%. An internal

standard (cotinine-d_4 from Sigma Aldrich, Stockholm, Sweden) was prepared using blank urine and urine analyses and a 50 µL human urine sample was mixed with 100 µL internal standard.

ECG Recordings

The digital ECG recordings were edited and a HRV analysis was made with Impressario software (Version 7 Del Mar Reynolds Medical, Hertford, UK). All 24-hour recordings were reviewed by a single analyst (FN) to ensure accurate detection of all QRS complexes (crucial because the quality of the HRV analysis depends upon accurate labeling of the QRS complexes). Editing was made on the full 24-hour data file and a 2-hour artifact-free period during night-time sleep (between 10:00 PM and 6:00 AM) was selected for frequency domain analysis.

At least 22 hours of edited data from the 24-hour recording were required for acceptable detection of extra systoles and arrhythmias. Complexes classified as ectopic or noise were rejected.

We performed spectral analysis of 5-minute epochs during the 2-hour period of sleep. Early beat-to-beat variation was set to < 40%.[21] The interval between the R waves in every normal QRS complex was measured; only normal-to-normal R-R intervals were used the HRV analysis (ie, extra systoles were excluded).

For the analysis of the frequency domain indexes, beat-to-beat fluctuations were transformed to the frequency domain by fast Fourier transformation, and the specific measures were computed as the square root of the areas under the power spectrum, expressed as ms^2/Hz.

Spectral indexes over three standard frequency regions of interest was determined; very low frequency (VLF, 0.017–0.05 Hz), low frequency (LF, 0.05–0.150 Hz), and high frequency (HF, 0.15–0.5 Hz). Total power (all frequencies between 0.017–0.5 Hz) was also determined as well as the ratio between low and high frequency (LF/HF). The spectral analysis was made in accordance with the standards of the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology.[21]

Ethical Approval

This study was approved by the local ethics committee in Stockholm, and written consent was obtained from the mother at the time of inclusion.

Statistics

Descriptive statistics were presented as mean and standard deviations and median (range) for numerical variables, or as frequencies for categorical variables. Differences between subject characteristics of the groups were examined using chi-square test or Fischer's Exact test for categorical variables and one-way ANOVA for continuous variables. Further analysis with Tukey's post hoc pairwise comparison was made if the overall ANOVA test was significant. A two-way ANOVA was used to examine the interaction between nicotine group and the sex of the infant. Simple main effects were used to evaluate heterogeneous effects in the presence of significant interactions. Effect size was examined by difference in mean, 95% confidence interval, partial eta-squared (η^2), *R*-squared, and the Spearman correlation coefficient. The Mann–Whitney *U*-test was used because of deviations from normality in the estimated nicotine exposure and urine cotinine levels. Spearman's correlation was used to examine the relationship between maternal nicotine exposure and measured levels of cotinine in the urine of the infants. Multiple linear regression was employed to control for potential confounders. The dependent variable was HRV and relevant clinical and demographic explanatory variables were included in the model, including maternal age, sex of the infant and breast feeding. We followed a systematic approach to identify the explanatory variables. At first, simple linear regression was used to examine the relationship between the predictor variables and the outcome variable in univariate models. After completing the univariate analyses, we selected variables for the multivariate analyses. Any variable whose univariate test had a *p*-value < .1 was considered as a candidate for the multivariate model, along with variables of known clinical importance. Variables identified were entered into a multivariate model. Potential outlier and multi-collinearity problems were checked. Residual plots, normal probability plot and Cook's distance were used to assess model assumptions. A Cook's distance value (D) > 1 was taken as a criterion to constitute a strong indication of outlier problems and $D > 4/n$ a criterion to indicate a possible problem, where n was the sample size. A Variation Inflation Factor (VIF) greater than 4 was considered as the cut-off criterion for deciding when a given independent variable displayed "too great" a multicollinearity problem. SPSS for Windows version 23 (IBM Corp, Armonk, NY) was used for all statistical analysis. The level of significance was specified at 0.05.

To attain 80% power and considering a 5% significance level and expecting a mean difference in LF/HF of 1.15 (between control and exposed groups) with a standard deviation of 0.95 and design effect of 1.5, we estimated that 54 subjects would be sufficient.[28]

Results

Participants

The 55 healthy pregnancies resulted in 56 infants, including one twin pregnancy (born preterm, week 34). Except for preterm birth, there was no morbidity or hospitalization reported for any of the infants. We did not observe significant differences between the groups except that tobacco-exposed mothers were younger than controls ($p = .009$), and that the estimated nicotine exposure of snus versus smokers during the first trimester and at the time of examination differed ($p < .001$ and $p = .009$, respectively). There was no reported use of drugs or alcohol in any of the groups. Descriptive data are displayed in Table 1.

Nicotine Consumption

Eleven women stopped using tobacco during pregnancy: six smokers quit during gestational week 6–10, and five snus users quit during gestational week 6–26. None of the mothers in the study changed their source of tobacco during the study, and none used nicotine gums or patches. In addition to maternal nicotine consumption, the postnatal exposure of the infants also depended on passive smoking and whether the child was breast fed or not. The reported maternal nicotine use was highly correlated to the urine cotinine levels of the infants (Spearman's correlation coefficient = 0.88, $p < .001$, in Supplementary Figure 1).

Cotinine Concentration in Urine

All controls had cotinine levels < 0.7 ng/mL, the snus group ranged from 0–408 ng/mL and the smoke group ranged from 0–129 ng/mL. All the infants in the snus and smoke groups without any reported postnatal exposure had cotinine levels < 0.7 ng/mL.

Electrocardiogram

Two ECG recordings were lost (one from the snus group due to technical problems and one from the smoke because the mother

disconnected the recording device), resulting in 54 recordings available for analysis. We did not observe statistically significant differences between controls, snus or smoke groups in R-R intervals (min, max or mean); the results are displayed in Table 2. There were occasional extra beats found in all groups, mostly supraventricular extra systoles and occasionally ventricular extra systoles, but no other arrhythmias were detected.

Heart Rate Variability

Spectral analysis of HRV showed that infants in the two tobacco-exposed groups had a comparable LF/HF ratio, which was significantly higher than that of the control group.

One-way ANOVA showed the statistically significant difference in LF/HF ($F_{2,51} = 6.97$, $p = .002$, partial $\eta^2 = 0.22$). The main differences for infants in the snus group (mean difference = 1.16, 95% CI = 0.29–2.02, $p = .006$) and the smoke group (mean difference = 1.36, 95% CI = 0.32–2.4, $p = .007$) were statistically significant higher compared to controls. However, we did not observe any difference between infants in the snus and smoke groups (mean difference = 0.20, 95% CI = −0.8 to 1.2, $p = .88$) (Table 2 and Figure 1).

We also observed a difference in HF ($F_{2,51} = 3.2$, $p = .049$, partial $\eta^2 = 0.11$) with a higher HF activity in the control group compared to smoke group (mean difference = 64, 95% CI = 0.14–128, $p = .049$). We were unable to detect any respiratory sinus arrhythmia (RSA) peak up to and including 0.8 Hz in the HF area in any of the groups.

Two-way ANOVA revealed a significant interaction between the tobacco group and the baby's sex ($p = .03$), which suggests that the effect of tobacco on LF/HF was heterogeneous. The mean differences for both the snus group (mean difference = 1.65, 95% CI = 0.56–2.7, $p = .003$) and smoke group (mean difference = 2.7, 95% CI = 1.4–4, $p < .001$) compared to controls were higher among boys. We did not observe significant difference amongst girls, with a difference of (0.84) amongst snus compared to controls (95% CI, -0.9–1.8, $p = .76$).

Table 1. Characteristics of Participants in Control, Snus and Smoke Group, Means and (*SD*)

	Control	Snus	Smoke
Women (*n* = 55)	*n* = 19	*n* = 23	*n* = 13
Age mother (years)	33.5 (4.7)	29 (6.3)	27.8 (4.8)
Weight mother (kg)	64.1 (9.6)	66 (11.3)	63.2 (9.1)
Height mother (cm)	167.4 (4.1)	165.5 (5.7)	164.8 (5.4)
Parity (*n*)	1.2 (0.5)	1.2 (1.1)	0.4 (0.5)
Nicotine first trimester (mg/day)	0	42.3 (11–56)	20 (6.7–40)
Nicotine dose at examination (mg/day)	0	17.5 (0–56)	2 (0–40)
Vaginal/cesarean delivery (*n*)	15 (79%)/4 (21%)	20 (87%)/3 (13%)	9 (69%)/4 (31%)
SHS exposure during pregnancy (*n*)	0	2 (9%)	3 (23%)
SHS exposure postpartum (*n*)	0	1 (4%)	2 (15%)
Infants (*n* = 56)	*n* = 19	*n* = 24	*n* = 13
Gestational (week)	40 (0.9)	39.1 (2.0)	39.9 (1.1)
Birthweight (g)	3727 (264)	3426 (555)	3501 (670)
SGA/LGA (*n*)	0/2 (10%)	0/0	2 (15%) /2 (15%)
Age at examination (week)	6 (1.4)	5.8 (1.6)	6.3 (1.5)
Weight at examination (g)	4833 (447)	4860 (665)	4841 (758)
Boy/girl (*n*)	7 (37%)/12 (63%)	12 (50%)/12 (50%)	5 (38%)/8 (62%)
Cotinine urine (ng/mL)	0.07 (0.2)	59.2 (95.9)	29.6 (44.0)
Breast fed/formula (*n*)	19 (100%)/0	19 (79%)/5 (21%)	11 (85%)/2 (15%)
Postnatal tobacco exposure (*n*)	0	14 (58%)	7 (54%)

SGA = Small for gestational age; LGA = large for gestational age.
Characteristics of women are data from the first visit to the maternity center. Second-hand smoke (SHS), defined as maternal exposure.

Table 2. Heart Rate Variability in Control, Snus and Smoke Groups and Prenatal Exposure "without" Postnatal Exposure (Prenatal Early Exposure and Continuous Prenatal Exposure Groups), in mean (*SD*)

	Control *n* = 19	Snus *n* = 23	Smoke *n* = 12	*p* ANOVA	Control *n* = 19	Prenatal-early *n* = 8	Prenatal-continuous *n* = 7	*p* ANOVA
max RR (ms)	733.5 (73.6)	728.3 (99.4)	670.6 (92.4)	.13	733.5 (73.6)	685.6 (108.4)	709.8 (95.4)	.42
min RR (ms)	303.5 (43.4)	304.4 (32.9)	296.9 (38.8)	.85	303.5 (43.4)	302.7 (44.1)	294.6 (22.9)	.88
mean RR (ms)	466.8 (26.5)	460.5 (36.7)	458.8 (24.6)	.73	466.8 (26.5)	462.7 (32.2)	438 (14.6)	.056
sd RR (ms)	40.6 (8)	39.9 (11.2)	37.2 (6.3)	.59	40.6 (8)	34.9 (7.6)	35.4 (6.7)	.14
VLF (ms^2)	322.8 (170.6)	362.5 (269.8)	314.4 (122.6)	.76	322.8 (170.6)	317.2 (160.1)	272.8 (81.4)	.76
LF (ms^2)	249.4 (100)	294.4 (198.4)	217.6 (101.9)	.34	249.4 (100)	217.4 (121.5)	231 (125.7)	.78
HF (ms^2)	136.2 (87.1)	96.9 (68)	72.2 (46.7)	.049	136.2 (87.1)	76.4 (57.1)	51.8 (23.1)	.023
total power (ms^2)	708.3 (309.4)	753.8 (500.8)	604.3 (232.9)	.57	708.3 (309.4)	611 (323.8)	555.7 (189.3)	.46
LF/HF	2.15 (0.81)	3.31 (1.2)	3.51(1.51)	.002	2.15 (0.81)	3.20 (0.77)	4.40(1.1)	< .001

HF = High frequency; LF = low frequency; VLF = very low frequency.

On merging the two tobacco groups for prenatal-early exposure ($n = 8$), prenatal-continuous exposure ($n = 7$) and controls ($n = 19$), we found statistically significant differences between the groups for the dependent variable LF/HF ($F_{2,31} = 18.06$, $p < .001$, partial $\eta^2 = 0.54$) and HF ($F_{2,31} = 4.3$, $p = .023$, partial $\eta^2 = 0.22$), respectively (Table 2). There was a higher LF/HF ratio in the early prenatal group than in the control group (mean difference = 1.04, 95% CI = 0.15–1.9, $p = .02$). The prenatal-continuous exposure group had an even higher LF/HF ratio than that of controls (mean difference = 2.24, 95% CI = 1.3–3.2, $p < .001$), with the difference significant compared to prenatal-early exposure group (mean difference = 1.2, 95% CI = 0.1–2.3, $p = .037$). The HF activity of the prenatal-continuous group was lower than that of the control group (mean difference = −84.4, 95% CI = −163.2 to −5.6, $p = .03$). The results of prenatal tobacco exposure are shown in Table 2 and Figure 2.

Infants with both pre- and postnatal exposure (total $n = 20$: snus $n = 13$, smoke $n = 7$) had higher LF/HF compared to controls (mean difference 0.94, 95% CI 0.06–1.81, $p = .033$), with no significant difference versus prenatal-only exposure ($p = .206$).

With respect to the acute dose–response relationship at the time of examination, we found no statistically significant relationship between cotinine levels in infants' urine or the reported nicotine dose and any HRV values.

We did not observe a statistically significant relationship between LF/HF and birth weight, baby's sex, mother's weight, parity, gestational week at delivery, infants' age and weight at examination in a multiple linear regression model. Simple linear regression revealed that breast feeding was a significant predictor of LF/HF (β = −0.53, 95% CI = −1.03 to −0.3, $p = .04$), accounting for 8% of the variance in LF/HF. However, multiple linear regression displayed non-significant findings when LF/HF was regressed on relevant clinical and demographic variables. We did not observe violations of assumptions of ANOVA and regression analysis and multicollinearity and outlier problems.

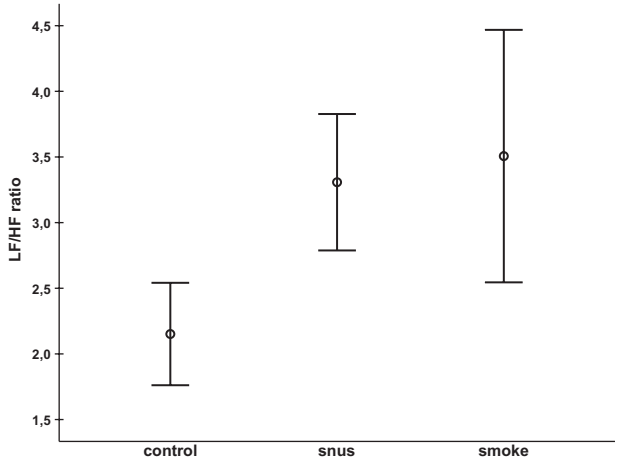

Figure 1. HRV in control, snus and smoke groups. LF/HF ratio in original groups based upon inclusion, mean and 95% CI. LF/HF ratio: low frequency/high frequency ratio made by spectral analysis of heart rate variability.

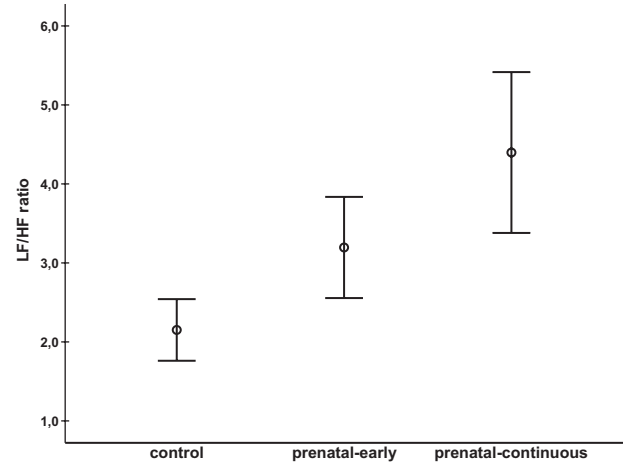

Figure 2. HRV in prenatal exposure without postnatal exposure. LF/HF ratio in control, prenatal early exposure without any further exposure and prenatal continuous exposure (whole gestation without postnatal exposure), mean and 95% CI. The snus and smoke groups are merged together in the exposure groups.

Discussion

We studied HRV in infants of mothers who used snus during pregnancy, which, to our knowledge, has not been done previously. Studies have shown reduced HRV (especially in parasympathetic activity) of infants exposed to cigarette smoke and those at higher risk of SIDS. In this study we demonstrate effects of prenatal tobacco exposure on cardiovascular regulation that extended well beyond the neonatal period. We found a higher LF/HF ratio in both snus- and smoke-exposed groups compared with controls, which was partly explained by lower HF activity, suggesting less vagal activity. The lack of RSA peak usually found in the HF area is conceivably due to the peak frequency of RSA lying above the 0.8 Hz cut-off or also possibly because RSA is low-to-absent at this early age, as suggested by human and animal data.[23,29,30] There was no significant difference between LF/HF ratios of the two tobacco-exposed groups, which suggests that nicotine may be the common constituent of tobacco mediating these effects regardless of the precise route by which it is absorbed. Cotinine concentrations in the urine of the infants of each group showed a good correlation with the reported nicotine doses by the mother and the highest values were observed in the infants of snus users.

Power spectral analysis of beat-to-beat variations in heart rate provides an indirect index of the autonomic modulation of the cardiovascular system. In the fetus and infant, sympathetic drive tends to dominate, with a gradual increase in parasympathetic activity and progressive decrease in sympathetic modulation of the autonomic system with advancing age.[31] Altered autonomic cardiorespiratory regulation is associated with an increased risk of SIDS. Infants who later succumbed to SIDS appeared to show reduced overall heart rate variability,[22] and lower variability due to reduced RSA during sleep.[32] An increased LF/HF ratio has been shown in infants born to smoking mothers during REM sleep.[33]

Nicotine per se may be of importance for the infants' health, as a large body of experimental animal data supports the concept that prenatal and perinatal nicotine exposure is detrimental to postnatal autonomic control. This autonomic dysfunction may be involved in SIDS, although the exact mechanism is not fully understood. Nicotine-exposed rat pups show enhanced expression of cardiac M2-muscarinic cholinergic receptors involved in inhibitory autonomic actions.[34] Mice lacking the b2-nAChR subtype exhibit autonomic control deficits (including unstable breathing, impaired arousal and catecholamine biosynthesis), much like nicotine-exposed mice.[35] Furthermore, nicotine-exposed animals exhibit

impaired cardiac regulation of sino-atrial reactivity,[7] augmented inhibitory cardio-vagal neural control,[36] and changes in medullary neurotransmitter receptors and altered heart rate variability.[23] Thus, a number of pathophysiological effects of prenatal nicotine exposure may interact.

We also found an increased LF/HF ratio in infants exposed to tobacco before but not after birth. This has been difficult to study before because most infants born to smokers receive a combination of prenatal and postnatal exposure. Most animal studies also include a combination of prenatal and perinatal exposure. Our study included infants whose exposure effectively ceased before or at birth, either because mother ceased tobacco use during pregnancy, or chose not to breast feed (snus group). The persistence of a relatively high LF/HF ratio for several months after exposure effectively ended suggests that there is a residual impact on autonomic system function. This may be due either to delayed maturation[29] or an altered trajectory of development, a finding which is supported by several studies. Human fetuses exposed early-on to tobacco show persistent changes in the expression of nicotinic and muscarinic acetylcholine receptors in the brainstem and cerebellum.[37] Infants of smokers who experience a combination of prenatal and postnatal exposure exhibit persistent mild dysfunction in blood pressure control during sleep.[38] Rat studies reveal long-lasting effects of prenatal nicotine exposure into adolescence and adulthood, such as persistent cholinergic hypoactivity and an altered sensitivity to nicotine.[39] The high levels of nicotine cholinergic receptors in the brainstem in mid-gestation, and rapid changes in the profile of receptor subtype expression during late gestation suggested that these are periods of particular vulnerability to nicotine,[40] in addition to any changes that occur earlier, for example, during the first trimester.[41] Our finding that autonomic regulation may be altered even if the infant is only exposed during first trimester suggests there is probably no time-point during development when exposure is "safe," that is to say, when the autonomic system is unaffected by constituents of tobacco, such as nicotine. Although we cannot establish causality, it is conceivable that prenatal nicotine exposure exerts a programming effect, altering the normal development of the autonomic regulation.

Boys may be more vulnerable to prenatal and postnatal tobacco exposure than girls, the former exhibiting a more pronounced rise in LF/HF ratio. This finding is supported by earlier studies of maternal smoking and secondhand exposure on RSA in infants (exposed boys had lower RSA vs. exposed girls).[42] Boys have an increased risk of SIDS,[43] and other studies also support the hypothesis that sex differences in HRV are associated with common risk factors such as preterm birth and intrauterine growth retardation.[44,45] Sex differences in HRV of healthy children is much more controversial.[46,47]

Our study has a number of strengths: it was a prospective study from early gestation to infancy, we conducted a high quality HRV analysis and carefully documented tobacco exposure at four longitudinal time points, and used an objective biomarker measures of nicotine exposure (cotinine analysis of the infants' urine). There were, however, limitations, the most important of which was the relatively small size of the groups, which also limits stratified analysis. Although we cannot definitively rule out the possibility of differences between the smoke-exposed and snus-exposed groups, our data suggest that this is unlikely. Secondly, the infants' actual nicotine exposure was estimated at only one time-point (ie, cotinine in urine at the time of investigation). Although highly correlated to the mothers' reported dose, a single urinary estimate may not be truly representative of the level of exposure at other times, or throughout gestation. Finally, although we did take into account passive smoking and the use of alcohol and other drugs, it is possible that there might be other factors in addition to nicotine that influence HRV.

In conclusion, we demonstrate an effect on autonomic regulation in infants of mothers using snus during pregnancy, in terms of an increase in LF/HF ratio comparable to that of infants of active smokers. Even very early prenatal exposure to tobacco has a long-lasting impact on autonomic cardiac regulation. It is apparent that tobacco can affect the developing infant regardless of the form, or route by which, or time-point during development when exposure actually occurs. Our data consequently emphasize the importance of abstaining from all forms of tobacco use during pregnancy, and suggest that there are no safe forms or safe periods for its use during pregnancy. Our findings may also have more general relevance to other routes by which nicotine can be delivered to a fetus and newborn, including NRT for smoking cessation.

Supplementary Material

Supplementary data are available at *Nicotine & Tobacco Research* online.

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Declaration of Interests

None declared.

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References

1. Lee YO, Hebert CJ, Nonnemaker JM, Kim AE. Youth tobacco product use in the United States. *Pediatrics*. 2015;135(3):409–415.
2. Kvaavik E, Lund I, Nygård M, Hansen BT. Lifestyle correlates of female snus use and smoking: a large population-based survey of women in Norway. *Nicotine Tob Res*. 2016;18(4):431–436.
3. Baba S, Wikström AK, Stephansson O, Cnattingius S. Changes in snuff and smoking habits in Swedish pregnant women and risk for small for gestational age births. *BJOG*. 2013;120(4):456–462.
4. The Swedish Medical Birth Register. *Swedish National Board of Health and Welfare*. 2014.
5. Mei-Dan E, Walfisch A, Weisz B, Hallak M, Brown R, Shrim A. The unborn smoker: association between smoking during pregnancy and adverse perinatal outcomes. *J Perinat Med*. 2015;43(5):553–558.
6. Haglund B, Cnattingius S. Cigarette smoking as a risk factor for sudden infant death syndrome: a population-based study. *Am J Public Health*. 1990;80(1):29–32.
7. Slotkin TA, Saleh JL, McCook EC, Seidler FJ. Impaired cardiac function during postnatal hypoxia in rats exposed to nicotine prenatally: implications for perinatal morbidity and mortality, and for sudden infant death syndrome. *Teratology*. 1997;55(3):177–184.
8. Luck W, Nau H, Hansen R, Steldinger R. Extent of nicotine and cotinine transfer to the human fetus, placenta and amniotic fluid of smoking mothers. *Dev Pharmacol Ther*. 1985;8(6):384–395.
9. Foulds J, Ramstrom L, Burke M, Fagerström K. Effect of smokeless tobacco (snus) on smoking and public health in Sweden. *Tob Control*. 2003;12(4):349–359.

10. Borgerding MF, Bodnar JA, Curtin GM, Swauger JE. The chemical composition of smokeless tobacco: a survey of products sold in the United States in 2006 and 2007. *Regul Toxicol Pharmacol*. 2012;64(3):367–387.
11. Baba S, Wikström AK, Stephansson O, Cnattingius S. Influence of smoking and snuff cessation on risk of preterm birth. *Eur J Epidemiol*. 2012;27(4):297–304.
12. Baba S, Wikström AK, Stephansson O, Cnattingius S. Influence of snuff and smoking habits in early pregnancy on risks for stillbirth and early neonatal mortality. *Nicotine Tob Res*. 2014;16(1):78–83.
13. Gunnerbeck A, Wikström AK, Bonamy AK, Wickström R, Cnattingius S. Relationship of maternal snuff use and cigarette smoking with neonatal apnea. *Pediatrics*. 2011;128(3):503–509.
14. England LJ, Levine RJ, Mills JL, Klebanoff MA, Yu KF, Cnattingius S. Adverse pregnancy outcomes in snuff users. *AJOG*. 2003;189(4):939–943.
15. Juárez SP, Merlo J. The effect of Swedish snuff (snus) on offspring birthweight: a sibling analysis. *PLoS One*. 2013;8(6):e65611.
16. Inamdar AS, Croucher RE, Chokhandre MK, Mashyakhy MH, Marinho VC. Maternal smokeless tobacco use in pregnancy and adverse health outcomes in newborns: a systematic review. *Nicotine Tob Res*. 2015;17(9):1058–1066.
17. Slotkin TA. If nicotine is a developmental neurotoxicant in animal studies, dare we recommend nicotine replacement therapy in pregnant women and adolescents? *Neurotoxicol Teratol*. 2008;30(1):1–19.
18. Dahlin S, Gunnerbeck A, Wikstrom AK, Cnattingius S, Edstedt Bonamy AK. Maternal tobacco use and extremely premature birth - a population-based cohort study. *BJOG*. 2016;123(12):1938–1946.
19. Wickström R. Effects of nicotine during pregnancy: human and experimental evidence. *Curr Neuropharmacol*. 2007;5(3):213–222.
20. Coleman T, Chamberlain C, Cooper S, Leonardi-Bee J. Efficacy and safety of nicotine replacement therapy for smoking cessation in pregnancy: systematic review and meta-analysis. *Addiction*. 2011;106(1):52–61.
21. Heart rate variability. Standards of measurement, physiological interpretation, and clinical use. Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology. *Eur Heart J*. 1996;17(3):354–381.
22. Schechtman VL, Raetz SL, Harper RK, et al. Dynamic analysis of cardiac R-R intervals in normal infants and in infants who subsequently succumbed to the sudden infant death syndrome. *Pediatr Res*. 1992;31(6):606–612.
23. Duncan JR, Garland M, Myers MM, et al. Prenatal nicotine-exposure alters fetal autonomic activity and medullary neurotransmitter receptors: implications for sudden infant death syndrome. *J Appl Physiol (1985)*. 2009;107(5):1579–1590.
24. Kahn A, Sawaguchi T, Sawaguchi A, et al. Sudden infant deaths: from epidemiology to physiology. *Forensic Sci Int*. 2002;130(suppl):S8–20.
25. Benowitz NL, Porchet H, Sheiner L, Jacob P III. Nicotine absorption and cardiovascular effects with smokeless tobacco use: comparison with cigarettes and nicotine gum. *Clin Pharmacol Ther*. 1988;44(1):23–28.
26. Digard H, Proctor C, Kulasekaran A, Malmqvist U, Richter A. Determination of nicotine absorption from multiple tobacco products and nicotine gum. *Nicotine Tob Res*. 2013;15(1):255–261.
27. Digard H, Errington G, Richter A, McAdam K. Patterns and behaviors of snus consumption in Sweden. *Nicotine Tob Res*. 2009;11(10):1175–1181.
28. Mehta SK, Super DM, Connuck D, et al. Heart rate variability in healthy newborn infants. *Am J Cardiol*. 2002;89(1):50–53.
29. Longin E, Gerstner T, Schaible T, Lenz T, König S. Maturation of the autonomic nervous system: differences in heart rate variability in premature vs. term infants. *J Perinat Med*. 2006;34(4):303–308.
30. Harper RM, Walter DO, Leake B, et al. Development of sinus arrhythmia during sleeping and waking states in normal infants. *Sleep*. 1978;1(1):33–48.
31. Yiallourou SR, Sands SA, Walker AM, Horne RS. Maturation of heart rate and blood pressure variability during sleep in term-born infants. *Sleep*. 2012;35(2):177–186.
32. Kluge KA, Harper RM, Schechtman VL, Wilson AJ, Hoffman HJ, Southall DP. Spectral analysis assessment of respiratory sinus arrhythmia in normal infants and infants who subsequently died of sudden infant death syndrome. *Pediatr Res*. 1988;24(6):677–682.
33. Franco P, Chabanski S, Szliwowski H, Dramaix M, Kahn A. Influence of maternal smoking on autonomic nervous system in healthy infants. *Pediatr Res*. 2000;47(2):215–220.
34. Slotkin TA, Epps TA, Stenger ML, Sawyer KJ, Seidler FJ. Cholinergic receptors in heart and brainstem of rats exposed to nicotine during development: implications for hypoxia tolerance and perinatal mortality. *Brain Res Dev Brain Res*. 1999;113(1–2):1–12.
35. Cohen G, Roux JC, Grailhe R, Malcolm G, Changeux JP, Lagercrantz H. Perinatal exposure to nicotine causes deficits associated with a loss of nicotinic receptor function. *Proc Natl Acad Sci U S A*. 2005;102(10):3817–3821.
36. Huang ZG, Wang X, Evans C, Gold A, Bouairi E, Mendelowitz D. Prenatal nicotine exposure alters the types of nicotinic receptors that facilitate excitatory inputs to cardiac vagal neurons. *J Neurophysiol*. 2004;92(4):2548–2554.
37. Falk L, Nordberg A, Seiger A, Kjaeldgaard A, Hellström-Lindahl E. Smoking during early pregnancy affects the expression pattern of both nicotinic and muscarinic acetylcholine receptors in human first trimester brainstem and cerebellum. *Neuroscience*. 2005;132(2):389–397.
38. Cohen G, Jeffery H, Lagercrantz H, Katz-Salamon M. Long-term reprogramming of cardiovascular function in infants of active smokers. *Hypertension*. 2010;55(3):722–728.
39. Abreu-Villaça Y, Seidler FJ, Tate CA, Cousins MM, Slotkin TA. Prenatal nicotine exposure alters the response to nicotine administration in adolescence: effects on cholinergic systems during exposure and withdrawal. *Neuropsychopharmacology*. 2004;29(5):879–890.
40. Kinney HC, O'Donnell TJ, Kriger P, White WF. Early developmental changes in [3H]nicotine binding in the human brainstem. *Neuroscience*. 1993;55(4):1127–1138.
41. Zeskind PS, Gingras JL. Maternal cigarette-smoking during pregnancy disrupts rhythms in fetal heart rate. *J Pediatr Psychol*. 2006;31(1):5–14.
42. Schuetze P, Eiden RD. The association between maternal smoking and secondhand exposure and autonomic functioning at 2-4 weeks of age. *Infant Behav Dev*. 2006;29(1):32–43.
43. Adams SM, Good MW, Defranco GM. Sudden infant death syndrome. *Am Fam Physician*. 2009;79(10):870–874.
44. Smith SL, Lux R, Haley S, Slater H, Beachy J, Moyer-Mileur LJ. The effect of massage on heart rate variability in preterm infants. *J Perinatol*. 2013;33(1):59–64.
45. Aziz W, Schlindwein FS, Wailoo M, Biala T, Rocha FC. Heart rate variability analysis of normal and growth restricted children. *Clin Auton Res*. 2012;22(2):91–97.
46. Longin E, Dimitriadis C, Shazi S, Gerstner T, Lenz T, König S. Autonomic nervous system function in infants and adolescents: impact of autonomic tests on heart rate variability. *Pediatr Cardiol*. 2009;30(3):311–324.
47. Silvetti MS, Drago F, Ragonese P. Heart rate variability in healthy children and adolescents is partially related to age and gender. *Int J Cardiol*. 2001;81(2–3):169–174.