Childhood exposure to passive smoking and bone health in adulthood. The Cardiovascular Risk in Young Finns Study

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Short title: Passive smoking and bone health

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Conflict of interest

There is no conflict of interest.

Disclosure statement

The authors have nothing to disclose.

Key Words: Passive Smoking, Cotinine, Bone Mineral Density

Precis

In this longitudinal 28-year follow-up study including 1422 individuals, parental smoking and elevated cotinine levels in childhood were associated with lower bone mineral density in adulthood.

Abstract

Context: Passive smoke exposure has been linked with the risk of osteoporosis in adults.

Objective: We aimed to examine the independent effects of exposure to passive smoking in childhood on adult bone health.

Design/Setting: Longitudinal, the Cardiovascular Risk in Young Finns Study

Participants: Study cohort included 1422 individuals followed up for 28 years since baseline in 1980 (age 3-18 years). Exposure to passive smoking was determined in childhood. In adulthood, peripheral bone traits were assessed with quantitative computed tomography (pQCT) at the tibia and radius, and calcaneal mineral density was estimated with quantitative ultrasound. Fracture data was gathered by questionnaires.

Results: Parental smoking in childhood was associated with lower pQCT derived bone sum index in adulthood (β±SE -0.064±0.023 per smoking parent, P=0.004) in multivariate models adjusted for age, sex, active smoking, BMI, serum 25-OH vitamin D concentration, physical activity, and parental socioeconomic position. Similarly, parental smoking was associated with lower heel ultrasound estimated bone mineral density in adulthood (β±SE -0.097±0.041 per smoking parent, P=0.02). Parental smoking was also associated with the incidence of low-energy fractures (odds ratio 1.28, 95% confidence interval 1.01-1.62). Individuals with elevated cotinine levels (3-20 ng/ml) in childhood had lower bone sum index with pQCT (β±SE -0.206±0.057, P=0.0003). Children whose parents smoked and had high cotinine levels (3-20 ng/ml) had significantly lower pQCT derived bone sum index compared to those with smoking parents but low cotinine levels (<3ng/ml) (β±SE -0.192±0.072, P=0.008).

Conclusions and relevance: Children of parents who smoke have evidence of impaired bone health in adulthood.

Introduction

Osteoporosis is a chronic systemic skeletal disease associated with elevated bone fracture risk due to a reduction in bone mass and alterations in bone quality. It is becoming an increasing public health concern along with aging populations[1]. Osteoporosis annually contributes to approximately 10 million fractures[2]. Although these fractures mostly occur in elderly people, the risk of osteoporosis may be influenced by early life exposures effecting the growing bone[3]. Therefore, it is important to identify the determinants of bone health. In prior studies, childhood growth/adiposity, physical activity and socioeconomic position have been related with bone quantity and quality[4-6].

An important environmental factor linked with osteoporosis in adults is exposure to tobacco smoke. The effects of smoking are cumulative over time, with an additional bone loss (independent of body weight and physical activity) of 4% by age 70, 6% by age 80, and 8% by age 90 years[7]. Meta-analyses have reported an increased fracture risk related to smoking in both men and women[7-9]. Importantly, exposure to secondhand smoke, i.e. passive smoking, [10-12] has also been associated with osteoporosis. However, little is known of the bone health effects of childhood exposure to passive smoking. One retrospective study in premenopausal women suggested a link between self-reported passive smoking in adolescence and reduced bone mass in adulthood[12].

In the present study, we aimed to examine passive smoking exposure in childhood (age 3-18 years) as a determinant of bone health at the skeletal maturity in mid-adulthood (age 31-46 years) among 1422 individuals. The analyses were performed in the longitudinal Cardiovascular Risk in Young Finns Study with data on peripheral bone traits of radius, tibia and calcaneus assessed with quantitative computed tomography[13] and heel ultrasonography[14], and questionnaire based information on low-energy fractures.

Materials and methods

Description of the Cardiovascular Risk in Young Finns Study has been published previously[15]. The study was approved by the institutional ethics committees, and written informed consent was obtained from all the study participants or their parents. Present analysis included 1422 participants who had a baseline evaluation during childhood in 1980 and a follow-up bone health examination 28 years later in adulthood. To control for active smoking in childhood and adolescence, the analyses were conducted after excluding the participants who were active smokers during the baseline evaluation. Detailed methods are provided in the Supplementary Appendix[16].

Childhood exposure measures – parental smoking and serum cotinine

Parents of participants self-reported their smoking habits at baseline[17]. One parent responding on behalf of both parents was asked to indicate the smoking status separately of the mother and father in the household from two questions. The first question was whether mother/father had ever smoked daily for at least one year (responses "Yes" or "No"), and the second question whether mother/father were currently smoking (responses "does not smoke", "occasionally", "daily"). Mothers or fathers indicating they had ever smoked daily for at least one year were designated as ever smokers. Mothers or fathers that indicated currently occasionally or daily smoking were designated as current smokers.

Serum samples were collected in 1980 and stored at -20°C until they were analyzed in 2014. During storage, samples were not thawed or refrozen. Serum cotinine measures were performed using standardized methods[18]. Cotinine values between 3-20 ng/ml in non-smokers were considered as indicative of positive nicotine exposure indicative of passive smoking (the concentration that could be detected reproducibly in the assay).

A three-level variable to indicate parental smoking hygiene was constructed as follows: 1 = no parental smoking and non-detectable cotinine level; 2 = parental smoking and non-detectable cotinine level (hygienic parental smoking); and 3 = parental smoking and detectable serum cotinine (non-hygienic parental smoking).

123

124 *Adult outcome variables - bone traits*

125 Peripheral quantitative computed tomography (pQCT) (Stratec XCT 2000R, Germany) was used to scan two
126 sites (distal and diaphysis) of radius and tibia[19]. This method provides data on volumetric bone mineral density
127 (vBMD). The following bone traits were measured: total bone mineral content (mass), total and cortical bone
128 areas, and trabecular and cortical bone densities. In addition, three bone strength indices were estimated. From
129 these data, four composite indices of bone mass, density, area and strength were calculated. Additionally, the
130 four indices were combined into one age- and sex-standardized bone sum index. In attrition analyses
131 comparing baseline characteristics between those attending (N=1800) and those non-attending (N=1796) the
132 bone study, it was observed that non-attendants were younger (10.0 vs 10.9 years, P<0.001) and more often
133 males (55 vs 43%, P<0.0001). However, there were no differences in baseline BMI (17.8 vs. 17.9 kg/m^2,
134 P=0.20) or parental school years (10.7 vs 10.7 years, P=0.97). As another variable, we measured heel bone
135 traits with a quantitative ultrasound device (Sahara Clinical Bone Sonometer, Hologic, Waltham, MA, USA)
136 among 1210 participants. It measures the speed of sound and ultrasound attenuation at the mid-calcaneus as
137 the sound waves traverse through bone tissue. The speed of sound (m/s) is linearly dependent on bone mineral
138 density[20]. Additionally, an estimate of heel bone mineral density Z-score was calculated as the difference from
139 the age- and sex-specific averages. Persons performing bone measurements were blinded to the parental
140 smoking status. Detailed description of measurement and calculation of bone traits is given in the
141 Supplementary appendix[16].

142 *Fractures*

143 During the bone study visit, participants were inquired their fracture history. Questionnaire information on
144 fracture site, fracture age, and how the fracture occurred were collected. Fractures were classified as low-
145 energy fractures if they were sustained in standing positions, and in the absence of excess strain due to falling
146 from heights greater than standing level or due to the high speed of a vehicle used (cycling, skiing, skating),
147 or if no other person or external factor was involved.

148

149 *Covariates*

150 To control for the effect of body size, we utilized serial data on height and weight collected in clinical
151 examinations in 1980, 1983 and 1986 to calculate the estimated area under the body mass index curve between
152 ages 6 and 24 years[21]. Data collected in study years 1980, 1983 and 1986 were used to estimate physical activity
153 in childhood, adolescence and young adulthood. At ages 3 and 6 years, a preschool children physical activity
154 index was calculated from the parents' ratings of the amount and vigorousness of their child's play time and
155 the child's general level of activity as compared with other children[22]. At ages 9-24 years data on frequency
156 and intensity of physical activity during leisure time were acquired with a self-administered questionnaire and
157 a sum index of physical activity was calculated[23]. The values for physical activity indices were standardized
158 and the average value was used as a measure of physical activity exposure during the time of peak bone mass
159 accrual. As a marker of nutritional vitamin D status, the baseline (year 1980) circulating 25-OH vitamin D
160 concentration was measured using radioimmunoassay (DiaSorin, Stillwater, MN). In adulthood, information
161 on smoking was collected with questionnaires in 2001 and 2007. Data on parental education (years) was used
162 as an indicator of the childhood socioeconomic status. Information on birth weight was collected using
163 questionnaires and confirmed from participants' records from well-baby clinics.

164

165 *Statistical analysis*

166 Statistical analysis was performed using SAS 9.3. Statistical significance was inferred by a P value <0.05.
167 Parental smoking, serum cotinine, and parental smoking hygiene were used as exposure variables in
168 multivariable linear regression models to examine effects of childhood passive smoking on pQCT and
169 ultrasound derived bone indices. Analyses using dichotomized fracture data as an outcome were performed
170 with logistic regression analyses. Stepwise multivariate regression with backward elimination was performed
171 to take into account the effects of possible intermediate or confounding factors. Variables in initial stepwise
172 multivariate models included age, sex, body mass index, physical activity, parental school years, and serum
173 25-OH vitamin D in childhood. Age and sex were forced into the final models. In addition, the effects of birth
174 weight and smoking in adulthood were controlled for in additional models.

175

We examined potential sex and age interactions by including interaction terms in logistic regression models. In addition, we investigated differences between the effects of a smoking mother and a smoking father. Replications of the analyses were done including only life-long non-smokers. Sensitivity analyses were performed using different cut-offs for serum cotinine (2.5, 3.0, 3.5 or 4.0 ng/L) to indicate passive smoking.

Results

Baseline characteristics (in 1980) stratified by parental smoking exposure are shown in Supplementary Table 1[16]. Bone measures, the calculation of bone indices and their mean values are shown in Supplementary Table 2[16].

Parental smoking and bone health

The effect of regular parental smoking during childhood on the pQCT derived bone indices in adulthood is shown in Table 1. In multivariable models, exposure to parental smoking was statistically significantly and inversely associated with the bone sum index, bone mass, bone density and bone strain, but not with bone area. The effect estimates of parental smoking remained essentially similar when the analyses were adjusted for birth weight (N=1214) or active smoking in adulthood (N=1345), or when active smokers in adulthood were excluded (N=1135). No evidence of statistically significant sex interactions was observed. Similarly, no significant age interactions were detected. The effect estimates were nearly identical for paternal smoking and maternal smoking.

Results for ultrasound measured bone indices are in Table 2. Parental smoking was inversely related with broadband attenuation, speed of sound and estimated bone mineral density Z-score. The findings remained essentially similar when additionally adjusted for birth weight (N=1067) or active smoking in adulthood (N=1171), or when active smokers in adulthood were excluded (N=961). There were no age-interactions, but statistically significant sex interactions were observed for all indices. In sex-stratified analyses, parental smoking was related with ultrasound derived bone indices among females (P always < 0.005), but not in males (P always > 0.6). The effect estimates were comparable for paternal and maternal smoking.

Questionnaire based low-energy fracture rates among individuals with 0, 1 and 2 smoking parents were 9.2, 12.0 and 13.7 %, respectively. Odds ratio per smoking parent was 1.28 (95% CI 1.01-1.62, P=0.04) in a logistic regression model adjusted for age, sex and childhood factors. The results remained essentially similar when

the analyses were additionally adjusted for birth weight or active smoking in adulthood, but the association was attenuated when active smokers in adulthood were excluded (P=0.24).

Cotinine exposure in childhood and bone health

Passive smoking in childhood, defined as a serum cotinine concentration between 3-20 ng/ml, was inversely associated with the pQCT derived bone sum index and the bone mass, density and strength indices in adulthood (Table 3). These associations remained similar after additional adjustment for birth weight (N=1047) or smoking in adulthood (N=1195), or when active smokers in adulthood were excluded (N=995). There were no significant age or sex interactions.

Concerning ultrasound measures, cotinine exposure was inversely associated with speed of sound (Table 4). Effect estimates were not altered after additional adjustments for birth weight (N=887) or active smoking in adulthood (N=1006), or when active smokers in adulthood were excluded (N=835). No significant age or sex interactions were observed.

Those individuals with low cotinine levels had a low-energy fracture rate of 11.3 % and among those with elevated cotinine it was 14.3% (P=0.15 in a logistic regression model adjusted for age, sex and childhood factors).

Parental smoking hygiene in childhood and bone health in adulthood

Figure 1 shows the association between parental smoking hygiene and bone indices. Children whose parents smoked non-hygienically (cotinine levels in children 3-20 ng/ml) had lower pQCT derived bone sum index compared with those whose parents smoked hygienically (cotinine levels in children <3ng/ml) or those whose parents did not smoke (Figure 1). Concerning ultrasound derived estimated bone mineral density Z-score, there were no differences between the groups (Figure 2).

Sensitivity analyses and replication

In analyses restricted to life-long non-smokers results were essentially similar to those shown in Tables 1-4. There were no significant differences in the effects of passive smoking in childhood on bone traits according to different cotinine concentration cut-offs (Supplementary Table 3[16]). Results remained similar when no upper limit to cotinine concentrations was applied.

Discussion

We observed that exposure to passive smoking in childhood, determined by parental smoking and serum cotinine concentrations, was a significant determinant of reduced bone mass, density and strength indices measured 28 years later in adulthood with two different methods. The effect of passive smoking in childhood was not attenuated after adjustments for age and sex and the possible intermediate or confounding factors, including BMI, active smoking, serum 25-OH vitamin D concentration, physical activity, parental school years and birth weight.

In adulthood, active smoking is a risk factor for osteoporosis and bone fractures[7,8,24-28]. Less is known of the effects of passive smoking on bone health. In adults, passive smoking has been inversely associated with phalangeal bone mineral density in a cohort of 15,038 adults aged 19-95 years[10]. Similar results were found in 2067 postmenopausal women, where passive smoking confirmed by urinary cotinine analysis was directly associated with osteoporosis[11]. Even less is known of passive smoking in childhood. In a retrospective study on 154 premenopausal women, self-reported exposure to passive smoking from age 10 onwards was negatively associated with total hip and femoral neck bone mineral density when aged 40-45 years[12]. The results of the present prospective study indicate that parental smoking exposure in childhood affects subsequent bone quality traits measured in mid-adulthood.

Concerning other childhood risk factors, the available prospective longitudinal studies demonstrating links between childhood exposures and adult bone health outcomes have mainly evaluated the effects of early growth, physical activity and socioeconomic position. It has been shown that poor fetal and infant growth and low levels of physical activity in childhood are associated with reduced peak bone mass later in life[29]. Direct relations have been observed between childhood overweight and adult bone density, supporting the hypothesis that excess weight during active growth imposes increased loading on the weight-bearing skeleton and leads to more robust bones in adulthood[19]. Among white males, socioeconomic disadvantage in childhood has been associated with lower adult femoral neck strength[6]. In the present analyses, the effects of childhood exposure

to secondhand smoke on bone health were independent of these factors, as well as other possible confounders, such as vitamin D concentrations and family socioeconomic status.

Most plausible mechanisms in smoking-induced bone loss may be increased bone resorption and a less efficient calcium absorption[30] and effects on circulating levels of sex hormones and 25-OH vitamin D[25]. Experimental nicotine exposure inhibited matrix synthesis and hypertrophic differentiation in human growth plate chondrocytes[31]. There is a large body of evidence from experimental studies that tobacco smoke has adverse effects on osteoneogenesis and osseointegration in bone cell culture and animal models via several mechanisms[32]. In animal models of bone biomechanical properties, tobacco smoke exposure decreased the structural strength, material properties, bone mass, and trabecular quality in the growing female mouse[33], and decreased bone mineral density through increased bone turnover in the female rat[34]. Nicotine has been suggested to have a direct toxic effect on osteoblasts[35]. However, experimental nicotine administration in rats caused no differences in bone mineral content or other bone traits between the low and high nicotine doses[36,37]. Thus, substances in smoke other than nicotine may also be responsible for the decreased bone density. In the present study, independent associations were seen with different indices of bone mineral density, bone mass and strength after a 28-year follow-up in both men and women, suggesting that tobacco smoke exposure may compromise the growing bone through multiple mechanisms.

From a clinical point of view, we observed in multivariable models that parental smoking in childhood was associated with up to 0.19 SD worse (estimated heel BMD, for both parents smoking) bone measures, and elevated cotinine levels (3-20 ng/L) were related with over 0.2 SD lower bone sum index. In addition, parental smoking was related with low-energy fractures. In prospective observational data, 1-SD decrease in bone mineral density with DXA has been related to approximately 1.4 times elevated total osteoporotic fracture risk at the age of 65 years[38]. However, for hip fractures the respective risk ratio for 1 SD change in bone mineral density is 2.9 at the age of 65 years and the relative risk significantly increases with decreasing age[38]. For these reasons, it would be essential to have increased public health awareness to the harms associated with secondhand tobacco smoke, especially in childhood. There would be several different ways to limit children's

291 exposure to environmental tobacco smoke, including restrictions to smoking in public places, in vehicles, and
292 at home. Smoking restrictions in public and work places have been shown to decrease hospitalizations for
293 cardiovascular and respiratory disease among adults[39]. However, there are observational data suggesting that
294 public smoking regulations may have increased passive smoke exposure in private places, such as at home[40].
295 Therefore it would be important to communicate to parents that their smoking has effects on their children's
296 health, both in short and long term.

297

298 Our study has limitations. There was only a single measurement of parental smoking and of serum cotinine
299 concentration in childhood at the age of 3 to 18 years. However, we did not detect age interactions, indicating
300 that a single measurement in childhood may be representative of exposure from childhood through youth. We
301 were unable to determine an age when exposure to parental smoking may have been most detrimental to bone
302 health. A limitation may also be that no data were available on smoking during pregnancy which may affect
303 birth weight, however all models were adjusted for birth weight. The present pQCT and ultrasound results
304 performed in peripheral bones, including calculations of bone sum index, provide epidemiological data and
305 they do not have instant clinical utility. The estimated BMDs and Z-scores are not comparable with DXA
306 measurements and they cannot be used for diagnostic classification. Another potential limitation is the non-
307 participation in the bone measurement study. However, even though non-participants were younger and more
308 often males, their baseline characteristics (BMI, parental education) were similar. Thus the present study cohort
309 seems to be representative of the original study population. The strengths of our study are the large, well
310 characterized population with a long clinical follow-up and the bone measurement methods of assessing
311 peripheral bone mass, density, area and strength from three different bones, radius, tibia and calcaneus. A
312 further strength is that exposure to secondhand smoke in childhood could be confirmed by serum cotinine
313 which is a biomarker of nicotine exposure. Furthermore, we performed the analyses excluding those who had
314 reported own smoking at the baseline.

315

316 Our results suggest that bone traits are persistently affected by exposure to passive smoking in childhood,
317 independent of potential confounding factors. Programs aimed at avoiding exposure to tobacco smoke early in
318 life could improve later bone health of children in risk to passive smoke exposure.

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Figure legends

Figure 1.

Effect of parental smoking hygiene at the offspring's age of 3-18 years on bone sum index measured with peripheral quantitative computed tomography in adulthood (age 31-46 years). Results are expressed as mean±SEM, p-values are from regression analyses adjusted for age, sex, childhood body mass index, physical activity, parental school year and 25-OH vitamin D-concentration. Serum cotinine concentration between 3 and 20 ng/ml was considered elevated.

Figure 2.

Effect of parental smoking hygiene at the offspring's age of 3-18 years on calcaneal bone mineral density Z-score estimated with ultrasound in adulthood (age 31-46 years). Results are expressed as mean±SEM, p-values are from regression analyses adjusted for age, sex, childhood body mass index, physical activity, parental school year and 25-OH vitamin D-concentration. Serum cotinine concentration between 3 and 20 ng/ml was considered elevated.

Table 1. Multivariable regression results of the independent effects of parental smoking in childhood (age of 3-18 years) on bone quality indices measured by peripheral quantitative computed tomography in adulthood (age 31-46 years) from 1422 participants (aged 31-46 years) of the Cardiovascular Risk in Young Finns Study.

	Bone sum index (z-score)		**Bone mass (mg)**		**Bone density (mg/cm^3)**		**Bone area (mm^2)**		**Bone strain (z-score)**	
	β±SE	P	β±SE	P	β±SE	P	β±SE	P	β±SE	P
Age (years)	0.016±0.003	<0.0001	5.8±1.0	<0.0001	-0.13±0.09	0.17	6.7±1.1	<0.0001	0.015±0.003	<0.0001
Male sex	1.509±0.033	<0.0001	511.2±9.4	<0.0001	4.76±0.91	<0.0001	408.5±10.4	<0.0001	1.614±0.029	<0.0001
Childhood body mass index	0.237±0.017	<0.0001	85.8±5.0	<0.0001			72.1±5.5	<0.0001	0.244±0.016	<0.0001
Childhood physical activity	0.129±0.019	<0.0001	47.4±5.4	<0.0001			35.1±6.0	<0.0001	0.110±0.017	<0.0001
Parental smoking *	-0.064±0.023	0.004	-17.1±6.4	0.008	-1.27±0.63	0.04	-11.1±7.4	0.12	-0.042±0.020	0.04

Results are from stepwise multivariable models. β = Parameter estimate for change in the outcome variable for 1-standard deviation / 1-category change in the exposure. SE = standard error. Initial models included data on age, sex, childhood body mass index, physical activity, parental school years, 25-OH vitamin D-concentration and parental smoking. Age and sex were forced into final models. * Effect per one smoking parent.

Table 2. Multivariable regression results of the independent effects of parental smoking in childhood (age of 3-18 years) on ultrasound derived bone quality indices in adulthood (age 31-46 years) from 1210 participants (aged 31-46 years) of the Cardiovascular Risk in Young Finns Study.

	Broadband attenuation (dB/MHz)		**Speed of sound (m/s)**		**Estimated bone mineral density (Z-score)**	
	β±SE	**P**	**β±SE**	**P**	**β±SE**	**P**
Age (years)	0.3±0.1	0.01	0.1±0.2	0.85	0.011±0.006	0.09
Male sex	2.3±1.0	0.02	-0.7±1.7	0.70	-0.075±0.060	0.21
Childhood body mass index	2.5±0.5	<0.0001	2.6±0.9	0.005	0.123±0.032	0.0001
Childhood physical activity	1.8±0.6	0.001	3.7±1.0	0.002	0.116±0.034	0.0009
Parental smoking *	-1.6±0.6	0.02	-2.7±1.1	0.02	-0.097±0.041	0.02

Results are from stepwise multivariable models. β = Parameter estimate for change in the outcome variable for 1-standard deviation / 1-category change in the exposure. SE = standard error. Initial models included data on age, sex, childhood body mass index, physical activity, parental school years, 25-OH vitamin D-concentration and parental smoking. Age and sex were forced into final models. * Effect per one smoking parent.

Table 3. Multivariable regression results of the independent effects childhood exposure to cotinine (age of 3-18 years) on bone quality indices measured by peripheral quantitative computed tomography in adulthood (age 31-46 years) in 1201 participants (aged 31-46 years) of the Cardiovascular Risk in Young Finns Study.

	Bone sum index (z-score)		**Bone mass (mg)**		**Bone density (mg/cm^3)**		**Bone area (mm^2)**		**Bone strain (z-score)**	
	β±SE	P	β±SE	P	β±SE	P	β±SE	P	β±SE	P
Age (years)	0.014±0.004	0.0003	5.4±1.1	<0.0001	-0.2±0.1	0.09	6.6±1.2	0.002	0.014±0.004	0.0002
Male sex	1.502±0.036	<0.0001	509.7±10.1	<0.0001	4.6±1.0	<0.0001	405.9±11.1	<0.0001	1.614±0.031	<0.0001
Childhood body mass index	0.215±0.019	<0.0001	79.4±5.4	<0.0001			73.2±5.9	<0.0001	0.226±0.017	<0.0001
Childhood physical activity	0.146±0.022	<0.0001	55.2±6.2	<0.0001			34.0±6.8	<0.0001	0.132±0.019	<0.0001
Elevated cotinine in childhood *	-0.206±0.057	0.0003	-47.6±16.0	0.003	-6.1±1.6	0.0001	-4.1±17.6	0.81	-0.149±0.050	0.003

Results are from stepwise multivariable models. β = Parameter estimate for change in the outcome variable for 1-standard deviation / 1-category change in the exposure. SE = standard error. Initial models included data on age, sex, childhood body mass index, physical activity, parental school years, 25-OH vitamin D-concentration and parental smoking. Age and sex were forced into final models. *Serum cotinine concentration between 3 and 20 ng/ml

Table 4. Multivariable regression results of the independent effects childhood exposure to cotinine (age of 3-18 years) on ultrasound derived bone quality indices in adulthood (age 31-46 years) in 1011 participants (aged 31-46 years) of the Cardiovascular Risk in Young Finns Study.

	Broadband attenuation (dB/MHz)		**Speed of sound (m/s)**		**Estimated bone mineral density (Z-score)**	
	β±SE	**P**	**β±SE**	**P**	**β±SE**	**P**
Age (years)	0.4±0.1	0.001	0.2±0.2	0.34	0.016±0.007	0.02
Male sex	2.0±1.0	0.05	-1.2±1.9	0.53	-0.079±0.065	0.22
Childhood body mass index	2.3±0.5	<0.0001	2.4±1.0	0.02	0.115±0.035	0.001
Childhood physical activity	3.0±0.6	<0.0001	5.8±1.2	<0.0001	0.187±0.041	<0.0001
Elevated cotinine in childhood *	-2.2±1.6	0.16	-7.0±2.9	0.01	-0.184±0.099	0.06

Results are from stepwise multivariable models. β = Parameter estimate for change in the outcome variable for 1-standard deviation / 1-category change in the exposure. SE = standard error. Initial models included data on age, sex, childhood body mass index, physical activity, parental school years, 25-OH vitamin D-concentration and parental smoking. Age and sex were forced into final models. *Serum cotinine concentration between 3 and 20 ng/ml

Supplementary Appendix for the article

Childhood exposure to passive smoking and bone health in adulthood. The Cardiovascular Risk in Young Finns Study

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Conflict of interest

There is no conflict of interest.

Disclosure statement

The authors have nothing to disclose.

Key Words: Passive Smoking, Cotinine, Bone Mineral Density

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Supplementary methods

Study population

The Cardiovascular Risk in Young Finns Study is a population-based cohort study on cardio-metabolic risk factors from childhood through adulthood. At baseline in 1980, 3,596 children and adolescents aged 3, 6, 9, 12, 15 and 18 years (83% of those invited) were examined at five study centers in Finland. Follow-up studies have been conducted in 1983, 1986, 2001, 2007, and 2011. In 2008, all original study participants now aged 31 to 46 years, alive and living in Finland (N = 3,386) were invited to peripheral quantitative computed tomography and heel ultrasound measurements that were organized in five study centers (cities of Turku, Helsinki, Tampere, Oulu and Kuopio) between February and December 2008. Peripheral quantitative computed tomography (pQCT) measurements were performed for 1,884 individuals and ultrasound measurements for 1,415 individuals. Pregnant women were excluded from the computed tomography studies. Complete data for the calculation composite indices of quantitative computed tomography measurements of bone mass, bone density, bone area and bone strength, and a combined bone sum index was available for N=1,800 participants. The primary statistical analyses included all individuals, who were non-smokers at baseline, and had data on bone traits, estimated area under the body mass index curve between ages 6 and 24 years, parental smoking, serum cotinine, parental education (school years), physical activity and serum vitamin D concentration. The number of individuals with complete data when assessing the effects of parental smoking on pQCT measures was 1422, and 1201 when assessing the effects of serum cotinine. For the ultrasound measures the respective numbers are 1210 and 1011.

Childhood exposure measures – parental smoking, serum cotinine and smoking hygiene

Parental smoking: Parents of participants self-reported their smoking habits at baseline. One parent responding on behalf of both parents was asked to indicate the smoking status separately of the mother and father in the household from two questions. The first question asked whether mother/father had ever smoked daily for at least one year (responses could be "Yes" or "No"), and the second question asked whether mother/father were currently smoking (responses could be "does not smoke", "occasionally", "daily"). Mothers or fathers that indicated they had ever smoked daily for at least one year were designated as ever smokers. Mothers or fathers that indicated they currently occasionally or daily smoked were designated as current smokers.

Serum cotinine: Child fasting serum samples were collected in 1980 and stored at -20°C until they were analyzed in 2014. During storage, samples were not thawed or refrozen. The assays were performed without knowing the smoking habits of the child or parents. Cotinine was extracted into dichloroethane from 0.2 ml of serum to which 0.2 ml of 5-methylcotinine (0.1 µg/ml in 0.01M HCl) was added by the method of Feyerabend and Russell[1]. Concentrated extract (2.0 µl) was injected into the Hewlett Packard FFAP silica capillary column (13 m, i.d. 0.32 mm, film thickness 0.52 µm) of the Shimadzu model GC-17 gas chromatograph, equipped with a nitrogen-sensitive Shimadzu FTD-17 flame-thermoionic detector and a Shimadzu AOC-20 auto-injector. The injector and detector temperatures were 220°C and 300°C, respectively. The retention times for nicotine, cotinine and 5-methylcotinine were 2.9 min, 10.8 min and 12.8 min, respectively. The peak areas were analyzed using Shimadzu Class-VP™ chromatography software.

The analytical sensitivity of the assay was determined from serum samples with known cotinine concentrations. The inter-assay coefficient of variation was 13.3% at a cotinine concentration of 1.56 ng/ml, 7.2% at a concentration of 3.125 ng/ml, 6.9% at a concentration of 6.25 ng/ml, 3.1% at a concentration of 12.5 ng/ml, 1.6% at a concentration of 25 ng/ml, 3.2% at a concentration of 50 ng/ml, and 1.56% at concentration of 100 ng/ml (6 samples at each concentration). Cotinine recovery from serum was 71–150% depending on its concentration. The estimated detection limit (mean of a blank sample + 3 standard deviations) was $1.7 + (3\times0.4)=2.9$ ng/ml. Values greater than 3.0 ng/ml were primarily used as an indicator of nicotine exposure.

Active smoking. The information on smoking habits was collected in 12- to 18-year-olds in connection with the medical examination in an isolated room where the participants could respond confidentially and undisturbed. The effect of active smoking in adolescence was primarily controlled by excluding smokers from the analyses, and secondarily by including adolescent smoking as covariate in multivariable models.

In adulthood, information on smoking was collected with questionnaires in 2001 and 2007. To replicate the know effect of active smoking exposure on bone health, we classified individuals who had reported ever being active smokers for a period of at least 5 years as exposed (regular smoking on daily bases for at least 5 years).

Parental smoking hygiene: A variable to indicate parental smoking hygiene was generated for those participants with both serum cotinine and parental reports of current or ever smoking in 1980. A three-level categorical variable was constructed: 1 = no parental smoking; 2 = children with a non-detectable (values between 0 and 3 ng /ml) cotinine level but whose parents smoked (hygienic

parental smoking); and 3 = children with a detectable serum cotinine level 3-20 ng/ml and whose parents smoked (non-hygienic parental smoking).

Measurements of the bone traits with computed tomography

Two sites (distal and diaphysis) of non-weight-bearing radius and weight-bearing tibia were measured with computed tomography device (XCT 2000R, Stratec Medizintechnik GmbH, Pforzheim, Germany). The distal sites of radius and tibia were scanned at 4% and 5% from the distal endplate, respectively. The proximal (shaft) sites of radius and tibia were scanned at 30% from the distal endplate. The length of ulna and tibia were measured with a tape measure from the proximal end to the distal end. The thickness of the tomographic slice was 2 mm, and the pixel size was 0.5×0.5 mm2 in all scans. The scan speed for the scout view was 40 mm/s and for the tomographic scan, 20 mm/s. Radius was measured on the non-writing side and tibia on the left side, except for those who had metal implants or previous injuries in the non-writing arm or left leg; they were measured from the opposite side. During the scanning, the subjects were asked to stay still. In case of movement artefacts, the scan was repeated. For the analysis of computed tomography scans, outer threshold value of 169 mg/cm3 was used for the separation of bone tissue from soft tissue. For the separation of trabecular and cortical bone tissues, an inner threshold of 480 mg/cm3 was applied at the distal bone sites and threshold of 710 mg/cm3 at the proximal measurement sites[2]. In all measurement analysis, iterative contour detection and filtration (contour mode 2 and peel mode 2) were used to define the total trabecular and cortical bone areas. The contour algorithm begins by searching for a voxel that represents the threshold defined by the operator, and the search continues until two neighboring voxels are found, and then the process continues all around the bone returning to the starting point. The trabecular and subcortical bone areas were separated with a filtration

algorithm that ignores isolated high attenuation voxels in the trabecular areas and in areas that are not continuous.

The following bone traits were obtained: total bone mineral content (in mg), total and cortical bone areas (in mm2), trabecular and cortical bone densities (in mg/cm3). In addition, three bone strength indices were estimated. The conventional stress-strain index (in mm3) that represents the resistance of the bone against torsional load was calculated for both distal and proximal bone sites. Additionally for the distal sites, bone strength index (in g2/cm4) that represents the bone strength against compressive loading was calculated as a product of volumetric bone mineral density squared and total cross-sectional area[3,4]. For distal and proximal sites of radius and tibia, cortical strength index was calculated as the ratio of cortical bone area and total bone area. For statistical analyses, four composite indices of bone mass, density, area and strength were calculated from the trabecular and cortical bone area, mineral content and mineral density measured at radius and tibia at both distal and shaft sites. Additionally, the four indices were combined to one bone sum index.

Measurements of the bone traits with ultrasound

A quantitative ultrasound technique (Sahara Clinical Bone Sonometer, Hologic, Waltham, MA, USA) was used to measure speed of sound (m/s) and broadband ultrasound attenuation (dB/MHz) at the left heel. The speed of sound value depends on the structural elasticity of the trabecular bone, and is linearly dependent on bone mineral density[5]. The broadband ultrasound attenuation reflects some aspects of trabecular architecture and bone mineral density[6]. The Sahara device measures both speed of sound and broadband attenuation at the mid-calcaneus, and combines these indices to

estimate heel bone mineral density (in g/cm2) using the following equation (= 0.0025926*(speed of sound + broadband ultrasound attenuation) - 3.687).

Quantitative ultrasound technique was used to measure ultrasound broadband attenuation (decibel per megahertz) and the speed of sound (m/s) at calcaneus, and the proportion of attenuation of the speed of sound was calculated to construct a quantitative ultrasound index (Supplementary Table 1). The speed of sound indicates the structural elasticity of the trabecular bone and it is linearly dependent on bone mineral density[7]. The broadband ultrasound attenuation indicates the frequency-dependent pattern of absorption reflecting the trabecular architecture and bone mineral density[8]. Bone mineral density Z-scores were calculated as the difference from the age and sex-specific averages.

To evaluate the precision of the ultrasound and computed tomography methods, repeated scans of volunteers were obtained in each centre before starting and after completing the measurements. In a group of 39 women and men (aged 24 to 64), either radius or tibia or both extremities and calcaneus were measured twice with repositioning and for pQCT scans also including the assessment of bone length. The in vivo coefficients of variation (CV%) for the radius were 2.5% for TotA at distal site and 3.9% at the shaft site, 4.4% for CorA at distal site and 1.1% at shaft site, 1.6% for the TraD at distal site, 3.2% for the CorD at the distal site and 0.5% at the shaft site. The CV%s for the tibial traits were 1.3%, 1.2%, 2.6%, 1.2%, 0.5%, 1.2% and 0.6%, respectively. The long-term performance of the pQCT scanner was assessed by daily phantom measurements, which showed no significant drift in the density levels during the study year. Each pQCT scan was individually analysed by the same researcher according to the analysis protocol. Another researcher repeated the measurement analyses for randomly selected scans (157; 8% of the total number of subjects; 76 females and 81males), and no significant differences were found in the repeated scan analyses. In

the repeated QUS measurements of the volunteers, the in vivo CV% was 0.3% for SOS and 4.8% for BUA. The long-term performance of the SAHARA device was assessed by daily phantom measurements that showed no significant changes in the measurement levels during the study year.

Supplementary results

Supplementary Table 1: Baseline characteristics

Supplementary Table 2: Bone metrics characteristics

Supplementary Table 3: Sensitivity analysis results for different cotinine level cut-off points

Supplementary Table 1. Baseline characteristics of the 1422 participants of the Cardiovascular Risk in Young Finns Study stratified by exposure to parental smoking*.

	Non-exposed N=410		**Exposed N=1012**			
			One parent smoking regularly N=703		Both parents smoking regularly N=309	
	Mean (SD) / %	Range	Mean (SD) / %	Range	Mean (SD) / %	Range
Age (years)	10.1 (5.1)	3-18	10.5 (4.8)	3-18	9.1 (4.6)	3-18
Males (%)	39		43		41	
Elevated cotinine**	3.6		9.2		17.1	
Birth weight (g)	3547 (499)	1870-5250	3495 (577)	1100-5450	3472 (532)	1300-4980
Weight (kg)	36.4 (17.3)	11.2-91.8	38.5 (17.3)	12.1-87.4	33.5 (16.5)	11.3-91.2
Height (cm)	139.5 (26.0)	86.8-189.2	142.3 (25.1)	90.4-192.5	134.5 (25.0)	89-190.1
Body mass index (kg/m^2)	17.4 (2.8)	11.9-29.3	17.8 (2.9)	9.2-28.7	17.3 (2.8)	12.8-30.4
Serum 25-OH-D (nmol/l)	54.1 (15.7)	12-122	51.0 (15.3)	17-114	54.0 (15.4)	18-96
Parental school years	11.2 (3.9)	1-28	10.6 (3.6)	1-25	11.3 (3.3)	4-22

SD = standard deviation * Those individuals who had reported own active smoking at baseline when aged 12-18 years were excluded from the analyses.
**Serum cotinine concentration between 3 and 20 ng/ml

Supplementary Table 2. Bone metrics measured by peripheral quantitative computed tomography and the bone trait indices calculated thereof, and the variables acquired by quantitative ultrasound at the age of 31-46 years.

Peripheral quantitative computed tomography measurements (N=1422)						
Bone quality metrics	**Bone**	**Mean (SD)**	**Range**	**Bone trait indices**	**Calculation of the bone trait indices**	**Mean (SD)**
Distal total mineral content (mg)	Radius	244 (64)	129-530	Bone mass (mg)	Distal total mineral content + shaft cortical bone mineral content; from radius and tibia	1698 (324)
	Tibia	602 (127)	344-1102			
Shaft cortical bone mineral content (mg)	Radius	213 (44)	132-359			
	Tibia	645 (110)	400-1084			
Distal total area (mm^2)	Radius	359 (75)	192-660	Bone area (mm^2)	Distal total area + shaft total area; from radius and tibia	1739 (290)
	Tibia	891 (150)	550-1522			
Shaft total area (mm^2)	Radius	113 (28)	53-224			
	Tibia	382 (66)	223-682			
Shaft cortical density (mg/cm^3)	Radius	1198 (26)	1073-1261	Bone mean density (mg/dm^3)	(Shaft cortical density from radius and tibia) / 2) + (Trabecular density from radius and tibia) / 2)	706 (17)
	Tibia	1159 (23)	1064-1220			
Trabecular density (mg/cm^3)	Radius	225 (37)	124-369			
	Tibia	241 (34)	153-361			
	Radius	-0.01 (1.001)	-1.76-4.46			

Bone strength index, standardized	Tibia	0.01(1.007)	-1.85-4.93	Bone strain (z-score)	Bone strength index + stress strain index; from radius and tibia	-0.08 (3.55)
Stress strain index, standardized	Radius	-0.02 (0.99)	-1.76-4.68			
	Tibia	-0.01 (1.00)	-2.28-4.51			
Bone sum index, standardized				Bone sum index (z-score)	Combined z-score of bone mass, area, density and strain	-0.02 (3.22)

Quantitative ultrasound measurements (N=1210)

Bone measure	**Bone**	**Mean (SD)**	**Range**
Ultrasound broadband attenuation (decibel/megahertz)	Calcaneus	81 (16)	6-155
Speed of sound (m/s)		1560 (29)	1494-1693
Estimated bone mineral density Z-score		0.00 (1.00)	-2.6-4.8

SD = standard deviation

Supplementary Table 3. Sensitivity analysis on the independent effects of passive smoking in childhood (age of 3-18 years) on bone quality indices in adulthood (age 31-46 years) in 1136 participants of the Cardiovascular Risk in Young Finns Study. Analyses were performed excluding those who had reported own smoking at baseline when aged 12-18 years.

	No. exposed/	**Bone sum index**		**Bone mass**		**Bone density**		**Bone area**		**Bone strain**	
Cotinine cut-offs	**non-exposed**	β±SE	P	β±SE	P	β±SE	P	β±SE	P	β±SE	P
>0 and <20 ng/L	268/868	-0.36	0.010	-28.92	0.021	-2.47	0.039	-10.23	0.44	-0.32	0.021
>1.0 and <20 ng/L	214/922	-0.38	0.115	-33.73	0.013	-2.04	0.11	-16.60	0.25	-0.36	0.017
>1.5and <20 ng/L	174/962	-0.37	0.022	-33.82	0.022	-1.83	0.189	-19.78	0.21	-0.33	0.04
>2.0 and <20 ng/L	136/1000	-0.34	0.06	-31.72	0.05	-1.81	0.24	-13.84	0.43	-0.33	0.07
>2.5 and <20 ng/L	105/1031	-0.56	0.004	-48.08	0.009	-3.73	0.03	-20.44	0.30	-0.53	0.008
>3.5 and <20 ng/L	66/1070	-0.65	0.01	-45.73	0.05	-5.99	0.005	-7.31	0.76	-0.46	0.06
>4.0 and <20 ng/L	56/1080	-0.73	0.008	-55.67	0.02	-6.19	0.008	-9.05	0.73	-0.57	0.03

β = Parameter estimate for change in the outcome variable for 1-standard deviation / 1-category change in the exposure. SE = standard error.

Models adjusted for age, sex, body mass index, parental education, vitamin D concentration and physical activity level.

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