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ORIGINAL ARTICLE ***Reproductive epidemiology***

Use of e-cigarettes associated with lower sperm counts in a cross-sectional study of young men from the general population

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STUDY QUESTION: Are use of e-cigarettes and snuff associated with testicular function as previously shown for conventional cigarettes and marijuana?

SUMMARY ANSWER: Use of e-cigarettes is associated with reduced semen quality but not with higher serum testosterone level as observed for conventional cigarette use. Snuff use was not associated with markers of testicular function.

WHAT IS KNOWN ALREADY: Cigarette smoking has previously been associated with higher testosterone levels and impaired semen quality, whereas it is unresolved whether use of e-cigarettes or snuff influence the testicular function.

STUDY DESIGN, SIZE, DURATION: This cross-sectional population-based study included 2008 men with information on cigarette and marijuana use (enrolled between 2012 and 2018), among whom 1221 men also had information on e-cigarette and snuff use (enrolled between 2015 and 2018).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Men (median age 19.0 years) from the general population provided a semen and blood sample and filled out a questionnaire on lifestyle including information on smoking behaviour. Associations between different types of smoking (e-cigarettes, snuff, marijuana and cigarettes) and reproductive hormones (total and free testosterone, sex hormone-binding globulin, LH, oestradiol and ratios of inhibin B/FSH, testosterone/LH and free testosterone/LH) and semen parameters (total sperm count and sperm concentration) were examined using multiple linear regression analyses adjusted for relevant confounders.

MAIN RESULTS AND THE ROLE OF CHANCE: Approximately half of the men (52%) were cigarette smokers, 13% used e-cigarettes, 25% used snuff and 33% used marijuana. Users of e-cigarettes and marijuana were often also cigarette smokers. Compared to non-users, daily e-cigarette users had significantly lower total sperm count (147 million vs 91 million) as did daily cigarette smokers (139 million vs 103 million), in adjusted analyses. Furthermore, significantly higher total and free testosterone levels were seen in cigarette smoking men (6.2% and 4.1% higher total testosterone and 6.2% and 6.2% higher free testosterone in daily smokers and occasional smokers, respectively, compared to non-smoking men), but not among e-cigarette users. Daily users of marijuana had 8.3% higher total testosterone levels compared to non-users. No associations were observed for snuff in relation to markers of testicular function.

LIMITATIONS, REASONS FOR CAUTION: We cannot exclude that our results can be influenced by residual confounding by behavioural factors not adjusted for. The number of daily e-cigarette users was limited and findings should be replicated in other studies.

WIDER IMPLICATIONS OF THE FINDINGS: This is the first human study to indicate that not only cigarette smoking but also use of e-cigarettes is associated with lower sperm counts. This could be important knowledge for men trying to achieve a pregnancy, as e-cigarettes are often considered to be less harmful than conventional cigarette smoking.

STUDY FUNDING/COMPETING INTEREST(S): Funding was received from the Danish Ministry of Health (1-1010-308/59), the Independent Research Fund Denmark (8020-00218B), ReproUnion (20200407) and the Research Fund of the Capital Region of Denmark (A6176). The authors have nothing to disclose.

TRIAL REGISTRATION NUMBER: NA

Key word: testosterone / smoking habits / sperm counts / semen quality / e-cigarettes

Introduction

It is estimated that more than 30% of the adult male population use tobacco products with the main type being cigarettes (World Health Organization, 2015). During the past decades, a decline in proportion of tobacco smokers has been observed in the Western world (Islami *et al.*, 2015). However, this decline has to some degree been paralleled by an increase in use of other types of tobacco products, especially among the young generations (Mantey *et al.*, 2019). Alternative tobacco products include smokeless and sometimes flavoured tobacco products such as electronic-cigarettes (e-cigarettes) and snuff. Use of multiple nicotine products is frequent, with the most common combination being cigarettes and e-cigarettes (Agaku *et al.*, 2014; Kasza *et al.*, 2017).

It is well known that male cigarette smokers have higher testosterone levels compared to non-smokers, however, the mechanism behind this is debated (Shiels *et al.*, 2009; Holmboe *et al.*, 2015). Furthermore, findings from a recent meta-analysis indicated that cigarette smoking has a negative impact on semen quality (Sharma *et al.*, 2016). Maternal cigarette smoking during pregnancy is also negatively associated with semen quality in offspring (Storgaard *et al.*, 2003; Jensen *et al.*, 2004; Ramlau-Hansen *et al.*, 2007; Ravnborg *et al.*, 2011). Recent studies on marijuana use and testicular function indicate that also marijuana users have higher testosterone levels compared to men with no use (Gundersen *et al.*, 2015; Nassan *et al.*, 2019). However, the studies show conflicting results regarding semen quality. In a study of young Danish men, similar to the population in the present study but with no overlap between cohorts, regular users of marijuana had a lower sperm concentration and total sperm count compared to men with no use (Gundersen *et al.*, 2015), whereas among American sub-fertile men, marijuana users were characterized by higher sperm concentration and total sperm count compared to never users (Nassan *et al.*, 2019). Few studies have investigated the association between male reproductive function and alternative tobacco products. In a small group of men ($n = 62$) attending a fertility clinic, use of snuff was adversely related to semen quality (Pärn *et al.*, 2015). As seen for conventional cigarette smoking men, it has been observed that men using smokeless tobacco have higher testosterone levels compared to men with no use (Shah *et al.*, 2019). To our knowledge, no human studies have investigated the association between use of e-cigarettes and reproductive function; however, animal studies have shown that intraperitoneal exposure to e-cigarette liquids was associated with impaired testis function including decreased testosterone levels independent of containing nicotine or not (El Golli *et al.*, 2016; Rahali *et al.*, 2018).

Thus, using a cohort of young men from the general population with information on use of various types of smoking/tobacco products, the aim of this study was to investigate whether use of e-cigarettes as well as other smoking habits were associated with reproductive hormone levels and semen quality.

Material and methods

Study population

In Denmark, all young men are called upon at age 18 years to undergo a compulsory physical examination to determine their fitness for military service, except men suffering from severe chronic disease. During the day of this examination, the draftees were approached and invited to participate in a cross-sectional study on testicular function without considering whether they were declared fit for military service or not. Those who consented were given an appointment for examination at the Department of Growth and Reproduction at Rigshospitalet (Copenhagen, Denmark). The overall participation rate was 25% (Priskorn *et al.*, 2018). All participants completed a questionnaire, delivered a semen sample, had a blood sample drawn and had a physical examination. Participants were compensated for their time (500 Danish Crowns ≈ 85 US$). A total of 2017 men participated between 2012 and 2018, but nine men were excluded from the present study due to current use of anabolic steroids (self-reported or indicated from reproductive hormone profile) leaving 2008 men eligible for analyses. Data on e-cigarette and snuff use was available from 2015 and onwards, leaving 1221 men in these analyses.

Ethical approval

Ethical approval was obtained from the local ethical committee (journal no. H-KF-289428).

Questionnaire

During the entire study period, the men were asked about whether and how often they smoked/used cigarettes or marijuana. From 2015, similar questions regarding use of e-cigarettes and snuff were included, and men reporting e-cigarette use also reported whether the e-liquid contained nicotine or not (for exact phrasing of questions and response categories, please see Supplementary Table SI). For each substance, the men were categorized as non-users, occasional users (i.e. any use less frequent than daily) or daily users.

The questionnaire furthermore included questions on demographics, reproductive and general health and health behaviour, as well as questions concerning the time of conception and pregnancy for which the men were encouraged to consult their mother when answering. At the examination day, questionnaire responses were reviewed with the participant to clarify missing or ambiguous information.

Physical examination

The physical examination included measurement of the men's weight and height from which BMI was calculated (kg/m^2).

Reproductive hormone analyses

Serum levels of FSH, LH and sex hormone-binding globulin (SHBG) were determined using a time-resolved immunofluorometric assay (Delfia, Wallac, Turku, Finland). Total testosterone levels were determined using time-resolved fluoroimmunoassays (Delfia, Wallac Oy, Turku, Finland). From 2014 and onwards, SHBG and total testosterone levels were assessed by chemiluminescent immunoassays (Access2, Beckman Coultier Ltd, High Wycombe, UK). Oestradiol was determined by radio-immune analysis (Pantex, USA) and inhibin-B by a specific two-sided enzyme-immunometric assay (Inhibin B gen II, Beckman Coulter Ltd, High Wycombe, UK). The hormones were analysed yearly in batches including reanalysis of a number of samples from the prior year. Free testosterone was calculated (cFT) based on the measured serum concentrations of total testosterone and SHBG and assuming a fixed albumin value according to Vermeulen *et al.* (1999). In addition, the ratios inhibin B/FSH, total testosterone/LH and cFT/LH were calculated.

Semen analysis

All men provided a semen sample by masturbation in a room close to the semen laboratory and the period of ejaculation abstinence was recorded. The men had been asked to abstain from ejaculation for at least 48 h before sampling but were still included if abstinence time was shorter. The semen sample was kept at 37°C until analysis, which was conducted according to the World Health Organization (WHO) guidelines (World Health Organization, 2010). In short, semen volume was assessed by weighing, sperm concentration was determined using a Bürker-Türk haemocytometer and the total sperm count was calculated (semen volume × sperm concentration) (Priskorn *et al.*, 2018).

Statistical analyses

Basic characteristics and levels of reproductive hormones and semen parameters for the total population and stratified by substance use are presented as medians and 5th and 95th percentiles or percentages. The proportion of daily users of different products are illustrated in a Venn diagram. The associations between use of cigarettes, e-cigarettes, marijuana or snuff and testicular function were investigated in univariate and multiple linear regression analyses. To obtain normally distributed residuals and homoscedasticity, reproductive hormones were transformed by natural logarithm and semen parameters were transformed by cubic root. Covariates were selected *a priori* based on the literature using causal diagrams including potential confounders for the association (lifestyle factors) as well as covariates closely related to variation in the outcome measurements (time of blood sampling, period of abstinence and examination year). Adjusted models all included examination year (continuous variable), BMI (continuous variable), units of alcohol the week prior to study participation (continuous variable) and self-rated physical fitness (poor, fair and good). Analyses with primary exposure other than cigarettes were furthermore adjusted for daily cigarette smoking (yes/no). Analyses with reproductive hormones as outcome also included time of blood sampling (continuous variable), while analyses with sperm counts as outcome included period of abstinence (continuous variable). In fully adjusted analyses, individuals with missing data on one or more of the included covariates were excluded (<3.5%). In all regression analyses, a trend test was performed across the three smoking categories (daily, occasional and no use).

In figures illustrating differences in levels of reproductive hormones, which were all transformed by use of the natural logarithm, the effect estimates and 95% confidence intervals were directly back-transformed to reflect the percentage difference according to smoking behaviour. Due to the cubic-root transformation used for sperm counts, these parameters were illustrated as the mean level in a standardized man [period of abstinence = 48 h, BMI = 20 kg/m^2, alcohol intake = 7 units, self-rated physical fitness = good, examination year = 2018, daily cigarette smoking = no (the latter not in analyses of cigarette smoking)].

Regression analyses were checked for interaction between cigarette smoking and each of the other products by including the interaction term in the adjusted models and furthermore by stratifying on daily cigarette smoking (yes/no) due to the frequent co-use of this product. Furthermore, adjusted analyses were repeated with further adjustment for maternal smoking during pregnancy. In subanalyses, e-cigarette use was stratified according to e-liquid containing nicotine or not. Finally, to enable a comparison with previous findings based on a similar study population (Gundersen *et al.*, 2015), the analyses of marijuana use were repeated using an alternative categorization (no use, monthly use, weekly use and daily use).

Statistical analyses were performed using PASW V.22.0 (IBM, Portsmouth, UK). *P*-values <0.05 were considered statistically significant. Findings were reported according to STROBE guidelines.

Results

Basic characteristics

The included men had a narrow age range with a median of 19 years. Median BMI was 22.1 kg/m^2. Approximately half of the men (52%) were cigarette smokers either on occasional or daily basis, 13% used e-cigarettes, 25% used snuff and 33% used marijuana (Table I). Users of other smoking types frequently also smoked cigarettes corresponding to 33% of e-cigarette users, 9% of snuff users and 71% of marijuana users (Fig. 1). In general, compared to non-smokers, cigarette smoking men were characterized by having a higher alcohol intake, more frequently reporting to have had a sexually transmitted disease, and a lower proportion reporting their physical fitness to be good or very good. For users of e-cigarettes, snuff and marijuana, the alcohol consumption was highest among occasional users. Finally, for all smoking types the proportion reporting that their mother smoked during pregnancy was highest among daily users (Table I).

Cigarette smoking

Cigarette smoking men were characterized by higher median levels of total testosterone (18.4 nmol/l, 17.8 nmol/l and 17.0 nmol/l for daily smokers, occasional smokers and non-smokers, respectively) and free

testosterone (414 pmol/l, 406 pmol/l and 382 pmol/l for daily smokers, occasional smokers and non-smokers, respectively) (Table II). Similar associations were seen in fully adjusted analysis (Supplementary Table SII). Thus, compared to non-smokers who served as the reference group, occasional and daily smokers had a 4.1% and 6.2% higher total testosterone level (*P*-trend <0.01) and 6.2% and 6.2% higher free testosterone level (*P*-trend <0.01), respectively (Fig. 2). Occasional and daily cigarette smokers had higher cFT/LH ratio compared to non-smokers (*P*-trend = 0.04) (Supplementary Table SII). Compared to non-smokers, smoking men had lower sperm concentrations (33 million/ml, 43 million/ml and 44 million/ml for daily smokers, occasional smokers and non-smokers, respectively, *P*-trend <0.01) and total sperm counts (103 million, 136 million and 139 million for daily smokers, occasional smokers and non-smokers, respectively, *P*-trend <0.01) in adjusted analyses (Fig. 3).

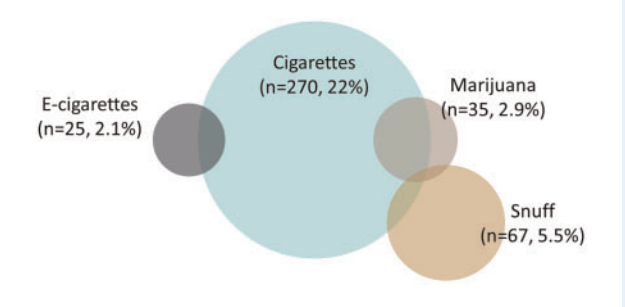

Figure 1. Overview of daily users of different types of smoking among men from 2015 to 2018 (*n* = 1219).

E-cigarette use

Only 25 men (2.0%) were using e-cigarettes daily, whereas 139 men (11.4%) reported occasional use and 1057 men (86.6%) reported no use. No consistent differences between these groups were seen for levels of reproductive hormones (Supplementary Table SIII). However, in fully adjusted analyses, a significant trend ($P < 0.01$) was seen across the categories in relation to semen quality with lower sperm concentration and total sperm count among e-cigarette users compared to non-users (sperm concentration: 33 million/ml, 39 million/ml and 45 million/ml and sperm count: 91 million, 128 million and 147 million for daily smokers, occasional smokers and non-smokers, respectively) (Fig. 3). The majority of daily e-cigarette users reported that the e-liquid contained nicotine and thus, the impact of daily e-cigarette use without nicotine content could not be examined. However, for the occasional users associations were similar irrespective of nicotine content.

Snuff use

As for e-cigarettes, only a small number of men (*n* = 68, 5.6%) reported to use snuff on daily basis, while 231 men (18.9%) used snuff occasionally and 922 men (75.5%) reported no use. Minor differences in hormone levels were seen according to snuff use. Descriptive medians indicated that daily users had higher inhibin B/FSH ratio

Table I Median levels (5–95 percentiles) or % (*n*) of basic characteristics according to smoking habits.

	N (%)	Age (years)	BMI (kg/m^2)	Alcohol (units/week)	Ejaculation abstinence (h)	Sexually transmitted disease,* % (*n*)	Good physical fitness,** % (*n*)	Mother smoked in pregnancy, % (*n*)
Total population	2008***	19.0 (18.4–22.4)	22.1 (18.2–27.7)	6 (0–33)	61 (36–133)	6.3 (126)	55.1 (1126)	11.7 (235)
Cigarettes								
Daily	467 (23)	19.0 (18.4–21.8)	21.6 (17.7–28.0)	10 (0–40)	60 (36–116)	11.1 (53)	37.5 (175)	15.6 (73)
Occasional	576 (29)	19.0 (18.4–22.4)	22.3 (18.8–27.6)	9 (0–32)	61 (36–133)	6.3 (36)	59.9 (345)	10.4 (60)
No	946 (48)	19.0 (18.4–23.0)	22.2 (18.3–27.8)	2 (0–27)	62 (36–152)	4.0 (38)	64.0 (605)	10.8 (102)
E-cigarettes								
Daily	25 (2)	19.0 (18.4–21.2)	22.5 (16.4–27.0)	4 (0–50)	60 (36–161)	8.0 (2)	68.0 (17)	16.0 (4)
Occasional	139 (11)	19.0 (18.5–20.6)	21.7 (18.0–29.2)	8 (0–39)	60 (35–110)	8.6 (12)	52.5 (73)	12.9 (18)
No	1057 (87)	19.0 (18.4–22.9)	22.1 (18.2–28.0)	5 (0–34)	61 (36–131)	6.0 (64)	55.8 (590)	9.9 (105)
Snuff								
Daily	68 (6)	19.0 (18.4–20.0)	22.7 (17.6–32.7)	7 (0–31)	60 (36–103)	13.2 (9)	66.2 (45)	11.8 (8)
Occasional	231 (19)	19.0 (18.4–20.6)	22.6 (18.8–27.4)	11 (0–44)	61 (35–132)	6.1 (14)	58.9 (136)	9.1 (21)
No	922 (76)	18.9 (18.4–23.2)	21.8 (18.0–28.1)	4 (0–33)	61 (37–132)	6.0 (55)	54.1 (499)	10.6 (98)
Marijuana								
Daily	52 (3)	19.0 (18.6–24.7)	20.9 (17.7–27.5)	5 (0–35)	59 (26–144)	11.5 (6)	28.8 (15)	15.4 (8)
Occasional	601 (30)	19.0 (18.4–21.7)	21.8 (18.2–26.9)	10 (0–36)	60 (37–117)	7.0 (42)	49.1 (295)	11.8 (71)
No	1334 (67)	19.0 (18.4–23.0)	22.3 (18.3–28.1)	4 (0–30)	61 (36–136)	5.8 (78)	60.9 (813)	11.7 (156)

*Defined as ever having had chlamydia or gonorrhoea.
**Good or very good self-rated physical fitness.
***Total numbers may vary due to missing values.

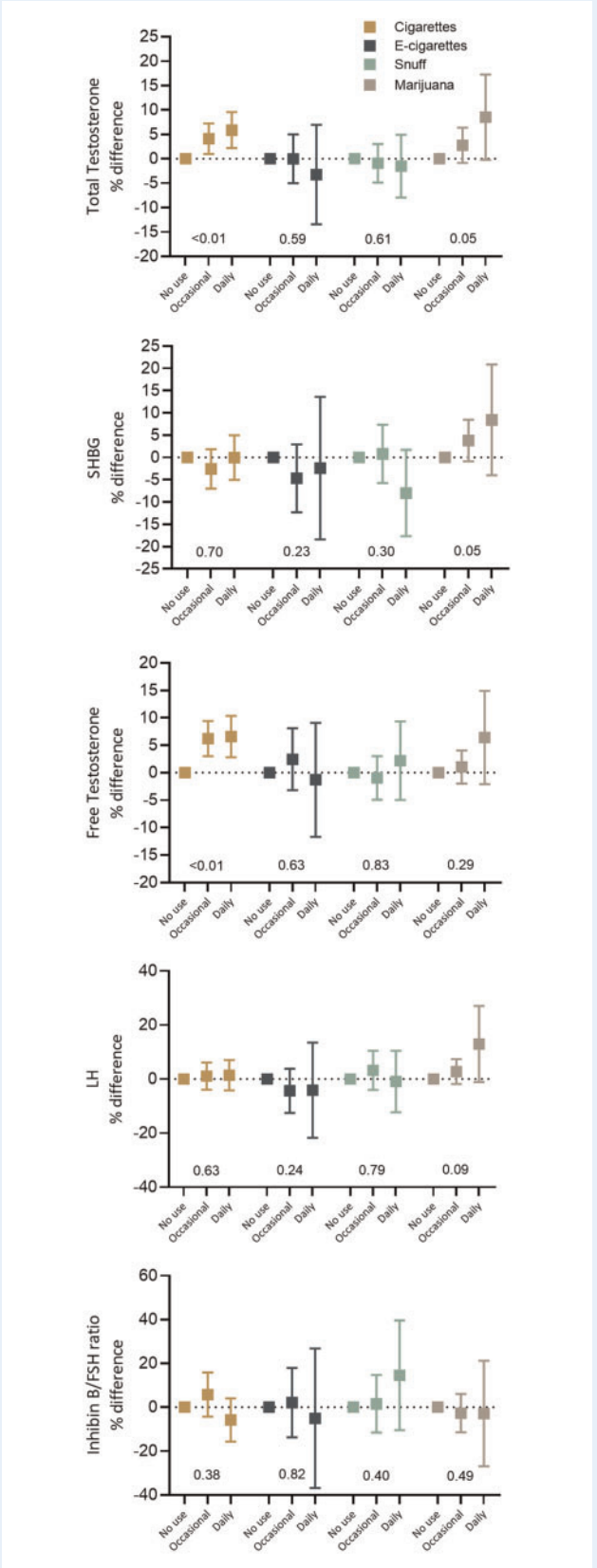

Figure 2. Percentage difference in hormone levels according to smoking habits adjusted for confounders. *P*-values indicate trend across categories.

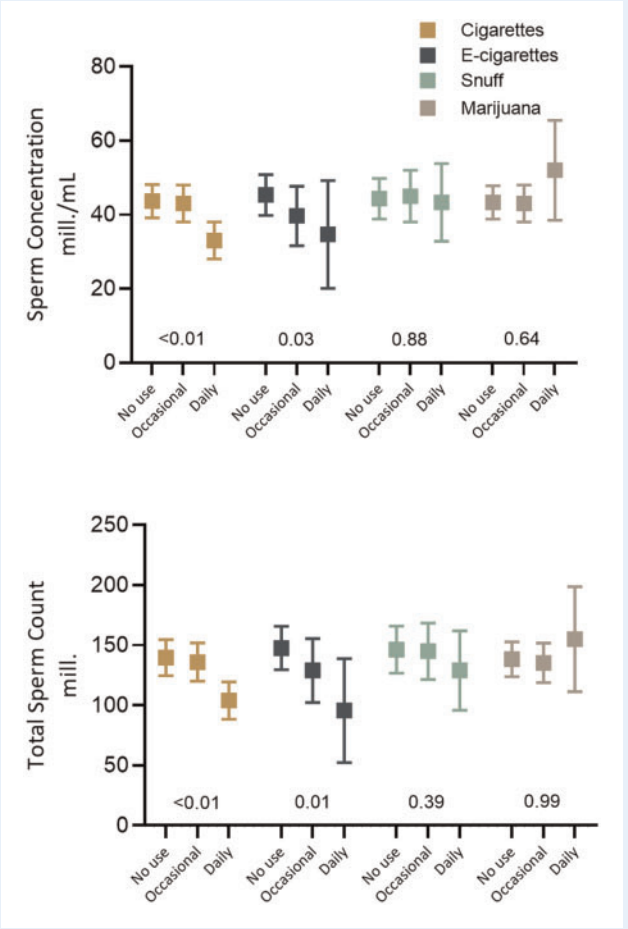

Figure 3. Back-transformed levels of semen parameters according to smoking habits adjusted for confounders. *P*-values indicate trend across categories.

compared to occasional users and non-users although it was insignificant in regression analyses (Fig. 2 and Supplementary Table SIV).

Marijuana use

In total, 52 men (2.6%) reported daily use of marijuana, 601 men (30.2%) reported to be occasional users and 1334 men (67.1%) reported no use of marijuana. Men who used marijuana on daily basis had higher total and free testosterone level as well as LH levels compared to non-smoking men (Fig. 2 and Supplementary Table SV); however, it was only significant for total testosterone. Thus, men smoking marijuana daily had a 8.3% higher total testosterone level (*P*-trend = 0.02), and 6.2% higher free testosterone level compared to non-smokers (*P*-trend = 0.29). In addition, LH levels were 12.7% higher among daily smokers of marijuana compared to non-smokers (*P*-trend = 0.09). There were no differences in sperm counts across the three groups. However, when using the alternative categorization, weekly but not daily users of marijuana had lower sperm concentrations and total sperm counts compared to non-users (Supplementary Fig. S1).

Table II Median levels (5–95 percentiles) of reproductive hormone levels and semen quality stratified according to smoking habits (unadjusted analysis).

		Reproductive hormones					Semen parameters	
	*n** (%)	Total T (nmol/L)	SHBG (nmol/L)	Free T (pmol/L)	Inhibin B/FSH ratio	LH (IU/L)	Sperm concentration (million/mL)	Total sperm count (million)
Total population	2008	17.6 (10.7–27.3)	28.7 (14.4–49.0)	396 (246–620)	66 (16–248)	3.3 (1.6–6.4)	42.0 (3.6–144.0)	132.6 (8.5–467.0)
Cigarettes								
Daily	467 (23)	18.4 (11.1–28.9)	29.1 (14.6–50.6)	414 (263–656)	62 (17–247)	3.3 (1.6–6.7)	38.0 (2.4–118.4)	106.0 (6.7–395.3)
Occasional	576 (29)	17.8 (11.7–26.7)	27.5 (14.4–47.2)	406 (262–625)	69 (17–249)	3.3 (1.5–6.3)	44.5 (4.2–156.0)	140.6 (8.9–471.0)
No	946 (48)	17.0 (10.0–27.3)	29.0 (14.2–50.8)	382 (231–602)	67 (15–240)	3.2 (1.5–6.4)	44.0 (3.6–150.0)	140.8 (10.9–516.0)
E-cigarettes								
Daily	25 (2)	16.2 (13.3–26.8)	27.3 (14.9–46.8)	402 (277–550)	64 (12–265)	2.7 (1.4–6.1)	39.0 (1.1–131.0)	91.8 (0.9–679.7)
Occasional	139 (11)	18.7 (11.7–26.1)	27.3 (12.2–48.1)	421 (285–618)	63 (14–267)	3.1 (1.5–6.3)	38.0 (1.2–127.1)	116.3 (3.7–441.5)
No	1057 (87)	17.7 (10.8–26.9)	28.7 (14.6–47.7)	396 (247–620)	67 (18–249)	3.2 (1.5–6.1)	43.0 (4.2–150.5)	137.2 (12.0–492.2)
Snuff								
Daily	68 (6)	17.9 (12.4–26.5)	27.4 (12.4–47.6)	406 (282–660)	81 (21–290)	2.9 (1.2–6.4)	43.5 (5.8–145.7)	142.8 (11.3–466.7)
Occasional	231 (19)	19.0 (11.0–28.1)	28.8 (13.9–49.3)	419 (259–648)	62 (19–250)	3.2 (1.6–6.0)	42.0 (4.4–160.5)	133.6 (11.5–532.8)
No	922 (76)	17.5 (10.8–26.7)	28.6 (14.8–47.6)	392 (246–605)	67 (17–249)	3.2 (1.5–6.2)	42.0 (2.3–144.0)	132.0 (7.6 470.9)
Marijuana								
Daily	52 (3)	19.5 (13.4–27.0)	29.9 (15.4–48.2)	413 (280–745)	59 (20–261)	3.6 (1.7–8.3)	37.5 (2.0–171.5)	97.5 (6.5–826.5)
Occasional	601 (30)	18.4 (11.5–27.5)	29.0 (15.8–49.8)	407 (256–647)	64 (16–221)	3.3 (1.6–6.5)	41.0 (3.7–130.0)	129.5 (9.1–439.4)
No	1334 (67)	17.2 (10.3–27.2)	28.4 (13.9–48.7)	389 (239–606)	68 (16–253)	3.2 (1.5–6.3)	43.0 (3.6–150.0)	135.5 (8.6–473.8)

SHBG, sex hormone-binding globulin.
*Total numbers may vary due to missing values.

Sensitivity analyses

Further adjustment for maternal smoking during pregnancy did not change the main findings for any of the outcomes.

No interaction was observed between cigarette smoking and any of the other tobacco products, neither when including an interaction term in the adjusted models nor when stratifying on daily cigarette smoking (yes/no). Thus, the observed effects in relation to testicular function were the same irrespective of whether the men were cigarette smokers or not.

Discussion

In young men, unselected regarding semen quality, the most frequent smoking type was cigarettes followed by marijuana and snuff. Cigarette smoking and e-cigarette use were associated with lower sperm counts. Cigarette smoking was, as expected, associated with higher total and free testosterone levels, but this was not found for e-cigarette use. We observed no significant associations between snuff and any of the markers of testicular function.

Reduced sperm counts in cigarette smoking men from the general population has been reported previously (Richthoff *et al.*, 2008). Dysfunction of the Sertoli and Leydig cells leading to alterations of intra-testicular reproductive hormone levels as well as histological changes and apoptosis have been suggested as explanations for the reduced semen quality in smokers [reviewed in Dai *et al.* (2015)]. E-cigarettes contain fewer constituents than traditional cigarettes: typically propylene glycol, glycerine, some additives and often also nicotine and flavouring agents. Estimations suggest availability of more than 7500 different flavours, the effects of which are likely to differ (Harrell *et al.*, 2017). Thus, the composition of the aerosol created from e-cigarettes, consisting of fine and ultrafine particles, depends on the e-liquid but also the device being used. Despite e-cigarettes not containing tars and many of the other potentially harmful substances found in cigarette smoke, we observed that compared to non-users, both daily cigarette smokers and users of e-cigarettes (adjusted for cigarette smoking) were characterized by lower sperm concentration and total sperm count with effect sizes of similar magnitude. The literature on reproductive effects of e-cigarette use is very limited. In one animal study impairment of testicular function was observed after exposure to e-liquid. Compared to controls, adult rats exposed to e-cigarette liquid with tobacco flavour with or without nicotine both showed reduced sperm concentration and histopathological changes in the testes suggesting adverse effects of the e-liquid *per se*. Exposed rats also showed lower plasma testosterone levels (El Golli *et al.*, 2016; Rahali *et al.*, 2018), whereas we found no difference in serum testosterone according to e-cigarette use. Importantly, the route of exposure in that study was not comparable to that in humans as the rats were exposed intraperitoneally and not via aerosol. We observed no association between use of snuff and any of the reproductive parameters. Nor was snuff associated with reproductive parameters in a Swedish study of men comparable to those in the present study (Richthoff *et al.*, 2008).

Previously, we have shown that men using marijuana at least once per week had significantly lower sperm counts compared to non-users in a similar cohort of men recruited from 2008 to 2012 (Gundersen *et al.*, 2015) with no overlap between the previous and current cohort. In our main analyses, we observed no differences in sperm counts in daily or occasional users of marijuana compared to non-users. However, in sensitivity analyses using the alternative categorization, weekly users, but not daily users, were characterized by lower sperm counts (Supplementary Fig. S1) which could explain the differences in findings as daily and weekly use was categorized into the same group in the previous study. Thus, there does not seem to be a dose-response association between marijuana use and semen quality. In contrast to daily users of marijuana, it could be speculated that regular users are more likely to be co-users of other types of recreational substances which could have an influence on testicular function. In fact, in the present study, median alcohol intake in daily users of marijuana was similar to that of non-users (5 units per week and 4 units per week, respectively), whereas the median alcohol intake for occasional users was 10 units per week. In adjusted regression models, we adjusted for alcohol intake, however, the pattern could be similar for other types of substance use not accounted for.

The observed higher total and free testosterone levels in cigarette smoking men are in accordance with other population-based studies (Travison *et al.*, 2007; Shiels *et al.*, 2009; Holmboe *et al.*, 2015). Tobacco smoke contains more than 4000 constituents, including nicotine, tar, carbonic monoxide and polycyclic aromatic hydrocarbons (PAHs) (Sharma *et al.*, 2016). It has been speculated whether cigarette smoke and nicotine may act as aromatase inhibitors reducing the conversion of testosterone to oestradiol and thus cause an elevated testosterone level (Biegon *et al.*, 2012). However, in the present study, daily cigarette smokers were characterized by higher total and free testosterone, and slightly higher levels of oestradiol (Supplementary Table SII) in line with observations from other population-based studies (Shiels *et al.*, 2009; Holmboe *et al.*, 2015), indicating sufficient aromatase activity. Furthermore, men using e-cigarettes, which in most cases were reported to include nicotine, did not have higher levels of testosterone compared to non-users, which would be expected if nicotine was the cause of higher testosterone. However, an association between e-cigarette use and testosterone cannot be excluded based on the present study, which had limited power. Alternatively, PAHs have been suggested to explain the difference in testosterone levels for smokers and non-smokers. PAHs are a class of ubiquitous toxic compounds produced by the incomplete combustion of organic materials and accordingly are highly present in tobacco smoke (Suwan-ampai *et al.*, 2009). *In vitro* studies of testis tissue from fish have shown an inverse U-shaped association between PAH levels and testosterone levels, indicating that lower doses of PAH can have a stimulatory effect on testosterone production, whereas inhibition was seen at high doses (Evanson and Van Der Kraak, 2001). Furthermore, several PAHs have been shown to exert antiandrogenic effects by modifying human androgen receptor activation (Vinggaard *et al.*, 2000). Thus, a competitive inhibition of the binding of testosterone to the androgen receptor by PAHs could lead to an attenuated negative feedback of the circulating testosterone on the LH release, resulting in an increase of LH, and subsequently higher circulating testosterone levels which could explain the observed higher levels of testosterone in smoking men. In line with this, a population-based study including 1102 adult men from NHANES (Wang *et al.*, 2017) observed that urinary PAH metabolites were positively associated with higher total testosterone level.

A strength of the study is the rather large sample size with information on various smoking behaviours, although the number of daily e-cigarette users was limited. Furthermore, detailed information on potentially related lifestyle factors was obtained, and the men were unaware of their testis function at time of recruitment. The included men are assumed to represent the general population in their age group regarding reproductive function and smoking habits. From 1996, where recruitment of military conscripts for monitoring semen quality among Danish men was initiated (which this study is part of), the proportion of men with known prior reproductive issues has been unchanged (Priskorn *et al.*, 2018). In addition, a previous study showed that concentrations of reproductive hormones did not differ between participants and 79% of the non-participants who agreed to have a blood sample drawn in a sub-study (Andersen *et al.*, 2000). According to the Danish Health Authorities, data from 2018 showed that 5.3% of Danes above 15 years of age reported to be using e-cigarettes of which 3% reported daily use (Danish Health Authorities, 2019). Numbers from the same survey show that 29% of daily e-cigarette users are co-using cigarettes which is in accordance with our study (33%). A limitation of the study is that information on smoking was obtained from questionnaire data with no objective measures of smoking exposure such as cotinine and no knowledge of the actual content in the different products, which would be needed to better understand the mechanisms behind the observed associations. Furthermore, the categorization into daily, occasional and non-users is a rather crude categorization but on the other hand, these categories could be used consistently across the different smoking types. It can also be debated whether it would have been reasonable to include information on doses of different types of smoking, although this type of information most often produces recall bias. However, it has previously been shown that testosterone levels were different for smokers and non-smokers but within the group of smokers, testosterone levels did not differ according to number of cigarettes smoked per day (Shiels *et al.*, 2009), indicating that a continuous exposure to cigarettes irrespective of dose is sufficient to affect markers of testicular function. In general, we observed frequent co-use of smoking products with cigarette smoking being the most frequent one and, for that reason, we adjusted for cigarette smoking in fully adjusted models. It could be relevant to study effects of different types of smoking in larger groups of single-users only to eliminate the effect from co-exposure. Due to lack of power regarding number of single-users, this was however not possible in the present study. Finally, due to the cross-sectional design, causal inferences cannot be made, and furthermore we cannot rule out that our results can be influenced by confounding by behavioural factors not included in the statistical analyses, including information on previous smoking habits.

This is the first human study to indicate that not only cigarette smoking but also use of e-cigarettes is associated with lower sperm counts, although the mechanisms are unknown. This could be important knowledge for clinicians and men trying to achieve a pregnancy as e-cigarettes are often considered to be less harmful than conventional cigarette smoking. Interestingly, e-cigarette use was not associated with higher serum testosterone levels as was cigarette smoking. However, the associations need to be confirmed in other studies and with more detailed information on the e-liquid content including information on the chemical profile of users.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Authors' roles

Substantial contribution to conception and design: S.A.H., L.P. and N.J. Data acquisition: L.P. and N.J. Data analysis: S.A.H. and L.P. Data interpretation: all authors. Drafting of the article: S.A.H. and L.P. Revision of the article critically for important intellectual content: all authors. Final approval of the article: all authors.

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Conflict of interest

The authors have nothing to disclose.

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