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Complete List of Authors:	Jensen, Tina; Rigshospitalet, University Department of Growth and Reproductio; University of Southern Denmark, Dep of Environmental medicine Gottschau, Mads; University of Southern Denmark, Dep of Environmental Medicine Madsen, Jens Otto; University of Southern Denmark, Dep of Environmental Medicine Andersson, Anna-Maria; Rigshospitalet, University Department of Growth and Reproduction Lassen, Tina; Rigshospitalet, University Department of Growth and Reproduction Skakkebaek, Niels; Rigshospitalet, University Department of Growth and Reproduction Swan, Shanna; Mount Sinai School of Medicine, Department of Preventive Medicine Priskorn, Lærke; Rigshospitalet, University Department of Growth and Reproduction Juul, Anders; Rigshospitalet, University Department of Growth and Reproduction; University of Copenhagen, Rigshospitalet, Dep. of Growth and Reproduction Jørgensen, Niels Erik; Rigshospitalet, University Department of Growth and Reproduction
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Habitual alcohol consumption associated with reduced semen quality and changes in reproductive hormones; a cross-sectional study among 1,221 young Danish men

Short running title: Alcohol consumption and semen quality

Professor Tina Kold Jensen[1-2]

MD Mads Gottschau[1]

MD Jens Otto Broby Madsen[1]

PhD Anne-Maria Andersson[2]

Cand.scient.san.publ Tina Harmer Lassen[2]

Professor Niels E. Skakkebæk[2]

Shanna H. Swan[3]

Lærke Priskorn[1]

Professor Anders Juul[2]

MD, PhD Niels Jørgensen[2]

1. Department of Environmental Medicine, University of Southern Denmark.
2. University Department of Growth and Reproduction, Rigshospitalet, The Faculty of Medical and Health Sciences, University of Copenhagen, Denmark
3. Department of Preventive Medicine, Icahn School of Medicine at Mount Sinai, 1 Gustave L. Levy Place, Box 1057, New York, NY 10029

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Corresponding author:
Tina Kold Jensen
University Department of Growth and Reproduction,
Rigshospitalet
Blegdamsvej 9
2100 Copenhagen Ø
tkjensen@health.sdu.dk

Abstract

Objective: Study associations between three measures of alcohol consumption (recent, typical/habitual, and binging), semen quality and serum reproductive hormones.

Design: Cross-sectional population based study.

Setting and participants: 1,221 young Danish men, aged 18-28 years were recruited when they attended a compulsory medical examination to determine their fitness for military service from 2008 to 2012. Total alcohol consumption; 1) in the week preceding the visit (recent alcohol intake), 2) in a typical week; and 3) frequency of "binge drinking" (consuming more than 5 units/day)) in the past 30 days was estimated.

Main outcome measures: Semen quality (volume, sperm concentration, total sperm count, and percentages of motile and morphologically normal spermatozoa) and serum concentration of reproductive hormones (FSH, LH, testosterone, SHBG, estradiol, free testosterone and inhibin B).

Results: Sperm concentration, total sperm count and percentage of spermatozoa with normal morphology were negatively associated with increasing habitual alcohol intake. This association was observed in men reporting at least 5 units in a typical week but was most pronounced for men with a typical intake of more than 25 units per week. Men with a typical weekly intake above 40 units had a 33% (95% CI -11%;59%) reduction in sperm concentration compared to men with an intake of 1-5 units/week (reference). We also saw a significant increase in serum free testosterone with an increase in alcohol consumption the week preceding the visit. Binging was not independently associated with semen quality or reproductive hormones.

Conclusions:

As the duration of spermatogenesis is approximately 72 days the typical alcohol intake is probably a more appropriate exposure measure than the intake the previous week. Contrary to this serum reproductive hormone levels fluctuate and are theoretically more susceptible towards recent alcohol

exposure. If confirmed, these results suggest that young men should be advised to avoid habitual high alcohol intake.

Article summary

Article focus

- High alcohol intake has been associated with a wide range of diseases. However, few studies have examined the correlation between alcohol and reproductive function and none have separated the effects of alcohol intake the week preceding the semen and blood sample, habitual alcohol intake and binging.

Key messages

- Habitual alcohol intake was a stronger predictor for reduced semen quality already from a weekly intake above 5 units in a typical week, but was most pronounced for men with a typical intake of more than 25 units per week, than alcohol intake the week preceding the visit.
- Last weeks intake was a stronger predictor for increase in serum free testosterone, supported by biology in which duration of spermatogenesis is approximately 72 days whereby the typical alcohol intake is probably a more appropriate exposure measure than the intake the previous week. Contrary, serum reproductive hormone levels fluctuate and are theoretically more susceptible towards recent alcohol exposure.
- Our finding are relevant to public health since young men in the western world have a high alcohol intake and this may be contributing to recent reports of poor semen quality. If confirmed, these results suggest that young men should be advised to avoid habitual high alcohol intake.

Strengths and limitations of the study

- Our study was large and consisted of young healthy men, the majority of our young men had no knowledge of their fertility potential and this is unlikely to have affected their motivation to participate.
- Our study was cross-sectional and reverse causation cannot be excluded, whereby men with poor semen quality have an unhealthier lifestyle and health behaviour and drink more alcohol even though we adjusted for these factors.
- The men in our study reported daily alcohol consumption the week preceding the visit, as we assumed that to be more accurate to recall than an average intake. This consumption may differ from the typical weekly intake, which can lead to misclassification of exposure.

Introduction

Alcohol consumption is widespread in the Western world, especially in Europe[1]. Drinking patterns have changed over time and binging (defined here as 5 units or more in a single day) is widespread among young Europeans[2]. Moderate alcohol consumption has been associated with reduced morbidity and mortality[3]. However, excessive alcohol intake has a negative impact on health (e.g. coronary heart disease, stroke and liver disease[4 5]).

Some studies found association between alcohol intake and semen quality[6-9], however other did not confirm these findings[10-18]. However, it is difficult to compare across studies, since populations as well as alcohol intake vary considerably between them. In addition, most studies only addressed average alcohol intake by use of only few questions, and within response categories consumption may vary considerably and is likely to be underreported. Only one study addressed the dose-response relationship between recent alcohol intake (during the past 5 days) and semen quality among 347 young Danish men. Poorer semen quality was found at higher levels of alcohol intake, although not statistical significant[16]. In an earlier multicenter study of over 8,000 American and European men, we found no adverse effects of alcohol intake in the week preceding the visit on semen quality. However, in that study most men reported only moderate intake of alcohol[19]. While some men in that study were similar to the men in this study, much less detailed information about drinking habits was collected prior to 2008. To our knowledge no studies have examined the effect of binging on male reproductive parameters nor have the effects of recent versus habitual alcohol intake been studied in healthy populations. We therefore investigated the association between semen quality and serum reproductive hormones and alcohol consumption during the week preceding the visit, in a typical week, and binging in a cross-sectional study of 1,221 young Danish men recruited between 2008-2012.

Materials and methods

Population

Because of the military draft in Denmark, all 18-year-old men, except those suffering from severe chronic disease, are required to undergo a compulsory physical examination to determine their fitness for military service. Since 1996, trained staffs from the Department of Growth and Reproduction at Copenhagen University Hospital (Rigshospitalet, Copenhagen, Denmark) have approached the draftees when they have appeared for their compulsory physical examination and have invited them to participate in a study of semen quality taking place at Rigshospitalet. Only men recruited from January 2008 to April 2012 were included in the present study, since the questionnaire they completed included detailed information about alcohol intake. All participants completed a questionnaire, delivered a semen sample, had a blood sample drawn, and underwent a physical examination. They received compensation for their time (DKK 500, equal to approximately US$85). Participants did not differ from nonparticipants with regard to age, but they were generally better educated than nonparticipants (data not shown). Ethical approval was obtained from the local ethical committee. A detailed description[20], and other aspects of the study have previously been published[21-24].

Semen analysis

All men provided a semen sample by masturbation in a room close to the semen laboratory. The period of ejaculation abstinence (time since last ejaculation) was recorded, and the semen sample was analyzed for volume, sperm concentration, total sperm count, percent motile spermatozoa, and percent morphologically normal spermatozoa as described by Jørgensen et al. al[20], which is in accordance with the most recent guideline from the World Health Organization[25]. Since 1996, our laboratory has led a quality control program for assessment of sperm concentration; the laboratory has kept the interlaboratory difference unchanged[26], and the variation between technicians was less

than 10%. The same 2 experienced technicians assessed the sperm morphology according to strict criteria for the first 904 men[27].

Serum samples

Serum levels of follicle-stimulating hormone (FSH), luteinizing hormone, and sex hormone-binding globulin (SHBG) were determined using a time-resolved immunofluorometric assay (Delfia; Wallac Oy, Turku, Finland). Testosterone and estradiol levels were determined using time-resolved fluoroimmunoassays (Delfia; Wallac Oy). Inhibin B level was determined by means of a specific 2-sided enzyme immunometric assay (Inhibin B Gen II; Beckman Coulter Ltd., High Wycombe, United Kingdom). The hormones were all measured within same time period and in the same assay batches. Free testosterone was calculated on the basis of the measured serum concentrations of total testosterone and SHBG using the method of Vermeulen et al[28] and a fixed albumin concentration of 43.8 g/L[28].

Physical examination

Physicians assessed genital development, the possible presence of a varicocele (grades 1–3) or hydrocele, and the location of the testes in the scrotum, and the consistency of the testis and epididymis were recorded. Weight and height was measured, and body mass index (BMI) was calculated as weight in kilograms divided by squared height in meters.

Questionnaire

Prior to the examination, all participants completed a questionnaire that collected information on previous and/or current diseases and genital diseases. Self-reported diseases of the reproductive organs affecting semen quality (torsion of testes, epididymitis, or inguinal hernia) were summarized

in 2 variables: “self-reported genital conditions” and “sexually transmitted diseases” (gonorrhea or chlamydia).

The mothers to the young men responded to questions about maternal education, which was coded as: less than 9, 9–10, and more than 10 years of schooling. Data on physical activity was converted to watts per week using the method of Craig et al[29]. Men were asked about current smoking habits and whether they were exposed to smoking in utero. Daily caffeine intake was estimated based on their reported intake of caffeine-containing beverages the week prior to the visit. Men completed a diary reporting their daily intake of red and white wine, beer, strong alcoholic drinks, alcopops and others during the week prior to participation and delivery of the semen and blood samples (recent intake). Men were told that 1 beer, 1 glass of wine or 40 mL of spirits contained 1 unit of alcohol (≈12 g of ethanol), whereas 1 strong beer or 1 alcopop contained 1.5 units of alcohol and 1 bottle of wine contained 6 units of alcohol and were asked to convert their intake to units. Alcohol intake was calculated as the sum of daily reported unit intakes within that week. In addition, the men were asked whether their alcohol intake in the week preceding the visit represented a typical week (typical/habitual intake). They were also asked how many times during the past 30 days that had been drunk or had consumed more than 5 units of alcohol on one occasion, which we defined as binging.

Statistics

Exposure variables were total number of alcohol units in the week preceding the visit (recent intake) and in a typical week (typical/habitual intake). Alcohol units were divided into 5 unit intervals. Because abstainers may differ from light-moderate drinkers we selected 1-5 drinks/week as the reference category. In addition, number of binge episodes and number of times being drunk during the past 30 days was categorized as; 0 (reference), 1-2, 3-5; 6-9; more than 9.

Sperm parameters and reproductive hormone levels were compared in relation to alcohol intake and binging and the distributions of the relevant covariates from the questionnaires and physical examinations among men with different alcohol intake were compared by χ^2 test in order to identify potential confounders. Finally, data were analyzed using multivariable linear regression models. Because of the non-normal (skewed) distributions of semen quality and serum reproductive hormones, semen parameters were transformed by cubic root and reproductive hormones by natural logarithmic scale and the latter back-transformed to obtain the expected percent change per unit increase in exposure. Covariates were then excluded stepwise if their exclusion did not change effect estimate by more than 10%. In final models, the same set of covariates was used for all semen parameters: period of abstinence, current smoking and BMI, except that period of abstinence was not included for sperm morphology and motility models and duration between the time of ejaculation and analysis of the sample was included only for models predicting sperm motility. Models predicting reproductive hormones were adjusted for time of blood sampling, current smoking and BMI. We initially adjusted alcohol intake for binge episodes, but as estimates were unchanged, binging was not included. Tests for linear trend were performed after excluding men with no alcohol intake. Finally, analyses were performed separately for beer adjusting for total alcohol intake, since beer was consumed by most men. We evaluated the fit of the regression models by testing the residuals for normality and by inspecting the residual plots. SPSS statistics version 19 was used and the results are presented with 95% confidence intervals (95% CI).

Results

A total of 1,221 men were included with a mean age of 19.1 years. The median alcohol intake the week preceding the visit was 11 units (25 and 75 percentiles 1-21 units) and 64% and 59% of men had binged or had been drunk more than twice during the past 30 days, respectively. Beer was the favorite alcoholic beverage and the median beer intake the week preceding the visit was 5 units (0-13 units). A total of 553 men (45%) reported that the week preceding the visit represented a typical week and these were used in the analyses of typical/habitual alcohol intake. These men did not differ from the total population (N=1,221) in semen or hormone parameters.

Semen quality decreased with increasing recent alcohol intake (data not shown) and binging (Table 1). Testosterone and cFT increased and SHBG decreased with increasing recent alcohol intake (Table 2) and binging already from an intake above 5 units per week. Men with a typical alcohol intake of 30 units in a typical week or binging were more often smokers, had a higher caffeine intake, more often reported having had STDs or fever, were younger and their mothers had a higher education (supplementary Table).

No clear association between recent alcohol intake (the week preceding the visit)(data not shown), binging (Table 3) and semen quality was found after control for confounders. A dose-response association with recent alcohol intake from one unit per week (abstainers excluded) and higher testosterone (p-trend=0.01) and cFT (p-trend<0.01) and lower SHBG (p-trend<0.01) was found (Table 3, Figure 1) after control for confounder. Similar associations were found with number of binge episodes and being drunk during the last 30 days (Table 3). Men with a weekly alcohol intake above 40 units the week preceding the visit had 20% (95%CI 9%;31%) higher cFT after control for confounders. No association with LH, FSH, inhibin B and estradiol was found (data not shown).

Among the 553 men with a habitual alcohol intake (alcohol intake the week preceding the visit represented a typical week) we found an inverse dose-response association between alcohol intake and sperm concentration (p-trend=0.02), total sperm count (p-trend=0.01) and percentage morphologically normal sperms (p-trend=0.01) (Table 3, Figure 2) after adjustment. The trend was more pronounced among men with a typical weekly alcohol intake above 25 units. Cubic root transformed sperm concentration and percentage morphologically normal spermatozoa were respectively 0.39 (95% CI -0.92;0.14) and 0.51 (95%CI: 1.03;0.01) lower among men with a typical alcohol intake of more than 40 units compared to men with an intake of 1-5 units in a typical week. No alcohol intake was also associated with reduced semen quality. Percentages of motile spermatoza and semen volume were not associated with habitual alcohol intake (data not shown). Habitual alcohol intake was also associated with serum reproductive hormones although not as strongly as the recent intake (data not shown). The associations between recent alcohol intake from beer was similar to that of total alcohol.

Discussion

Findings

In this cohort of more than 1,200 young healthy men with detailed questionnaire information on alcohol intake we found that a habitual alcohol intake was associated with a reduction in semen quality already from more than 5 units per week in a typical week although the decreasing trend was most apparent for men with a typical weekly intake above 25 units. In addition, recent alcohol intake (the week preceding the visit) was associated with increase in serum testosterone and reduction in SHBG. No independent adverse effect of binging was found. The negative association between alcohol intake and semen quality may be attributed to a direct adverse effect of alcohol on spermatogenesis or it may be a result of differences in lifestyle, health behavior and diet found among high alcohol consumers, despite adjustment for these factors.

This is to our knowledge the first study to separate the effects of recent versus habitual alcohol exposure, and as the duration of spermatogenesis is approximately 72 days[30] the typical intake is probably a more appropriate exposure measure than the recent intake during the week preceding the delivery of the semen sample. Contrary to this serum reproductive hormone levels fluctuate[31] and are theoretically more susceptible towards recent changes (within days) induced by recent alcohol exposure (the week preceding the blood sampling).

Comparison with previous studies

Our findings are in accordance with a recent study among 347 young Danish men in which a non-significant dose-response association between recent alcohol intake (5 days preceding the delivery of the sample) and semen quality was found[16]. The study did not obtain information on typical alcohol exposure nor on binging. However, a Chinese study among 1,346 men did not find association between semen quality or alcohol intake neither in high doses (more than 120 units per months)[18]. Other previous studies of association between alcohol intake and semen quality have

shown contradictory results[6 7 9-15 17], but have been conducted in small selected population and not been able to address dose-response associations and none have been able to separate the effect of recent versus habitual intake. A previous multicenter study including young and fertile men did not find adverse effect of recent alcohol intake (the week preceding the visit) on semen quality, however most men only had a moderate alcohol intake[19]. The young Danish men in that study were also conscripts but included from 1996 to 2007 after which the questionnaire included more detailed information on alcohol intake. The men in this study were therefore included from 2008. No alcohol consumption was also associated with reduced semen quality, which may be attributed to social or health parameters differentiating non-drinkers from drinkers.

We found no independent adverse effect of binging on semen quality, which to our knowledge has not previously been reported. It was, however, difficult to separate binging from typical alcohol intake as most young men who binged also had a high alcohol intake. The percentage of Danes drinking 5 units or more in a typical drinking occasion has been reported to be 23%. Furthermore, young people aged 15-24 years are more likely (25%) to drink 5 units or more on one occasion compared to people above 55 years of age (11%)[1].

Animal studies have suggested that alcohol may affect the hypothalamic-pituitary-gonadal axis, change sperm morphology and directly negatively affect the testis[32 33]. In addition, analysis of histological samples from 195 deceased men showed that high alcohol consumption (>80 g alcohol/>7 units per day) was associated with significantly reduced spermatogenesis, including spermatogenic arrest and sertoli-cell-only syndrome[34].

Our observed association between alcohol intake, testosterone and cFT is in accordance with previous studies showing increased total testosterone and cFT or increased cFT in combination with decreased SHBG[16 19 35 36], whereas other studies found no association with cFT[37-40]. If SHBG levels are affected this could explain the observed increase in cFT. Otherwise, it may be explained by

alcohol detoxification leading to a changed metabolism of steroids in the liver. In contrast, decreased testosterone levels have been reported in male alcoholics suggesting that habitual alcohol abuse may damage Leydig cells or impair the hypothalamic-pituitary-gonadal axis[6 41].

Strengths and weaknesses

Our study has several strengths. It was large and consisted of young healthy men and the participation rate was approximately 30%, which is higher than in other population-based semen-quality studies [42-44]. The drinking habits of these men resembled those of Danish men aged 16-20 years in 2008[45], suggesting that they are not selected. In addition, the majority of our young men had no knowledge of their fertility potential and this is unlikely to have affected their motivation to participate. Our study was, however, cross-sectional and reverse causation cannot be excluded, whereby men with poor semen quality have an unhealthier lifestyle and health behaviour and drink more alcohol even though we adjusted for these factors.

The men in our study reported daily alcohol consumption the week preceding the visit, as we assumed that to be more accurate to recall than an average intake. This consumption may differ from the typical weekly intake, which can lead to misclassification of exposure, and we therefore repeated the analyses among men stating that that week represented a typical week. We used diary information on alcohol consumption, which makes it easier to recall the units consumed, but it may still be underreported. Further, the definition of a unit may vary according to size, method of preparation and brand. We defined binging as an intake of 5 units or more in a single day, which is also the definition used by The Danish National Board of Health[46]. These potential sources of exposure misclassification are likely to be unrelated to semen quality, since the men responded to the questionnaire, before they knew the result of their semen and blood analysis. Such non-differential misclassifications would underestimate the associations between alcohol habits and semen quality and reproductive hormones and cannot explain our findings.

Conclusion and implications

In conclusion, we found an adverse dose-response association between semen quality and habitual alcohol intake most pronounced among men with an alcohol intake above 25 units in a typical week. In addition, men with a high alcohol intake the week preceding the visit had increased free testosterone. This is to our knowledge the first study among healthy young men with detailed information on alcohol intake and given the fact that young men in the western world have a high alcohol intake, this is of public health concern and could be a contributing factor to the low sperm count reported among young men[20]. It remains to be seen whether semen quality is restored if alcohol intake is reduced, but young men should be advised that high habitual alcohol intake may affect their reproductive health.

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Contributors: TKJ performed data analysis and interpretation and wrote the manuscript. MG and JOBM provided assistance with data analysis and revised and edited the manuscript. THL, LP, NJ performed data collection, provided assistance with data analysis and interpretation, revised and edited the manuscript. NES, SHS, AJ provided assistance with data analysis and interpretation, and revised and edited the manuscript.

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Competing interests: All authors have completed the ICMJE uniform disclosure form at www.icmje.org/coi_disclosure.pdf and declare: no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted

work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.

Data sharing: Additional data regarding technical details, statistical code, and derivative data is available from the principal investigator at tkjensen@health.sdu.dk. Data access for further analyses is possible through direct collaborative agreement or through locally managed access arranged through the study's principal investigator.

Ethical approval: All studies have ethical approval in their respective countries and participants have provided informed consent.

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Table 1 Semen quality according to typical (last week represented a typical week) alcohol intake and binging during the past 30 days among respectively 553 and 1,221 healthy, young Danish men. Presented as median (M) and 5 and 95 percentiles (5-95).

Alcohol intake	N	Semen volume mL		Sperm concentration mill/mL		Total sperm count mill		Motility %		Morphology[a] %	
		M	5-95	M	5-95	M	5-95	M	5-95	M	5-95
Units in a typical week, N=553											
0	122	3.3	0.9;6.7	39	5;140	132	17;554	55	26;79	7.8	0.5;15.9
1-5	93	3.3	1.5;5.8	50	6;159	175	18;529	58	29;81	7.8	2.0;15.5
6-10	72	3.2	1.5;6.2	50	6;204	187	20;574	58	20;79	5.8	0;21
11-15	82	3.2	2.0;6.3	41	3;180	152	10;604	55	19;78	7.0	1.9;18.3
16-20	64	3.0	1.1;7.5	51	6;148	163	13;514	58	29;80	7.5	1.8;19.5
21-25	47	2.9	1.1;5.6	50	13;205	141	45;541	60	28;78	7.0	1.9;15.8
26-30	27	3.4	1.0;6.9	34	1;243	147	2;574	53	0;78	8.0	;
31-35	14	3.0	0.8;	43	6;	96	6;	55	0;	6.3	3.5;
36-40	11	3.6	1.4;	41	3;	108	9;	67	23;	8.0	1.0;
>40	21	3.6	1.4;8.4	33	4;162	86	23;507	62	38;79	6.5	0;
Number of binge episodes during the past 30 days[b] N=1,221											
0	176	3.2	1.0;6.1	49	5;160	145	17;583	57	29;79	7.5	0.5;15.8
1-2	255	3.3	1.4;7.0	47	3;160	153	9;535	59	13;80	7.5	0.5;15.5
3-5	425	3.3	1.2;6.2	48	3;176	164	11;610	58	27;79	7.3	1.0;17.0
6-9	258	3.3	1.4;6.0	43	5;158	137	11;446	57	22;78	6.5	0.5;15.5
>9	92	3.2	1.2;7.9	41	5;163	130	16;469	56	19;78	6.0	0.5;12.6

[a]Counted for 904 men of whom 397 stated that last weeks alcohol intake represented a typical week.
[b]Binging defined as alcohol intake of more than 5 units on one occasion.

Table 2 Reproductive hormones according to recent (the week preceding the visit) alcohol intake and binging during the past 30 days among 1,194 healthy, young Danish men. Presented as median (M) and 5 and 95 percentiles (5-95).

Alcohol intake	N	FSH (IU/L)		LH (IU/L)		Testosterone (nmol/L)		SHBG (nmol/L)		Free testosterone pmol/L		Inhibin B (pg/mL)		Estradiol (Nmol/L)	
		M	5-95	M	5-95	M	5-95	M	5-95	M	5-95	M	5-95	M	5-95
Units the week preceding the visit N=1,194															
0	243	2.4	0.9;5.2	3.4	1.6;6.8	19.1	10.2;35.4	29	13;52	439	249;749	166	81;271	81	48;128
1-5	198	2.4	1.0;5.8	3.4	1.4;6.1	19.5	12.1;32.5	28	15;51	446	288;694	169	73;275	81	48;134
6-10	154	2.5	0.9;5.9	3.3	1.7;7.1	20.8	12.1;32.3	28	12;46	486	260;742	162	98;283	79	46;128
11-15	162	2.3	0.9;6.2	3.3	1.3;6.4	20.8	12.4;32.8	29	14;48	483	283;770	169	67;295	80	32;125
16-20	131	2.3	1.0;6.0	3.1	1.4;6.7	21.1	11.8;34.5	28	14;50	480	298;752	163	76;266	80	54;120
21-25	92	2.7	0.8;6.3	3.4	1.6;7.4	22.1	11.8;36.2	30	14;56	473	283;883	159	72;290	88	42;141
26-30	72	2.5	1.0;6.1	3.4	1.7;5.8	21.4	12.3;30.5	27	14;47	476	285;779	164	76;257	81	39;143
31-35	48	2.4	0.6;5.6	3.5	1.7;7.0	21.2	13.0;36.7	28	12;55	526	334;858	185	93;321	83	44;131
36-40	28	2.5	0.8;6.1	3.6	1.7;7.0	21.1	11.5;36.6	26	12;44	497	326;900	157	86;226	75	38;133
>40	66	2.4	0.8;6.6	3.3	1.8;7.8	22.9	13.8;36.1	27	11;47	541	335;920	158	82;290	84	41;140
Number of binge episodes during the past 30 days[a] N=1,194															
0	171	2.4	0.9;5.8	3.6	1.6;7.4	17.9	10.9;32.9	27	12;51	440	260;740	166	84;281	83	50;127
1-2	250	2.6	1.0;5.7	3.2	1.3;6.5	19.9	11.4;32.3	28	13;54	449	257;737	164	71;266	80	43;131
3-5	405	2.3	1.0;5.8	3.2	1.4;6.5	20.8	12.0;33.1	28	14;49	476	286;765	170	86;277	80	46;125
6-9	254	2.4	0.9;6.5	3.4	1.7;6.6	21.3	12.0;35.6	28	14;48	496	300;842	164	73;297	80	41;140
>9	90	2.5	0.8;6.1	3.3	1.8;7.0	21.9	12.5;37.2	27	13;55	523	310;858	164	79;291	85	41;137

[a]Binging defined as alcohol intake of more than 5 units on one occasion.

Table 3 Results from linear regression analyses of semen quality (adjusted β-coefficients) and serum reproductive hormones (percent change) among young, Danish men according to habitual alcohol intake (last week represented a typical week) or recent (the week preceding the visit) or binging during the past 30 days.

Alcohol intake	N	Sperm concentration[a,b] mill/mL		Total sperm count[a,b] mill		Morphology[b,c] %		N	Testosterone[d,e] nmol/l		SHBG[d,e] nmol/l		Free testosterone[d,e] pmol/l	
		B	95% CI	β	95% CI	β	95% CI		%	95% CI	%	95% CI	%	95% CI
Units in a typical week, N=553								**Units the week preceding the visit, N=1,194**						
0	121	-0.32	-0.62;-0.03	-0.42	-0.86;0.01	-0.21	-0.54;0.12	242	-2.7	-8.5;3.5	-0.6	-8.6;6.9	-3.3	-9.1;2.6
1-5	92	Reference		Reference		Reference		193	Reference		Reference		Reference	
6-10	71	-0.04	-0.38;0.3	-0.06	-0.56;0.45	-0.12	-0.49;0.26	154	3.3	-3.6;10.6	-3.0	-10.6;5.2	5.5	-1.4;12.9
11-15	80	-0.21	-0.54;0.12	-0.29	-0.77;0.20	-0.19	-0.56;0.18	160	2.0	-4.9;9.2	-3.1	-10.6;5.1	3.6	-3.1;10.7
16-20	62	-0.03	-0.39;0.33	-0.18	-0.70;0.34	-0.09	-0.46;0.29	130	3.0	-4.2;10.8	-2.1	-10.1;6.7	4.3	-3.0;12.0
21-25	45	0.25	-0.15;0.65	0.07	-0.52;0.65	-0.13	-0.55;0.29	92	7.3	-1.1;16.4	-0.2	-9.4;9.9	7.9	-0.3;16.9
26-30	25	-0.35	-0.83;0.14	-0.65	-1.37;0.08	-0.19	-0.71;0.34	71	1.0	-7.7;10.4	-9.0	-18.1;1.1	5.9	-3.1;15.5
31-35	14	-0.29	-0.92;0.33	-0.60	-1.51;0.31	-0.56	-1.19;0.06	47	6.1	-4.5;17.8	-9.8	-20.3;2.1	11.9	0.8;23.9
36-40	11	-0.33	-1.02;0.35	-0.73	-1.73;0.28	-0.54	-1.20;0.13	28	9.1	-4.2;17.8	-9.3	-22.2;5.7	16.0	2.1;31.7
>40	21	-0.39	-0.92;0.14	-0.54	-1.32;0.23	-0.46	-0.99;0.08	66	10.6	0.8;21.3	-12.0	-21.1;-1.9	19.5	9.2;30.9
p-trend[f]		0.02		0.01		0.02			0.01		<0.01		<0.01	
Number of binge episodes during the past 30 days[g]														
0	174	Reference		Reference		Reference		171	Reference		Reference		Reference	
1-2	248	-0.01	-0.23;0.22	0.15	-0.18;0.48	0.07	-0.16;0.31	246	4.0	-2.5;10.8	7.9	0.1;16.4	1.0	-5.2;7.6
3-5	407	0.08	-0.13;0.29	0.26	-0.05;0.56	0.10	-0.12;0.31	401	6.2	0.1;12.6	4.1	-2.9;11.6	6.1	0.1;12.4
6-9	253	0.00	-0.23;0.23	0.08	-0.26;0.41	-0.04	-0.28;0.19	252	8.4	1.6;15.6	1.1	-6.7;9.2	10.2	3.4;17.5
>9	88	-0.02	-0.32;0.29	0.03	-0.41;0.47	-0.22	-0.52;0.08	89	13.0	3.8;23.1	-2.9	-12.1;7.5	17.2	7.8;27.6
p-trend[f]		0.93		0.87		0.16			<0.01		0.31		0.01	

[a] Adjusted for period of abstinence, smoking and BMI categorized according to Table 2 (sperm morphology not adjusted for period of abstinence).
[b] Transformed by cubic root.
[c] Counted for 904 men of whom 397 stated that last weeks alcohol intake represented a typical week.
[d] Adjusted to a time at 8.00 AM, BMI and smoking.
[e] Transformed by the use of natural logarithm and back transformed giving the percentages change.
[f] Test for trend was performed by inserting the categorical alcohol variable into the model assuming the association to be linear with 1-5 units weekly as reference and 0 units excluded.
[g] Binging defined as alcohol intake for more than 5 units on one occasion.

Supplementary table Information (%) obtained from questionnaires and physical examination according to typical alcohol intake and binging among healthy, young Danish men.

Variable distribution in percent	N	%	Alcohol intake (units /week) N=553			Number of binge episodes during the past 30 days[a] N=1,221		
			0-15 N=369	16-30 N=138	>30 N=46	0-2 N=431	3-9 N=673	>10 N=92
Information obtained at the physical examination								
Season of examination between October and March	315	57	58	57	54	57	58	58
Varicocele found at the physical examinations[b]	40	7	7	8	7	8	9	8
Fever above 38°C within the last 3 months	31	6	4	11	10*	7	7	8
BMI (kg/m^2)								
< 20	111	20	22	15	20	20	16	13
20 – 24.99	327	60	56	67	61	62	66	70
≥ 25	112	20	21	18	20	19	18	17
Information obtained from questionnaire								
Above 20 years of age at time of examination	134	24	27	20	15*	31	17	14*
Physical activity 400 ≥ Watt per week	547	45	45	42	41	46	44	45
Total caffeine intake>300 (mg/day)	284	23	22	24	33	22	22	27
Maternal education								
<9 years	17	3	5	2	0*	7	2	0*
9-10 years	104	22	24	17	18	25	20	15
>10 years	362	75	71	81	82	68	78	86
Current smoking	252	46	36	61	76*	31	54	74*
Exposure to mother's smoking in utero	136	25	27	29	28	22	27	25
Self-reported genital conditions[c]	37	7	6	7	13	6	6	9
Sexual transmitted diseases[d]	60	11	8	12	33*	9	10	13
Born with cryptorchidism[e]	31	6	5	6	9	7	6	2
Number of binge episodes during the past 30 days								
0	176	15	23	0	1			
1-2	255	21	31	5	2			
3-5	415	35	33	45	25			
6-9	258	22	11	39	43			
>9	92	8	3	10	29			

[a]Binging defined as alcohol intake for more than 5 units on one occasion.
[b] Varicocele grade 2 or 3 found at physical examination.
[c] Self-reported information about torsion of testes, epididymitis or inguinal hernia.
[d]Sexually transmitted diseases; gonorrhea and chlamydia.
[e] If information was missing the man was categorized as not having cryptorchidism.
* p<0.05 by chi-square test.

Figure 1 Adjusted (for period of abstinence, BMI and smoking) changes in sperm concentration (%) according to habitual alcohol intake (reference 1-5 units in a typical week) among 553 young, Danish men. The p-value refers to the linear trend from the reference alcohol intake to the highest intake (abstainers excluded).

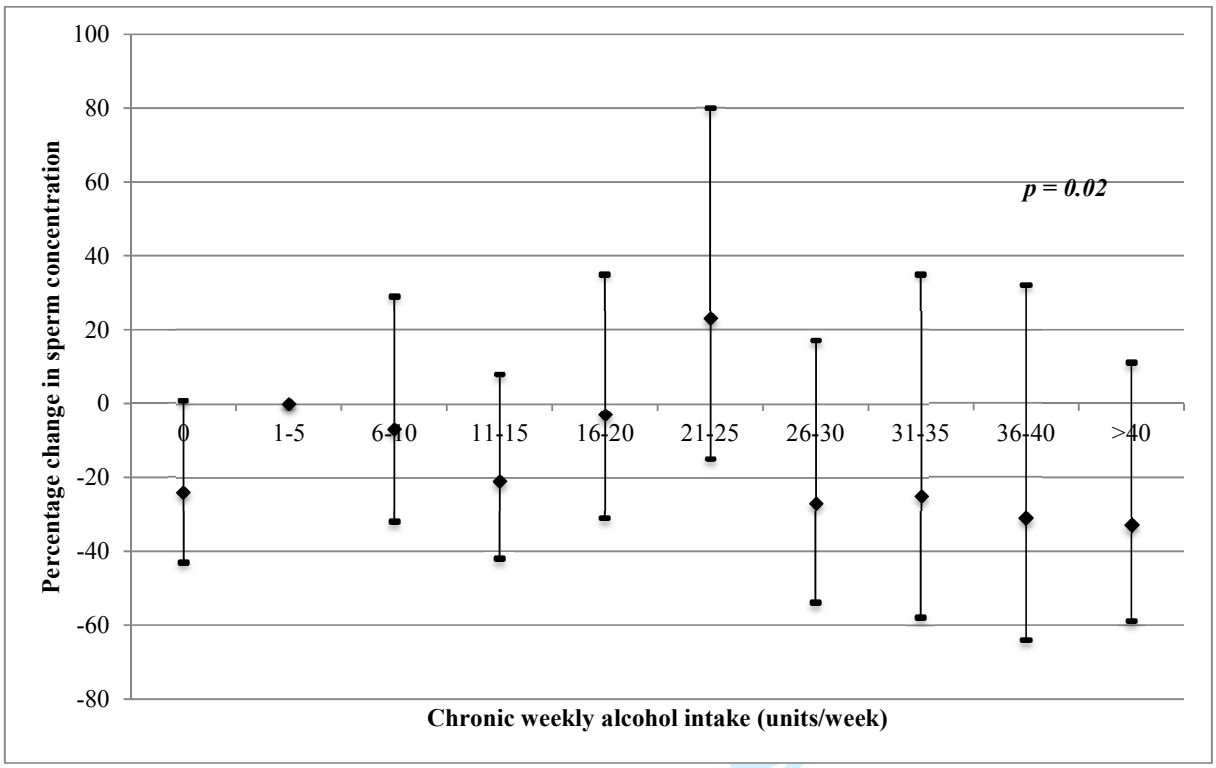
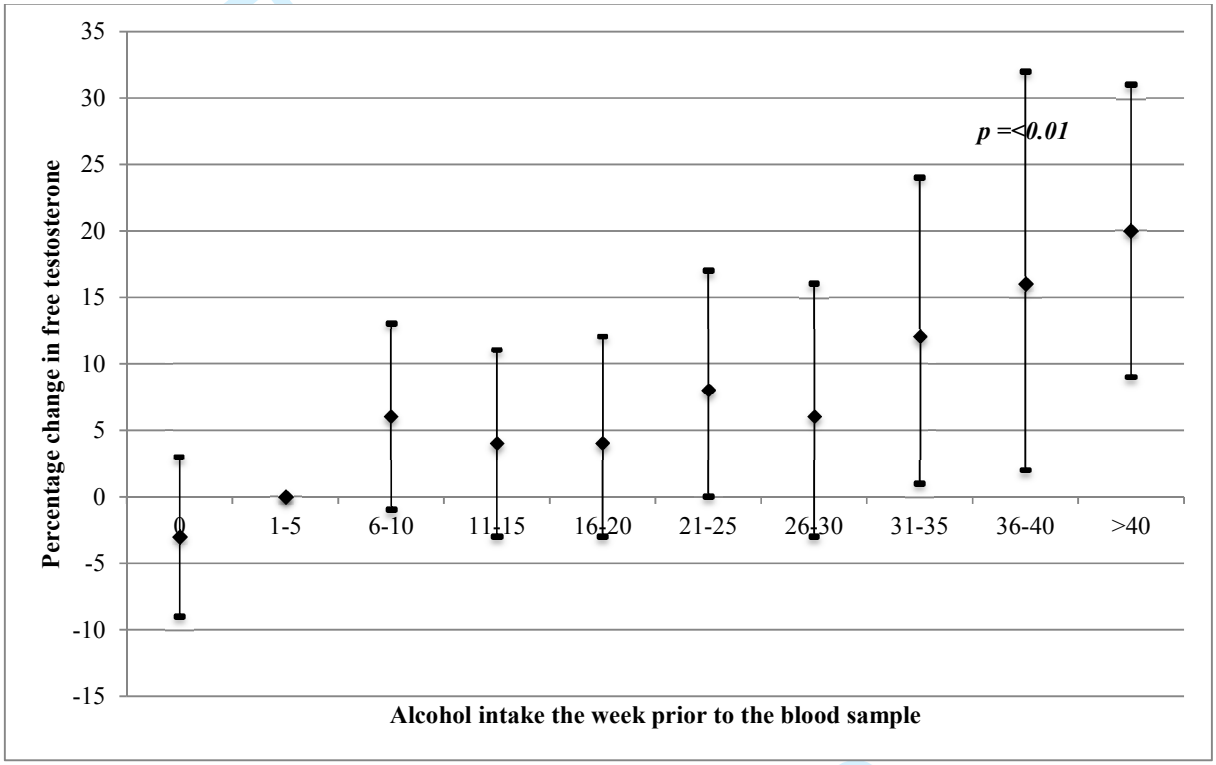


Figure 2 Adjusted (for BMI, time 8:00 AM and smoking) changes in free testosterone (%) according to recent alcohol intake (reference 1-5 units the week preceding the visit) among 1,194 young Danish men. The p-value refers to the linear trend from the reference alcohol intake to the highest intake.



STROBE Statement—Checklist of items that should be included in reports of ***cross-sectional studies***

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract p 1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found p2-3
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported p5
Objectives	3	State specific objectives, including any prespecified hypotheses p5
Methods		
Study design	4	Present key elements of study design early in the paper p6
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection p6
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants p6
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable p6-8
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group p6-8
Bias	9	Describe any efforts to address potential sources of bias p14-15
Study size	10	Explain how the study size was arrived at p6
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why p7-9
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding p8-9
		(*b*) Describe any methods used to examine subgroups and interactions p9
		(*c*) Explain how missing data were addressed p9
		(*d*) If applicable, describe analytical methods taking account of sampling strategy
		(*e*) Describe any sensitivity analyses p9
Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed p10
		(b) Give reasons for non-participation at each stage seen in earlier publications p10
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders p10 suppl table
		(b) Indicate number of participants with missing data for each variable of interest suppl table
Outcome data	15*	Report numbers of outcome events or summary measures Table 1-2
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included Table 3
		(*b*) Report category boundaries when continuous variables were categorized Table 3

		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses p11
Discussion		
Key results	18	Summarise key results with reference to study objectives p12
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias p14-15
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence p15
Generalisability	21	Discuss the generalisability (external validity) of the study results p15
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based p16

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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Habitual alcohol consumption associated with reduced semen quality and changes in reproductive hormones; a cross-sectional study among 1,221 young Danish men

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Complete List of Authors:	Jensen, Tina; Rigshospitalet, University Department of Growth and Reproductio; University of Southern Denmark, Dep of Environmental medicine Gottschau, Mads; University of Southern Denmark, Dep of Environmental Medicine Madsen, Jens Otto; University of Southern Denmark, Dep of Environmental Medicine Andersson, Anna-Maria; Rigshospitalet, University Department of Growth and Reproduction Lassen, Tina; Rigshospitalet, University Department of Growth and Reproduction Skakkebaek, Niels; Rigshospitalet, University Department of Growth and Reproduction Swan, Shanna; Mount Sinai School of Medicine, Department of Preventive Medicine Priskorn, Lærke; Rigshospitalet, University Department of Growth and Reproduction Juul, Anders; University of Copenhagen, Rigshospitalet, Dep. of Growth and Reproduction; Rigshospitalet, University Department of Growth and Reproduction Jørgensen, Niels Erik; Rigshospitalet, University Department of Growth and Reproduction
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Habitual alcohol consumption associated with reduced semen quality and changes in reproductive hormones; a cross-sectional study among 1,221 young Danish men

Short running title: Alcohol consumption and semen quality

Professor Tina Kold Jensen[1-2]

MD Mads Gottschau[1]

MD Jens Otto Broby Madsen[1]

PhD Anne-Maria Andersson[2]

Tina Harmer Lassen[2]

Professor Niels E. Skakkebæk[2]

Shanna H. Swan[3]

Lærke Priskorn[1]

Professor Anders Juul[2]

MD, PhD Niels Jørgensen[2]

1. Department of Environmental Medicine, University of Southern Denmark.
2. University Department of Growth and Reproduction, Rigshospitalet, The Faculty of Medical and Health Sciences, University of Copenhagen, Denmark
3. Department of Preventive Medicine, Icahn School of Medicine at Mount Sinai, 1 Gustave L. Levy Place, Box 1057, New York, NY 10029

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Corresponding author:
Tina Kold Jensen
University Department of Growth and Reproduction,
Rigshospitalet
Blegdamsvej 9
2100 Copenhagen Ø
tkjensen@health.sdu.dk

Abstract

Objective: Study associations between three measures of alcohol consumption (recent, typical/habitual, binging), semen quality and serum reproductive hormones.

Design: Cross-sectional population based study.

Setting and participants: 1,221 young Danish men, aged 18-28 years were recruited when they attended a compulsory medical examination to determine their fitness for military service from 2008 to 2012. Total alcohol consumption; 1) in the week preceding the visit (recent alcohol intake), 2) in a typical week; and 3) frequency of "binge drinking" (consuming more than 5 units/day)) in the past 30 days was estimated.

Main outcome measures: Semen quality (volume, sperm concentration, total sperm count, and percentages of motile and morphologically normal spermatozoa) and serum concentration of reproductive hormones (FSH, LH, testosterone, SHBG, estradiol, free testosterone and inhibin B).

Results: Sperm concentration, total sperm count and percentage of spermatozoa with normal morphology were negatively associated with increasing habitual alcohol intake. This association was observed in men reporting at least 5 units in a typical week but was most pronounced for men with a typical intake of more than 25 units per week. Men with a typical weekly intake above 40 units had a 33% (95% CI 11%;59%) reduction in sperm concentration compared to men with an intake of 1-5 units/week. A significant increase in serum free testosterone with increasing alcohol consumption the week preceding the visit was found. Binging was not independently associated with semen quality.

Conclusions: As the duration of spermatogenesis is approximately 72 days the typical alcohol intake is probably a more appropriate exposure measure than the intake the previous week. Contrary to this serum reproductive hormone levels fluctuate and are theoretically more susceptible towards

recent alcohol exposure. These results suggest that young men should be advised to avoid habitual high alcohol intake, which may be beneficial both for general and reproductive health.

Article summary

Article focus

- High alcohol intake has been associated with a wide range of diseases. However, few studies have examined the correlation between alcohol and reproductive function and none have separated the effects of alcohol intake the week preceding the semen and blood sample, habitual alcohol intake and binging.

Key messages

- Habitual alcohol intake was a stronger predictor for reduced semen quality (WHO manual), but not according to proven fertilizing capacity, already from a weekly intake above 5 units in a typical week, but was most pronounced for men with a typical intake of more than 25 units per week, than alcohol intake the week preceding the visit.
- Last weeks intake was a stronger predictor for increase in serum free testosterone, supported by biology in which duration of spermatogenesis is approximately 72 days whereby the typical alcohol intake is probably a more appropriate exposure measure than the intake the previous week. Contrary, serum reproductive hormone levels fluctuate and are theoretically more susceptible towards recent alcohol exposure.
- Our finding are relevant to public health since young men in the western world have a high alcohol intake and this may be contributing to recent reports of poor semen quality. These results suggest that young men should be advised to avoid habitual high alcohol intake, which may be beneficial both for general and reproductive health.

Strengths and limitations of the study

- Our study was large and consisted of young healthy men, of whom the majority had no knowledge of their fertility. It is therefore unlikely to have affected their motivation to participate.
- Our study was cross-sectional and reverse causation cannot be excluded, whereby men with poor semen quality have an unhealthier lifestyle and health behaviour and drink more alcohol even though we adjusted for these factors.
- The men in our study reported daily alcohol consumption the week preceding the visit, as we assumed that to be more accurate to recall than an average intake. This consumption may differ from the typical weekly intake, which can lead to misclassification of exposure.

Introduction

Alcohol consumption is widespread in the Western world, especially in Europe[1]. Drinking patterns have changed over time and binging (defined here as 5 units or more in a single day) is widespread among young Europeans[2]. Moderate alcohol consumption has been associated with reduced morbidity and mortality however not confirmed in all studies[3]. However, excessive alcohol intake has a negative impact on health (e.g. coronary heart disease, stroke and liver disease[4 5]).

Some studies found association between alcohol intake and semen quality[6-9], however other did not confirm these findings[10-18]. However, it is difficult to compare across studies, since populations as well as alcohol intake vary considerably between them. In addition, most studies only addressed average alcohol intake by use of only few questions, and within response categories consumption may vary considerably and is likely to be underreported. Only one study addressed the dose-response relationship between recent alcohol intake (during the past 5 days) and semen quality among 347 young Danish men. Poorer semen quality was found at higher levels of alcohol intake, although not statistical significant[16]. In an earlier multicenter study of over 8,000 American and European men, we found no adverse effects of alcohol intake in the week preceding the visit on semen quality. However, in that study most men reported only moderate intake of alcohol[19]. While some men in that study, were similar to the men in this study, much less detailed information about drinking habits was collected prior to 2008. To our knowledge no studies have examined the effect of binging on male reproductive parameters nor have the effects of recent versus habitual alcohol intake been studied in healthy populations. We therefore investigated the association between semen quality and serum reproductive hormones and alcohol consumption during the week preceding the visit, in a typical week, and binging in a cross-sectional study of 1,221 young Danish men recruited between 2008-2012.

Materials and methods

Population

Because of the military draft in Denmark, all 18-year-old men, except those suffering from severe chronic disease, are required to undergo a compulsory physical examination to determine their fitness for military service. Since 1996, trained staffs from the Department of Growth and Reproduction at Copenhagen University Hospital (Rigshospitalet, Copenhagen, Denmark) have approached the draftees when they have appeared for their compulsory physical examination and have invited them to participate in a study of semen quality taking place at Rigshospitalet. Only men recruited from January 2008 to April 2012 were included in the present study, since the questionnaire they completed included detailed information about alcohol intake. All participants completed a questionnaire, delivered a semen sample, had a blood sample drawn, and underwent a physical examination. They received compensation for their time (DKK 500, equal to approximately US$85). Participants did not differ from nonparticipants with regard to age, but they were generally better educated than nonparticipants (data not shown). Ethical approval was obtained from the local ethical committee. A detailed description[20], and other aspects of the study have previously been published[21-24].

Semen analysis

All men provided a semen sample by masturbation in a room close to the semen laboratory. The period of ejaculation abstinence (time since last ejaculation) was recorded, and the semen sample was analyzed for volume, sperm concentration, total sperm count, percent motile spermatozoa, and percent morphologically normal spermatozoa as described by Jørgensen et al. al[20], which is in accordance with the most recent guideline from the World Health Organization[25]. Since 1996, our laboratory has led a quality control program for assessment of sperm concentration; the laboratory has kept the interlaboratory difference unchanged[26], and the variation between technicians was less

than 10%. The same 2 experienced technicians assessed the sperm morphology according to strict criteria for the first 904 men[27].

Serum samples

Serum levels of follicle-stimulating hormone (FSH), luteinizing hormone, and sex hormone-binding globulin (SHBG) were determined using a time-resolved immunofluorometric assay (Delfia; Wallac Oy, Turku, Finland). Testosterone and estradiol levels were determined using time-resolved fluoroimmunoassays (Delfia; Wallac Oy). Inhibin B level was determined by means of a specific 2-sided enzyme immunometric assay (Inhibin B Gen II; Beckman Coulter Ltd., High Wycombe, United Kingdom). The hormones were all measured within same time period and in the same assay batches. Free testosterone was calculated on the basis of the measured serum concentrations of total testosterone and SHBG using the method of Vermeulen et al[28] and a fixed albumin concentration of 43.8 g/L[28].

Physical examination

Physicians assessed genital development, the possible presence of a varicocele (grades 1–3) or hydrocele, and the location of the testes in the scrotum, and the consistency of the testis and epididymis were recorded. Weight and height was measured, and body mass index (BMI) was calculated as weight in kilograms divided by squared height in meters.

Questionnaire

Prior to the examination, all participants completed a questionnaire that collected information on previous and/or current diseases and genital diseases. Self-reported diseases of the reproductive organs affecting semen quality (torsion of testes, epididymitis, or inguinal hernia) were summarized

in 2 variables: “self-reported genital conditions” and “sexually transmitted diseases” (gonorrhea or chlamydia).

The mothers to the young men responded to questions about maternal education, which was coded as: less than 9, 9–10, and more than 10 years of schooling. Data on physical activity was converted to watts per week using the method of Craig et al[29]. Men were asked about current smoking habits and whether they were exposed to smoking in utero. Daily caffeine intake was estimated based on their reported intake of caffeine-containing beverages the week prior to the visit. Men completed a diary reporting their daily intake of red and white wine, beer, strong alcoholic drinks, alcopops and others during the week prior to participation and delivery of the semen and blood samples (recent intake). Men were told that 1 beer, 1 glass of wine or 40 mL of spirits contained 1 unit of alcohol (≈12 g of ethanol), whereas 1 strong beer or 1 alcopop contained 1.5 units of alcohol and 1 bottle of wine contained 6 units of alcohol and were asked to convert their intake to units. Alcohol intake was calculated as the sum of daily reported unit intakes within that week. In addition, the men were asked whether their alcohol intake in the week preceding the visit represented a typical week (typical/habitual intake). They were also asked how many times during the past 30 days that had been drunk or had consumed more than 5 units of alcohol on one occasion, which we defined as binging.

Statistics

Exposure variables were total number of alcohol units in the week preceding the visit (recent intake) and in a typical week (typical/habitual intake). Alcohol units were divided into 5 unit intervals. Because abstainers may differ from light-moderate drinkers we selected 1-5 drinks/week as the reference category. In addition, number of binge episodes and number of times being drunk during the past 30 days was categorized as; 0 (reference), 1-2, 3-5; 6-9; more than 9.

Sperm parameters and reproductive hormone levels were compared in relation to alcohol intake and binging and the distributions of the relevant covariates from the questionnaires and physical examinations among men with different alcohol intake were compared by χ^2 test in order to identify potential confounders. Finally, data were analyzed using multivariable linear regression models. Because of the non-normal (skewed) distributions of semen quality and serum reproductive hormones, semen parameters were transformed by cubic root and reproductive hormones by natural logarithmic scale and the latter back-transformed to obtain the expected percent change per unit increase in exposure. Covariates were then excluded stepwise if their exclusion did not change effect estimate by more than 10%. In final models, the same set of covariates was used for all semen parameters: period of abstinence, current smoking and BMI, except that period of abstinence was not included for sperm morphology and motility models and duration between the time of ejaculation and analysis of the sample was included only for models predicting sperm motility. Models predicting reproductive hormones were adjusted for time of blood sampling, current smoking and BMI. We initially adjusted alcohol intake for binge episodes, but as estimates were unchanged, binging was not included. Tests for linear trend were performed after excluding men with no alcohol intake. Finally, analyses were performed separately for beer adjusting for total alcohol intake, since beer was consumed by most men. We evaluated the fit of the regression models by testing the residuals for normality and by inspecting the residual plots. SPSS statistics version 19 was used and the results are presented with 95% confidence intervals (95% CI).

Results

A total of 1,221 men were included with a mean age of 19.1 years. The median alcohol intake the week preceding the visit was 11 units (25 and 75 percentiles 1-21 units) and 64% and 59% of men had binged or had been drunk more than twice during the past 30 days, respectively. Beer was the favorite alcoholic beverage and the median beer intake the week preceding the visit was 5 units (0-13 units). A total of 553 men (45%) reported that the week preceding the visit represented a typical week and these were used in the analyses of typical/habitual alcohol intake. These men did not differ from the total population (N=1,221) in semen or hormone parameters.

Semen quality decreased with increasing recent alcohol intake (data not shown) and binging (Table 1). Testosterone and cFT increased and SHBG decreased with increasing recent alcohol intake (Table 2) and binging already from an intake above 5 units per week. Men with a typical alcohol intake of 30 units in a typical week or binging were more often smokers, had a higher caffeine intake, more often reported having had STDs or fever, were younger and their mothers had a higher education (supplementary Table).

No clear association between recent alcohol intake (the week preceding the visit)(data not shown), binging (Table 3) and semen quality was found after control for confounders. A dose-response association with recent alcohol intake from one unit per week (abstainers excluded) and higher testosterone (p-trend=0.01) and cFT (p-trend<0.01) and lower SHBG (p-trend<0.01) was found (Table 3, Figure 1) after control for confounder. Similar associations were found with number of binge episodes and being drunk during the last 30 days (Table 3). Men with a weekly alcohol intake above 40 units the week preceding the visit had 20% (95%CI 9%;31%) higher cFT after control for confounders. No association with LH, FSH, inhibin B and estradiol was found (data not shown).

Among the 553 men with a habitual alcohol intake (alcohol intake the week preceding the visit represented a typical week) we found an inverse dose-response association between alcohol intake and sperm concentration (p-trend=0.02), total sperm count (p-trend=0.01) and percentage morphologically normal sperms (p-trend=0.01) (Table 3, Figure 2) after adjustment. The trend was more pronounced among men with a typical weekly alcohol intake above 25 units. Cubic root transformed sperm concentration and percentage morphologically normal spermatozoa were respectively 0.39 (95% CI -0.92;0.14) and 0.51 (95%CI: 1.03;0.01) lower among men with a typical alcohol intake of more than 40 units compared to men with an intake of 1-5 units in a typical week. No alcohol intake was also associated with reduced semen quality. Percentages of motile spermatoza and semen volume were not associated with habitual alcohol intake (data not shown). Habitual alcohol intake was also associated with serum reproductive hormones although not as strongly as the recent intake (data not shown). The associations between recent alcohol intake from beer was similar to that of total alcohol.

Discussion

Findings

In this cohort of more than 1,200 young healthy men with detailed questionnaire information on alcohol intake we found that a habitual alcohol intake was associated with a reduction in semen quality already from more than 5 units per week in a typical week although the decreasing trend was most apparent for men with a typical weekly intake above 25 units. In addition, recent alcohol intake (the week preceding the visit) was associated with increase in serum testosterone and reduction in SHBG. No independent adverse effect of binging was found. The negative association between alcohol intake and semen quality may be attributed to a direct adverse effect of alcohol on spermatogenesis or it may be a result of differences in lifestyle, health behavior and diet found among high alcohol consumers, despite adjustment for these factors.

This is to our knowledge the first study to separate the effects of recent versus habitual alcohol exposure, and as the duration of spermatogenesis is approximately 72 days[30] the typical intake is probably a more appropriate exposure measure than the recent intake during the week preceding the delivery of the semen sample. Contrary to this serum reproductive hormone levels fluctuate[31] and are theoretically more susceptible towards recent changes (within days) induced by recent alcohol exposure (the week preceding the blood sampling).

Comparison with previous studies

Our findings are in accordance with a recent study among 347 young Danish men in which a non-significant dose-response association between recent alcohol intake (5 days preceding the delivery of the sample) and semen quality was found[16]. The study did not obtain information on typical alcohol exposure nor on binging. However, a Chinese study among 1,346 men did not find association between semen quality or alcohol intake neither in high doses (more than 120 units per months)[18]. Other previous studies of association between alcohol intake and semen quality have

shown contradictory results[6 7 9-15 17], but have been conducted in small selected population and not been able to address dose-response associations and none have been able to separate the effect of recent versus habitual intake. A previous multicenter study including young and fertile men did not find adverse effect of recent alcohol intake (the week preceding the visit) on semen quality, however most men only had a moderate alcohol intake[19]. The young Danish men in that study were also conscripts but included from 1996 to 2007 after which the questionnaire included more detailed information on alcohol intake. The men in this study were therefore included from 2008. No alcohol consumption was also associated with reduced semen quality, which may be attributed to social or health parameters differentiating non-drinkers from drinkers.

We found no independent adverse effect of binging on semen quality, which to our knowledge has not previously been reported. It was, however, difficult to separate binging from typical alcohol intake as most young men who binged also had a high alcohol intake. The percentage of Danes drinking 5 units or more in a typical drinking occasion has been reported to be 23%. Furthermore, young people aged 15-24 years are more likely (25%) to drink 5 units or more on one occasion compared to people above 55 years of age (11%)[1].

Animal studies have suggested that alcohol may affect the hypothalamic-pituitary-gonadal axis, change sperm morphology and directly negatively affect the testis[32 33]. In addition, analysis of histological samples from 195 deceased men showed that high alcohol consumption (>80 g alcohol/>7 units per day) was associated with significantly reduced spermatogenesis, including spermatogenic arrest and sertoli-cell-only syndrome[34].

Our observed association between alcohol intake, testosterone and cFT is in accordance with previous studies showing increased total testosterone and cFT or increased cFT in combination with decreased SHBG[16 19 35 36], whereas other studies found no association with cFT[37-40]. If SHBG levels are affected this could explain the observed increase in cFT. Otherwise, it may be explained by

alcohol detoxification leading to a changed metabolism of steroids in the liver. In contrast, decreased testosterone levels have been reported in male alcoholics suggesting that habitual alcohol abuse may damage Leydig cells or impair the hypothalamic-pituitary-gonadal axis[6 41].

Strengths and weaknesses

Our study has several strengths. It was large and consisted of young healthy men and the participation rate was approximately 30%, which is higher than in other population-based semen-quality studies [42-44]. The drinking habits of these men resembled those of Danish men aged 16-20 years in 2008[45], suggesting that they are not selected. In addition, the majority of our young men had no knowledge of their fertility potential and this is unlikely to have affected their motivation to participate. Our study was, however, cross-sectional and reverse causation cannot be excluded, whereby men with poor semen quality have an unhealthier lifestyle and health behaviour and drink more alcohol even though we adjusted for these factors.

The men in our study reported daily alcohol consumption the week preceding the visit, as we assumed that to be more accurate to recall than an average intake. This consumption may differ from the typical weekly intake, which can lead to misclassification of exposure, and we therefore repeated the analyses among men stating that that week represented a typical week. We used diary information on alcohol consumption, which makes it easier to recall the units consumed, but it may still be underreported. Further, the definition of a unit may vary according to size, method of preparation and brand. We defined binging as an intake of 5 units or more in a single day, which is also the definition used by The Danish National Board of Health[46]. These potential sources of exposure misclassification are likely to be unrelated to semen quality, since the men responded to the questionnaire, before they knew the result of their semen and blood analysis. Such non-differential misclassifications would underestimate the associations between alcohol habits and semen quality and reproductive hormones and cannot explain our findings.

Conclusion and implications

In conclusion, we found an adverse dose-response association between semen quality and habitual alcohol intake most pronounced among men with an alcohol intake above 25 units in a typical week. In addition, men with a high alcohol intake the week preceding the visit had increased free testosterone. This is to our knowledge the first study among healthy young men with detailed information on alcohol intake and given the fact that young men in the western world have a high alcohol intake, this is of public health concern and could be a contributing factor to the low sperm count reported among young men[20]. It remains to be seen whether semen quality is restored if alcohol intake is reduced, but young men should be advised that high habitual alcohol intake may affect not only their general but also their reproductive health.

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Contributors: TKJ performed data analysis and interpretation and wrote the manuscript. MG and JOBM provided assistance with data analysis and revised and edited the manuscript. THL, LP, NJ performed data collection, provided assistance with data analysis and interpretation, revised and edited the manuscript. NES, SHS, AJ provided assistance with data analysis and interpretation, and revised and edited the manuscript.

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work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.

Data sharing: Additional data regarding technical details, statistical code, and derivative data is available from the principal investigator at tkjensen@health.sdu.dk. Data access for further analyses is possible through direct collaborative agreement or through locally managed access arranged through the study's principal investigator.

Ethical approval: All studies have ethical approval in their respective countries and participants have provided informed consent.

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Table 1 Semen quality according to typical (last week represented a typical week) alcohol intake and binging during the past 30 days among respectively 553 and 1,221 healthy, young Danish men. Presented as median (M) and 25 and 75 percentiles (25-75).

Alcohol intake	N	Semen volume mL		Sperm concentration mill/mL		Total sperm count mill		Motility %		Morphology[a] %	
		M	25-75	M	25-75	M	25-75	M	25-75	M	25-75
Units in a typical week, N=553											
0	122	3.3	2.6;4.4	39	19;68	132	62;249	55	46;67	7.8	3.9;12.0
1-5	93	3.3	2.3;4.2	50	28;87	175	82;291	58	48;68	7.8	4.5;12.0
6-10	72	3.2	2.3;4.1	50	23;89	187	64;302	58	47;67	5.8	3.0;11.1
11-15	82	3.2	2.6;4.1	41	21;73	152	70;260	55	46;69	7.0	4.1;11.6
16-20	64	3.0	2.2;4.1	51	32;83	163	81;254	58	48;69	7.5	5.0;11.0
21-25	47	2.9	2.1;4.0	50	30;97	141	98;262	60	50;71	7.0	5.9;8.8
26-30	27	3.4	2.2;4.8	34	13;81	147	18;285	53	39;66	8.0	4.5;10.5
31-35	14	3.0	1.7;4.7	43	18;59	96	41;176	55	46;61	6.3	4.0;8.8
36-40	11	3.6	2.2;4.0	41	13;77	108	57;158	67	52;79	8.0	2.3;11.0
>40	21	3.6	2.6;4.7	33	12;70	86	48;245	62	50;71	6.5	2.5;11.0
Number of binge episodes during the past 30 days[b] N=1,221											
0	176	3.2	2.3;4.2	49	23;81	145	67;257	57	46;68	7.5	3.5;11.1
1-2	255	3.3	2.4;4.5	47	24;80	153	76;282	59	47;68	7.5	4.0;11.5
3-5	425	3.3	2.4;4.4	48	22;88	164	69;300	58	47;68	7.3	4.0;11.5
6-9	258	3.3	2.3;4.2	43	22;77	137	69;259	57	47;69	6.5	4.0;9.5
>9	92	3.2	2.3;4.2	41	20;73	130	61;239	56	44;70	6.0	3.5;9.1

[a]Counted for 904 men of whom 397 stated that last weeks alcohol intake represented a typical week.
[b]Binging defined as alcohol intake of more than 5 units on one occasion.

Table 2 Reproductive hormones according to recent (the week preceding the visit) alcohol intake and binging during the past 30 days among 1,194 healthy, young Danish men. Presented as median (M) and 5 and 95 percentiles (5-95).

Alcohol intake	N	FSH (IU/L)		LH (IU/L)		Testosterone (nmol/L)		SHBG (nmol/L)		Free testosterone pmol/L		Inhibin B (pg/mL)		Estradiol (Nmol/L)	
		M	5-95	M	5-95	M	5-95	M	5-95	M	5-95	M	5-95	M	5-95
Units the week preceding the visit N=1,194															
0	243	2.4	0.9;5.2	3.4	1.6;6.8	19.1	10.2;35.4	29	13;52	439	249;749	166	81;271	81	48;128
1-5	198	2.4	1.0;5.8	3.4	1.4;6.1	19.5	12.1;32.5	28	15;51	446	288;694	169	73;275	81	48;134
6-10	154	2.5	0.9;5.9	3.3	1.7;7.1	20.8	12.1;32.3	28	12;46	486	260;742	162	98;283	79	46;128
11-15	162	2.3	0.9;6.2	3.3	1.3;6.4	20.8	12.4;32.8	29	14;48	483	283;770	169	67;295	80	32;125
16-20	131	2.3	1.0;6.0	3.1	1.4;6.7	21.1	11.8;34.5	28	14;50	480	298;752	163	76;266	80	54;120
21-25	92	2.7	0.8;6.3	3.4	1.6;7.4	22.1	11.8;36.2	30	14;56	473	283;883	159	72;290	88	42;141
26-30	72	2.5	1.0;6.1	3.4	1.7;5.8	21.4	12.3;30.5	27	14;47	476	285;779	164	76;257	81	39;143
31-35	48	2.4	0.6;5.6	3.5	1.7;7.0	21.2	13.0;36.7	28	12;55	526	334;858	185	93;321	83	44;131
36-40	28	2.5	0.8;6.1	3.6	1.7;7.0	21.1	11.5;36.6	26	12;44	497	326;900	157	86;226	75	38;133
>40	66	2.4	0.8;6.6	3.3	1.8;7.8	22.9	13.8;36.1	27	11;47	541	335;920	158	82;290	84	41;140
Number of binge episodes during the past 30 days[a] N=1,194															
0	171	2.4	0.9;5.8	3.6	1.6;7.4	17.9	10.9;32.9	27	12;51	440	260;740	166	84;281	83	50;127
1-2	250	2.6	1.0;5.7	3.2	1.3;6.5	19.9	11.4;32.3	28	13;54	449	257;737	164	71;266	80	43;131
3-5	405	2.3	1.0;5.8	3.2	1.4;6.5	20.8	12.0;33.1	28	14;49	476	286;765	170	86;277	80	46;125
6-9	254	2.4	0.9;6.5	3.4	1.7;6.6	21.3	12.0;35.6	28	14;48	496	300;842	164	73;297	80	41;140
>9	90	2.5	0.8;6.1	3.3	1.8;7.0	21.9	12.5;37.2	27	13;55	523	310;858	164	79;291	85	41;137

[a]Binging defined as alcohol intake of more than 5 units on one occasion.

Table 3 Results from linear regression analyses of semen quality (adjusted β-coefficients) and serum reproductive hormones (percent change) among young, Danish men according to habitual alcohol intake (last week represented a typical week) or recent (the week preceding the visit) or binging during the past 30 days.

Alcohol intake	N	Sperm concentration[a,b] mill/mL		Total sperm count[a,b] mill		Morphology[b,c] %		N	Testosterone[d,e] nmol/l		SHBG[d,e] nmol/l		Free testosterone[d,e] pmol/l	
		B	95% CI	β	95% CI	β	95% CI		%	95% CI	%	95% CI	%	95% CI
Units in a typical week, N=553								**Units the week preceding the visit, N=1,194**						
0	121	-0.32	-0.62;-0.03	-0.42	-0.86;0.01	-0.21	-0.54;0.12	242	-2.7	-8.5;3.5	-0.6	-8.6;6.9	-3.3	-9.1;2.6
1-5	92	Reference		Reference		Reference		193	Reference		Reference		Reference	
6-10	71	-0.04	-0.38;0.3	-0.06	-0.56;0.45	-0.12	-0.49;0.26	154	3.3	-3.6;10.6	-3.0	-10.6;5.2	5.5	-1.4;12.9
11-15	80	-0.21	-0.54;0.12	-0.29	-0.77;0.20	-0.19	-0.56;0.18	160	2.0	-4.9;9.2	-3.1	-10.6;5.1	3.6	-3.1;10.7
16-20	62	-0.03	-0.39;0.33	-0.18	-0.70;0.34	-0.09	-0.46;0.29	130	3.0	-4.2;10.8	-2.1	-10.1;6.7	4.3	-3.0;12.0
21-25	45	0.25	-0.15;0.65	0.07	-0.52;0.65	-0.13	-0.55;0.29	92	7.3	-1.1;16.4	-0.2	-9.4;9.9	7.9	-0.3;16.9
26-30	25	-0.35	-0.83;0.14	-0.65	-1.37;0.08	-0.19	-0.71;0.34	71	1.0	-7.7;10.4	-9.0	-18.1;1.1	5.9	-3.1;15.5
31-35	14	-0.29	-0.92;0.33	-0.60	-1.51;0.31	-0.56	-1.19;0.06	47	6.1	-4.5;17.8	-9.8	-20.3;2.1	11.9	0.8;23.9
36-40	11	-0.33	-1.02;0.35	-0.73	-1.73;0.28	-0.54	-1.20;0.13	28	9.1	-4.2;17.8	-9.3	-22.2;5.7	16.0	2.1;31.7
>40	21	-0.39	-0.92;0.14	-0.54	-1.32;0.23	-0.46	-0.99;0.08	66	10.6	0.8;21.3	-12.0	-21.1;-1.9	19.5	9.2;30.9
p-trend[f]		0.02		0.01		0.02			0.01		<0.01		<0.01	
Number of binge episodes during the past 30 days[g]														
0	174	Reference		Reference		Reference		171	Reference		Reference		Reference	
1-2	248	-0.01	-0.23;0.22	0.15	-0.18;0.48	0.07	-0.16;0.31	246	4.0	-2.5;10.8	7.9	0.1;16.4	1.0	-5.2;7.6
3-5	407	0.08	-0.13;0.29	0.26	-0.05;0.56	0.10	-0.12;0.31	401	6.2	0.1;12.6	4.1	-2.9;11.6	6.1	0.1;12.4
6-9	253	0.00	-0.23;0.23	0.08	-0.26;0.41	-0.04	-0.28;0.19	252	8.4	1.6;15.6	1.1	-6.7;9.2	10.2	3.4;17.5
>9	88	-0.02	-0.32;0.29	0.03	-0.41;0.47	-0.22	-0.52;0.08	89	13.0	3.8;23.1	-2.9	-12.1;7.5	17.2	7.8;27.6
p-trend[f]		0.93		0.87		0.16			<0.01		0.31		0.01	

[a] Adjusted for period of abstinence, smoking and BMI categorized according to Table 2 (sperm morphology not adjusted for period of abstinence).
[b] Transformed by cubic root.
[c] Counted for 904 men of whom 397 stated that last weeks alcohol intake represented a typical week.
[d] Adjusted to a time at 8.00 AM, BMI and smoking.
[e] Transformed by the use of natural logarithm and back transformed giving the percentages change.
[f] Test for trend was performed by inserting the categorical alcohol variable into the model assuming the association to be linear with 1-5 units weekly as reference and 0 units excluded.
[g] Binging defined as alcohol intake for more than 5 units on one occasion..

Figure 1 Adjusted (for period of abstinence, BMI and smoking) changes in sperm concentration (%) according to habitual alcohol intake (reference 1-5 units in a typical week) among 553 young, Danish men. The p-value refers to the linear trend from the reference alcohol intake to the highest intake (abstainers excluded).

Figure 2 Adjusted (for BMI, time 8:00 AM and smoking) changes in free testosterone (%) according to recent alcohol intake (reference 1-5 units the week preceding the visit) among 1,194 young Danish men. The p-value refers to the linear trend from the reference alcohol intake to the highest intake.

Habitual alcohol consumption associated with reduced semen quality and changes in reproductive hormones; a cross-sectional study among 1,221 young Danish men

Short running title: Alcohol consumption and semen quality

Professor Tina Kold Jensen[1-2]

MD Mads Gottschau[1]

MD Jens Otto Broby Madsen[1]

PhD Anne-Maria Andersson[2]

Cand.scient.san.publ Tina Harmer Lassen[2]

Professor Niels E. Skakkebæk[2]

Shanna H. Swan[3]

Lærke Priskorn[1]

Professor Anders Juul[2]

MD, PhD Niels Jørgensen[2]

1. Department of Environmental Medicine, University of Southern Denmark.
2. University Department of Growth and Reproduction, Rigshospitalet, The Faculty of Medical and Health Sciences, University of Copenhagen, Denmark
3. Department of Preventive Medicine, Icahn School of Medicine at Mount Sinai, 1 Gustave L. Levy Place, Box 1057, New York, NY 10029

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Corresponding author:
Tina Kold Jensen
University Department of Growth and Reproduction,
Rigshospitalet
Blegdamsvej 9
2100 Copenhagen Ø
tkjensen@health.sdu.dk

Abstract

Objective: Study associations between three measures of alcohol consumption (recent, typical/habitual, binging), semen quality and serum reproductive hormones.

Design: Cross-sectional population based study.

Setting and participants: 1,221 young Danish men, aged 18-28 years were recruited when they attended a compulsory medical examination to determine their fitness for military service from 2008 to 2012. Total alcohol consumption; 1) in the week preceding the visit (recent alcohol intake), 2) in a typical week; and 3) frequency of "binge drinking" (consuming more than 5 units/day)) in the past 30 days was estimated.

Main outcome measures: Semen quality (volume, sperm concentration, total sperm count, and percentages of motile and morphologically normal spermatozoa) and serum concentration of reproductive hormones (FSH, LH, testosterone, SHBG, estradiol, free testosterone and inhibin B).

Results: Sperm concentration, total sperm count and percentage of spermatozoa with normal morphology were negatively associated with increasing habitual alcohol intake. This association was observed in men reporting at least 5 units in a typical week but was most pronounced for men with a typical intake of more than 25 units per week. Men with a typical weekly intake above 40 units had a 33% (95% CI 11%;59%) reduction in sperm concentration compared to men with an intake of 1-5 units/week. A significant increase in serum free testosterone with increasing alcohol consumption the week preceding the visit was found. Binging was not independently associated with semen quality.

Conclusions: As the duration of spermatogenesis is approximately 72 days the typical alcohol intake is probably a more appropriate exposure measure than the intake the previous week. Contrary to this serum reproductive hormone levels fluctuate and are theoretically more susceptible towards

recent alcohol exposure. These results suggest that young men should be advised to avoid habitual high alcohol intake, which may be beneficial both for general and reproductive health.

Article summary

Article focus

- High alcohol intake has been associated with a wide range of diseases. However, few studies have examined the correlation between alcohol and reproductive function and none have separated the effects of alcohol intake the week preceding the semen and blood sample, habitual alcohol intake and binging.

Key messages

- Habitual alcohol intake was a stronger predictor for reduced semen quality (WHO manual), but not according to proven fertilizing capacity, already from a weekly intake above 5 units in a typical week, but was most pronounced for men with a typical intake of more than 25 units per week, than alcohol intake the week preceding the visit.
- Last weeks intake was a stronger predictor for increase in serum free testosterone, supported by biology in which duration of spermatogenesis is approximately 72 days whereby the typical alcohol intake is probably a more appropriate exposure measure than the intake the previous week. Contrary, serum reproductive hormone levels fluctuate and are theoretically more susceptible towards recent alcohol exposure.
- Our finding are relevant to public health since young men in the western world have a high alcohol intake and this may be contributing to recent reports of poor semen quality. These results suggest that young men should be advised to avoid habitual high alcohol intake, which may be beneficial both for general and reproductive health.

Strengths and limitations of the study

- Our study was large and consisted of young healthy men, of whom the majority had no knowledge of their fertility. It is therefore unlikely to have affected their motivation to participate.
- Our study was cross-sectional and reverse causation cannot be excluded, whereby men with poor semen quality have an unhealthier lifestyle and health behaviour and drink more alcohol even though we adjusted for these factors.
- The men in our study reported daily alcohol consumption the week preceding the visit, as we assumed that to be more accurate to recall than an average intake. This consumption may differ from the typical weekly intake, which can lead to misclassification of exposure.

Introduction

Alcohol consumption is widespread in the Western world, especially in Europe[1]. Drinking patterns have changed over time and binging (defined here as 5 units or more in a single day) is widespread among young Europeans[2]. Moderate alcohol consumption has been associated with reduced morbidity and mortality however not confirmed in all studies[3]. However, excessive alcohol intake has a negative impact on health (e.g. coronary heart disease, stroke and liver disease[4 5]).

Some studies found association between alcohol intake and semen quality[6-9], however other did not confirm these findings[10-18]. However, it is difficult to compare across studies, since populations as well as alcohol intake vary considerably between them. In addition, most studies only addressed average alcohol intake by use of only few questions, and within response categories consumption may vary considerably and is likely to be underreported. Only one study addressed the dose-response relationship between recent alcohol intake (during the past 5 days) and semen quality among 347 young Danish men. Poorer semen quality was found at higher levels of alcohol intake, although not statistical significant[16]. In an earlier multicenter study of over 8,000 American and European men, we found no adverse effects of alcohol intake in the week preceding the visit on semen quality. However, in that study most men reported only moderate intake of alcohol[19]. While some men in that study, were similar to the men in this study, much less detailed information about drinking habits was collected prior to 2008. To our knowledge no studies have examined the effect of binging on male reproductive parameters nor have the effects of recent versus habitual alcohol intake been studied in healthy populations. We therefore investigated the association between semen quality and serum reproductive hormones and alcohol consumption during the week preceding the visit, in a typical week, and binging in a cross-sectional study of 1,221 young Danish men recruited between 2008-2012.

Materials and methods

Population

Because of the military draft in Denmark, all 18-year-old men, except those suffering from severe chronic disease, are required to undergo a compulsory physical examination to determine their fitness for military service. Since 1996, trained staffs from the Department of Growth and Reproduction at Copenhagen University Hospital (Rigshospitalet, Copenhagen, Denmark) have approached the draftees when they have appeared for their compulsory physical examination and have invited them to participate in a study of semen quality taking place at Rigshospitalet. Only men recruited from January 2008 to April 2012 were included in the present study, since the questionnaire they completed included detailed information about alcohol intake. All participants completed a questionnaire, delivered a semen sample, had a blood sample drawn, and underwent a physical examination. They received compensation for their time (DKK 500, equal to approximately US$85). Participants did not differ from nonparticipants with regard to age, but they were generally better educated than nonparticipants (data not shown). Ethical approval was obtained from the local ethical committee. A detailed description[20], and other aspects of the study have previously been published[21-24].

Semen analysis

All men provided a semen sample by masturbation in a room close to the semen laboratory. The period of ejaculation abstinence (time since last ejaculation) was recorded, and the semen sample was analyzed for volume, sperm concentration, total sperm count, percent motile spermatozoa, and percent morphologically normal spermatozoa as described by Jørgensen et al. al[20], which is in accordance with the most recent guideline from the World Health Organization[25]. Since 1996, our laboratory has led a quality control program for assessment of sperm concentration; the laboratory has kept the interlaboratory difference unchanged[26], and the variation between technicians was less

than 10%. The same 2 experienced technicians assessed the sperm morphology according to strict criteria for the first 904 men[27].

Serum samples

Serum levels of follicle-stimulating hormone (FSH), luteinizing hormone, and sex hormone-binding globulin (SHBG) were determined using a time-resolved immunofluorometric assay (Delfia; Wallac Oy, Turku, Finland). Testosterone and estradiol levels were determined using time-resolved fluoroimmunoassays (Delfia; Wallac Oy). Inhibin B level was determined by means of a specific 2-sided enzyme immunometric assay (Inhibin B Gen II; Beckman Coulter Ltd., High Wycombe, United Kingdom). The hormones were all measured within same time period and in the same assay batches. Free testosterone was calculated on the basis of the measured serum concentrations of total testosterone and SHBG using the method of Vermeulen et al[28] and a fixed albumin concentration of 43.8 g/L[28].

Physical examination

Physicians assessed genital development, the possible presence of a varicocele (grades 1–3) or hydrocele, and the location of the testes in the scrotum, and the consistency of the testis and epididymis were recorded. Weight and height was measured, and body mass index (BMI) was calculated as weight in kilograms divided by squared height in meters.

Questionnaire

Prior to the examination, all participants completed a questionnaire that collected information on previous and/or current diseases and genital diseases. Self-reported diseases of the reproductive organs affecting semen quality (torsion of testes, epididymitis, or inguinal hernia) were summarized

in 2 variables: "self-reported genital conditions" and "sexually transmitted diseases" (gonorrhea or chlamydia).

The mothers to the young men responded to questions about maternal education, which was coded as: less than 9, 9–10, and more than 10 years of schooling. Data on physical activity was converted to watts per week using the method of Craig et al[29]. Men were asked about current smoking habits and whether they were exposed to smoking in utero. Daily caffeine intake was estimated based on their reported intake of caffeine-containing beverages the week prior to the visit. Men completed a diary reporting their daily intake of red and white wine, beer, strong alcoholic drinks, alcopops and others during the week prior to participation and delivery of the semen and blood samples (recent intake). Men were told that 1 beer, 1 glass of wine or 40 mL of spirits contained 1 unit of alcohol (≈12 g of ethanol), whereas 1 strong beer or 1 alcopop contained 1.5 units of alcohol and 1 bottle of wine contained 6 units of alcohol and were asked to convert their intake to units. Alcohol intake was calculated as the sum of daily reported unit intakes within that week. In addition, the men were asked whether their alcohol intake in the week preceding the visit represented a typical week (typical/habitual intake). They were also asked how many times during the past 30 days that had been drunk or had consumed more than 5 units of alcohol on one occasion, which we defined as binging.

Statistics

Exposure variables were total number of alcohol units in the week preceding the visit (recent intake) and in a typical week (typical/habitual intake). Alcohol units were divided into 5 unit intervals. Because abstainers may differ from light-moderate drinkers we selected 1-5 drinks/week as the reference category. In addition, number of binge episodes and number of times being drunk during the past 30 days was categorized as; 0 (reference), 1-2, 3-5; 6-9; more than 9.

Sperm parameters and reproductive hormone levels were compared in relation to alcohol intake and binging and the distributions of the relevant covariates from the questionnaires and physical examinations among men with different alcohol intake were compared by χ^2 test in order to identify potential confounders. Finally, data were analyzed using multivariable linear regression models. Because of the non-normal (skewed) distributions of semen quality and serum reproductive hormones, semen parameters were transformed by cubic root and reproductive hormones by natural logarithmic scale and the latter back-transformed to obtain the expected percent change per unit increase in exposure. Covariates were then excluded stepwise if their exclusion did not change effect estimate by more than 10%. In final models, the same set of covariates was used for all semen parameters: period of abstinence, current smoking and BMI, except that period of abstinence was not included for sperm morphology and motility models and duration between the time of ejaculation and analysis of the sample was included only for models predicting sperm motility. Models predicting reproductive hormones were adjusted for time of blood sampling, current smoking and BMI. We initially adjusted alcohol intake for binge episodes, but as estimates were unchanged, binging was not included. Tests for linear trend were performed after excluding men with no alcohol intake. Finally, analyses were performed separately for beer adjusting for total alcohol intake, since beer was consumed by most men. We evaluated the fit of the regression models by testing the residuals for normality and by inspecting the residual plots. SPSS statistics version 19 was used and the results are presented with 95% confidence intervals (95% CI).

Results

A total of 1,221 men were included with a mean age of 19.1 years. The median alcohol intake the week preceding the visit was 11 units (25 and 75 percentiles 1-21 units) and 64% and 59% of men had binged or had been drunk more than twice during the past 30 days, respectively. Beer was the favorite alcoholic beverage and the median beer intake the week preceding the visit was 5 units (0-13 units). A total of 553 men (45%) reported that the week preceding the visit represented a typical week and these were used in the analyses of typical/habitual alcohol intake. These men did not differ from the total population (N=1,221) in semen or hormone parameters.

Semen quality decreased with increasing recent alcohol intake (data not shown) and binging (Table 1). Testosterone and cFT increased and SHBG decreased with increasing recent alcohol intake (Table 2) and binging already from an intake above 5 units per week. Men with a typical alcohol intake of 30 units in a typical week or binging were more often smokers, had a higher caffeine intake, more often reported having had STDs or fever, were younger and their mothers had a higher education (supplementary Table).

No clear association between recent alcohol intake (the week preceding the visit)(data not shown), binging (Table 3) and semen quality was found after control for confounders. A dose-response association with recent alcohol intake from one unit per week (abstainers excluded) and higher testosterone (p-trend=0.01) and cFT (p-trend<0.01) and lower SHBG (p-trend<0.01) was found (Table 3, Figure 1) after control for confounder. Similar associations were found with number of binge episodes and being drunk during the last 30 days (Table 3). Men with a weekly alcohol intake above 40 units the week preceding the visit had 20% (95%CI 9%;31%) higher cFT after control for confounders. No association with LH, FSH, inhibin B and estradiol was found (data not shown).

Among the 553 men with a habitual alcohol intake (alcohol intake the week preceding the visit represented a typical week) we found an inverse dose-response association between alcohol intake and sperm concentration (p-trend=0.02), total sperm count (p-trend=0.01) and percentage morphologically normal sperms (p-trend=0.01) (Table 3, Figure 2) after adjustment. The trend was more pronounced among men with a typical weekly alcohol intake above 25 units. Cubic root transformed sperm concentration and percentage morphologically normal spermatozoa were respectively 0.39 (95% CI -0.92;0.14) and 0.51 (95%CI: 1.03;0.01) lower among men with a typical alcohol intake of more than 40 units compared to men with an intake of 1-5 units in a typical week. No alcohol intake was also associated with reduced semen quality. Percentages of motile spermatoza and semen volume were not associated with habitual alcohol intake (data not shown). Habitual alcohol intake was also associated with serum reproductive hormones although not as strongly as the recent intake (data not shown). The associations between recent alcohol intake from beer was similar to that of total alcohol.

Discussion

Findings

In this cohort of more than 1,200 young healthy men with detailed questionnaire information on alcohol intake we found that a habitual alcohol intake was associated with a reduction in semen quality already from more than 5 units per week in a typical week although the decreasing trend was most apparent for men with a typical weekly intake above 25 units. In addition, recent alcohol intake (the week preceding the visit) was associated with increase in serum testosterone and reduction in SHBG. No independent adverse effect of binging was found. The negative association between alcohol intake and semen quality may be attributed to a direct adverse effect of alcohol on spermatogenesis or it may be a result of differences in lifestyle, health behavior and diet found among high alcohol consumers, despite adjustment for these factors.

This is to our knowledge the first study to separate the effects of recent versus habitual alcohol exposure, and as the duration of spermatogenesis is approximately 72 days[30] the typical intake is probably a more appropriate exposure measure than the recent intake during the week preceding the delivery of the semen sample. Contrary to this serum reproductive hormone levels fluctuate[31] and are theoretically more susceptible towards recent changes (within days) induced by recent alcohol exposure (the week preceding the blood sampling).

Comparison with previous studies

Our findings are in accordance with a recent study among 347 young Danish men in which a non-significant dose-response association between recent alcohol intake (5 days preceding the delivery of the sample) and semen quality was found[16]. The study did not obtain information on typical alcohol exposure nor on binging. However, a Chinese study among 1,346 men did not find association between semen quality or alcohol intake neither in high doses (more than 120 units per months)[18]. Other previous studies of association between alcohol intake and semen quality have

shown contradictory results[6 7 9-15 17], but have been conducted in small selected population and not been able to address dose-response associations and none have been able to separate the effect of recent versus habitual intake. A previous multicenter study including young and fertile men did not find adverse effect of recent alcohol intake (the week preceding the visit) on semen quality, however most men only had a moderate alcohol intake[19]. The young Danish men in that study were also conscripts but included from 1996 to 2007 after which the questionnaire included more detailed information on alcohol intake. The men in this study were therefore included from 2008. No alcohol consumption was also associated with reduced semen quality, which may be attributed to social or health parameters differentiating non-drinkers from drinkers.

We found no independent adverse effect of binging on semen quality, which to our knowledge has not previously been reported. It was, however, difficult to separate binging from typical alcohol intake as most young men who binged also had a high alcohol intake. The percentage of Danes drinking 5 units or more in a typical drinking occasion has been reported to be 23%. Furthermore, young people aged 15-24 years are more likely (25%) to drink 5 units or more on one occasion compared to people above 55 years of age (11%)[1].

Animal studies have suggested that alcohol may affect the hypothalamic-pituitary-gonadal axis, change sperm morphology and directly negatively affect the testis[32 33]. In addition, analysis of histological samples from 195 deceased men showed that high alcohol consumption (>80 g alcohol/>7 units per day) was associated with significantly reduced spermatogenesis, including spermatogenic arrest and sertoli-cell-only syndrome[34].

Our observed association between alcohol intake, testosterone and cFT is in accordance with previous studies showing increased total testosterone and cFT or increased cFT in combination with decreased SHBG[16 19 35 36], whereas other studies found no association with cFT[37-40]. If SHBG levels are affected this could explain the observed increase in cFT. Otherwise, it may be explained by

alcohol detoxification leading to a changed metabolism of steroids in the liver. In contrast, decreased testosterone levels have been reported in male alcoholics suggesting that habitual alcohol abuse may damage Leydig cells or impair the hypothalamic-pituitary-gonadal axis[6 41].

Strengths and weaknesses

Our study has several strengths. It was large and consisted of young healthy men and the participation rate was approximately 30%, which is higher than in other population-based semen-quality studies [42-44]. The drinking habits of these men resembled those of Danish men aged 16-20 years in 2008[45], suggesting that they are not selected. In addition, the majority of our young men had no knowledge of their fertility potential and this is unlikely to have affected their motivation to participate. Our study was, however, cross-sectional and reverse causation cannot be excluded, whereby men with poor semen quality have an unhealthier lifestyle and health behaviour and drink more alcohol even though we adjusted for these factors.

The men in our study reported daily alcohol consumption the week preceding the visit, as we assumed that to be more accurate to recall than an average intake. This consumption may differ from the typical weekly intake, which can lead to misclassification of exposure, and we therefore repeated the analyses among men stating that that week represented a typical week. We used diary information on alcohol consumption, which makes it easier to recall the units consumed, but it may still be underreported. Further, the definition of a unit may vary according to size, method of preparation and brand. We defined binging as an intake of 5 units or more in a single day, which is also the definition used by The Danish National Board of Health[46]. These potential sources of exposure misclassification are likely to be unrelated to semen quality, since the men responded to the questionnaire, before they knew the result of their semen and blood analysis. Such non-differential misclassifications would underestimate the associations between alcohol habits and semen quality and reproductive hormones and cannot explain our findings.

Conclusion and implications

In conclusion, we found an adverse dose-response association between semen quality and habitual alcohol intake most pronounced among men with an alcohol intake above 25 units in a typical week. In addition, men with a high alcohol intake the week preceding the visit had increased free testosterone. This is to our knowledge the first study among healthy young men with detailed information on alcohol intake and given the fact that young men in the western world have a high alcohol intake, this is of public health concern and could be a contributing factor to the low sperm count reported among young men[20]. It remains to be seen whether semen quality is restored if alcohol intake is reduced, but young men should be advised that high habitual alcohol intake may affect not only their general but also their reproductive health.

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Contributors: TKJ performed data analysis and interpretation and wrote the manuscript. MG and JOBM provided assistance with data analysis and revised and edited the manuscript. THL, LP, NJ performed data collection, provided assistance with data analysis and interpretation, revised and edited the manuscript. NES, SHS, AJ provided assistance with data analysis and interpretation, and revised and edited the manuscript.

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Competing interests: All authors have completed the ICMJE uniform disclosure form at www.icmje.org/coi_disclosure.pdf and declare: no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted

work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.

Data sharing: Additional data regarding technical details, statistical code, and derivative data is available from the principal investigator at tkjensen@health.sdu.dk. Data access for further analyses is possible through direct collaborative agreement or through locally managed access arranged through the study's principal investigator.

Ethical approval: All studies have ethical approval in their respective countries and participants have provided informed consent.

Figure 1 Adjusted (for period of abstinence, BMI and smoking) changes in sperm concentration (%) according to habitual alcohol intake (reference 1-5 units in a typical week) among 553 young, Danish men. The p-value refers to the linear trend from the reference alcohol intake to the highest intake (abstainers excluded).

Figure 2 Adjusted (for BMI, time 8:00 AM and smoking) changes in free testosterone (%) according to recent alcohol intake (reference 1-5 units the week preceding the visit) among 1,194 young Danish men. The p-value refers to the linear trend from the reference alcohol intake to the highest intake.

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Table 1 Semen quality according to typical (last week represented a typical week) alcohol intake and binging during the past 30 days among respectively 553 and 1,221 healthy, young Danish men. Presented as median (M) and 25 and 75 percentiles (25-75).

Alcohol intake	N	Semen volume mL		Sperm concentration mill/mL		Total sperm count mill		Motility %		Morphology[a] %	
		M	25-75	M	25-75	M	25-75	M	25-75	M	25-75
Units in a typical week, N=553											
0	122	3.3	2.6;4.4	39	19;68	132	62;249	55	46;67	7.8	3.9;12.0
1-5	93	3.3	2.3;4.2	50	28;87	175	82;291	58	48;68	7.8	4.5;12.0
6-10	72	3.2	2.3;4.1	50	23;89	187	64;302	58	47;67	5.8	3.0;11.1
11-15	82	3.2	2.6;4.1	41	21;73	152	70;260	55	46;69	7.0	4.1;11.6
16-20	64	3.0	2.2;4.1	51	32;83	163	81;254	58	48;69	7.5	5.0;11.0
21-25	47	2.9	2.1;4.0	50	30;97	141	98;262	60	50;71	7.0	5.9;8.8
26-30	27	3.4	2.2;4.8	34	13;81	147	18;285	53	39;66	8.0	4.5;10.5
31-35	14	3.0	1.7;4.7	43	18;59	96	41;176	55	46;61	6.3	4.0;8.8
36-40	11	3.6	2.2;4.0	41	13;77	108	57;158	67	52;79	8.0	2.3;11.0
>40	21	3.6	2.6;4.7	33	12;70	86	48;245	62	50;71	6.5	2.5;11.0
Number of binge episodes during the past 30 days[b] N=1,221											
0	176	3.2	2.3;4.2	49	23;81	145	67;257	57	46;68	7.5	3.5;11.1
1-2	255	3.3	2.4;4.5	47	24;80	153	76;282	59	47;68	7.5	4.0;11.5
3-5	425	3.3	2.4;4.4	48	22;88	164	69;300	58	47;68	7.3	4.0;11.5
6-9	258	3.3	2.3;4.2	43	22;77	137	69;259	57	47;69	6.5	4.0;9.5
>9	92	3.2	2.3;4.2	41	20;73	130	61;239	56	44;70	6.0	3.5;9.1

[a]Counted for 904 men of whom 397 stated that last weeks alcohol intake represented a typical week.
[b]Binging defined as alcohol intake of more than 5 units on one occasion.

Table 2 Reproductive hormones according to recent (the week preceding the visit) alcohol intake and binging during the past 30 days among 1,194 healthy, young Danish men. Presented as median (M) and 5 and 95 percentiles (5-95).

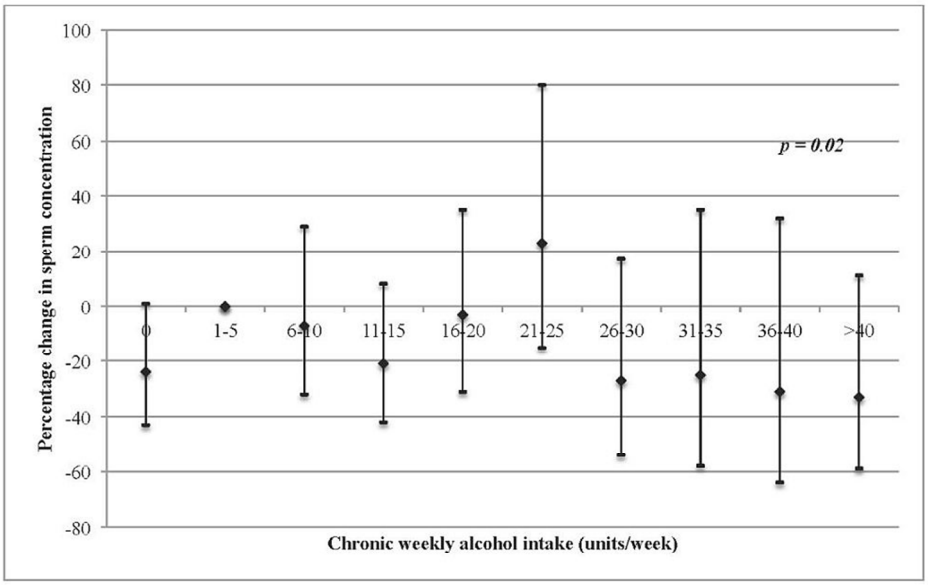
Alcohol intake	N	FSH (IU/L)		LH (IU/L)		Testosterone (nmol/L)		SHBG (nmol/L)		Free testosterone pmol/L		Inhibin B (pg/mL)		Estradiol (Nmol/L)	
		M	5-95	M	5-95	M	5-95	M	5-95	M	5-95	M	5-95	M	5-95
Units the week preceding the visit N=1,194															
0	243	2.4	0.9;5.2	3.4	1.6;6.8	19.1	10.2;35.4	29	13;52	439	249;749	166	81;271	81	48;128
1-5	198	2.4	1.0;5.8	3.4	1.4;6.1	19.5	12.1;32.5	28	15;51	446	288;694	169	73;275	81	48;134
6-10	154	2.5	0.9;5.9	3.3	1.7;7.1	20.8	12.1;32.3	28	12;46	486	260;742	162	98;283	79	46;128
11-15	162	2.3	0.9;6.2	3.3	1.3;6.4	20.8	12.4;32.8	29	14;48	483	283;770	169	67;295	80	32;125
16-20	131	2.3	1.0;6.0	3.1	1.4;6.7	21.1	11.8;34.5	28	14;50	480	298;752	163	76;266	80	54;120
21-25	92	2.7	0.8;6.3	3.4	1.6;7.4	22.1	11.8;36.2	30	14;56	473	283;883	159	72;290	88	42;141
26-30	72	2.5	1.0;6.1	3.4	1.7;5.8	21.4	12.3;30.5	27	14;47	476	285;779	164	76;257	81	39;143
31-35	48	2.4	0.6;5.6	3.5	1.7;7.0	21.2	13.0;36.7	28	12;55	526	334;858	185	93;321	83	44;131
36-40	28	2.5	0.8;6.1	3.6	1.7;7.0	21.1	11.5;36.6	26	12;44	497	326;900	157	86;226	75	38;133
>40	66	2.4	0.8;6.6	3.3	1.8;7.8	22.9	13.8;36.1	27	11;47	541	335;920	158	82;290	84	41;140
Number of binge episodes during the past 30 days[a] N=1,194															
0	171	2.4	0.9;5.8	3.6	1.6;7.4	17.9	10.9;32.9	27	12;51	440	260;740	166	84;281	83	50;127
1-2	250	2.6	1.0;5.7	3.2	1.3;6.5	19.9	11.4;32.3	28	13;54	449	257;737	164	71;266	80	43;131
3-5	405	2.3	1.0;5.8	3.2	1.4;6.5	20.8	12.0;33.1	28	14;49	476	286;765	170	86;277	80	46;125
6-9	254	2.4	0.9;6.5	3.4	1.7;6.6	21.3	12.0;35.6	28	14;48	496	300;842	164	73;297	80	41;140
>9	90	2.5	0.8;6.1	3.3	1.8;7.0	21.9	12.5;37.2	27	13;55	523	310;858	164	79;291	85	41;137

[a]Binging defined as alcohol intake of more than 5 units on one occasion.

Table 3 Results from linear regression analyses of semen quality (adjusted β-coefficients) and serum reproductive hormones (percent change) among young, Danish men according to habitual alcohol intake (last week represented a typical week) or recent (the week preceding the visit) or binging during the past 30 days.

Alcohol intake	N	Sperm concentration[a,b] mill/mL		Total sperm count[a,b] mill		Morphology[b,c] %		N	Testosterone[d,e] nmol/l		SHBG[d,e] nmol/l		Free testosterone[d,e] pmol/l	
		B	95% CI	β	95% CI	β	95% CI		%	95% CI	%	95% CI	%	95% CI
Units in a typical week, N=553								**Units the week preceding the visit, N=1,194**						
0	121	-0.32	-0.62;-0.03	-0.42	-0.86;0.01	-0.21	-0.54;0.12	242	-2.7	-8.5;3.5	-0.6	-8.6;6.9	-3.3	-9.1;2.6
1-5	92	Reference		Reference		Reference		193	Reference		Reference		Reference	
6-10	71	-0.04	-0.38;0.3	-0.06	-0.56;0.45	-0.12	-0.49;0.26	154	3.3	-3.6;10.6	-3.0	-10.6;5.2	5.5	-1.4;12.9
11-15	80	-0.21	-0.54;0.12	-0.29	-0.77;0.20	-0.19	-0.56;0.18	160	2.0	-4.9;9.2	-3.1	-10.6;5.1	3.6	-3.1;10.7
16-20	62	-0.03	-0.39;0.33	-0.18	-0.70;0.34	-0.09	-0.46;0.29	130	3.0	-4.2;10.8	-2.1	-10.1;6.7	4.3	-3.0;12.0
21-25	45	0.25	-0.15;0.65	0.07	-0.52;0.65	-0.13	-0.55;0.29	92	7.3	-1.1;16.4	-0.2	-9.4;9.9	7.9	-0.3;16.9
26-30	25	-0.35	-0.83;0.14	-0.65	-1.37;0.08	-0.19	-0.71;0.34	71	1.0	-7.7;10.4	-9.0	-18.1;1.1	5.9	-3.1;15.5
31-35	14	-0.29	-0.92;0.33	-0.60	-1.51;0.31	-0.56	-1.19;0.06	47	6.1	-4.5;17.8	-9.8	-20.3;2.1	11.9	0.8;23.9
36-40	11	-0.33	-1.02;0.35	-0.73	-1.73;0.28	-0.54	-1.20;0.13	28	9.1	-4.2;17.8	-9.3	-22.2;5.7	16.0	2.1;31.7
>40	21	-0.39	-0.92;0.14	-0.54	-1.32;0.23	-0.46	-0.99;0.08	66	10.6	0.8;21.3	-12.0	-21.1;-1.9	19.5	9.2;30.9
p-trend[f]		0.02		0.01		0.02			0.01		<0.01		<0.01	
Number of binge episodes during the past 30 days[g]														
0	174	Reference		Reference		Reference		171	Reference		Reference		Reference	
1-2	248	-0.01	-0.23;0.22	0.15	-0.18;0.48	0.07	-0.16;0.31	246	4.0	-2.5;10.8	7.9	0.1;16.4	1.0	-5.2;7.6
3-5	407	0.08	-0.13;0.29	0.26	-0.05;0.56	0.10	-0.12;0.31	401	6.2	0.1;12.6	4.1	-2.9;11.6	6.1	0.1;12.4
6-9	253	0.00	-0.23;0.23	0.08	-0.26;0.41	-0.04	-0.28;0.19	252	8.4	1.6;15.6	1.1	-6.7;9.2	10.2	3.4;17.5
>9	88	-0.02	-0.32;0.29	0.03	-0.41;0.47	-0.22	-0.52;0.08	89	13.0	3.8;23.1	-2.9	-12.1;7.5	17.2	7.8;27.6
p-trend[f]		0.93		0.87		0.16			<0.01		0.31		0.01	

[a] Adjusted for period of abstinence, smoking and BMI categorized according to Table 2 (sperm morphology not adjusted for period of abstinence).
[b] Transformed by cubic root.
[c] Counted for 904 men of whom 397 stated that last weeks alcohol intake represented a typical week.
[d] Adjusted to a time at 8.00 AM, BMI and smoking.
[e] Transformed by the use of natural logarithm and back transformed giving the percentages change.
[f] Test for trend was performed by inserting the categorical alcohol variable into the model assuming the association to be linear with 1-5 units weekly as reference and 0 units excluded.
[g] Binging defined as alcohol intake for more than 5 units on one occasion.



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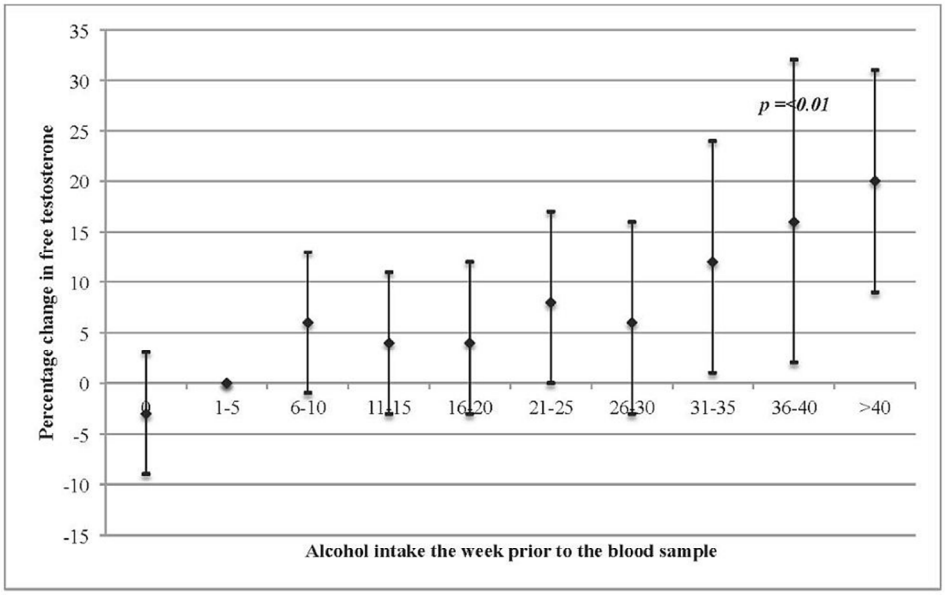

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Supplementary table Information (%) obtained from questionnaires and physical examination according to typical alcohol intake and binging among healthy, young Danish men.

Variable distribution in percent	N	%	Alcohol intake (units /week) N=553			Number of binge episodes during the past 30 days[a] N=1,221		
			0-15 N=369	16-30 N=138	>30 N=46	0-2 N=431	3-9 N=673	>10 N=92
Information obtained at the physical examination								
Season of examination between October and March	315	57	58	57	54	57	58	58
Varicocele found at the physical examinations[b]	40	7	7	8	7	8	9	8
Fever above 38°C within the last 3 months	31	6	4	11	10*	7	7	8
BMI (kg/m^2)								
< 20	111	20	22	15	20	20	16	13
20 – 24.99	327	60	56	67	61	62	66	70
≥ 25	112	20	21	18	20	19	18	17
Information obtained from questionnaire								
Above 20 years of age at time of examination	134	24	27	20	15*	31	17	14*
Physical activity 400 ≥ Watt per week	547	45	45	42	41	46	44	45
Total caffeine intake>300 (mg/day)	284	23	22	24	33	22	22	27
Maternal education								
<9 years	17	3	5	2	0*	7	2	0*
9-10 years	104	22	24	17	18	25	20	15
>10 years	362	75	71	81	82	68	78	86
Current smoking	252	46	36	61	76*	31	54	74*
Exposure to mother's smoking in utero	136	25	27	29	28	22	27	25
Self-reported genital conditions[c]	37	7	6	7	13	6	6	9
Sexual transmitted diseases[d]	60	11	8	12	33*	9	10	13
Born with cryptorchidism[e]	31	6	5	6	9	7	6	2
Number of binge episodes during the past 30 days								
0	176	15	23	0	1	100	0	0
1-2	255	21	31	5	2	100	0	0
3-5	415	35	33	45	25	0	100	0
6-9	258	22	11	39	43	0	100	0
>9	92	8	3	10	29	0	0	100

[a]Binging defined as alcohol intake for more than 5 units on one occasion.
[b] Varicocele grade 2 or 3 found at physical examination.
[c] Self-reported information about torsion of testes, epididymitis or inguinal hernia.
[d]Sexually transmitted diseases; gonorrhea and chlamydia.
[e] If information was missing the man was categorized as not having cryptorchidism.
* p<0.05 by chi-square test.

STROBE Statement—Checklist of items that should be included in reports of ***cross-sectional studies***

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract p 1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found p2-3
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported p5
Objectives	3	State specific objectives, including any prespecified hypotheses p5
Methods		
Study design	4	Present key elements of study design early in the paper p6
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection p6
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants p6
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable p6-8
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group p6-8
Bias	9	Describe any efforts to address potential sources of bias p14-15
Study size	10	Explain how the study size was arrived at p6
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why p7-9
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding p8-9
		(*b*) Describe any methods used to examine subgroups and interactions p9
		(*c*) Explain how missing data were addressed p9
		(*d*) If applicable, describe analytical methods taking account of sampling strategy
		(*e*) Describe any sensitivity analyses p9
Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed p10
		(b) Give reasons for non-participation at each stage seen in earlier publications p10
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders p10 suppl table
		(b) Indicate number of participants with missing data for each variable of interest suppl table
Outcome data	15*	Report numbers of outcome events or summary measures Table 1-2
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included Table 3
		(*b*) Report category boundaries when continuous variables were categorized Table 3

		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses p11
Discussion		
Key results	18	Summarise key results with reference to study objectives p12
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias p14-15
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence p15
Generalisability	21	Discuss the generalisability (external validity) of the study results p15
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based p16

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.