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ORIGINAL ARTICLE

Male Infertility

Heavy cigarette smoking and alcohol consumption are associated with impaired sperm parameters in primary infertile men

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We assessed the concomitant impact of cigarette smoking and alcohol consumption in men presenting for primary couple's infertility. Data from 189 infertile men were analyzed. Semen analysis, serum hormones, and sperm DNA fragmentation (SDF) were obtained. Smoking status was categorized as follows: current nonsmoker (–S), moderate smoker (+MS), and heavy smoker (+HS). Alcohol consumption was categorized as follows: abstainer (–D), moderate drinker (+MD), and heavy drinker (+HD). Descriptive statistics and logistic regression models were applied. Among all the participants, 132 (69.8%), 30 (15.9%), and 27 (14.3%) patients were –S, +MS, and +HS, respectively. In addition, 67 (35.4%), 77 (40.7%) and 45 (23.8%) men were -D, +MD and +HD, respectively. Regarding concomitant habits, 52 (27.5%) patients were nonsmokers and abstainers (–S/–D: Group 1), 91 (48.1%) had at least one recreational habit (–S/+D or +S/–D: Group 2), and 46 (24.3%) were both smokers and drinkers (+S/+D: Group 3). Sperm concentration and progressive motility were lower in +HS and +HD, compared with –S and –D (all $P < 0.05$), respectively. Similarly, both parameters were significantly lower in Group 3 than Groups 1 and 2 (all $P < 0.05$). SDF values were higher in Group 3 than Groups 1 and 2 (both $P < 0.05$). In multivariate analysis, follicle-stimulating hormone (FSH) levels and concomitant +S/+D status were independent predictors of impaired sperm concentration and progressive motility (all $P < 0.05$). Heavy smoking and heavy drinking were associated with worse seminal parameters than moderate smoking/drinking and nonsmoking/abstaining. When concomitant, +S/+D status has an even greater detrimental effect on semen parameters.

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INTRODUCTION

Despite the well-known deleterious impact that cigarette smoking exerts on general health, approximately 37% of male adults worldwide use tobacco.[1] Europe, in particular, has the highest rate of tobacco use among all World Health Organization (WHO) regions, with men of reproductive age (20–39 years) representing nearly 46% of all smokers.[2]

Cigarette smoke contains several toxic compounds, including nicotine, carbon monoxide, and cadmium, which may have detrimental effects on male germ cells.[3] Chronic exposure of spermatozoa to toxic components has been associated with decreased sperm mitochondrial activity and damage of the chromatin structure and sperm DNA, eventually leading to impaired fertilization capacity both *in vivo* and *in vitro*.[4,5] Moreover, cigarette smoking may lead to sperm damage via a local increase of the reactive oxygen species (ROS) and the simultaneous decline of antioxidant molecules.[6] Nevertheless, the current literature reports controversial results regarding the effect of cigarette smoking on sperm quality. While it has been shown that smoking has a negative effect on semen volume, sperm concentration, sperm motility, and sperm morphology,[7–9] other studies have found no effect of cigarette smoking on sperm parameters.[2,10,11]

Similarly, alcohol consumption has been considered a potential detrimental factor for male fertility due to its deleterious effects on semen parameters and reproductive hormonal levels.[12,13] However, there is a paucity of evidence regarding possible associations between alcohol intake and semen quality.[14,15]

Two recent studies have reported a negative impact of smoking and alcohol consumption on sperm DNA fragmentation (SDF),[8,16] a parameter currently measured in assisted reproductive technology (ART) outcomes.[17–19] However, other studies have failed to find any association between these recreational habits and SDF value.[20]

Overall, the individual impacts of either alcohol or cigarette smoking on sperm parameters have been extensively investigated, but findings are conflicting. In contrast, few studies have considered the concomitant impact of alcohol consumption and smoking on semen quality and SDF.[8,16] Therefore, we sought to cross-sectionally investigate the impact of concomitant alcohol consumption and cigarette smoking on sperm parameters and sperm DNA quality in a homogeneous cohort of Caucasian-European men seeking medical help for primary couple's infertility associated with male-only factors.

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PATIENTS AND METHODS

Study population

We analyzed data from 189 Caucasian-European men (age range: 18–55 years) evaluated at a single academic center (IRCCS San Raffaele Hospital, Milan, Italy) for primary couple's infertility between September 2015 and September 2017. Primary infertility is defined as when a couple has never had a pregnancy and has tried for more than 1 year without success.[21] Infertile patients were included in the study if they had only male factor infertility (MFI), which was defined after a comprehensive gynecological evaluation of the female partners. Patients underwent at least two consecutive semen analyses, both showing below-standard values for normal semen parameters according to the WHO criteria.[22]

Semen analysis

Semen samples were collected by masturbation after a sexual abstinence of 2–7 days and analyzed within 2 h of ejaculation, in accordance with the WHO criteria. For the specific purposes of this analysis, we considered semen volume, sperm concentration, sperm progressive motility, and sperm morphology. The improved Neubauer hemocytometer chamber (100-μm-deep; Brand™ Blaubrand™ Neubauer Improved Counting Chambers, Fisher Scientific, Loughborough, UK) was used for the calculation of the sperm number and concentration.

Sperm morphology was assessed through the following steps: preparation of a smear of semen on a slide; fixing and staining (a combination of methylene blue-N and cresyl violet acetate; Sigma-Aldrich, Darmstadt, Germany) the slide (Testsimplets® Prestained Slides, Waldeck GmbH & Co. KG, Münster, Germany); examination with brightfield optics at ×1000 magnification (Nikon Eclipse E 200, Nikon Instruments Europe B.V., Rome, Italy) with oil immersion; and assessment of approximately 200 spermatozoa per replicate for the percentage of normal or abnormal forms.

The sperm DNA fragmentation index was measured by sperm chromatin structure assay (SCSA). This assay measures the susceptibility of sperm nuclear DNA to *in situ* acid-induced DNA denaturation. As previously described,[23] frozen seminal samples containing 1×10^6 spermatozoa were thawed and treated immediately with detergent solution (pH 1.2) containing 0.1% Triton X-100 (Sigma-Aldrich), 0.15 mol l^{-1} NaCl, and 0.08 mol l^{-1} HCl (Sigma-Aldrich). Spermatozoa were stained after 30 s with 6 mg ml^{-1} acridine orange (AO) in a phosphate citrate buffer (pH 6) (Sigma-Aldrich). A BD FACScalibur™ flow cytometer (BD Bioscience, San Jose, CA, USA) was used to analyze the stained cells. The raw data of the intensity value of coordinates of red and green fluorescence for each spermatozoon were plotted on a scattergram using standard Becton Dickinson software (FACSDiva Software, BD Bioscience). The percentage of spermatozoa with abnormal chromatin structure was represented by the SDF (%), which was calculated as the ratio of red fluorescence to the total of red and green fluorescence. SDF was considered pathological if ≥30%.[23]

In the laboratory for semen analysis (Laboratory Medicine Service, IRCCS Ospedale San Raffaele, Milan, Italy), a continuous quality assurance program has been developed and maintained for several years. It relies on a quality manual containing standard operating procedures and a detailed set of instructions for the different processes and methods used in the laboratory. Internal quality control (IQC) is implemented with the inclusion of IQC materials in the laboratory's regular workload, and the results for these materials are monitored using quality control charts. External quality control (EQA) is regularly performed through peer comparison and proficiency testing programs (Italian EQA program). Results are sent to a central facility (QualiMedLab, Milan, Italy) that assesses the performance of the laboratory. Continuous training and education of the laboratory personnel is also undertaken.

Clinical and hormonal characteristics of the whole cohort

The baseline assessment included a detailed medical history and physical examination. Comorbidities were scored using the Charlson Comorbidity Index (CCI).[24] The CCI was categorized as 0 or ≥1. Body mass index (BMI, in kg m^{-2}) was calculated for every patient. Smoking habits were assessed as pack-year history (*i.e.*, a man with a 2 pack-year history smokes 2 packs of cigarettes per day) and then categorized into three groups, namely nonsmokers, moderate smokers (0–1 pack-year history), and heavy smokers (>1 pack-year history), as previously reported.[16] Patients were considered active smokers if they reported smoking for at least 1 year or if their date of quitting was within 3 months of the clinical evaluation. Similarly, alcohol consumption was categorized as abstainer (no alcohol consumption), moderate drinkers (up to 2 drinks per day), and heavy drinker (>2 drinks per day).[25]

Testicular volume was assessed with a Prader orchidometer (Bayer, Müllerstraße 178, Berlin, Germany). Venous blood samples were drawn from every patient between 7 a.m. and 11 a.m. after an overnight fast. Follicle-stimulating hormone (FSH), luteinizing hormone (LH), 17-β-estradiol (E2), inhibin B (InhB), total testosterone (tT), and sex hormone-binding globulin (SHBG) levels were measured, and chromosomes were analyzed for every patient.[26] The same laboratory analyzed all parameters for the entire cohort. Color-Duplex ultrasound (Hitachi Hi Vision 5500, Hitachi Medical Systems America Inc., Twinsburg, OH, USA) was used to detect spermatic vein reflux and to classify the grade of varicocele for every patient.[27]

We excluded men who were ex-smokers (*i.e.*, men who had stopped smoking more than 3 months before the study) and those with a history of occupational exposure to chemicals (such as heavy metals, organic solvents, polychlorinated biphenyls, dichlorodiphenyltrichloroethane [DDT], and dichlorodiphenyldichloroethylene [DDE]), or a history of cryptorchidism, or abnormal karyotyping (any type). This was done to reduce the effect of these potential confounders (previous smoking history and chemical exposure), which previous literature has suggested may affect the association between smoking/alcohol consumption and reproductive parameters.

Data collection followed the principles outlined in the Declaration of Helsinki. All patients signed informed consent agreeing to share their own anonymous information for future studies. The study was approved by the IRCCS San Raffaele Hospital Ethical Committee, Milan, Italy (Prot. 2014 – Pazienti Ambulatoriali).

Statistical analyses

Statistical analyses were performed using SPSS software version 19 (IBM Corp., Armonk, NY, USA). Normality of data distribution was assessed with the Shapiro–Wilk test and the value of the skewness and kurtosis test. Descriptive statistics were used to assess potential differences in clinical parameters, as well as hormonal and seminal values across the groups based on smoking and alcohol intake categorization. We used the one-way analysis of variance (ANOVA) or the Kruskal–Wallis test to assess differences in continuous variables between groups, as appropriate. The Pearson's Chi-squared test was used to test the statistical significance of differences in proportions between groups. Univariate (UVA) and multivariate (MVA) logistic regression models were fit to test the associations between clinical variables (*e.g.*, age, BMI, CCI, FSH, and smoking and alcohol status) and semen parameters. Similarly, UVA and MVA logistic regression analyses were used to identify the potential

predictors of pathologic SDF score (*i.e.*, SDF ≥30%). All tests were two sided, and statistical significance level was determined at $P < 0.05$.

RESULTS

Table 1 illustrates the descriptive and clinical characteristics of the study population. Overall, 132 (69.8%), 30 (15.9%), and 27 (14.3%) patients were nonsmokers (−S), moderate smokers (+MS), and heavy smokers (+HS), respectively. Similarly, 67 (35.4%), 77 (40.7%), and 45 (23.8%) men were abstainers (−D), moderate drinkers (+MD), and heavy drinkers (+HD), respectively. In terms of concomitant recreational habits, 52 (27.5%) patients were nonsmokers and abstainers (−S/−D; Group 1), 91 (48.1%) had at least one recreational habit (−S/+D or +S/−D; Group 2), and 46 (24.3%) were both smokers and drinkers (+S/+D; Group 3).

Population segregated according to smoking status

Smokers (+MS/+HS) had higher BMI, FSH, and LH levels than nonsmokers (−S) (all $P < 0.05$). In particular, men in the +HS group had a higher mean BMI than the reference group of −S patients ($P = 0.02$). No other differences were observed among groups in terms of clinical parameters. Compared to the −S group, LH and FSH levels were higher in the +HS group (all $P < 0.05$), but did not differ from those of the +MS group. No differences were observed among groups in terms of tT and SHBG values. Multiple comparison analysis showed that the +HS group had worse, but not significantly different, clinical and hormonal values than the +MS group. Overall, lower sperm concentration ($P < 0.01$) and sperm motility ($P < 0.01$) values were observed in smokers compared to nonsmokers. Multiple comparison analysis showed that sperm concentration ($P = 0.04$) and progressive motility ($P = 0.03$) were lower in the +HS than in the −S group. No differences were found between the +MS and +HS groups or between the +MS and −S groups in semen parameter values. Higher SDF values were observed in smokers (+MS and +HS) than in the −S group ($P = 0.02$). Specifically, SDF values were higher in the +HS group compared to the −S group ($P = 0.03$), whereas no differences were observed between the −S and +MS groups or between the +HS and +MS groups (**Table 2**).

Table 1: Characteristics and descriptive statistics of the whole cohort

Clinical characteristics	*The whole cohort (n=189)*
Age (year), mean±s.d. (range)	38.1±5.6 (27–55)
BMI (kg m^{-2}), mean±s.d. (range)	25.3±2.6 (18.4–34.4)
CCI score, mean±s.d. (range)	0.1±0.5 (0–3)
CCI, *n* (%)	
CCI=0	177 (93.7)
CCI ≥1	12 (6.3)
Left testis volume (Prader estimation, cm^3), mean±s.d. (range)	14.8±4.7 (4–25)
Smoking status, *n* (%)	
Nonsmokers	132 (69.8)
Moderate smokers	30 (15.9)
Heavy smokers	27 (14.3)
Alcohol status, *n* (%)	
Abstainers	67 (35.4)
Moderate drinkers	77 (40.7)
Heavy drinkers	45 (23.8)
Smoking and alcohol status, *n* (%)	
Nonsmokers and abstainers	52 (27.5)
At least one recreational habit	91 (48.1)
Smokers and drinkers	46 (24.3)
Hormonal parameters	
FSH (mUI ml^{-1}), mean±s.d. (range)	7.3±6.4 (0.8–45.8)
LH (mUI ml^{-1}), mean±s.d. (range)	4.5±2.2 (0.9–17.4)
InhB (pg ml^{-1}), mean±s.d. (range)	130.9±76.3 (6.0–328.0)
tT (ng ml^{-1}), mean±s.d. (range)	4.6±1.6 (0.1–8.8)
E2 (pg ml^{-1}), mean±s.d. (range)	27.5±10.4 (5.0–94.0)
SHBG (nmol l^{-1}), mean±s.d. (range)	41.8±6.9 (6.0–228.0)
Semen parameters	
Semen volume (ml), mean±s.d. (range)	3.3±1.6 (1.0–9.0)
Sperm concentration (×10^6 ml^{-1}), mean±s.d. (range)	20.1±28.4 (0.5–140.0)
Sperm concentration ≤15 × 10^6 ml^{-1}, *n* (%)	114 (60.3)
Progressive motility (%)	20.0±16.9 (0–68.0)
Progressive motility ≤32%, *n* (%)	142 (75.1)
Normal morphology (%), mean±s.d. (range)	8.5±15.8 (0–94.0)
Normal morphology ≤4%, *n* (%)	112 (59.3)
SDF (%), mean±s.d. (range)	38.7±24.3 (1.4–99.8)
SDF ≤30%, *n* (%)	104 (55.0)

s.d.: standard deviation; BMI: body mass index; CCI: Charlson Comorbidity Index; FSH: follicle-stimulating hormone; LH: luteinizing hormone; InhB: inhibin B; tT: total testosterone; E2: 17-β-estradiol; SHBG: sex hormone-binding globulin; SDF: sperm DNA fragmentation

Population segregated according to alcohol consumption status

Clinical characteristics were similar among the three alcohol consumption groups. FSH levels were higher in drinkers (+MD/+HD) than abstainers ($P = 0.03$). Similarly, sperm concentration ($P < 0.01$) and sperm motility ($P < 0.01$) were lower in drinkers than abstainers. Multiple comparison analysis revealed that sperm concentration was lower in both the +HD ($P = 0.02$) and +MD ($P = 0.02$) groups compared to abstainers. Conversely, only the +HD group had lower sperm motility ($P = 0.04$) than the reference group. Heavy drinkers had higher SDF values than abstainers ($P = 0.04$), but did not differ from the +MD group (**Table 3**).

The concomitant impact of alcohol consumption and smoking habits on patient characteristics

The groups did not differ in age or CCI scores. Group 3 (+S/+D) patients showed higher BMI levels than Group 1 (−S/−D) ($P = 0.031$), but were similar to Group 2 (−S/+D and +S/−D). Moreover, Group 3 had higher FSH and LH values than both Groups 1 and 2 (all $P < 0.05$). The groups did not differ in terms of tT, SHBG, InhB, and E2 levels. Sperm concentration and sperm motility were significantly lower in Group 3 compared to Groups 1 and 2 (all $P < 0.05$). Similarly, SDF values were higher in Group 3 than in Groups 1 and 2 (both $P < 0.05$) (**Table 4**).

Logistic regression analyses tested the association between clinical and hormonal predictors and impaired sperm parameters or SDF values

Table 5 shows results from logistic regression models examining the association between clinical variables (*i.e.*, age, BMI, CCI, FSH, and smoking and alcohol status) and pathological sperm parameters or impaired SDF values (≥30%). In univariate models, higher FSH ($P < 0.001$) and being categorized as either +HS ($P = 0.04$) or +HD ($P = 0.003$) were associated with pathologically lower sperm concentrations. Similarly, +HS status ($P = 0.023$) and high FSH ($P = 0.02$) were associated with impaired progressive motility in univariate models. No variables were associated with normal sperm morphology. Higher FSH and being categorized as either +HS or +HD were also associated with pathological SDF (all $P = 0.01$). In multivariate models, FSH levels (odds ratio [OR] = 1.23, $P < 0.001$) and +HS (OR = 1.23, $P < 0.001$) or +HD (OR = 2.32, $P = 0.03$) status were independently associated with pathologic sperm concentration. FSH (OR = 1.04, $P = 0.02$) and +HS status (OR = 6.17, $P = 0.019$) were the only variables independently associated with impaired sperm motility. No variables

Table 2: Descriptive statistics according to smoking status for the entire cohort

Clinical, hormonal and seminal characteristics	*Nonsmokers (n=132)*	*Smokers (+MS/+HS) (n=57)*	*P*[a]	*Moderate smokers (n=30)*	*P*[b]	*Heavy smokers (n=27)*	*P*[c]
Age (year)	38.2 (5.8)	37.8 (5.3)	0.67	37.3 (4.6)	0.71	38.4 (6.0)	0.98
BMI (kg m^{-2})	25.0 (2.6)	26.2 (2.7)	<0.01	25.8 (2.5)	0.28	26.5 (2.9)	0.02
CCI score	0.16 (0.5)	0.1 (0.1)	0.36	0.1 (0.1)	0.19	0.1 (0.1)	0.22
Left testis volume (Prader estimation, cm^3)	14.6 (4.7)	15.0 (4.7)	0.62	14.4 (4.1)	0.96	15.7 (5.3)	0.54
FSH (mUI ml^{-1})	6.4 (5.1)	8.3 (5.9)	0.03	7.7 (6.0)	0.62	10.9 (10.3)	<0.01
LH (mUI ml^{-1})	4.2 (1.7)	5.1 (1.9)	0.04	4.5 (1.6)	0.66	5.4 (3.8)	0.02
InhB (pg ml^{-1})	129.2 (76.5)	135.3 (76.1)	0.66	113.1 (56.6)	0.63	162.2 (88.9)	0.19
tT (ng ml^{-1})	4.6 (1.6)	4.5 (1.5)	0.79	5.0 (1.6)	0.44	4.0 (1.6)	0.20
E2 (pg ml^{-1})	27.1 (11.0)	27.5 (9.0)	0.82	27.6 (7.8)	0.97	27.4 (10.5)	0.98
SHBG (nmol l^{-1})	38.2 (15.1)	40.6 (14.6)	0.23	34.5 (11.4)	0.96	38.3 (16.5)	0.11
Semen volume (ml)	3.4 (1.5)	3.3 (1.7)	0.53	3.1 (1.4)	0.55	3.4 (2.0)	0.99
Sperm concentration (×10^6 ml^{-1})	23.6 (31.6)	11.9 (16.5)	<0.01	14.3 (20.4)	0.23	9.2 (10.5)	0.04
Sperm concentration ≤15 × 10^6 ml^{-1}, *n* (%)	74 (56.1)	41 (71.9)	<0.05	21 (70.0)	0.16	20 (74.1)	0.08
Progressive motility (%)	22.2 (17.6)	15.0 (14.4)	<0.01	16.7 (16.4)	0.24	13.1 (11.8)	0.03
Progressive motility ≤32%, *n* (%)	91 (68.9)	51 (89.5)	<0.01	26 (86.7)	0.05	25 (92.6)	0.01
Normal morphology (%)	8.5 (16.3)	8.4 (14.6)	0.97	9.5 (17.2)	0.94	7.3 (11.5)	0.92
Normal morphology ≤4%, *n* (%)	77 (58.3)	35 (61.4)	0.69	17 (56.7)	0.86	18 (66.7)	0.42
SDF (%)	36.1 (23.4)	45.1 (25.1)	0.02	41.3 (23.6)	0.52	49.3 (26.3)	0.03
SDF ≥30%, *n* (%)	66 (50.0)	38 (66.7)	0.03	17 (56.7)	0.51	21 (77.8)	<0.01

Data presented as mean (s.d.) if not indicated. Moderate smokers: 0–1 pack-year history; heavy smokers: >1 pack-year history. *P* values were calculated according to Chi-squared test or ANOVA or the Kruskal–Wallis test, as appropriate; [a]*P*: smokers (moderate + heavy) versus nonsmokers; [b]*P*: moderate smokers versus nonsmokers; [c]*P*: heavy smokers versus nonsmokers; s.d.: standard deviation; BMI: body mass index; CCI: Charlson Comorbidity Index; tT: total testosterone; SDF: sperm DNA fragmentation index; E2: 17-β-estradiol; SHBG: sex hormone-binding globulin; +MS: moderate smoker; +HS: heavy smoker; ANOVA: analysis of variance

Table 3: Descriptive statistics according to alcohol status for the entire cohort

Clinical, hormonal and seminal characteristics	*Abstainers (n=67)*	*Drinkers (+MD/+HD) (n=122)*	*P*[a]	*Moderate drinkers (n=77)*	*P*[b]	*Heavy drinkers (n=45)*	*P*[c]
Age (year)	37.6 (5.5)	38.3 (5.7)	0.45	38.5 (6.1)	0.58	37.8 (5.1)	0.98
BMI (kg m^{-2})	25.0 (2.6)	25.5 (2.7)	0.25	25.7 (2.6)	0.28	25.1 (2.8)	0.96
CCI score	0.16 (0.5)	0.1 (0.4)	0.31	0.1 (0.4)	0.65	0.1 (0.4)	0.64
Left testis volume (Prader estimation, cm^3)	14.8 (4.3)	14.7 (5.0)	0.90	14.4 (4.5)	0.83	15.3 (5.6)	0.84
FSH (mUI ml^{-1})	5.9 (4.4)	8.1 (7.1)	0.03	7.7 (7.4)	0.28	8.5 (7.2)	0.06
LH (mUI ml^{-1})	4.2 (1.8)	4.3 (2.1)	0.47	4.4 (2.7)	0.81	4.7 (2.1)	0.59
InhB (pg ml^{-1})	134.5 (76.4)	128.7 (77.7)	0.59	129.3 (77.3)	0.94	141.7 (87.2)	0.92
tT (ng ml^{-1})	4.6 (1.7)	4.5 (1.6)	0.65	4.6 (1.6)	0.99	3.9 (1.3)	0.11
E2 (pg ml^{-1})	28.1 (13.7)	26.6 (8.8)	0.24	27.2 (9.9)	0.92	25.8 (7.8)	0.68
SHBG (nmol l^{-1})	37.1 (14.9)	43.0 (16.3)	0.54	43.6 (11.4)	0.45	34.6 (15.3)	0.98
Semen volume (ml)	3.2 (1.4)	3.4 (1.6)	0.28	3.4 (1.7)	0.67	3.7 (1.6)	0.25
Sperm concentration (×10^6 ml^{-1})	29.1 (34.8)	15.5 (23.1)	<0.01	16.1 (27.3)	0.02	14.6 (15.8)	0.02
Sperm concentration ≤15 × 10^6 ml^{-1}, *n* (%)	33 (49.3)	82 (67.2)	<0.01	44 (57.1)	0.02	31 (68.9)	0.01
Progressive motility (%)	23.7 (16.5)	18.2 (16.9)	<0.01	19.5 (17.2)	0.31	16.1 (16.2)	0.04
Progressive motility ≤32%, *n* (%)	47 (70.1)	95 (77.9)	0.23	56 (72.7)	0.27	34 (75.6)	0.03
Normal morphology (%)	8.4 (14.4)	8.6 (16.5)	0.93	8.4 (16.0)	0.99	9.0 (17.5)	0.97
Normal morphology ≤4%, *n* (%)	41 (61.2)	72 (59.0)	0.83	44 (57.1)	0.70	28 (62.2)	0.94
SDF (%)	32.3 (21.6)	42.1 (24.6)	<0.01	39.8 (22.3)	0.19	44.7 (30.0)	0.04
SDF ≥30%, *n* (%)	29 (43.3)	77 (63.1)	<0.01	44 (57.1)	0.09	28 (62.2)	0.02

Data presented as mean (s.d.) if not indicated. Moderate drinkers: up to 2 drinks per day; heavy drinkers: >2 drinks per day. *P* values were calculated according to Chi-squared test or ANOVA or the Kruskal–Wallis test, as appropriate; [a]*P*: drinkers (moderate + heavy) versus abstainers; [b]*P*: moderate alcohol users versus abstainers; [c]*P*: heavy alcohol users versus abstainers. s.d.: standard deviation; BMI: body mass index; CCI: Charlson Comorbidity Index; tT: total testosterone; SDF: sperm DNA fragmentation; E2: 17-β-estradiol; SHBG: sex hormone-binding globulin; +MD: moderate drinker; +HD: heavy drinker; ANOVA: analysis of variance

were associated with pathological normal sperm morphology in these analyses. Both FSH (OR = 1.11, $P = 0.04$) and +HS status (OR = 3.96, $P = 0.01$) were associated with higher SDF values (**Table 5**).

Table 6 shows results from logistic regression models investigating the association between concomitant recreational habits and semen parameters or SDF values. In univariate models, FSH values and +S/+D status were associated with pathological sperm concentrations, sperm motility, and impaired SDF value. After adjusting for age, BMI, and CCI score, FSH levels (OR = 1.14, $P < 0.001$) and +S/+D status (OR = 2.78, $P = 0.036$) were associated with lower sperm concentration. There was no association between Group 2 (−S/+D or +S/−D) and abnormal sperm concentration. However, when comparing Group 3 (+S/+D) and Group 2 (−S/+D or +S/−D), Group 3 had 2.2 times greater odds ($P < 0.03$) of having impaired sperm concentration. Similarly, FSH levels (OR = 1.14, $P = 0.02$) and +S/+D status (OR = 9.72, $P = 0.005$) were independently associated with pathological sperm motility. Indeed, patients in Group 3

Table 4: Descriptive statistics for the entire cohort segregated according to the combination of recreational habits

Clinical, hormonal and seminal characteristics	*Group 1 (n=52)*	*Group 2 (n=91)*	*P*[a]	*Group 3 (n=46)*	*P*[b]
Age (year)	37.7 (5.4)	38.2 (6.1)	0.86	38.0 (5.1)	0.97
BMI (kg m^{-2})	24.8 (2.6)	25.2 (2.5)	0.65	26.2 (2.9)	0.031
CCI score	0.19 (0.5)	0.1 (0.4)	0.63	0.1 (0.3)	0.32
Left testis volume (Prader estimation, cm^3)	14.8 (4.4)	14.6 (4.7)	0.96	15.1 (5.1)	0.95
FSH (mUI ml^{-1})	5.9 (4.6)	6.5 (5.2)	0.87	10.5 (9.0)	<0.001**
LH (mUI ml^{-1})	4.2 (1.8)	4.1 (1.6)	0.86	5.3 (3.1)	0.03**
InhB (pg ml^{-1})	133.4 (78.6)	129.1 (73.4)	0.95	132.2 (81.8)	0.98
tT (ng ml^{-1})	4.7 (1.7)	4.6 (1.6)	0.91	4.5 (1.6)	0.89
E2 (pg ml^{-1})	28.6 (13.7)	26.5 (9.4)	0.58	27.2 (8.4)	0.81
SHBG (nmol l^{-1})	37.9 (15.3)	37.7 (14.7)	0.88	44.4 (16.6)	0.39
Semen volume (ml)	3.1 (1.3)	3.5 (1.6)	0.38	3.2 (1.6)	0.86
Sperm concentration (×10^6 ml^{-1})	31.4 (36.8)	20.3 (25.4)	0.04	8.7 (12.1)	<0.001*
Sperm concentration ≤15 × 10^6 ml^{-1}, *n* (%)	24 (46.2)	56 (61.5)	0.07	35 (76.1)	0.003*
Progressive motility (%)	23.3 (16.9)	21.9 (17.6)	0.89	12.4 (13.2)	0.004**
Progressive motility ≤32%, *n* (%)	36 (69.2)	63 (69.2)	0.99	43 (93.5)	0.002**
Normal morphology (%)	7.8 (13.9)	7.8 (17.5)	0.86	7.2 (14.3)	0.99
Normal morphology ≤4%, *n* (%)	31 (59.6)	53 (58.2)	0.87	28 (60.9)	0.89
SDF (%)	31.4 (20.6)	38.4 (24.9)	0.21	49.2 (23.1)	<0.001*
SDF ≥30%, *n* (%)	23 (44.2)	47 (51.6)	0.39	34 (73.9)	0.003*

Data presented as mean (s.d.) if not indicated. Group 1: nonsmokers and abstainers; Group 2: at least one recreational habit; Group 3: smokers and drinkers. *P* values were calculated according to Chi-squared test or ANOVA or the Kruskal–Wallis test, as appropriate. [a]*P*: Group 2 versus Group 1; [b]*P*: Group 3 versus Group 1; *$P<0.05$ for Group 3 versus Group 2; **$P<0.01$ for Group 3 versus Group 2. Group 1: –S/–D; Group 2: either –S/+D or +S/–D; Group 3: +S/+D; s.d.: standard deviation; BMI: body mass index; CCI: Charlson Comorbidity Index; tT: total testosterone; SDF: sperm DNA fragmentation; E2: 17-β-estradiol; SHBG: sex hormone-binding globulin; ANOVA: analysis of variance

Table 5: Logistic regression models examining the association of clinical variables with pathologic sperm parameters according to the World Health Organization 2010 criteria and pathologic sperm DNA fragmentation in the whole cohort (*n*=189)

Clinical predictors of impaired sperm parameters	*Sperm concentration <15×10^6 ml^{-1}*		*Progressive motility <32%*		*Normal morphology <4%*		*SDF ≥30%*	
	UVA model	*MVA model*	*UVA model*	*MVA model*	*UVA model*	*MVA model*	*UVA model*	*MVA model*
Age	1.02 (0.9–1.0), 0.49	1.16 (0.90–1.0), 0.29	1.03 (0.9–1.1), 0.29	1.01 (0.9–1.1), 0.85	0.99 (0.9–1.1), 0.86	0.94 (0.9–1.0), 0.39	1.1 (0.9–1.0), 0.06	1.04 (0.9–1.1), 0.17
BMI	1.07 (0.9–1.2), 0.22	1.00 (0.88–1.1), 0.97	1.06 (0.9–1.3), 0.33	0.99 (0.8–1.1), 0.97	0.96 (0.9–1.1), 0.49	0.96 (0.8–1.1), 0.49	0.94 (0.8–1.0), 0.31	0.89 (0.8–1.1), 0.08
CCI ≥1	3.4 (0.7–6.4), 0.12	4.10 (0.75–5.3), 0.10	3.8 (0.5–3.7), 0.20	4.66 (0.5–9.5), 0.16	3.67 (0.7–7.3), 0.09	4.0 (0.7–9.5), 0.10	0.81 (0.3–2.6), 0.71	0.56 (0.1–2.3), 0.49
FSH	1.2 (1.1–1.3), <0.001	1.23 (1.11–1.3), <0.001	1.07 (1.0–1.1); 0.02	1.04 (1.0–1.2), 0.02	1.04 (0.9–1.1), 0.16	1.04 (0.9–1.1), 0.13	1.12 (1.0–1.2), 0.01	1.11 (1.0–1.1), 0.04
Smoking status								
Nonsmokers	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Moderate smokers	1.82 (0.8–4.2), 0.16	1.83 (0.6–4.9), 0.23	2.92 (0.9–8.9), 0.06	1.02 (0.9–8.8), 0.23	0.93 (0.5–2.1), 0.86	0.95 (0.4–2.2), 0.97	1.3 (0.5–2.9), 0.51	1.22 (0.5–2.9), 0.65
Heavy smokers	2.23 (1.0–5.3), 0.04	1.23 (1.1–7.9), <0.001	5.63 (1.3–11.9), 0.023	6.17 (1.3–14.8), 0.019	1.42 (1.0–1.1), 0.43	1.85 (0.7–4.6), 0.19	3.5 (1.3–9.2), 0.011	3.96 (1.4–11.2), 0.01
Alcohol consumption status								
Abstainers	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Moderate drinkers	1.5 (0.7–3.3), 0.22	1.01 (0.4–2.4), 0.96	1.51 (0.7–3.2), 0.27	1.29 (0.6–2.9), 0.53	0.87 (0.4–1.7), 0.70	0.82 (0.4–1.6), 0.67	1.63 (0.7–3.4), 0.19	1.27 (0.5–2.9), 0.57
Heavy drinkers	2.91 (1.4–5.9), 0.003	2.32 (1.1–5.2), 0.03	1.49 (0.6–3.5), 0.36	1.09 (0.4–2.8), 0.83	1.04 (0.5–2.2), 0.92	0.87 (0.4–1.9), 0.74	2.49 (1.2–4.8), 0.01	1.93 (0.93–4.1), 0.07

All the data are expressed as OR (95% CI), *P*. UVA: univariate model; MVA: multivariate model; BMI: body mass index; FSH: follicle-stimulating hormone; CCI: Charlson Comorbidity Index; SDF: sperm DNA fragmentation; OR: odds ratio; CI: confidence interval; WHO: World Health Organization

had a 9.4-fold increased probability ($P = 0.04$) of having impaired sperm motility compared to those in Group 2. No variables were independently associated with normal sperm morphology. Age, FSH, and concomitant +S/+D status were associated with higher SDF value (all $P < 0.05$).

DISCUSSION

The aim of this study was to investigate the concomitant impact of cigarette smoking and alcohol consumption on semen quality and sperm DNA integrity in a cohort of Caucasian-European men seeking medical help for primary couple's infertility. We found that heavy smoking and heavy drinking were associated with worse seminal parameters than moderate smoking/drinking and nonsmoking/abstaining. When concomitant, +S/+D status has an even greater detrimental effect on semen parameters.

Our results should be considered in the context of the limitations of the study. Overall, the cross-sectional design of the study and the

Table 6: Logistic regression models examining the association of clinical variables with sperm parameters according to the World Health Organization 2010 criteria and pathologic sperm DNA fragmentation in the whole cohort (*n*=189)

Clinical predictors of impaired sperm parameters	*Sperm concentration <15x10⁶ ml⁻¹*		*Progressive motility <32%*		*Normal morphology <4%*		*SDF ≥30%*	
	UVA model	*MVA model*	*UVA model*	*MVA model*	*UVA model*	*MVA model*	*UVA model*	*MVA model*
Age	1.01 (0.95–1.06), 0.89	1.11 (0.91–3.41), 0.34	1.03 (0.97–1.10), 0.29	1.01 (0.94–1.08), 0.74	0.99 (0.95–1.04), 0.85	0.98 (0.92–1.04), 0.52	1.05 (0.99–1.11), 0.06	1.06 (1.01–1.12), 0.04
BMI	1.07 (0.96–1.19), 0.21	1.04 (0.92–1.68), 0.55	1.06 (0.94–1.21), 0.33	1.01 (0.88–1.15), 0.88	0.93 (0.86–1.07), 0.48	0.97 (0.86–1.09), 0.63	0.95 (0.85-1.05), 0.30	0.91 (0.81–1.03), 0.12
CCI ≥1	2.4 (0.63–9.23), 0.19	3.48 (0.78–4.89), 0.13	3.8 (0.48–5.75), 0.20	4.50 (0.53–8.21), 0.16	3.67 (0.78–7.32), 0.09	3.7 (0.72–9.52), 0.11	0.81 (0.25-2.59), 0.72	0.55 (0.14–2.23), 0.41
FSH	1.15 (1.07–1.24), <0.001	1.14 (1.06–1.22), <0.001	1.05 (1.01–1.23), 0.03	1.14 (1.05–1.33), 0.02	1.07 (1.01–1.13), 0.02	1.07 (1.01–1.13), 0.021	1.11 (1.04–1.18), 0.01	1.09 (1.03–1.17), 0.007
Smoking and alcohol status								
Group 1	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Group 2	1.30 (0.66–2.57), 0.45	1.28 (0.61–2.68), 0.51	1.01 (0.47–2.09), 0.98	1.03 (0.48–2.21), 0.94	0.94 (0.47–1.89), 0.87	0.96 (0.46–1.96), 0.91	1.34 (0.68–2.67), 0.39	1.29 (0.62–2.67), 0.48
Group 3	3.71 (1.55–8.85), 0.003	2.78 (1.07–7.24), 0.036	6.37 (1.72–8.91), 0.006	9.72 (2.01–12.18), 0.005	1.05 (0.46–2.37), 0.89	0.89 (0.36–2.17), 0.79	3.57 (1.51–8.44), 0.004	2.83 (1.09–7.35), 0.032

All the data are expressed as OR (95% CI), *P*. Group 1: nonsmokers and abstainers; Group 2: at least one recreational habit; Group 3: smokers and drinkers. UVA: univariate model; MVA: multivariate model; BMI: body mass index; FSH: follicle-stimulating hormone; CCI: Charlson Comorbidity Index; SDF: sperm DNA fragmentation; OR: odds ratio; CI: confidence interval; WHO: World Health Organization

lack of a control group of fertile men are the main flaws. Moreover, we were unable to evaluate a history of passive smoking and dietary habits, which may influence the oxidative state of the spermatozoa.[28] Lastly, abstinence time and antisperm antibodies, which are known to potentially affect semen parameters, were not assessed in our cohort.

Our interest in this topic was fuelled by the extensive controversies throughout the recent literature regarding the impact of cigarette smoking and alcohol consumption on semen parameters and, more specifically, the substantial lack of research exploring the concomitant effect of these habits in primary infertile men. Indeed, despite the well-known associations of cigarette smoking and alcohol consumption with poorer general health, and a growing body of literature focusing on the effects these behaviors have on male fertility,[4,9,12,13] these habits are still not listed among the potential risk factors for male infertility, for instance, in the current European Association of Urology guidelines.[29]

A systematic review and meta-analysis showed that cigarette smoking was associated with a reduction of sperm concentration, motility, and normal morphology.[7] Moreover, this association was more robust in infertile men than in the general population and in moderate and heavy smokers, compared to mild smokers.[7] Similarly, a recent study showed that cigarette smoking had detrimental effects on all conventional semen parameters, in addition to sperm chromatin condensation and sperm viability.[9] These abnormalities were also proportional to the number of cigarettes smoked per day and to the duration of smoking.[9]

Our current findings corroborate previous observations showing that smoking, in general, was associated with lower sperm concentration and sperm motility than nonsmoking among infertile men. More precisely, we showed that heavy smokers had the lowest values of sperm concentration and motility compared to moderate and nonsmokers.

The mechanisms by which cigarette smoking affects semen quality are not fully understood. Calogero *et al*.[5] showed, for the first time, a detrimental effect of cigarette smoking on sperm mitochondrial activity, which resulted in reduced sperm motility. Cigarette smoking also promotes the formation of DNA and protein adducts, mutations, and chromosomal abnormalities during anaphase and telophase, micronucleus formation, sister chromatid exchange (SCE), and promoter methylation in sperm cells.[3] Both micronucleus, which are typically found during the anaphase of meiosis and mitosis, and SCE are more frequent in smokers than nonsmokers and have been associated with chromosomal instability and infertility.[3] Smoking-associated epigenetic modifications in the sperm genome have also been reported. Specifically, Besingi and Johansson[30] demonstrated alterations in the methylation profile of 95 sites in smokers and suggested a possible association between methylation status and infertility. Moreover, other studies have found higher rates of histone abnormalities in smokers compared to nonsmokers.[31,32] Nicotine can also alter the hypothalamic–pituitary axis by stimulating the release of growth hormone, cortisol, vasopressin, and oxytocin, leading to Leydig cell failure and energy imbalance.[33] While the seminal plasma supports and protects spermatozoa from pathological levels of ROS through free radical scavengers and ROS-metabolizing enzymes,[34] sperm concentration may be affected by the oxidative stress caused by either the increased levels of oxidants originating from the smoke or by decreased levels of antioxidants in seminal plasma.[6] Moreover, toxins in cigarette smoke also affect seminal fluid components and accessory glands, leading to increased viscosity and reduced volume, thus reducing progressive sperm motility.[35]

The association between cigarette smoking and impaired SDF value is controversial. While previous reports have shown a negative

impact of smoking on SDF,[8,16] others have found no such effect.[20,36] We found that cigarette smoking had a detrimental impact on SDF value in infertile men. This effect was even more marked in heavy smokers than in moderate smokers and nonsmokers.

Our results are not surprising and support previous findings.[7–9,16] However, it is challenging to directly compare findings from studies that have looked at the association between smoking, as a potential risk habit, and sperm quality, owing to the difficulty of adjusting for confounders such as exposure to alcohol use, medical illnesses and health comorbidities, toxins, and hormonal status.[3]

Alcohol, indeed, is a risk factor that can affect semen values. Kucheria *et al*.,[13] for instance, reported that heavy alcohol users or men with alcohol dependence syndrome had decreased semen volume and sperm concentration. Our results, which showed that heavy drinkers, as compared to abstainers, had higher SDF value and lower sperm concentration and progressive motility, support the potential negative effect of alcohol consumption on semen parameters. Furthermore, moderate drinkers had lower sperm concentration than abstainers.

In terms of hormonal milieu, we found that heavy smokers had higher LH and FSH values than moderate smokers and nonsmokers. Smoking had no impact on tT and SHBG values. Similarly, alcohol intake did not promote significant effects on hormonal parameters. Our findings partially confirmed previous observations that showed a positive association of smoking with tT, LH, and FSH values, thus suggesting that tobacco smoking might interrupt regular hypothalamic–pituitary–gonadal axis functioning, eventually leading to Leydig cell failure.[33]

Our study sought to reduce biases that may have impinged on the findings of previous studies through several methodological approaches. First, we investigated a relatively large homogeneous cohort of patients with a comprehensive hormonal evaluation, which was not considered in most of the previous literatures, and an accurate assessment of possible confounders for impaired semen parameters (such as cigarette smoking, alcohol consumption, and health comorbidities).

The current study also advances the current literature by examining the concomitant impact of smoking and alcohol consumption as potential predictors of alteration in semen composition. Indeed, few previous studies have considered the combination of these factors in infertile men.[8,16] Anifandis *et al*.,[16] for instance, revealed that these two factors exert their action independently, but may also operate synergistically to result in reduced semen volume, increased percentage of degenerated spermatozoa, and increased percentage of SDF. Similarly, the combination of alcohol and smoking has been associated with higher levels of oxidative stress and higher SDF.[8] Our findings confirm these previous observations, with the concomitant +S/+D status being associated with worse semen parameters and higher levels of SDF than both −S/−D status and the condition of only one recreational habit (either −S/+D or +S/−D).

Of relevance, the present analyses included only primary infertile men seeking medical help for couple's infertility in an outpatient setting. In fact, the discrepancy in study outcomes[2,7–11] may also be attributable to studies combining fertile and infertile men.[37]

CONCLUSION

Both heavy smokers and heavy drinkers had worse seminal parameters than moderate smokers/drinkers and nonsmokers/abstainers. Concomitant heavy smoking and heavy drinking had an even more detrimental impact on semen parameters, supporting more severe forms of male infertility. Overall, these observations indicate the importance of an accurate investigation of lifestyle factors during the everyday diagnostic workup of primary infertile men. Moreover, it may be beneficial for clinicians to advise male patients who are seeking paternity to avoid these habits. Similarly, clinicians should facilitate smoking and alcohol cessation through education, monitoring, and constant support.

AUTHOR CONTRIBUTIONS

LB participated in study design and data collection, performed statistical analyses, and wrote the article. EV collected and interpreted the data. FP and FC collected the data. WC collected the data and participated in study design. PC collected the data, performed statistical analyses, and interpreted the results. FD was involved in study concept and design and data interpretation. AS was involved in study design, collected data, and revised the manuscript critically. EM and FM revised the manuscript critically. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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