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Title

Effect of cigarette smoking on serum anti-Mullerian hormone and antral follicle count in women seeking fertility treatment – a prospective cross-sectional study

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Abstract page

Objectives

The relationship between smoking and ovarian reserve markers is inconclusive. The primary objective of our study was to assess the effect of cigarette smoking on the quantitative ovarian reserve parameters, serum anti-Mullerian hormone (AMH) and antral follicle count (AFC) in women seeking fertility treatment. Our secondary aims were to validate self-reported smoking behaviour using biomarkers and evaluate the association between biomarkers of ovarian reverse (serum AMH and AFC) with biomarkers of smoking exposure (breath carbon monoxide (CO) and urine cotinine levels).

Design

Prospective, cross-sectional study

Setting

Single tertiary care centre

Participants

Women ≤ 35 years seeking fertility treatment

Primary outcome measures

Serum AMH and AFC

Results

Significant differences were found amongst current smokers, ex-smokers and never smokers for breath CO ($F(2,97)=33.32$, $p< 0.0001$) and urine cotinine levels ($p< 0.001$). However, no significant differences were found either for serum AMH ($F(2,91)=1.19$, $p=0.309$) or total AFC ($F(2,81)=0.403$, $p=0.670$) among the three groups. There was no significant correlation between pack years of smoking and serum AMH ($r=- 0.212$, $n=23$, $p=0.166$) or total AFC ($r=-0.276$, $n=19$, $p=0.126$). No significant correlation was demonstrated. between breath CO and serum AMH ($r=0.082$, $n=94$, $p=0.216$) or total AFC ($r=0.096$, $n=83$, $p=0.195$). Similarly, no significant correlation was demonstrated between urine cotinine levels and serum AMH ($r=0.146$, $n=83$, $p=0.095$) or total AFC ($r=-0.027$, $n=77$, $p=0.386$).

Conclusion

We did not find a statistically significant difference in quantitative ovarian reserve markers between current, ex- and never smokers in our study population. We confirmed that self-reported smoking correlates well with quantitatively measured biomarkers of smoking, validating the comparison groups based on self-reported smoking history to ensure a valid comparison of outcome measures. There was no significant association between biomarkers of smoking and biomarkers of ovarian reserve. We were also unable to demonstrate a correlation between the lifetime smoking exposure and ovarian reserve.

Strengths and limitations of this study

- We used a comprehensive and detailed self-reported questionnaire to assess smoking exposure.
- We used biomarkers of smoking exposure; breath CO and urine cotinine concentrations to validate our self-reported study groups.
- We recruited an unselected population of women seeking fertility treatment in an attempt to improve generalisability of results.
- We have included only women 35 years and younger to reduce bias due to the impact of advancing age.
- Our study was powered to detect differences in ovarian reserve markers of relatively large magnitude that we considered to have a clinical significance in the management of young women seeking fertility treatment.

1 Introduction

Anti-Mullerian hormone (AMH) and antral follicle count (AFC) are well established biomarkers of ovarian reserve, commonly used in the context of fertility treatment(1, 2). Estimation of the size of the primordial follicle pool is difficult and impractical for routine clinical application as there is no known biochemical marker for estimating the number of primordial follicles, and their small size makes in-vivo imaging with sufficient resolution impossible using currently available technology. A subsection of the true ovarian reserve is the pool of pre-antral and antral follicles which are responsive to pituitary gonadotropins and are clinically relevant for menstruation, ovulation and fertility. The currently available biomarkers, AMH and AFC, measure the antral follicle pool. AMH is expressed exclusively by the granulosa cells of pre-antral and small antral follicles in the ovary and hence an excellent quantitative marker of the ovarian reserve(3). Antral follicle counts assessed by ultrasound scan measure the same biological entity and show a strong positive correlation with serum AMH levels(4).

Age remains one of the most important determinants of ovarian reserve and fertility (5), with a natural decline due to a decrease in the number of oocytes and a reduction in oocyte quality. Additionally, genetic, life-style and environmental factors are also recognised to affect variation in ovarian reserve(5, 6). The relationship between smoking and serum AMH and AFC reported in literature is inconsistent. Some studies suggest that smoking may negatively impact the ovarian reserve(7, 8), whereas the others have failed to corroborate this association(9).Differences in ascertainment of cigarette smoking exposure, potential inaccuracies in self-reported smoking history and selection biases in studies may have led to discrepancies in the results. The role of passive smoking has also not been well investigated.

Thus, the primary objective of our study was to assess the effect of cigarette smoking on the quantitative ovarian reserve parameters, AMH and AFC. Our secondary aims were to validate self-reported smoking behaviour using biomarkers and evaluate the association between biomarkers of ovarian reverse (serum AMH and AFC) with biomarkers of smoking exposure (i.e. breath carbon monoxide and urine cotinine levels).

2 Materials and methods

2.1 Study design, setting and population

We conducted a single-centre prospective cross-sectional study from July 2019 to February 2020. The study population comprised of couples referred to the fertility centre for investigations and treatment of subfertility. We compared the levels of serum AMH and AFC among current smokers, ex-smokers and never-smokers based on a self-reported smoking history and validated by the measurements of breath carbon monoxide (CO) and urine cotinine levels. We also explored the association between biomarkers of ovarian reserve (AMH and AFC) and biomarkers of smoking (breath CO and urine cotinine) and correlated the lifetime smoking exposure quantified as "pack years" with levels of serum AMH and AFC.

2.2 Patient and public involvement

The study question and design were discussed with patients attending the fertility clinic who agreed that the research question was important and the outcomes appropriate. Patients helped with design and language of the participant information leaflets and questionnaires. Patients were not involved in recruitment or conduct of the study. We plan to involve patients in dissemination of findings through patient networks such as the East London Katherine Twining Network.

2.3 Inclusion and exclusion criteria

We included women aged ≤35 years attending the fertility unit for investigations and treatment. We excluded women on long term oral contraceptive pills or GnRH analogues, those not having both ovaries and with a history of previous chemotherapy, abdominal/pelvic radiotherapy or major ovarian surgery.

2.4 Study procedures, screening, consent, care pathway, study intervention, laboratory procedures

We screened and invited eligible participants to participate in the study. Following informed consent we assessed the participants for markers of smoking. This included a short self-reported questionnaire about the participant's current and past smoking history, a non-invasive breath test to detect the levels of carbon monoxide and a urine test to detect the levels of cotinine. Based on the smoking history we classified

participants into one of three categories; current smokers, ex-smokers and never-smokers. The smoking history also accounted for passive smokers and smoking details aimed to quantify the smoking exposure in terms of “pack years”. We measured serum AMH and AFC as a part of the standard fertility work up done for all fertility patients. We also collected baseline demographic and clinical data for confounding variables. We followed up all participants for the results of their tests.

2.5 Products, devices, techniques and tools

A bespoke questionnaire was used to obtain self-reported smoking history. This was designed with the input of clinical and research members of the team to ensure content validity and reliability. The questionnaire was tested on a pilot sample of the target population. This highlighted deficiencies and allowed improvements in the final questionnaire used. The questionnaire details are provided in Appendix S1.

The device used to measure the breath CO (Smokelyser) is a CE marked, commercially available, non-invasive CO breath test that uses an electrochemical sensor to measure the breath concentration of CO with a concentration range of 0-150 ppm with a sensor sensitivity of 1 ppm and an accuracy of ±2 ppm. The instrument was used within the specified warranty period and used and serviced according to manufacturer’s specifications.

The urine cotinine was measured using the DRI®Cotinine assay (Thermo Fisher Scientific). The DRI® Cotinine Assay is an in vitro diagnostic medical device intended for the qualitative and semi-quantitative determination of cotinine in human urine at a cut off level of 500 ng/mL. The accuracy of the assay has been confirmed by gas chromatography /mass spectrometry. According to manufacturer, the sensitivity, defined as the lowest concentration that can be differentiated from the negative urine calibrator with 95% confidence, is 34 ng/mL.

All serum AMH assays were performed in an on-site clinical laboratory using the bench-top fully automated assay Access 2 immunoassay system (Beckman-Coulter) and values were expressed as pmol/l. Inter-assay coefficients of variation for a low and high control were 0.056 and 0.44, respectively. Venous blood samples were obtained and delivered to the laboratory immediately, centrifuged, and stored at 2-8°C, and analysed every day.

Ultrasound imaging of ovaries was performed using a Voluson S10 diagnostic ultrasound system (GE Healthcare) equipped with a multi-frequency transvaginal probe (RIC5-9W-RS: 9-5MHz) to visualize antral follicles systematically. AFC was obtained automatically using the sono-AVC™ software. Manual image post-processing was done if required. A total AFC was calculated as the sum of total number of follicles between 2-9 mm on each ovary. This measurement was not restricted to a particular time of the cycle.

2.6 Outcome measures

The primary outcome measures were serum AMH and total AFC.

2.7 Data collection

Data were recorded onto study specific paper Case Report Forms (CRFs) and subsequently transferred to a study database. We collected baseline demographic characteristics of the study population (age, ethnicity), baseline clinical data (BMI, presence of PCO/PCOS, history of ovarian surgery), data for on smoking parameters (type of smoker, passive smoking, smoking in pack years, breath CO and urine cotinine levels) and data for primary outcomes (serum AMH, AFC).

Data for smoking parameters were collected by members of the research team directly from the participant. All other data were collected from the participants' medical records and electronic hospital records.

2.8 Statistical considerations, sample size, analysis

The sample size calculation was based upon the primary outcome of serum AMH. Approximately 13% of women in the UK are current smokers (10) and the number of ex-smokers exceeds that of smokers. The proportion of never smokers in the UK population is increasing and reported at 59% in 2014 (11). Hence we estimated that at the fertility clinic approximately one third of our population would be either smokers or ex-smokers. We have previously found the mean serum AMH to be 28.28 pmol/l and a significantly lower pregnancy rates among women in the lowest quartile of AMH, i.e. below 10.28 pmol/l.(12) To detect an absolute decrease in AMH from 28.28 to 10.28 pmol/l with 80% power at a 5% significance level with an enrolment ratio of 0.5, we would require 96 participants (32 smokers/ex-smokers and 64 non-smokers). We planned to recruit approximately 100 participants to compensate for dropout and loss

to follow up. Appropriate descriptive statistics were used to describe the baseline variables in the dataset. Normality of data was checked using Shapiro-Wilk test and skewed data were log transformed to achieve normal distribution before using parametric test. Nonparametric tests were used for data analysis if normal distribution was not achieved. An one-way between-groups analysis of variance (ANOVA), a Chi-squared test or a Kruskal-Wallis test were used to assess differences between baseline variables and smoking markers between current smokers, ex -smokers and never smokers. An ANOVA was used to assess differences in outcome variables between the three study groups. When the P-value was <.05, the difference was considered statistically significant. When a difference was found to be significant, a post-hoc Tukey multiple comparison test was performed. A one-way between-groups analysis of co-variance (ANCOVA) was performed to assess the differences between groups taking into account the variability of other confounding variables. Differences in breath CO concentrations and urine cotinine levels in the three comparison groups were used to validate group stratification and the results for the primary outcome variables. Pearsons correlation test was used to explore the relationship between lifetime exposure to smoking (pack years), breath CO or urine cotinine and outcome variables. Statistical analysis was done using the Statistical Package for Social Sciences (SPSS version 26).

3 Results

101 women were recruited to the study over a period of nine months. Based on a self-reported smoking history women were classified into three comparison groups: current smokers, ex-smokers and never smokers. The baseline clinical characteristics of the participants are summarised in Table 1. There were no significant differences in the baseline variables amongst the three groups.

Table 1: Baseline variables

	Current smokers (n=12)	Ex-smokers (n=25)	Never smokers (n=64)	p
Age (years)	30 (25.5-33.0)	32.5 (31.0-33.5)	31.0 (28.0-33.0)	0.057
BMI	23.2 (21.8-26.2)	25.3 (20.8-28.3)	25.1 (22.1-27.8)	0.632
Ethnicity				0.208

White European	8	21	35	
Asian	2	4	16	
Afro-Caribbean	1	0	8	
Others	1	0	5	
Category of infertility				0.077
Anovulatory	4	1	11	
Male	4	5	14	
Tubal	2	0	9	
Unexplained	1	14	20	
Other	0	4	4	
Ovarian surgery				0.659
No	12	23	60	
Yes	0	1	1	
PCOS/PCOM				0.351
N	7	17	42	
Y	5	4	20	

Values expressed as median (IQR) or n

The smoking markers for the three groups are detailed in table 2. The pack years of smoking, quantifying exposure to cigarette smoking, were not significantly different between current and ex-smokers ($F(1,25) = 0.547$, $p=0.467$). The breath CO levels were significantly different amongst current, ex- and never smokers ($F(2,97) = 33.32$, $p< 0.0001$). Urine cotinine levels were also significantly higher in current smokers as compared to ex-smokers and never smokers. ($p< 0.001$). Current smokers reported to be more exposed to passive smoking (75%, 9/12) as compared to ex-smokers (20%, 5/25) and never smokers (25%, 16/64) ($p=0.001$).

Table 2: Smoking markers

	Current smokers (n=12)	Ex-smokers (n=25)	Never smokers (n=64)	p
Pack years of smoking	2.13 (0.59-3.48)	2.13 (0.05-5.40)	0.00 (0.00-0.00)	0.467*

Breath CO (ppm)	9 (3.5-21)	2 (2-3)	1 (1-2)	<0.001
Urine Cotinine (ng/ml)	837 (22.42 – 1571.8)	22.42 (22.42-22.42)	22.42 (22.42-22.42)	<0.001

comparison between current and ex-smokers only. Values presented as median (IQR)

The primary outcomes are detailed in Table 3. No significant difference was observed amongst current, ex- and never smokers either for serum AMH (F(2,91) = 1.19, p=0.309) or total AFC (F(2,81) = 0.403, p= 0.670). When comparing baseline variables, age showed borderline non-significance between the groups (p=0.057). Hence, we performed an analysis of covariance (ANCOVA) to explore the impact of smoking status on serum AMH using age as a covariate. No significant difference was demonstrated among the three groups (F(2,90) = 0.398, p = 0.673).

Table 3: Outcomes

	Current smokers (n=12)	Ex-smokers (n=25)	Never smokers (n=64)	p
Serum AMH (pmol/l)	38.9 (20.4-66.2)	26.0 (14.7-32.2)	27.6 (16.4-39.7)	0.309
Total AFC (n)	30.5 (16-41.5)	22.5 (13-30)	21.5 (15-35.5)	0.670

Values presented as median (IQR)

No significant correlation was demonstrated between the pack years of smoking and serum AMH (r= -0.212, n=23, p=0.166) or total AFC (r= -0.276, n=19, p=0.126). No significant correlation was found between breath CO and serum AMH (r= 0.082, n=94, p=0.216) or total AFC (r= 0.096, n=83, p=0.195). Similarly, no significant correlation was found between urine cotinine levels and serum AMH (r= 0.146, n=83, p=0.095) or total AFC (r= -0.027, n=77, p=0.386).

4 Discussion

4.1 Main results

We did not find a statistically significant difference in quantitative ovarian reserve markers serum AMH and AFC between current, ex and never smokers in our study population. By demonstrating significant differences in breath CO and urine cotinine levels among the groups, we confirmed that self-reported smoking correlates well with

quantitatively measured markers of smoking. We were hence able to validate the comparison groups created by a self-reported history to ensure a valid comparison of outcome measures. We were unable to demonstrate a significant correlation between the pack years smoked and serum AMH and AFC. We did not find a significant association between biomarkers of smoking and biomarkers of ovarian reserve.

4.2 Interpretation of results

Biological plausibility exists for the effect of smoking on ovarian reserve and ovarian ageing. Animal studies have suggested adverse effects of cigarette smoking on ovarian reserve (13, 14). Several mechanisms have been postulated, which may affect quality, quantity or both. Gannon et al in 2012 (15) hypothesised a mechanism of direct toxicity to ovarian follicles resulting in an accelerated follicle loss. An indirect effect on ovarian follicle numbers has been suggested through an action on the hypothalamic pituitary axis (16) . These effects are however not evident in our study population of younger women based on serum AMH and AFC. This may be because the natural decline of ovarian reserve with age does not follow a linear function but shows a rapid decline with increasing age(5).It has also been suggested that ovarian follicles may differ in susceptibility to the effects of smoking at different ages with older oocytes being more susceptible to negative effects of smoking.

The effect of smoking may be dose related. The pack years of smoking in our study population was relatively low at 2.13 pack years. It is possible that the deleterious effects are evident only at higher levels of smoking exposure or smoking is associated with smaller magnitude of reduction in ovarian reserve markers. Although it may be possible to demonstrate such small differences with a larger sample size, the clinical implications of such findings would be questionable. Serum AMH and AFC are largely used in young women in the context of fertility treatment, to predict ovarian response to treatment and pregnancy rates. Hence in younger women seeking fertility treatment , a clinically relevant decrease in ovarian reserve may be considered one which significantly reduces the probability of the most important outcome for this group of women; the pregnancy rate. Significantly lower pregnancy rates have been reported in the lowest quartile of AMH below 10.28 pmol/l(12). Pregnancy rates in women with serum AMH in the upper three quartiles are not statistically different from each other.(12). The absence of an association between smoking and serum AMH and AFC also argues for a mechanism against follicular atresia. This is strengthened by

the finding of no association between ex-smokers and lower AMH values in our study and also in other studies such as Dolleman et al(7).

Our results are in agreement with those of Bressler et al, 2016 (9). They were unable to demonstrate an association between smoking exposure and serum AMH in a population based cross-sectional analysis. The age of their study population was women aged 23-35 years which is similar to that of our study. However, exposure ascertainment was done using only a self-reported questionnaire. Similarly, Kline et al in 2016 reported no association between AMH and smoking in a cross-sectional study using self-reported smoking to ascertain exposure. Dolleman et al in 2013 in a large population based study reported lower serum AMH in current smokers but not in ex-smokers as compared to never smokers. The study population was however significantly older (mean 37.3, SD 9.2) than our study population, which may explain a difference in the results. It has been suggested that the increase in follicular decline may be accelerated and more evident with advancing age(16). Also, the smoking exposure in pack years was higher in this population (mean 10.2, SD 9.1) as compared to our study (median 2.13 (IQR 0.59-3.48)) which could account for the differences. Dolleman also reported a threshold after which the linear association of pack years and serum AMH was significant. They reported this at 10 pack years of smoking below which there was no significant association with serum AMH. Hence, these results could be considered to be in agreement with our study.

We have used breath CO and urine cotinine as biomarkers of smoking to validate self -reported smoking history. This is in agreement with previously reported studies. Marrone et al report significantly higher breath CO and cotinine levels in smokers compared with non-smokers ($P<0.001$), with 100% specificity and sensitivity at a concentration of 5ppm(17). Similarly, MacLaren et al reported a strong agreement between self-reported smoking and breath CO levels with a sensitivity of 96% and specificity of 93.3% using a cut off of 7ppm(18).

4.3 Strengths and limitations

A major strength of our study is that we used a comprehensive and detailed self-reported questionnaire to assess smoking exposure, which allowed estimation of lifetime smoking exposure in terms of pack years and also accounted for passive smoking. Furthermore, we also used breath CO and urine cotinine concentrations to

validate our study groups. The CO breath test shows the amount of CO in the breath (ppm), as an indirect, non-invasive measure of blood carboxyhemoglobin (%COHb). CO leaves the body rapidly and the half-life is about 5 hours. Within 24 to 48 hours of not smoking, smokers will be at non-smoker levels. Cotinine is the predominant metabolite of nicotine. It has a half-life of 20 hours and is detectable for up to one week after the use of tobacco. This is useful to identify smokers who have abstained from smoking for several hours.

The participants included an unselected population of women attending the clinic for various investigations and treatments. There were wide variations in the baseline characteristics of participants such as ethnicity, cause of infertility and diagnosis. By using a wide-ranging unselected population of women we have attempted to improve the generalisability of the results.

Age remains a major determinant of ovarian reserve. We have included only women 35 years and younger to reduce bias due to the impact of advancing age. The participants included only sub-fertile women with a limited range of BMI and age. This is because fertility treatment within the UK and funded by the National Health Service is restricted by limits on age and BMI. Therefore, caution should be exercised when extrapolating these results to other populations. Our study was powered only to detect differences in ovarian markers of relatively large magnitude that we considered to have a clinical significance in the management of young women seeking fertility treatment. However, a much larger sample size would be required to detect statistically significant differences of smaller magnitude which may be relevant to different study populations and research questions.

5 Conclusion

We did not find a quantitative change in the antral follicle pool following exposure to cigarette smoking in women ≤35 years seeking fertility treatment. We confirmed that self-reported smoking correlates well with quantitatively measured biomarkers of smoking. There was no significant association between biomarkers of smoking and biomarkers of ovarian reserve. We were also unable to demonstrate a correlation between the lifetime smoking exposure and ovarian reserve parameters

6 Competing interests

None declared.

7 Contribution to authorship

PB: Study concept and design, participant recruitment, data collection, data analysis and interpretation, drafting the article, critical review and final approval.

ET: participant recruitment, data collection, critical review and final approval.

AK: participant recruitment, data collection, critical review and final approval.

AG: critical review and final approval

AS: critical review and final approval

RH: critical review and final approval

GA: Study concept and design, critical review and final approval.

All authors read the manuscript critically, commented on the draft and approved the final version before submission.

8 Details of ethics approval

The study was approved by Health Research Authority and Health and Research Care Wales- Central Research Ethics Committee on 10/Apr/2019. (REC reference: 19/WA/0089)

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Effect of smoking on ovarian reserve parameters, sperm parameters and embryo quality in sub-fertile couples.

We would be grateful if you could complete this short questionnaire.
This information will be confidential and accessed only by the research team.

1) Are you
   - Male
   - Female
2) As regards cigarette smoking, do you consider yourself a
   - Current smoker
   - Ex-smoker
   - Never smoker
3) If you are a current smoker
   - How often do you smoke?
     - Daily
     - 3-6 days in a week
     - 1-2 days a week
     - less than once a week
   - How many cigarettes do you smoke per day?
   - How long have you been smoking?
4) If you are an ex-smoker,
   - When did you stop smoking? (mm/yyyy)
   - How often did you smoke?
     - Daily
     - 3-6 days in a week
     - 1-2 days a week
     - Less than once a week
   - How many cigarettes did you smoke per day?
   - How long had you been smoking before you stopped?
5) Does anyone living/working closely with you smoke in your presence (are you a passive smoker)?
   - Yes
   - No
6) Do you use electronic cigarettes/vaping?
   - Yes
   - No

Thank you for taking part in the study and taking time to complete this questionnaire.

Dr Priya Bhide
Principal investigator

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 Page 1, line 3
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found Page 2-3, lines 23-59
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported page 4, lines 75-97
Objectives	3	State specific objectives, including any prespecified hypotheses Page 4, lines 98-103
Methods		
Study design	4	Present key elements of study design early in the paper Page 5, lines 106-114
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection Page 5, lines 106-114
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants page 5, lines 116-119
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable page 7, line 166
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 Pages 6-7, lines 134-164, 168-176
Bias	9	Describe any efforts to address potential sources of bias Page 11, lines 318-319, Page 7-8, line 189-207
Study size	10	Explain how the study size was arrived at Page 7, lines 178-189
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why Page 7-8, line 189-207
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding Page 7-8, lines 189-207
		(*b*) Describe any methods used to examine subgroups and interactions Page 7-8, line 189-207
		(*c*) Explain how missing data were addressed Page 7-8, line 189-207
		(*d*) If applicable, describe analytical methods taking account of sampling strategy Not applicable
		(*e*) Describe any sensitivity analyses Not applicable
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 Page 8, line 209 and table 1
		(b) Give reasons for non-participation at each stage Not applicable
		(c) Consider use of a flow diagram Not applicable
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders Table 1
		(b) Indicate number of participants with missing data for each variable of interest
Outcome data	15*	Report numbers of outcome events or summary measures Tables 2 and 3
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 Pages 8-9, lines 209-234, Table 2 and 3
		(*b*) Report category boundaries when continuous variables were categorized Not applicable
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period Not applicable
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses Not applicable
Discussion		
Key results	18	Summarise key results with reference to study objectives Page 9, lines 237-245
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 Pages 11-12, lines 302-328
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence Pages 9-11, lines 247-300
Generalisability	21	Discuss the generalisability (external validity) of the study results page 11, lines 313-317
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 page 13, line 354-355

*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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Effect of cigarette smoking on serum anti-Mullerian hormone and antral follicle count in women seeking fertility treatment – a prospective cross-sectional study

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Title

Effect of cigarette smoking on serum anti-Mullerian hormone and antral follicle count in women seeking fertility treatment – a prospective cross-sectional study

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Abstract page

Objectives

The relationship between smoking and ovarian reserve markers is inconclusive. The primary objective of our study was to assess the effect of cigarette smoking on the quantitative ovarian reserve parameters, serum anti-Mullerian hormone (AMH) and antral follicle count (AFC) as relevant to prediction of fertility outcomes in women seeking fertility treatment. Our secondary aims were to validate self-reported smoking behaviour using biomarkers and evaluate the association between biomarkers of ovarian reverse (serum AMH and AFC) with biomarkers of smoking exposure (breath carbon monoxide (CO) and urine cotinine levels).

Design

Prospective, cross-sectional study

Setting

Single tertiary care fertility centre

Participants

Women ≤ 35 years seeking fertility treatment

Primary outcome measures

Serum AMH and AFC

Results

Significant differences were found amongst current smokers, ex-smokers and never smokers for breath CO ($F(2,97)=33.32$, $p< 0.0001$) and urine cotinine levels ($p< 0.001$). However, no significant differences were found either for serum AMH ($F(2,91)=1.19$, $p=0.309$) or total AFC ($F(2,81)=0.403$, $p=0.670$) among the three groups. There was no significant correlation between pack years of smoking and serum AMH ($r=- 0.212$, $n=23$, $p=0.166$) or total AFC ($r=-0.276$, $n=19$, $p=0.126$). No significant correlation was demonstrated between breath CO and serum AMH ($r=0.082$, $n=94$, $p=0.216$) or total AFC ($r=0.096$, $n=83$, $p=0.195$). Similarly, no significant correlation was demonstrated between urine cotinine levels and serum AMH ($r=0.146$, $n=83$, $p=0.095$) or total AFC ($r=-0.027$, $n=77$, $p=0.386$).

Conclusion

We did not find a statistically significant difference in quantitative ovarian reserve markers between current, ex- and never smokers which would be clinically meaningful in our study population. We confirmed that self-reported smoking correlates well with quantitatively measured biomarkers of smoking. This validated the self-reported comparison groups to ensure a valid comparison of outcome measures. There was no significant association between biomarkers of smoking and biomarkers of ovarian reserve. We were also unable to demonstrate a correlation between the lifetime smoking exposure and ovarian reserve.

Strengths and limitations of this study

- We used a comprehensive and detailed self-reported questionnaire to assess smoking exposure.
- We used biomarkers of smoking exposure; breath CO and urine cotinine concentrations to validate our self-reported study groups.
- We recruited an unselected population of women seeking fertility treatment in an attempt to improve generalisability of results.
- We have included only women 35 years and younger to reduce bias due to the impact of advancing age.
- Our study was powered to detect differences in ovarian reserve markers of relatively large magnitude that we considered to have a clinical significance in the management of young women seeking fertility treatment.

1 Introduction

Anti-Mullerian hormone (AMH) and antral follicle count (AFC) are well established biomarkers of ovarian reserve, commonly used in the context of fertility treatment(1, 2). Estimation of the size of the primordial follicle pool is difficult and impractical for routine clinical application as there is no known biochemical marker for estimating the number of primordial follicles, and their small size makes in-vivo imaging with sufficient resolution impossible using currently available technology. A subsection of the true ovarian reserve is the pool of pre-antral and antral follicles which are responsive to pituitary gonadotropins and are clinically relevant for menstruation, ovulation and fertility. The currently available biomarkers, AMH and AFC, measure the antral follicle pool. AMH is expressed exclusively by the granulosa cells of pre-antral and small antral follicles in the ovary and hence an excellent quantitative marker of the ovarian reserve(3). Antral follicle counts assessed by ultrasound scan measure the same biological entity and show a strong positive correlation with serum AMH levels(4).

Age remains one of the most important determinants of ovarian reserve and fertility (5), with a natural decline due to a decrease in the number of oocytes and a reduction in oocyte quality. Additionally, genetic, life-style and environmental factors are also recognised to affect variation in ovarian reserve(5, 6). The relationship between smoking and serum AMH and AFC reported in literature is inconsistent. Some studies suggest that smoking may negatively impact the ovarian reserve(7, 8), whereas the others have failed to corroborate this association(9).Differences in ascertainment of cigarette smoking exposure, potential inaccuracies in self-reported smoking history and selection biases in studies may have led to discrepancies in the results. The role of passive smoking has also not been well investigated.

Thus, the primary objective of our study was to assess the effect of cigarette smoking on the quantitative ovarian reserve parameters, AMH and AFC. Our secondary aims were to validate self-reported smoking behaviour using biomarkers and evaluate the association between biomarkers of ovarian reverse (serum AMH and AFC) with biomarkers of smoking exposure (i.e. breath carbon monoxide and urine cotinine levels).

2 Materials and methods

2.1 Study design, setting and population

We conducted a single-centre prospective cross-sectional study from July 2019 to February 2020. The study population comprised of couples referred to the fertility centre for investigations and treatment of subfertility. We compared the levels of serum AMH and AFC among current smokers, ex-smokers and never-smokers based on a self-reported smoking history and validated by the measurements of breath carbon monoxide (CO) and urine cotinine levels. We also explored the association between biomarkers of ovarian reserve (AMH and AFC) and biomarkers of smoking (breath CO and urine cotinine) and correlated the lifetime smoking exposure quantified as "pack years" with levels of serum AMH and AFC.

2.2 Patient and public involvement

The study question and design were discussed with patients attending the fertility clinic who agreed that the research question was important and the outcomes appropriate. Patients helped with design and language of the participant information leaflets and questionnaires. Patients were not involved in recruitment or conduct of the study. We plan to involve patients in dissemination of findings through patient networks such as the East London Katherine Twining Network.

2.3 Inclusion and exclusion criteria

We included women aged ≤35 years attending the fertility unit for investigations and treatment. We excluded women on long term oral contraceptive pills or GnRH analogues, those not having both ovaries and with a history of previous chemotherapy, abdominal/pelvic radiotherapy or major ovarian surgery.

2.4 Study procedures, screening, consent, care pathway, study intervention, laboratory procedures

We screened and invited eligible participants to participate in the study. Following informed consent we assessed the participants for markers of smoking. This included a short self-reported questionnaire about the participant's current and past smoking history, a non-invasive breath test to detect the levels of carbon monoxide and a urine test to detect the levels of cotinine. Based on the smoking history we classified

participants into one of three categories; current smokers, ex-smokers and never-smokers. The smoking history also accounted for passive smokers and smoking details aimed to quantify the smoking exposure in terms of “pack years”. We measured serum AMH and AFC as a part of the standard fertility work up done for all fertility patients. We also collected baseline demographic and clinical data for confounding variables. We followed up all participants for the results of their tests.

2.5 Products, devices, techniques and tools

A bespoke questionnaire was used to obtain self-reported smoking history. This was designed with the input of clinical and research members of the team to ensure content validity and reliability. The questionnaire was tested on a pilot sample of the target population. This highlighted deficiencies and allowed improvements in the final questionnaire used. The questionnaire details are provided in Appendix S1.

The device used to measure the breath CO (Smokelyser) is a CE marked, commercially available, non-invasive CO breath test that uses an electrochemical sensor to measure the breath concentration of CO with a concentration range of 0-150 ppm with a sensor sensitivity of 1 ppm and an accuracy of ±2 ppm. The instrument was used within the specified warranty period and used and serviced according to manufacturer’s specifications.

The urine cotinine was measured using the DRI®Cotinine assay (Thermo Fisher Scientific). The DRI® Cotinine Assay is an in vitro diagnostic medical device intended for the qualitative and semi-quantitative determination of cotinine in human urine at a cut off level of 500 ng/mL. The accuracy of the assay has been confirmed by gas chromatography /mass spectrometry. According to manufacturer, the sensitivity, defined as the lowest concentration that can be differentiated from the negative urine calibrator with 95% confidence, is 34 ng/mL.

All serum AMH assays were performed in an on-site clinical laboratory using the bench-top fully automated assay Access 2 immunoassay system (Beckman-Coulter) and values were expressed as pmol/l. Inter-assay coefficients of variation for a low and high control were 0.056 and 0.44, respectively. Venous blood samples were obtained and delivered to the laboratory immediately, centrifuged, and stored at 2-8°C, and analysed every day.

Ultrasound imaging of ovaries was performed using a Voluson S10 diagnostic ultrasound system (GE Healthcare) equipped with a multi-frequency transvaginal probe (RIC5-9W-RS: 9-5MHz) to visualize antral follicles systematically. AFC was obtained automatically using the sono-AVC™ software. Manual image post-processing was done if required. A total AFC was calculated as the sum of total number of follicles between 2-9 mm on each ovary. This measurement was not restricted to a particular time of the cycle.

2.6 Outcome measures

The primary outcome measures were serum AMH and total AFC.

2.7 Data collection

Data were recorded onto study specific paper Case Report Forms (CRFs) and subsequently transferred to a study database. We collected baseline demographic characteristics of the study population (age, ethnicity), baseline clinical data (BMI, presence of PCO/PCOS, history of ovarian surgery), data for on smoking parameters (type of smoker, passive smoking, smoking in pack years, breath CO and urine cotinine levels) and data for primary outcomes (serum AMH, AFC).

Data for smoking parameters were collected by members of the research team directly from the participant. All other data were collected from the participants' medical records and electronic hospital records.

2.8 Statistical considerations, sample size, analysis

The sample size calculation was based upon the primary outcome of serum AMH. Approximately 13% of women in the UK are current smokers (10) and the number of ex-smokers exceeds that of smokers. The proportion of never smokers in the UK population is increasing and reported at 59% in 2014 (11). Hence we estimated that at the fertility clinic approximately one third of our population would be either smokers or ex-smokers. We have previously found the mean serum AMH to be 28.28 pmol/l and a significantly lower pregnancy rates among women in the lowest quartile of AMH, i.e. below 10.28 pmol/l.(12) To detect an absolute decrease in AMH from 28.28 to 10.28 pmol/l with 80% power at a 5% significance level with an enrolment ratio of 0.5, we would require 96 participants (32 smokers/ex-smokers and 64 non-smokers). We planned to recruit approximately 100 participants to compensate for dropout and loss

to follow up. Appropriate descriptive statistics were used to describe the baseline variables in the dataset. Normality of data was checked using Shapiro-Wilk test and skewed data were log transformed to achieve normal distribution before using parametric test. Nonparametric tests were used for data analysis if normal distribution was not achieved. An one-way between-groups analysis of variance (ANOVA), a Chi-squared test or a Kruskal-Wallis test were used to assess differences between baseline variables and smoking markers between current smokers, ex -smokers and never smokers. An ANOVA was used to assess differences in outcome variables between the three study groups. When the P-value was <.05, the difference was considered statistically significant. When a difference was found to be significant, a post-hoc Tukey multiple comparison test was performed. A one-way between-groups analysis of co-variance (ANCOVA) was performed to assess the differences between groups taking into account the variability of other confounding variables. Differences in breath CO concentrations and urine cotinine levels in the three comparison groups were used to validate group stratification and the results for the primary outcome variables. Pearsons correlation test was used to explore the relationship between lifetime exposure to smoking (pack years), breath CO or urine cotinine and outcome variables. Statistical analysis was done using the Statistical Package for Social Sciences (SPSS version 26).

3 Results

101 women were recruited to the study over a period of nine months. Based on a self-reported smoking history women were classified into three comparison groups: current smokers, ex-smokers and never smokers. We included 12 smokers, 25 ex-smokers and 64 non-smokers to the study. The baseline clinical characteristics of the participants are summarised in Table 1. The median age (IQR) for the three groups was 30 (25.5-33.0), 32.5 (31.0-33.5) and 31 (28.0- 33.0). There were no significant differences in the other baseline variables amongst the three groups.

Table 1: Baseline variables

	Current smokers (n=12)	Ex-smokers (n=25)	Never smokers (n=64)	p
Age (years)	30 (25.5-33.0)	32.5 (31.0-33.5)	31.0 (28.0-33.0)	0.057

BMI	23.2 (21.8-26.2)	25.3 (20.8-28.3)	25.1 (22.1-27.8)	0.632
Ethnicity				0.208
White European	8	21	35	
Asian	2	4	16	
Afro-Caribbean	1	0	8	
Others	1	0	5	
Category of infertility				0.077
Anovulatory	4	1	11	
Male	4	5	14	
Tubal	2	0	9	
Unexplained	1	14	20	
Other	0	4	4	
Ovarian surgery				0.659
No	12	23	60	
Yes	0	1	1	
PCOS/PCOM				0.351
N	7	17	42	
Y	5	4	20	

Values expressed as median (IQR) or n

The smoking markers for the three groups are detailed in table 2. The pack years of smoking, quantifying exposure to cigarette smoking, were not significantly different between current and ex-smokers ($F(1,25) = 0.547$, $p=0.467$). The breath CO levels were significantly different amongst current, ex- and never smokers ($F(2,97) = 33.32$, $p< 0.0001$). Urine cotinine levels were also significantly higher in current smokers as compared to ex-smokers and never smokers. ($p< 0.001$). Current smokers reported to be more exposed to passive smoking (75%, 9/12) as compared to ex-smokers (20%, 5/25) and never smokers (25%, 16/64) ($p=0.001$).

Table 2: Smoking markers

	Current smokers (n=12)	Ex-smokers (n=25)	Never smokers (n=64)	p

Pack years of smoking	2.13 (0.59-3.48)	2.13 (0.05-5.40)	0.00 (0.00-0.00)	0.467*
Breath CO (ppm)	9 (3.5-21)	2 (2-3)	1 (1-2)	<0.001
Urine Cotinine (ng/ml)	837 (22.42 – 1571.8)	22.42 (22.42-22.42)	22.42 (22.42-22.42)	<0.001

comparison between current and ex-smokers only. Values presented as median (IQR)

The primary outcomes are detailed in Table 3. No significant difference was observed amongst current, ex- and never smokers either for serum AMH (F(2,91) = 1.19, p=0.309) or total AFC (F(2,81) = 0.403, p= 0.670). When comparing baseline variables, age showed borderline non-significance between the groups (p=0.057). Hence, we performed an analysis of covariance (ANCOVA) to explore the impact of smoking status on serum AMH using age as a covariate. No significant difference was demonstrated among the three groups (F(2,90) = 0.398, p = 0.673).

Table 3: Outcomes

	Current smokers (n=12)	Ex-smokers (n=25)	Never smokers (n=64)	p
Serum AMH (pmol/l)	38.9 (20.4-66.2)	26.0 (14.7-32.2)	27.6 (16.4-39.7)	0.309
Total AFC (n)	30.5 (16-41.5)	22.5 (13-30)	21.5 (15-35.5)	0.670

Values presented as median (IQR)

No significant correlation was demonstrated between the pack years of smoking and serum AMH (r= -0.212, n=23, p=0.166) or total AFC (r= -0.276, n=19, p=0.126). No significant correlation was found between breath CO and serum AMH (r= 0.082, n=94, p=0.216) or total AFC (r= 0.096, n=83, p=0.195). Similarly, no significant correlation was found between urine cotinine levels and serum AMH (r= 0.146, n=83, p=0.095) or total AFC (r= -0.027, n=77, p=0.386).

4 Discussion

4.1 Main results

We did not find a statistically significant difference in quantitative ovarian reserve markers serum AMH and AFC between current, ex and never smokers in our study

population. By demonstrating significant differences in breath CO and urine cotinine levels among the groups, we confirmed that self-reported smoking correlates well with quantitatively measured markers of smoking. We were hence able to validate the comparison groups created by a self-reported history to ensure a valid comparison of outcome measures. We were unable to demonstrate a significant correlation between the pack years smoked and serum AMH and AFC. We did not find a significant association between biomarkers of smoking and biomarkers of ovarian reserve.

4.2 Interpretation of results

Biological plausibility exists for the effect of smoking on ovarian reserve and ovarian ageing. Animal studies have suggested adverse effects of cigarette smoking on ovarian reserve (13, 14). Several mechanisms have been postulated, which may affect quality, quantity or both. Gannon et al in 2012 (15) hypothesised a mechanism of direct toxicity to ovarian follicles resulting in an accelerated follicle loss. An indirect effect on ovarian follicle numbers has been suggested through an action on the hypothalamic pituitary axis (16) . These effects are however not evident in our study population of younger women based on serum AMH and AFC. This may be because the natural decline of ovarian reserve with age does not follow a linear function but shows a rapid decline with increasing age(5).It has also been suggested that ovarian follicles may differ in susceptibility to the effects of smoking at different ages with older oocytes being more susceptible to negative effects of smoking.

The effect of smoking may be dose related. The pack years of smoking in our study population was relatively low at 2.13 pack years. It is possible that the deleterious effects are evident only at higher levels of smoking exposure or smoking is associated with smaller magnitude of reduction in ovarian reserve markers. Although it may be possible to demonstrate such small differences with a larger sample size, the clinical implications of such findings would be questionable. Serum AMH and AFC are largely used in young women in the context of fertility treatment, to predict ovarian response to treatment and pregnancy rates. Hence in younger women seeking fertility treatment , a clinically relevant decrease in ovarian reserve may be considered one which significantly reduces the probability of the most important outcome for this group of women; the pregnancy rate. Significantly lower pregnancy rates have been reported in the lowest quartile of AMH below 10.28 pmol/l(12). Pregnancy rates in women with serum AMH in the upper three quartiles are not statistically different from each

other.(12). The absence of an association between smoking and serum AMH and AFC also argues for a mechanism against follicular atresia. This is strengthened by the finding of no association between ex-smokers and lower AMH values in our study and also in other studies such as Dolleman et al(7).

Our results are in agreement with those of Bressler et al, 2016 (9). They were unable to demonstrate an association between smoking exposure and serum AMH in a population based cross-sectional analysis. The age of their study population was women aged 23-35 years which is similar to that of our study. However, exposure ascertainment was done using only a self-reported questionnaire. Similarly, Kline et al in 2016 reported no association between AMH and smoking in a cross-sectional study using self-reported smoking to ascertain exposure(17). Dolleman et al in 2013 in a large population based study reported lower serum AMH in current smokers but not in ex-smokers as compared to never smokers(7). The study population was however significantly older (mean 37.3, SD 9.2) than our study population, which may explain a difference in the results. It has been suggested that the increase in follicular decline may be accelerated and more evident with advancing age(16). Also, the smoking exposure in pack years was higher in this population (mean 10.2, SD 9.1) as compared to our study (median 2.13 (IQR 0.59-3.48)) which could account for the differences. Dolleman also reported a threshold after which the linear association of pack years and serum AMH was significant. They reported this at 10 pack years of smoking below which there was no significant association with serum AMH. Hence, these results could be considered to be in agreement with our study.

We have used breath CO and urine cotinine as biomarkers of smoking to validate self -reported smoking history. This is in agreement with previously reported studies. Marrone et al report significantly higher breath CO and cotinine levels in smokers compared with non-smokers (P<0.001), with 100% specificity and sensitivity at a concentration of 5ppm(18). Similarly, MacLaren et al reported a strong agreement between self-reported smoking and breath CO levels with a sensitivity of 96% and specificity of 93.3% using a cut off of 7ppm(19).

4.3 Strengths and limitations

A major strength of our study is that we used a comprehensive and detailed self-reported questionnaire to assess smoking exposure, which allowed estimation of

lifetime smoking exposure in terms of pack years and also accounted for passive smoking. Furthermore, we also used breath CO and urine cotinine concentrations to validate our study groups. The CO breath test shows the amount of CO in the breath (ppm), as an indirect, non-invasive measure of blood carboxyhemoglobin (%COHb). CO leaves the body rapidly and the half-life is about 5 hours. Within 24 to 48 hours of not smoking, smokers will be at non-smoker levels. Cotinine is the predominant metabolite of nicotine. It has a half-life of 20 hours and is detectable for up to one week after the use of tobacco. This is useful to identify smokers who have abstained from smoking for several hours.

The participants included an unselected population of women attending the clinic for various investigations and treatments. There were wide variations in the baseline characteristics of participants such as ethnicity, cause of infertility and diagnosis. By using a wide-ranging unselected population of women we have attempted to improve the generalisability of the results.

Age remains a major determinant of ovarian reserve. We have included only women 35 years and younger to reduce bias due to the impact of advancing age. The participants included only sub-fertile women with a limited range of BMI and age. This is because fertility treatment within the UK and funded by the National Health Service is restricted by limits on age and BMI. Therefore, caution should be exercised when extrapolating these results to other populations. Pregnancy rates following assisted reproduction treatments are influenced primarily by age but also indirectly by the number of eggs. Serum AMH and AFC are excellent predictors for the number of eggs retrieved, and in young women < 35 years only a large decrease in quantitative reserve would significantly impact pregnancy rates(20). Our study was hence powered only to detect differences in ovarian markers of relatively large magnitude that we considered to have a clinical significance in the management of young women seeking fertility treatment. A much larger sample size would be be able to detect statistically significant differences of smaller magnitude which may be relevant to different study populations and research questions but clinically less meaningful for fertility.

5 Conclusion

We did not find a significant quantitative change in the antral follicle pool following exposure to cigarette smoking in women ≤35 years seeking fertility treatment. We

confirmed that self-reported smoking correlates well with quantitatively measured biomarkers of smoking. There was no significant association between biomarkers of smoking and biomarkers of ovarian reserve. We were also unable to demonstrate a correlation between the lifetime smoking exposure and ovarian reserve parameters

6 Competing interests

None declared.

7 Contribution to authorship

PB: Study concept and design, participant recruitment, data collection, data analysis and interpretation, drafting the article, critical review and final approval.

ET: participant recruitment, data collection, critical review and final approval.

AK: participant recruitment, data collection, critical review and final approval.

AG: critical review and final approval

AS: critical review and final approval

RH: critical review and final approval

GA: Study concept and design, critical review and final approval.

All authors read the manuscript critically, commented on the draft and approved the final version before submission.

8 Details of ethics approval

The study was approved by Health Research Authority and Health and Research Care Wales- Central Research Ethics Committee on 10/Apr/2019. (REC reference: 19/WA/0089)

9 Funding

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

10 Data availability statement

The fully anonymized study data can be made available to interested researchers on request to the corresponding author.

11 Acknowledgements

We would like to thank all our patient advisors for their contributions to this study.

12 References

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UIN: ☐☐☐

Effect of smoking on ovarian reserve parameters, sperm parameters and embryo quality in sub-fertile couples.

We would be grateful if you could complete this short questionnaire.
This information will be confidential and accessed only by the research team.

1) Are you
   - Male
   - Female
2) As regards cigarette smoking, do you consider yourself a
   - Current smoker
   - Ex-smoker
   - Never smoker
3) If you are a current smoker
   - How often do you smoke?
     - Daily
     - 3-6 days in a week
     - 1-2 days a week
     - less than once a week
   - How many cigarettes do you smoke per day?
   - How long have you been smoking?
4) If you are an ex-smoker,
   - When did you stop smoking? (mm/yyyy)
   - How often did you smoke?
     - Daily
     - 3-6 days in a week
     - 1-2 days a week
     - Less than once a week
   - How many cigarettes did you smoke per day?
   - How long had you been smoking before you stopped?
5) Does anyone living/working closely with you smoke in your presence (are you a passive smoker)?
   - Yes
   - No
6) Do you use electronic cigarettes/vaping?
   - Yes
   - No

Thank you for taking part in the study and taking time to complete this questionnaire.

Dr Priya Bhide
Principal investigator

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 Page 1, line 3
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found Page 2-3, lines 23-59
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported page 4, lines 75-97
Objectives	3	State specific objectives, including any prespecified hypotheses Page 4, lines 98-103
Methods		
Study design	4	Present key elements of study design early in the paper Page 5, lines 106-114
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection Page 5, lines 106-114
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants page 5, lines 116-119
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable page 7, line 166
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 Pages 6-7, lines 134-164, 168-176
Bias	9	Describe any efforts to address potential sources of bias Page 11, lines 318-319, Page 7-8, line 189-207
Study size	10	Explain how the study size was arrived at Page 7, lines 178-189
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why Page 7-8, line 189-207
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding Page 7-8, lines 189-207
		(*b*) Describe any methods used to examine subgroups and interactions Page 7-8, line 189-207
		(*c*) Explain how missing data were addressed Page 7-8, line 189-207
		(*d*) If applicable, describe analytical methods taking account of sampling strategy Not applicable
		(*e*) Describe any sensitivity analyses Not applicable
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 Page 8, line 209 and table 1
		(b) Give reasons for non-participation at each stage Not applicable
		(c) Consider use of a flow diagram Not applicable
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders Table 1
		(b) Indicate number of participants with missing data for each variable of interest
Outcome data	15*	Report numbers of outcome events or summary measures Tables 2 and 3
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 Pages 8-9, lines 209-234, Table 2 and 3
		(*b*) Report category boundaries when continuous variables were categorized Not applicable
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period Not applicable
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses Not applicable
Discussion		
Key results	18	Summarise key results with reference to study objectives Page 9, lines 237-245
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 Pages 11-12, lines 302-328
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence Pages 9-11, lines 247-300
Generalisability	21	Discuss the generalisability (external validity) of the study results page 11, lines 313-317
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 page 13, line 354-355

*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.