


BMJ Open 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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To cite: Bhide P, Timlick E, Kulkarni A, *et al.* Effect of cigarette smoking on serum anti-Mullerian hormone and antral follicle count in women seeking fertility treatment: a prospective cross-sectional study. *BMJ Open* 2022;**12**:e049646. doi:10.1136/bmjopen-2021-049646

► Prepublication history and additional supplemental material for this paper are available online. To view these files, please visit the journal online (<http://dx.doi.org/10.1136/bmjopen-2021-049646>).

Received 31 January 2021
Accepted 18 February 2022



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ABSTRACT

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 reserve (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 among 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 smokers, ex-smokers 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

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 carbon monoxide 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.

to demonstrate a correlation between the lifetime smoking exposure and ovarian reserve.

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 AFCs 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, lifestyle 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 reserve (serum AMH and AFC) with biomarkers of smoking exposure (ie, breath carbon monoxide (CO) and urine cotinine levels).

MATERIALS AND METHODS

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 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.

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.

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 contraceptives or Gonadotropin Releasing Hormone (GnRH) analogues, those not having both ovaries and with a history of chemotherapy, abdominal/pelvic radiotherapy or major ovarian surgery.

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 smoking history, a non-invasive breath test to detect the levels of CO 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.

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 online supplemental appendix 1.

The device used to measure the breath CO (Smoke-lyser) 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 semiquantitative 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. Interassay 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°C–8°C, and analysed every day.

Ultrasound imaging of ovaries was performed using a Voluson S10 diagnostic ultrasound system (GE Healthcare) equipped with a multifrequency transvaginal probe (RIC5-9W-RS: 9–5 MHz) to visualise 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 and 9 mm on each ovary. This measurement was not restricted to a particular time of the cycle.

Outcome measures

The primary outcome measures were serum AMH and total AFC.

Data collection

Data were recorded onto study-specific paper Case Report Forms and subsequently transferred to a study database. We collected baseline demographic characteristics of the study population (age, ethnicity), baseline clinical data (body mass index (BMI), presence of polycystic ovaries (PCO)/polycystic ovary syndrome (PCOS), history of ovarian surgery), data 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.

Statistical considerations, sample size, analysis

The sample size calculation was based on the primary outcome of serum AMH. Approximately 13% of women in the UK are current smokers¹⁰ 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, that is, 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. Non-parametric tests

were used for data analysis if normal distribution was not achieved. A one-way between-groups analysis of variance (ANOVA), a X^2 test or a Kruskal-Wallis test was 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 <0.05, 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 covariance (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. Pearson's 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 SPSS (V.26).

RESULTS

One hundred one women were recruited to the study over a period of 9 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 ages (IQR) for the three groups were 30 (25.5–33.0), 32.5 (31.0–33.5) and 31 (28.0–33.0) years. There were no significant differences in the other baseline variables among the three groups.

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 among current smokers, ex-smokers and never smokers ($F(2,97)=33.32$, $p<0.0001$). Urine cotinine levels were also significantly higher in current smokers as compared with ex-smokers and never smokers ($p<0.001$). Current smokers reported to be more exposed to passive smoking (75%, 9 of 12) as compared with ex-smokers (20%, 5 of 25) and never smokers (25%, 16 of 64) ($p=0.001$).

The primary outcomes are detailed in table 3. No significant difference was observed among current smokers, ex-smokers 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 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 1 Baseline variables

	Current smokers (n=12)	Ex-smokers (n=25)	Never smokers (n=64)	P value
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
No	7	17	42	
Yes	5	4	20	

Values expressed as median (IQR) or n.

BMI, body mass index; PCOM, polycystic ovarian morphology; PCOS, polycystic ovary syndrome.

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 ($r=-0.027$, $n=77$, $p=0.386$).

DISCUSSION

Main results

We did not find a statistically significant difference in quantitative ovarian reserve markers serum AMH and AFC between current smokers, ex-smokers 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.

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*¹⁵

Table 2 Smoking markers

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

Values presented as median (IQR).

*Comparison between current and ex-smokers only.

CO, carbon monoxide.

Table 3 Outcomes

	Current smokers (n=12)	Ex-smokers (n=25)	Never smokers (n=64)	P value
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).

AFC, antral follicle count; AMH, anti-Müllerian hormone.

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 Hawkins Bressler *et al.*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* reported no association between AMH and smoking in a cross-sectional study using self-reported smoking to ascertain exposure.¹⁷ Dolleman *et al*, in a large population-based study, reported lower serum AMH in current smokers but not in ex-smokers as compared with 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 with our study (median 2.13 (IQR 0.59–3.48)) which could account for the differences. Dolleman *et al* 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 5 ppm.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 7 ppm.¹⁹

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 carboxyhaemoglobin. CO leaves the body rapidly and the half-life is about 5 hours. Within 24–48 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 1 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 subfertile 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 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.

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.

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

Contributors 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. PB is responsible for the overall content as the guarantor.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests None declared.

Patient consent for publication Not required.

Ethics approval This study involves human participants and was approved by the Health Research Authority and Health and Research Care Wales-Central Research Ethics Committee on 10 April 2019 (REC reference: 19/WA/0089). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request.

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