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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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Complete List of Authors:	Bhide, Priya; UiT The Arctic University of Norway Faculty of Health Sciences, Faculty of Health Sciences; Homerton University Hospital, Timlick, Elizabeth; Homerton University Hospital, Kulkarni, Abhijit; Homerton University Hospital, Gudi, Anil; Homerton University Hospital, Shah, Amit; Homerton University Hospital, Homburg, Roy; Homerton University Hospital, Acharya, Ganesh; UiT The Arctic University of Norway Faculty of Health Sciences, Faculty of Health Sciences; Karolinska Institute
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4 1 Title

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6 2 Effect of cigarette smoking on serum anti-Mullerian hormone and antral follicle count
7
8 3 in women seeking fertility treatment – a prospective cross-sectional study
9

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12 5 P Bhide^{a,b}, E Timlick^b, A Kulkarni^b, A Gudi^b, A Shah^b, R Homburg^b, G Acharya^{a,c}

13
14
15 6 ^aWomen's Health and Perinatology Research Group, Department of Clinical Medicine,
16 7 Faculty of Health Sciences, University of Tromsø, Tromsø, Norway.

17
18
19 8 ^b Homerton Fertility Centre, Homerton University Hospital NHS Foundation Trust,
20 9 London, UK.

21
22
23 10 ^c Department of Clinical Science, Intervention and Technology, Karolinska Institute,
24 11 Stockholm, Sweden.
25
26
27 12

28
29 13 Corresponding author

30
31 14 Dr. Priya Bhide

32
33 15 Homerton Fertility Centre,

34
35 16 Homerton University Hospital NHS Foundation Trust,

36
37 17 London E9 6SR, UK.

38
39 18 Tel: +44 2085107660

40
41 19 Email: priya.bhide@nhs.net

42
43 20 ORCID ID: <https://orcid.org/0000-0003-0871-6508>
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22 Abstract page

23 Objectives

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

32 Design

33 Prospective, cross-sectional study

34 Setting

35 Single tertiary care centre

36 Participants

37 Women \leq 35 years seeking fertility treatment

38 Primary outcome measures

39 Serum AMH and AFC

40 Results

41 Significant differences were found amongst current smokers, ex-smokers and never
42 smokers for breath CO ($F(2,97)=33.32$, $p < 0.0001$) and urine cotinine levels ($p <$
43 0.001). However, no significant differences were found either for serum AMH
44 ($F(2,91)=1.19$, $p=0.309$) or total AFC ($F(2,81)=0.403$, $p=0.670$) among the three
45 groups. There was no significant correlation between pack years of smoking and
46 serum AMH ($r=-0.212$, $n=23$, $p=0.166$) or total AFC ($r=-0.276$, $n=19$, $p=0.126$). No
47 significant correlation was demonstrated between breath CO and serum AMH
48 ($r=0.082$, $n=94$, $p=0.216$) or total AFC ($r=0.096$, $n=83$, $p=0.195$). Similarly, no
49 significant correlation was demonstrated between urine cotinine levels and serum
50 AMH ($r=0.146$, $n=83$, $p=0.095$) or total AFC ($r=-0.027$, $n=77$, $p=0.386$).

51 Conclusion

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

60 Strengths and limitations of this study

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

72

73

74 1 Introduction

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

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

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

104 2 Materials and methods

105 2.1 Study design, setting and population

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

115 2.2 Patient and public involvement

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

122 2.3 Inclusion and exclusion criteria

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

127 2.4 Study procedures, screening, consent, care pathway, study intervention, 128 laboratory procedures

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

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2
3 134 participants into one of three categories; current smokers, ex-smokers and never-
4
5 135 smokers. The smoking history also accounted for passive smokers and smoking
6
7 136 details aimed to quantify the smoking exposure in terms of “pack years”. We measured
8
9 137 serum AMH and AFC as a part of the standard fertility work up done for all fertility
10
11 138 patients. We also collected baseline demographic and clinical data for confounding
12
13 139 variables. We followed up all participants for the results of their tests.

14 2.5 Products, devices, techniques and tools

15
16 141 A bespoke questionnaire was used to obtain self-reported smoking history. This was
17
18 142 designed with the input of clinical and research members of the team to ensure content
19
20 143 validity and reliability. The questionnaire was tested on a pilot sample of the target
21
22 144 population. This highlighted deficiencies and allowed improvements in the final
23
24 145 questionnaire used. The questionnaire details are provided in Appendix S1.

25
26 146 The device used to measure the breath CO (Smokelyser) is a CE marked,
27
28 147 commercially available, non-invasive CO breath test that uses an electrochemical
29
30 148 sensor to measure the breath concentration of CO with a concentration range of 0-
31
32 149 150 ppm with a sensor sensitivity of 1 ppm and an accuracy of ± 2 ppm. The instrument
33
34 150 was used within the specified warranty period and used and serviced according to
35
36 151 manufacturer’s specifications.

37
38 152 The urine cotinine was measured using the DRI®Cotinine assay (Thermo Fisher
39
40 153 Scientific). The DRI® Cotinine Assay is an in vitro diagnostic medical device intended
41
42 154 for the qualitative and semi-quantitative determination of cotinine in human urine at a
43
44 155 cut off level of 500 ng/mL. The accuracy of the assay has been confirmed by gas
45
46 156 chromatography /mass spectrometry. According to manufacturer, the sensitivity,
47
48 157 defined as the lowest concentration that can be differentiated from the negative urine
49
50 158 calibrator with 95% confidence, is 34 ng/mL.

51
52 159 All serum AMH assays were performed in an on-site clinical laboratory using the
53
54 160 bench-top fully automated assay Access 2 immunoassay system (Beckman-Coulter)
55
56 161 and values were expressed as pmol/l. Inter-assay coefficients of variation for a low
57
58 162 and high control were 0.056 and 0.44, respectively. Venous blood samples were
59
60 163 obtained and delivered to the laboratory immediately, centrifuged, and stored at 2-
164 8°C, and analysed every day.

1
2
3 165 Ultrasound imaging of ovaries was performed using a Voluson S10 diagnostic
4 166 ultrasound system (GE Healthcare) equipped with a multi-frequency transvaginal
5 167 probe (RIC5-9W-RS: 9-5MHz) to visualize antral follicles systematically. AFC was
6 168 obtained automatically using the sono-AVC™ software. Manual image post-
7 169 processing was done if required. A total AFC was calculated as the sum of total
8 170 number of follicles between 2-9 mm on each ovary. This measurement was not
9 171 restricted to a particular time of the cycle.

16 172 2.6 Outcome measures

17 173 The primary outcome measures were serum AMH and total AFC.

20 174 2.7 Data collection

21 175 Data were recorded onto study specific paper Case Report Forms (CRFs) and
22 176 subsequently transferred to a study database. We collected baseline demographic
23 177 characteristics of the study population (age, ethnicity), baseline clinical data (BMI,
24 178 presence of PCO/PCOS, history of ovarian surgery), data for on smoking parameters
25 179 (type of smoker, passive smoking, smoking in pack years, breath CO and urine
26 180 cotinine levels) and data for primary outcomes (serum AMH, AFC).

27 181 Data for smoking parameters were collected by members of the research team directly
28 182 from the participant. All other data were collected from the participants' medical
29 183 records and electronic hospital records.

32 184 2.8 Statistical considerations, sample size, analysis

33 185 The sample size calculation was based upon the primary outcome of serum AMH.
34 186 Approximately 13% of women in the UK are current smokers (10) and the number of
35 187 ex-smokers exceeds that of smokers. The proportion of never smokers in the UK
36 188 population is increasing and reported at 59% in 2014 (11). Hence we estimated that
37 189 at the fertility clinic approximately one third of our population would be either smokers
38 190 or ex-smokers. We have previously found the mean serum AMH to be 28.28 pmol/l
39 191 and a significantly lower pregnancy rates among women in the lowest quartile of AMH,
40 192 i.e. below 10.28 pmol/l.(12) To detect an absolute decrease in AMH from 28.28 to
41 193 10.28 pmol/l with 80% power at a 5% significance level with an enrolment ratio of 0.5,
42 194 we would require 96 participants (32 smokers/ex-smokers and 64 non-smokers). We
43 195 planned to recruit approximately 100 participants to compensate for dropout and loss

1
2
3 196 to follow up. Appropriate descriptive statistics were used to describe the baseline
4
5 197 variables in the dataset. Normality of data was checked using Shapiro-Wilk test and
6
7 198 skewed data were log transformed to achieve normal distribution before using
8
9 199 parametric test. Nonparametric tests were used for data analysis if normal distribution
10
11 200 was not achieved. An one-way between-groups analysis of variance (ANOVA), a Chi-
12
13 201 squared test or a Kruskal-Wallis test were used to assess differences between
14
15 202 baseline variables and smoking markers between current smokers, ex -smokers and
16
17 203 never smokers. An ANOVA was used to assess differences in outcome variables
18
19 204 between the three study groups. When the P-value was <.05, the difference was
20
21 205 considered statistically significant. When a difference was found to be significant, a
22
23 206 post-hoc Tukey multiple comparison test was performed. A one-way between-groups
24
25 207 analysis of co-variance (ANCOVA) was performed to assess the differences between
26
27 208 groups taking into account the variability of other confounding variables. Differences
28
29 209 in breath CO concentrations and urine cotinine levels in the three comparison groups
30
31 210 were used to validate group stratification and the results for the primary outcome
32
33 211 variables. Pearsons correlation test was used to explore the relationship between
34
35 212 lifetime exposure to smoking (pack years), breath CO or urine cotinine and outcome
36
37 213 variables. Statistical analysis was done using the Statistical Package for Social
38
39 214 Sciences (SPSS version 26).

215 3 Results

216 101 women were recruited to the study over a period of nine months. Based on a self-
217
218 reported smoking history women were classified into three comparison groups: current
219
220 smokers, ex-smokers and never smokers. The baseline clinical characteristics of the
221
222 participants are summarised in Table 1. There were no significant differences in the
223
224 baseline variables amongst the three groups.

225 **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	

222 Values expressed as median (IQR) or n

223

224 The smoking markers for the three groups are detailed in table 2. The pack years of
 225 smoking, quantifying exposure to cigarette smoking, were not significantly different
 226 between current and ex-smokers ($F(1,25) = 0.547$, $p=0.467$). The breath CO levels
 227 were significantly different amongst current, ex- and never smokers ($F(2,97) = 33.32$,
 228 $p < 0.0001$). Urine cotinine levels were also significantly higher in current smokers as
 229 compared to ex-smokers and never smokers. ($p < 0.001$). Current smokers reported
 230 to be more exposed to passive smoking (75%, 9/12) as compared to ex-smokers
 231 (20%, 5/25) and never smokers (25%, 16/64) ($p=0.001$).

232 **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

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

234 The primary outcomes are detailed in Table 3. No significant difference was observed
 235 amongst current, ex- and never smokers either for serum AMH ($F(2,91) = 1.19$,
 236 $p=0.309$) or total AFC ($F(2,81) = 0.403$, $p= 0.670$). When comparing baseline
 237 variables, age showed borderline non-significance between the groups ($p=0.057$).
 238 Hence, we performed an analysis of covariance (ANCOVA) to explore the impact of
 239 smoking status on serum AMH using age as a covariate. No significant difference was
 240 demonstrated among the three groups ($F(2,90) = 0.398$, $p = 0.673$).

241 **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

242 Values presented as median (IQR)

243
 244 No significant correlation was demonstrated between the pack years of smoking and
 245 serum AMH ($r= -0.212$, $n=23$, $p=0.166$) or total AFC ($r= -0.276$, $n=19$, $p=0.126$). No
 246 significant correlation was found between breath CO and serum AMH ($r= 0.082$, $n=94$,
 247 $p=0.216$) or total AFC ($r= 0.096$, $n=83$, $p=0.195$). Similarly, no significant correlation
 248 was found between urine cotinine levels and serum AMH ($r= 0.146$, $n=83$, $p=0.095$)
 249 or total AFC ($r= -0.027$, $n=77$, $p=0.386$).

250 4 Discussion

251 4.1 Main results

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

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2
3 256 quantitatively measured markers of smoking. We were hence able to validate the
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5 257 comparison groups created by a self-reported history to ensure a valid comparison of
6
7 258 outcome measures. We were unable to demonstrate a significant correlation between
8
9 259 the pack years smoked and serum AMH and AFC. We did not find a significant
10 260 association between biomarkers of smoking and biomarkers of ovarian reserve.

11 12 261 4.2 Interpretation of results

13
14 262 Biological plausibility exists for the effect of smoking on ovarian reserve and ovarian
15 263 ageing. Animal studies have suggested adverse effects of cigarette smoking on
16 264 ovarian reserve (13, 14). Several mechanisms have been postulated, which may affect
17 265 quality, quantity or both. Gannon et al in 2012 (15) hypothesised a mechanism of direct
18 266 toxicity to ovarian follicles resulting in an accelerated follicle loss. An indirect effect on
19 267 ovarian follicle numbers has been suggested through an action on the hypothalamic
20 268 pituitary axis (16) . These effects are however not evident in our study population of
21 269 younger women based on serum AMH and AFC. This may be because the natural
22 270 decline of ovarian reserve with age does not follow a linear function but shows a rapid
23 271 decline with increasing age(5).It has also been suggested that ovarian follicles may
24 272 differ in susceptibility to the effects of smoking at different ages with older oocytes
25 273 being more susceptible to negative effects of smoking.

26 274 The effect of smoking may be dose related. The pack years of smoking in our study
27 275 population was relatively low at 2.13 pack years. It is possible that the deleterious
28 276 effects are evident only at higher levels of smoking exposure or smoking is associated
29 277 with smaller magnitude of reduction in ovarian reserve markers. Although it may be
30 278 possible to demonstrate such small differences with a larger sample size, the clinical
31 279 implications of such findings would be questionable. Serum AMH and AFC are largely
32 280 used in young women in the context of fertility treatment, to predict ovarian response
33 281 to treatment and pregnancy rates. Hence in younger women seeking fertility treatment
34 282 , a clinically relevant decrease in ovarian reserve may be considered one which
35 283 significantly reduces the probability of the most important outcome for this group of
36 284 women; the pregnancy rate. Significantly lower pregnancy rates have been reported
37 285 in the lowest quartile of AMH below 10.28 pmol/l(12). Pregnancy rates in women with
38 286 serum AMH in the upper three quartiles are not statistically different from each
39 287 other.(12). The absence of an association between smoking and serum AMH and
40 288 AFC also argues for a mechanism against follicular atresia. This is strengthened by

1
2
3 289 the finding of no association between ex-smokers and lower AMH values in our study
4
5 290 and also in other studies such as Dolleman et al(7).
6

7 291 Our results are in agreement with those of Bressler et al, 2016 (9). They were unable
8
9 292 to demonstrate an association between smoking exposure and serum AMH in a
10
11 293 population based cross-sectional analysis. The age of their study population was
12
13 294 women aged 23-35 years which is similar to that of our study. However, exposure
14
15 295 ascertainment was done using only a self-reported questionnaire. Similarly, Kline et al
16
17 296 in 2016 reported no association between AMH and smoking in a cross-sectional study
18
19 297 using self-reported smoking to ascertain exposure. Dolleman et al in 2013 in a large
20
21 298 population based study reported lower serum AMH in current smokers but not in ex-
22
23 299 smokers as compared to never smokers. The study population was however
24
25 300 significantly older (mean 37.3, SD 9.2) than our study population, which may explain
26
27 301 a difference in the results. It has been suggested that the increase in follicular decline
28
29 302 may be accelerated and more evident with advancing age(16). Also, the smoking
30
31 303 exposure in pack years was higher in this population (mean 10.2, SD 9.1) as compared
32
33 304 to our study (median 2.13 (IQR 0.59-3.48)) which could account for the differences.
34
35 305 Dolleman also reported a threshold after which the linear association of pack years
36
37 306 and serum AMH was significant. They reported this at 10 pack years of smoking below
38
39 307 which there was no significant association with serum AMH. Hence, these results
40
41 308 could be considered to be in agreement with our study.

42
43 309 We have used breath CO and urine cotinine as biomarkers of smoking to validate self
44
45 310 -reported smoking history. This is in agreement with previously reported studies.
46
47 311 Marrone et al report significantly higher breath CO and cotinine levels in smokers
48
49 312 compared with non-smokers ($P < 0.001$), with 100% specificity and sensitivity at a
50
51 313 concentration of 5ppm(17). Similarly, MacLaren et al reported a strong agreement
52
53 314 between self-reported smoking and breath CO levels with a sensitivity of 96% and
54
55 315 specificity of 93.3% using a cut off of 7ppm(18).

51 316 4.3 Strengths and limitations

52
53 317 A major strength of our study is that we used a comprehensive and detailed self-
54
55 318 reported questionnaire to assess smoking exposure, which allowed estimation of
56
57 319 lifetime smoking exposure in terms of pack years and also accounted for passive
58
59 320 smoking. Furthermore, we also used breath CO and urine cotinine concentrations to
60

1
2
3 321 validate our study groups. The CO breath test shows the amount of CO in the breath
4 322 (ppm), as an indirect, non-invasive measure of blood carboxyhemoglobin (%COHb).
5
6 323 CO leaves the body rapidly and the half-life is about 5 hours. Within 24 to 48 hours of
7
8 324 not smoking, smokers will be at non-smoker levels. Cotinine is the predominant
9
10 325 metabolite of nicotine. It has a half-life of 20 hours and is detectable for up to one week
11
12 326 after the use of tobacco. This is useful to identify smokers who have abstained from
13
14 327 smoking for several hours.

15
16 328 The participants included an unselected population of women attending the clinic for
17
18 329 various investigations and treatments. There were wide variations in the baseline
19
20 330 characteristics of participants such as ethnicity, cause of infertility and diagnosis. By
21
22 331 using a wide-ranging unselected population of women we have attempted to improve
23
24 332 the generalisability of the results.

25 333 Age remains a major determinant of ovarian reserve. We have included only women
26
27 334 35 years and younger to reduce bias due to the impact of advancing age. The
28
29 335 participants included only sub-fertile women with a limited range of BMI and age. This
30
31 336 is because fertility treatment within the UK and funded by the National Health Service
32
33 337 is restricted by limits on age and BMI. Therefore, caution should be exercised when
34
35 338 extrapolating these results to other populations. Our study was powered only to detect
36
37 339 differences in ovarian markers of relatively large magnitude that we considered to have
38
39 340 a clinical significance in the management of young women seeking fertility treatment.
40
41 341 However, a much larger sample size would be required to detect statistically significant
42
43 342 differences of smaller magnitude which may be relevant to different study populations
44
45 343 and research questions.

46 344 5 Conclusion

47 345 We did not find a quantitative change in the antral follicle pool following exposure to
48
49 346 cigarette smoking in women ≤ 35 years seeking fertility treatment. We confirmed that
50
51 347 self-reported smoking correlates well with quantitatively measured biomarkers of
52
53 348 smoking. There was no significant association between biomarkers of smoking and
54
55 349 biomarkers of ovarian reserve. We were also unable to demonstrate a correlation
56
57 350 between the lifetime smoking exposure and ovarian reserve parameters
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351 6 Competing interests

352 None declared.

353 7 Contribution to authorship

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

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

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

358 AG: critical review and final approval

359 AS: critical review and final approval

360 RH: critical review and final approval

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

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

364 8 Details of ethics approval

365 The study was approved by Health Research Authority and Health and Research Care
366 Wales- Central Research Ethics Committee on 10/Apr/2019. (REC reference:
367 19/WA/0089)

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373 11 References

374 1. Laven JS, Mulders AG, Visser JA, Themmen AP, De Jong FH, Fauser BC. Anti-
375 Mullerian hormone serum concentrations in normoovulatory and anovulatory women
376 of reproductive age. J Clin Endocrinol Metab. 2004;89(1):318-23.

- 1
2
3 377 2. Fanchin R, Schonauer LM, Righini C, Guibourdenche J, Frydman R, Taieb J.
4
5 378 Serum anti-Mullerian hormone is more strongly related to ovarian follicular status than
6
7 379 serum inhibin B, estradiol, FSH and LH on day 3. *Hum Reprod.* 2003;18(2):323-7.
- 8
9 380 3. Weenen C, Laven JS, Von Bergh AR, Cranfield M, Groome NP, Visser JA, et
10
11 381 al. Anti-Mullerian hormone expression pattern in the human ovary: potential
12
13 382 implications for initial and cyclic follicle recruitment. *Mol Hum Reprod.* 2004;10(2):77-
14
15 383 83.
- 16
17 384 4. Pigny P, Jonard S, Robert Y, Dewailly D. Serum anti-Mullerian hormone as a
18
19 385 surrogate for antral follicle count for definition of the polycystic ovary syndrome. *J Clin*
20
21 386 *Endocrinol Metab.* 2006;91(3):941-5.
- 22
23 387 5. Wallace WH, Kelsey TW. Human ovarian reserve from conception to the
24
25 388 menopause. *PLoS One.* 2010;5(1):e8772.
- 26
27 389 6. Kelsey TW, Wright P, Nelson SM, Anderson RA, Wallace WH. A validated
28
29 390 model of serum anti-mullerian hormone from conception to menopause. *PLoS One.*
30
31 391 2011;6(7):e22024.
- 32
33 392 7. Dolleman M, Verschuren WM, Eijkemans MJ, Dolle ME, Jansen EH,
34
35 393 Broekmans FJ, et al. Reproductive and lifestyle determinants of anti-Mullerian
36
37 394 hormone in a large population-based study. *J Clin Endocrinol Metab.*
38
39 395 2013;98(5):2106-15.
- 40
41 396 8. Plante BJ, Cooper GS, Baird DD, Steiner AZ. The impact of smoking on
42
43 397 antimullerian hormone levels in women aged 38 to 50 years. *Menopause.*
44
45 398 2010;17(3):571-6.
- 46
47 399 9. Hawkins Bressler L, Bernardi LA, De Chavez PJ, Baird DD, Carnethon MR,
48
49 400 Marsh EE. Alcohol, cigarette smoking, and ovarian reserve in reproductive-age
50
51 401 African-American women. *Am J Obstet Gynecol.* 2016;215(6):758 e1- e9.
- 52
53 402 10. Statistics OfN. Adult smoking habits in the UK 2019 [Available from:
54
55 403 <https://www.ons.gov.uk/peoplepopulationandcommunity/healthandsocialcare/healthandlifeexpectancies/bulletins/adultsmokinghabitsingreatbritain/previousReleases>].
- 56
57 404
58 405 11. Statistics OfN. Opinions and Lifestyle Survey 2014 2016
59
60 406 [https://www.ons.gov.uk/peoplepopulationandcommunity/healthandsocialcare/healthandlifeexpectancies/bulletins/adultsmokinghabitsingreatbritain/2014]
407

- 1
2
3 408 12. Bhide P, Gudi A, Shah A, Timms P, Grayson K, Homburg R. Anti-Mullerian
4 409 hormone as a predictor of pregnancy following IVF. *Reprod Biomed Online*.
5 410 2013;26(3):247-52.
6
7
8
9 411 13. Bordel R, Laschke MW, Menger MD, Vollmar B. Nicotine does not affect
10 412 vascularization but inhibits growth of freely transplanted ovarian follicles by inducing
11 413 granulosa cell apoptosis. *Hum Reprod*. 2006;21(3):610-7.
12
13
14 414 14. Paszkowski T, Clarke RN, Hornstein MD. Smoking induces oxidative stress
15 415 inside the Graafian follicle. *Hum Reprod*. 2002;17(4):921-5.
16
17
18 416 15. Gannon AM, Stampfli MR, Foster WG. Cigarette smoke exposure leads to
19 417 follicle loss via an alternative ovarian cell death pathway in a mouse model. *Toxicol*
20 418 *Sci*. 2012;125(1):274-84.
21
22
23 419 16. Schuh-Huerta SM, Johnson NA, Rosen MP, Sternfeld B, Cedars MI, Reijo Pera
24 420 RA. Genetic markers of ovarian follicle number and menopause in women of multiple
25 421 ethnicities. *Hum Genet*. 2012;131(11):1709-24.
26
27
28 422 17. Marrone GF, Paulpillai M, Evans RJ, Singleton EG, Heishman SJ. Breath
29 423 carbon monoxide and semiquantitative saliva cotinine as biomarkers for smoking.
30 424 *Hum Psychopharmacol*. 2010;25(1):80-3.
31
32
33 425 18. Maclaren DJ, Conigrave KM, Robertson JA, Ivers RG, Eades S, Clough AR.
34 426 Using breath carbon monoxide to validate self-reported tobacco smoking in remote
35 427 Australian Indigenous communities. *Popul Health Metr*. 2010;8(1):2.
36
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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

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60STROBE 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.

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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4 1 Title

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6 2 Effect of cigarette smoking on serum anti-Mullerian hormone and antral follicle count
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8 3 in women seeking fertility treatment – a prospective cross-sectional study
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12 5 P Bhide^{a,b}, E Timlick^b, A Kulkarni^b, A Gudi^b, A Shah^b, R Homburg^b, G Acharya^{a,c}

13
14
15 6 ^aWomen's Health and Perinatology Research Group, Department of Clinical Medicine,
16 7 Faculty of Health Sciences, University of Tromsø, Tromsø, Norway.

17
18
19 8 ^b Homerton Fertility Centre, Homerton University Hospital NHS Foundation Trust,
20 9 London, UK.

21
22
23 10 ^c Department of Clinical Science, Intervention and Technology, Karolinska Institute,
24 11 Stockholm, Sweden.
25
26
27 12

28
29 13 Corresponding author

30
31 14 Dr. Priya Bhide

32
33 15 Homerton Fertility Centre,

34
35 16 Homerton University Hospital NHS Foundation Trust,

36
37 17 London E9 6SR, UK.

38
39 18 Tel: +44 2085107660

40
41 19 Email: priya.bhide@nhs.net

42
43 20 ORCID ID: <https://orcid.org/0000-0003-0871-6508>
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22 Abstract page

23 Objectives

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

32 Design

33 Prospective, cross-sectional study

34 Setting

35 Single tertiary care fertility centre

36 Participants

37 Women \leq 35 years seeking fertility treatment

38 Primary outcome measures

39 Serum AMH and AFC

40 Results

41 Significant differences were found amongst current smokers, ex-smokers and never
42 smokers for breath CO ($F(2,97)=33.32$, $p < 0.0001$) and urine cotinine levels ($p <$
43 0.001). However, no significant differences were found either for serum AMH
44 ($F(2,91)=1.19$, $p=0.309$) or total AFC ($F(2,81)=0.403$, $p=0.670$) among the three
45 groups. There was no significant correlation between pack years of smoking and
46 serum AMH ($r=-0.212$, $n=23$, $p=0.166$) or total AFC ($r=-0.276$, $n=19$, $p=0.126$). No
47 significant correlation was demonstrated between breath CO and serum AMH
48 ($r=0.082$, $n=94$, $p=0.216$) or total AFC ($r=0.096$, $n=83$, $p=0.195$). Similarly, no
49 significant correlation was demonstrated between urine cotinine levels and serum
50 AMH ($r=0.146$, $n=83$, $p=0.095$) or total AFC ($r=-0.027$, $n=77$, $p=0.386$).

51 Conclusion

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

60 Strengths and limitations of this study

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

72

73

74 1 Introduction

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

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

98 Thus, the primary objective of our study was to assess the effect of cigarette smoking
99 on the quantitative ovarian reserve parameters, AMH and AFC. Our secondary aims
100 were to validate self-reported smoking behaviour using biomarkers and evaluate the
101 association between biomarkers of ovarian reserve (serum AMH and AFC) with
102 biomarkers of smoking exposure (i.e. breath carbon monoxide and urine cotinine
103 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

1
2
3 134 participants into one of three categories; current smokers, ex-smokers and never-
4
5 135 smokers. The smoking history also accounted for passive smokers and smoking
6
7 136 details aimed to quantify the smoking exposure in terms of “pack years”. We measured
8
9 137 serum AMH and AFC as a part of the standard fertility work up done for all fertility
10
11 138 patients. We also collected baseline demographic and clinical data for confounding
12
13 139 variables. We followed up all participants for the results of their tests.

14 2.5 Products, devices, techniques and tools

15
16 141 A bespoke questionnaire was used to obtain self-reported smoking history. This was
17
18 142 designed with the input of clinical and research members of the team to ensure content
19
20 143 validity and reliability. The questionnaire was tested on a pilot sample of the target
21
22 144 population. This highlighted deficiencies and allowed improvements in the final
23
24 145 questionnaire used. The questionnaire details are provided in Appendix S1.

25
26 146 The device used to measure the breath CO (Smokelyser) is a CE marked,
27
28 147 commercially available, non-invasive CO breath test that uses an electrochemical
29
30 148 sensor to measure the breath concentration of CO with a concentration range of 0-
31
32 149 150 ppm with a sensor sensitivity of 1 ppm and an accuracy of ± 2 ppm. The instrument
33
34 150 was used within the specified warranty period and used and serviced according to
35
36 151 manufacturer’s specifications.

37
38 152 The urine cotinine was measured using the DRI®Cotinine assay (Thermo Fisher
39
40 153 Scientific). The DRI® Cotinine Assay is an in vitro diagnostic medical device intended
41
42 154 for the qualitative and semi-quantitative determination of cotinine in human urine at a
43
44 155 cut off level of 500 ng/mL. The accuracy of the assay has been confirmed by gas
45
46 156 chromatography /mass spectrometry. According to manufacturer, the sensitivity,
47
48 157 defined as the lowest concentration that can be differentiated from the negative urine
49
50 158 calibrator with 95% confidence, is 34 ng/mL.

51
52 159 All serum AMH assays were performed in an on-site clinical laboratory using the
53
54 160 bench-top fully automated assay Access 2 immunoassay system (Beckman-Coulter)
55
56 161 and values were expressed as pmol/l. Inter-assay coefficients of variation for a low
57
58 162 and high control were 0.056 and 0.44, respectively. Venous blood samples were
59
60 163 obtained and delivered to the laboratory immediately, centrifuged, and stored at 2-
164 8°C, and analysed every day.

1
2
3 165 Ultrasound imaging of ovaries was performed using a Voluson S10 diagnostic
4 166 ultrasound system (GE Healthcare) equipped with a multi-frequency transvaginal
5 167 probe (RIC5-9W-RS: 9-5MHz) to visualize antral follicles systematically. AFC was
6 168 obtained automatically using the sono-AVC™ software. Manual image post-
7 169 processing was done if required. A total AFC was calculated as the sum of total
8 170 number of follicles between 2-9 mm on each ovary. This measurement was not
9 171 restricted to a particular time of the cycle.

16 172 2.6 Outcome measures

17 173 The primary outcome measures were serum AMH and total AFC.

20 174 2.7 Data collection

21 175 Data were recorded onto study specific paper Case Report Forms (CRFs) and
22 176 subsequently transferred to a study database. We collected baseline demographic
23 177 characteristics of the study population (age, ethnicity), baseline clinical data (BMI,
24 178 presence of PCO/PCOS, history of ovarian surgery), data for on smoking parameters
25 179 (type of smoker, passive smoking, smoking in pack years, breath CO and urine
26 180 cotinine levels) and data for primary outcomes (serum AMH, AFC).

27 181 Data for smoking parameters were collected by members of the research team directly
28 182 from the participant. All other data were collected from the participants' medical
29 183 records and electronic hospital records.

32 184 2.8 Statistical considerations, sample size, analysis

33 185 The sample size calculation was based upon the primary outcome of serum AMH.
34 186 Approximately 13% of women in the UK are current smokers (10) and the number of
35 187 ex-smokers exceeds that of smokers. The proportion of never smokers in the UK
36 188 population is increasing and reported at 59% in 2014 (11). Hence we estimated that
37 189 at the fertility clinic approximately one third of our population would be either smokers
38 190 or ex-smokers. We have previously found the mean serum AMH to be 28.28 pmol/l
39 191 and a significantly lower pregnancy rates among women in the lowest quartile of AMH,
40 192 i.e. below 10.28 pmol/l.(12) To detect an absolute decrease in AMH from 28.28 to
41 193 10.28 pmol/l with 80% power at a 5% significance level with an enrolment ratio of 0.5,
42 194 we would require 96 participants (32 smokers/ex-smokers and 64 non-smokers). We
43 195 planned to recruit approximately 100 participants to compensate for dropout and loss

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2
3 196 to follow up. Appropriate descriptive statistics were used to describe the baseline
4
5 197 variables in the dataset. Normality of data was checked using Shapiro-Wilk test and
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7 198 skewed data were log transformed to achieve normal distribution before using
8
9 199 parametric test. Nonparametric tests were used for data analysis if normal distribution
10
11 200 was not achieved. An one-way between-groups analysis of variance (ANOVA), a Chi-
12
13 201 squared test or a Kruskal-Wallis test were used to assess differences between
14
15 202 baseline variables and smoking markers between current smokers, ex -smokers and
16
17 203 never smokers. An ANOVA was used to assess differences in outcome variables
18
19 204 between the three study groups. When the P-value was <.05, the difference was
20
21 205 considered statistically significant. When a difference was found to be significant, a
22
23 206 post-hoc Tukey multiple comparison test was performed. A one-way between-groups
24
25 207 analysis of co-variance (ANCOVA) was performed to assess the differences between
26
27 208 groups taking into account the variability of other confounding variables. Differences
28
29 209 in breath CO concentrations and urine cotinine levels in the three comparison groups
30
31 210 were used to validate group stratification and the results for the primary outcome
32
33 211 variables. Pearsons correlation test was used to explore the relationship between
34
35 212 lifetime exposure to smoking (pack years), breath CO or urine cotinine and outcome
36
37 213 variables. Statistical analysis was done using the Statistical Package for Social
38
39 214 Sciences (SPSS version 26).

215 3 Results

216 101 women were recruited to the study over a period of nine months. Based on a self-
217
218 reported smoking history women were classified into three comparison groups: current
219
220 smokers, ex-smokers and never smokers. We included 12 smokers, 25 ex-smokers
221
222 and 64 non-smokers to the study. The baseline clinical characteristics of the
223
224 participants are summarised in Table 1. The median age (IQR) for the three groups
225
226 was 30 (25.5-33.0), 32.5 (31.0-33.5) and 31 (28.0- 33.0). There were no significant
227
228 differences in the other baseline variables amongst the three groups.

229 **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	

224 Values expressed as median (IQR) or n

225

226 The smoking markers for the three groups are detailed in table 2. The pack years of
 227 smoking, quantifying exposure to cigarette smoking, were not significantly different
 228 between current and ex-smokers ($F(1,25) = 0.547$, $p=0.467$). The breath CO levels
 229 were significantly different amongst current, ex- and never smokers ($F(2,97) = 33.32$,
 230 $p < 0.0001$). Urine cotinine levels were also significantly higher in current smokers as
 231 compared to ex-smokers and never smokers. ($p < 0.001$). Current smokers reported
 232 to be more exposed to passive smoking (75%, 9/12) as compared to ex-smokers
 233 (20%, 5/25) and never smokers (25%, 16/64) ($p=0.001$).

234 **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

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

236 The primary outcomes are detailed in Table 3. No significant difference was observed
 237 amongst current, ex- and never smokers either for serum AMH ($F(2,91) = 1.19$,
 238 $p=0.309$) or total AFC ($F(2,81) = 0.403$, $p= 0.670$). When comparing baseline
 239 variables, age showed borderline non-significance between the groups ($p=0.057$).
 240 Hence, we performed an analysis of covariance (ANCOVA) to explore the impact of
 241 smoking status on serum AMH using age as a covariate. No significant difference was
 242 demonstrated among the three groups ($F(2,90) = 0.398$, $p = 0.673$).

243 **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

244 Values presented as median (IQR)

245
 246 No significant correlation was demonstrated between the pack years of smoking and
 247 serum AMH ($r= -0.212$, $n=23$, $p=0.166$) or total AFC ($r= -0.276$, $n=19$, $p=0.126$). No
 248 significant correlation was found between breath CO and serum AMH ($r= 0.082$, $n=94$,
 249 $p=0.216$) or total AFC ($r= 0.096$, $n=83$, $p=0.195$). Similarly, no significant correlation
 250 was found between urine cotinine levels and serum AMH ($r= 0.146$, $n=83$, $p=0.095$)
 251 or total AFC ($r= -0.027$, $n=77$, $p=0.386$).

252 4 Discussion

253 4.1 Main results

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

1
2
3 256 population. By demonstrating significant differences in breath CO and urine cotinine
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5 257 levels among the groups, we confirmed that self-reported smoking correlates well with
6
7 258 quantitatively measured markers of smoking. We were hence able to validate the
8
9 259 comparison groups created by a self-reported history to ensure a valid comparison of
10
11 260 outcome measures. We were unable to demonstrate a significant correlation between
12
13 261 the pack years smoked and serum AMH and AFC. We did not find a significant
14
15 262 association between biomarkers of smoking and biomarkers of ovarian reserve.

16 263 4.2 Interpretation of results

17
18 264 Biological plausibility exists for the effect of smoking on ovarian reserve and ovarian
19
20 265 ageing. Animal studies have suggested adverse effects of cigarette smoking on
21
22 266 ovarian reserve (13, 14). Several mechanisms have been postulated, which may affect
23
24 267 quality, quantity or both. Gannon et al in 2012 (15) hypothesised a mechanism of direct
25
26 268 toxicity to ovarian follicles resulting in an accelerated follicle loss. An indirect effect on
27
28 269 ovarian follicle numbers has been suggested through an action on the hypothalamic
29
30 270 pituitary axis (16) . These effects are however not evident in our study population of
31
32 271 younger women based on serum AMH and AFC. This may be because the natural
33
34 272 decline of ovarian reserve with age does not follow a linear function but shows a rapid
35
36 273 decline with increasing age(5).It has also been suggested that ovarian follicles may
37
38 274 differ in susceptibility to the effects of smoking at different ages with older oocytes
39
40 275 being more susceptible to negative effects of smoking.

41 276 The effect of smoking may be dose related. The pack years of smoking in our study
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43 277 population was relatively low at 2.13 pack years. It is possible that the deleterious
44
45 278 effects are evident only at higher levels of smoking exposure or smoking is associated
46
47 279 with smaller magnitude of reduction in ovarian reserve markers. Although it may be
48
49 280 possible to demonstrate such small differences with a larger sample size, the clinical
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51 281 implications of such findings would be questionable. Serum AMH and AFC are largely
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53 282 used in young women in the context of fertility treatment, to predict ovarian response
54
55 283 to treatment and pregnancy rates. Hence in younger women seeking fertility treatment
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57 284 , a clinically relevant decrease in ovarian reserve may be considered one which
58
59 285 significantly reduces the probability of the most important outcome for this group of
60
286 women; the pregnancy rate. Significantly lower pregnancy rates have been reported
287 in the lowest quartile of AMH below 10.28 pmol/l(12). Pregnancy rates in women with
288 serum AMH in the upper three quartiles are not statistically different from each

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3 289 other.(12). The absence of an association between smoking and serum AMH and
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5 290 AFC also argues for a mechanism against follicular atresia. This is strengthened by
6
7 291 the finding of no association between ex-smokers and lower AMH values in our study
8
9 292 and also in other studies such as Dolleman et al(7).

10
11 293 Our results are in agreement with those of Bressler et al, 2016 (9). They were unable
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13 294 to demonstrate an association between smoking exposure and serum AMH in a
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15 295 population based cross-sectional analysis. The age of their study population was
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17 296 women aged 23-35 years which is similar to that of our study. However, exposure
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19 297 ascertainment was done using only a self-reported questionnaire. Similarly, Kline et al
20
21 298 in 2016 reported no association between AMH and smoking in a cross-sectional study
22
23 299 using self-reported smoking to ascertain exposure(17). Dolleman et al in 2013 in a
24
25 300 large population based study reported lower serum AMH in current smokers but not in
26
27 301 ex-smokers as compared to never smokers(7). The study population was however
28
29 302 significantly older (mean 37.3, SD 9.2) than our study population, which may explain
30
31 303 a difference in the results. It has been suggested that the increase in follicular decline
32
33 304 may be accelerated and more evident with advancing age(16). Also, the smoking
34
35 305 exposure in pack years was higher in this population (mean 10.2, SD 9.1) as compared
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37 306 to our study (median 2.13 (IQR 0.59-3.48)) which could account for the differences.
38
39 307 Dolleman also reported a threshold after which the linear association of pack years
40
41 308 and serum AMH was significant. They reported this at 10 pack years of smoking below
42
43 309 which there was no significant association with serum AMH. Hence, these results
44
45 310 could be considered to be in agreement with our study.

46
47 311 We have used breath CO and urine cotinine as biomarkers of smoking to validate self
48
49 312 -reported smoking history. This is in agreement with previously reported studies.
50
51 313 Marrone et al report significantly higher breath CO and cotinine levels in smokers
52
53 314 compared with non-smokers ($P < 0.001$), with 100% specificity and sensitivity at a
54
55 315 concentration of 5ppm(18). Similarly, MacLaren et al reported a strong agreement
56
57 316 between self-reported smoking and breath CO levels with a sensitivity of 96% and
58
59 317 specificity of 93.3% using a cut off of 7ppm(19).

318 4.3 Strengths and limitations

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

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3 321 lifetime smoking exposure in terms of pack years and also accounted for passive
4 322 smoking. Furthermore, we also used breath CO and urine cotinine concentrations to
5 323 validate our study groups. The CO breath test shows the amount of CO in the breath
6 324 (ppm), as an indirect, non-invasive measure of blood carboxyhemoglobin (%COHb).
7 325 CO leaves the body rapidly and the half-life is about 5 hours. Within 24 to 48 hours of
8 326 not smoking, smokers will be at non-smoker levels. Cotinine is the predominant
9 327 metabolite of nicotine. It has a half-life of 20 hours and is detectable for up to one week
10 328 after the use of tobacco. This is useful to identify smokers who have abstained from
11 329 smoking for several hours.

12 330 The participants included an unselected population of women attending the clinic for
13 331 various investigations and treatments. There were wide variations in the baseline
14 332 characteristics of participants such as ethnicity, cause of infertility and diagnosis. By
15 333 using a wide-ranging unselected population of women we have attempted to improve
16 334 the generalisability of the results.

17 335 Age remains a major determinant of ovarian reserve. We have included only women
18 336 35 years and younger to reduce bias due to the impact of advancing age. The
19 337 participants included only sub-fertile women with a limited range of BMI and age. This
20 338 is because fertility treatment within the UK and funded by the National Health Service
21 339 is restricted by limits on age and BMI. Therefore, caution should be exercised when
22 340 extrapolating these results to other populations. Pregnancy rates following assisted
23 341 reproduction treatments are influenced primarily by age but also indirectly by the
24 342 number of eggs. Serum AMH and AFC are excellent predictors for the number of eggs
25 343 retrieved, and in young women < 35 years only a large decrease in quantitative reserve
26 344 would significantly impact pregnancy rates(20). Our study was hence powered only to
27 345 detect differences in ovarian markers of relatively large magnitude that we considered
28 346 to have a clinical significance in the management of young women seeking fertility
29 347 treatment. A much larger sample size would be able to detect statistically significant
30 348 differences of smaller magnitude which may be relevant to different study populations
31 349 and research questions but clinically less meaningful for fertility.

32 350 5 Conclusion

33 351 We did not find a significant quantitative change in the antral follicle pool following
34 352 exposure to cigarette smoking in women ≤ 35 years seeking fertility treatment. We

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3 353 confirmed that self-reported smoking correlates well with quantitatively measured
4 354 biomarkers of smoking. There was no significant association between biomarkers of
5 355 smoking and biomarkers of ovarian reserve. We were also unable to demonstrate a
6 356 correlation between the lifetime smoking exposure and ovarian reserve parameters

10 357 6 Competing interests

11
12
13 358 None declared.

15 359 7 Contribution to authorship

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17
18 360 PB: Study concept and design, participant recruitment, data collection, data analysis
19 361 and interpretation, drafting the article, critical review and final approval.

20
21
22 362 ET: participant recruitment, data collection, critical review and final approval.

23
24
25 363 AK: participant recruitment, data collection, critical review and final approval.

26
27 364 AG: critical review and final approval

28
29 365 AS: critical review and final approval

30
31 366 RH: critical review and final approval

32
33 367 GA: Study concept and design, critical review and final approval.

34
35
36 368 All authors read the manuscript critically, commented on the draft and approved the
37 369 final version before submission.

39 370 8 Details of ethics approval

40
41
42 371 The study was approved by Health Research Authority and Health and Research Care
43 372 Wales- Central Research Ethics Committee on 10/Apr/2019. (REC reference:
44 373 19/WA/0089)

47 374 9 Funding

48
49
50
51 375 This research received no specific grant from any funding agency in the public,
52 376 commercial or not-for-profit sectors.

54 377 10 Data availability statement

55
56
57 378 The fully anonymized study data can be made available to interested researchers on request
58 379 to the corresponding author.

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8
9 383 We would like to thank all our patient advisors for their contributions to this study.
10

12 References

- 11
12 384
13
14 385 1. Laven JS, Mulders AG, Visser JA, Themmen AP, De Jong FH, Fauser BC. Anti-Mullerian
15 386 hormone serum concentrations in normoovulatory and anovulatory women of reproductive
16 387 age. *J Clin Endocrinol Metab.* 2004;89(1):318-23.
17
18 388 2. Fanchin R, Schonauer LM, Righini C, Guibourdenche J, Frydman R, Taieb J. Serum
19 389 anti-Mullerian hormone is more strongly related to ovarian follicular status than serum
20 390 inhibin B, estradiol, FSH and LH on day 3. *Hum Reprod.* 2003;18(2):323-7.
21
22 391 3. Weenen C, Laven JS, Von Bergh AR, Cranfield M, Groome NP, Visser JA, et al. Anti-
23 392 Mullerian hormone expression pattern in the human ovary: potential implications for initial
24 393 and cyclic follicle recruitment. *Mol Hum Reprod.* 2004;10(2):77-83.
25
26 394 4. Pigny P, Jonard S, Robert Y, Dewailly D. Serum anti-Mullerian hormone as a
27 395 surrogate for antral follicle count for definition of the polycystic ovary syndrome. *J Clin*
28 396 *Endocrinol Metab.* 2006;91(3):941-5.
29
30 397 5. Wallace WH, Kelsey TW. Human ovarian reserve from conception to the menopause.
31 398 *PLoS One.* 2010;5(1):e8772.
32
33 399 6. Kelsey TW, Wright P, Nelson SM, Anderson RA, Wallace WH. A validated model of
34 400 serum anti-mullerian hormone from conception to menopause. *PLoS One.*
35 401 2011;6(7):e22024.
36
37 402 7. Dolleman M, Verschuren WM, Eijkemans MJ, Dolle ME, Jansen EH, Broekmans FJ, et
38 403 al. Reproductive and lifestyle determinants of anti-Mullerian hormone in a large population-
39 404 based study. *J Clin Endocrinol Metab.* 2013;98(5):2106-15.
40
41 405 8. Plante BJ, Cooper GS, Baird DD, Steiner AZ. The impact of smoking on antimullerian
42 406 hormone levels in women aged 38 to 50 years. *Menopause.* 2010;17(3):571-6.
43
44 407 9. Hawkins Bressler L, Bernardi LA, De Chavez PJ, Baird DD, Carnethon MR, Marsh EE.
45 408 Alcohol, cigarette smoking, and ovarian reserve in reproductive-age African-American
46 409 women. *Am J Obstet Gynecol.* 2016;215(6):758 e1- e9.
47
48 410 10. Statistics OfN. Adult smoking habits in the UK 2019 [Available from:
49 411 51 413 53 415 eexpectancies/bulletins/adultsmokinghabitsingreatbritain/previousReleases.
54 416 eexpectancies/bulletins/adultsmokinghabitsingreatbritain/previousReleases.
55 417 eexpectancies/bulletins/adultsmokinghabitsingreatbritain/previousReleases.
56 418 eexpectancies/bulletins/adultsmokinghabitsingreatbritain/previousReleases.
57 419 eexpectancies/bulletins/adultsmokinghabitsingreatbritain/previousReleases.
58 420 eexpectancies/bulletins/adultsmokinghabitsingreatbritain/previousReleases.
59 421 eexpectancies/bulletins/adultsmokinghabitsingreatbritain/previousReleases.
60 422 eexpectancies/bulletins/adultsmokinghabitsingreatbritain/previousReleases.

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2
3 416 13. Bordel R, Laschke MW, Menger MD, Vollmar B. Nicotine does not affect
4 417 vascularization but inhibits growth of freely transplanted ovarian follicles by inducing
5 418 granulosa cell apoptosis. *Hum Reprod.* 2006;21(3):610-7.
- 7 419 14. Paszkowski T, Clarke RN, Hornstein MD. Smoking induces oxidative stress inside the
8 420 Graafian follicle. *Hum Reprod.* 2002;17(4):921-5.
- 10 421 15. Gannon AM, Stampfli MR, Foster WG. Cigarette smoke exposure leads to follicle loss
11 422 via an alternative ovarian cell death pathway in a mouse model. *Toxicol Sci.*
12 423 2012;125(1):274-84.
- 14 424 16. Schuh-Huerta SM, Johnson NA, Rosen MP, Sternfeld B, Cedars MI, Reijo Pera RA.
15 425 Genetic markers of ovarian follicle number and menopause in women of multiple
16 426 ethnicities. *Hum Genet.* 2012;131(11):1709-24.
- 18 427 17. Kline J, Tang A, Levin B. Smoking, alcohol and caffeine in relation to two hormonal
19 428 indicators of ovarian age during the reproductive years. *Maturitas.* 2016;92:115-22.
- 21 429 18. Marrone GF, Paulpillai M, Evans RJ, Singleton EG, Heishman SJ. Breath carbon
22 430 monoxide and semiquantitative saliva cotinine as biomarkers for smoking. *Hum*
23 431 *Psychopharmacol.* 2010;25(1):80-3.
- 25 432 19. Maclaren DJ, Conigrave KM, Robertson JA, Ivers RG, Eades S, Clough AR. Using
26 433 breath carbon monoxide to validate self-reported tobacco smoking in remote Australian
27 434 Indigenous communities. *Popul Health Metr.* 2010;8(1):2.
- 29 435 20. Sunkara SK, Rittenberg V, Raine-Fenning N, Bhattacharya S, Zamora J, Coomarasamy
30 436 A. Association between the number of eggs and live birth in IVF treatment: an analysis of
31 437 400 135 treatment cycles. *Hum Reprod.* 2011;26(7):1768-74.

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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

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60STROBE 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.