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ORIGINAL ARTICLE *Infertility*

Infertile women below the age of 40 have similar anti-Müllerian hormone levels and antral follicle count compared with women of the same age with no history of infertility

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STUDY QUESTION: Do infertile patients below the age of 40 years have a lower ovarian reserve, estimated by anti-Müllerian hormone (AMH) and total antral follicle count (AFC), than women of the same age with no history of infertility?

SUMMARY ANSWER: Serum AMH and AFC were not lower in infertile patients aged 20–39 years compared with a control group of the same age with no history of infertility.

WHAT IS KNOWN ALREADY?: The management of patients with a low ovarian reserve and a poor response to controlled ovarian stimulation (COS) remains a challenge in assisted reproductive technologies (ART). Both AMH levels and AFC reflect the ovarian reserve and are valuable predictors of the ovarian response to exogenous gonadotrophins. However, there is a large inter-individual variation in the age-related depletion of the ovarian reserve and a broad variability in the levels of AMH and AFC compatible with conception. Women with an early depletion of the ovarian reserve may experience infertility as a consequence of postponement of childbearing. Thus, low ovarian reserve is considered to be overrepresented among infertile patients.

STUDY DESIGN, SIZE, DURATION: A prospective cohort study including 382 women with a male partner referred to fertility treatment at Rigshospitalet, Copenhagen, Denmark during 2011–2013 compared with a control group of 350 non-users of hormonal contraception with no history of infertility recruited during 2008–2010.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Included patients and controls were aged 20–39 years. Women with polycystic ovary syndrome were excluded. On Cycle Days 2–5, AFC and ovarian volume were measured by transvaginal sonography, and serum levels of AMH, FSH and LH were assessed.

MAIN RESULTS AND THE ROLE OF CHANCE: Infertile patients had similar AMH levels (11%, 95% confidence interval (CI): −1;24%) and AFC (1%, 95% CI: −7;8%) compared with controls with no history of infertility in an age-adjusted linear regression analysis. The prevalence of very low AMH levels (<5 pmol/l) was similar in the two cohorts (age-adjusted odds ratio: 0.9, 95% CI: 0.5;1.7). The findings persisted after adjustment for smoking status, body mass index, gestational age at birth, previous conception and chronic disease in addition to age.

LIMITATIONS, REASON FOR CAUTION: The comparison of ovarian reserve parameters in women recruited at different time intervals could be a reason for caution. However, all women were examined at the same centre using the same sonographic algorithm and AMH immunoassay.

WIDER IMPLICATIONS OF THE FINDINGS: This study indicates that the frequent observation of patients with a poor response to COS in ART may not be due to an overrepresentation of women with an early depletion of the ovarian reserve but rather a result of the expected age-related decline in fertility.

STUDY FUNDING/COMPETING INTEREST(S): The study received funding from MSD and the Interregional European Union (EU) projects 'ReproSund' and 'ReproHigh'. The authors have no conflict of interest.

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Key words: anti-Müllerian hormone / antral follicle count / infertility / low ovarian reserve / ovarian aging

Introduction

The management of patients with a poor response to controlled ovarian stimulation in IVF remains a clinical challenge (Ferraretti *et al.*, 2011; Busnelli *et al.*, 2015). Poor ovarian response is associated with advanced female age and a low ovarian reserve, all of which result in reduced pregnancy rates in assisted reproductive technologies (ART) (Ferraretti *et al.*, 2011; Iliodromiti *et al.*, 2015; Busnelli *et al.*, 2015).

The ovarian reserve can be estimated by serum concentrations of anti-Müllerian hormone (AMH) and total antral follicle count (AFC) (Visser *et al.*, 2006; Hansen *et al.*, 2011; Fleming *et al.*, 2012; Dewailly *et al.*, 2014). According to the Bologna Criteria, a low ovarian reserve is defined by an AFC <5–7 follicles or an AMH level <0.5–1.1 ng/ml (~3.6–7.9 pmol/l) (Ferraretti *et al.*, 2011). It has recently been suggested to adjust the cut off levels of AMH to 0.7–1.3 ng/ml (~5.0–9.3 pmol/l) (Ferraretti and Gianaroli, 2014; La Marca and Sunkara, 2014).

A significant decline in female fecundity is observed 10–12 years prior to the menopausal transition due to a decrease in the follicular pool and an increased prevalence of aneuploidy (teVelde and Pearsson, 2002; Broekmans *et al.*, 2007). The average age at menopause is 51 years with a broad normal range from 40 to 60 years implying that the age-related decline in fertility varies considerably between women (teVelde and Pearson, 2002; Broekmans *et al.*, 2009). Prospective population-based studies indicate that women with a low age-specific AMH may experience an early age-related decline in fertility related to the ovarian reserve depletion *per se* resulting in a shift towards early menopause (Broer *et al.*, 2011; Tehrani *et al.*, 2011, 2013; Freeman *et al.*, 2012; Dólléman *et al.*, 2015; Depman *et al.*, 2016). Postponement of childbearing is associated with an increased risk of infertility (Schmidt *et al.*, 2012). Women with an early age-related depletion of the ovarian reserve may be at particular risk and could thus be overrepresented among infertile patients.

The present study aimed to investigate to what extent impaired ovarian reserve contributes to infertility in newly referred patients at a tertiary fertility centre. We hypothesised that the frequent reports of infertile patients with a poor response to ovarian stimulation in ART reflect an increased prevalence of women with an early age-related exhaustion of the follicular pool. If this hypothesis holds true, we would expect AMH levels and AFC to be lower in infertile patients compared with women of similar age with no history of infertility.

Materials and Methods

Study design

This study was designed as a prospective cohort study with a historical control group.

Study population

The study population included 382 infertile patients referred for fertility treatment at The Fertility Clinic, Rigshospitalet, at Copenhagen University Hospital from September 2011 to October 2013. From September 2011, the Fertility Clinic offered newly referred infertile patients an assessment of ovarian and endocrine parameters prior to the first treatment cycle. Patients identified as eligible for the present study were examined on Cycle Days (CD) 2–5 and interviewed to obtain relevant background information.

The following patients were considered non-eligible: (i) patients referred for preimplantation genetic diagnosis, (ii) patients referred due to HIV or contagious hepatitis B or C infection and (iii) single and homosexual women, as they were *per se* not considered infertile. Furthermore, patients referred directly for oocyte donation (OD) from other fertility centres were not examined on CD 2–5 and thus not included as they had already been diagnosed with a diminished ovarian reserve and most had started hormone replacement therapy or treatment with estradiol to prepare for the OD. The control group comprised 350 non-users of hormonal contraception with no history of infertility recruited in a prospective cross sectional study conducted at the Fertility Clinic, Rigshospitalet, from August 2008 to February 2010. All participants were health care workers employed at Copenhagen University Hospital, Rigshospitalet. The study has previously been described in detail (Bentzen *et al.*, 2013).

We excluded patients and controls with polycystic ovary syndrome (PCOS) defined as oligo- or amenorrhoea in addition to AFC ≥12 and/or an ovarian volume >10 ml^3 in at least one ovary in accordance with the Rotterdam Criteria (The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004). Figure 1 provides a flow chart of the study population.

Collection of background information

Data on menstrual cycle pattern, reproductive and medical history, maternal menopause, lifestyle factors and demography were collected. The infertile patients were interviewed using a standardized registration form. The control group provided similar background information in an internet-based questionnaire at inclusion.

Ovarian sonography

A transvaginal ultrasonography was performed on CD 2–5. The ovarian volume was calculated as described by Rosendahl *et al.* (2010). If endometriomas or cysts were present in one ovary, the mean ovarian volume (MOV) was estimated as the volume of the contralateral ovary. Antral follicles were counted and grouped according to size: 2–4 mm (small), 5–7 mm (intermediate) and 8–10 mm (large).

Endocrinology

Blood samples were taken on CD 2–5 (prior to stimulation start in patients). The serum concentrations of FSH and LH were analysed by electrochemiluminescence immunoassays (Roche Diagnostics, Mannheim, Germany). The analytical sensitivity, intra-assay and inter-assay coefficient of variation were for FSH: <0.1 IU/l, 2.8 and 4.5%, respectively; and for LH: <0.1 IU/l, 1.2 and 2.2%, respectively. The serum concentrations of AMH were analysed

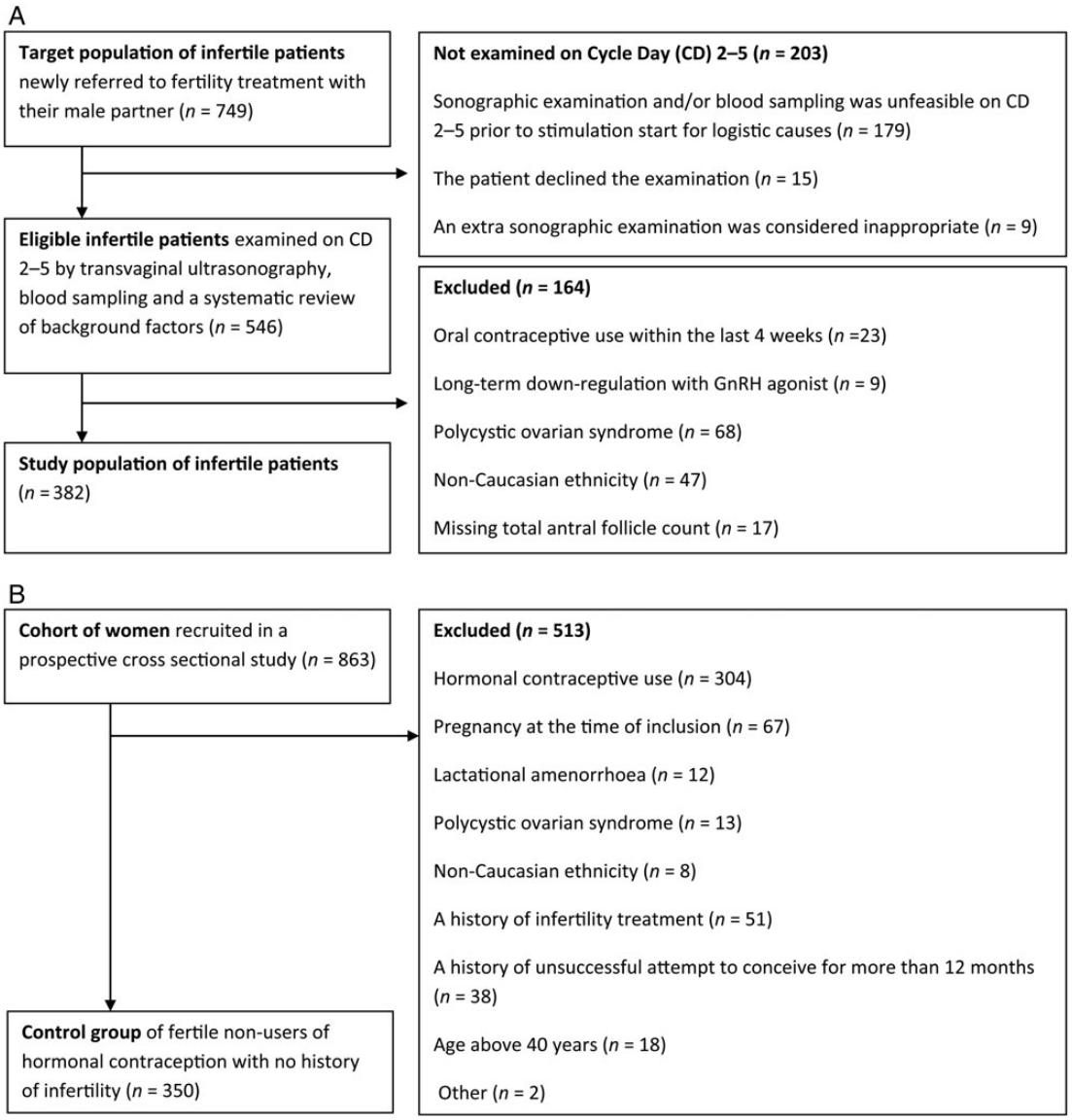

Figure 1 Flow chart of the study population. (**A**) The prospective cohort of infertile patients included at the Fertility Clinic, Rigshospitalet, Copenhagen University Hospital from September 2011 to October 2013. (**B**) The control group of women with no history of infertility recruited in a prospective cross sectional study among female health care workers conducted at the Fertility Clinic, Rigshospitalet, University Hospital Copenhagen, from September 2008 to February 2010.

by an ELISA Generation I (Immunotech; Beckman Coulter, Marseilles, France). The analytical sensitivity, intra-assay and inter-assay coefficient of variation for AMH were: 0.7 pmol/l, 12.3 and 14.2%, respectively. AMH levels were estimated to be 1.5 pmol/l if measurements were below the clinically applied detection level of 3 pmol/l due to poor reproducibility of measurements below this level.

Statistics

Baseline characteristics were presented as number (percentage), mean ± SD or median (90% population limits) where appropriate. Participants were categorised into three age groups: 20.0–29.9, 30.0–34.9 and 35.0–39.9 years. Differences between the two cohorts and across age groups within each cohort were tested using one-way ANOVA for normally distributed continuous data, the Kruskal–Wallis test for non-normally distributed continuous data and the χ^2 test for categorical data. Differences in the composition of small, intermediate and large size antral follicles between the groups were tested using permutation tests (Pesarin and Salmosa, 2010). Confidence intervals (CIs) for mean follicle counts and proportions were computed by bootstrapping (Davison and Hinkley, 1997). Logarithmic transformation was applied to AMH and AFC prior to further analysis. Thus, differences between groups were reported as percentage differences. After transformation, both variables showed an approximately normal distribution of residuals. The correlation between AMH levels and AFC was assessed by Spearman's rank correlation coefficient (r_s). To visualize the age-related changes in ovarian reserve parameters, data were plotted in scatter diagrams stratified according to fertility status (infertile versus control). We applied the non-linear model proposed by Hansen *et al.* (2008) to test whether the

Table I Clinical and demographic characteristics of the study population comprising 382 infertile patients and 350 controls.

	Infertile patients					Controls					Infertile versus controls
	Age group (years)				Total	Age group (years)				Total	*P*-value
	20–29	30–34	35–39	*P*-value		20–29	30–34	35–39	*P*-value		
Number of women [*n* (%)]	77 (20.2)	166 (43.5)	139 (36.4)	–	382 (100)	72 (20.6)	166 (47.4)	112 (32.0)	–	350 (100)	–
Age (years) [mean ± SD]	27.5 ± 1.8	32.4 ± 1.4	37.3 ± 1.4	–	33.2 ± 3.9	27.7 ± 1.7	32.4 ± 1.4	37.2 ± 1.5	–	33.0 ± 3.7	0.5[‡]
BMI [median (90% population limits)]	22.0 (18.3;27.3)	22.0 (18.6;29.7)	22.4 (19.4;30.5)	0.049[¶]	22.2 (18.8;30.0)	22.0 (19.1;30.0)	21.7 (18.9;31.7)	22.8 (19.1;32.0)	0.02[¶]	22.1 (18.9;31.1)	0.7[¶]
Age at menarche (mean ± SD)	13.2 ± 1.2	12.9 ± 1.3	13.2 ± 1.3	0.07[‡]	13.1 ± 1.2	13.0 ± 1.2	13.2 ± 1.3	13.2 ± 1.4	0.5[‡]	13.1 ± 1.3	0.5[‡]
Cycle length (days) (mean ± SD)[a]	29.1 ± 2.2	28.7 ± 2.0	28.2 ± 2.4	0.01[‡]	28.6 ± 2.2	29.0 ± 2.6	28.5 ± 2.2	28.8 ± 2.1	0.2[‡]	28.7 ± 2.3	0.4[¶]
Previously conceived [*n* (%)]	22 (28.6)	48 (28.9)	45 (32.4)	0.8[†]	115 (30.1)	26 (36.1)	106 (63.9)	86 (76.8)	<0.001[†]	218 (62.3)	<0.001[†]
Maternal age at menopause (mean ± SD)[b]	49.2 ± 4.7	51.0 ± 4.5	50.8 ± 4.6	0.09[‡]	50.7 ± 4.6	49.0 ± 4.4	50.0 ± 4.6	49.6 ± 5.4	0.5[‡]	49.7 ± 4.9	0.03
Birthweight (kilogram) [*n* (%)][c]											
<2500	2 (2.6)	7 (4.2)	6 (4.3)	0.8[†,*]	15 (3.9)	2 (2.8)	13 (7.8)	7 (6.3)	0.6[†,*]	22 (6.3)	0.4[†,*]
2500–4000	52 (67.5)	123 (74.1)	91 (65.5)		266 (69.6)	50 (69.4)	125 (73.3)	79 (79.5)		254 (72.6)	
>4000	8 (10.4)	12 (7.2)	12 (8.6)		32 (8.4)	7 (9.7)	14 (8.4)	6 (5.4)		27 (7.7)	
Gestational age at birth [*n* (%)][d]											
37 + 0–41 + 6	62 (80.5)	129 (77.7)	93 (66.9)	0.1[†,*]	284 (74.4)	63 (87.5)	135 (81.3)	94 (83.9)	0.3[†,*]	292 (83.4)	0.02[†,*]
<37 + 0	5 (6.5)	8 (4.8)	15 (10.8)		28 (7.3)	3 (4.2)	12 (7.2)	8 (7.1)		23 (6.6)	
≥42 + 0	4 (5.2)	15 (9.0)	15 (10.8)		34 (8.9)	2 (2.8)	11 (6.6)	2 (1.8)		15 (4.3)	
Intrauterine exposure to tobacco [*n* (%)][e]	24 (31.2)	47 (28.3)	39 (26.6)	0.9[†,*]	108 (31.4)	13 (18.3)	35 (22.6)	35 (33.3)	0.048[†,*]	83 (25.1)	0.07[†,*]
Smoking status [*n* (%)][f]											
Present smoker	9 (11.7)	11 (6.6)	9 (6.5)	0.4[†,*]	29 (7.6)	16 (22.2)	37 (22.3)	20 (17.8)	0.03[†]	73 (20.9)	<0.001[†,*]
Former smoker	28 (36.4)	54 (32.5)	56 (40.3)		138 (36.1)	14 (19.4)	44 (26.5)	45 (40.2)		103 (29.4)	
Never smoker	39 (50.7)	96 (57.8)	73 (52.5)		208 (54.5)	42 (58.3)	85 (51.2)	47 (42.0)		174 (49.7)	
Number of cigarettes per day [*n* (%)][g]											
Not daily	9 (24.3)	24 (36.9)	15 (23.1)	0.4[†,*]	48 (28.7)	17 (56.7)	33 (40.7)	19 (29.2)	0.1[†]	69 (39.2)	0.08[†,*]
1–10	15 (40.5)	19 (29.2)	24 (36.9)		58 (34.7)	10 (33.3)	30 (37.0)	27 (41.5)		67 (38.1)	
>10	13 (35.1)	17 (26.2)	22 (33.9)		52 (31.1)	3 (10.0)	18 (22.2)	19 (29.3)		40 (22.7)	
Duration of smoking (years) [median (90% p.limits)][g]	9 (2;14)	10 (1;20)	10 (1.5;22)	0.02[¶]	10 (1;21)	8 (1;14)	8 (0;17)	10 (1;22)	0.01[¶]	10 (0;20)	0.3[¶]
Alcohol (units/week) [*n* (%)]											
0–6	76 (98.7)	143 (86.1)	122 (87.8)	0.01[†]	341 (89.3)	63 (87.5)	152 (91.6)	97 (86.6)	0.4[†]	312 (89.1)	1.0[†]
≥7	1 (1.3)	23 (13.9)	17 (12.2)		41 (10.7)	9 (12.5)	14 (8.4)	15 (13.4)		38 (10.9)	

Continued

Table I *Continued*

	Infertile patients					Controls					Infertile versus controls
	Age group (years)				Total	Age group (years)				Total	
	20–29	30–34	35–39	*P*-value		20–29	30–34	35–39	*P*-value		*P*-value
Pelvic inflammatory disease [*n* (%)]	18 (23.4)	52 (31.3)	42 (30.2)	0.4[†]	112 (29.3)	26 (36.1)	48 (28.9)	42 (38.4)	0.2[†]	117 (33.4)	0.2[†]
Chronic disease [*n* (%)]	24 (31.2)	49 (29.5)	56 (40.3)	0.1[†]	129 (33.8)	21 (29.2)	40 (24.1)	29 (25.9)	0.7[†]	90 (25.7)	0.02[†]
Abdominal surgery [*n* (%)]	12 (15.6)	40 (24.1)	35 (25.2)	0.2[†]	87 (22.8)	11 (15.3)	35 (21.1)	26 (23.2)	0.4[†]	72 (20.6)	0.5[†]

Data are presented as number (percent), mean ± SD and median (90% population limits). For both cohorts, data are stratified according to age group and statistical difference across age groups tested. Data for the total infertile patients and controls are also presented and statistical differences between the two cohorts tested (the *P*-value to the right).

[a]Cycle length unknown in eight cases.
[b]Natural age at menopause unknown in 215 cases.
[c]Birthweight unknown in 116 cases.
[d]Gestational age at birth unknown in 56 cases.
[e]Intrauterine exposure to tobacco unknown in 57 cases.
[f]Smoking status missing in seven infertile cases.
[g]Data presented for present and former smokers
[†]χ^2 test.
[‡]One-way ANOVA.
[¶]Kruskal–Wallis test.
*Excluding individuals with missing values from statistical analysis.

age-related decline accelerated with increasing age. Median values of AMH and AFC as functions of age were estimated by non-linear least squares and compared with the corresponding linear fit. As the non-linear model did not prove superior to the linear fit, linear regression analyses were performed to test for differences between the two cohorts. Age was used as a continuous variable for adjustment. Similar subgroup analyses were carried out firstly excluding infertile patients with male infertility, and secondly including only infertile patients with unexplained infertility.

Differences in the prevalence of low ovarian reserve between the two cohorts were analysed using logistic regression analysis. We defined low ovarian reserve as an AMH level below 5 pmol/l, which is equivalent to the fifth percentile of AMH in women in their early 30s from the background population (unpublished data) and in accordance with the recently suggested lower cut off level for an abnormal ovarian reserve (Ferraretti and Gianaroli, 2014; La Marca and Sunkara, 2014). In addition, we tested for differences in the prevalence of an AMH level <9.3 pmol/l and an AFC <7 suggested as the upper cut off levels for a low ovarian reserve (Ferraretti and Gianaroli, 2014; La Marca and Sunkara, 2014).

A non-response test was conducted to compare the AMH level between infertile patients included in the present study and eligible infertile patients who were not included as they had not undergone the sonographic examination at CD 2–5.

Descriptive statistics and statistical analyses were performed using STATA MP, version 13.1 (StataCorp, Texas, USA), and permutation tests and bootstrapping performed using R version 3.1.2 (R Development Core Team, Vienna, Austria). A two-sided *P*-value below 0.05 was considered statistically significant.

Ethical approval

As data on the infertile patients were collected as part of the daily practice at the Fertility Clinic, ethical approval was not required according to the Ethical Committee of the Capital Region of Denmark. Data collection was approved by the Danish Data Protection Agency (journal number: 2007-58-0015). All patients gave informed consent to the examination and systematic review of their background factors and were informed that collected data would be used for research.

The control group was recruited in a study approved by the Ethical Committee of the Capital Region of Denmark (H-B-2007-129) and the Danish Data Protection Agency (journal number: 2008-41-1881).

Results

Baseline characteristics

The distribution across the three age groups was the same in the two cohorts ($P = 0.4$, χ^2 test). As seen in Table I, the mean female age was 33 years in both cohorts and the age distribution was the same in the two cohorts ($P = 0.5$). The two cohorts had similar body mass index (BMI), age at menarche, cycle length, birthweight, intrauterine exposure to tobacco, alcohol consumption, pelvic inflammatory disease and abdominal surgery. More infertile patients reported to have a chronic disease due to a higher prevalence of thyroid disease (6.5 versus 3.4%, $P = 0.05$) and endometriosis (7.1 versus 0.6%, $P < 0.001$) compared with the control group (data not shown). Fewer infertile patients reported to be present smokers (7.6 versus 20.9%, $P < 0.001$). However, the proportion of present smokers who smoked on a daily basis was higher among infertile patients (82 versus 55%, $P = 0.01$) (data not shown). The proportion of never smokers was the same in the two cohorts (54.5 versus 49.7%, $P = 0.1$), but more infertile patients had ceased smoking (36.1 versus 29.4%, $P = 0.04$). As seen in Table I,

Table II Endocrine and sonographic characteristic of the study population comprising 382 infertile patients and 350 controls assessed on CD 2–5.

Age groups	Infertile women					Controls					Infertile versus Controls
	Age group (years)				Total	Age group (years)				Total	*P*-value
	20–29	30–34	35–39	*P*-value		20–29	30–34	35–39	*P*-value		
Number of women [*n* (%)]	77 (20.2)	166 (43.5)	139 (36.4)		382 (100)	72 (20.6)	166 (47.4)	112 (32.0)		350 (100)	
Endocrine parameters											
AMH (pmol/l) [median (90% population limits)]	26.0 (12.0;76.0)	22.0 (6.7;66.0)	17.0 (3.9;59.0)	<0.001¶	22.0 (5.6;62.0)	24.0 (7.4;96.8)	19.8 (6.0;62.3)	15.5 (3.3;56.0)	<0.001¶	20.0 (4.6;66.2)	0.07¶
AMH categories [*n* (%)]											
<5.0	2 (2.6)	6 (3.6)	10 (7.2)	0.001†	18 (4.7)	2 (2.8)	4 (2.4)	12 (10.7)	<0.001†	18 (5.1)	0.2†
5.0–9.29	0 (0.0)	13 (7.8)	23 (16.5)		36 (9.4)	2 (2.8)	24 (14.5)	24 (21.4)		50 (14.3)	
9.3–20.99	20 (26.0)	60 (36.1)	42 (30.2)		122 (31.9)	27 (37.5)	59 (35.5)	31 (27.7)		117 (33.4)	
21.0–51.99	42 (54.5)	70 (42.2)	54 (38.9)		166 (43.5)	27 (37.5)	65 (39.2)	38 (33.9)		130 (37.2)	
≥52.0	13 (16.9)	17 (10.2)	10 (7.2)		40 (10.5)	14 (19.4)	14 (8.4)	7 (6.3)		35 (10.0)	
FSH (IU/l) [median (90% population limits)]	6.9 (4.1;9.6)	7.1 (4.7;12.6)	7.3 (4.9;14.3)	0.2¶	7.1 (4.7;12.5)	6.5 (4.1;9.6)	6.8 (4.8;10.7)	6.8 (4.2;11.9)	0.6¶	6.7 (4.5;10.8)	0.02¶
LH (IU/l) [median (90% population limits)]	5.9 (3.3;10.2)	6.1 (3.4;9.3)	6.3 (3.8;11.8)	0.3¶	6.1 (3.5;10.5)	5.8 (2.8;9.7)	5.5 (3.1;8.8)	5.4 (3.0;10.9)	0.2¶	5.5 (3.0; 9.3)	<0.001¶
LH/FSH-ratio [median (90% population limits)]	0.9 (0.6;1.6)	0.9 (0.5;1.5)	0.8 (0.5;1.6)	0.6¶	0.9 (0.5;1.5)	0.9 (0.5;1.8)	0.8 (0.4;1.3)	0.8 (0.4;1.5)	0.1¶	0.8 (0.4;1.5)	0.02¶
Sonographic characteristics											
Total AFC [median (90% population limits)]	26 (12;54)	21 (10;48)	16 (5;36)	<0.001¶	19 (7;44)	26 (12;50)	20 (8;41)	17 (5;38)	<0.001¶	20 (7;43)	0.6¶
AFC categories [*n* (%)]											
<7	0 (0.0)	3 (1.8)	13 (9.4)	<0.001†	16 (4.2)	1 (1.4)	3 (1.8)	11 (9.8)	<0.001†	15 (4.3)	0.6†
7–19	21 (27.3)	74 (44.6)	79 (56.8)		174 (45.5)	21 (29.2)	71 (42.8)	53 (47.3)		145 (41.4)	
20–44	50 (64.9)	78 (47.0)	43 (30.9)		171 (44.8)	43 (59.7)	85 (51.2)	46 (41.1)		174 (49.7)	
>44	6 (7.8)	11 (6.6)	4 (2.9)		21 (5.5)	7 (9.7)	7 (4.2)	2 (1.8)		16 (4.6)	
Ovarian cysts/ endometriomas [*n* (%)]	15 (19.5)	25 (15.1)	28 (20.1)	0.5†	68 (17.8)	3 (4.2)	8 (4.8)	3 (2.7)	0.7†	14 (4.0)	<0.001†
Ovarian volume [median (90% population limits)]	5.7 (3.3;12.9)	5.3 (2.8;10.3)	5.2 (2.4;12.0)	0.1¶	5.3 (2.7;11.2)	5.5 (2.6;9.1)	5.0 (2.8;8.9)	4.6 (2.2;9.6)	0.004¶	5.0 (2.6;9.0)	0.007¶
PCO morphology [*n* (%)]	55 (71.4)	92 (55.4)	54 (38.9)	<0.001†	201 (52.6)	51 (70.8)	85 (51.2)	49 (43.8)	0.001†	185 (52.9)	1.0†
AFC (2–10 mm) ≥12 [*n* (%)]	55 (71.4)	90 (54.2)	49 (35.3)	<0.001†	194 (50.8)	50 (69.4)	85 (51.2)	47 (42.0)	0.001†	182 (52.0)	0.7†
Ovarian volume >10 ml [*n* (%)]	14 (18.2)	15 (9.0)	18 (13.0)	0.1†	47 (12.3)	7 (9.7)	10 (6.0)	6 (5.4)	0.5†	23 (6.6)	0.008†
Uterine myomas [*n* (%)]	2 (2.6)	14 (8.4)	26 (18.7)	0.001†	42 (11.0)	1 (1.4)	5 (3.0)	9 (8.0)	0.05†	15 (4.3)	0.001†

Data are presented as number (percent) and median (90% population limits). For both cohorts, data are stratified according to age group and statistical difference across age groups tested. Data for the total infertile patients and controls are also presented and statistical differences between the two cohorts tested (the *P*-value to the right).

†χ^2 test.

¶Kruskal–Wallis test.

the number of cigarettes per day and the duration of smoking was the same in the two cohorts. The mean maternal age at menopause reported by infertile patients was higher compared with controls, although the mean difference was only 0.79 years (95% CI: 0.03;1.55). However, as the age at natural maternal menopause was unknown in many cases, this finding should be interpreted with caution. We found no association between AMH levels and any of the clinical and demographic baseline characteristics in the age-adjusted analyses with the exception of maternal menopause; when maternal age at menopause increased with 1 year, AMH levels increased by 3% (95% CI: 2;5%) (data not shown). The same association was found between AFC and maternal age at menopause (2%, 95% CI: 1;3%).

As seen in Table II, infertile patients had significantly higher levels of FSH and LH, and a higher LH/FSH-ratio than controls. The prevalence of polycystic ovarian morphology, defined by an AFC $\geq$12 and/or an ovarian volume $>$10 ml in at least one ovary (The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004), was the same in the two cohorts although infertile patients had a higher MOV than controls. FSH levels remained higher among infertile patients after age-adjustment (6%, 95% CI: 1;10%), and the per year age increase in serum FSH was 1% (95% CI: 0.5;2%) in both cohorts (data not shown).

Ovarian reserve parameters and age in infertile patients versus controls

As seen in Fig. 2, the power model was not superior to the linear model in predicting the age-related changed in neither AMH nor AFC within the age range of our cohort. The age-related depletion of the ovarian reserve was the same in the two cohorts; AMH levels decreased by 5.5% (95% CI: 4;7%) and AFC decreased by 5% (95% CI: 4;6%) per year age increase.

Figure 3A illustrates the linear correlation between AMH levels and AFC (for the total population $r_s = 0.83$, $P < 0.001$). As illustrated in Fig. 3B and C, no significant differences in neither AMH levels (11%, 95% CI: −1;24%) nor AFC (1%, 95% CI: −7;8%) were observed between the two cohorts after age-adjustment. After adjustment for smoking status, BMI, chronic disease, gestational age at birth and previous conception in addition to age, these findings persisted for both AMH (7%, 95% CI: −6;21%) and AFC (0%, 95% CI: −9;9%). As seen in Table III, the proportion of small follicles was significantly lower in infertile patients compared with controls with a mean difference of 5% ($P < 0.001$), whereas the proportion of large follicles was significantly higher in infertile patients with a mean difference of 3% ($P < 0.001$).

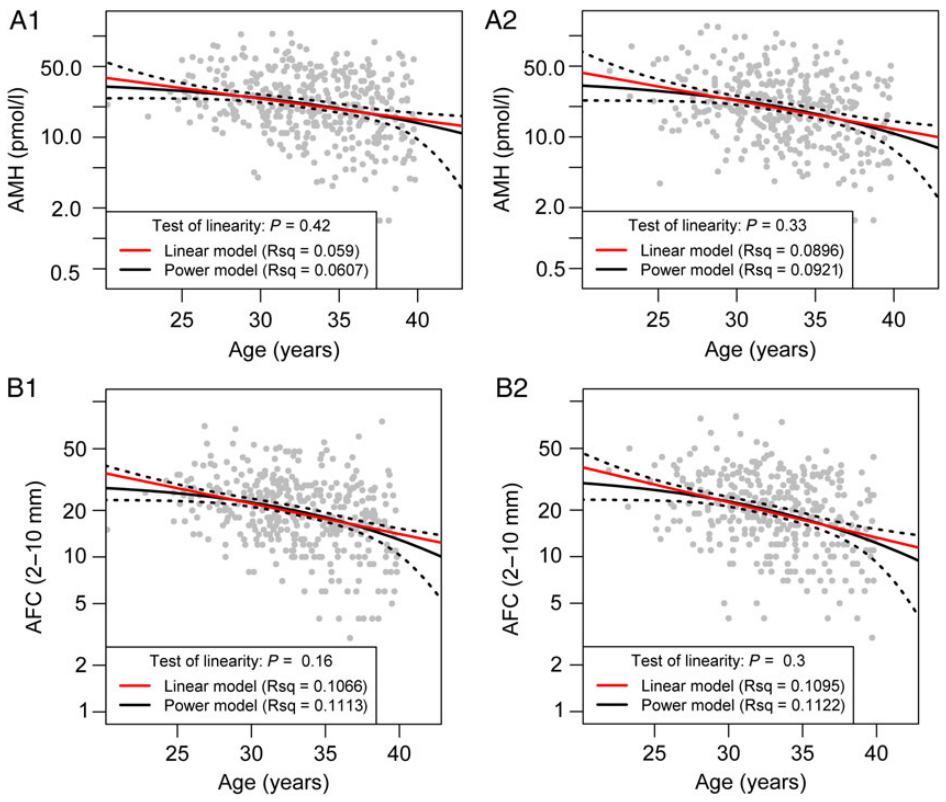

Figure 2 Relation between chronological age and ovarian reserve markers: (**A**) Serum AMH concentrations, and (**B**) total AFC (2–10 mm) in (1) 382 infertile patients aged below 40 newly referred to fertility treatment, and (2) 350 controls of the same age with no history of infertility. Estimated median lines of the linear (solid red lines) and the power model (solid black lines) are shown over the raw data (grey dots). The dotted lines represent 95% confidence limits. As seen, there was no significant difference between the linear and power model for neither AMH nor AFC in the age range (20–39 years) of the two cohorts.

Prevalence of low ovarian reserve

As seen in Table II, the prevalence of a very low serum AMH <5 pmol/l was 4.7% in patients versus 5.1% in controls (age-adjusted odds ratio (aOR): 0.9, 95% CI: 0.4;1.7) and highly age-dependent; women aged 35–39 years had a 3.1 (95% CI: 1.4;6.7) times higher risk of having an AMH level <5 pmol/l compared with women aged 30–34 years, whereas no difference was observed between the two younger age groups ($P = 0.8$). We found that AMH levels <9.3 pmol/l were less prevalent in infertile patients (14.1 versus 19.4%, age-aOR: 0.6, 95% CI: 0.4;0.9), whereas the prevalence of AFC <7 was the same in the two cohorts as seen in Table II (age-aOR: 0.9, 95% CI: 0.4;1.9). Irrespective of the cut off level applied, the prevalence of low ovarian reserve was the same in the two cohorts after adjustment for smoking status, BMI, chronic disease, gestational age at birth and previous conception in addition to age. Never smokers tended to have a lower risk of having an AMH <5.0 pmol/l (aOR: 0.4, 95% CI: 0.1;1.0) (data not shown).

Ovarian reserve and infertility diagnoses

As seen in Table IV, the most frequent infertility diagnoses were male infertility and unexplained infertility. Male infertility was more prevalent in the younger age groups ($P < 0.001$). We observed a significant interaction between age and male infertility in relation to both AMH and AFC. Male infertility was associated with higher AMH levels in patients aged 30–34 years (28%, 95% CI: 2;60%) and 35–39 years (42%, 95% CI: 7;88%). For AFC the coefficients were 17% (95% CI: 1;36%) for patients aged 30–34 years and 30% (95% CI: 6;58%) for patients aged 35–39 years. No differences in AMH levels and AFC were observed in patients aged 20–29 years with or without male infertility. AMH levels (2%, 95% CI: −11;17%) and AFC (−7%, 95% CI: −15;27%) remained identical in the two cohorts after the exclusion of patients with male infertility; so did the prevalence of low ovarian reserve irrespective of the cut off level applied, although the proportion of patients with AMH levels <5 pmol/l and AFC <7 had increased to 7.7% (age-aOR: 1.3, 95% CI: 0.6;2.7) and 7.2% (age-aOR: 1.4, 95% CI: 0.6;3.0), respectively.

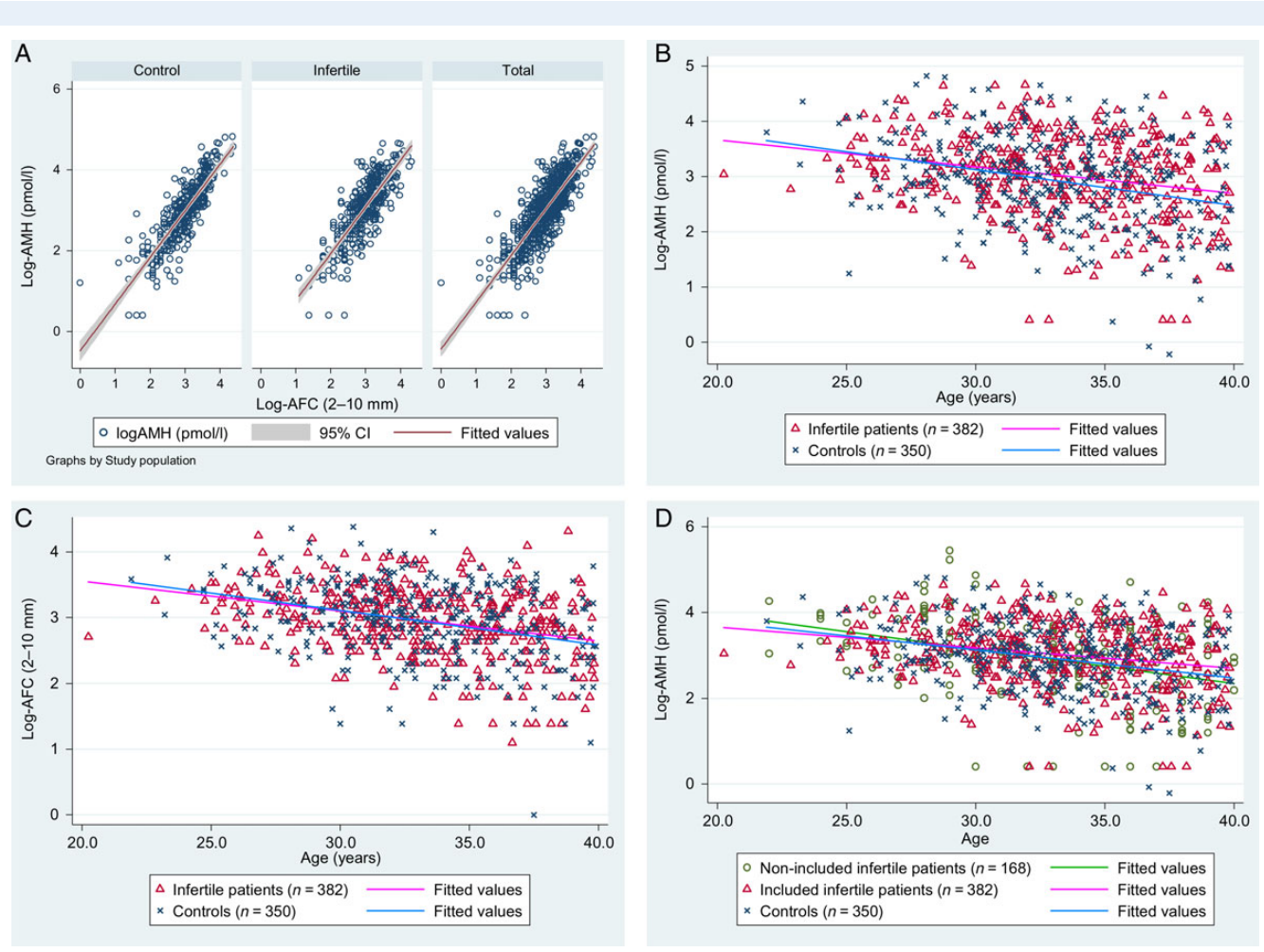

Figure 3 Scatter plots to illustrate the linear association between ovarian reserve parameters and age according to fertility status (infertile patients versus controls). (**A**) The correlation between AMH and AFC in controls ($n = 350$, $r^2 = 0.85$, $P < 0.001$), infertile patients ($n = 382$, $r^2 = 0.81$, $P < 0.001$) and the total study population ($n = 732$, $r^2 = 0.83$, $P < 0.001$). (**B**) The age-related decline in AMH according to the fertility status. (**C**) The age-related decline in AFC according to the fertility status. (**D**) AMH levels in 350 controls, 382 infertile patients included in the study and 168 infertile patients who had not been included as examination was not conducted at CD 2–5.

Table III Distribution of small (2–4 mm), intermediate (5–7 mm) and large (8–10 mm) antral follicles across age groups in 382 infertile patients and 350 controls examined on CD 2–5.

Age groups	Infertile (n = 382)			Controls (n = 350)			Mean difference (95% CI)		*P*-value
	20–29	30–34	35–39	20–29	30–34	35–39			
n (%)	77 (20.2)	166 (43.5)	139 (36.4)	82 (22.8)	175 (47.2)	114 (30.7)			
AFC, mean (90% population limits)									
Total AFC	26.9 (24.4;29.6)	22.8 (21.2;24.4)	18.2 (16.5;20.1)	27.8 (25.0;30.8)	22.6 (20.8;24.5)	18.8 (16.9;20.8)	−0.30 (−1.93;1.35)		0.7
AFC 2–4	18.0 (15.8;20.5)	14.6 (13.3;16.0)	NA (9.7;12.4)	19.9 (17.5;22.6)	15.6 (14.1;17.4)	12.5 (11.0;14.0)	−1.36 (−2.75;0.03)		0.06
AFC 5–7	7.7 (6.7;8.7)	6.9 (6.2;7.6)	NA (5.1;7.0)	7.3 (6.3;8.3)	6.4 (5.9;7.0)	5.6 (4.8;6.4)	0.42 (−0.23;1.06)		0.2
AFC 8–10	1.2 (0.8;1.6)	1.2 (0.9;1.5)	NA (1.0;1.5)	0.5 (0.4;0.7)	0.5 (0.4;0.7)	0.8 (0.6;1.0)	0.6 (0.41;0.83)		<0.001
AFC, mean proportion (90% population limits)									
AFC 2–4	0.65 (0.61;0.69)	0.63 (0.60;0.66)	0.59 (0.55;0.62)	0.7 (0.67;0.73)2	0.67 (0.65;0.70)	0.64 (0.61;0.67)	−0.05 (−0.08;−0.02)		<0.001
AFC 5–7	0.30 (0.27;0.33)	0.31 (0.28;0.34)	0.32 (0.29;0.35)	0.27 (0.24;0.30)	0.29 (0.27;0.31)	0.30 (0.27;0.33)	0.02 (−0.004;0.044)		0.1
AFC 8–10	0.05 (0.04;0.07)	0.06 (0.04;0.07)	0.09 (0.07;0.11)	0.03 (0.02;0.05)	0.03 (0.02;0.04)	0.06 (0.04;0.08)	0.03 (0.02;0.04)		<0.001

Data are presented as mean follicle number and mean proportion (90% population limits). Mean differences was estimated by permutation tests, and *P*-values calculated by bootstrapping.

One in four patients had unexplained infertility (Table IV). The prevalence of unexplained infertility was the same in the three age groups ($P = 0.5$). Patients with unexplained infertility had similar AMH levels (age-adjusted: −8%, 95% CI: −23;10%) and AFC (age-adjusted: −5%, 95% CI: −16;7%) compared with other patients. In an age-adjusted subgroup analysis comparing patients with unexplained infertility with controls, no differences in neither AMH levels (5%, 95% CI: −22;25%) nor AFC (−2%, 95% CI: −14;11%) were observed.

Non-response analysis

A serum AMH was available in 168 (83%) of the 203 eligible infertile patients who had not undergone a sonographic examination on CD 2–5. These 168 patients had a mean age ± SD of 32.6 ± 4.4 year and a median AMH level (90% population limits) of 20.5 (3.3; 74.0) pmol/l. The mean age was the same ($P = 0.1$), and the AMH level equivalent to that of the included patients (age-adjusted: −11%, 95% CI: −23;3%). If the 168 patients were included in the comparison between infertile patients and controls, AMH levels remained similar after age-adjustment (7%, 95% CI: −4;19%) as illustrated in Fig. 3D. In addition, no differences in the prevalence of AMH levels <5 pmol/l (age-aOR: 1.29, 95% CI: 0.7;2.3) and AMH levels <9.3 pmol/l (age-aOR: 0.7, 95% CI: 0.5;1.1) were observed.

Discussion

Our cohort of newly referred infertile patients aged 20–39 years did not have a lower ovarian reserve estimated by serum AMH levels and AFC than a control group of women of the same age with no history of infertility. Furthermore, low ovarian reserve, irrespective of the cut off level applied, was not overrepresented in the cohort of infertile patients, and the age-related decline in AMH levels and AFC were identical in the two cohorts. Interestingly, the distribution of follicle sizes in the infertile patients mimicked that of older women with a higher proportion of large follicles and a lower proportion of small follicles in addition to marginally higher FSH levels (Bentzen *et al.*, 2013). In line with our results, a study found similar AFC and FSH levels in 53 fertile and 62 infertile ovulatory women aged 35–45 years (Erdem *et al.*, 2003). Another study reported a steeper age-dependent decrease in AMH in 197 infertile women compared with 176 controls aged 19–47 years (Raeissi *et al.*, 2015). However, the infertile population in this study had an endocrine profile compatible with the menopausal transition with high FSH and low AMH levels in accordance with a diminished ovarian reserve and thus not compatible with the infertile patients included in our study (Raeissi *et al.*, 2015). Erdem *et al.* (2003) found MOV, AFC and FSH levels to be similar in infertile patients with tubal disease, unexplained or male infertility. In our study, male infertility was associated with higher AMH levels and AFC in infertile patients above 30 years of age. If patients with male infertility were excluded, the prevalence of low ovarian reserve increased, but AMH levels and AFC as well as the prevalence of low ovarian reserve remained the same in patients and controls. We found no association between unexplained infertility and a reduced ovarian reserve. Based on our findings, we conclude that an early age-related loss of oocytes is a minor contributing factor to infertility in the vast majority of patients of reproductive age. Evidently, as we did not include infertile patients referred directly for OD, our results do

Table IV Infertility diagnosis and duration in a cohort of 382 infertile patients newly referred to fertility treatment at The Fertility Clinic, Rigshospitalet, Copenhagen University Hospital from September 2011 to October 2013.

	Age group			Total
	20–29	30–34	35–39	
n (%)	77 (20.2)	166 (43.5)	139 (36.4)	382 (100)
Months of infertility [median (90% population limits)]	22 (10;48)	24 (7;54)	25 (12;58)	24 (10;56)
Previous fertility treatment [*n* (%)]	31 (40.3)	66 (39.8)	68 (48.9)	165 (43.2)
Infertility diagnosis [*n* (%)]				
Anovulation	2 (2.6)	2 (1.2)	0 (0.0)	4 (1.0)
Tubal factor infertility	1 (11.3)	6 (3.6)	10 (7.2)	17 (4.4)
Male infertility	50 (64.9)	87 (52.4)	51 (36.7)	188 (49.2)
Endometriosis	3 (3.9)	4 (2.4)	1 (0.7)	8 (2.1)
Multiple female factors	1 (1.3)	3 (1.8)	10 (7.2)	14 (3.7)
Male and female factors	5 (6.5)	18 (10.8)	20 (14.4)	43 (11.3)
Unexplained infertility	15 (19.5)	42 (25.3)	36 (25.9)	93 (24.4)
Other	0 (0.0)	4 (2.4)	11 (7.9)	15 (3.9)

A proportion of the patients had received fertility treatment at another centre prior to the referral. Data are presented for each age group and for the total cohort of infertile patients. Data are presented as number (percent) and median (90% population limits).

not exclude premature ovarian insufficiency (POI) as an important cause of female infertility.

Both AMH and AFC are quantitative makers of the ovarian reserve representing the available number of growing antral follicles. The AFC includes only visible antral follicles >2 mm, and serum AMH is a measure of the total mass of AMH-producing granulosa cells; the largest contribution to circulating AMH per follicle appears to come from antral follicles measuring 5–8 mm (Weenen *et al.*, 2004; Andersen *et al.*, 2010; Jeppesen *et al.*, 2013). Whether there is a correlation between quantity and quality remains uncertain. Spontaneous and treatment related pregnancies do occur in women with a diminished ovarian reserve (Fraisse *et al.*, 2008; Weghofer *et al.*, 2011). A recently published study found the same prevalence of AMH <1.1 ng/ml (~7.9 pmol/l) in women who had conceived naturally within <12 versus >12 months (Somigliani *et al.*, 2015). A study among young fertile women found no correlation between low AMH levels and reduced fecundity (Hagen *et al.*, 2012), whereas fecundity was found to be reduced in women aged 35–45 with AMH levels <10 pmol/l (Steiner *et al.*, 2011). AMH levels were associated with live birth rate irrespective of the oocyte yield (Brodin *et al.*, 2013), and in large multicentre trials serum AMH did predict cumulative birth rates after ART treatments (Arce *et al.*, 2013). However, recent meta-analyses conclude that AMH is a poor predictor of implantation, on-going pregnancy and live birth in ART (Broer *et al.*, 2013; Iliodromiti *et al.*, 2014; Tal *et al.*, 2015). According to our findings, the available number of growing antral follicles and their capability to produce AMH in the early follicular phase is the same in infertile patients and controls. We cannot rule out that infertility might be caused by a poor oocyte quality irrespective of the size of the follicular pool in some patients.

The major strength of the present study is the relative large number of participants who underwent a thorough examination and data collection. Nonetheless, in some of the subgroup analyses, the numbers may have been insufficient to show a difference. Infertile patients and controls were recruited during different time periods, but the maximal time interval was 3 years, and all participants were examined at the same centre using the same algorithm and ultrasound equipment, laboratory and AMH assay.

Not all eligible patients were included, as they had not undergone the examination at CD 2–5 primarily for logistic reasons. The AMH levels of the non-included patients were similar to that of the included patients and the controls. Thus, a major selection bias is unlikely. We did not include infertile patients referred directly for OD from other fertility clinics since they had already been diagnosed with a diminished ovarian reserve as the cause of their infertility. During the study period, this patient group comprised only 20 patients of whom many had iatrogenic POI or chromosomal abnormalities. The inclusion of such patients could have biased the result.

The fact that the control group comprised health care workers may have introduced a selection bias, as their risk profile may not be representative of the background population. More patients than controls were diagnosed with an ovarian cyst. The majority of the observed cysts were small (<4 cm) corpus luteum cysts and unlikely to impact the AFC. As endometrioses is a cause of infertility and infertile patients are often screened for thyroid disease, it is not surprising that the two cohorts differ in these respects. The smoking status in the two cohorts was different. However, we concluded that the overall exposure to smoking was similar in patients and controls as the number of cigarettes smoked per day and the total duration of smoking were the same in the two cohorts. Nonetheless, as current smoking is associated with suppressed AMH levels (Dólleman *et al.*, 2013; Fleming *et al.*, 2015), we included smoking status in addition to other possible confounders in the multiple regression analyses.

In conclusion, our results indicate that infertile women aged 20–39 years have the same age-related depletion of the ovarian reserve as women of the same age with no history of infertility. In addition, women with a low ovarian reserve were not overrepresented among newly referred infertile patients. Thus, the apparent frequent observation of poor responders in assisted reproduction may not be due to an overrepresentation of patients with an early age-related decline in the

ovarian reserve, but rather a result of the expected age-related decline in the pool of growing antral follicles at the time of follicular recruitment and selection.

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Authors' roles

The study was established at the initiative of A.N.A. H.W.H. was project coordinator and recruited and examined the majority of the infertile patients. L.L.T., A.N.A., A.L. and M.P.L. participated in the recruitment and examination of infertile patients. J.G.B. was principal investigator of the control study. A.L. and A.P. planned the project together with A.N.A., J.G.B. and H.W.H. H.W.H. carried out the statistical analysis with the assistance of J.L.F. and drafted the paper. All authors contributed to the interpretation of the results, critically revised the manuscript and approved the final version.

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Conflict of interest

None declared.

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