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ORIGINAL ARTICLE ***Reproductive endocrinology***

Serum anti-Müllerian hormone concentration and follicle density throughout reproductive life and in different diseases—implications in fertility preservation

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STUDY QUESTION: How do anti-Müllerian hormone (AMH) serum concentrations and follicle densities (FDs) change with age and disease and what are the implications for fertility preservation?

SUMMARY ANSWER: AMH concentrations and FD do not correlate in young women, and AMH but not FD is reduced in some diseases, limiting the value of AMH as a predictive parameter of ovarian tissue transplantation.

WHAT IS KNOWN ALREADY: AMH is widely used as a parameter to estimate the ovarian reserve. However, the reliability of AMH to predict total number of follicles and the FD is questionable. Women with lymphoma and leukaemia have been shown to have reduced AMH concentrations, but it is unknown if the FD is also reduced. In fertility preservation it is essential to estimate the correct total number of follicles and the FD, as ovarian tissue should only be cryopreserved if ovarian reserve is high. Furthermore, the amount of tissue to be transplanted should be based on the estimation of the real FD.

STUDY DESIGN, SIZE, DURATION: This retrospective observational study included 830 women (mean ± SD age, 28.2 ± 6.81 years; range, 4–43 years) with malignant ($n = 806$) and benign ($n = 24$) diseases who cryopreserved tissue in a single centre as part of a national fertility preservation programme. Females with ovarian surgery or known predispositions for a reduced ovarian reserve were excluded. AMH concentrations and FD were evaluated from March 2011 to September 2016.

PARTICIPANTS/MATERIALS, SETTING, METHODS: AMH concentrations were analysed before gonadotoxic therapies. Standardized biopsies, obtained from different areas of ovarian cortex, were collected. FD was analysed after tissue digestion and calcein staining and was expressed as average number of primordial and primary follicles count per 3 mm biopsy and per cubic millimeter tissue. AMH concentrations and FD were analysed in relation to age and diagnosis group. Both parameters were age adjusted, and associations between the different diagnosis groups and AMH versus FD were assessed.

MAIN RESULTS AND THE ROLE OF CHANCE: Mean ± SD AMH concentration was 3.1 ± 2.81 g/ml, mean FD per 3 mm biopsy was 137 ± 173.9 and 19.4 ± 24.60 per mm^3. Maximum AMH concentrations were found in children and teenagers at the age of 6–10 years (5.71 ng/ml) and in adults at the age of 21–25 years (3.33 ng/ml). FD was highest in young children up to an age of 15 years and decreased with increasing age. AMH and FD were not correlated in women ≤20 years and weakly to moderately correlated in women 21–40 years ($r = 0.24$–0.39). Age-adjusted correlations between AMH and FD were demonstrated in several diagnosis groups such as breast cancer, leukaemia, sarcoma, gastrointestinal cancer and gynaecological cancer but not in the groups exhibiting Hodgkin's and non-Hodgkin's lymphoma, cerebral cancer, other types of malignancies and other types of benign diseases. Further statistical analysis supported the finding that, in some diagnosis

groups such as Hodgkin's lymphoma and in gynaecological cancer, AMH concentrations but not FDs are reduced, questioning the prognostic accuracy of AMH for the FD in these diseases.

LIMITATIONS, REASONS FOR CAUTION: Even though biopsies were taken from different sites, heterogenous distribution of follicles might have had some effect on the accuracy of the analysis.

WIDER IMPLICATION OF THE FINDINGS: AMH should be used with care to estimate the total ovarian reserve and FD of cancer patients in young women in some diseases. Therefore, calculating the amount of ovarian tissue to be transplanted based solely on AMH might be inaccurate whereas FD might be a better parameter.

STUDY FUNDING/COMPETING INTEREST(S): The study did not receive any exterior funding.

Key words: ovarian tissue / ovarian reserve / anti-Müllerian hormone / primordial follicle / primary follicle / fertility preservation / cancer

Introduction

Precise information about the ovarian reserve from childhood to the end of the reproductive phase is of great clinical importance. For example, it is necessary to predict the ovarian response during IVF treatment as precisely as possible (Broekmans *et al.*, 2006; Nelson *et al.*, 2015) to estimate the risk of chemotherapy-induced reduction of the follicular pool (Brougham *et al.*, 2012), to calculate the required amount of ovarian tissue for transplantation (Beckmann *et al.*, 2018; Liebenthron *et al.*, 2019) and to allow fertility prognosis with a low ovarian reserve (Broer *et al.*, 2011).

A wide variety of parameters have been tested in the past to estimate ovarian reserve. These include female age, levels of FSH and inhibin B, antral follicle count (AFC) and the concentration of anti-Müllerian hormone (AMH) (Haadsma *et al.*, 2007; Broer *et al.*, 2010; Wallace and Kelsey, 2010; Dólleman *et al.*, 2013; Jeppesen *et al.*, 2013). Comparative studies have shown that AMH and AFC best reflect the response to gonadotropins (Broekmans *et al.*, 2006; Broer *et al.*, 2013; Hvidman *et al.*, 2016; Sermondade *et al.*, 2018). Automated and therefore more accurate AMH assays have enabled the introduction of age-related reference levels for AMH (Lee *et al.*, 2017), so that AMH is regarded as the most reliable marker for determining ovarian reserve. Accordingly, mainly AMH is used as a parameter for estimating the ovarian reserve (Helden and Weiskirchen, 2017).

However, the AMH concentration only reflects the number of AMH-secreting follicles, i.e. the predominantly antral secondary follicles, and is therefore only a kind of surrogate parameter for the total ovarian reserve, which is defined by the total number of all follicles, including the primordial and primary follicles. The total ovarian reserve and the follicle density (FD) are decisive for questions in the field of fertility protection, both play a role in the decision for or against cryopreservation of ovarian tissue (von Wolff *et al.*, 2018a) and may also be relevant in the calculation of the amount of ovarian tissue to be transplanted (Beckmann *et al.*, 2018; Liebenthron *et al.*, 2019). It has also been postulated that the ovarian reserve is reduced in various oncological diseases such as Hodgkin's lymphoma, as the AMH concentration and ovarian response are less during gonadotropin stimulation (Lawrenz *et al.*, 2011; Lekovich *et al.*, 2016; von Wolff *et al.*, 2018b). It is unclear, however, whether the total ovarian reserve and the FD are also reduced.

In 2010, Wallace and Kelsey developed a model of the age-dependent number of non-growing follicles based on 8 studies with a total of 325 women. Furthermore, Hansen *et al.* (2011) conducted a correlation analysis of the number of primordial follicles among 42 women aged 26–52 years with AMH, AFC and FSH, among others. In all these studies, however, only small patient collectives or studies from multiple centres were combined, and it remained unclear whether the analyses also have clinical significance, especially in the area of fertility protection.

As a result of this, we performed a large-scale analysis in a centre with patients from childhood to the end of the reproductive phase. The FD was determined by means of standardized ovarian biopsies for the determination of the FD and determination of the serum AMH concentration using ELISA in 830 patients whose ovarian tissue and blood serum were cryopreserved prior to gonadotoxic therapy. Both parameters were presented age dependent, correlated with each other and analysed in different malignant and benign diseases.

Materials and Methods

Prior to initiation of ovarian tissue cryopreservation an ethical vote, approved by the institutional review board of the University of Bonn (no. 007/09), was obtained to offer a central cryobanking service and to use up to 10% of the incoming tissue for quality control measures and patient-related research. Ovarian tissue was collected from centres of the network *Ferti*PROTEKT, a German, Austrian and Swiss network for fertility preservation (www.fertiprotekt.com) and transported to the ovarian tissue cryobank in Bonn, Germany.

Retrieval, transportation and preparation of ovarian tissue and serum

Eight hundred and thirty women (mean ± SD age, 28.24 ± 6.81 years; range, 4–43 years) underwent ovarian tissue freezing prior to gonadotoxic therapy from March 2011 to September 2016. For each patient, around half an ovary, corresponding to 25% of the total ovarian tissue, was removed by laparoscopy. The entire tissue was immediately transferred into a tube with Custodiol medium (Dr Franz Köhler Chemie GmbH, Bensheim, Germany). Transportation to the cryobank in Bonn was performed either by direct or by overnight transportation as described previously (Liebenthron *et al.*, 2019). The tissue reached the cryobank within a maximum of 22 hours. Tissue preparation was immediately performed in a sterile Class II lamina air flow cabinet in a contamination-free environment. The entire ovarian piece was placed in a culture dish containing fresh Custodiol on a convolution cooling plate (UKH602, FRYKA Kältetechnik GmbH, Esslingen, Germany) that was pre-cooled to a temperature of 2°C to 8°C. Cortex strips (~8 × 4 × 1mm) were prepared for potential transplantation

and cryopreserved. From the remaining cortical tissue, standardized biopsies were obtained from different areas of the prepared cortex using a biopsy punch (PFM Medical AG, Cologne, Germany). Tissue was cryopreserved and stored in the vapour phase of liquid nitrogen as described previously (van der Ven *et al.*, 2016; Beckmann *et al.*, 2018; Liebenthron *et al.*, 2019).

AMH analysis

For determination of AMH serum levels, blood samples were drawn prior to laparoscopy and transported with the ovarian tissue to the cryobank at 2–8°C. Samples were centrifuged at 1500*g* for 10 minutes and the obtained serum was stored in 0.5 ml aliquots at −80°C until further analysis. AMH concentration (ng/ml) was analysed using the AMH Gen II ELISA Kit (Beckman Coulter GmbH, Krefeld, Germany) according to manufacturer's instructions (Kumar *et al.*, 2009). The intra- and inter-assay coefficients of variation were less than 5.4% and 5.6%, respectively. The limit of quantification was 0.16 ng/ml.

Analysis of FD

Concentration of viable primordial and primary follicles were analysed in standardized biopsies—initially in one standardized 3 mm biopsy with 1 mm thickness (volume per biopsy 7.02 mm^3), later, to reduce the influence of follicle heterogenicity, the protocol was adapted and three 2 mm standardized biopsies with 1 mm thickness (volume per biopsy 3.12mm^3) were taken from different areas of the cortex. To ensure that all values determined remained comparable, the follicle numbers counted in 3 × 2 mm biopsies were converted to the volume of a 3 mm biopsy and to a volume of 1 mm^3. For statistical analysis concentrations per 3 mm biopsies and per cubic millimeter were used.

Variation of FD in different biopsies was analysed in 20 women (mean ± SD age, 30.2 ± 5.37 years; range, 18–38 years). The coefficient of variation of FD (per 3 mm biopsy) was 75.4 ± 29.03 years (range, 21–128 years).

Tissue biopsies were incubated for 1.5 hours in CTS AIM-V culture medium (GIBCO® CTS™ AIM V® SFM, Fisher Scientific GmbH, Schwerte, Germany) supplemented with 0.2% calcein AM/CTS DPBS (V/V) (GIBCO® CTS™ DPBS, Fisher Scientific GmbH, Schwerte, Germany) and 1 mg/ml collagenase Type 1A (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) at 37°C. Digestion was mechanically enhanced by pipetting every 30 minutes; the reaction was terminated at room temperature and by the addition of an equal volume of cold CTS DPBS supplemented with serum albumin supplement substitute (SSS; Irvine Scientific, Santa Ana, USA) (1 g serum albumin per 100 ml CTS DPBS). The follicles descended to the bottom of the dishes and were analysed from below using an inverse fluorescence microscope (Fig. 1). Three freshly prepared standardized 2 mm biopsies from different cortex areas were used for counting of viable follicles after digestion.

Calcein and cleaved Calcein-AM, cleaved by cell esterases found in viable cells, accumulated in oocytes and surrounding granulosa cells. Viable follicles were recognizable by a layer-like, close association of granulosa cells surrounding the oocyte with a uniform green fluorescence (495 nm). Primordial follicles were identified by a single flattened layer and primary follicles by a single cuboidal layer of granulosa cells. The evaluation was carried out at a 10-fold magnification and the whole number of follicles in the entire well of three digested biopsies was determined by meander-shaped counting (Liebenthron *et al.*, 2013; Bastings *et al.*, 2014; Beckmann *et al.*, 2018; Liebenthron et al., 2019). The FD was expressed as average follicle count per biopsy.

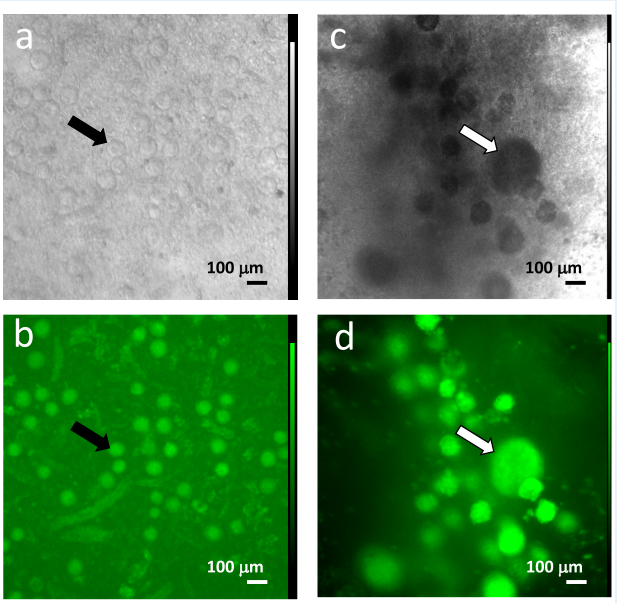

Figure 1 Standardized cortical tissue biopsies samples after digestion with collagenase and mechanical enhancement and staining with calcein from a prepubertal girl (8 years) (a and b) and an adult woman (27 years) (c and d). Top row with inverse light and bottom row uniform green fluorescence light (495 nm). Primordial follicles with single flattened layer (black arrow) and growing secondary follicle (white arrow).

Statistical analyses

AMH and FD were summarized as mean, minimum (Min), maximum (Max), 10th, 25th, 75th and 90th percentile, standard error of mean (SEM) and SD (Figs 1 and 2 and Table II). Due to the skewed distribution of AMH and FD the log-transformed values were used in further analysis. The association between AMH and FD were estimated by Pearson's correlation coefficient (Fig. 4 and Table III). Analyses of covariance were performed to compare the age-adjusted AMH and FD between breast cancer patients (reference group) and other patients (Supplementary Tables SI and SII); breast cancer patients had already served as a control group in previous study (von Wolff *et al.*, 2018b). In case of multiple pairwise comparisons, Bonferoni corrections were applied. All calculations were performed with SPSS Statistics 25 (IBM Corp., Armonk, NY, USA). *P* values < 0.05 were considered as statistically significant.

Results

Characteristics of patients are shown in Table I. Serum AMH concentration and FD was analysed in 830 women (mean ± SD age, 28.2 ± 6.81 years; range, 4–43 years). A total of 469 women were diagnosed with breast cancer, 165 women with Hodgkin's lymphoma, 40 women with non-Hodgkin's lymphoma, 16 with leukaemia, 43

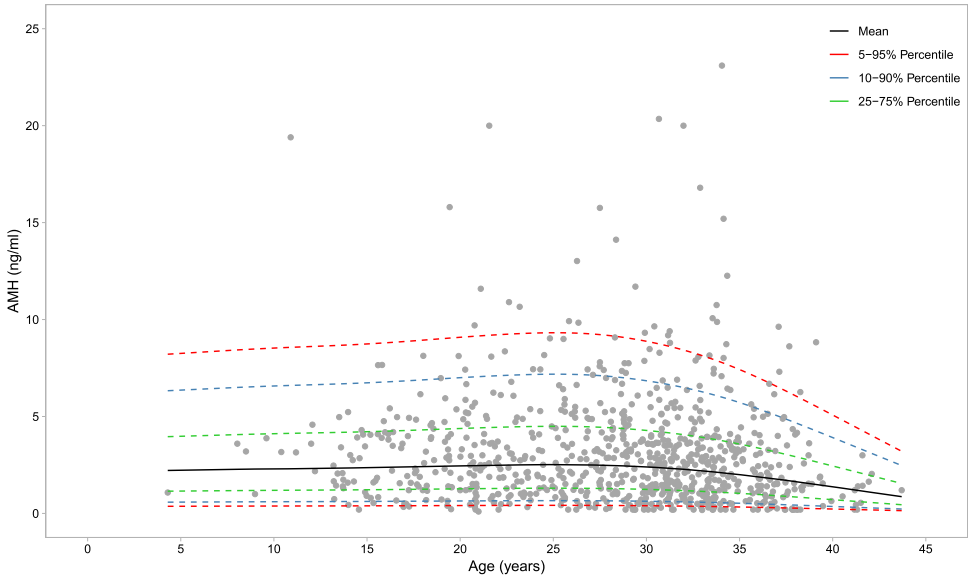

Figure 2 Scatterplot of serum anti-Müllerian hormone (AMH) concentration (ng/ml) in relation to age.

Table I Patient characteristics. Data are mean ± SD (range) or *n* (%)

Age, years	28.2 ± 6.8 (4-43)
Anti Müllerian hormone (AMH) ng/Ml	3.1 ± 2.8 (0.1-23.1)
Primordial and primary follicle density (FD) per 3 mm biopsy	137 ± 174.0 (1-1751)
FD per mm^3	19.4 ± 24.6 (0-248)
Diagnosis groups (per 830 patients)	
Breast cancer	469 (56.5)
Hodgkin's lymphoma	165 (19.9)
non-Hodgkin's lymphoma	40 (4.8)
Leukaemia	16 (1.9)
Sarcoma	43 (5.2)
Cerebral cancer	17 (2.0)
Gastrointestinal cancer	23 (2.8)
Gynaecological cancer[1]	21 (2.5)
Other types of malignancies[2]	12 (1.4)
Other types of benign diseases[3]	24 (2.9)

[1] Endometrial cancer, cervical cancer, vulva cancer, chorion cancer (without ovarian malignancies)
[2] 4x Myelodysplastic syndromes, 2x Malignant nerve sheath tumors, 1x Mediastinal tumor, 1x Metachromatic leukodystrophy, 1x Myelofibrosis, 1x Pseudomyxoma peritonei, 1x Ear, Nose, Throat (ENT) cancer, 1x Langerhans cell histiocytosis
[3] 18x Hemoglobinopathies, 4x Hydatidiform moles, 2x Desmoid fibromatosis

with sarcoma, 17 with cerebral cancer, 23 with gastrointestinal cancer, 21 with gynaecological cancer, 12 with other types of malignancies and 24 women with benign diseases, mainly hemoglobinopathies (18/24).

Scatterplots of AMH and FD were drawn in relation to age (Figs 2 and 3). AMH concentrations and FD of different age groups are shown in Table II. Mean ± SD AMH concentrations were highest in age groups 6–10 years (5.7 ± 6.78 ng/ml), 21–25 years (3.4 ± 2.90 ng/ml) and 26–30 years (3.3 ± 2.88 ng/ml). Concentrations were lowest in one child at the age of 4 years (1.1 ng/ml) and in women 41–45 years (1.2 ± 0.85 ng/ml). FD was highest in one child at the age of 8 years (1751 per 3 mm biopsy, 248 per mm^3) and in the age group 6–10 years (mean 678 ± 646.4 per 3 mm biopsy and 96 per mm^3; Table II).

AMH and FD correlation were weakly but statistically significantly correlated in women aged 21-35y ($r = 0.24$–0.269) and more strongly in women aged 36-40y ($r = 0.394$) (Table III). In contrast, AMH concentrations and FD was not correlated in women ≤20y ($r = -0.038$) (Fig. 4).

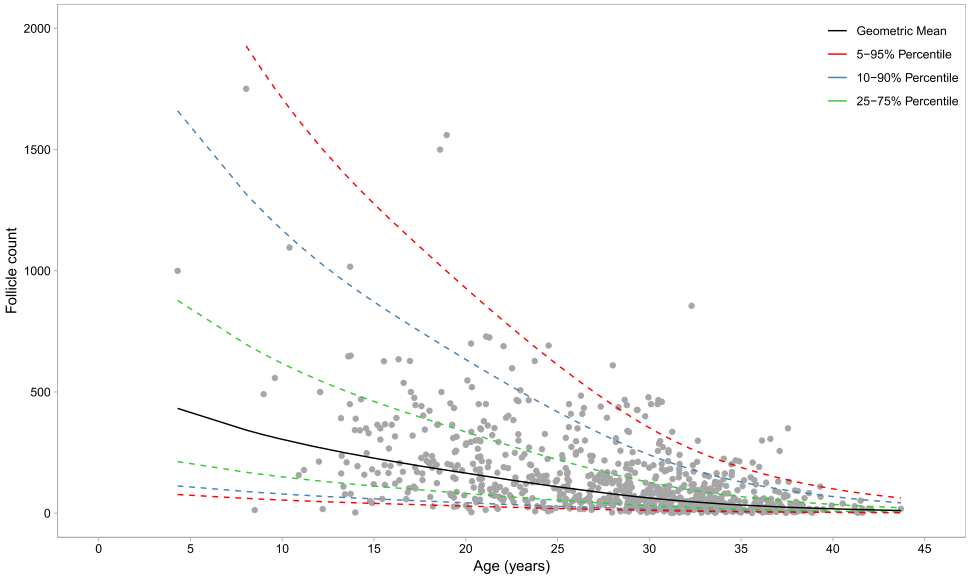

Figure 3 Scatterplot of follicle density (FD; primordial and primary follicles) in relation to age.

Table II AMH concentration and follicle density by age.

Age group	n	AMH ng/ml								
		Mean	Min	Max	SD	10th percentile	25th percentile	50th percentile	75th percentile	90th percentile
0-5	1	1.08	-	-	-	-	-	-	-	-
6-10	6	5.71	1.00	19.40	6.78	-	-	-	-	-
11-15	42	2.76	0.20	7.66	1.78	0.76	1.43	2.33	4.06	4.91
16-20	101	3.03	0.10	15.80	2.42	0.50	1.30	2.49	4.29	5.83
21-25	124	3.36	0.20	20.00	2.90	0.83	1.32	2.55	4.47	7.11
26-30	219	3.33	0.20	20.35	2.88	0.50	1.23	2.81	4.49	6.99
31-35	242	3.05	0.18	23.10	3.04	0.53	1.17	2.39	3.71	6.37
36-40	84	2.26	0.20	9.63	2.12	0.20	0.84	1.57	3.19	5.07
41-45	11	1.21	0.20	3.00	0.85	0.20	0.45	1.20	1.64	2.81
Age group	n	Follicle density (follicles/3 mm biopsy)								
		Mean	Min	Max	SD	10th percentile	25th percentile	50th percentile	75th percentile	90th percentile
0-5	1	1000.00	-	-	-	-	-	-	-	-
6-10	6	677.67	13.00	1751.00	646.39	-	-	-	-	-
11-15	42	2783.86	3.00	1017.00	203.02	52.70	149.25	225.00	365.50	588.90
16-20	101	261.22	4.00	1560.00	237.62	59.00	113.50	204.00	345.50	495.20
21-25	124	181.28	8.00	729.00	164.28	28.00	62.50	130.00	241.75	430.00
26-30	219	125.45	3.00	610.00	121.94	18.00	46.00	81.00	161.00	300.00
31-35	242	67.42	1.00	856.00	79.72	10.30	20.00	42.00	91.00	159.80
36-40	84	48.17	2.00	350.00	65.84	6.00	12.00	25.00	59.00	102.00
41-45	11	16.27	2.00	52.00	14.40	2.00	5.00	14.00	19.00	47.40

Table III Correlation between serum anti-Müllerian hormone concentration and follicle density by age group

Age group (y)	n	Pearson correlation coefficient	P
6–10	6	−0.177	0.738
11–15	42	−0.206	0.190
16–20	101	0.001	0.989
21–25	124	0.240	0.007
26–30	219	0.266	<0.001
31–35	242	0.269	<0.001
36–40	84	0.394	<0.001
41–45	11	0.080	0.81

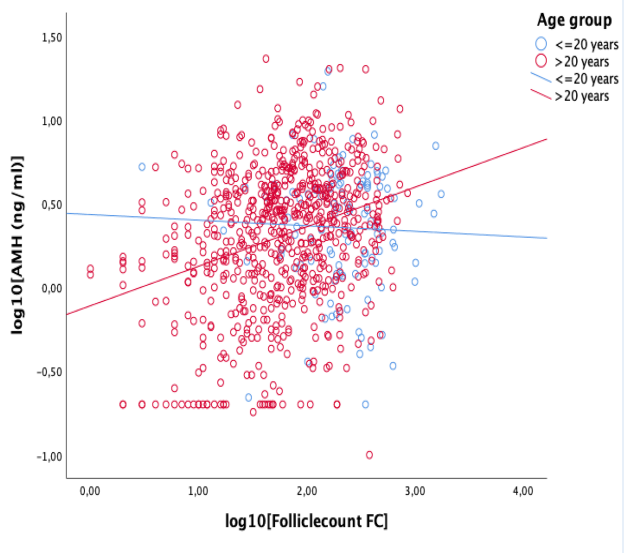

Figure 4 Correlation analysis and linear regression analysis of serum AMH values (ng/ml) and FD (primordial and primary follicles) in women ≤20 years ($r = -0.038$) and >20 years ($r = 0.289$).

AMH concentrations and FD were age adjusted and analysed in different diseases (Table IV). AMH concentration was highest in gastrointestinal cancer (4.09 ng/ml, SE 0.58) and lowest in leukaemia (1.79 ng/ml, SE 0.69). FD was highest in cerebral cancer (192.67, SE 36,77, per 3 mm biopsy and 27.26 per mm^3) and lowest in non-Hodgkin's lymphoma (91.67, SE 23.80, per 3 mm biopsy and 12.97 per mm^3) (Table IV). Furthermore, in different diagnosis groups AMH concentrations and FD suggested different characteristics. For instance, the FD in the breast cancer group was 135.76, SE 7.88, per 3 mm biopsy and in the Hodgkin's-lymphoma group (134.79, SE 13.04, per cubic millimeter), therefore almost equal, whereby the AMH concentration in the Hodgkin's-lymphoma group (2.09 ng/ml, SE 0.24) was much lower compared to the breast cancer (3.49 ng/ml, SE 0.15). Subsequently age-adjusted correlations of AMH concentrations and FD per diagnosis group were conducted (Supplementary Table SI) and although they showed an overall relationship ($r = 0.255$, $P < 0.05$), differences were noticeable in individual diagnosis groups. Significant correlations ($P < 0.05$) were found in the groups breast cancer, leukaemia, sarcoma, gastrointestinal cancer and gynaecological cancer, but not in the groups Hodgkin's and non-Hodgkin's lymphoma, cerebral cancer, other types of malignancies and other types of benign diseases, included predominantly patients with hemoglobinopathies ($n = 18/24$).

In order to show more clearly that AMH is limited as a prognostic factor for the real FD in some diseases, breast cancer women were defined as a reference group as a previous study had suggested that the ovarian reserve was not negatively affected in women with breast cancer (von Wolff *et al.*, 2018b). Pairwise correlations between AMH and FD differed significantly ($P < 0.05$) between the breast cancer group and the groups Hodgkin's lymphoma, gynaecological cancer and the group 'other types of benign diseases' with predominantly hemoglobinopathical patients ($n = 18/24$) (Supplementary Table II), caused by significant lower AMH levels in contrast to the FD, which was equivalent—supporting the finding that some malignant diseases may be associated with reduced AMH concentrations—but with still normal follicle densities. These correlations were independent of whether the relationship in the respective group was significant or not.

Discussion

In our study, we created scatterplots for the serum AMH concentration and FD of the primordial and primary follicles as a function of age. The serum AMH concentration did not correlate with the FD in females ≤20 years but did to a certain degree in females >20 years of age. The poor correlation between the two parameters was also evident in individual diseases such as Hodgkin's and non-Hodgkin's lymphomas, leukaemia and several benign diseases.

The large number of patients and the analysis of the ovarian reserve with FD are the strengths of this study, since the AMH concentration is only one parameter for the total number of growing, predominantly antral secondary follicles and therefore does not completely reflect either the FD or the total ovarian reserve.

However, analysing the FD also has some limitations. Even though FD was analysed in most cases in different cortex regions, heterogeneity of FD might still have affected the analysis. This was confirmed by the coefficient of variation, calculated in 20 women. Furthermore, inaccuracies could have resulted from a bias by the involved researchers. As the analysis was part of a daily routine procedure, we were not able to set up a double-blinded analysis. However, in case the analysis was

Table IV Age adjusted anti Müllerian hormone and follicle densities by diagnosis group.

Diagnosis group	Mean	SE	95% CI
Anti-Müllerian hormone (ng/ml)			
Breast cancer	3.5	0.15	3.2;3.8
Hodgkin's lymphoma	2.1	0.24	1.6;2.6
non-Hodgkin's lymphoma	2.6	0.44	1.8;3.5
Leukaemia	1.8	0.69	0.4;3.2
Sarcoma	2.9	0.45	2.0;3.8
Cerebral cancer	3.5	0.68	2.2;4.9
Gastrointestinal cancer	4.1	0.58	3.0;5.2
Gynaecological cancer	3.7	0.61	2.5;4.9
Other types of malignancies	2.0	0.81	0.4;3.6
Other types of benign diseases	2.2	0.61	1.0;3.4
Follicle density/3 mm biopsy			
Breast cancer	135.8	7.9	120.3;151.2
Hodgkin's lymphoma	134.8	13.0	109.2;160.4
non-Hodgkin's lymphoma	91.7	23.8	45.0;138.4
Leukaemia	94.7	37.6	21.0;168.4
Sarcoma	181.1	24.6	132.8;229.3
Cerebral cancer	192.7	36.8	120.5;264.8
Gastrointestinal cancer	145.5	31.4	84.0;2017.1
Gynaecological cancer	149.1	33.0	84.4;213.9
Other types of malignancies	148.7	43.6	63.1;234.3
Other types of benign diseases	134.8	32.8	70.4;199.2
Follicle density/mm^3			
Breast cancer	19.2	1.1	17.0;21.4
Hodgkin's lymphoma	19.1	1.8	15.5;22.7
non-Hodgkin's lymphoma	13.0	3.4	6.4;19.6
Leukaemia	13.4	5.3	3.0;23.8
Sarcoma	25.6	3.5	18.8;32.5
Cerebral cancer	27.3	5.2	17.1;37.5
Gastrointestinal cancer	20.6	4.4	11.9;29.3
Gynaecological cancer	21.1	4.7	11.9;30.3
Other types of malignancies	21.0	6.2	8.9;33.2
Other types of benign diseases	19.1	4.6	9.9;28.2

difficult to interpret, the second of our two trained researchers was involved in the analysis.

When data are interpreted, it should also be kept in mind that ovarian volume and ovarian surface area are smaller during childhood and at the end of the reproductive phase, which means that the total number of follicles can only be deduced to a limited extent from the cortical FD. Thus, ovarian volume changes with age (Orsini *et al.*, 1984; Cohen *et al.*, 1993; Goldberg and McGahan, 2006; Perven *et al.*, 2014; El Issaoui *et al.*, 2016).

In young infants (1 years), ovarian volume is ~1 cm^3 and decreases slightly at the age of 2–6 years. Ovarian volume increases in prepubertal girls (6–10 years) to 1.2–2.3 cm^3, in premenarchal girls (11–12 years) to 2–4 cm^3 and in postmenarchal girls to ~8cm^3 (2.5–20 cm^3). The maximum ovarian volume is reached in young adults (8–14 cm^3) and declines thereafter. In a cadaver study the average ovary size in females aged 14–45 years was determined as 5.95 ± 1.37 cm^3 (right ovary) and 4.24 ± 1.05 cm^3 (left ovary) (Perven *et al.*, 2014).

As the follicles are located just underneath the cortical surface, the ovarian surface might be a better parameter to calculate the number of follicles per ovary. However, the formula V (ovarian volume) = (transverse × anteroposterior × craniocaudal length) × $\pi/6$ (Rosendahl *et al.*, 2010; Kelsey *et al.*, 2013) reveals that the relative differences of the ovarian volume and the ovarian surface in relation to age are similar, and therefore the ovarian volume can be used to calculate the total number of follicles. Assuming that the ovarian volume measures 2 cm^3 at the age of 6 years, 8 cm^3 at the age of 15 years, 12 cm^3 at the age of 25 years and 8 cm^3 at the age of 40 years, the calculated FD per cubic millimeter would need to be corrected at the age of 15 years and 40 years by ×0.67 and at the age of 6 years by ×0.17 to compare the

total number of primordial and primary follicles with women aged 25 years.

Although these relationships are based only on mathematical calculations and are therefore only conditionally applicable to an organ system, they show that correlations of the AMH concentration, as a secretory product of the predominantly antral follicles and the FD as markers for primordial and primary follicles, should only be interpreted with caution, bearing in mind the diversity of these parameters and in a comparative analysis after age adjustment (Table IV; Supplementary Tables I and II).

Our study shows that both FD and AMH levels decrease at the end of the reproductive phase. In childhood, however, the FD and AMH concentrations show a different tendency. FD is higher in adults, whereas AMH concentrations tend to be lower apart from the small group of children aged 6–10 years, which might be due to random fluctuations. Lower concentration in childhood is in line with previous studies (Kelsey *et al.*, 2011). The decreased AMH levels were explained in previous studies by the fact that the ovaries in childhood are not yet subject to any relevant gonadotrophic stimulation, which makes the number of AMH-secreting follicles even lower.

The FD is higher in our study in all age groups of children and adolescents than in adulthood. If, however, the lower ovarian volume and the correction factor mentioned above in childhood are taken into account, the FD is higher in childhood, but the total number of primordial and primary follicles should differ only insignificantly from the total number in young adulthood, considering the correction factors mentioned above. Our research therefore questions whether the ovarian reserve really does decrease in a relevant way during childhood and adolescence, as shown in other studies (Wallace and Kelsey, 2010).

We have also shown the FD as a function of various oncological diseases. Previous studies have shown that the AMH concentration (Lawrenz *et al.*, 2012; Lekovich *et al.*, 2016) and the number of oocytes recovered after gonadotrophic stimulation (von Wolff *et al.*, 2018b) are reduced in patients with lymphomas and also in leukaemias (van Dorp *et al.*, 2014). Based on the AMH concentration, it has been speculated that the underlying disease may lead to a reduction in ovarian reserve, which is relevant in the decision to cryopreserve ovarian tissue for which a high ovarian reserve is recommended (von Wolff *et al.*, 2018a). Our study has confirmed that after age adjustment, the AMH concentration is reduced in lymphomas, leukaemias and benign diseases (predominantly hemoglobinopathies). However, FD was not statistically reduced in these diseases. This means that possibly diseases of the haematopoietic system may have an effect on follicles and, however, that this affects only growing and thus mainly AMH-producing antral secondary follicles, but not the FD of the primordial and primary follicles. Thus, it is possible that the reduction in the AMH concentration is only temporary and that the AMH concentration returns to normal after the disease has been cured.

For fertility-protective therapies, this insight plays an important role. It is generally recommended to remove and preserve ovarian tissue only when there is a high ovarian reserve and otherwise to perform ovarian stimulation to preserve oocytes (von Wolff *et al.*, 2018a). According to our study, the decision for or against cryopreservation must include the possibility that the AMH concentration, but not the FD, is lowered due to illness.

A disease-related reduction in AMH concentrations has already been described in connection with other diseases. For example, there is a reduction in AMH concentration in HIV infections. Reduced AMH concentrations have also been measured in autoimmune diseases such as lupus erythematodes, rheumatoid arthritis, etc. (Lawrenz *et al.*, 2011; Henes *et al.*, 2015). It has been shown in a rat model that even diabetes mellitus leads to a reduction in the AMH concentration. It is therefore plausible that severe disease can lead to a reduction in the serum AMH concentration, at least temporarily.

The discrepancy between AMH concentration and total FD has a further implication in the area of fertility protection. It is largely unclear which factors are crucial for the success of ovarian tissue transplantation. The only certainty so far is that a lower age of the woman during cryopreservation is associated with higher pregnancy rates (van der Ven *et al.*, 2016). It can be assumed that the FD of the transplant also plays a relevant role. Jensen *et al.* (2015) showed that in those women who became pregnant, FD of the transplanted tissue had been higher. However, the difference was not significant. Sermondade *et al.* (2018) described a positive correlation between the FD and number of mature oocytes after controlled ovarian stimulation and a positive correlation of AMH and FD, suggesting AMH to be a prognostic maker for the FD. This finding is in line with our study regarding the analysis of the total group of patients. However, our subanalysis focusing on different diagnosis groups revealed that this correlation does not apply to all diseases. Furthermore, own data revealed that FD appears to be a better prognostic factor for a pregnancy after ovarian tissue transplantation than AMH (J. Liebenthron, Duesseldorf, Germany, personal communication). Therefore, FD rather than serum AMH concentration should be used to calculate the amount of tissue to be transplanted. Accordingly, in most cases, in which 50% of one ovary was cryopreserved, roughly 15–25% of the volume of one ovary (one-third to one-half of the cryopreserved tissue) is transplanted. If the ovarian reserve is reduced, 25% of tissue (half of the cryopreserved tissue) is transplanted, leaving exactly the same amount for a second transplantation.

In conclusion, this systematic analysis of the correlation of serum AMH concentrations with FD provides insights into the FD and ovarian reserve throughout childhood and reproductive life. AMH concentrations, but not FD, tend to be reduced at young age, indicating that AMH analysis is of limited value to estimate FD up to the age of 20 years. In women >20 years, AMH correlates with FD but this correlation is rather low. Accordingly, the prognostic accuracy of AMH to estimate the FD and the total ovarian reserve is limited, which has implications in fertility preservation. Firstly, estimating the individual FD and total ovarian reserve by analysing serum AMH might lead to misinterpretations in some diseases. Secondly, the decision to freeze or not to freeze ovarian tissue, based solely on AMH concentration, should be taken with care. Thirdly and most importantly, the amount of ovarian tissue to be transplanted should be based on individual FD calculated from several standardized small cortical biopsies at time of preparation for cryopreservation.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

JL designed the study and contributed to the collection of samples, the preparation and analysis of biopsies, analysis of the data and preparation and revision of the manuscript. JR collected samples, prepared and analysed the biopsies and performed the AMH analysis. NS and JSK contributed to the proofreading and revision of the manuscript. MvW contributed to the data analysis, the writing and the revision of the manuscript. All authors revised the final manuscript.

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Conflict of interest

The authors of this manuscript have nothing to declare and no conflicts of interest.

References

Bastings L, Liebenthron J, Westphal JR, Beerendonk CC, van der Ven H, Meinecke B, Montag M, Braat DD, Peek R. Efficacy of ovarian tissue cryopreservation in a major European center. *J Assist Reprod Genet* 2014;**31**:1003–1012.

Beckmann MW, Lotz L, Toth B, Baston-Büst DM, Fehm T, Frambach T, Germeyer A, Goeckenjan M, Häberlin F, Henes M *et al.* Concept paper on the technique of cryopreservation, removal and transplantation of ovarian tissue for fertility preservation. GebFra science review. *Geburtsh Frauenheilk* 2018;**78**:1–10.

Broekmans FJ, Kwee J, Hendriks DJ, Mol BW, Lambalk CB. A systematic review of tests predicting ovarian reserve and IVF outcome. *Hum Reprod Update* 2006;**12**:685–718.

Brougham MF, Crofton PM, Johnson EJ, Evans N, Anderson RA, Wallace WH. Anti-Müllerian hormone is a marker of gonadotoxicity in pre- and postpubertal girls treated for cancer: a prospective study. *J Clin Endocrinol Metab* 2012;**97**:2059–2067.

Broer SL, Mol B, Dólleman M, Fauser BC, Broekmans FJ. The role of anti-Müllerian hormone assessment in assisted reproductive technology outcome. *Curr Opin Obstet Gynecol* 2010;**22**:193–201.

Broer SL, Eijkemans MJ, Scheffer GJ, van Rooij IA, de Vet A, Themmen AP, Laven JS, de Jong FH, Te Velde ER, Fauser BC *et al.* Anti-mullerian hormone predicts menopause: a long-term follow-up study in normoovulatory women. *J Clin Endocrinol Metab* 2011;**96**:2532–2539.

Broer SL, Dólleman M, van Disseldorp J, Broeze KA, Opmeer BC, Bossuyt PM, Eijkemans MJ, Mol BW, Broekmans FJ, IPD-EXPORT Study Group. Prediction of an excessive response in in vitro fertilization from patient characteristics and ovarian reserve tests and comparison in subgroups: an individual patient data meta-analysis. *Fertil Steril* 2013;**100**:420–9.e7.

Cohen HL, Shapiro MA, Mandel FS, Shapiro ML. Normal ovaries in neonates and infants: a sonographic study of 77 patients 1 day to 24 months old. *Am J Roentgenol* 1993;**160**:583–586.

Dólleman M, Faddy MJ, van Disseldorp J, van der Schouw YT, Messow CM, Leader B *et al.* The relationship between anti-Müllerian hormone in women receiving fertility assessments and age at menopause in subfertile women: evidence from large population studies. *J Clin Endocrinol Metab* 2013;**98**:1946–1953.

El Issaoui M, Giorgione V, Mamsen LS, Rechnitzer C, Birkebæk N, Clausen N, Kelsey TW, Andersen CY. Effect of first line cancer treatment on the ovarian reserve and follicular density in girls under the age of 18 years. *Fertil Steril* 2016;**106**:1757–62.e1.

Goldberg BB, McGahan JP. *Atlas of Ultrasound Measurements*. Philadelphia: Elsevier Health Sciences, 2006

Haadsma ML, Bukman A, Groen H, Roeloffzen EM, Groenewoud ER, Heineman MJ, Hoek A. The number of small antral follicles (2-6 mm) determines the outcome of endocrine ovarian reserve tests in a subfertile population. *Hum Reprod* 2007;**22**:1925–1931.

Hansen KR, Hodnett GM, Knowlton N, Craig LB. Correlation of ovarian reserve tests with histologically determined primordial follicle number. *Fertil Steril* 2011;**95**:170–175.

Helden JV, Weiskirchen R. Age-independent anti-Müllerian hormone (AMH) standard deviation scores to estimate ovarian function. *Eur J Obstet Gynecol Reprod Biol* 2017;**213**:64–70.

Henes M, Froeschlin J, Taran FA, Brucker S, Rall KK, Xenitidis T, Igney-Oertel A, Lawrenz B, Henes JC. Ovarian reserve alterations in premenopausal women with chronic inflammatory rheumatic diseases: impact of rheumatoid arthritis, Behçet's disease and spondyloarthritis on anti-Müllerian hormone levels. *Rheumatology (Oxford)* 2015;**54**:1709–1712.

Hvidman HW, Bentzen JG, Thuesen LL, Lauritsen MP, Forman JL, Loft A, Pinborg A, Nyboe Andersen A. 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. *Hum Reprod* 2016;**31**:1034–1045.

Jensen AK, Kristensen SG, Macklon KT, Jeppesen JV, Fedder J, Ernst E, Anderse CY. Outcomes of transplantations of cryopreserved ovarian tissue to 41 women in Denmark. *Hum Reprod* 2015;**30**:2838–2845.

Jeppesen JV, Anderson RA, Kelsey TW, Christiansen SL, Kristensen SG, Jayaprakasan K, Raine-Fenning N, Campbell BK, Andersen YC. Which follicles make the most anti-Mullerian hormone in humans? Evidence for an abrupt decline in AMH production at the time of follicle selection. *Mol Hum Reprod* 2013;**19**:519–527.

Kelsey TW, Wright P, Nelson SM, Anderson RA, Wallace WH. A validated model of serum anti-müllerian hormone from conception to menopause. *PLoS One* 2011;**6**:e22024.

Kelsey TW, Dodwell SK, Wilkinson AG, Greve T, Andersen CY, Anderson RA, Wallace WH. Ovarian volume throughout life: a validated normative model. *PLoS One* 2013;**8**:e71465.

Kumar A, Kalra B, Patel AS, McDavid L. Development of a second generation anti-Mullerian hormone (AMH) ELISA. *Clin Chem* 2009;**55**:A175.

Lawrenz B, Fehm T, von Wolff M, Soekler M, Huebner S, Henes J, Henes M. Centers of FertiPROTEKT network. Reduced pretreatment ovarian reserve in premenopausal female patients with Hodgkin lymphoma or non-Hodgkin-lymphoma–evaluation by using antimüllerian hormone and retrieved oocytes. *Fertil Steril* 2012; **98**:141–144.

Lawrenz B, Henes J, Henes M, Neunhoeffer E, Schmalzing M, Fehm T, Kitter I. Impact of systemic lupus erythematosus on ovarian reserve in premenopausal women: evaluation by using anti-Muellerian hormone. *Lupus* 2011;**20**:1193–1197.

Lee JY, Ahn S, Lee JR, Jee BC, Kim CH, Seo S, Seo S, Suh CS, Kim SH. Reference values for the revised anti-Müllerian hormone generation II assay: infertile population-based study. *J Korean Med Sci* 2017;**32**:825–829.

Lekovich J, Lobel ALS, Stewart JD, Pereira N, Kligman I, Rosenwaks Z. Female patients with lymphoma demonstrate diminished ovarian reserve even before initiation of chemotherapy when compared with healthy controls and patients with other malignancies. *J Assist Reprod Genet* 2016;**33**:657–662.

Liebenthron J, Montag M, Reinsberg J, Köster M, Isachenko V, van der Ven K, van der Ven H, Krüssel JS, von Wolff M. Overnight ovarian tissue transportation for centralized cryobanking – a feasible option. *Reprod Biomed Online* 2019;**38**:740–749.

Liebenthron J, Köster M, Drengner C, Reinsberg J, van der Ven H, Montag M. The impact of culture conditions on early follicle recruitment and growth from human ovarian cortex biopsies in vitro. *Fertil Steril* 2013;**100**:483–491:e5.

Nelson SM, Klein BM, Arce JC. Comparison of antimüllerian hormone levels and antral follicle count as predictor of ovarian response to controlled ovarian stimulation in good-prognosis patients at individual fertility clinics in two multicenter trials. *Fertil Steril* 2015;**103**: 923–30.e1.

Orsini LF, Salardi S, Pilu G, Bovicelli L, Cacciari E. Pelvic organs in premenarcheal girls: real-time ultrasonography. *Radiology* 1984;**153**: 113–116.

Perven HA, Nurunnabi AS, Ara S, Jahan MU. Cadaver study of the volume of the ovary in Bangladeshi women. *Bangladesh Med Res Counc Bull* 2014;**40**:15–17.

Rosendahl M, Ernst E, Rasmussen PE, Andersen CY. True ovarian volume is underestimated by two-dimensional transvaginal ultrasound measurement. *Fertil Steril* 2010;**93**:995–998.

Sermondade N, Sonigo C, Sifer C, Valtat S, Ziol M, Eustache F, Grynberg M. Serum antimüllerian hormone is associated with the number of oocytes matured in vitro and with primordial follicle density in candidates for fertility preservation. *Fertil Steril* 2019;**111**:357–362.

van der Ven H, Liebenthron J, Beckmann M, Toth B, Korell M, Krüssel J, Frambach T, Kupka M, Hohl MK, Winkler-Crepaz K. Ninety-five orthotopic transplantations in 74 women of ovarian tissue after cytotoxic treatment in a fertility preservation network: tissue activity, pregnancy and delivery rates. *Hum Reprod* 2016;**31**:2031–2041.

van Dorp W, van den Heuvel-Eibrink MM, de Vries AC, Pluijm SM, Visser JA, Pieters R, Laven JS. Decreased serum anti-Müllerian hormone levels in girls with newly diagnosed cancer. *Hum Reprod* 2014; **29**:337–342.

von Wolff M, Germeyer A, Liebenthron J, Korell M, Nawroth F. Practical recommendations for fertility preservation in women by the FertiPROTEKT network. Part II: fertility preservation techniques. *Arch Gynecol Obstet* 2018a;**297**:257–267.

von Wolff M, Bruckner T, Strowitzki T, Germeyer A. Fertility preservation: ovarian response to freeze oocytes is not affected by different malignant diseases-an analysis of 992 stimulations. *J Assist Reprod Genet* 2018b;**35**:1713–1719.

Wallace WH, Kelsey TW. Human ovarian reserve from conception to the menopause. *PLoS One* 2010;**5**:e8772.