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Abundance of Anti-Muellerian hormone in cat ovaries and correlation of its plasma concentration with animal age, weight and stage of the estrous cycle

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ABSTRACT

In female animals of different species, Anti-Müllerian hormone (AMH) is produced by follicular granulosa cells and has been associated with the ovarian follicle pool. Because concentration of AMH in plasma of ovary-intact female cats is apparently more variable than previously assumed, we have analysed AMH concentration in blood of cats (n = 93) presented for routine ovariectomy and assessed ovarian histology and AMH protein expression in the surgically removed ovaries. We hypothesised that AMH is synthesized only in preantral and small antral follicles and that plasma AMH concentration reflects the antral follicle count (AFC). Corpora lutea were detected in 35% of the female cats, whereas plasma progesterone concentration was ≥1 ng/mL in 57% of the cats. Follicular cysts were present in 15 cats (16%). Positive immunostaining for AMH protein was detected in close to all primordial and antral follicles, ovarian cysts, 70% of corpora lutea and 28% of atretic follicles. Concentration of AMH in plasma averaged 6.8 ± 0.5 ng/mL (range 1.3–21.7 ng/mL). The AFC increased with increasing AMH concentration with a moderate positive correlation between AFC and AMH ($r = 0.286$, $p < 0.01$). Plasma AMH concentration was not affected by season or cats' age, weight, stage of the estrous cycle and presence of follicular cysts. In conclusion, AMH protein is expressed in all endocrine structures of the cat ovary. While AMH is a marker for the presence of ovarian tissue, its usefulness to assess ovarian function in individual female cats is of limited value.

1. Introduction

Anti-Muellerian Hormone (AMH) is a glycoprotein in the transforming growth factor-β family and has long been associated with its role for fetal sex differentiation first described in rabbits [1]. Only much later its role as a marker for the presence of gonadal tissue in postnatal male and female animals and as a diagnostic aid to assess gonadal pathologies has been described. In male animals, as in men, AMH is expressed by Sertoli cells in fetal, neonatal, prepubertal and postpubertal testes and in non-descended testes of cryptorchids (e.g., bull: [2,3]; stallion: [4,5]). In females, AMH is expressed by the granulosa cells of preantral and small antral follicles, is correlated with the ovarian follicle pool and AMH concentration in plasma allows predicting the ovarian reserve of gonadotrophin-responsive follicles in women [6–9], cows [10], does [11], ewes [12] and female cats [13]. Although AMH concentration is associated with the number of follicles and thus oocytes, its concentration is not correlated with per cycle pregnancy rate in women (reviewed by Ref. [9]). The concentration of AMH in plasma of women has been suggested to be relatively stable across the estrous cycle and among consecutive cycles [14]. More recently, however, this stability has been questioned [9]. Similarly, variations in plasma AMH concentration throughout the estrous cycle and between cycles in individual animals have been described for cats [13,15]. Serum AMH concentration was also higher in pregnant compared to cyclic cats [16] and in prepubertal female cats [13].

Irrespective of fertility, in female dogs and cats, AMH can be determined when it is desirable to assess the presence of ovarian tissue. In animals abandoned by their owners, it may be difficult to diagnose

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whether they are already spayed. Females are also presented with the suspicion of the ovarian remnant syndrome, i.e., incomplete ovariectomy [15,17–19]. In cats, plasma AMH concentration decreases when gonadal function is downregulated with implants releasing the GnRH agonist deslorelin. In this context, determination of AMH has been suggested as a useful tool to determine resumption of ovarian activity following cessation of deslorelin effects [20].

Although AMH is increasingly determined for diagnostic purposes in female cats, its concentration in plasma of ovary-intact, healthy females is apparently more variable than previously assumed. Therefore, ovarian remnant syndrome cannot always be diagnosed accurately by AMH determination [21]. Previous studies on AMH in cats, however, often included only a limited number of animals acquired by convenience sampling. Experimental studies in cats are restricted and, in many countries, legal regulations allow such studies only with cats originating from a source explicitly approved for breeding experimental animals. If possible, descriptive studies must include a larger number of cases to allow meaningful conclusions. To gain more insight into the ovarian source of AMH and its release into the circulation, we have determined AMH concentration in blood of female cats presented for routine ovariectomy together with AMH protein expression in follicles, corpora lutea and cysts of the surgically removed ovaries. Cases were selected from all female cats presented to a large animal hospital over a one-year period. We hypothesised that (i) AMH is near-exclusively synthesized in the granulosa cells of preantral and small antral follicles, (ii) plasma AMH concentration reflects the number of ovarian follicles, (iii) plasma AMH concentration decreases with age of the animal but is not affected by the presence or absence of corpora lutea and ovarian cysts.

2. Materials and methods

2.1. Animals

For this study, both ovaries from 98 healthy cats (*Felis catus*) were collected over a one-year period at random stages of the estrous cycle. Cats were presented for routine ovariectomy at Vetmeduni Vienna, Austria. From the 98 cats, 88 came from a local animal shelter, 54 of which had already been rehomed and their new owners had been issued a voucher for a castration by the animal shelter. At the time of castration, they had been with their new owners for 8–378 days. A total of 34 cats were presented directly by the animal shelter before being rehomed and ten cats came from private owners independent from the animal shelter. According to their current owners, none of the cats had received any hormonal treatment such as progestins or GnRH agonists. Before castration, a clinical examination of the reproductive tract including transabdominal ultrasonography and collection of a blood sample for pre-surgery determination of hematocrit and total protein concentration was carried out, to ensure general health of the cat, normal development of the reproductive tract, absence of a visible pregnancy and absence of abnormalities of ovaries and/or uterus. Furthermore, recent parturition was excluded. Cats belonged to the breeds European shorthair (n = 78), European Longhair (n = 4), British Shorthair (n = 3), Persian (n = 1), Burmese (n = 1), Scottish Fold (n = 2), Sphynx (n = 3) and Maine Coon (n = 6).

Because of processing-related damage to some very small ovaries, complete histological evaluation was not possible in five cats. Therefore, 93 out of 98 cats were included into the analysis. Age of the 93 cats included for statistical analysis ranged from 4 months to 9 years (19.3 ± 18.4 months) and their weight from 1.5 to 4.7 kg (3.1 ± 0.6 kg). Out of 93 cats, four were kept together with gonad-intact males but none of these females was confirmed pregnant.

The study was approved by the Ethics and Animal Welfare Committee of Vetmeduni Vienna (ETK-020/02/2021). Because the reason for ovariectomy was not connected to our study and AMH analysis in blood plasma was performed from a routine pre-surgery blood sample, no further animal experimentation license by the competent Austrian authority was required. All owners were provided with a study information sheet and consented to participate in the study.

2.2. Surgical procedures

During preparation for surgery, a venous catheter (Vasofix Safety G22, Braun, Melsungen, Germany) was placed into one cephalic vein and one blood sample per animal was collected into an EDTA tube (S-Monovette EDTA 1.3 ml, Sarstedt, Nümbrecht, Germany). After determination of hematocrit and total protein, the remaining blood was centrifuged and the plasma aliquoted into two tubes and stored at −20 °C until determination of progesterone and AMH. Routine ovariectomy with partial resection of the uterine horns was performed via a small ventral midline incision. In case of macroscopic abnormalities of the uterus (thickening of the uterine wall in nine cats), the incision was extended, and an ovariohysterectomy was performed. The ovaries and uterus (in case of the nine ovariohysterectomized cats) were collected and fixed in 4% neutral buffered formalin immediately after surgery. After 48 h of fixation at room temperature they were placed into 70% ethanol solution.

2.3. AMH and progesterone analysis

Plasma progesterone and AMH concentrations were determined with enzyme linked immunosorbent assays (ELISA) validated for cats in our laboratory (Progesterone ELISA DE1561, Demeditec Diagnostics, Kiel, Germany; Canine AMH ELISA, AL-116, Ansh Labs, Webster, TX, USA). For the progesterone ELISA, the intra-assay coefficient of variation was 7.8%, the interassay coefficient of variation was 13.2% and the minimal detectable concentration was 10 pg/mL. For the AMH ELISA, the intra-assay variation was 5.3%, interassay variation was 19.2% and the minimal detectable concentration was 5 pg/mL.

2.4. Histopathological examination

The fixed ovaries were cut longitudinally. Several transverse sections of both uterine horns were produced where applicable. The tissue specimens were embedded in paraffin wax after preparation. Sections (3 μm thick) were excised from the resulting tissue blocks using a microtome. The sections were transferred to slides, dried overnight at 37 °C and then stained with hematoxylin and eosin. The slides were scanned with the Fritz Microscopy Slide Scanner (PreciPoint, Garching, Germany) at 20x magnification. For histologic evaluation, antral follicles, corpora lutea, atretic follicles and follicular cysts were counted on both sections of each ovary. Antral follicles were defined as fluid-filled structures with an intact follicular wall that allowed differentiation between a zona granulosa, basement membrane, theca interna and theca externa. In atretic follicles, a clear differentiation of follicular wall layers was absent, and the theca was increasingly replaced by collagenous tissue. Follicular cysts were defined as fluid-filled structures >3.5 mm in diameter and either the follicular wall covered by a monolayer of granulosa cells (n = 12) or multiple layers of luteinised theca cells (n = 3). For all ovarian structures, the mean of the counts from both ovaries was then calculated and used for statistical analyses. Cysts of the Wolffian or Muellerian ducts (n = 4 cats) and rete ovarii cysts (n = 5 cats) classified as published previously by our group [22] were recorded but not included into further analysis.

2.5. Immunohistochemistry

For immunohistochemistry, 3 μM paraffin sections were cut with the microtome. These were deparaffinized by xylol and rehydrated in a graduated alcohol series (100, 95 and 70% ethanol). The sections were then placed in distilled water and the endogenous peroxidase activity was blocked with 30% H_2O_2 in distilled water. After rinsing with tap water, the tissue sections for antigen masking were steamed for 30 min

in Tris-EDTA at a pH of 9.0 and then cooled for 20 min. The slides were rinsed in PBS and treated for the protein block with 1.5% Normal Goat Serum (Sigma Aldrich, Vienna, Austria) in PBS for 30min (150 µl goat serum in 10 ml PBS). After blockage, the sections were incubated overnight at 4 °C with a primary rabbit polyclonal AMH antibody (1:5000; Genetex, Hsinchu City, Taiwan, cat. no. GTX129593). Simultaneously, IgG controls (1:15000) were performed. After incubation with the primary antibody, slides were rinsed in PBS for 2 × 5 min and incubated with a second antibody (BrightVision Poly-HRP-*anti*-rabbit, ImmunoLogic, Duiven, The Netherlands) for 30 min. After rinsing with PBS, the samples were incubated with DAB solution (Quanto, Richard Allan Scientific, Kalamazoo, MI, USA; TA-125-QHDX) for 5 min and then washed with distilled water. The slides were counterstained with hematoxylin, then rinsed with aqua fontis, thereafter dehydratated with 96% and 100% ethanol and finally with xylol. Slides were covered with DPX (Fluka, Buchs, Switzerland) and cover glass. All stains were positive and all IgG controls negative. All immunohistochemistry evaluations were made by a single observer and AMH staining was classified as either positive or negative and no attempt was made to score staining intensity in case of positive staining. Specificity of antibody binding was confirmed by Western blot in the authors' laboratory as described recently [16].

2.6. Statistical analysis

Data were analysed with the SPSS statistics software (version 28, IBM-SPSS, Armonk, NY, USA). Data for AMH were not normally distributed (Kolmogorov-Smirnov test) and therefore non-parametric tests were used throughout. The AMH concentration in different groups of cats was compared by Kruskal-Wallis test (>two groups) or Mann-Whitney test (two groups). To analyse correlations between AMH concentration in plasma and antral follicle count, Spearman's coefficient of variation was calculated. The number of cats in different age groups with plasma progesterone concentration ≥1 ng/mL and <1 ng/mL was compared by chi^2 test. All values given are means and standard error of mean. A p-value <0.05 was considered statistically significant.

3. Results

One or more corpora lutea were detected on histology in 32% of the cats, whereas plasma progesterone concentration was ≥1 ng/mL in 55% of the cats (Fig. 1a). The percentage of cats with plasma progesterone concentration ≥1 ng/mL increased with increasing age (Fig. 1b). None of the cats was visibly pregnant using abdominal ultrasonography. Follicular cysts were present in 15 out of 93 cats and the average number of cysts in these cats was 3.3 ± 3.1 (±SD; range 1–13) per cat. Representative structures determined on cat ovaries are shown in Fig. 2.

Positive immunostaining for AMH was present in close to all primordial, primary, secondary and tertiary follicles and in all follicular cysts, but only in 28% of atretic follicles. Out of the corpora lutea, 79% stained positive for AMH (Fig. 3).

In all follicles, AMH immunostaining was restricted to granulosa cells and in antral follicles was evident in granulosa cells of the follicular wall and cumulus oophorus (Fig. 4a and b). Immunostaining was, however, negative in the majority of atretic follicles (Fig. 4c). Lutein cells in most corpora lutea stained positive for AMH, but immunostaining was visually less intense than in follicular granulosa cells and not always evenly distributed within the corpus luteum (Fig. 4d). In the 15 follicular cysts, AMH protein expression was restricted to a monolayer of cells covering the wall of the cyst (n = 12; Fig. 4e) or multiple layers of luteinized cells (n = 3; Fig. 4f).

For all 93 female cats, the mean AMH concentration in plasma was 6.8 ± 0.5 ng/mL (median 5.5 ng/mL). The concentration of AMH ranged from 1.3 to 21.7 ng/mL with the highest concentration in a 4-months-old cat. Out of the 93 cats, 39 (41.9%) had a plasma AMH concentrations <5 ng/mL, 78 (83.9%) a plasma AMH concentrations <10 ng/mL. The distribution of plasma AMH concentration in all 93 cats is summarized in Fig. 5. The antral follicle count (average of two sections from both ovaries) ranged from 1.3 to 35.5 (7.1 ± 0.5) and was 20 in the 4-months-old cat with the highest AMH concentration. Antral follicle count increased with increasing AMH concentration in plasma (Fig. 6a) and there was a significant positive correlation between antral follicle count and plasma AMH concentration in cats (Fig. 6b).

The concentration of AMH in plasma did not differ among female cats of different age groups, different weight and included into the study at different times of the year (Fig. 7a–c).

Concentration of AMH in plasma of cats was not affected by stage of the estrous cycle when cats were assigned to the follicular and luteal phase either by the presence or absence of corpora lutea on histology or by progesterone concentration ≥1 ng/mL or <1 ng/mL (Fig. 8a and b). Plasma AMH concentration did also not differ significantly between cats with and without follicular cysts (p = 0.41; Fig. 8c).

4. Discussion

The results of this study indicate that in cat ovaries, AMH is synthesized not only by preantral and small antral follicles but also by luteal cells after ovulation and formation of corpora lutea. Whereas AMH protein expression in cat preantral and antral follicles has been reported previously [16] and is in agreement with findings in other species (e.g., dog: [23]; cow: [24]; horse: [4]; pig: [25], human: [26]), AMH protein expression in corpora lutea is not a general finding across species. Its expression after ovulation in luteal cells has been considered a phenomenon unique to the polyovulatory pig [25] whereas luteal AMH protein expression has been excluded in the mono-ovulatory horse [4]. Recently, an expression of AMH in interstitial cells of cat corpora lutea at mid pregnancy has been described but no interpretation of this finding was given [16]. The present study provides the first evidence for AMH

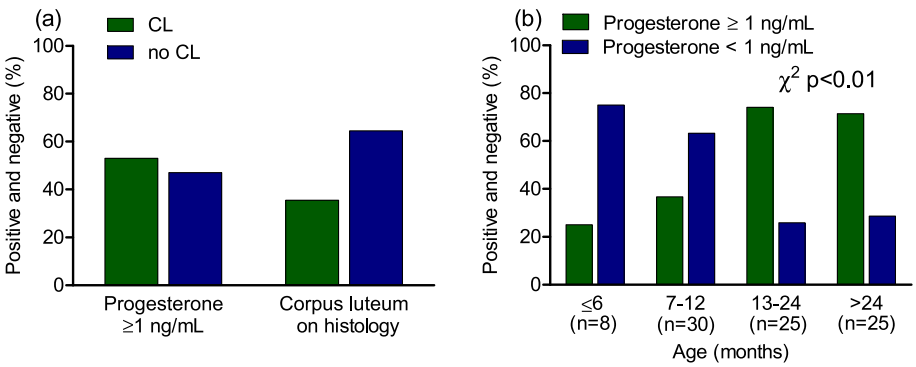

Fig. 1. (a) Percentage of cats with and without corpora lutea determined by histology or by plasma progesterone concentration (≥1 ng/mL = CL and <1 ng/mL = no CL); (b) percentage of cats with plasma progesterone concentration ≥1 ng/mL and <1 ng/mL by age group (total number of cats included into analysis n = 93)

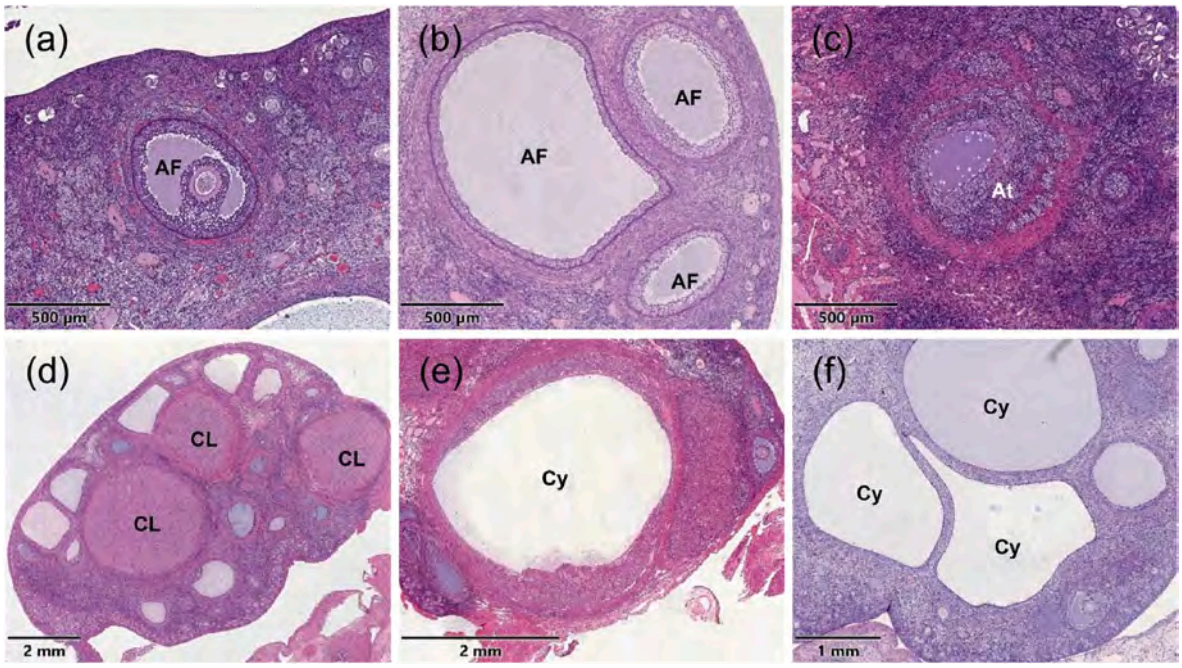

Fig. 2. Hematoxylin and eosin staining of representative cat ovaries with different structures: (a) small antral follicle (AF) with oocyte (b) multiple antral follicles (AF) (c) atretic follicle (At) (d) multiple corpora lutea (CL) and (e) luteinized cyst (Cy) and (f) multiple follicular cysts (Cy).

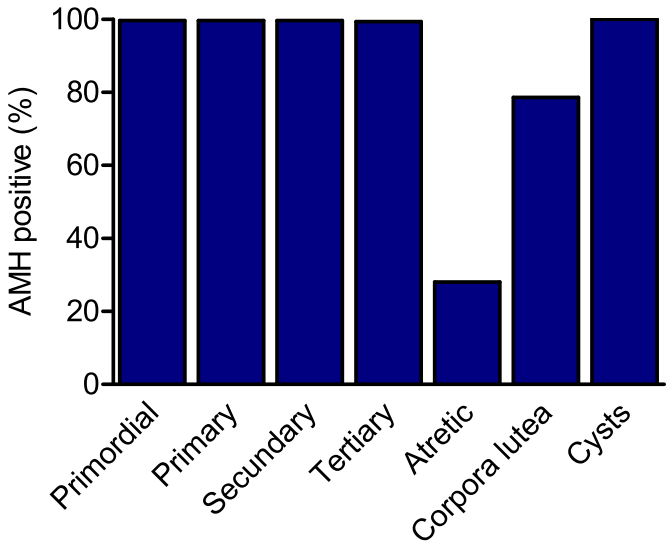

Fig. 3. Percentage of primordial, primary, secondary, tertiary and atretic follicles, corpora lutea and follicular cysts staining positive for AMH in cats (total number of cats included into analysis n = 93).

expression in corpora lutea of non-pregnant cyclic cats, although with our experimental design, non-detected early pregnancy in a small percentage of these cats cannot be ruled out. Most interestingly, AMH was expressed only in 80% of the corpora lutea and not always evenly throughout an individual corpus luteum. Because of our experimental design, the exact day of ovulation and thus age of the corpora lutea could not be determined. If the expression of AMH in cat luteal tissue is anyhow related to corpus luteum age can only be speculated. It can be assumed that AMH synthesis may continue after ovulation but gradually cease in the aging corpus luteum. The physiological function of luteal AMH is at present not clear. Based on its expression in the corpus luteum of cyclic sows, it has been suggested that AMH contributes to regulation of the cyclic recruitment of small antral follicles and prevents premature exhaustion of the ovarian follicular reserve [25]. Although AMH is not expressed in the corpus luteum of women, the hormone inhibits the gonadotrophin-induced expression of the steroidogenic enzyme CYP19A1 aromatase and thus exerts an inhibitory role on early stages of follicular development [27]. In the female cats from our study, AMH expression was markedly reduced only in atretic follicles which is in agreement with findings in cows [24].

Similar to follicles, AMH expression occurred also in granulosa cells and luteinized cells of follicular cysts, indicating that these are capable of producing AMH. Although the term ovarian cyst is widely used in clinical small animal reproduction, we have recently suggested that ovarian cysts in cats are mostly incidental findings without clinical relevance [22]. Such structures in cats may more resemble (hemorrhagic) anovulatory follicles in mares which delay the next estrus [28,29] than follicular cysts in cattle which can be the cause of a prolonged anestrus (e.g., Refs. [30,31]). This interpretation is supported by the unchanged AMH expression in cysts in the cats of our study when compared to physiological ovarian follicles. With regard to AMH protein expression, in horse ovaries strong AMH immunolabelling was observed in granulosa cells of growing follicles, whereas only faint expression was detected in granulosa cells of dominant follicles [32]. Although to the best of our knowledge, AMH expression has not been studied in horse anovulatory follicles, cats apparently differ from mares with a continuous AMH expression in growing large follicles.

Concentration of AMH in plasma of ovary-intact female cats varied markedly among animals, but AMH was always detectable and well above the minimal detection threshold of our assay. In healthy cows, it has been suggested that AMH concentrations are to some extent characteristic for the individual animal [24] and the same may be true for female cats. In contrast to cats, however, 40% of postpartum cows had undetectable plasma AMH concentrations not associated with obvious ovarian pathologies [33].

When cats were grouped by antral follicle count, AMH concentration was higher in cats with more antral follicles compared to those with fewer antral follicles detected on histology. There was, however, only a loose linear correlation between AMH concentration in plasma and antral follicle count. Although at first glance, our data may only partially agree with findings in other species, it must be considered that although

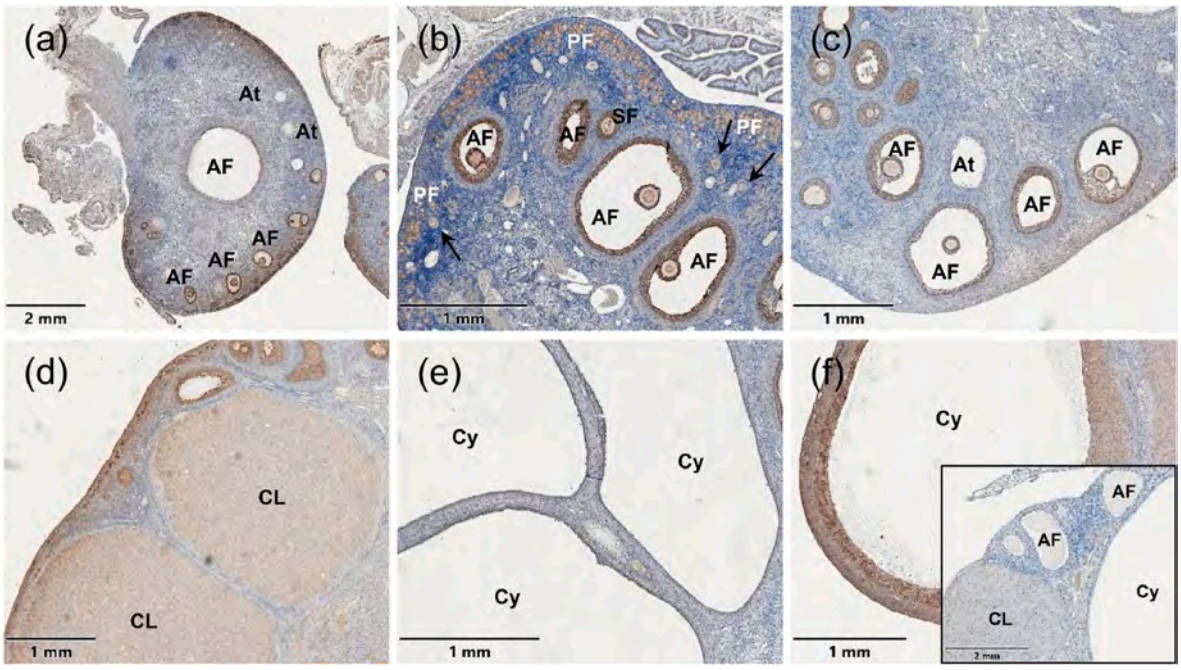

Fig. 4. Immunohistochemical staining for AMH: (a,b,c) ovaries with multiple positively stained primordial (PF), primary (black arrows), secondary (SF) and antral follicles (AF) and non-stained atretic follicles (At), (d) positively stained corpora lutea (CL) besides positively stained follicles and (e) follicular cysts with a single layer of positively stained cells covering the wall of the cyst (Cy), (f) lutein cyst with positively stained cells (Cy) and inserted negative control with corpus luteum (CL), antral follicles (AF) and a cyst (Cy).

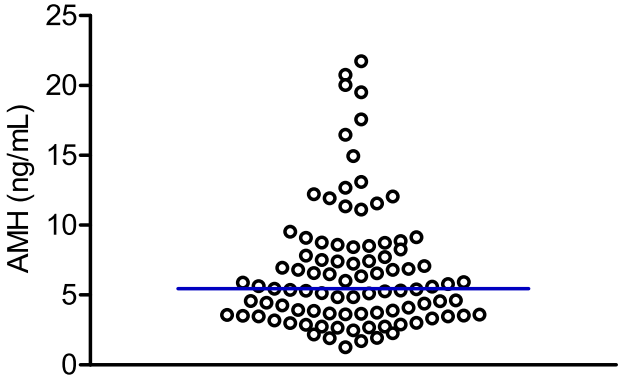

Fig. 5. Distribution of individual AMH concentration and their median (solid blue line) in plasma of all cats included into the study (n = 93). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the oldest cats in our study were 9 years old, only 10 were older than 3 years. With a life expectancy in cats of over 15 years [34], old cats were thus underrepresented in our study. In horse mares, an overall positive correlation between antral follicle count and plasma AMH concentration exists but varied by mare age with a strong correlation in older mares, a moderate correlation in middle-aged mares and no correlation in young mares [35]. Our findings do not exclude that the relationship between AMH and antral follicle count varies across age groups also in cats.

Previous studies have reported higher plasma AMH concentration in younger and particular in prepubertal cats compared to older animals [13,15,17,20]. The same has been demonstrated in female dogs [36,37], mice [38] and horses [35]. Puberty in cats often occurs when they are between four and six months old [39]. Because prepubertal cats are rarely presented for ovariectomy at the authors' institution, it is possible that increased plasma AMH concentrations in cats of this young age group were missed in our study. Although statistical significance was not reached, plasma AMH concentration in cats up to one year of age, however, tended to be higher than in older cats.

The concentration of AMH in plasma of cats was not affected by the presence or absence of corpora lutea which is in agreement with previous studies in women [14] and pigs [40] and horses [35]. Some variations in plasma AMH concentration throughout the estrous cycle and between cycles in individual animals have been described previously for cats [13,15] but based on the larger cat population with individual cats only included once cannot be confirmed in the present study. Concentration of AMH in plasma was not affected by the presence or absence of follicular cysts which was to be expected because of unchanged AMH protein expression in these apparently anovulatory structures follicles and ovarian cysts.

For a long time, cats have been considered a species with mating-induced ovulations only, but recently, regularly occurring spontaneous ovulations have been described in cats based on ovarian histology [41]. Based on plasma progesterone concentration, the percentage of cats with active luteal tissue was even higher in the present study. Because only two sections per ovaries were accessed, apparently not all corpora lutea present were detected by histological examination. None of the cats was visibly pregnant and – except for four females - contact to tomcats was excluded by the owners. Our findings thus confirm and extend previous studies on spontaneous ovulations or luteinisation of follicles in female cats.

5. Conclusion

The AMH protein is expressed in granulosa and luteal cells and thus in all functional structures of the cat ovary. Therefore, in ovary-intact, postpubertal cats, AMH concentrations do not differ among stages of the estrous cycle or between cats with and without ovarian cysts. While AMH clearly is a marker for the absence of ovarian tissue in cats, its usefulness to assess ovarian function in non-spayed cats is of limited value.

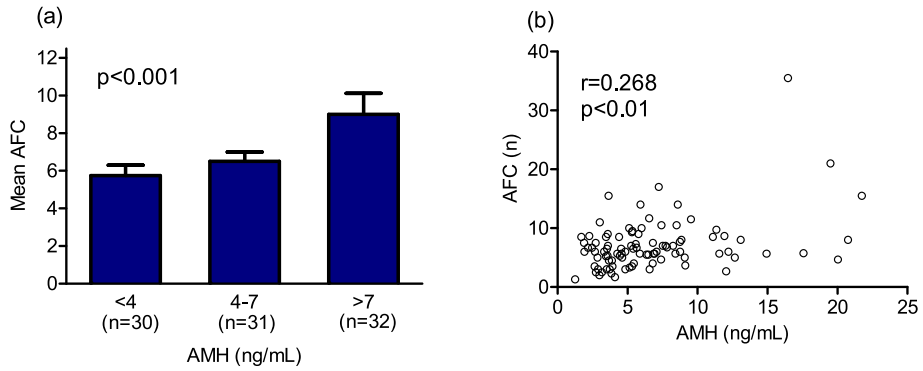

Fig. 6. (a) Mean antral follicle count (AFC) per ovary in cats with different AMH concentration in plasma and (b) correlation between AFC and plasma AMH concentration (n = 93 cats).

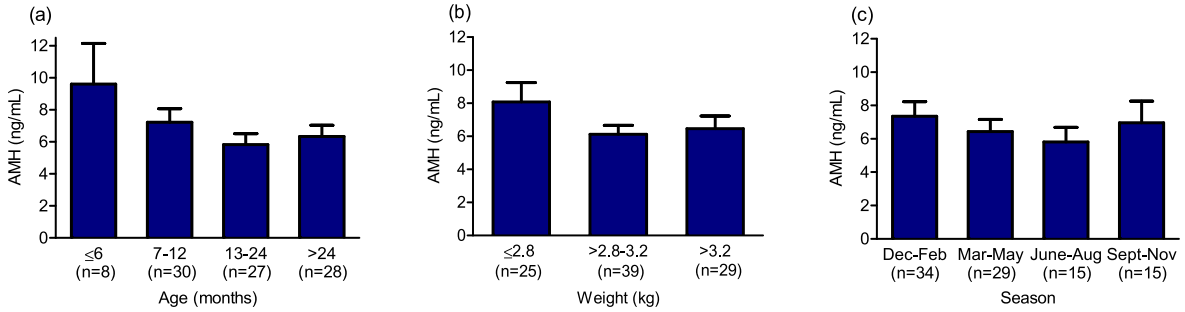

Fig. 7. Concentration of AMH in plasma of cats (n = 93) grouped by (a) age and (b) weight and (c) ovariectomized at different times of the year.

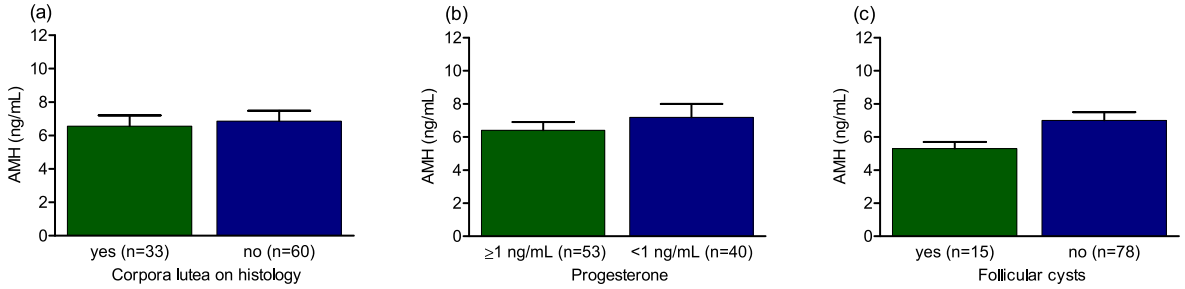

Fig. 8. Concentration of AMH in plasma of cats (a) with and without corpora lutea on histology, (b) plasma progesterone concentration ≥1 ng/mL and <1 ng/mL and (c) with and without follicular cysts determined by histology.

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CRediT authorship contribution statement

Svenja Claaßen: Formal analysis, Investigation, Methodology, Software, Validation, Writing – original draft. **Jörg Aurich:** Conceptualization, Formal analysis, Investigation, Methodology, Funding acquisition, Writing – original draft, and revision. **Ingrid Walter:** Methodology, Validation, Formal analysis. **Camille Gautier:** Methodology, Validation, Formal analysis. **Christine Aurich:** Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – review & editing.

Declaration of competing interest

None of the authors has any conflict of interest to declare.

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