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Anti-Müllerian hormone in canine pyometra

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AMH vid pyometra hos tik

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SUMMARY

The anti-Müllerian hormone (AMH) determines whether a foetus develops a male or female phenotype but the hormone is also produced in the adult; in the testes in males and in ovaries in females. In the ovaries, it is produced by the granulosa cells surrounding the pre-antral to small antral stage follicles. This means that the concentrations of AMH in the blood varies with the presence of these follicles. Pyometra is a common and serious disease that affects many bitches with clinical signs like depression, fever, anorexia and even peritonitis or death. Endocrinological aberrations have been suggested to play a role in the pathogenesis.

The aim of the present study was to compare the concentration of AMH in bitches with pyometra with that in healthy bitches in the corresponding cyclus stage. The hypothesis was that there would be a difference in AMH concentrations between the two groups, which would indicate aberrant follicular dynamics in bitches with pyometra, possibly contributing to the pathogenesis of the disease.

Blood was collected from 14 bitches suffering from pyometra and 19 control bitches. The results showed that the AMH concentration in bitches is affected by both age and weight; the older and heavier, the lower concentration of AMH. No association was detected between AMH and uterine disease. This could possibly be due to the low number of bitches but it could also indicate that the development of pyometra is not due to early aberrant follicular dynamics. However, this study does not rule out any aberrant functions in later follicular dynamics that could include cysts or persistent follicles.

SAMMANFATTNING

Det anti-Mülleriska hormonet (AMH) bestämmer om ett foster ska utveckla en hanlig eller honlig fenotyp men hormonet produceras även hos det vuxna djuret, i testiklarna hos hanar och äggstockarna hos honor. Hos honorna produceras AMH i granulosa cellerna som omger de pre-antrala till små antrala folliklarna. Detta innebär att koncentrationen av AMH i blodet varierar med närvaron av dessa folliklar. Pyometra är en vanlig och allvarlig sjukdom som drabbar många tikar med kliniska fynd så som nedsatt allmäntillstånd, feber, inappetens och i vissa fall bukhinneinflammation och död. Endokrinologiska avvikelser har föreslagits utgöra en del av patogenesen.

Målet med denna studie var att jämföra AMH-koncentrationen mellan tikar med pyometra och friska kontroller under samma cyklusstadium. Frågeställningen var om det fanns en skillnad i AMH-koncentration mellan de olika grupperna, vilket då skulle indikera en avvikande follikeldynamik hos tikar med pyometra och på så sätt bidra till att förstå patogenesen bakom pyometra.

Blod samlades från 14 tikar med pyometra och 19 kontrolltikar. Resultatet visade att AMH-koncentrationen hos tik påverkas av ålder och vikt (sjunker med stigande ålder och högre vikt). Inget samband med livmodersjukdom kunde påvisas. Det är möjligt att för få tikar undersöktes för att få fram ett sådant samband, men det kan också bero på att det inte är någon störning i den tidiga follikelutvecklingen hos tikar som gör att de utvecklar pyometra. Det utesluter dock inte att det kan vara störningar i de senare follikelstadierna, vilket kan ge upphov till cystor eller persisterande folliklar.

Contents

Introduction

Anti-Müllerian hormone (AMH) or Müllerian inhibiting substance (MIS) has got its name from its effect in sex differentiation during the foetal stage of young mammals: it inhibits the development of the Müllerian ducts. In the soon to be male phenotype the Sertoli cells in the abdominal testes produce high concentration of this hormone, preventing the Müllerian ducts to develop into oviducts, the uterus and the upper part of the vagina as they do in a female phenotype (Broekmans *et al* 2008; Broer *et al* 2014; Place *et al* 2011). The female equivalent to the Sertoli cells are the granulosa cells. Both Sertoli cells and granulosa cells surround the germ cells in the respective phenotype and control development of the germ cell by converting androgens to oestradiol and creating a protective barrier to the rest of the body. Though similar the granulosa cells enclose the oocyte until ovulation while the Sertoli cells pass the developing sperm between them as they grow (Sjaastad *et al* 2010). The main difference regarding AMH production, although not studied specifically in dogs, is that it starts in the foetal stage in the male but not until just before birth in the female and then in much lower concentration (Rajpert-De Mayets *et al* 1999).

Knowledge of the clinical potential of AMH analysis in the female dog is limited. In bitches AMH is only produced in the granulosa cells surrounding the primary, secondary and pre-antral stage follicles (Nagashima *et al* 2016; Themmen *et al* 2016). Some areas of interest are the use in *in vitro* fertilization (IVF) in both horses (Claes *et al* 2015), dogs (Nagashima *et al* 2016) and cattle (Monniaux *et al* 2013), diagnosing and follow up of granulosa cell carcinomas in humans (La Marca & Volpe 2007), cattle and horses. A potential usefulness for diagnosing granulosa cell tumours has also been described in bitches (Holst 2016). Analysis of AMH concentration has also been used for quantification of the ovarian reserve in both women and cattle (Baldrighi *et al* 2014).

Analysis of AMH could potentially help understand the pathogenesis of the complex, common and dangerous disease pyometra in the female dog. Most cases of pyometra occur during the two months following heat; during the luteal phase (Dąbrowski *et al* 2013; Enginler *et al* 2014; England & von Heimendahl 2010). The high concentrations of oestradiol during heat followed by two months with high progesterone concentrations result in changes within the endometrium. These changes include increased secretion into the uterine lumen, less muscle contractions and leukocyte inhibition, giving incidental bacteria a good environment to grow in (Smith 2006).

The concentration of AMH in bitches is basal from around 30 up to 9 days prior to ovulation and then peaks up to three times the basal concentration at approximately a week prior to ovulation, thereafter it quickly decreases again. This seems to be correlated with the increased and later decreased number of early antral stage follicles (Nagashima *et al* 2016). AMH concentrations are thus related to follicle dynamics in the bitch. A sign of aberrant follicular dynamics in bitches suffering from pyometra is that the simultaneous presence of both follicles and *corpus luteum* is more common than in healthy bitches (Ström Holst *et al* 2001). Follicular cysts may also be found in the removed ovaries after ovariohysterectomy following a pyometra (England & von Heimendahl 2010).

The aim of the present study was to compare the concentration of AMH in bitches with or without pyometra in the corresponding cyclus stage. We hypothesized that bitches with pyometra might have

higher AMH concentrations, than bitches without pyometra, due to presence of growing follicles even after ovulation. Such follicles could contribute to higher oestradiol concentrations that, along with high progesterone concentrations could increase the risk of pyometra. A higher concentration of AMH in bitches with pyometra would thus indicate aberrant follicular dynamics in bitches with pyometra, possibly contributing to the pathogenesis of the disease.

Literature review

Anti-Müllerian hormone

The Anti-Müllerian hormone, also known as Müllerian inhibiting substance (MIS), is a member of the transforming growth factor-β (TGF-β) family which includes many types of growth and differentiation factors (Broer *et al* 2014; Holst 2016; Monniaux *et al* 2013). During the foetal stage of development, it has an important function in sex differentiation. In the male foetus, the Sertoli cells produce high concentrations of AMH resulting in inhibition of the Müllerian ducts and allowing the Wolffian ducts to develop with help of testosterone, resulting in a male phenotype (Broer *et al* 2014). This secretion is not seen in foetal female gonads, resulting in development of the inner female genitalia instead (Meyers-Wallen 2005).

In both the bitch and in other female mammals, AMH is produced and secreted by the granulosa cells surrounding the pre-antral to early antral staged follicles (Broekmans *et al* 2008; Monnaiux *et al* 2013; Nagashima *et al* 2016; Themmen *et al* 2016). In women, this production is not present when follicle enters the preovulatory phase, become FSH- (follicular stimulation hormone) susceptible and start producing oestradiol, see also Fig. 1 below. There is also a correlation between the number of AMH producing follicles and the concentration of AMH in the circulating blood (Broekmans *et al* 2008; Durlinger *et al* 2002; Fanchin *et al* 2003; Gigli *et al* 2005). In mice, the *corpus luteum* has been shown not to produce AMH (Myers *et al* 2009).

In the bitch, it has been described that the concentrations of AMH increase 8-9 days prior to the LH-surge (luteinizing hormone), up to three times the normal baseline for the individual, and then decrease back to normal at around 4 days prior to the LH-surge. This is presumably due to the growth of follicles and the variation in AMH secretion between different follicle stages, and coincides with the increase and decrease of early-antral stage follicles described in a study by England *et al* (2009). The concentrations, both baseline and increase, may also vary between individuals (Nagashima *et al* 2016). In humans, the concentration of AMH can predict the onset of menopause, as the concentrations of AMH become undetectable when there are no primordial follicles left (Broer *et al* 2014; Nelson & La Marca 2011). AMH analysis has also been used to diagnose granulosa cell tumours in humans (La Marca & Volpe 2007) and Sertoli cell tumours in dogs (Ström Holst & Dreimanis 2015).

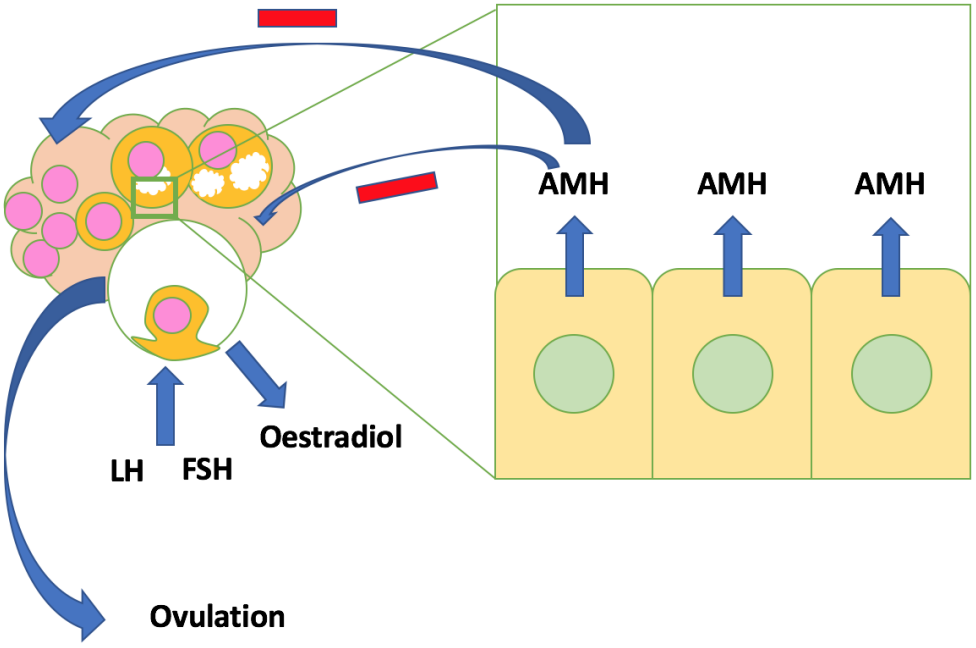

Fig. 1 *Schematic view of AMHs secretion from the granulosa cells surrounding the pre-antral to antral stage follicles, its negative effect on follicular recruitment and growth, and later the lack of secretion from the following follicle stages, at the stage of LH and FSH susceptibility and start of oestradiol secretion, resulting in an ovulation. Free sketch from Holst 2016.*

AMH is present throughout the life of an intact male dog as it is continuously secreted from the Sertoli cells in the testis. Studies have shown that the concentrations of AMH is generally higher in males than in females. Other studies have shown that after a gonadectomy AMH concentrations were significantly lower or absent in both female and male dogs, compared to intact individuals; in accordance with our present knowledge, that AMH is only produced in the gonads (Place *et al* 2011; Themmen *et al* 2016). This has also been shown in cats (Axnér & Ström Holst 2015).

Analysing AMH

Most commonly an ELISA (enzyme-linked immunosorbent assay) is used to analyse AMH. In human endocrinology, this assay was first developed in the 1990s by Hudson *et al* and has been used by both Diagnostic Systems Laboratories and Immunotech (later Immunotech Beckman Coulter) (Holst 2016; Rustamov *et al* 2014), followed by the development of the AMH Gen II assay (Beckman Coulter) (Nelson & Couto 2014; Nelson & La Marca 2011). There are also assays available specially designed for dogs (Pir Yagci *et al* 2016; Themmen *et al* 2016; Ylimaz *et al* 2015).

The AMH Gen II assay has been used for both cats and dogs (Axnér & Ström Holst 2015; Nagashima *et al* 2016; Ström Holst & Dreimanis 2015) and has been shown to result in undetectable concentrations in spayed cats, both females and males (Axnér & Ström Holst 2015). The AMH Canine ELISA has been used and validated in two recent studies (Themmen *et al* 2016; Ylimaz *et al* 2015).

The normal oestrous cycle

The domestic bitch is typically monoestral and not seasonal (Concannon 2011) and has one or two oestrous cycles annually (England & von Heimendahl 2010). This cycle is divided in proestrus, lasting 3 days to 3 weeks, oestrus for approximately 1 week, followed by two months of metoestrus, or luteal phase, and then the inactive anoestrus (Concannon 2011).

During proestrus, the follicular stimulating hormone (FSH) secreted from the anterior pituitary gland in minor pulses leads to an increased secretion of oestradiol from the follicles (se fig. 1 below) and a following thickening of the endometrium. The follicles grow from primordial follicles into bigger pre-antral to antral stage follicles (Concannon 2011). When the follicles reach the pre-antral stage, approximately 9 days before ovulation, they start secreting AMH (Nagashima *et al* 2016; Reynaud *et al* 2012), se Fig. 2 below. More pre-antral follicles lead to an increased production of AMH, with a peak at around seven days pre-ovulation (Nagashima *et al* 2016).

Just before the oestrus starts the FSH-pulses occur more frequent and the concentration increases. The start of oestrus is characterised by the luteinizing hormone- (LH) peak that induces ovulation of the pre-ovulatory follicles (Concannon 2011). Just before the ovulation a pre-ovulatory luteinisation starts resulting in a progesterone secretion. After ovulation, the ovulated oocytes need another 48-72 hours to mature in the oviduct before they are ready for fertilization and then they stay fertile for approximately 2-3 days. At this stage the *corpora lutea* are formed resulting in higher concentrations of progesterone (se fig. 1 below). Progesterone, the pregnancy maintaining hormone, is then secreted from the *corpus luteum* (Concannon 2011). Progesterone inhibits the immune system in the uterus to allow the embryos to attach to the uterine wall (Nelson & Couto 2014).

Metoestrus, also known as the luteal phase, is characterised by high concentration of progesterone. These high concentrations are present in all bitches, pregnant or not (Gultiken *et al* 2016).

At the end of metoestrus, the concentration of progesterone decreases. If the bitch is pregnant, this prepares both the pups and the bitch for partum. If she is not pregnant the decreasing progesterone concentrations and the following increase of prolactin concentrations may instead lead to pseudo pregnancy with signs such as imaginary pups, lactation and changes in behaviour. Clinical signs of pseudo pregnancy may also be seen in early anoestrus. During anoestrus, the reproductive system is relatively dormant until the next proestrus (Concannon 2011). The AMH-concentrations during the luteal phase and the early anoestrus phase in female canines has to the authors' knowledge not yet been studied.

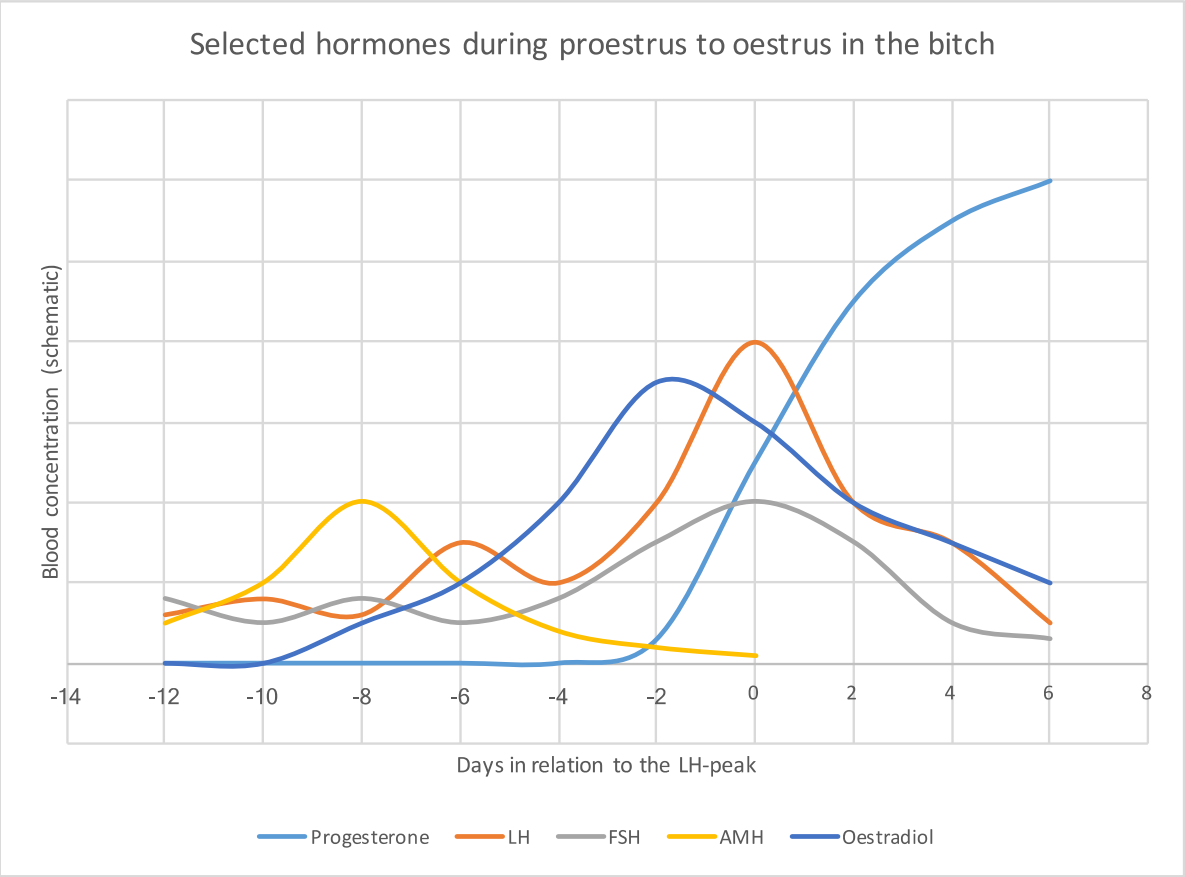

Fig. 2 *Schematic image of selected hormones during the bitch proestrus to oestrus period. The anti-Müllerian hormone concentrations past ovulation are not known, therefore the AMH-curve ends at day 0, the day of ovulation. Free sketch from Concannon 2011 and Nagashima et al 2016.*

Pyometra

Pyometra is characterised by inflammation and infection within the uterus resulting in suppuration into the uterine lumen. It is mostly seen in middle-aged or older dogs (Hagman *et al* 2011). The disease is normally diagnosed in the metoestrus period (Smith 2006) during the time of progesterone and residual oestradiol influence. The oestradiol thickens the endometrium and prepares it for a supposed pregnancy (Sjaastad *et al* 2010; Smith 2006), and the high progesterone concentrations result in proliferation of the endometrium, increased secretion from uterine glands and inhibited contractions within the myometrium. That along with removal of otherwise naturally occurring leukocytes gives the oocytes but also the incidental bacteria a perfect environment and time to attach to the uterine wall (Nelson & Couto 2014). Although the aetiology of pyometra is not fully known, it is suggested that it is related to the hormonal changes following oestrus, a secondary opportunistic infection (Dąbrowski *et al* 2013) and possibly with a genetic predisposition, as the disease is more common in certain breeds (England & von Heimendahl 2010; Hagman *et al* 2011; Jitpean *et al* 2012).

Further, oestradiol and progesterone receptors within the uterus are also believed to play a role in the pathogenesis of pyometra. It has been suggested that the progesterone receptors in a uterus suffering

from pyometra become over active, compared to those in healthy controls (Ververidis *et al* 2004). This over activation has been described to occurs despite normal (for the metoestrus) concentrations of progesterone, resulting in a down-regulation of oestradiol receptors, thus minimizing the oestradiol effect. Higher oestradiol concentrations in the blood of bitches with pyometra than in control bitches have also been reported by Ververidis *et al* (2004). Possible factors behind a high oestradiol concentration and over activation of progesterone receptors are not yet known but an aberrant follicular dynamic may contribute and thus trigger the development of pyometra (Ververidis *et al* 2004).

As the bitch is ageing it is common to detect macroscopic changes in both the uterus (such as cystic endometrial hyperplasia) and the ovaries. In the ovaries, it is not uncommon to find both follicles and *corpora lutea* simultaneously, more commonly in bitches suffering from a pyometra than in healthy bitches (Ström Holst *et al* 2001). Follicular cysts may also be found in bitches with pyometra (England & von Heimendahl 2010).

The clinical signs vary between individuals, from only subtle changes to heavy depression, fever, anorexia, vomiting, polyuria and polydipsia (Dąbrowski *et al* 2013; Nelson & Couto 2014). In closed-cervix pyometra there is no vaginal discharge, in contrast to open cervix pyometra, when pus accumulated in the uterus leak out through the cervix (Dąbrowski *et al* 2007; Enginler *et al* 2014). A closed-cervix pyometra is considered more urgent since the pus may leak out via the fallopian tubes or even lead to rupture the uterine wall and cause peritonitis (Jitpean *et al* 2014a; Nelson & Couto 2014). A female dog with closed or open pyometra may show dehydration, fever and enlargement of the uterus on ultrasonography or radiography (Dąbrowski *et al* 2007 and 2013). The enlarged uterus may be palpated via the abdominal wall.

To evaluate the bitch further blood analyses are usually performed, depending on the clinical status. Pyometra is an inflammatory process that usually induces systemic effects (Sjaastad *et al* 2010) which may be measured by analysing the acute phase protein C-reactive protein (CRP) that is produced by the liver during a systemic inflammation (Hillström *et al* 2015). Studies have shown that concentrations of CRP are significantly higher in bitches suffering from a uterine disease than in healthy bitches (Dąbrowski *et al* 2007 and 2013; Enginler *et al* 2014; Jitpean *et al* 2014b). In addition, it is not uncommon to find an elevated white blood cell count in response to the inflammation (Jitpean *et al* 2014b; Smith 2006).

Material and Method

Animals

Blood samples were taken from bitches (N=14, Table 1) with spontaneous pyometra at the University Animal Hospital, SLU, Uppsala. The pyometra was diagnosed from during the actual heat and up to 16 weeks after the end of heat according to the owners' observations. The pyometra were diagnosed by imaging techniques, ocular inspection of the uterus post-surgery and/or histological examination of the uterus (P2). Based on these examinations, all the sick bitches were considered as having pyometra. Other diseases or deviations were recorded, one individual was treated with 100mg cyclosporine daily (P1) for allergic dermatitis, another with 5 mg prednisolone daily (P4) for mite allergy, a third with 30 mg cimocoxib daily (P7) for arthritis and a fourth with 100 mg toceranib every two days (P12) for mastocytoma.

The control group consisted of 19 female dogs (Table 2), all included within two months after heat. A clinical examination was performed by the author. Information such as numbers of previous litters, signs of pseudo pregnancy and number of oestrous cycles annually was recorded. None of the control bitches showed clinical signs of uterine disease nor were they diagnosed with a uterine disease within two months after the sample was taken.

Blood samples

Blood was collected by venipuncture from a peripheral vein, all pyometra bitches by staff at the UDS, SLU, and all control bitches by the author. Two samples from bitches with pyometra were collected from left over blood emitted for analysis (P14, P7). All blood samples were allowed to cool and/or set before centrifuging at 4500 rpm for 5 minutes. Serum and EDTA-plasma was separated and transferred into cryotubes and stored at -80°C until analysis. The sample from one bitch was stored in the fridge (+8°C) for 12 hours before frozen in -80°C (P10), another was stored in -20°C for 2 hours before being moved to -80°C (P9) and two samples were stored in room temperature for a few hours before they were moved to -20°C for 24 vs 72 hours and then moved to -80°C (P14, P7).

Bitches with pyometra

	Breed	Age	Weight	Time since observed end of heat	Clinical signs	AMH ng/mL	CRP mg/l	Progesterone nmol/L
P1	German Shepherd	8,5 years	34 kg	14 days	Mild depression, fever, vaginal disscharge, anorexia	0,09	208,2	25,3
P2	German Shepherd	1,5 years	29 kg	0 days	Moderate depression, fever, pu/pd, vaginal disscharge, red mucosal membranes, anorexia	0,17	184,3	7,28
P3	American staffordshire terrier	5 years	22,5 kg	0 days	Mild depression, fever, anorexia, pu/pd	0,11	>290	37,8
P4	Mixed breed	9 years	26,5 kg	28 days	Moderate depression, fever	0,16	>290	36,3
P5	Welsh Springer Spaniel	10,5 years	19 kg	5 days	Mild depression, fever, pu/pd, possibly icteric mucosal membranes, mammary tumors	0,07	>290	3,91
P6	Miniature schnauzer	6 years	10 kg	0 days	Moderate depression, vaginal discharge, anorexia, reddend and sticky mucosal membranes, cystitis	0,79	<7	52,5
P7	Cairnterrier	10 years	10 kg	0 days	Severe depression, fever, mild anorexia, reddend mucosal membranes with short CRT	0,21	197	56,3
P8	Swedish Elkhound	7,5 years	46 kg	45 days	Mild depression, vaginal discharge, sticky mucosal membranes, obese	0,47	51,94	4,93
P9	Cocker Spaniel	8 years	15,5 kg	45 days	No depression, sticky mucosal membranes, no vaginal discharge	0,22	>290	35
P10	Eurasier	4,5 years	25 kg	Aprox. 14 days	Moderate depression, pu/pd, vaginal discharge, anorexia, sticky mucosal membranes	0,2	107,1	3,21
P11	German Shepherd	11,5 years	33,5 kg	Unknown	Severe depression, fever, short CRT	0,11	50	8,75
P12	Bernese Mountain Dog	6 years	36,5 kg	Unknown, min. 16 weeks	Mild depression, vaginal discharge, mild anorexia, reddend mucosal membranes	0,26	219	1,5
P13	Australian Kelpie	5 years	16 kg	32 days	Moderate depression, fever, vaginal discharge, sticky mucosal membranes	0,12	>290	9,7
P14	Mixed breed	10 years	42,5 kg	0 days	No depression, sticky mucosal membranes, vaginal discharge, mammary tumors, obese	0,05	196,1	41,7

Table 1 *Included bitches with uterine disease. With individual results for Anti-Müllerian hormone, C-reactive protein and progesterone.*

Control group

	Breed	Age	Weight	Time since observed end of heat	AMH ng/mL	CRP mg/l	Progesterone nmol/L
K1	Jack Russell Terrier	10,5 years	7 kg	47 days	0,53	11,13	22,2
K2	Jack Russell Terrier	2 years	7 kg	31 days	1,23	6,98	20,5
K3	Beagle	9 years	12 kg	22 days	0,08	12,46	10,6
K4	Beagle	9 years	11,5 kg	25 days	0,14	6,62	21,1
K5	Beagle	9 years	11,5 kg	25 days	0,25	6,77	27
K6	Beagle	5 years	13,5 kg	24 days	0,19	10,57	25,1
K7	Beagle	5,5 years	11,5 kg	24 days	0,21	7,4	27,9
K8	Poodle	2 years	7 kg	43 days	0,68	7,03	24,7
K9	Am Staff Terrier	5 years	18,5 kg	14 days	0,25	6,23	69,3
K10	Poodle	5 years	7 kg	47 days	1,11	7,42	6,77
K11	Border Collie	2 years	15 kg	47 days	0,41	6,44	10,6
K12	Shetland Sheepdog	8 years	8,5 kg	9 days	0,3	5,78	82
K13	Mixed breed	11,5 years	12,7 kg	15 days	0,14	15,15	33,4
K14	Groenendal	2 years	17 kg	8 days	0,35	13,37	83,3
K15	Eng Springer Spaniel	2,5 years	20 kg	14 days	0,61	6,31	71,2
K16	Working Kelpie	4,5 years	19 kg	26 days	0,12	6,04	24,9
K17	Australian Terrier	11 years	9 kg	17 days	0,28	6,85	63,9
K18	Bichon Havanais	7 years	4,5 kg	17 days	0,53	8,51	60,4
K19	Australian Shepherd	8 years	19 kg	11 days	0,23	7,09	104

Table 2 *Bitches in the control group. With individual results for Anti-Müllerian hormone, C-reactive protein and progesterone.*

AMH

AMH was analysed at the Clinical Chemistry Laboratory, UDS, SLU, Uppsala, Sweden, in one batch using an enzyme-linked immunosorbent assay (AMH Gene II ELISA; Beckman Coulter), validated for dogs, per the instructions from the manufacturer.

In short, 20 mL of controls, samples and standards were diluted with buffer and added to an anti-AMH antibody-coated microtitration plate. After a washing step, anti-AMH biotin conjugate was added to each well. Another incubation and washing was performed before adding streptavidin-horseradish peroxidase. A third incubation and washing was performed. The substrate tetramethylbenzidine was added and briefly incubated before the addition of an acidic stopping solution. The enzymatic turnover was then determined by a dual-wavelength absorbance measurement at 450nm and 620nm.

CRP and progesterone

Progesterone and CRP were analysed in one batch at the Clinical Chemistry Laboratory, UDS, SLU, Uppsala, Sweden. For CRP, a validated immunoturbidimetric canine CRP method (Gentian) (Hillström *et al* 2015), was used. The reference interval in healthy dogs for CRP is <7 mg/l, a value of 7-20 mg/l can be seen without clinical illness and a value >200 mg/l is considered a strong response. Samples from four of the bitches (P3, P6, P7, P11, Table 1) with pyometra had already been analysed for CRP at the same laboratory with the same method and were not tested again. Progesterone was analysed using a chemiluminescence method (Immulite Siemens).

Ethical consideration

The study was approved by the local animal ethical committee, permit number C136/13. Participating dog owners gave their informed written consent.

Statistics

The data was analysed using Minitab Express and showcased via boxplots and a scatterplot. A linear regression analysis was performed, and since the residuals for AMH and weight were not normally distributed, logarithms were used to achieve normality. In the regression analysis, log AMH was the outcome, and presence or not of pyometra, age, days since the end of the last heat and log weight were explanatory variables. Two owners did not know the onset or end of the last heat so these two individuals had missing values, the individuals that were still in heat, or just after, were analysed as 0 days since end of heat. Interactions between these parameters were not significant, and thus not included in the final model. Other comparisons between bitches and without uterine disease were made using the Mann-Whitney test, because of non-normally distributed data. The level of significance was set at $P<0.05$.

Results

Bitches of 22 different breeds and two mixed breed bitches were included in the study. In the pyometra-group eleven different breeds were included and no breed was included more than three times (German Shepherd). The age varied from 1.5 to 11.5 years with a median age of 7.75 years and the weight varied from 10 to 46 kg with a median of 26 kg. In the control group eleven different breeds were included and no breed was included more than five times (Beagle), other breeds no more than two. The age varied from 2 to 11.5 years with a median age of 5 years and the weight varied from 4.5 to 20 kg with a median of 11.5 kg. 10% of the bitches (2/19) were known to be pseudo pregnant post heat (K1, K2), 31% (6/19) had had previous litters (K4, K6, K7, K9, K12, K17) and 100% (19/19) of the controls had approximately two heat cycles annually.

In the 14 dogs with pyometra 38% (5/14) had mild depression, 38% (5/14) had moderate depression and 15% (2/14) were severely depressed. A vaginal discharge was present in 47% (8/14) of the bitches with pyometra, 7% (1/14) did not have vaginal discharge and for 38% (5/14) no information on discharge was given in the medical records.

The control bitches and bitches with pyometra did not differ significantly concerning age (p=0.314), or progesterone concentrations (p=0.074) but bitches with pyometra were significantly heavier (p<0.0001).

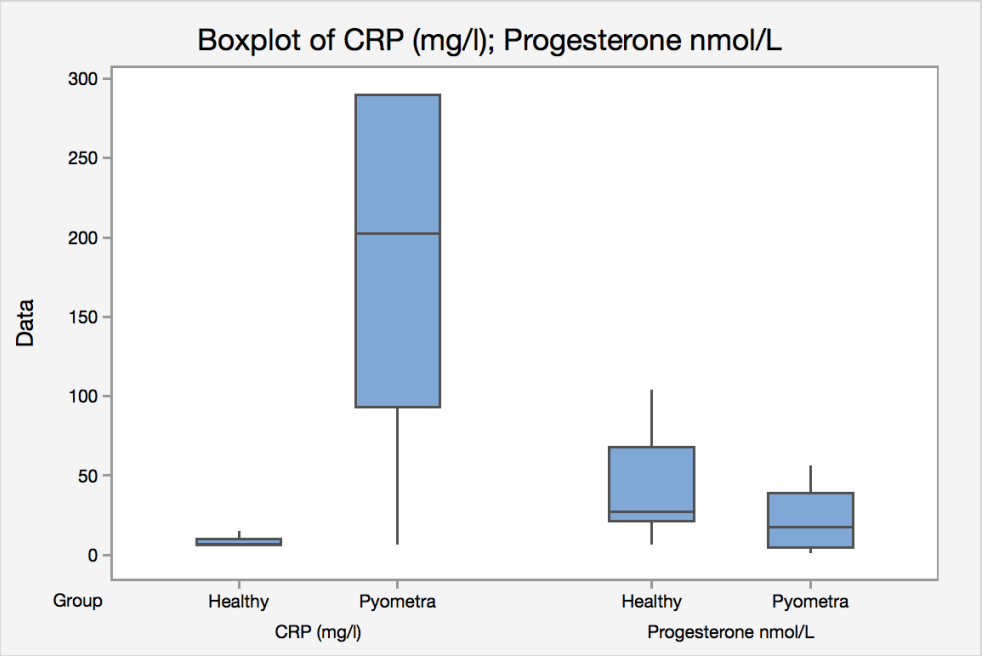

Fig. 3 *Boxplot of CRP, divided in control group (7,0 mg/l, 5,78-15,15) and a pyometra group (202,6 mg/l, 7,0-290). Boxplot of progesterone divided in control group (27,4 nmol/L, 6,77-104,0) and a pyometra group (17,5 nmol/L, 1,5-56,3).*

Bitches with pyometra had significantly higher CRP concentrations than the control bitches (p<0.0001, Fig. 3). Of these bitches 93% (13/14) showed concentrations in the region of moderate to severe systemic inflammation while one (7%, P6, see Table 1) had a concentration of <7 mg/l, indicating no inflammation. Of the controls 47% (9/19) showed concentrations <7 mg/l while 53% (10/19) showed CRP concentrations above the reference limit for healthy dogs, though still in the region of mild/subclinical inflammation. According to the clinical examination findings 53% (10/19)

had mild gingivitis, 16% (3/19) had moderate to severe gingivitis while 32% (6/19) showed no gingivitis. 80% (8/10) of the individuals with CRP concentration above the reference limit for healthy dogs had gingivitis, while 33% (2/6) of the individuals without elevated CRP showed gingivitis. Ten per cent (2/20) showed slight pain from limb or back along with gingivitis, both with elevated CRP concentrations.

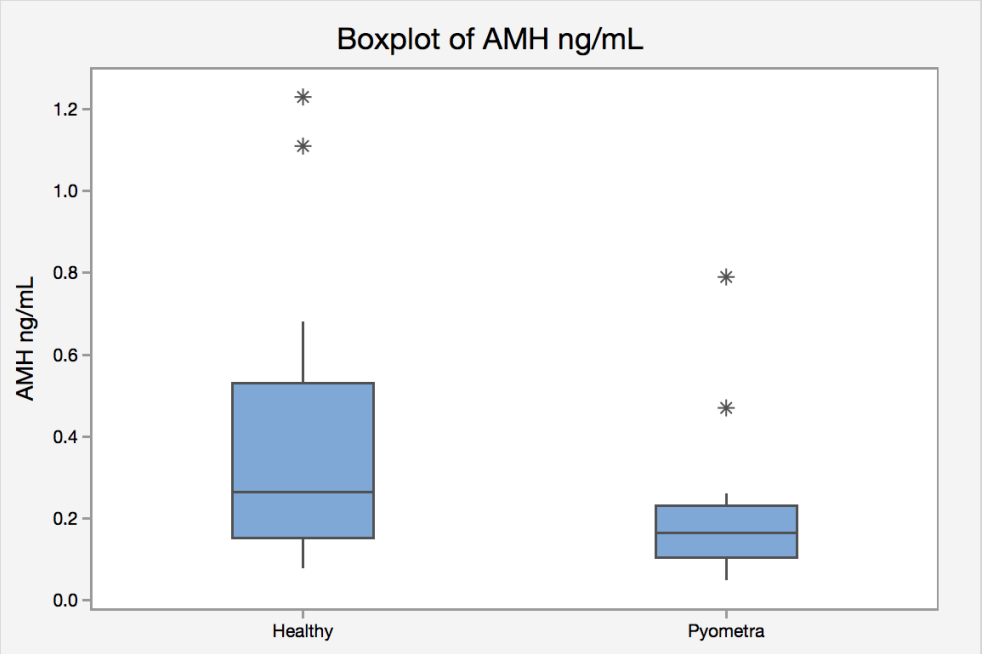

Fig. 4 *Boxplot showing concentrations of AMH in the control group (0,265 ng/mL, 0,08-1,23) and a pyometra group (0,165 ng/mL, 0,05-0,79).*

All bitches had detectable values of AMH (Fig. 4). The regression analysis showed a significant association between log AMH and age (p=0.003) and log weight (p=0.003). There was no significant association between log AMH and if the bitch had pyometra or not (p=0.598).

Discussion

All bitches had detectable AMH concentrations, in accordance with the results of earlier studies in dogs and cats (Axnér & Ström Holst 2015; Themmen *et al* 2016; Ylimaz *et al* 2015). There was no significant association between pyometra and AMH concentrations, but age and weight had a significant effect; the older and heavier the lower concentration of AMH. The bitches with pyometra were significantly heavier and were also older although not significantly so and because of this had lower AMH concentrations in the present study.

As previous studies have described the AMH concentration is dependent on the number of pre-antral to antral staged follicles (Broekmans *et al* 2008; Monnaiux *et al* 2013; Nagashima *et al* 2016; Themmen *et al* 2016). In both women (Nelson & La Marca 2011), mares (Claes *et al* 2015) and bitches (Hollinstead *et al* 2016) the concentration of AMH decrease as the individual is ageing. In

women, AMH becomes undetectable when undergoing menopause, having no active follicles left (Broer *et al* 2014; Nelson & La Marca 2011). The age-related decrease in AMH concentration seen in this study indicate fewer AMH-producing follicles in older bitches. This correlation between fewer follicles and decreased AMH concentrations with high age is also seen in mares (Claes *et al* 2015). A lower fertility in older dogs, resulting in smaller litter seizes, has been described in a study by Gravilovic *et al* (2008) and Hollinstead *et al* (2016). This is an indication that the older the individual, the lower the concentrations of AMH and in turn fewer active follicles.

In the present study, the AMH concentrations were dependent on the weight of the bitch, the heavier the dog the lower the concentration of AMH. Such a relation has recently been described (Hollinstead *et al* 2016) in a population of 155 intact bitches of various ages and breeds (weights). Especially giant breeds had significantly lower AMH concentrations than any other breeds. Because two dogs of the same breed can vary a lot in weight, it would be interesting to relate the weight to a body condition score or similar assessment of body mass. Though an association between AMH concentrations and BMI is not seen in young or adult women (Bertrand *et al* 2016) the individual with the lowest concentration of AMH in this study was also the next heaviest and stated as obese per the clinician (P14, Table 1).

The lack of association between AMH concentration and presence of pyometra indicates that the development of pyometra is not due to early aberrant follicular dynamics. It is unlikely that there is any difference between individuals with or without pyometra regarding the number of pre-antral to antral stage follicles and its AMH-producing granulosa cells. However, this study does not rule out any aberrant dynamics in later follicular development, including follicular cysts, or persistent follicles, or other hormonal imbalances within the ovary. Since both pyometra and such changes in the ovaries have been seen more commonly in older bitches (Ström Holst *et al* 2001), such aberrations in later follicular dynamics might be of importance in the pathogenesis of pyometra. The results suggest that the inflammation caused by pyometra does not affect the AMH concentration. This is interesting since women with an inflammation caused by Crohn's disease have lower concentrations of AMH than healthy women (Senates *et al* 2013). The lack of association between AMH concentration and presence of pyometra could also be due to the limited number of dogs included.

Preferably, should this study be done again it may profit if more criteria were set for both the controls and the bitches suffering from pyometra. Firstly, they should be in the same age range, or several age ranges, as the AMH concentrations is seen to decrease the older the bitch becomes. Secondly, the same arrangement should be set for weight to determine whether the weight dependent AMH concentrations are applicable in all weight ranges. And thirdly, more individuals should be included in both the pyometra and control groups.

In this study, all bitches were diagnosed with pyometra by clinical examination with special regard to depression, fever and vaginal discharge, diagnostic imaging, ocular inspection of the uterus post-surgery and/or by histological examination of the uterus (Table 1). The amount of fluid within the uterus varied from minimal to severe. These criteria exclude several other diagnoses. No bacteriological cultures were performed, which would have been beneficial in differing pyometra from uterine diseases such as haemo- or mucometra, even though not all pyometra have positive uterine

bacterial cultures (Enginler *et al* 2014). To validate the diagnosis further a histological examination of the uterus in all ovariohysterectomised bitches would have been beneficial.

In some cases, CRP was analysed by the clinician in the diagnostic work-up, and the other bitches were tested specifically for the study. All bitches with pyometra, except P6, had circulating concentrations well over the clinical decision limit for subclinical inflammation (20 mg/l) in accordance with many previous studies (Dąbrowski *et al* 2007 and 2013; Enginler *et al* 2014; Jitpean *et al* 2014b). The outlier (P6) was not expected and could be due to an enclosed inflammation localized within the uterus or that the dog was sampled just at the beginning of the pyometra and therefore not yet showing increased CRP concentrations. Several (53%) of the bitches in the control group showed concentrations that were within, but not above, the limits of subclinical inflammation. This was not expected. In most of these cases (80%, 8/10) the dogs had gingivitis. It cannot be excluded that the gingivitis may have contributed to the slightly elevated concentrations. The presence of mild inflammation in the control bitches is thought not to have affected the concentrations of AMH due to the low concentrations of CRP detected, and since there was no effect of the severe inflammation caused by pyometra on AMH concentrations.

Along with AMH and CRP, progesterone was analysed to determine whether all individuals were in the metoestrus period. Some bitches with pyometra had relatively low progesterone concentrations. That pyometra had developed with low concentrations could be due to the increased sensitivity of the uterine progesterone receptors as has been reported earlier in pyometra (Ververidis *et al* 2004) resulting in a substantial influence of progesterone despite relatively low circulating concentrations or that the disease was diagnosed relatively late due to a more chronic development. It has been suggested that the high oestrogen concentrations during oestrus followed by the high progesterone concentrations during metoestrus is an important part in the pathogenesis of pyometra (Smith 2006). To further clarify the development of pyometra, regarding the cyclus stage, and progesterone concentrations, it would be advantageous to determine the end of oestrus with greater precision, for example using vaginal cytology.

Conclusion

In this study, AMH concentrations were not associated with presence of pyometra. However, AMH was affected by both age and weight; the heavier and older the individual the lower concentrations of AMH.

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