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RESEARCH ARTICLE

# Anti-Müllerian hormone levels in relation to ovarian structures, season and age in Lipizzaner broodmares

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**ABSTRACT**

The anti-Müllerian hormone (AMH) is a granulosa cell-derived hormone that has been associated with female fertility and reflects the population of growing follicles. This study aimed to evaluate the average concentration of AMH in Lipizzaner mares, as well as to determine the relationship between AMH concentration and follicle number and size. We also investigated the relationship between the age of mares and their AMH levels. The possible effect of seasonality of AMH levels was also assessed. Twenty-three mares between 6 and 24 years of age were included in the experiment. Mares were divided into two groups: Group 1 included mares aged 6 – 15 years ($n$ = 11), while Group 2 included individuals older than 15 years of age ($n$ = 12). Venous blood was collected and ovarian activity was monitored parallelly by transrectal ultrasonography. Serum AMH concentrations varied widely between the two different groups. AMH concentrations were significantly lower in old mares than in younger animals. A positive relationship was detected between AMH concentration and the number of medium-sized follicles ($P$ = 0.022), large follicles ($P$ = 0.016) and the total follicle count ($P$ = 0.026). No seasonal effect was detected.

**KEYWORDS**

anti-Müllerian hormone, Lipizzaner, fertility, ovary, aging

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## INTRODUCTION

The presence of the anti-Müllerian hormone (AMH) was recognized after the middle of the 20th century. The AMH, also known as the Müllerian inhibiting substance (MIS), is a glycoprotein hormone in terms of its biochemical structure (Papas et al., 2021). Its molecular

weight is 140 kDa, and its biological half-life is 1.5 days (Umer et al., 2019). Together with inhibins, activins, bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs), the AMH is part of the TGFβ superfamily. Factors that can be classified into this superfamily have a wide range of physiological functions, they play a role in mesenchymal-epithelial interactions, cell growth, extracellular matrix production and tissue remodelling. Unlike the other members of this family, which elicit multiple functions in many tissues, the main role of the AMH is to induce the regression of the Müllerian duct during male sexual differentiation (Durlinger et al., 2002). In mammals, the AMH is solely expressed by male and female gonadal somatic cells. In males, its key function is to inhibit the development of organs originating from the Müllerian duct (paramesonephric duct) during sexual differentiation, which occurs during the foetal period (Papas et al., 2021). However, in females, the AMH is not expressed at the time of sexual differentiation, ensuring the appropriate development of the female genital tract (Paes de Almeida Ferreira Braga et al., 2021).

In women and female animals, AMH expression can be detected in the granulosa cells of early primary, preantral and small antral follicles after birth (Papas et al., 2021). The AMH regulates follicle number and the selection of the dominant follicle during follicular waves (Umer et al., 2019). In the postnatal ovary, the AMH plays a key role in the recruitment of primordial follicles by preventing these follicles from joining the pool of growing follicles before the selection process. Thus, the AMH prevents premature exhaustion of the ovarian follicle reserve. Additionally, the AMH also regulates follicular development by reducing the sensitivity of pre-antral follicles to the follicle-stimulating hormone or FSH (Papas et al., 2021). The reason behind this process is that the AMH suppresses FSH receptors in gonadotropin-dependent small antral follicles (Umer et al., 2019).

Nowadays, the AMH is often the subject of research in human reproductive biology, thanks to the fact that it is an indicator of fertility and reproductive ageing, and it can also be used to estimate the size of ovarian follicle populations in women (Uliani et al., 2019). In domestic animals, examinations in this field are especially important because fertility is often identified as the primary factor that hinders the efficiency of livestock systems. With the help of assisted reproduction techniques (ART), reproductive performance can be optimized in domestic animals and many reproductive disorders can be eliminated. However, it is important to emphasize that the effectiveness of these techniques depends largely on the characteristics, especially the physiological parameters, of the individual, such as the antral follicle population (AFP) of the ovary. The efficiency of ART can be significantly diminished by the great variability in the number of antral follicles of the donors. Therefore, strict selection of animals prior to the application of these techniques is crucial. For this purpose, two main selection tools are available, genomic evaluation and the use of endocrine markers. Markers for determining AFP can also be classified in the latter group. The size of the pool of healthy follicles and oocytes (ovarian reserve) differs greatly at birth in humans and domestic animals and their number and quality rapidly decrease with age. Endocrine markers are used in conjunction with ultrasound techniques to estimate ovarian reserve capacity (Readhead, 2017).

In contrast to cattle and women, information is relatively scarce on the AMH in mares (Claes and Ball, 2016). Moreover, the evaluation of follicular populations together with AMH concentration is unusually difficult in mares due to the size, shape and pattern of follicular arrangement in the ovary. Unfortunately, due to the size and peculiar asymmetry of the ovary of these animals, accurate histological evaluation of small follicular populations is rather problematic. However, it is important to mention that these small follicles likely have the highest contribution to AMH secretion (Uliani et al., 2019). According to previous observations, the circulating AMH concentration is related to the population of follicles with a diameter between 6 and 20 mm (Papas et al., 2021). AMH expression decreases in the granulosa cells of follicles larger than 30 mm. Although the serum AMH concentration does not change notably during the oestrous cycle, significant differences can be observed between individual mares in this regard (Claes and Ball, 2016). Papas et al. (2021) observed remarkably high variability in AMH levels in individual mares (0.6 – 4.1 $\mu g^{*}L^{-1}$). These results are similar to previous research, where the concentration of serum AMH varied between 0.22 $ng^{*}mL^{-1}$ and 2.94 $ng^{*}mL^{-1}$ in mares with normal oestrous cycles and before ovulation this value ranged between 0.07 and 3.56 $ng^{*}mL^{-1}$ (Papas et al., 2021).

Circulating AMH, as a marker of ovarian function, is often examined in relation to maternal age (Papas et al., 2021). Age is a significant factor for women in terms of reproduction, as the chance of spontaneous conception decreases considerably with advancing age. In mares, this difficulty also exists. In this species, genital tract dysfunctions, the reduction of the ovarian reserve and poor oocyte quality are all age-related factors that significantly affect fertility. The degeneration of the reproductive tract takes place gradually, as the age of mares advances, the probability of the occurrence of endometrial cysts in the uterus and tissue growths in the fallopian tubes increases (Benammar et al., 2021). The results of recent research show that significantly lower AMH concentrations can be measured in geriatric mares (16–27 years old), which can be paralleled by the exhaustion of the ovarian reserve. Furthermore, it is also an important observation that there is a higher correlation between the ovarian capacity and the circulating AMH level in older mares (Papas et al., 2021).

The objectives of the study reported here were to:

1. Evaluate the average AMH concentration in Lipizzaner mares of different ages
2. Analyse the relationship between the number of follicles of different sizes and AMH levels
3. Assess a possible seasonal effect

Mares have many characteristics that make them suitable model animals in human research. The anatomy of the equine

reproductive system, the long follicular phase of the cycle, and the fact that one dominant follicle reaches ovulation provide a suitable basis for the study of oocytes and follicle development. As the mare ages, the changes in hormonal and reproductive cycle are very similar to those seen in ageing women (Carnevale, 2008). On the grounds of these similarities, our hypothesis was that as with ovarian exhaustion in women, AMH levels decline with age in Lipizzaner mare horses. We also hypothesized that the AMH level shows a positive correlation with the population of antral follicles.

## MATERIALS AND METHODS

### Ethical authorization of animal experiment

All methods and the applied procedures on the animals were carried out in accordance with the European Union Directive of the European Parliament and the Council on the protection of animals assessed for scientific purposes. The study was approved by the Department of Epidemiology and Animal Protection of the Directorate of Food Chain Safety and Animal Health at the Central Agricultural Office (Permit Number: PE/EA/91e2/2017). All procedures involving animals were approved by the Ethics Committee of the University of Veterinary Medicine.

***Mares in the study.*** All Lipizzaner mares were owned by the National Stud Farm Szilvásvárad and were kept and fed under the same circumstances (their intake was approximately 2 kg of oats and 6 kg grass hay per day with unlimited access to water). The mares were moved to the pasture (located approximately 3 km from the stables) twice a day. All animals were clinically healthy (6–24 years of age, 16 ± 5 years [mean ± standard deviation {SD}]) asymptomatic with no detectable disease or abnormality affecting the reproductive organs with body condition scores ranging between 5 and 6 (Body Condition Score Chart, Kentucky Equine Research, USA) at the beginning of the study.

In our study, we also assessed whether the time of the year might have some influence on AMH levels. The samples from the winter season were from January and the samples for the spring-summer season from March, April and June 2022.

***Blood sampling.*** Venous blood samples have been collected during the 2022 study period. At the beginning of the study, 14 mares have been involved and sampled until they have been inseminated. A total of 1–4 blood samples have been collected and analysed per mare.

In all cases, venipuncture was preformed from the external jugular vein. The blood sampling site was disinfected with 70% diluted ethyl alcohol (Materia Medica Pharmacy Budapest, Hungary) prior to blood sampling. Considering the biological variation of blood parameters within in the day, blood draws were always taken between 9 am and 12 pm. Blood was collected via serum vacutainer tubes (Vacutainer Serum Tube®, BD Medical, USA) with a 18G single-use, sterile needle (Vacutainer® Needle, BD Medical, USA). Blood tubes were immediately labelled and transported to the ELISA Laboratory (University of Veterinary Medicine Budapest, Department of Obstetrics and Food Animal Medicine) within 4 h. Blood collection tubes were centrifuged (3,000 g, 10 min) and clear sera was pipetted into 2 mL Eppendorf tubes and placed into a −80 °C refrigerator until further processing to avoid loss of bioactivity and contamination. There were no additional freeze–thaw cycles.

***Transrectal ultrasonography.*** After blood sampling, transrectal palpation and ultrasonographic examination were carried out as described by Schönbom et al. (2014). Both ovaries and the entire uterus were scanned thoroughly and 10 s recordings were saved onto a portable ultrasound machine (Draminski ultrasound scanner 4Vet Slim, endorectal probe 5 MHz). Structures on the ovaries were counted as follows: follicles smaller than 10 mm in diameter were referred to as "small", follicles between 10 and 20 mm were called "medium-sized" follicles and "large" follicles were those larger than 20 mm. All data was entered into a Microsoft Excel spreadsheet.

***ELISA test.*** An equine AMH ELISA test kit (Ansh Labs, Webster, Texas, USA) was implemented to measure AMH levels in frozen-thawed serum with a detection range of 0.06–14 $ng^{*}mL^{-1}$. All samples were left to thaw for 25–35 min at room temperature (22–25 degrees Celsius) prior to testing. The enzyme-linked immunosorbent assay (Equine AMH ELISA) was performed according to the guidelines recommended by the manufacturer (https://www.anshlabs.com/product/equine-and-ovine-amh-elisa/).

***Statistics.*** The results were entered into a Microsoft Excel 2019 (Microsoft, California, USA) spreadsheet and the statistical analysis was performed using the IBM SPSS Statistics software package. The available data was analysed by Pearson's correlation analysis, bivariate linear regression, analysis of variance, Welch's test, paired sample *t*-test and two independent sample *t*-tests. The correlations between AMH levels and follicle number, and between AMH levels and age of mares was examined using Pearson's correlation analysis for the three age groups established (young, middle-aged, old). Subsequently, a bivariate linear regression analysis was also implemented to explore the relationship between the variables more precisely.

## RESULTS AND DISCUSSION

The mean serum AMH concentration calculated for all the Lipizzaner mares in this study was 3.02 ± 1.88 $ng^{*}mL^{-1}$ (between 0.46 and 8.21 $ng^{*}mL^{-1}$), with the median value of 2.44 $ng^{*}mL^{-1}$. The younger animals in Group 1 (mares aged 6 – 15; $n = 11$) had considerably higher AMH concentrations and their values varied over a substantially wider range. In younger mares (Group 1), the mean concentration was 3.93 ± 2.01 $ng^{*}mL^{-1}$, while in the older group (Group 2, aged 16 – 24, $n = 12$), this value was 2.26 ± 1.41 $ng^{*}mL^{-1}$.

Traversari et al. (2019) studied mares of a similar age range, namely 12.9 ± 4.5 years of age (range: 3–23 years) and found that the AMH concentration showed a high variability between individuals; AMH levels were 0.07–3.56 ng*mL$^{-1}$ with a median value of 0.59 ng*mL$^{-1}$ (Traversari et al., 2019). Hanlon et al. (2018) in a different study found a mean AMH concentration of 2.4 ± 1.7 ng*mL$^{-1}$ and the range was 0.07–15.3 ng*mL$^{-1}$ in this instance (Hanlon et al., 2018). Comparing these results with our findings, the average AMH levels differed and were higher in this study. Uliani et al. (2019) also examined AMH in elderly mares where the mean concentration of AMH was 2.2 ng*mL$^{-1}$ in animals between 15 and 20 years of age and 1.8 ng*mL$^{-1}$ in mares in the 20- to 25-year-old group, but gave results up to 12 ng*mL$^{-1}$ in different age groups. These results demonstrate that a pronounced variability between horse populations exists. Fouché et al. (2022) observed that AMH concentrations ranged between 0.01 and 2.44 ng*mL$^{-1}$ with a median concentration of 0.64 ng*mL$^{-1}$. The latter experiment, similar to our own study, involved an older population of horses between 12 and 21 years of age (Fouché et al., 2022). Claes et al. (2015) concluded that AMH concentrations were significantly lower in old mares (19–27 years of age; median, 0.21 ng*mL$^{-1}$) as compared to their middle-aged conspecifics (9–18 years of age; median, 0.47 ng*mL$^{-1}$) (Claes et al., 2015). In the study by Claes et al. (2015), significantly lower values were measured compared to our own results. Ball and colleagues studied mares between 4 and 23 years of age, with AMH concentrations ranging from 0 to 2.98 ng*mL$^{-1}$, the mean ±SD concentration were found to be 0.61 ± 0.45 ng*mL$^{-1}$ (Ball et al., 2019).

Regarding individual AMH values in the present study, no significant differences have been detected between individual mares associated with season (Fig. 1), however, considerable differences have been detected between individual mares in non-seasonal AMH concentrations.

Based on the findings of our experiment and comparison with similar studies, it can be stated that it is worth determining a reference interval for AMH levels in mares for the different horse breeds because a breed-dependant effect can be suspected; Hanlon et al. (2018) found a significant effect of breed on AMH concentrations in thoroughbreds, which had lower AMH concentrations when compared to standardbred mares. The determined reference intervals in

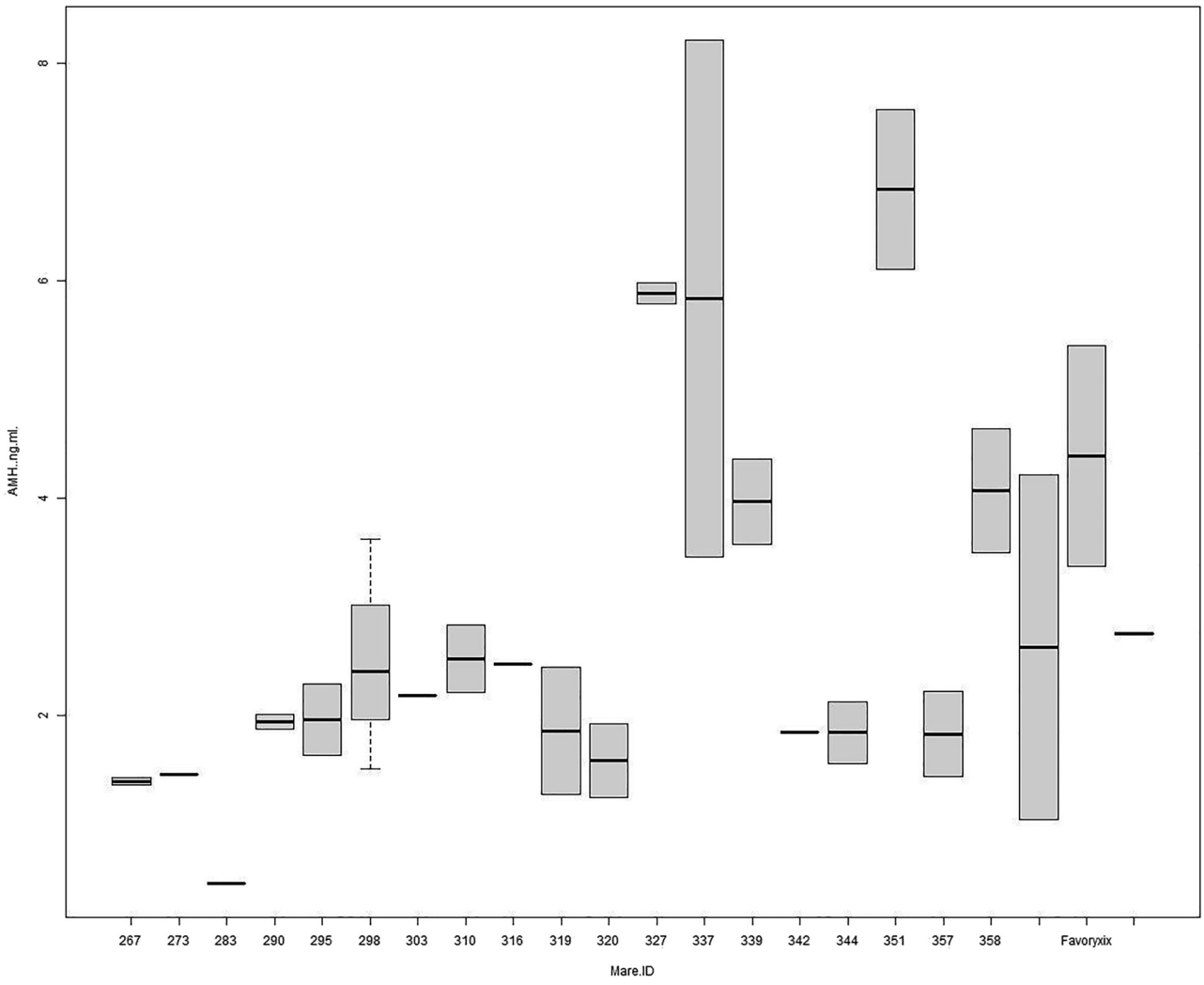


*Fig. 1.* Individual AMH-levels showing great variances between mares

that specific study were 0.3–4.6 $ng^{*}mL^{-1}$ for the thoroughbreds and 0.3 – 5.6 $ng^{*}mL^{-1}$ for standardbred mares (Hanlon et al., 2018). Korkmaz et al. (2020) examined serum AMH levels in purebred Arabian mares and found a mean serum AMH concentration in Arabian mares to be 0.515 ± 0.324 $ng^{*}mL^{-1}$ (Korkmaz et al., 2020). Possible breed-, and subspecific differences were also investigated in cattle; Baldrighi et al. (2014) examined Murrah (*Bubalus bubalis*) and Holstein (*Bos taurus*) heifers compared to Gyr (*Bos indicus*), while Batista et al. (2014) designed a study to compare AMH concentrations in Nelore (*Bos indicus*) and Holstein (*Bos taurus*) heifers and their observations displayed a difference between the respective species. Overall AMH concentrations were higher in Nelore than in Holstein heifers (Batista et al., 2014).

When counting and analysing ovarian follicles in relation to blood AMH, the findings showed significant correlation in three associations: between the total number of medium sized follicles and AMH levels ($P = 0.022$), between the total number of large follicles and AMH concentration ($P = 0.016$) (Fig. 2) and between total follicle count and AMH levels ($P = 0.026$) (Fig. 3). No significant correlation was found between the number of small follicles and AMH levels ($P = 0.869$).

Claes et al. (2015) aimed to determine plasma AMH concentrations and antral follicle counts (AFC) in mares of

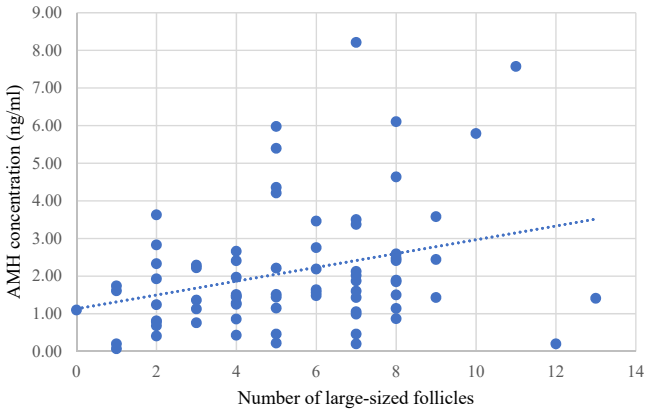


*Fig. 2.* Correlation diagram of AMH level and total number of large follicles in Lipizzaner mares (y = 1.05 + 0.32*x; $R^2 = 0.148$)

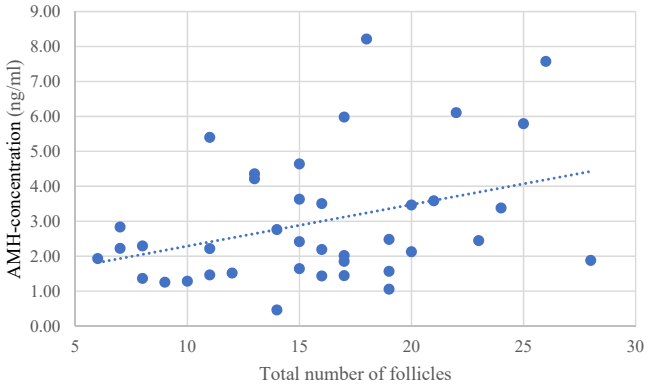


*Fig. 3.* Correlation diagram of AMH level and total follicle count in Lipizzaner mares (y = 1.1 + 0.12*x; $R^2 = 0.127$)

different age groups. These authors found that plasma AMH concentrations were positively associated with follicles between 6 and 20 mm diameter in size while there was no evident association between the number of follicles between 2 and 5 mm, and >20 mm in size and plasma AMH concentrations. Traversari et al. (2019) came to a slightly different conclusion, as they observed that serum AMH concentration and AFC were positively correlated in the case of follicles up to 30 mm in diameter (Traversari et al., 2019).

The results of the current study can be explained by the theory that the number of granulosa cells gradually increases parallel with follicle development. Given that granulosa cells of the follicle are the sole source of AMH in mares, we hypothesized that AMH production increases with the increase in the number of granulosa cells. Granulosa cells play a crucial role in the ovaries, undergoing major morphological and physiological changes during proliferation and differentiation, ovulation, luteinisation and atresia. The oocyte controls the proliferation and differentiation of granulosa cells, while granulosa cells influence oocyte maturation (Lu et al., 2005). In primordial follicles, the initiation of growth is marked by an increase in the size of the oocyte and the proliferation of surrounding somatic cells, which is accompanied by the formation of avascular cell layers called granulosa. The further stages of development can be recognised by an increase in the size of the oocyte and a change in the shape of the granulosa cells. Within the oocyte, the volume of the cytoplasm and nucleus increases dramatically and the granulosa cells, which are usually crescent-shaped in primordial follicles at rest, take on a cuboidal character during proliferation (Hirshfield, 1991). When the oocyte is surrounded by a complete layer of cuboidal granulosa cells, it is called a primary follicle. Thereafter, granulosa cell proliferation continues, and more and more granulosa cell layers are formed (Da Silva-Buttkus et al., 2008). Granulosa cell proliferation continues even after oocyte growth has ceased (Hirshfield, 1991).

In younger mares, the findings of this study show that AMH concentrations increased with age, while a negative correlation was observed in the older Lipizzaner mares: the greater the age of the animals, the lower their AMH levels. Furthermore, there was a statistically significant correlation between age and AMH levels in the group of older individuals ($P = 0.005$) (Fig. 4).

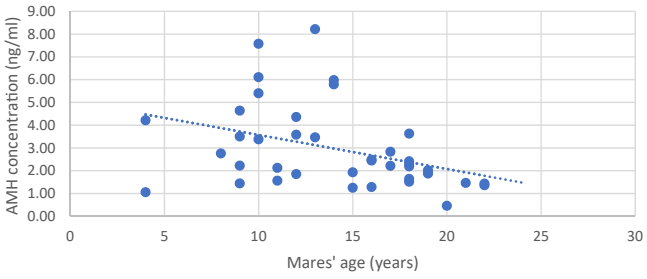


*Fig. 4.* Correlation diagram of AMH levels and age in old Lipizzaner mares (y = 9.1–0.35*x; $R^2 = 0.349$)

Our results are in line with those of other research groups, as previous investigation has shown that older mares (16–27 years of age) have significantly lower AMH concentrations, which can be paralleled with a decrease in ovarian follicle supply. In addition, the correlation between ovarian reserve capacity and circulating AMH levels in the blood was closer in the group of older individuals. However, in young and middle-aged individuals, a positive correlation was found between the number of oocytes retrieved and the measured AMH concentrations (Papas et al., 2021). In studies of fillies, AMH concentrations were fairly constant during the first two years of life. Despite the fact that in mammals, the number of follicles gradually decreases with age, AMH levels in horses do not start to decline significantly until after the age of 20, after which their number drops very rapidly (Uliani et al., 2019).

Korkmaz and colleagues made an interesting observation regarding the relationship between age and AMH levels in female dogs. The aim of their experiment was to evaluate the relationship between the follicular population present in the ovary and AMH levels in young and elderly female dogs. In the case of the preantral follicles, the number of granulosa cells ranged between 301.31 ± 4.16 in the group of young bitches, while in older animals, it was 270.25 ± 3.54. Their results indicated that the numbers of granulosa cells in preantral follicles decreased with advancing age resulting in lower serum AMH levels in aged individuals (Korkmaz et al., 2016).

In our study, 14 mares were tested during the winter and during this season, their average AMH level was 3.00 $ng^*mL^{-1}$, the minimum value was 1.28 $ng^*mL^{-1}$, the maximum was 7.57 $ng^*mL^{-1}$. The highest value (7.57 $ng^*mL^{-1}$) was measured in a 12-year-old mare, while the lowest value (1.28 $ng^*mL^{-1}$) was in an 18-year-old horse. In the spring-summer period the average AMH level was 3.04 $ng^*mL^{-1}$, the minimum value was 0.46 $ng^*mL^{-1}$, the maximum was 8.21 $ng^*mL^{-1}$. The lowest value (0.46 $ng^*mL^{-1}$) was measured in a 22-year-old mare, while the highest value (8.21 $ng^*mL^{-1}$) was measured in a 15-year-old individual. Thus, from these results the statement can be made that in Lipizzaner mares, the mean AMH level during the spring-summer period was higher than the mean level in the autumn-winter months.

To the knowledge of the authors, this is the first report of the analysis of serum AMH concentrations in Lipizzaner mares. In this study, serum AMH was measured and its relationship with follicle number, mare age and season was explored.

The relationship between AMH levels and follicle populations is species-specific. AMH levels show a strong correlation with the number of 2–6 mm diameter follicles in women, 3–7 mm in cattle, and 1–5 mm in goats (Papas et al., 2021). In this study, the follicles present in the ovary were grouped according to size, namely their diameter. Small follicles were those smaller than 10 mm, medium-sized follicles ranged between 10 and 20 mm, and large follicles were denoted as those greater than 20 mm in size. We found that the total follicle count ($P = 0.026$), the number of medium follicles ($P = 0.022$) and the number of large follicles ($P = 0.016$) showed a positive correlation with serum AMH concentrations. However, further results show, that the number of small follicles present in the ovary at a given time had no effect on serum AMH levels.

Increasing age is inversely related to mammalian fertility in females (Hanlon et al., 2018). Our present-day understanding of female reproductive longevity assumes that the ovaries contain a limited number of oocytes, comprising the pool of primordial and developing follicles, that is often referred to as the 'ovarian reserve'. Ovarian reserve diminishes with age as follicles are lost (Uliani et al., 2019). The total number of primordial follicles in mares at two to four years of age is approximately 35,000, however, great variation can be observed between mares. At first glance, this number seems abundant, but it is smaller than similar estimates for cattle (120,000). On this basis, we can conclude that the ovarian reserve in mares is smaller and more variable than in other domestic species (Ball et al., 2019). Although menopause does not occur in horses, older mares undergo ovarian senescence leading to the cessation of ovulation and follicular growth (Claes et al., 2015). It is important to note, that the depletion of oocytes occurs at a variable age in this species. Accordingly, the reproductive and calendar age of a mare may well differ from its conspecifics. Ovarian depletion occurs at an earlier age in some, which is an important phenomenon from a clinical standpoint (Ball et al., 2019). Unlike other domestic animal breeds, there is very often a demand for the reproduction of older mares in the horse breeding industry. A horse breeder may have several reasons for producing foals from older individuals, including reasons such as the given mare has produced valuable foals in the past, or if the performance and/or genetic or sentimental value of the mare is important. In women, AMH levels steadily decline with age, reflecting the decrease in 'growing' follicle populations, with concentrations finally being undetectable from menopause. Therefore, the measurement of AMH levels in women is used to determine the onset of fertility decline (Hanlon et al., 2018). Research has been conducted on the relationship between AMH levels and fertility in mares, however, the results are often contradictory (Fouché et al., 2022). One of the objectives of our study was to analyse the relationship between age and AMH levels in Lipizzaner mares. The mare population studied can be considered relatively old, as the average age of the animals in the experiment was 16 ± 5 years. Consistent with human research, we observed that serum AMH levels in mares decreased after a certain age. The highest values were measured in middle-aged mares, while the concentration of the hormone began to decline afterwards.

As for the influence of season, we observed that the mean AMH levels in Lipizzaner mares were higher in the spring-summer months (3.04 $ng^*mL^{-1}$) than in the autumn-winter period (3.00 $ng^*mL^{-1}$). The maximum value was higher in the spring-summer timeframe (8.21 $ng^*mL^{-1}$) than in the autumn-winter period (7.57 $ng^*mL^{-1}$). However, further studies would be needed to prove the influence of seasons in this breed.

The relationships and implications we have established may help to better map the function of AMH and its practicality in studying the reproductive biology of mares.

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