Pathohistological findings after bilateral ovariectomy in mares with behavioral problems

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Meiner Familie

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
AMH	Anti-Müllerian hormone
AR	Aromatase
BID	Twice a day
BO	Bilateral ovariectomy
bOE	Mares with bilaterally removed, clinically unremarkable ovaries (group)
CAL	Calretinin
CBC	Complete blood count
CL	Corpus luteum
CRI	Constant rate infusion
E-Cad	Epithelial cadherin
EGFR	Epidermal growth factor receptor
EMH	Extramedullary hematopoiesis
ENC	Early neoplastic changes
ExP	Pathologists from an external laboratory
FEI	Federation Equestre Internationale
FN	Federation nationale
FSH	Follicle-stimulating hormone
GC	Granulosa cells
GCT	Granulosa cell tumor
GCT-uOE	Mares with unilaterally removed granulosa cell tumors (group)
GnRH	Gonadotropin releasing hormone
HE	Hematoxylin and eosin
HAF	Hemorrhagic anovulatory follicle
IU	International Units
i.m.	Intramuscular
i.v.	Intravenous
Ki67	Ki-67
LLC	Leydig-like cells
NSAID	Non-steroidal anti-inflammatory drugs
PI	Proliferation index of Ki-67
p.o.	Per os, orally
SID	Once a day
SLC	Sertoli-like cells
WNL	Within normal limits
WOAH	World Organization for Animal Health

I. INTRODUCTION

Mares with behavioral problems not obviously related to ovarian pathology are common and represent a challenge for owners, trainers and veterinarians. Presented problems range from normal, estrous-related sexual behavior to management-related problems and medical issues caused by disease or pain of unknown origin (PRYOR and TIBARY, 2005; MCDONNELL, 2017; MELGAARD et al., 2020). Behavioral patterns include moodiness, stressed or aggressive manner towards people or other horses, stallion-like behavior with nymphomania, problems under the saddle, or even recurrent colic signs of unknown gastrointestinal origin. Estrus is an assumed reported cause for behavioral disorders, especially when riders and trainers demand a high concentration of performance in athletic mares (JORGENSEN 1996; TROEDSSON et al., 2003; KAMM and HENDRICKSON, 2007). Mares in estrus consequently might not achieve their demanded performance in professional sport and are difficult or even dangerous in daily handling.

Granulosa cell or granulosa theca cell tumors, summarized as granulosa cell tumors (GCTs) in this study, are known to cause behavioral changes due to their excessive hormonal activity (KENNEDY and MILLER, 1993; MCCUE et al., 2006). However, assuming that behavioral problems are associated with cyclicity of the mare in absence of ovarian neoplasia, owners demand for methods to suppress ovarian function and estrous behavior. AURICH and KAPS (2022) as well as CRABTREE (2022) described a variety of conservative treatment options for estrous suppression, but most of these options are cost-intensive, might have severe side effects, or their reversibility is not clearly determined yet. Furthermore, legal limitations in Germany restrict the use of the majority of conservative possibilities (DIETZE et al., 2017).

Therefore, bilateral ovariectomy (BO) is often chosen for mares that are not intended for breeding due to the minimally invasive surgical approach and reported safe method (RÖCKEN et al., 2011). Bilateral ovariectomy resulted in a high success rate with significant improvement of behavior from the owner's perspective in several studies (83%, (KAMM and HENDRICKSON, 2007); 89%, (DEVICK et al., 2020); 40%, (MELGAARD et al., 2020); 90%, (COLLAR et al., 2021); 91%, (STRATICÓ et al., 2023)). Removal of both ovaries could have a positive effect on behavior due to elimination of estrogen-producing theca and granulosa cells (GCs) of ovarian follicles (CHRISTENSEN, 2011).

However, a pathohistological reason for this phenomenon of behavioral improvement after BO in mares with clinically unremarkable ovaries remains unknown. Gonadectomy in male horses in order to facilitate daily handling, rideability, and cohabitation with other horses is common practice. Indeed, castration of mares especially in case of a missing ovarian pathology or a lack of an understandable link to the ovaries (MCDONNELL, 2017) is discussed due to animal welfare aspects regarding removal of healthy organs (DIETZE et al., 2017). As the equine hospital in Starnberg is renowned for minimally invasive

surgical procedures, mares with diagnosed GCTs but also mares with normal ovaries are frequently referred from veterinarians for laparoscopic ovarian removal. We consequently questioned whether bilaterally removed, clinically unremarkable ovaries of mares with behavioral problems might demonstrate other, non-neoplastic abnormalities that could explain the fact of successful BO.

Therefore, the aim of this doctor thesis was to pathohistologically evaluate bilaterally removed, clinically unremarkable ovaries and to compare them with unilaterally removed GCTs by means of immunohistology. Studies on bilaterally ovariectomized mares with behavioral problems are published, but a detailed pathohistological evaluation other than the presence or absence of GCTs in removed ovaries is missing (DEVICK et al., 2020; STRATICÓ et al., 2023). Moreover, studies with pathohistological analyses of healthy ovaries and GCTs did not involve clinical data of the mares (ELLENBERGER et al., 2007; MÜLLER et al., 2009, 2012; DOLIN et al., 2023).

Our study aimed to involve a complete data set of each mare with bilaterally removed, clinically unremarkable ovaries and with unilaterally removed GCTs as basis for the pathohistological evaluation. The clinical data set included clinical history with patterns of behavioral problems and conservative treatment before surgery, clinical examination, analysis of serum anti-Müllerian hormone (AMH) and testosterone concentrations, and surgical outcome of BO.

II. LITERATURE REVIEW

1. Behavioral disorders

Behavior is a complex trait and influenced by many factors. An association to the ovaries is assumed, when behavioral abnormalities in mares are related to estrus and may affect the mare's performance in competitions or even lead to difficulties or danger in daily handling (JORGENSEN, 1996; PRYOR and TIBARY, 2005). Mares exhibit variable behavioral problems that range from moodiness, stressed manner, hyperexcitability, change of attitude, unwillingness to be ridden, flank sensitivity, and colic-like discomfort to more precise abnormal manner as aggression towards people or other horses, stallion-like or nymphomaniac behavior (JORGENSEN, 1996; KAMM and HENDRICKSON, 2007; ROESSNER et al., 2015; STRATICÓ et al., 2023). Stallion-like or nymphomaniac behavior are reported pathognomonic signs for GCT presence, together with prolonged or absent estrous signs (MEAGHER et al., 1977; SHERLOCK et al., 2016).

Some behavioral problems might be accompanied by afflictions not related to the reproduction tract like neurological, ophthalmic or musculoskeletal issues, metabolic or endocrine diseases, gastrointestinal problems causing abdominal discomfort, or management factors as social environment and inappropriate handling of inexperienced owners and trainers (NOUT-LOMAS and BEACOM, 2015). However, ovaries tend to be a main cause of behavioral problems, as several studies reported highly successful outcomes of behavioral improvement after BO (KAMM and HENDRICKSON, 2007; DEVICK et al., 2020; MELGAARD et al., 2020; COLLAR et al., 2021; STRATICÓ et al., 2023).

2. Evaluating the mare with behavioral problems

2.1. Diagnostic challenge

Mares frequently present behavioral problems that are associated to estrous-related hormonal activity or ovarian abnormalities like GCTs. In some cases, however, behavioral problems might be unrelated to the gonads and are therefore misattributed to the ovaries (PRYOR and TIBARY, 2005; MCDONNELL, 2017). Several other abnormalities of other body systems like the gastrointestinal tract might further affect the mare's behavior and should be regarded as differential diagnosis (NOUT-LOMAS and BEACOM, 2015). Management factors like poor riding technique or unrealistic expectations of the horse can be moreover responsible for inappropriate behavior (MCGREEVY, 2012). Not-ovarian related problems should be ruled out by a full diagnostic work-up to find possible alternative sources of behavioral abnormalities in mares (NOUT-LOMAS and BEACOM, 2015; MCDONNELL, 2017). If other abnormalities can be excluded, the diagnosis of ovarian pathology is typically based on a combination of clinical history, clinical signs, transrectal palpation,

ultrasonographical examination, and hormone analysis (MCCUE et al., 2006).

Positive response to oral Altrenogest (Allyltrenbolone) application is a further diagnostic aid, as Altrenogest effectively eliminates estrous signs within 2-3 days and therefore indicates the ovaries as source of behavioral problems (MCCUE, 2003). Nevertheless, a positive correlation between Altrenogest treatment and positive outcome of BO could not be found (COLLAR et al., 2012; MELGAARD et al., 2020).

Granulosa cell tumors are a common pathological cause for behavioral disorders (MEAGHER et al., 1977; CRABTREE et al., 2013; SHERLOCK et al., 2016). However, some mares with untypical GCTs might reveal unclear diagnostic statements, as those tumors can show a wide variety of clinical signs. RENAUDIN et al. (2021) emphasized the diagnostic challenge of GCT detection, especially if they do not fit in the definition of a 'classic' GCT and concluded an unpredictable endocrinological behavior with variable clinical signs in some mares with GCTs. Therefore, detection of the source of behavioral problems can be in general challenging, especially in mares with clinically unremarkable ovaries.

2.2. Clinical history

NOUT-LOMAS and BEACOM (2015) advised to start evaluation of behavioral problems with gathering detailed information about management factors and behavioral patterns. Management factors include feeding, social environment and training. Being kept in stables, high intensity exercises, stressful transports to competitions, or keeping horses in mixed groups might compromise the mare's health, could have an influence on behavior, or should be even criticized regarding animal welfare at some point (MCGREEVY, 2012). MCDONNELL (2017) suggested a detailed description of the specific behavior pattern and advised several assessments of behavioral change over time and in various situations as the most informative part of clinical history. Therefore, long-term observations by owners, trainers, and riders might be necessary in order to rule out other health issues (MCDONNELL, 2017). This includes the mare's response to social challenges (for example their reaction to a stallion) or even a 24-hours video of behavioral evaluation. Other authors advised to distinguish between learned behavior and abnormal behavior due to a disease process (MCGREEVY, 2012) and to document all reproductive events (PRYOR and TIBARY, 2005).

The clinical history in published studies evaluating the effect of BO included the reason for pursuing ovariectomy, a description of specific misbehavior and the time from first presentation to referral (DEVICK et al., 2020; MELGAARD et al., 2020; STRATICÓ et al., 2023). Recorded, abnormal behavior patterns ranged from general problems when ridden, including bucking or rearing and increased flank sensitivity, to aggressive manner with attempts to bite or kick other horses or humans (STRATICÓ et al., 2023). Stallion-like behavior and abnormal estrous signs were further reported by the owners (DEVICK et al., 2020) and are commonly associated with GCTs (MEAGHER et al., 1977; CRABTREE et al., 2013;

SHERLOCK et al., 2016).

2.3. Clinical examination

A multimodal approach to evaluate the cause for behavioral problems should contain a complete physical examination with diligent assessment of all body systems (**Figure 1**, NOUT-LOMAS and BEACOM, 2015; MCDONNELL, 2017). This includes disease factors of neurological, endocrinological, musculoskeletal, gastrointestinal, ophthalmic, or reproductive systems and should help to determine, if evidence for a pathological process is present or not. A rectal examination is recommended to detect caudal abdominal or reproductive issues. This procedure is commonly followed by a transrectal ultrasonographical examination to determine morphological abnormalities on uterus or ovaries, such as hemorrhagic anovulatory follicles (CRABTREE, 2020). A transvaginal examination followed by endometrial biopsy is further indicative for abnormalities of the reproductive tract, as BARTMANN et al. (2001) reported a pathohistological irregular endometrial differentiation in mares with GCTs.

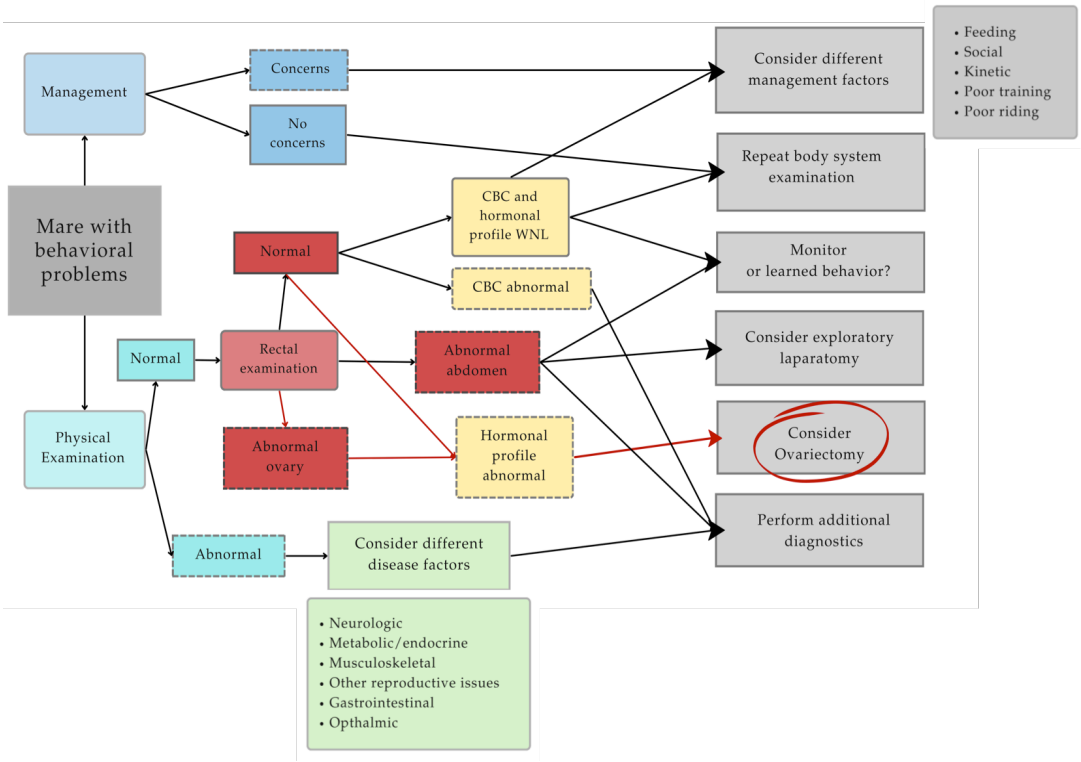

Figure 1. Multimodal approach for a mare with behavioral problems modified according to NOUT-LOMAS and BEACOM (2015). WNL=within normal limits, CBC=complete blood count.

Laboratory tests like analysis of a complete blood count and serum biochemistry are helpful to indicate inflammatory processes or organ dysfunctions (NOUT-LOMAS and BEACOM, 2015). Evaluations of endocrine panels are of high diagnostic value regarding detection of ovarian dysfunctions (MCCUE, 2014; CONLEY and BALL, 2017) and are further described below. Some authors moreover suggested advanced diagnostic assessments like neurological

examinations or nuclear scintigraphy in case of not expedient physical examinations or blood work (NOUT-LOMAS and BEACOM, 2015). Moreover, reevaluation of body system assessment might be necessary, if unclear findings on clinical examination or change of behavioral patterns are present (NOUT-LOMAS and BEACOM, 2015).

2.4. Serum hormone assays

Several serum hormone assays are available to determine estrous cycle stage and to evaluate corpus luteum (CL) function. In terms of reproductive disorders in the mare, endocrine tests are a valuable tool to confirm a presumptive clinical diagnosis of ovarian abnormalities (MCCUE, 2014; CONLEY and BALL, 2017). Hormonal assays include estradiol, progesterone, testosterone, inhibin, AMH, and moreover adrenocorticotrophic hormone (ACTH) and cortisol. Serum progesterone concentrations are indicative for the presence or absence of active luteal tissue (MCCUE, 2014) and high concentrations of testosterone, inhibin, and AMH are associated with the presence of GCTs (STABENFELDT et al., 1979; BAILEY et al., 2002; MCCUE et al., 2006; BALL et al., 2013). However, there is a lack of information regarding endocrinological abnormalities of ovarian pathologies other than GCTs.

2.4.1. Estradiol and progesterone

Estradiol concentrations range from 20-45 pg/mL (69-121 nmol/L) in normal cycling mares (MCCUE et al., 2006). Serum estradiol is variable in GCTs and not clearly related to the expressed predominant behavior (STABENFELDT et al., 1979; MEINECKE and GIPS, 1987). A low progesterone concentration of <1.0 ng/mL (<3.18 nmol/L) is consistent with absent luteal tissue and acyclicity and therefore almost invariably low in mares with GCTs (STABENFELDT et al., 1979; MCCUE, 2014). However, mares with GCTs and simultaneous active CLs with increased progesterone concentrations are reported and explained by the fact of an early disease process or the possibility of pregnancy (HINRICHS et al., 1990; MCCUE et al., 1991; BALL et al., 2014; RENAUDIN et al., 2021).

2.4.2. Inhibin

The glycoprotein inhibin is produced by GCs of female gonads (CUEVAS et al., 1987) and is elevated in 85-95% of GCT-affected mares (BAILEY et al., 2002; BALL et al., 2014). Normal inhibin ranges from 0.1-0.7 ng/mL (0.32-2.23 nmol/L), but fluctuates during estrous cycle and is increased in pregnant mares (NAMBO, 1996). Moreover, inhibin has an inhibitory effect on the contralateral ovary by suppression of the pituitary Follicle-stimulating-Hormone (FSH) release, leading to follicular inactivation and downsizing of the ovary (MCCUE, 1998).

2.4.3. Testosterone

Testosterone is a steroid hormone and produced by theca cells (NETO et al., 2010). Granulosa cell tumors with elevated serum testosterone concentrations are

usually present with a substantial theca cell component and termed as granulosa theca cell tumors by some authors (MCCUE et al., 2006; NETO et al., 2010). Follicles of such tumors are not capable of converting testosterone into estrogens due to a lack of aromatase capacity (HOQUE et al., 2003), leading to increased testosterone concentrations in serum. This elevation of testosterone is commonly associated with stallion-like behavior (STABENFELDT et al., 1979; HINRICHS et al., 1990; ELLENBERGER et al., 2007; CRABTREE, 2011).

HUGGINS et al. (2023) recently reported an association between stallion-like behavior and increased serum hormone concentrations, including testosterone, inhibin and AMH, but concluded a poor association between increased hormones and other behavioral problems. Testosterone ranges from 20-45 pg/mL (69-121 nmol/L) in normal cycling mares (MCCUE, 2014) and has a reported sensitivity of 48-50% in GCTs (MCCUE et al., 2006; BALL et al., 2013). This hormone is variable in the individual, as it fluctuates over a short period of time and throughout the year and is further increased during pregnancy (BALL et al., 2013; CONLEY and BALL, 2017).

2.4.4. Anti-Müllerian hormone

Anti-Müllerian hormone has become a popular marker for the diagnosis of GCTs and is almost replacing inhibin (BALL et al., 2008; ALMEIDA et al., 2011; CRABTREE, 2011). This homodimeric glycoprotein is produced by Sertoli cells of the testis from sexual differentiation in fetal stage to puberty and is involved in regression of the Müllerian ducts in the male fetus (JOSSO et al., 2001). Anti-Müllerian hormone plays an important role in folliculogenesis, as it has an inhibitory function on growth of FSH-responsive follicles in humans (PELLAT et al., 2011). The hormone has a relatively long biological half-life of 1.5 days, which was evaluated in castrated stallions after surgery (CLAES et al., 2013).

Anti-Müllerian hormone is produced by GCs of antral follicles in the ovary of postnatal females (VISSER et al., 2006) and is expressed in neoplastic GCs of GCTs (BALL et al., 2008). Unlike inhibin or testosterone, AMH concentrations do not significantly vary throughout the year, during estrous cycle, or pregnancy (ALMEIDA et al., 2011; BALL et al., 2013; CRABTREE et al., 2013). However, AMH concentrations differ among mares and a possible association to ovarian reserves in each individual is therefore assumed (ALMEIDA et al., 2011). CLAES et al. (2015) could find a correlation between the presence of antral follicles and plasma AMH concentration. Anti-Müllerian hormone might therefore reflect the mare's reproductive age, as older mares had significantly less antral follicles and lower serum AMH concentrations compared to young and middle-aged mares in the study of CLAES et al. (2015).

The highest sensitivity to detect GCTs in mares of all available hormonal tests is attributable to AMH (BALL et al., 2008, 2013; ALMEIDA et al., 2011). It's sensitivity of 98% is greater than inhibin, testosterone, or a combination of both (BALL et al., 2014). Reported AMH concentrations range from 0.22-2.94 ng/mL (1.57-21.0 pmol/L) in normal cycling mares and from 0.26-2.61 ng/mL (1.86-18.6

pmol/L) in pregnant mares (ALMEIDA et al., 2011). Reported cut-off values in GCT-affected mares differ in literature, ranging from 4 ng/mL (28.6 pmol/L; BALL et al., 2013, 2014) to 4.7 ng/mL (33.6 pmol/L; MURASE et al., 2018) or even 6.9 ng/mL (49.3 pmol/L), which was recently revised (ULIANI et al., 2019).

The diagnostic accuracy of serum hormone assays, especially of AMH, is high in clinically obvious and pathohistologically confirmed GCT cases (ALMEIDA et al., 2011). Thus, reliability of diagnostic endocrinology remains unclear in cases of early tumor growth (DEVICK et al., 2020) or GCTs, that are not described as 'classic' (RENAUDIN et al., 2021).

3. Characteristics of equine ovaries

3.1. Physiological equine ovaries

Equine ovaries have peculiar characteristics compared to other mammalian species. They are characterized by an extremely large size (35-120 cm^3) and weight (40-80 g) with varying diameters from 2-4 cm in anestrus to 6-8 cm during sexual activity (KIMURA et al., 2005; DAVIS MOREL, 2015; GONZÁLEZ et al., 2015). The inverted location of cortex and medulla and the presence of the ovulation fossa are unique for equine ovaries. Compared to other mammals, equine ovaries contain a lower number of follicles with 35,000-40,000 primordial and 100 growing follicles (DRIANCOURT et al., 1982).

Follicles are heterogeneously distributed in cluster formations and mainly located in the inner portion of the ovary next to the ovulation fossa (GONZÁLEZ et al., 2017). Some authors could find preantral follicles in all parts of the equine ovarian stroma (SZLACHTA and TISCHNER, 2002; HAAG et al., 2013a). The special distribution and low follicular intensity in the equine ovary causes difficulties to detect follicular structures, especially if ovarian fragments or biopsies are analyzed (HAAG et al., 2013a, 2013b). Equine antral follicles reach a large diameter of up to 55 mm (EVANS, 2003) and "migrate" through the ovarian stroma towards the ovulation fossa, where the germinal epithelium of the cortex reaches the surface and the thick tunica albuginea is absent (DAVIS MOREL, 2015). These characteristic features might explain the unusually long and variable estrus in mares (TROEDSSON et al., 2003).

In contrast to other species, large luteal cells of CLs are reported to solely derive from GCs of the preovulatory follicle in equine ovaries (VAN NIEKERK et al., 1975). Equine CLs have a pear-like, roughly shaped surface, and consist of microscopically separated insular compartments (KIMURA et al., 2005). Forming CLs enlarge within the inner portion and do not protrude to the ovarian surface as in other species, which is explained by the specific anatomy of equine ovaries (DAVIS MOREL, 2015).

3.2. Ovarian abnormalities

Several pathological conditions occur in the mare's ovary, including neoplastic

and non-neoplastic abnormalities. Ovarian pathologies are commonly detected by transrectal palpatory and ultrasonographical enlargement of >100 mm in size (TROEDSSON et al., 2003). Unilateral enlargement has to be differentiated from bilateral enlargement, which is physiological in pregnant mares from 40-150 days of gestation due to formation of accessory CLs (MCCUE, 1998). However, bilateral ovarian enlargement can be also associated with bilaterally occurring pathologies, as DANIEL et al. (2015a) reported leiomyomas in both ovaries of a mare with aggressive behavior. Unilateral ovarian enlargement is a common finding in mares with GCTs (TROEDSSON et al., 2003), but also observed in mares with anovulatory follicles or ovarian hematomas (CRABTREE, 2020).

3.2.1. Non-neoplastic abnormalities

Non-neoplastic ovarian abnormalities as ovarian abscesses or hematomas are common in mares and might cause abdominal discomfort due to their large size or inflammation (RAMIREZ et al., 1999; CURTIN, 2003). Ovarian hematomas are the most frequent cause for ovarian enlargement (BOSU et al., 1982; MCCUE et al., 1991) and originate from excessive bleeding into the follicular lumen after ovulation (MCCUE, 1998). Diagnosis of hematomas might result in confusion with the diagnosis of a neoplastic event due to enlargement up to 100 mm and similar honeycomb-like appearance on ultrasound (CURTIN, 2003). However, in contrast to GCTs, hematomas regress over time due to reabsorption of the fluid (CURTIN, 2003).

Cyst-like structures are reported in equine ovaries, but there is a lack of agreement on their classification (BOSU et al., 1982). The term "cyst" represents a controversial debate for years in equine literature. In contrast, ovarian and follicular cysts are well described in bovine ovaries and a common problem for veterinarians due to blockage of the estrus cycle (ZERBE et al., 1999). ENGLAND (1996) emphasized the dogma of non-existing cysts in equine ovaries decades ago, but other authors described cyst-like, persistent anovulatory follicles in equine ovaries, that are comparable with the cystic ovarian syndrome in cattle (HUGHES et al., 1980; BOSU et al., 1982; KESLER and GARVERICK, 1982; MCCUE, 1998; MCCUE and SQUIRES, 2002). MCCUE (1998) reported a case with bilaterally occurring polycystic ovaries, but a detailed histomorphological description was lacking.

More recently, CRABTREE (2020) published an update on anovulatory follicles in the mare. The author defined them as "follicles that reach the correct size of >35 mm but fail to ovulate". Moreover, he differentiated between persistent anovulatory follicles and hemorrhagic anovulatory follicles. The incidence of anovulatory follicles increases with age and is with 13.1% higher in sixteen-to-twenty-year-old mares compared to 4.4% in six-to-ten-year-old mares (MCCUE and SQUIRES, 2002). Fifteen percent of anovulatory follicles present a cystic appearance with a missing luteinization and are considered as persistent anovulatory follicles (MCCUE and SQUIRES, 2002). Such anovulatory follicles regress spontaneously after persistence for several cycles, do not influence reproductive cyclicity, and have a limited clinical significance in mares

(CRABTREE, 2020).

However, the remaining 85% of anovulatory follicles luteinize and are regarded as pathological ovarian abnormalities (MCCUE and SQUIRES, 2002). They are termed as luteinized unruptured follicles or hemorrhagic anovulatory follicles (HAFs), although bleeding does not always occur (CRABTREE, 2020). Hemorrhagic anovulatory follicles have the potential of progesterone synthesis and are present in 4.5-8.2% of cycling mares (CHOPIN et al., 2001; MCCUE and SQUIRES, 2002). Such HAFs mainly occur during the transition phases in spring and autumn (GINTHER, 1992; NUNES et al., 2002), and can cause estrous problems with reproductive infertility (MCCUE and SQUIRES, 2002). Some mares are more susceptible to HAFs than others and the likelihood of consecutive HAFs in an individual mare is estimated with 31.5 % (CUERVO-ARANGO and NEWCOMBE, 2010). Estrous induction with prostaglandin and administration of high doses of flunixin meglumine (2 mg/kg BID) before or during ovulation are supposed to cause HAF formation in mares (CUERVO-ARANGO and NEWCOMBE, 2010). Hemorrhagic anovulatory follicles are another cause for ovarian enlargement, as they increase up to 100 mm (CRABTREE, 2011). They should be moreover regarded as differential diagnoses for GCTs due to their confusing similar appearance (CRABTREE, 2011).

Non-follicular cyst formations within the region of the ovulation fossa are termed as fossa cysts (O'SHEA, 1968; BOSU et al., 1982). Fossa cysts are thought to arise from remaining structures of the Müllerian and Wolffian ducts due to their similar epithelial appearance (O'SHEA, 1968; MCCUE, 1998) and are commonly found in the equine ovary (ELLENBERGER et al., 2009). They increase in number with the age and might be related to infertility, if they enlarge in size and consequently block the ovulation fossa or oocyte transport into the oviduct (KENNEDY et al., 1998). Fossa cysts might moreover trigger the development of HAFs (KÖLLING and ALLEN, 2006).

Behavioral abnormalities, especially aggressive or stallion-like behavior, are commonly attributable to the presence of GCTs (SHERLOCK et al., 2016), but rarely reported in other ovarian abnormalities like leiomyomas (DANIEL et al., 2015a). However, discomfort or colic signs are frequently observed by owners to be related to estrus. Ovarian pain might not be classified as pathologic, but plays an important role and is termed as "painful ovary syndrome" (CRABTREE, 2016, 2022). According to menstruating women, who experience pelvic pain associated to ovulation and menstrual cramps, mares might also face pain in relation to ovulation and large follicular structures. Colic of ovarian origin with possible additional sensitivity in the back or hindlimb lameness is described by several authors (HOOPER et al., 1993; PRYOR and TIBARY, 2005; CHENIER, 2009; VANDERWALL and NIE, 2011; ROESSNER et al., 2015; CRABTREE, 2022). Estrous- or ovarian-related pain is assessed by a transrectal palpation (CHENIER, 2009; VANDERWALL and NIE, 2011). The painful ovary syndrome can be considered in case of repeated estrous-related pain over time with large preovulatory or anovulatory structures present on one of the ovaries (CRABTREE, 2022).

3.2.2. Neoplastic abnormalities

Classification of gonadal tumors in domestic animals is mainly based on similarities of neoplastic cells to cellular constituents of the normal gonads (MACLACHLAN, 1987). Neoplastic changes in equine ovaries are classified as sex-chord stromal tumors, epithelial tumors, germ cell tumors, and non-gonadal tumors like hemangiomas, leiomyomas, or metastatic tumors (JUBB et al., 1985; KENNEDY et al., 1998).

Sex-chord stromal tumors are characterized by coexistence of multiple cell types in the same tumor and share the potential of hormonal activity and secretion of steroid hormones (MACLACHLAN, 1987). They account for 6% of all equine neoplasms and include GCTs, thecomas, and fibromas (KNOWLES et al., 2016). In contrast to equines, human ovarian sex-chord stromal tumors are further divided into juvenile and adult GCTs, thecomas, fibromas, Sertoli cell tumors, Sertoli-Leydig cell tumors, and steroid cell tumors (CHEN et al., 2003). Thecomas are rarely occurring in equine ovaries and consist of cells with theca cell phenotype (RAOOFI et al., 2006; PRESTES et al., 2013).

Granulosa cell tumors represent the most common form of ovarian neoplasia in mares (MEAGHER et al., 1977; KENNEDY et al., 1998; MCCUE, 1998) and account for 2.5% of all equine neoplasms (SUNDBERG et al., 1977). The term granulosa-theca cell tumor is further used, if a significant theca cell component is additionally affected (KENNEDY and MILLER, 1993; KENNEDY et al., 1998; MCCUE, 1998). Granulosa cell tumors are usually described as multicystic, honey-comb like structures, but also appear as solid mass or single large fluid-filled cyst (MCCUE et al., 2006). These ovarian tumors, which mainly occur unilaterally and rarely bilaterally (TURNER and MANNO, 1983; MCCOY, 1986; FREDERICO et al., 2007; CASTILLO et al., 2019), affect mares of all ages (MEAGHER et al., 1977). Granulosa cell tumors were reported in neonates (GREEN et al., 1988), older foals (HULTGREN et al., 1987), yearlings (CHARMAN and MCKINNON, 2007), and even pregnant mares (MEAGHER et al., 1977; BOSU et al., 1982; CRABTREE, 2011; CRABTREE et al., 2013). In general, GCTs are benign (KENNEDY and MILLER, 1993), but malignancy with a metastatic character was also reported (MEAGHER et al., 1977; ABBOTT et al., 2004; ELLENBERGER et al., 2007).

Stallion-like, aggressive, or nymphomaniac behavior and prolonged or absent estrous signs are typical behavioral changes for mares with GCTs (SHERLOCK et al., 2016). Such behavioral disorders are caused by increased hormonal activity of the tumor (KENNEDY and MILLER, 1993; MCCUE et al., 2006). Excessive inhibin production by neoplastic cells (BAILEY et al., 2000) has a suppressive effect on FSH release, which leads to reduction in size and inactivation of the contralateral ovary (TROEDSSON et al., 2003; MCCUE et al., 2006). Consequently, GCTs are usually detected with unilateral enlargement and a small, inactive ovary on the contralateral side (HINRICHS and HUNT, 1990; SHERLOCK et al., 2016). However, GCT-affected mares with a functional contralateral ovary and normal behavior (MCCUE et al., 1991; CASTILLO et al.,

2019) or pregnant mares with unilateral GCTs and subsequent foaling without complications were also reported (VAN DER ZAAG et al., 1996; CHOPIN et al., 2002; CRABTREE et al., 2013).

Beside GCTs, further tumors including cystadenomas, teratomas, and dysgerminomas are present in the mare's ovary. Cystadenomas originate from the epithelium, are benign and unilaterally occurring with a normal sized and functional contralateral ovary (MCCUE, 1998). They present cyst-like structures on ultrasound and are not considered to be hormonally active (MCCUE, 1998). Dysgerminomas and teratomas are tumors of germ cell origin, might contain ovarian-foreign tissue like hair, bone, or muscle and are growing unilaterally (MCENTEE, 1990). Both tumors are hormonally inactive with a normal sized contralateral ovary and do not alter behavior or estrous cycle (MCCUE, 1998). Dysgerminomas are malign with a high potential of metastasis (BOSU et al., 1982; MCCUE, 1998), whereas teratomas are usually benign, but malignancy was also reported (BARTMANN et al., 2001). Teratomas are occasionally found in equine ovaries (CATONE et al., 2004; RENAUDIN et al., 2021) and present the second most common ovarian tumor in the mare (HUGHES et al., 1980).

Other neoplastic tumors of non-gonadal origin rarely occur in equine ovaries. These include hemangiomas, epitheliomas, fibromas, leiomyomas, or fibroleiomyomas, and metastatic tumors as adenocarcinomas and lymphosarcomas (LOCK and MACY, 1979; VAN CAMP et al., 1989; CARSTANJEN et al., 2009; PAUWELS et al., 2012; DANIEL et al., 2015a).

3.3. Pathohistological evaluation

Clinical examination and analysis of serum hormones is often evident for the presence or absence of ovarian pathologies. A subsequent pathohistological examination can be further helpful in case of unclear or changing clinical results and moreover confirms the presence of clinically detected ovarian abnormalities like GCTs. A pathohistological evaluation includes histomorphological and immunohistochemical analyses of removed ovaries by means of diagnostic markers like AMH, inhibin, or tumor markers, as reported in equine GCTs by ELLENBERGER et al. (2007) and other authors (HOQUE et al., 2003; MÜLLER et al., 2009, 2012; NETO et al., 2010; IBRAHIM, 2019; DOLIN et al., 2023). This procedure usually requires excision of the organs in total through ovariectomy. Alternatively, a transvaginal ovarian biopsy method in the standing mare was reported by use of an ultrasound-guided biopsy-pick up device (HAAG et al., 2013a, 2013b). This method was established to determine the practicability of retrieving preantral follicles from mares in vivo (HAAG et al., 2013a). BARTMANN et al. (2001) further described a pathohistological analysis of the mare's endometrium by a transcervical biopsy technique. The authors could find a high association to irregular endometrial differentiation in mares diagnosed with GCTs and emphasized further pathohistological value of endometrial samples, besides pathohistological analyses of removed ovaries.

3.3.1. Histomorphology

3.3.1.1. Histomorphological characteristics of physiological equine ovaries

Equine ovaries are characterized by special features, which were previously mentioned (see 3.1.). The large ovaries mainly consist of stroma composed of spindle shaped cells and collagen fibers. The inverted cortex inside the ovary contains follicles with oocytes in various developmental stages, atretic follicles, and CLs and is enclosed within a highly vascularized connective tissue. This is corresponding to the ovarian medulla of other species (STABENFELDT et al., 1975; GINTHER, 1992). As already mentioned, the density of heterogeneously distributed follicles is low in equine ovaries (DRIANCOURT et al., 1982; GONZÁLEZ et al., 2017).

Different follicular and luteal developmental and atretic stages in equine ovaries are classified according to other species (TEH et al., 2018). Developing stages include primordial, primary, secondary, tertiary, and Graafian follicles, whereas follicular atresia is categorized in stage I-III (VAN NIEKERK et al., 1973; KENNEY et al., 1979; DRIANCOURT et al., 1982). Cyclic luteal development and regression is categorized in CLs, regressing CLs, corpora hemorrhagica, and corpora albicans (MÜLLER et al., 2009).

Preantral follicles, including primordial, primary, and secondary follicles, are histomorphologically classified according to the stage of development (MÜLLER et al., 2009; ALVES et al., 2015). Primordial follicles contain a small non-growing oocyte, which is surrounded by flattened, ellipsoid GCs. Primary follicles are characterized by an enlarging oocyte with a cuboidal GC layer and a forming zona pellucida. The oocyte of secondary follicles is surrounded by a clear zona pellucida with two or more layers of cuboidal GCs. Secondary follicles contain an additional theca cell layer for their own vascular supply.

Antral follicles include tertiary and Graafian/preovulatory follicles and present multiple GC layers with a single basal layer of cuboidal GCs arranged on the basement membrane, which separates the avascular GC layer from the vascular theca cell layer (VAN NIEKERK et al., 1973). The theca cell layer of antral follicles is further distinguished in an external and internal layer. Large polyhedral cells with hyper- or hypochromatic nuclei and plentiful eosinophilic cytoplasm are typical for the theca interna layer with additional activated endothelial cells in capillaries, arterioles, and venules (WATSON and AL-ZI'ABI, 2002; MÜLLER et al., 2009). Those vascular structures are essential for luteal vascularization, which is mainly present in the theca interna of the mare's ovary (MÜLLER et al., 2012).

Atretic follicular stages are categorized based on different follicular sizes and amounts of pycnotic GCs (VAN NIEKERK et al., 1973; KENNEY et al., 1979; DRIANCOURT et al., 1982). Follicular atresia is characterized by an "increasing disarrangement of thecal capillaries followed by endothelial cell apoptosis" (MÜLLER et al., 2009). In stage I, early atretic follicles present only a few nuclei with karyorrhexis and apoptosis. In stage II, advanced atretic follicles show

extensive pyknosis, high amounts of detected apoptotic bodies in the follicle antrum, and a gradual thickening of the basement. In stage III of late atretic follicles, GCs and theca cells are no more distinguishable and the basement membrane is presented as a thick hyaline membrane (DRIANCOURT et al., 1982; MLODAWSKA and SLOMCZYNSKA, 2010).

As atresia occurs in every stage of follicular development, follicles can be further categorized in normal/viable and abnormal/atretic follicles (MLODAWSKA and SLOMCZYNSKA, 2010; HAAG et al., 2013b). Normal follicles contain an intact oocyte and are covered by one or more GC layers, whereas abnormal follicles are characterized by a deteriorated oocyte and detached cells from the basement membrane (HAAG et al., 2013b).

IBRAHIM (2019) recently published a further classification in 4 different stages of folliculogenesis: very healthy follicles, healthy follicles, early atretic, and late atretic follicles. This classification is mainly based on the presence of three types of GCs: basal, intermediate, and antral GCs. Very healthy follicles present a uniform thickness of the GC layer with palisade arrangement of basal GCs, only few intermediate GCs, and partly interrupted palisade arrangement of antral GCs. Their theca interna is characterized by a heterogeneous mixture of large and small theca cells. Healthy follicles show a slight disarray of basal GCs with otherwise similar features as very healthy follicles. In early atretic follicles, the GC layer is decreased in thickness with a higher portion of intermediate GCs, more disorganized basal GCs, and an increased degree of pyknosis in the GC layer. Early atretic follicles show a higher amount of small theca cells with vascular supply in the theca interna cell layer and already a thin amorphous basement membrane. Late atretic follicles present a complete absent GC layer and a thick amorphous eosinophilic band. Their theca cell layer mainly contains small spindle-like cells with darkly stained nucleus, which are considered as small theca cells. The purpose of this follicular categorization in the study of IBRAHIM (2019) was to find a possible association to GCT development, which could not be observed.

Corpora lutea in equine ovaries consist of small insular compartments, which are formed by trabecular strands of small luteal cells (KIMURA et al., 2005). Luteal cells are distinguished into large, steroidogenic cells and small luteal cells (VAN NIEKERK et al., 1975). However, compared to other species, equine luteal cells are reported to solely derive from GCs of the preovulatory follicle and might therefore not be of thecal origin (VAN NIEKERK et al., 1975). At time of ovulation, theca cells are at various stages of degeneration and replaced by hypertrophied fibroblasts (VAN NIEKERK et al., 1975). Equine CLs contain fibroblasts, smooth muscle cells, immune cells, and endothelial cells, which present 85% of the proliferating cells (REYNOLDS et al., 1994). Developing luteal cells are characterized by pronounced mitotic activity, which indicates high progesterone production. Synthesis of progesterone is restricted to large luteal cells, as progesterone receptors or steroidogenic enzymes were nondetectable in small luteal cells (ROBERTO DA COSTA et al., 2005; FERREIRA-DIAS et al., 2007). However, small luteal cells might be precursors of large luteal cells, which

develop in size and function during CL growth in equine ovaries (VAN NIEKERK et al., 1975; ROBERTO DA COSTA et al., 2005).

3.3.1.2. Histomorphological characteristics of pathological equine ovaries

TROEDSSON et al. (2003) provided an overview of clinical aspects of ovarian pathologies in the mare and included anovulatory follicles, ovarian hematomas, and ovarian tumors like GCTs, dysgerminomas, or teratomas. The ultrasonographical and macroscopical appearance of GCTs is in general well described, but a histomorphological characterization of abnormal structures in equine ovaries apart from these pathologies is mostly lacking in equine literature.

Anovulatory follicles are classified as persistent anovulatory follicles or HAFs and present increased echogenic particles or "snow storm" appearance on transrectal ultrasound examination (CRABTREE, 2020). Hemorrhagic anovulatory follicles are histomorphologically characterized as large follicles with a blood-filled central cavity. Their inner layer contains fibrous tissue and their outer layer is composed of vacuolated, polygonal shaped, luteinized cells, which are surrounded by stromal cells (ELLENBERGER et al., 2009). WATSON and AL-ZI'ABI (2002) described anovulatory follicles as structures with well organized, multiple layers of GCs attached to the basement membrane with a poorly developed theca interna cell layer and only few polyhedral cells. In contrast to others (ELLENBERGER et al., 2009; CRABTREE, 2020), WATSON and AL-ZI'ABI (2002) did not differ between spontaneously regressing, persistent anovulatory follicles, and HAFs. Hemorrhagic anovulatory follicles are regarded as pathological ovarian abnormalities (CRABTREE, 2020).

Morphological descriptions of other cyst-like structures are rarely reported in equine ovaries. As previously mentioned, MCCUE (1998) published a case with bilaterally occurring polycystic ovaries, that reached a diameter of 15 cm, but a detailed histomorphological description other than a "polycystic" appearance was missing. A recent case report of GOTO et al. (2021) described a single large ovarian structure of >25 cm diameter in a mare with the size of a basketball. Pathohistological analysis in their study revealed an unclear defined epithelial lining of the thickened wall, which contained layers of smooth muscle cells, collagenous tissue, and glandular structures with cuboidal epithelial cells. An ovarian cyst was diagnosed in this case.

The histomorphological appearance of non-follicular cyst formations was reported in more detail (O'SHEA, 1968; BOSU et al., 1982). Non-follicular cysts are termed fossa cysts, which are located at the region of the ovulation fossa, and moreover paraovarian cysts, which are adjacent to the oviduct (BOSU et al., 1982). They are histomorphologically described as blind, epithelium-lined tubules, vesicles, or gross cysts in the ovarian tissue next to the ovulation fossa and might contain secretory material (O'SHEA, 1968). Their epithelium is either squamous, columnar, or cuboidal with additional cilia present (O'SHEA, 1968). However, both non-follicular cyst formations are not regarded as pathological, as they usually do not affect fertility and are commonly incidentally found in mares

(MCCUE, 1998; ELLENBERGER et al., 2009).

Detailed histomorphological characteristics are provided for GCTs in equine literature. Granulosa cell tumors are classified as sex-chord stromal tumors, as their neoplastic cells resemble endocrine cells of the normal ovary on histomorphological examination (MCCUE, 1998). A multicystic, honeycomb-like appearance on gross section is also present on histomorphological examination in form of macro- or microfollicular patterns (MÜLLER et al., 2012). However, GCTs can also exist as a solid mass or a single large cyst (MCCUE, 1998). The tumors resemble a disorganized follicle formation, as they consist of irregular accumulations of GCs, which are more or less separated by a stroma of spindle cells (KENNEDY and MILLER, 1993; KENNEDY et al., 1998).

Granulosa cell tumors are pathohistologically classified according to the current World Organization for Animal Health (WOAH) tumor classification in microfollicular, macrofollicular, trabecular, tubular, insular, diffuse, or mixed patterns (KENNEDY et al., 1998). Those different pathohistological growth patterns were further described by MÜLLER et al. (2012) in equine GCTs and in more detail by TEH et al. (2021) in bovine GCTs. Macrofollicular growth patterns resemble normal Graafian follicles, whereas microfollicular patterns show rosette-like formations and are called Call-Exner bodies. Trabecular or tubular patterns resemble Sertoli-like structures and are separated by fibrous strands. Insular patterns consist of islands or nests of neoplastic GCs. Diffuse growth patterns are described with a diffuse proliferation of GCs. The most commonly occurring mixed type contains parts of different growth patterns. TEH et al. (2021) additionally defined a pseudopapillary growth pattern in bovine GCTs, in which pseudopapillae are formed by intracystic cellular projections. This growth pattern is not present in equine GCTs (MÜLLER et al., 2012).

TEH et al. (2021) characterized different patterns of GC formations, but an association to clinical appearance or endocrinological function of GCTs was not determined. The authors could further not find a correlation between different histological growth patterns and nuclear atypia, anisokaryosis, and mitotic count in neoplastic GCs of bovine GCTs. The presence of a substantial theca cell component in granulosa theca cell tumors, however, is associated to stallion-like behavior and/or increased serum testosterone concentrations (STABENFELDT et al., 1979; BAILEY et al., 2000; MCCUE et al., 2006; ELLENBERGER et al., 2007). A prominent theca cell layer is characterized by large numbers of Leydig-like cells (LLCs), which were first described in 1979 by STABENFELDT et al. and frequently reported in GCTs (ELLENBERGER et al., 2007; BALL et al., 2008, 2013; NETO et al., 2010; CRABTREE, 2011; MÜLLER et al., 2012; DOLIN et al., 2023). Leydig-like cells are defined as luteinized derivatives of thecal cells (STABENFELDT et al., 1979; MACLACHLAN, 1987; MCCUE et al., 2006) and appear as large polyhedral cells with eosinophilic, finely granular cytoplasm (CRABTREE, 2011). STABENFELDT et al. (1979) observed LLCs most commonly in GCTs of mares with high serum testosterone concentrations. Accordingly, BAILEY et al. (2000) confirmed a relation between the abundant presence of LLCs and high testosterone concentration in tumor fluid. Moreover,

ELLENBERGER et al. (2007) and MÜLLER et al. (2012) reported from the presence of numerous LLCs in mares with stallion-like behavior and/or increased serum testosterone concentrations.

Granulosa cells with Sertoli-cell morphology are termed as Sertoli-like cells (SLCs) and are also commonly reported in equine GCTs (BALL et al., 2008; CRABTREE, 2011; MÜLLER et al., 2012; DOLIN et al., 2023). They are described as "closely packed solid tubules lined by columnar and cuboidal GCs" (TSOGTGEREL et al., 2021), but not included in tumor classification. In human medicine, sex-chord stromal tumors are believed to originate from theca cells, other stromal cells, GCs, or their testicular sex cord counterparts (Sertoli and Leydig cells). Human sex-chord stromal tumors are therefore subdivided into adult and juvenile GCTs, thecomas, fibromas, Sertoli cell tumors, Sertoli-Leydig cell tumors, and steroid cell tumors based on the most presented neoplastic cell type (CHEN et al., 2003). In contrast, sex-chord stromal tumors other than GCTs, granulosa-theca cell tumors, thecomas, and fibromas by means of clinic, pathology and tumor character are not further classified in equine medicine.

3.3.2. Immunohistochemistry – Diagnostic markers

Immunohistochemistry gives the possibility to visualize specific antigens in tissues or cells based on antibody-antigen recognition. Various immunohistological studies in healthy equine ovaries and GCTs are published. Several authors tested hormonal diagnostic markers like AMH (BALL et al., 2008; CLAES et al., 2016; TSOGTGEREL et al., 2021; NELISSEN and MILLER, 2022; DOLIN et al., 2023) and inhibin (NAGAMINE et al., 1998; BAILEY et al., 2000; HOQUE et al., 2003; DAVIS et al., 2005; ELLENBERGER et al., 2007), steroidogenetic enzymes like aromatase (AR), cytochrome P450, and glutathione S-transferase α (WATSON and THOMSON, 1996; HOQUE et al., 2003; MLODAWSKA and SLOMCZYNSKA, 2010; NETO et al., 2010; DOLIN et al., 2023), angiogenic factors (MÜLLER et al., 2009, 2012), and tumor markers like Ki-67 (Ki67), calretinin (CAL), epithelial cadherin (E-Cad), and c-erbB-2 oncoprotein (WATSON and AL-ZI'ABI, 2002; ELLENBERGER et al., 2007; DOLIN et al., 2023). Moreover, CD56, GATA-4, and FOXL2 were recently reported as diagnostic markers in human medicine with a suggested prognostic indicator for human GCTs (JUNG et al., 2023).

Regarding the published diagnostic value in humans, some of those immunohistochemical markers might be also helpful to detect and classify ovarian abnormalities in equine ovaries. Most of the immunohistochemical markers like Ki67, AMH, AR, CAL, epidermal growth factor receptor (EGFR), and E-Cad revealed promising results in a single equine GCT (DOLIN et al., 2023) and are therefore further mentioned in detail.

3.3.2.1. Ki-67

Ki-67 is a nuclear protein, which is involved in cell cycle activity, and a marker for determination of tumor proliferation activity in humans (GERDES et al.,

1983). The proliferation marker is associated with tumor progression, metastasis, and prognosis and indicative for tumor growth speed (BALAN et al., 2017; GIL and VAGNARELLI, 2018; AKHTER et al., 2019).

In animals, Ki67 is minimally expressed in neoplastic GCs of GCTs in bitches and mares (MATOS et al., 2021; DOLIN et al., 2023). Expression of Ki67 is scored as "percentage of positively stained cells" (KING et al., 1996). The Ki67 proliferation index (PI) is classified in grade 0 (0-25% stained cells), grade 1 (26-50% stained cells), grade 2 (51-75% stained cells), and grade 3 (76-100% stained cells) and a PI >1 (>25%) is defined as highly proliferative (KING et al., 1996; SILVA-FILHO et al., 2005; MATOS et al., 2021). DOLIN et al. (2023) reported a mean PI of 3.87% in neoplastic GCs of one examined GCT and MATOS et al. (2021) revealed a similar low Ki67 PI in 97% of 39 examined GCTs in bitches. In healthy ovaries, Ki67 PI is higher in GCs and endothelial cells of preovulatory follicles compared to transitional anovulatory follicles, but still regarded as minimally proliferative in both follicular structures (WATSON and AL-ZI'ABI, 2002). Further literature of Ki67 expression in healthy ovaries of mares is lacking.

3.3.2.2. Anti-Müllerian hormone

The homodimeric glycoprotein AMH is produced by Sertoli cells of the testis from sexual differentiation in fetal stage to puberty and is later additionally produced by GCs in the ovary of postnatal females (JOSSO et al., 2001; VISSER et al., 2006). Expression of AMH is restricted to GCs in the ovary and varies with follicular development (BALL et al., 2008). Granulosa cells of preantral and small antral follicles show growing intensity of AMH expression, which correlates to the number of GC layers (BALL et al., 2008). However, AMH expression decreases in large antral follicles >30 mm with absence in atretic follicles (BALL et al., 2008). Anti-Müllerian hormone is further not expressed in stromal and theca cells (BALL et al., 2008).

In GCTs, some authors could determine general AMH expression in neoplastic GCs (TSOGTGEREL et al., 2021; NELISSEN and MILLER, 2022), whereas others reported heterogeneous expression patterns within and between the tumors (BALL et al., 2008). BALL et al. (2008) revealed higher AMH expression in neoplastic GCs compared to polyhedral LLCs in the interstitial tissue. DOLIN et al. (2023) published distinct AMH expression in one GCT with clear expression in LLCs and GCs with specifically highlighting SLCs.

3.3.2.3. Aromatase

Aromatase is an enzyme complex, which contains aromatase cytochrome P450 as an essential component and catalyzes the synthesis of androgens into estrogens in GCs of mammals (CONLEY and HINSHELWOOD, 2001). Healthy ovaries are characterized by heterogeneous AR expression in GCs of viable, non-atretic follicles >5 mm diameter and intense expression in GCs of preovulatory follicles (WATSON and THOMSON, 1996; MLODAWSKA and SLOMCZYNSKA, 2010). MLODAWSKA and SLOMCZYNSKA (2010) could further detect this enzyme in

theca interna cells and large lutein cells of CLs in normal ovaries of mares during estrous cycle and pregnancy. Atretic follicles showed heterogeneous expression patterns with disappearing AR expression correlated to ongoing atresia in their study. Aromatase is not expressed in preantral follicles, stromal cells, and small lutein cells of CLs (WATSON and THOMSON, 1996; MLODAWSKA and SLOMCZYNSKA, 2010).

Regarding equine GCTs, immunohistochemical analysis of AR is published controversially. DOLIN et al. (2023) reported weak AR expression in neoplastic GCs and high expression in LLCs, according to WATSON and AL-ZI'ABI (2002). The authors therefore suggested that LLCs might be capable of converting testosterone into estrogens. In contrast, others negated AR expression in GCs or LLCs in GCTs and consequently concluded a failure of testosterone conversation into estrogens (HOQUE et al., 2003; IBRAHIM, 2019). NETO et al. (2010) investigated the steroidogenic phenotype of equine GCTs by immunohistochemical analysis of 17α -hydroxylase/17,20-lyase cytochrome P450, "the enzyme most directly responsible for androgen synthesis". They could detect this enzyme in 50% of GCTs with the highest expression in polyhedral LLCs and concluded the potential of synthesis and secretion of androgens by neoplastic GCs and LLCs.

3.3.2.4. Epidermal growth factor receptor

Epidermal growth factor receptor is a transmembrane glycoprotein and with signal transduction pathways involved in the regulation of normal cell proliferation, differentiation, migration, and survival (JOST et al., 2000). This glycoprotein has moreover an essential role in the ovulation process together with its ligands (PARK et al., 2004). In human tumors, overexpression of EGFR is correlated with poor tumor differentiation, a high metastatic rate, tumor growth, and a low survival rate (PAVELIC et al., 1993; VEALE et al., 1993; HERBST, 2004). Immunohistochemical studies on EGFR in equine healthy ovaries and GCTs were not reported yet.

3.3.2.5. Calretinin

Calretinin is a 29-kDa calcium binding protein and expressed in mesothelial cells, in Leydig cells of the testis, and further in theca lutein cells, theca interna cells, and stromal cells of human ovaries (BERTSCHY et al., 1997; DEAVERS et al., 2003; LUGLI et al., 2003). Calretinin is a sensitive marker for sex-chord stromal tumors and mesotheliomas in humans (DOGLIONI et al., 1996; CAO et al., 2001; MOVAHEDI-LANKARANI and KURMAN, 2002; DEAVERS et al., 2003; YOUSEFI et al., 2009; BALAN et al., 2017). Expression of CAL is weak in GCs of human GCTs, but intense in Leydig-cell components of Sertoli-Leydig cell tumors, suggesting an association to androgen production (CAO et al., 2001). Calretinin is also expressed in equine GCTs and there predominantly in GCs, LLCs, and the theca interna cell layer (DOLIN et al., 2023). Expression of CAL was moreover reported in a diffuse malignant metastatic mesothelioma in an Arabian mare (STOICA et al., 2004).

3.3.2.6. Epithelial cadherin

Epithelial cadherin is a member of the cadherin superfamily and a calcium-dependent transmembrane glycoprotein. Epithelial cadherin has an important impact as adhesion molecule in the apical zonula and therefore regulatory characteristics in the epithelial structure as cell-cell adhesion, cell stabilization, and intercellular exchange (KOURTIDIS et al., 2017). This glycoprotein is further involved in tumor metastasis due to its tumor suppression activity (BRACKE et al., 1996). Loss of E-Cad function, especially at the apical zonula, is a crucial step in cancer progression in humans (KOURTIDIS et al., 2017).

In veterinary medicine, research on E-Cad is only in its infancy (KASZAK et al., 2020). Immunohistochemical expression is reported in healthy and tumorous ovaries of rats and pigs (RYAN et al., 1996; SUNDFELDT et al., 2000). RYAN et al. (1996) could find high E-Cad expression in GCs of healthy follicles with decreased expression in GCs of atretic follicles in pig ovaries. The authors therefore suggested an important role of E-Cad in maintaining the structural integrity of the ovarian follicle during growth and development. However, E-Cad expression was determined by immunoblot in their study without a detailed immunohistochemical analysis. Neoplastic processes like tumor progression and metastasis are associated with a decreased E-Cad expression in different neoplasia of dogs and humans (KASZAK et al., 2020). In contrast, DOLIN et al. (2023) recently reported intense expression of E-Cad in LLCs of an equine GCTs and weak expression in GCs and theca externa cells of healthy ovarian control tissue. The authors therefore recommended E-Cad as potential marker for LLCs in equine GCTs.

4. Treatment options

As previously mentioned, behavioral problems in mares commonly represent a diagnostic challenge for veterinarians. Even if a full diagnostic work-up is targeted (PRYOR and TIBARY, 2005; NOUT-LOMAS and BEACOM, 2015), clinicians often end up with a missing diagnosis for abnormal behavior and might consequently struggle with the correct individual treatment method (MELGAARD et al., 2020). However, ovaries tend to be a root cause for behavioral problems, as several authors reported a significant behavioral improvement after bilateral removal, even in case of a missing ovarian pathology in some mares (KAMM and HENDRICKSON, 2007; DEVICK et al., 2020; MELGAARD et al., 2020; COLLAR et al., 2021; STRATICÓ et al., 2023). The following treatment options aim to modify behavior based on ovarian-related problems and are differentiated between estrous-related behavioral problems and behavioral abnormalities caused by GCT presence.

4.1. Treatment of estrous-related behavioral problems

Conservative, medical treatment options are usually regarded as first choice to treat estrous-related behavioral problems in mares (JORGENSEN, 1996; MCCUE, 2003). As some owners intend to breed with their mares, surgical ovarian

removal is considered as the last solution due to elimination of any future reproductive potential (AURICH and KAPS, 2022). Therefore, many conservative treatment options with the goal of estrous suppression are available (PRYOR and TIBARY, 2005; AURICH and KAPS, 2022; CRABTREE, 2022). However, legal limitations, like the veterinary association for animal welfare in Germany, restrict the use of most of the conservative medications due to missing medical indications, therapeutic emergencies, and animal welfare aspects (DIETZE et al., 2017).

4.1.1. Conservative treatment options

Conservative treatment options for estrous suppression are summarized in **Figure 2** and are based on the imitation or prolongation of the luteal phase and suppression of ovarian activity (PRYOR and TIBARY, 2005; AURICH and KAPS, 2022; CRABTREE, 2022).

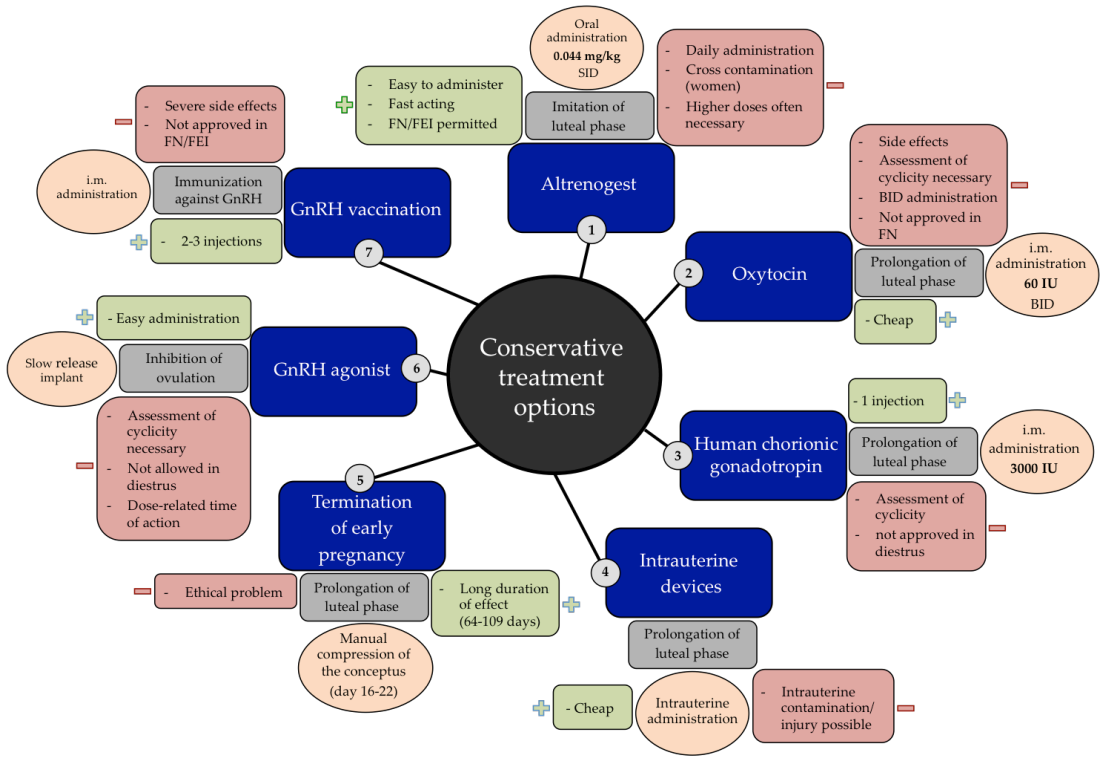

Figure 2: Overview over conservative treatment options to suppress estrus in mares with function, approach, pro (green box) and contra (red box). FN=Federation Nationale, FEI=Federation Equestre Internationale, SID=once a day, BID=twice a day, GnRH=Gonadotropin releasing hormone.

Daily Altrenogest administration (**1**) (Allyltrenbolone, Regumate®, 2.2mg/mL) suppresses estrus within 2-3 days for as long as it is given by causing an artificial luteal phase. However, follicular development and ovulation might not be reliably suppressed with the standard dose of 0.044 mg/kg and symptoms as the "painful ovary syndrome" might continue (PRYOR and TIBARY, 2005;

CRABTREE, 2022). The dose can be increased up to 0.088 mg/kg (CRABTREE, 2022). Possible contamination of other horses or humans (via skin contact) is described and needs to be aware of the person, who administers the drug orally. Altrenogest is permitted in the international sport (Federation Equestre Internationale, FEI) and in national competitions in Germany (Federation Nationale, FN), but is not approved for long-term treatment over 10 days (DIETZE et al., 2017).

A standard protocol for prolonging the natural luteal phase is the i.m. injection of 60 IU oxytocin (**2**) twice daily 7-14 days after ovulation (VANDERWALL, 2015). The anti-luteolytic effect of oxytocin will last for about 70 days (VANDERWALL et al., 2012; VANDERWALL, 2015), but requires assessment of the cycle stage by means of transrectal palpation and ultrasonographical examination. Oxytocin is banned in FN rules, but "controlled medication" under FEI rules. Oxytocin is not approved for prolonging the luteal phase and not recommended in this high dose of 60 IU due to possible side effects such as colic symptoms or sweating (DIETZE et al., 2017).

A prolonged luteal phase of 58-82 days by induction of diestrous ovulations can be achieved by i.m. injection of 3000 IU human chorionic gonadotropin (**3**) (hCG, Ovogest®, HEDBERG et al., 2006). This treatment option requires availability of a mature follicle over 30 mm diameter in diestrus (HEDBERG et al., 2006). In Germany, Ovogest is allowed for induction of ovulation from day two in estrus, but prohibited to use in diestrus (DIETZE et al., 2017).

Several intrauterine devices (**4**), such as glass marbles or other spheres out of plastic, or polymethyl-methacrylate, and special magnetic rings are reported to obtain a mechanical prolonged natural luteal phase, but with a lack of information regarding withdrawal time (CRABTREE, 2022). Such devices might cause complications like urogenital discomfort and colic in individual mares (VANDERWALL et al., 2012).

Another reported conservative treatment option is the termination of early pregnancy (**5**) between day 16 and 22. This debatable method prolongs luteal phase for 64-109 days (LEFRANC and ALLEN, 2003), but is restricted due to obvious ethical considerations for this purpose (DIETZE et al., 2017).

Application of long-acting GnRH-Agonists (**6**) (Deslorelin implants) inhibits ovulation and suppresses estrous behavior for a short time of 20-40 days (KAPS et al., 2021). However, deslorelin application failed to induce luteinization or ovulation of follicles in diestrus in one study (GLAZAR et al., 2004). GnRH-Agonists are generally not permitted for use in diestrus (DIETZE et al., 2017).

Suppression of ovarian activity is achieved by application of GnRH-vaccination (**7**). The vaccination provides an immunization against GnRH and leads to suppression of sex hormone secretion (GARZA et al., 1986). Mares with a two-dose GnRH vaccination stop their ovarian activity with reduction in progesterone and 17β-estradiol concentrations within four weeks (IMBODEN et

al., 2006; ELHAY et al., 2007). They remain suppressed for a variable amount of time, but at least for a minimum of three months (ELHAY et al., 2007). The GnRH- vaccine Equity® is unapproved and under doping control in Germany. Improvac® is available for medical treatment in porcine, but restrictively used in equines due to a lack of medical indication (DIETZE et al., 2017). Side effects especially after the booster injection are reported as severe, including swollen and painful injection site, neck stiffness, pyrexia, and apathy (IMBODEN et al., 2006).

4.1.2. Surgical treatment options

Surgical removal of both ovaries is in general considered as the last resort of estrous suppression, as it eliminates any further reproductive potential of the mare (AURICH and KAPS, 2022). Ovariectomy was originally used as treatment of choice for GCT-affected mares (MEAGHER et al., 1977) and gained nowadays popularity as long-term solution for mares with behavioral problems. Laparoscopic BO is a minimally invasive surgical procedure in the standing mare and is reported as a safe method (RÖCKEN et al., 2011). The surgical option resulted in successful outcomes in several independent studies regarding improvement of behavioral problems when owners were surveyed (KAMM and HENDRICKSON, 2007; DEVICK et al., 2020; MELGAARD et al., 2020; COLLAR et al., 2021; STRATICÓ et al., 2023). The positive outcome of BO ranged from 40-80% (MELGAARD et al., 2020) to 83% (KAMM and HENDRICKSON, 2007), 89% (DEVICK et al., 2020), 90% (COLLAR et al., 2021), and 91% (STRATICÓ et al., 2023). Even 10 mule mares with behavioral problems showed 100% improvement after BO (PETRIZZI et al., 2020). Most of the studies also included cases with neoplastic ovaries, but reported no difference in behavioral improvement between GCT-affected mares and mares with no ovarian pathology present (MELGAARD et al., 2020; STRATICÓ et al., 2023).

A reason for behavioral improvement after removal of both ovaries could be based on hormonal changes, as BO leads to omission of estrogen-producing theca cells and GCs of ovarian follicles (CHRISTENSEN, 2011). However, residual estrous signs might still be present in bilaterally ovariectomized mares, as reported in 35% (HOOPER et al., 1993), 27% (COLLAR et al., 2021), and 20% (ROESSNER et al., 2015). An extragonadal production of sex steroids, most likely from the adrenal cortex, is assumed as source of persistent estrous signs (ASA et al., 1980; SILBERZAHN et al., 1984; HEDBERG et al., 2007a, 2007b). The fact of absent luteal tissue as primary source of progesterone production is moreover discussed, as progesterone might have a more important role as inhibitory effect on estrous behavior than the presence of estrogens itself (NETT et al., 1976; WATSON and HINRICHS, 1989; ROESSNER et al., 2015).

4.2. Treatment of ovarian neoplasia

Several different neoplastic abnormalities occur in the equine ovary, as described previously (see 3.2.2). Regarding treatment options, emphasis was put on GCT, the most common neoplasia in the equine ovary (MEAGHER et al., 1977;

KENNEDY et al., 1998; MCCUE, 1998).

4.2.1. Conservative treatment options

In case of evident results for GCT presence by means of rectal and ultrasonographical examination and hormonal analysis, surgical removal is regarded as the treatment of choice (MEAGHER et al., 1977; MCCUE, 1998; HOQUE et al., 2002; MCCUE et al., 2006). Malignancy and metastatic processes of GCTs are rarely occurring (MEAGHER et al., 1977; ABBOTT et al., 2004; ELLENBERGER et al., 2007). However, surgical removal is indeed recommended due to possible sequelae caused by the tumor apart from behavioral disorders (SHERLOCK et al., 2016). Secondary problems include gastrointestinal issues and lameness due to progressive enlargement (MEAGHER et al., 1977; VAN DER ZAAG et al., 1996), adhesions between the tumor and other organs (RAMBAGS et al., 2003), granulomatous dermatitis (ABBOTT et al., 2004), and weight loss (MCCUE, 1998). More severe sequelae like small colon impaction (MAIR, 2002) or hemoperitoneum due to rupture of advanced GCTs were further reported (GREEN et al., 1988; ALEXANDER et al., 2004).

A non-surgical approach via GnRH vaccination was recently published (BEHRENDT et al., 2021). Three consecutive GnRH vaccinations (Improvac®) were administered to GCT-affected mares (n=4) with typical GCT-like behavior and elevated serum hormone concentrations. The vaccination resulted in resolution of behavioral problems, downsize of the neoplastic ovaries, and decrease of serum testosterone concentrations. One mare was observed for seven years with no additional enlargement of the ovary afterwards. However, severe side effects after vaccination are reported and include swollen and painful injection site, neck stiffness, pyrexia, and apathy (IMBODEN et al., 2006) and also anaphylactic shock with following death (BURGER et al., 2010). Nevertheless, GnRH vaccination could be an alternative for mares with no surgery option.

4.2.2. Surgical treatment options

Surgical removal of GCTs is regarded as treatment of choice with an excellent survival rate and restoration of fertility (MEAGHER et al., 1977; RAGLE and SCHNEIDER, 1995; HOQUE et al., 2002; MCCUE et al., 2006; MCKINNON and BARKER, 2010; CRABTREE, 2011; SHERLOCK et al., 2016). Removal of the tumor results in rapid decrease of serum hormone concentrations. Serum testosterone and inhibin decline to basal concentrations within 24 hours (MEAGHER et al., 1977; HUGHES et al., 1980; MCCUE et al., 2006; ELLENBERGER et al., 2007) and serum AMH decreases to 50% of the basal concentration within 1.9 days after surgery (ALMEIDA et al., 2011). Omission of excessive hormonal production, i.e. inhibin, results in a reshown follicular activity on the contralateral ovary in an average of 8.5 months after GCT removal (MEAGHER et al., 1977).

Surgical techniques for tumor removal include the initially established approach via colpotomy (COLBERN and REAGAN, 1987), a flank or ventral midline

laparotomy (GREET and BATHE, 1993; EMBERTSON, 2006), and laparoscopic removal (LEE and HENDRICKSON, 2008; RÖCKEN et al., 2011; HENDRICKSON, 2012). The first standing BO was described in 1993 via colpotomy by HOOPER et al. Laparoscopic minimally invasive surgical procedures have prevailed as technique of choice in recent decades due to several advantages compared to laparotomy or colpotomy. Laparoscopic ovariectomy is performed in the standing horse (RÖCKEN et al., 2011), but also in dorsal recumbency (RAGLE and SCHNEIDER, 1995), or in Trendelenburg position (HENDRICKSON, 2012). This depends on individual surgeon's preferences and on the mare's tolerance. For owners, standing laparoscopic ovariectomy offers the advantage of avoidance of general anesthesia risks, provides fast healing, and consequently shortens hospitalization (FISCHER, 1991). For surgeons, laparoscopic ovariectomy enables full intraoperative visibility, secure hemostasis, and less invasiveness and is therefore regarded as a save method (RÖCKEN et al., 2011). Compared, serious perioperative complications were described with the technique of laparotomy or colpotomy, like fatal hemorrhage of the mesovarium, mesenteric trauma, peritonitis, adhesions, infection and dehiscence, myopathy, and postoperative colic or ileus (MEAGHER et al., 1977; NICKELS, 1988; RODGERSON et al., 2001).

A routine protocol of the minimally invasive, laparoscopic technique was published by RÖCKEN et al. (2011). Laparoscopic ovariectomy in the standing horse is performed in sedation with detomidine (0.01 mg/kg i.v.) and butorphanol (0.01 mg/kg i.v.) and analgesia with flunixin meglumine (1.1 mg/kg i.v.). Sedation is adjusted effect-depending during the surgical procedure by consecutive administration of detomidine and butorphanol (0.005-0.01 mg/kg i.v.). The paralumbar fossa of one or both sides, according to the diagnosis, is aseptically prepared. This area and the 17th intercostal space at tuber coxae level are locally infiltrated with 2% lidocaine with additional subperitoneal anesthesia. Two ipsilateral instrumental and one optic portal per side are placed in the 17th intercostal space or caudal to the last rib. After visualization of the ovary, another infiltration anesthesia of the ovarian pedicle is necessary. Fluid suction of large follicles achieves reduction of tumor size and facilitates tumor removal over the flank. Transection of the ovary with secure hemostasis of the mesovarium is performed with a linear stapling devise, bipolar electrosurgical instruments, a diode-laser with ligation, or a vessel-sealing system (LigaSure® Force TriadTM). Large-sized GCTs can be crushed into pieces by means of an intraabdominal morcellator and sterile retrieval bag (DANIEL et al., 2015b). Removal of the ovaries is performed by enlargement of the instrumental ports or connecting two ports up to a length of 15 cm. Flank incisions and portals are sutured after ovarian removal. In case of bilateral ovariectomy, the same procedure is continued on the other side.

For ovarian tumors larger than 20 cm diameter, a two-step procedure is recommended (RÖCKEN et al., 2011). This starts similar with a standing laparoscopic dissection of the mesovarium and is followed by removal of the ovary via ventral midline celiotomy under general anesthesia (RÖCKEN, 2000). Aftercare includes variable administration of antibiotic treatment and necessary

administration of NSAIDs for two to six days post-surgery. Clinical examinations and white blood cell count are suggested within the first three days postoperatively. Major intraoperative complications of this laparoscopic procedure were not described by RÖCKEN et al. (2011). Reported postoperative complications included dehydration after surgery, subcutaneous emphysema, fevers, mild colic signs, wound swelling, hematoma at the resection site, and seroma formation with following dehiscence of the flank incision. RÖCKEN et al. (2011) reported an overall postoperative morbidity of 10.8% and mortality of 0% in mares with standing laparoscopic BO, suggesting this surgical treatment option as a safe method.

III. OWN INVESTIGATIONS

1. Objective

The objective of the present study was to find a pathohistological explanation for the high success rate of BO in mares with behavioral problems but clinically unremarkable ovaries. Therefore, bilaterally removed ovaries of mares with behavioral problems (group "bOE", n=20) were histomorphologically and immunohistochemically evaluated by means of the diagnostic markers Ki67, AMH, AR, EGFR, CAL, and E-Cad. Those ovaries of bOE were further compared with unilaterally removed ovaries of GCT-affected mares (group "GCT-uOE", n=10). Moreover, we aimed to involve a complete dataset of each individual case as basis for pathohistological evaluation and included clinical history with behavioral patterns and preoperative conservative treatment, clinical examination with rectal and ultrasonographical evaluation, analysis of serum AMH and testosterone concentrations, and outcome of surgery by means of an owner's questionnaire.

We hypothesized that GCTs are not the only ovarian source of behavioral problems in mares and that other, pathohistologically detectable changes might explain the reported success of BO. We moreover hypothesized that clinically unremarkable ovaries of bOE are differentiated from neoplastic changes of GCT-uOE by means of the immunohistochemical markers Ki67, AMH, AR, EGFR, CAL, and E-Cad.

2. Publication

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Article

Pathohistological Findings after Bilateral Ovariectomy in Mares with Behavioral Problems

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Simple Summary: Bilateral ovariectomy in mares with behavioral problems is a common long-term solution with high owner satisfaction. However, a pathohistological explanation for behavioral improvement after surgery is lacking. Therefore, bilaterally removed, clinically unremarkable ovaries from mares with behavioral problems were immunohistologically evaluated and compared with pathohistologically confirmed granulosa cell tumors. A complete data set including clinical history, clinical examination, serum anti-Müllerian hormone (AMH), and testosterone concentrations was analyzed as the basis for the pathohistological study. Immunohistochemical evaluation of Ki-67, AMH, aromatase, epidermal growth factor receptor, calretinin, and epithelial cadherin revealed no clear differentiation between large follicular structures of clinically unremarkable ovaries and cyst-like structures of neoplastic ovaries. Clinical data and success rate after bilateral ovariectomy of 85% were comparable with previous studies. Preoperatively measured serum AMH and testosterone concentrations were indicative of advanced granulosa cell tumors but were variable in mares with clinically unremarkable ovaries. Ultrasonographically nondetectable early neoplastic changes could be determined in 15% of mares and anovulatory-like follicles in 30% of mares with bilaterally removed ovaries. These changes might be a pathohistological explanation for behavioral problems of ovarian origin and a reason for the high success rate of bilateral ovariectomy.

Abstract: Behavioral problems in reproductively healthy mares are a challenging issue that is successfully treated with bilateral ovariectomy (BO). This laparoscopic procedure represents an alternative to conservative treatment for mares not intended for breeding and results in high owner satisfaction regarding behavioral improvement. However, a pathohistological explanation to justify surgical ovarian removal regarding animal welfare is lacking. Therefore, the objective of this study was to pathohistologically evaluate bilaterally removed, clinically unremarkable ovaries of mares with behavioral problems (bOE, $n = 20$) and to compare them with pathohistologically confirmed granulosa cell tumors of mares with neoplastic ovaries (GCT-uOE, $n = 10$). A complete data set including preliminary presentation, clinical examination, and serum anti-Müllerian hormone (AMH) and testosterone was further analyzed in both groups. Both hormones were significantly higher in GCT-uOE compared with bOE. Immunohistochemical expression of Ki-67, AMH, aromatase, epidermal growth factor receptor, calretinin, and epithelial cadherin in granulosa cells of large follicular structures in bOE did not differ from neoplastic granulosa cells in GCT-uOE. Ultrasonographically nondetectable early neoplastic changes were pathohistologically evaluated in 15% of mares and anovulatory-like follicles in 30% of mares in bOE and might be one explanation for the high success rate of BO in 85% of bOE in this study.

Keywords: equine ovary; behavior; immunohistochemical marker; anti-Müllerian hormone; granulosa cell tumor

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1. Introduction

Mares with behavioral problems not obviously related to ovarian pathology are common and represent a challenge for owners, trainers, and veterinarians. As behavioral problems are assumed to be related to estrus, an association with ovarian hormonal secretion and anatomic changes is also assumed, especially in high-performance athletic mares. In some cases, even daily handling leads to difficulties and danger during estrus [1–3]. Consequently, veterinarians are expected to diagnose the cause of behavioral problems in order to find a successful solution for owners, riders, and the mares themselves. Behavioral problems range from normal, estrous-related sexual behavior due to physiological steroid hormone production, to management-related problems and medical issues caused by disease or pain of unknown origin [3–5]. Therefore, a multimodal approach with an assessment of all body systems with additional appraisal of management factors is recommended [6]. A possible medical cause for behavioral problems is the commonly occurring granulosa cell or granulosa-theca cell tumor, abbreviated as GCT in this study, due to excessive hormonal activity [7,8]. However, mares frequently present behavioral problems despite clinically unremarkable ovaries and a missing obvious ovarian neoplasia. Therefore, these cases pose a diagnostic and therapeutic challenge.

Presuming an association of behavioral problems with the cyclicity of the mare in the absence of ovarian pathology, owners ask for methods to suppress ovarian function and estrous behavior. Aurich and Kaps (2022) [9], as well as Crabtree (2022) [10], currently summarize a variety of conservative treatment options for estrous suppression, but most of them are cost-intensive, might have severe side effects, or their reversibility is not clearly determined yet. Furthermore, legal limitations in Germany restrict the use of the majority of the conservative possibilities [11]. Therefore, bilateral ovariectomy (BO) is often inquired due to the minimally invasive surgical approach and safe method [12] for mares that are not intended for breeding. Although persistent estrous signs in mares after BO are described (in 35% [13], 27% [14], 20% [15], respectively), BO revealed a significant improvement of behavior after surgery from the owner's perspective (success rate of 40% [5], 83% [16], 89% [17], 90% [14], 91% [18], respectively). However, an explanation for BO as a successful solution for mares with behavioral problems not obviously related to estrus or ovarian pathology is still lacking.

Gonadectomy in male horses in order to facilitate daily handling, rideability, and cohabitation with other horses is common practice. However, the indication for BO in mares especially in case of a missing ovarian pathology or a lack of an understandable link to the ovaries [4] is discussed due to animal welfare aspects regarding removal of healthy organs [11]. Abdominal discomfort associated with physiological estrus is reported as "painful ovary syndrome" [3,10,19] and a justified reason for BO as a long-term solution. Further problems such as aggressive or stallion-like behavior are commonly associated with GCTs and treated by unilateral removal of the affected ovary [8,20]. However, these behavioral abnormalities were also found in mares with normal ovaries [17]. Therefore, the question arose as to whether bilaterally removed ovaries of clinically and reproductively unremarkable mares with behavioral problems might demonstrate other, non-neoplastic abnormalities that could explain the success and owner's satisfaction after BO.

Pathohistological examination using diagnostic markers is reported to effectively determine ovarian abnormalities, especially equine GCTs [21–26]. Dolin et al. (2023) [21] evaluated different immunohistochemical markers in a single equine GCT case, including Ki-67 (Ki67), anti-Müllerian hormone (AMH), aromatase (AR), calretinin (CAL), and epithelial cadherin (E-Cad), and suggested most of them as potential markers for tumor diagnosis. Ki-67 has tumor proliferation activity and is correlated to tumor progression, metastasis, and prognosis in humans [27–29]. Anti-Müllerian hormone is produced by granulosa cells (GCs) of postnatal females [30]. It is expressed in growing follicles of healthy equine ovaries and to a higher extent in GCTs [31–33]. Therefore, serum AMH is regarded as a sensitive marker for GCTs in mares [34,35]. The enzyme complex AR is expressed in healthy ovarian tissue and suggested as a further valuable diagnostic marker

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for equine GCTs [21,23,24,36,37]. Moreover, CAL, E-Cad, and epidermal growth factor receptor (EGFR) represent essential tumor markers, originally used in human tumor diagnostic [28,38,39]. Next to those tumor markers, CD56, GATA-4, and FOXL2 pose additional new diagnostic markers in human medicine as indicators for prognosis in GCTs [40]. Regarding the reported diagnostic value in humans, some of those immunohistochemical markers might also be helpful in detecting ovarian abnormalities other than GCTs in equine ovaries.

Therefore, the aim of the present study was to examine bilaterally removed ovaries of clinically unremarkable mares with behavioral problems (bOE) by means of histomorphology and immunohistochemistry. We hypothesized that GCTs are not the only ovarian source of behavioral problems in mares and that other, pathohistologically detectable changes might explain the reported success of BO. Moreover, we compared findings in bOE with findings in unilaterally removed, pathohistologically confirmed GCTs (GCT-uOE) and therefore hypothesized that clinically unremarkable ovaries of bOE are differentiated from neoplastic changes in GCT-uOE by means of the immunohistochemical markers Ki67, AMH, AR, EGFR, CAL, and E-Cad.

2. Materials and Methods

This pathohistological study was conducted in 2022 in accordance with national laws for animal use and approved by the ethics committee of the veterinary department of the LMU Munich (reference number: AZ 295-04-01-2022). The clinical part was performed from July 2019 to June 2022 and included clinical history, clinical examination, serum hormone analysis and surgical procedure. The clinical data set was retrospectively evaluated and implemented as basis for the pathohistological study. Surgical removal of the ovaries was suggested by referring veterinarians and performed with owner's declaration of consent.

2.1. Animals, Group Determination, Clinical History, Premedication, and Preoperative Examination

Ovaries of 30 mares were collected after standing minimally invasive laparoscopic ovariectomy at the Equine Hospital in Starnberg. Mares were divided into two groups based on uni- or bilateral ovarian removal. In mares with unilateral ovariectomy (group GCT-uOE, n = 10 mares), GCT presence was suspected on clinical examination before surgery. In mares with BO (group bOE, n = 20 mares), both ovaries were unremarkable on clinical examination. This classification was further confirmed by routine pathohistological evaluation of removed ovaries by an external laboratory. Therefore, bilaterally removed ovaries (n = 40) of clinically unremarkable mares with behavioral problems (bOE) were compared with unilaterally removed, pathohistologically confirmed GCTs (GCT-uOE, n = 10).

A detailed history was taken by means of an owner questionnaire prior to surgery (see Supplementary Data SQ1). The information included age, breed, duration of behavioral problems, behavioral patterns, and weather the mare had received any conservative treatment before surgery like Altrenogest (allyltrenbolone) or a GnRH vaccine (see Supplementary Data, Table S1). A selection option of different behavioral patterns was provided according to commonly occurring behavioral abnormalities in mares (Supplementary Data, SQ1) [5,17,18,41]. Further specifically mentioned behavioral problems observed by the owners themselves were also considered. All mares underwent a transrectal palpation and ultrasonographical examination of the reproductive tract before surgery to evaluate ovarian size, clinical presence of GCT, and stage of cycle determined by the presence of follicular structures and corpora lutea (CL). Estrus was defined if follicles > 30 mm were present in absence of a CL, diestrus if at least one CL was present [42], and an intermediate estrous stage if follicles > 30 mm and CL were ultrasonographically not detectable. The cyclic state was adapted if follicular structures or CL not identified on clinical examination were detected retrospectively by macroscopical or microscopical evaluation of the removed ovaries.

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2.2. Hormone Concentrations

Routine blood collection was performed from a catheter immediately before surgery, centrifuged, and left over sera were frozen for further hormonal analysis. Serum AMH concentrations of all (n = 30) and testosterone concentrations of 18 cases (n = 11 in bOE, n = 7 in GCT-uOE) were determined by an external laboratory (Antech Lab Germany, formerly SYNLAB Vet) by means of a standardized and validated ELISA and LC-MS, respectively. In brief, AMH concentrations were measured using the Tecan Sunrise Absorbance Reader (AMH Gen II ELISA, Tecan, Männedorf, Switzerland), which works with a noncompetitive, two-side immunoassay. Analyses were performed according to the manufacturer's instructions. Serum AMH concentrations between 14.3 pmol/L and 28.6 pmol/L were regarded as suspicious for GCT presence, while the cut-off value of 28.6 pmol/L was proving a GCT presence [35]. Testosterone concentrations were measured with the Sciex Triple Quad ^{TM} 5500+ System (Sciex, Framingham, MA, USA) using inductively coupled plasma mass spectrometry (ICP-MS) according to the manufacturer's instructions. Serum testosterone concentrations above 0.35 nmol/L were indicating the presence of GCT.

2.3. Surgical Procedure and Owner Consult

Standing laparoscopic ovariectomy was performed by the same surgeon using routine procedures [12]. In brief, mares were prepared for surgery 48 h in advance with no access to hay but to a special diet with haycobs, mash, and laxatives and were lunged several times a day. Mares received flunixin meglumine (1.1 mg/kg BW i.v., Flumeg Nova, Serumwerk Bernburg AG, Bernburg, Germany) before and until 5 days after surgery and were sedated (Detomidine 0.01 mg/kg BW i.v., Eurovet Animal Health B.V., Bladel, The Netherlands, and Butorphanol 0.01 mg/kg BW i.v., CP-Pharma, Burgdorf, Germany). After aseptic preparation, the incisional side on one (GCT-uOE) or both flanks (bOE) was infiltrated with local anesthetics (20 mL 2% lidocainhydrochloride, bela-pharm GmbH & Co. KG, Vechta, Germany). Three incisions for two instrumental portals and one optic portal were created on each side. The ovaries were detached at the anesthetized mesovarium using a vessel-sealing system (LigaSure^{TM}, Medtronic, Meerbusch, Germany) and extracted through the extended flank incision, followed by suturing the incisions in the routine manner. All mares of bOE underwent a bilateral ovariectomy, whereas only the neoplastic ovary was removed in GCT-uOE. In the case of large-sized GCTs > 20 cm in diameter in GCT-uOE, a two-step procedure was performed with laparoscopic standing detachment followed by removal of the enlarged ovary in dorsal recumbency under general anesthesia [12]. Those mares were additionally treated with systemic antibiotics (Procain-Penicillin, 20.000 IE/kg BW i.m. SID, Dechra, Aulendorf, Germany, and Gentamicin, 6.6 mg/kg BW i.v. SID, CP-Pharma, Burgdorf, Germany) for 3 days.

In both groups, a routine retrospective telephone survey was carried out 6 to 12 months after surgery regarding the improvement of behavioral problems, reoccurrence of behavioral problems, and possible signs of estrus, as well as owner's satisfaction by means of a questionnaire (see Supplementary Data SQ2).

2.4. Macro- and Microscopical Evaluation of the Ovaries

Macroscopical examination of the removed ovaries including measurement of the size (length × width × height), gross section, and cycle stage determination was carried out immediately after the surgical procedure. Ovaries with 50–80 mm length and 20–40 mm width were regarded as normal sized [43], under 50 × 20 mm as small, and over 80 × 40 mm as large sized. Routinely, one representative sample of ovarian tissue with macroscopically visible follicles of each ovary was sent to a commercial pathohistological laboratory (Antech Lab Germany, formerly SYNLAB Vet Tierpathologie München, Munich, Germany) for routine pathohistological confirmation or exclusion of GCT. Additional samples with primarily visible follicles of the removed ovaries were fixed in 4% buffered formaldehyde and restored in 70% ethanol until further pathohistological analysis was conducted at

the University of Veterinary Medicine Vienna (Vienna, Austria). There, specimens were embedded in paraffin, sectioned at 3–4 µm, and stained with hematoxylin and eosin (HE) for general tissue assessment. Slides of two different locations with primarily follicular structures were prepared for each ovary. Based on light microscopical examination, different stages of developing and regressing follicles of each ovary in bOE (n = 40) were categorized into preantral follicles (including primordial, primary, and secondary follicles), antral follicles (including tertiary and preovulatory follicles), atretic follicles (including early and late atretic follicles), and CL according to others [26,44–46]. Different GCT types of each ovary in GCT-uOE (n = 10) were routinely determined [25] and categorized according to the current World Organization for Animal Health (WOAH) tumor classification [47] but were not further included in the interpretation of results in this study. Macroscopical and pathohistological evaluation of removed ovaries was performed by the same trained observer advised by an experienced histologist and board-certified pathologists of an external laboratory.

2.5. Immunohistochemical Staining and Evaluation

Consecutive serial sections of two different locations of each ovary were prepared for immunohistochemical examination with Ki67, AMH, AR, EGFR, CAL, and E-Cad. An indirect method with secondary antibodies conjugated with horseradish peroxidase was used. Therefore, paraffin sections of bOE and GCT-uOE were dewaxed through graded alcohol series (xylene, 100, 96, and 70% ethanol) and endogenous peroxidase activity was blocked by incubation in H_2O_2 with methanol at room temperature (RT) for 15 min with rinsing in tap water afterward. Table 1 summarizes functional and diagnostic relevance, sources, pretreatments, and dilutions of the primary antibodies, as well as equine positive controls used in this study.

Table 1. Function and diagnostic relevance, clone, sources, dilution, pretreatments, and positive controls of used immunohistochemical markers: Ki-67 (Ki67), anti-Müllerian hormone (AMH), aromatase (AR), epidermal growth factor receptor (EGFR), calretinin (CAL), and epithelial cadherin (E-Cad).

Antibody	Function—Diagnostic Relevance	Clone/Host	Source	Dilution	Pretreatment	Positive Control
Ki67	Cell proliferation—Tumor marker	MIB-1 Mouse	Agilent Technologies, Santa Clara, CA, USA	1:1000 in PBS	0.01 M Citrate buffer pH 6.0	Lymph node
AMH	Hormone—Produced in granulosa cells	Polyclonal Rabbit	Genetex, Irvine, CA, USA	1:5000 in PBS	Tris EDTA pH 9.0	Fetal gonads
AR	Enzyme complex—Conversion of androgens into estrogens	Polyclonal Rabbit	BioVision, Waltham, MA, USA	1:1000 in PBS	Tris EDTA pH 9.0	Testes
EGFR	Transmembrane protein with function in ovulation—Tumor marker	4575 Mouse	NeoBiotechnologies, Aachen, Germany	1:200 in PBS	Tris EDTA pH 9.0	Skin
CAL	Calcium-binding protein—Tumor marker	Polyclonal Rabbit	Chemicon, Temecula, CA, USA	1:5000 in PBS	0.01 M Citrate buffer pH 6.0	Cerebrum
E-Cad	Adhering cell junction—Tumor suppression activity, Tumor marker	Polyclonal Rabbit	Sigma Prestige, St. Louis, MI, USA	1:500 in PBS	0.01 M Citrate buffer pH 6.0	Kidney

Antigen retrieval was processed by steaming in the presence of antigen unmasking solution (Tris-EDTA pH 9, or citrate buffer pH 6) for 30 min before cooling down to RT. Slides were rinsed in PBS and blocked with 1.5% normal goat serum (Sigma Aldrich, Merck, Darmstadt, Germany) for 30 min to minimize unspecific binding of the primary antibody. After blocking, sections were incubated with the primary antibody overnight at 4 °C, rinsed in PBS, and applied with BrightVision Poly-HRP-anti-rabbit second antibody (ImmunoLogic-Duiven-The-Netherlands) for 30 min at RT. After rinsing in PBS, slides were

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incubated with DAB-solution (Qanto, Richard Allan Scientific, TA-125-QHDX, Kalamazoo, MI, USA) according to the manufacturer's protocol. Finally, nuclei were counterstained with haemalaun, rinsed in tap water, dehydrated in graded alcohol series (96 and 100% ethanol, xylene), and mounted with DPX medium (Fluka, Buchs, Switzerland) and cover glasses. Negative controls were obtained by omission of the primary antibodies to demonstrate the specificity of the secondary system. Sections of the equine lymph node, fetus, testes, skin, cerebrum, and kidney served as positive controls. Moreover, Western blots were performed to validate the specificity of the used antibodies for equine tissue (Supplementary Data, Figure S1).

Immunolabeled slides were examined via light microscopy, and images were captured using a digital camera (UC90, Olympus, Munich, Germany) and imaging software (cellSense 2.3, Olympus). Immunoreactivity was assessed by evaluating four representative high-power fields per slide (200× magnification), and the number of positive cells of previously described different functional components of bOE and GCT-uOE was estimated. Expression of the proliferation marker Ki67 was evaluated by counting the number of positive stained nuclei in cells among a total of 100 cells, and the proliferation index (PI) was graded according to King et al. [27] as follows: 0 (PI 0–25%), 1 (PI 26–50%), 2 (PI 51–75%), and 3 (PI 76–100%). A high PI of Ki67 was set at >25% stained cells, including grade 1–3 [48]. Expression of AMH, AR, EGFR, CAL, and E-Cad was defined according to Ball et al. (2008) [31] with −, +, ++, and +++ for negative, mild, moderate, and high expression, respectively.

For statistical evaluation of comparative immunohistochemistry, only GCs of detected large follicles with multiple GC layers (antral, early atretic, and anovulatory follicles) were included in bOE (n = 23) and compared with neoplastic GCs of GCT-uOE (n = 10), independent of the individual case. Expression of Ki67 was compared by means of the PI in percentage, whereas expression of the remaining markers was compared by means of their intensity. Moreover, immunohistochemical AMH expression was compared with serum AMH concentrations. Therefore, the highest detectable intensity of AMH in GCs of follicular structures in each case of bOE and in neoplastic GCs of cyst-like structures in each case of GCT-uOE was compared with the corresponding mare's serum AMH concentration.

2.6. Statistical Analysis

Statistical analyses were performed using R version 4.3.3. (The R Foundation for Statistical Computing, Vienna, Austria). All parameters were tested for normality of distribution with the Shapiro–Wilk normality test. In cases where data were not normally distributed, the Mann–Whitney U test (Wilcoxon rank-sum test) for two groups and the Kruskal–Wallis test for more than two groups were conducted. In cases where the Kruskal–Wallis omnibus test was significant, additional Dunn pairwise tests were performed to demonstrate which groups differed significantly.

For normally distributed parameters, Levene's test was used to test the homogeneity of variances among groups. Student's t-test for two groups and Fisher's One-Way ANOVA for more than two groups were applied when variances were similar. Welch's t-test and Welch's ANOVA were used when variances differed. Subsequently, a pairwise Student's t-test was additionally performed with Fischer's ANOVA, and pairwise Games–Howell tests were used for Welch's ANOVA to demonstrate which groups differed significantly.

A chi-square test was performed to compare categorical parameters. In cases where one of the categorical variables had more than two categories, pairwise Fisher tests were performed. Statistical significance was set at $p < 0.05$. In the case of multiple comparisons (e.g., ANOVA, Kruskal–Wallis, pairwise Fisher tests), the p-values were adjusted with the Holm correction method for multiple testing.

3. Results

Findings of clinical history (behavioral patterns, duration of behavioral problems, and conservative treatment before surgery), clinical examination (serum AMH and testosterone

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concentration, rectal palpation, and ultrasonographical examination with determination of cyclic stage), outcome of surgery, and pathohistological evaluation of each case in bOE and GCT-uOE are summarized in Supplementary Data (Tables S1 and S2). Furthermore, parts of the study were previously published on the ISER (International Symposium on Equine Reproduction 2023) conference as a poster and published as an abstract in the Journal of Equine Veterinary Science [49].

3.1. Animals, Clinical History, and Pre-OP Examinations

Included mares were of different breeds, mainly German Warmblood (n = 16) and Polish Warmblood (n = 3), in addition to Arab, Haflinger, Frisian, Quarter Horse, Pure Raza Española, Trotter, Icelandic Pony, and other Ponies. In bOE, mares were 4–20 years old at presentation (mean 13.0 years, SD 3.7), whereas mares in GCT-uOE were aged from 8–16 years (mean 13.2 years, SD 5.0).

Nineteen of twenty mares in bOE (95%) presented one or more specific behavioral problems (Table S1). One mare in bOE was bilaterally ovariectomized due to an increased serum AMH concentration without reported behavioral problems (Case 16). Nine of 10 mares in GCT-uOE demonstrated at least one specific behavior pattern (Table S1). One mare in GCT-uOE was ovariectomized due to a randomly detected neoplastic ovary on rectal palpation with normal cyclicity and behavior (Case 12). Table 2 provides an overview of the frequency of behavioral problems in bOE and GCT-uOE from the owner's perspective. Moodiness and stressed manner were additionally mentioned as further behavioral disorders by owners and included in the classification. Moodiness was the most frequently complained about behavioral problem in mares of bOE, whereas stallion-like behavior was most commonly present in mares of GCT-uOE. As demonstrated in Table 2, behavior patterns differed among bOE and GCT-uOE. Aggressive behavior and increased flank sensitivity were not reported in any case of GCT-uOE, in contrast to bOE, with an incidence of 35% and 25%, respectively.

Table 2. Frequency of behavioral problems in mares with bilaterally removed, clinically unremarkable ovaries (bOE; n = 20) and mares with unilaterally removed granulosa cell tumors (GCT-uOE; n = 10) as referred by the owner. Mares showed in general more than one specific behavioral problem. Values are expressed as percentages and ratios (in parentheses).

Behavioral Problem	Frequency of Occurrence	
	bOE	**GCT-uOE**
Moodiness	50% (10/20)	40% (4/10)
Stressed manner	30% (6/20)	20% (2/10)
Unwillingness to be ridden	45% (9/20)	30% (3/10)
Aggressiveness towards people	15% (3/20)	0% (0/10)
Aggressiveness towards other horses	30% (6/20)	0% (0/10)
Stallion-like behavior	5% (1/20)	60% (6/20)
Increased flank sensitivity	25% (5/20)	0% (0/20)
Colic symptoms	40% (8/20)	10% (1/10)
Prolonged or constant estrous signs	20% (4/20)	10% (1/10)
No estrous signs	5% (1/20)	40% (4/10)
No abnormal behavior	5% (1/20)	10% (1/10)

The time from the first presentation of behavioral problems to referral was under 12 months in 63% and over 12 months in 37% in bOE. In GCT-uOE, 67% of the mares showed behavioral problems under 12 months and 33% over 12 months before surgery. One case of each group was not incorporated due to no behavioral problems present before

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surgery (Case 16, 12). Eleven mares in bOE (55%) were conservatively treated either with Altrenogest (n = 5), GnRH-vaccine (n = 3), or Altrenogest following GnRH vaccination (n = 3) before surgery. All but one of the mares (91%) responded well to conservative treatment. In GCT-uOE, no mare was treated with Altrenogest or GnRH vaccination before surgery.

In bOE, rectal palpation prior to and macroscopical evaluation after surgery revealed symmetric ovaries in 16 mares (80%) with normal size (65%) and small size (15%) but asymmetric ovaries in 4 mares (20%). Four mares were assigned to estrus (20%), ten mares to diestrus (50%), and six mares to the intermediate estrous stage (30%). In GCT-uOE, all neoplastic ovaries were unilaterally enlarged on rectal palpation and macroscopical evaluation. All GCT-affected ovaries presented a honeycomb-like appearance on ultrasound and a small, inactive contralateral ovary. One mare presented a second GCT four years after the surgical removal of the first one (Case 37).

3.2. Results of Hormone Measurements

Prior to surgery, serum AMH concentrations in bOE ranged from 0.4–43.5 pmol/L with a median of 11.8 pmol/L (interquartile range (IQR) 9.5 pmol/L). Seventy percent of serum AMH concentrations were within normal limits in bOE, whereas fifteen percent were in the suspicious range of 14.3–28.6 pmol/L and fifteen percent above the cut-off limit of 28.6 pmol/L. Serum AMH concentrations in GCT-uOE ranged from 123.0–150.0 pmol/L with a median of 143.0 pmol/L (IQR 19.3 pmol/L). Mares of GCT-uOE showed significantly higher serum AMH concentrations compared with bOE ($p < 0.001$), which is demonstrated in Figure 1.

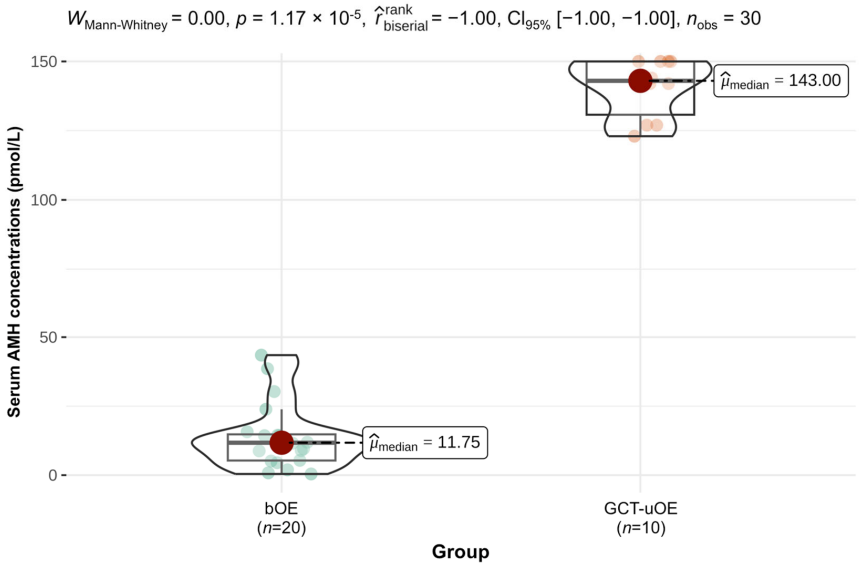

Figure 1. Comparison of serum anti-Müllerian hormone (AMH) concentrations between bilaterally ovariectomized mares (bOE, n = 20) and mares with granulosa cell tumors (GCT-uOE, n = 10). Mann–Whitney U (Wilcoxon rank-sum) nonparametric test was used due to not-normally distributed data (Shapiro–Wilk normality test). p-value < 0.001 shows very strong evidence for the (rank-sum) difference in serum AMH concentrations between bOE and GCT-uOE. Rank biserial correlation coefficient of −1.00 with 95% confidence interval indicates a very large effect size of serum AMH concentrations between the two groups. The red bullets show the median AMH concentrations; the green bullets show AMH concentrations of single cases. n_{obs} = total number of tested mares; x-axis: analyzed different groups, bOE = mares with bilaterally removed, clinically unremarkable ovaries, n = 20; GCT-uOE = mares with unilaterally removed granulosa cell tumors, n = 10; y-axis: measured serum AMH concentrations in pmol/L of each case with a range of 0.4–150 pmol/L.

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Serum testosterone of bOE was measured in 11/20 cases (55%) before surgery and was within normal limits (<0.14 nmol/L) in 10 of 11 mares (95%). The only mare with increased serum testosterone of 0.45 nmol/L in bOE additionally presented increased serum AMH (38.6 pmol/L, Case 29). In GCT-uOE, serum testosterone concentrations were measured in 7/10 cases (70%) and were increased in 20% of those cases with a median of 0.21 nmol/L (range 0.14–1.25 nmol/L, IQR 0.42). Serum testosterone concentrations were significantly higher in GCT-uOE compared with bOE ($p = 0.04$).

3.3. Outcome of Surgery and Improvement of Behavior

Surgeries in both groups were conducted throughout the year. Standing laparoscopic ovariectomy was performed in all mares ($n = 30$). Additional laparotomy under general anesthesia was necessary in five cases of GCT-uOE (50%) due to the large size of the neoplastic ovary. Overall complications were uncommon and present in 16.7% of all mares with fevers up to 39.7 °C in the first two days after surgery (16.7%) and seroma formation (6.7%) in both groups. Mild-to-moderate subcutaneous emphysema around the incision was found in all cases. Mares with standing surgery in bOE were discharged from the hospital after 5 to 7 days, whereas mares with enlarged GCTs in GCT-uOE following a two-step procedure or mares with complications in both groups were discharged after 7 to 10 days in a healthy condition.

Routine owner follow-up 6 to 12 months after surgery resulted in an improvement in behavioral problems in 85% of bOE and in 100% of GCT-uOE (overall improvement in both groups 90%). Two mares of bOE (Case 39, 49) demonstrated colic signs before surgery and were euthanized due to recurrence of colic 1 and 6 months after surgery, respectively. One mare of bOE (Case 40) showed a reoccurrence of initial behavioral problems 6 months after surgery with aggressiveness towards other horses, unwillingness to be ridden, and additional persistent estrous signs. Persistent estrous signs after BO occurred in 10% of the mares and included Case 40 of bOE and Case 37 of GCT-uOE. Case 37 was ovariectomized due to a second GCT. Both owners described the residual estrous signs as mild and well manageable. Of all conservatively treated mares before surgery in bOE, 4/5 (80%) were treated with Altrenogest, 2/3 (67%) were treated with GnRH-vaccination, and 2/3 (67%) were treated with both further improved after BO.

3.4. Macro- and Microscopical Findings on the Ovaries

Size determination of extracted ovaries in bOE revealed the largest ovary with 80 × 40 × 30 mm and the smallest ovary with 35 × 20 × 15 mm. Normal-sized symmetric ovaries were determined in 65%, small-sized symmetric ovaries in 15%, and asymmetric ovaries of normal and small size in 20% in bOE. In gross sections, numerous multiple follicles, antral follicles, and CL (Figure 2(A1–A3)) were found. Moreover, a small pale area near the ovulation fossa in one ovary in bOE (Case 29, Figure 3A, red circle) was detected. All GCT-affected ovaries in GCT-uOE were large-sized with a small-sized contralateral ovary. The largest GCT was not measurable due to intraoperative fragmentation but weighed approximately 5 kg (Case 28, Figure 2(B1)). The smallest ovary of GCT-uOE measured 85 × 65 × 55 mm. Gross sections revealed a multicystic appearance in all neoplastic ovaries of GCT-uOE (Figure 2(B1–B3)).

In bOE, microscopical evaluation of removed ovaries ($n = 40$) revealed preantral follicles (primordial, primary, and secondary follicles) in 40%, antral follicles (tertiary and preovulatory follicles; Figure 4(A1–B7)) in 30%, early atretic follicles in 28%, late atretic follicles in 40%, and CL in 28%. We could additionally determine large follicular structures with the size of preovulatory follicles and multiple GC layers but a poorly developed theca cell layer (Figure 5D). This layer was mainly formed by theca cells with a retained fibroblast-type appearance in contrast to preovulatory follicles, which contained polyhedral cells in a well-developed and vascularized theca interna cell layer (Figure 5A). These large follicles were defined as anovulatory-like follicles according to the histomorphological definition of anovulatory follicles [50] but without clinical examination of persistence. These structures

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were present in 15% of all examined ovaries and in 30% of all mares of bOE. All follicles with multiple GC layers were summarized as large follicles and included antral follicles, early atretic follicles, and anovulatory-like follicles. Polyhedral cells with a pale nucleus and foamy cytoplasm could occasionally be seen in the theca interna of preovulatory follicles (Figure 4(B1–B7), asterisks) and early atretic follicles. Fossa cysts with flattened, partially ciliated epithelial cells were present in 25% of all examined ovaries and in total in 35% of all mares in bOE. Small areas of GC nests (Figure 3(B1,B2)) or spindle-shaped GCs in the GC layer (Figure 3(C1–C6)) were detected in four ovaries (10%) of three mares in bOE (15%). Moreover, polyhedral cells with foamy cytoplasm (similar to Leydig-like cells in GCTs) could be found in the theca interna cell layer (Figure 3(C1–C6)) or within GC nests (Figure 3(B1,B2), asterisks) in those cases. Such histological abnormalities were defined as early neoplastic changes (ENCs) in this study and regarded as the onset of ovarian degeneration. Early neoplastic changes occurred unilaterally (Case 10, 36), as well as bilaterally (Case 29), and were growing from large follicular structures and mainly from anovulatory-like follicles (75% of ENC).

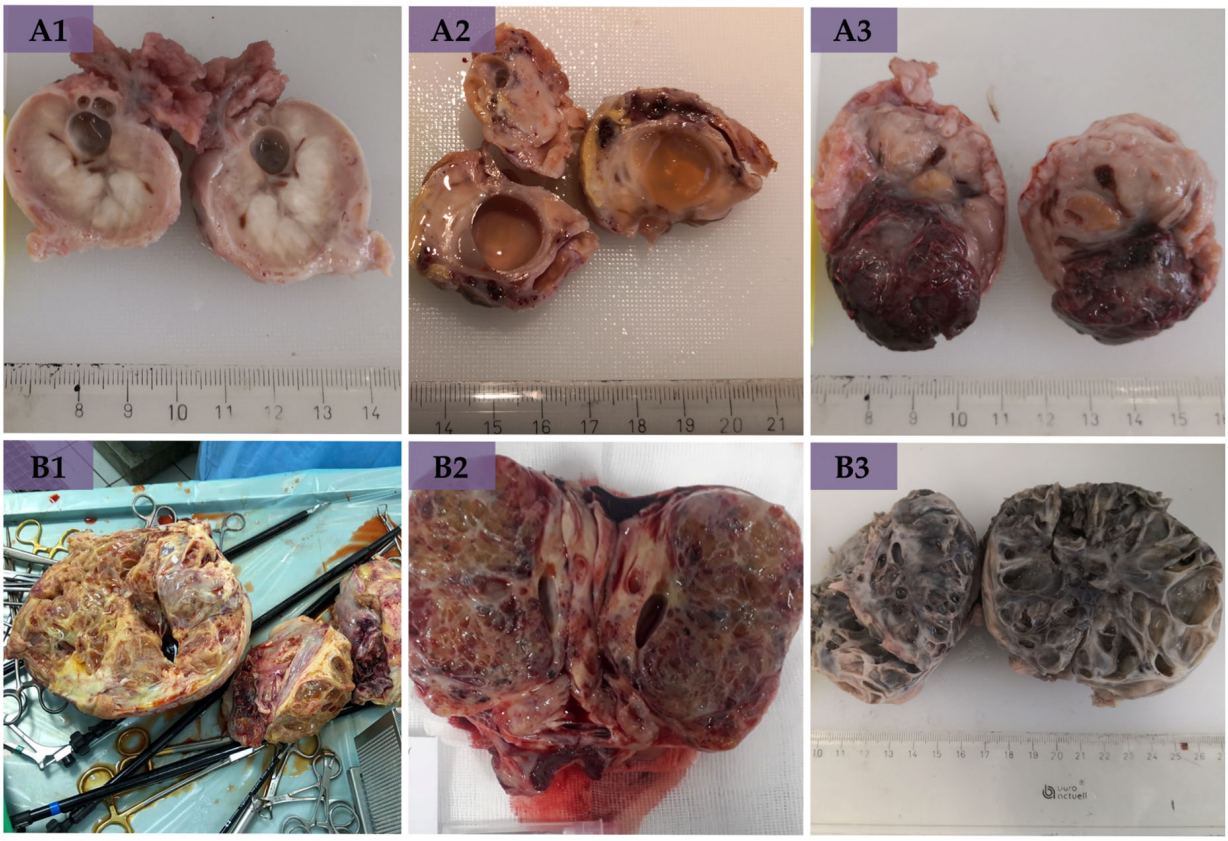

Figure 2. Ovarian gross sections of bilaterally ovariectomized mares (bOE, (**A**)) and mares with granulosa cell tumors (GCT-uOE, (**B**)). (**A1**,**A2**) Ovary after fixation with preantral and tertiary follicles. (**A3**) Ovary with a corpus luteum (CL). (**B1**) Fragmented 5 kg granulosa cell tumor (GCT) immediately after removal via laparotomy. (**B2**,**B3**) GCT with typical multicystic appearance.

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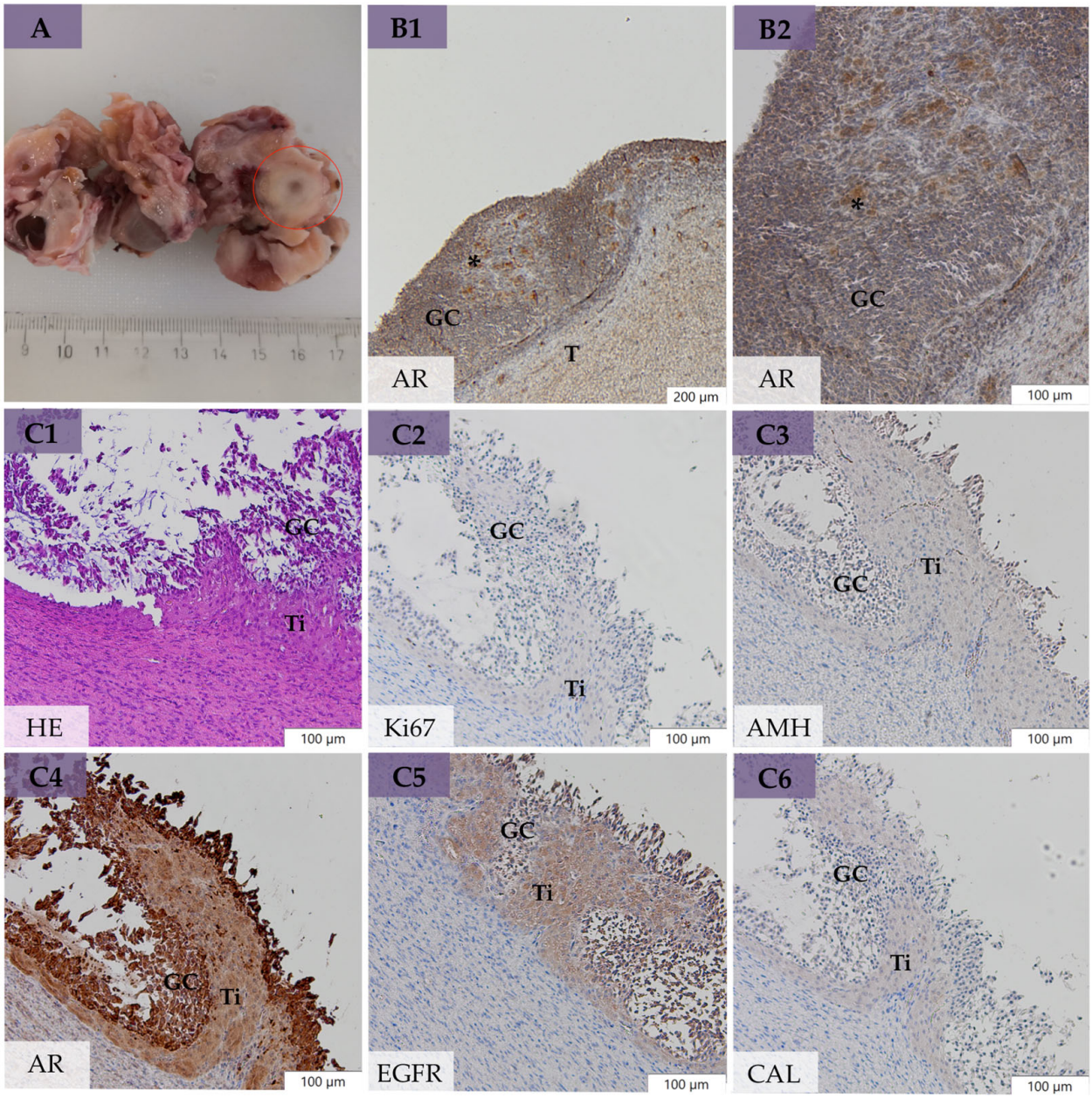

Figure 3. Early neoplastic changes (ENCs) in different ovaries of bilaterally ovariectomized mares (bOE, Case 29 and Case 10): (**A**) Gross section of a clinically unremarkable ovary with a pale area (red circle) near the ovulation fossa suspicious for ENCs (Case 29). (**B**) Immunohistochemical evaluation of this area in aromatase (AR) staining in different magnifications (**B1**,**B2**); note the granulosa cell (GC) nests with AR-positive cells resembling Leydig-like cells (LLCs) in between (asterisk), defined as ENC; Bars 200 µm, 100 µm. (**C**) Pathohistological findings in a clinically unremarkable ovary with detected ENCs (Case 10): spindle-shaped, neoplastic GCs with polyhedral, foamy cells in the theca interna cell layer resembling LLC. Figures are presented in hematoxylin and eosin (HE, (**C1**)) and different immunohistochemical staining with Ki-67 (Ki67, (**C2**)), anti-Müllerian hormone (AMH, (**C3**)), AR (**C4**), epidermal growth factor receptor (EGFR, (**C5**)), and calretinin (CAL, (**C6**)); bars 100 µm; GCs = granulosa cells, T = theca cell layer, Ti = theca interna cells.

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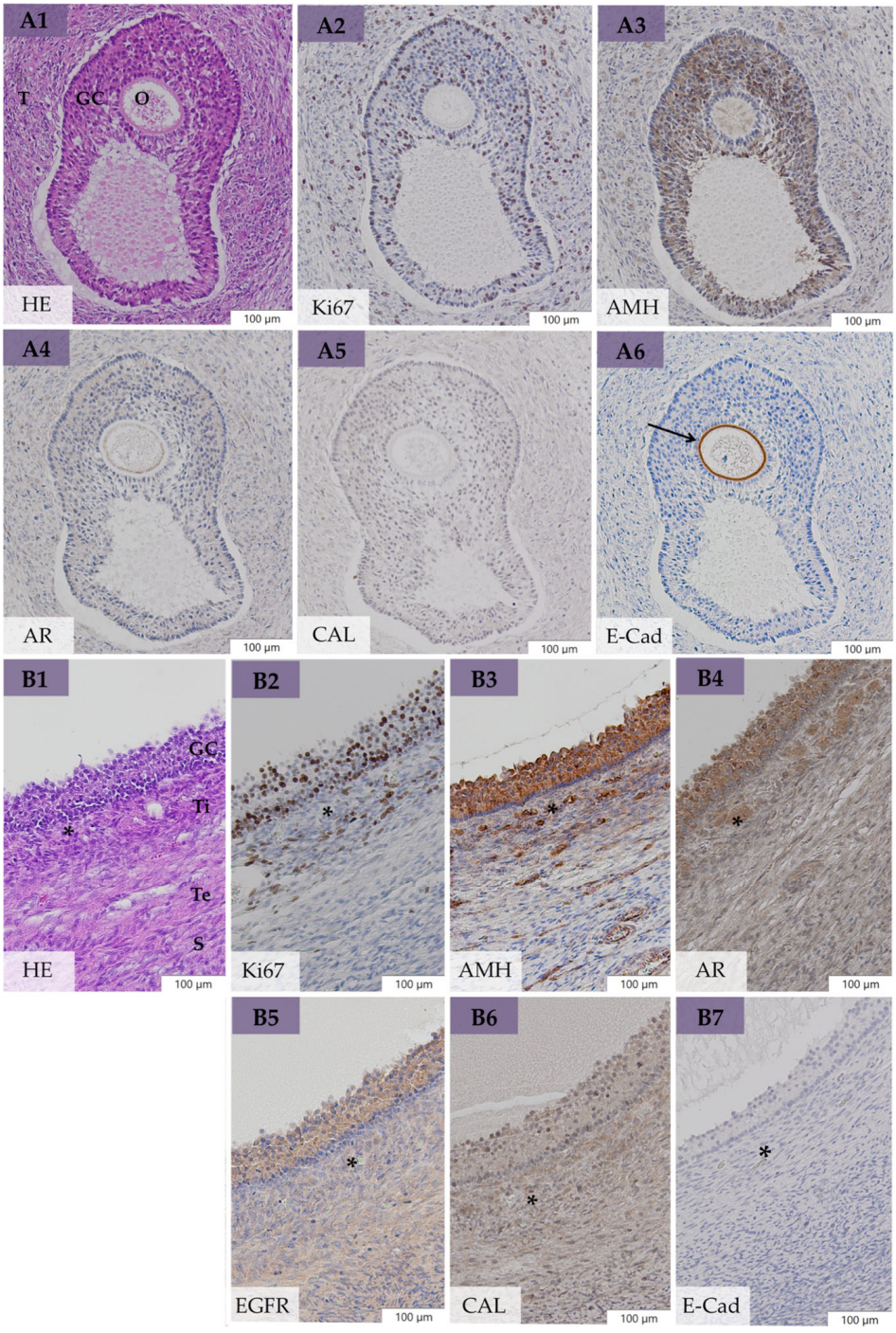

Figure 4. Antral follicles in ovaries of bilaterally ovariectomized mares (bOE) in different immunohistochemical staining with hematoxylin (HE (**A1**,**B1**)), Ki-67 (Ki67 (**A2**,**B2**)), anti-Müllerian hormone (AMH (**A3**,**B3**)), aromatase (AR (**A4**,**B4**)), epidermal growth factor receptor (EGFR (**B5**)), calretinin (CAL (**A5**,**B6**)), and epithelial cadherin (E-Cad (**A6**,**B7**)): (**A**) Tertiary follicle with oocyte, granulosa cell (GC) layer, and theca cell layer (T); note the positive E-Cad staining of the zona pellucida (**A6**, arrow). (**B**) Preovulatory follicle with GC, theca interna (Ti), and theca externa cell layer (Te) and stroma (S); note the polyhedral cells with foamy cytoplasm in the theca interna cell layer (asterisks); bars 100 µm; GCs = granulosa cells, O = oocyte, S = stroma.

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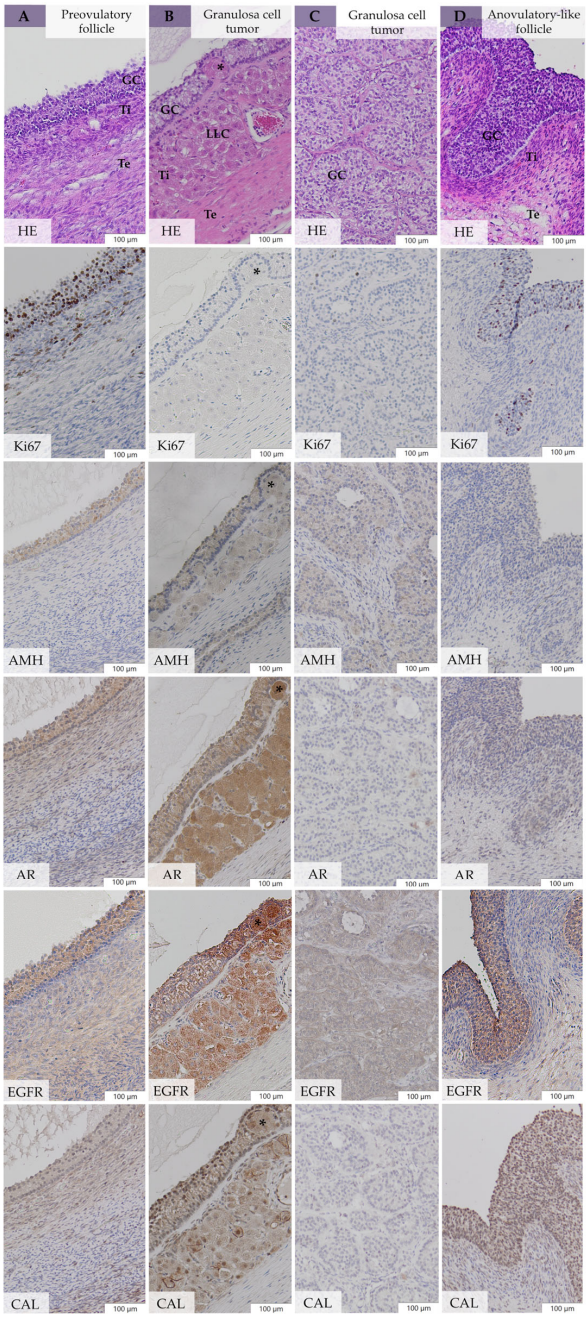

Figure 5. Large follicular structures in ovaries of bilaterally ovariectomized mares (bOE; (**A**,**D**)) compared with cyst-like structures in two different granulosa cell tumors (GCTs) of unilaterally ovariectomized mares (GCT-uOE; (**B**,**C**)) in hematoxylin and eosin (HE) and different immunohistochemical staining with Ki-67 (Ki67), anti-Müllerian hormone (AMH), aromatase (AR), epidermal growth factor receptor (EGFR), and calretinin (CAL). (**A**) Preovulatory follicle with granulosa cell (GC) layer, theca interna, and theca externa cell layer. (**B**) GCT with a multiple GC layer and numerous Leydig-like cells in the theca interna cell layer, some show an invasive growing character (asterisk). (**C**) GCT with microfollicular pattern of the GC layer. (**D**) Anovulatory-like follicle with a multiple GC layer, poorly developed theca interna, and theca externa cell layer; bars 100 µm; GCs = granulosa cells, Ti = theca interna cell layer, Te = theca externa cell layer, LLCs = Leydig-like cells.

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All tissue samples of GCT-uOE demonstrated a cyst-like appearance with variable structural compositions of macrofollicular, microfollicular, insular, trabecular, and diffuse types of patterns. Slim, prismatic GCs with Sertoli-cell morphology, so-called Sertoli-like cells (SLCs), and numerous polyhedral LLCs with foamy cytoplasm in the theca cell layer were detected in 70% of GCT-uOE. Leydig-like cells were invasive growing by penetrating the basement membrane into the GC layer in one ovary of GCT-uOE (Case 24, Figure 5B, asterisk).

3.5. Immunohistochemical Evaluation

The PI grade of Ki67 and intensity of immunohistochemical expression of AMH, AR, EGFR, and CAL in different cell populations of ovarian structures in bOE and GCT-uOE are summarized in Table S3 (Supplementary Data). Evaluated cell populations included GCs, theca cells, and lutein cells in bOE and GC, LLC, SLC, and theca cells in GCT-uOE. In brief, all tested markers, with the exception of E-Cad, were expressed in the ovaries of bOE and GCT-uOE. The proliferation marker Ki67 was mainly expressed in nuclei, whereas AMH, AR, EGFR, and CAL were generally allocated to nuclei and/or cytoplasm. Epithelial cadherin showed delicate cell membrane-associated staining and was restricted to epithelial cells of fossa cysts and the zona pellucida of oocytes (Figure 4(A6), arrow). Epithelial cadherin was not expressed in other structures of bOE or neoplastic tissue of GCT-uOE and was consequently not further evaluated.

To differentiate between clinically unremarkable ovaries of bOE and neoplastic ovaries of GCT-uOE, the GCs of large follicular structures of bOE, including antral follicles, early atretic follicles, and anovulatory-like follicles, were statistically compared with neoplastic GCs of cyst-like structures of GCT-uOE. Therefore, only evaluation of those cells was included for further discussion. A complete immunohistochemical analysis of all detected structures in bOE and GCT-uOE is provided in Table S3.

3.5.1. Expression of Ki67, AMH, AR, EGFR, and CAL in Large Follicular Structures of bOE

Granulosa cells of tertiary follicles (Figure 4(A1–A6)) showed a varying Ki67 PI (grade 1–3), moderate AR and CAL expression, and intense expression of AMH and EGFR. Granulosa cells of preovulatory follicles (Figure 4(B1–B7)) presented a high Ki67 PI (grade 2–3), moderate CAL, and intense expression of AMH, AR, and EGFR. Polyhedral cells of the well-developed theca interna layer of preovulatory follicles (Figure 4(B1–B7), asterisks) revealed high AR and CAL and mild AMH and EGFR expression. Granulosa cells of early atretic follicles showed a high Ki67 PI (grade 2–3) and high expression of AMH, AR, EGFR, and CAL, similar to preovulatory follicles. Granulosa cells of anovulatory-like follicles (Figure 5D) presented a varying Ki67 PI, and AMH, AR, EGFR, and CAL were less expressed compared with GC of preovulatory follicles. Early neoplastic changes showed a low Ki67 PI, mild AMH, moderate CAL, high AR, and high EGFR expression in spindle-shaped GCs, as well as in LLCs in the theca interna cell layer (Figure 3(C1–C6)) or in between GC nests (Figure 3(B1,B2)), with no difference to adherent healthy GC tissue.

3.5.2. Expression of Ki67, AMH, AR, EGFR, and CAL in Neoplastic Cells of GCT-uOE

Neoplastic GCs of GCT-uOE were characterized by high expression of EGFR and, in general, moderate AR, AMH, and CAL coexpression with partly heterogeneous patterns within and between the tumors. The proliferation marker Ki67 was varyingly expressed in neoplastic GCs with a high PI in 30% and a low PI in the remaining 70% of GCT-uOE (Figure 5B,C, Ki67). Leydig-like cells (Figure 5B) demonstrated high coexpression of AR, EGFR, and CAL and moderate AMH expression. Sertoli-like cells presented an expression pattern equal to neoplastic GC.

3.5.3. Comparative Immunohistochemistry between bOE and GCT-uOE

Comparative immunohistochemistry between GCs of large follicular structures in bOE (n = 23, Figure 5A,D) and neoplastic GCs of cyst-like structures in GCT-uOE (n = 10,

Figure 5B,C) revealed a statistically significant higher Ki67 PI ($p = 0.02$) in bOE (median 51.0%, IQR 52.0) compared with GCT-uOE (median 4.5%, IQR 27.3%). Immunohistochemical analysis of AMH, AR, EGFR, and CAL revealed no significant difference between the two groups ($p > 0.05$).

3.5.4. Correlation of Serum AMH and Immunohistochemical AMH Expression

In bOE, a significant correlation between serum AMH concentrations and AMH expression of follicular structures detected by immunohistochemistry was evaluated ($p = 0.02$). No correlation was found between serum AMH concentrations and immunohistochemical AMH expression in neoplastic GCs of GCT-uOE ($p = 0.25$).

4. Discussion

Bilateral ovariectomy in mares with clinically unremarkable ovaries but behavioral problems is known to result in high improvement of behavior from the owner's perspective [5,15–18]. However, an underlying pathohistological reason for this phenomenon has not been identified yet. To our knowledge, this is the first pathohistological study characterizing bilaterally removed, clinically unremarkable ovaries of mares with behavioral problems in comparison with pathohistologically confirmed GCTs by means of histomorphology and immunohistochemistry. We further involved a complete clinical data set to confirm the positive outcome of bilateral ovariectomy in 85% of mares in our study, which is in accordance with recent studies [5,15–18]. We, therefore, included a detailed clinical history, clinical examination, and analysis of serum AMH and testosterone concentrations as the basis for the pathohistological study. Evaluation of the complete clinical data resulted in a wide variety of each examined mare in bOE and GCT-uOE and is therefore summarized in Tables S1 and S2 of Supplementary Data.

Conservative treatment with Altrenogest and/or GnRH vaccination before surgery resulted in a good response in 91% (10/11) of treated mares of bOE. Therefore, ovaries are suggested as the source of behavioral problems in these mares. Commonly, both treatment options are used in mares with estrous-related behavioral problems but are restricted in Germany as a long-term solution [11]. Moreover, Altrenogest was reported to be less effective compared with BO regarding behavioral improvement, but a correlation with a positive outcome of BO could be seen [15]. In contrast, Collar et al. (2012) [14] found no efficient prediction for the behavioral outcome of BO by means of Altrenogest administration before surgery. In our study, 73% (8/11) of the conservatively treated mares showed a positive outcome of BO, but this included mares that were treated with Altrenogest, GnRH vaccination, or both, and the exact date and duration of administration remained unknown. GnRH vaccination has a reported significant impact on ovarian activity and size [51,52] and was even reported to decrease serum testosterone and ovarian size in three GCT-affected mares with additional resolution of behavioral problems [53]. Therefore, GnRH vaccination in six mares of bOE in our study might have suppressed presumptive present abnormalities, as histomorphological evaluation revealed no pathological findings in those mares.

The time from onset of behavioral problems to referral was less than 12 months in 63% of bOE and in 67% of GCT-uOE and was therefore comparable to outcomes of Straticò et al. (2023) [18], who found no difference in time of presentation and severity of behavioral problems of mares with normal ovaries and mares with GCTs. These results, moreover, emphasize that behavioral problems occurred recently and suggest the development of a pathological event in those mares. In the case of long-term behavioral problems in 37% of bOE, the possibility of learned behavior or even a pre-existing problem [4], especially in older mares, should be considered as well. However, we also determined long-lasting behavioral problems in young mares (Case 10, 4 years old) with detected ENCs as pathological events, which was uncommon in our results.

As already detected by others [17,18], the mares in our study presented in general more than one specific behavioral problem, with moodiness and unwillingness to be ridden

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most commonly occurring in bOE and stallion-like behavior predominantly present in GCT-uOE (Table 2). Stallion-like behavior is characterized by attempts to mount other mares [4,8] and was observed in one case of bOE with pathohistologically determined ENCs (Case 10) and in 60% of GCT-uOE. This pattern is regarded as a testosterone-driven-sexual behavior and, together with an aggressive manner, is reported as pathognomonic for GCTs [8,54]. However, serum testosterone concentrations were evaluated only in single mares and therefore associated with stallion-like behavior in only one mare of GCT-uOE (Case 32). Aggressive behavior was clearly separated between aggression towards people and towards other horses in our study (Table 2, Table S1), but neither was observed in any case of GCT-uOE, including cases with increased serum testosterone concentrations. These findings therefore support the outcome of Huggins et al. (2023) [41], who could not find a significant trend between aggressive behavior and increased serum hormone concentrations.

Serum AMH concentrations of clinically unremarkable mares in bOE varied from 0.4 to 43.5 pmol/L. With a median of 11.8 pmol/L in bOE, serum AMH concentrations in our study were higher compared with reported concentrations of 6.9 pmol/L (mean) in cyclic mares [34] and 2.14 pmol/L (median) in reproductively normal mares [35]. Serum AMH is a highly sensitive marker for GCTs and independent of daily fluctuations, cyclic stage, or pregnancy [34,35,55–57]. However, the inclusion of mares in all cyclic stages and with constant or prolonged estrus (15%) or anovulatory-like follicles (30%) and ultrasonographically nondetectable, small nests of neoplastic GCs (ENCs, 15%) in one or both ovaries of bOE might explain the deviating median serum AMH in our study compared with others [34,35]. Early neoplastic changes might contribute to variable serum AMH concentrations in bOE, as two mares with pathohistologically detected ENCs presented increased serum AMH concentrations (Case 29, 36), whereas the third mare (Case 10) showed the lowest measured AMH concentration of 0.4 pmol/L in bOE. Wide fluctuations of periodically measured serum AMH within specific cases have been reported [58], and serum AMH might not be reliable in the case of early tumor growth [17]. Devick et al. (2020) [17] reported an elevation of AMH above 27.0 pmol/L in only 44% and above 57.0 pmol/L in only 11% of pathohistologically confirmed GCTs and concluded there was a low sensitivity of AMH in early detected GCTs, which is in accordance with our findings. Therefore, repeated measurement of serum AMH is recommended in ambiguous cases [59]. Furthermore, Devick et al. (2020) reported perceived false positive serum AMH measurements, as investigated mares with pathohistologically normal ovaries had increased concentrations above 27.0 pmol/L in 53% and above 57.0 pmol/L in 24% in their study. Comparably, 15% of bOE in our study were in the suspicious range above 14.3 pmol/L and 15% above the cut-off value of 28.6 pmol/L. However, the suspicious range and the cut-off value used by our laboratory were lower compared with others [17,41]. Ball et al. (2013) [35] determined concentrations of over 28.6 pmol/L, indicating GCT presence, which was in accordance with our cut-off value, but the authors did not include a suspicious range. Five of the six mares with increased serum AMH in bOE showed behavioral abnormalities with pathohistologically detected ENCs (n = 2), anovulatory-like follicles (n = 4), and fossa cysts (n = 1). The remaining mare with increased serum AMH (Case 16), however, showed no behavioral problems and no abnormalities on pathohistological evaluation. Regarding the cut-off value of 28.6 pmol/L [35], an increased AMH of 43.5 pmol/L in Case 16 would therefore prove GCT presence. Consequently, based on our results, the reference limits of 14.3 pmol/L and 28.6 pmol/L used in this study were adjusted by the laboratory, with concentrations above 71.4 pmol/L clearly indicating the existence of GCTs and less than 30.7 pmol/L suggesting healthy ovaries. These new reference values are comparable to values used by Huggins et al. (2023) [41]. As all mares in GCT-uOE showed significantly higher serum AMH concentrations compared with bOE, with a range of 127.0–150.0 pmol/L (Figure 1), we assume neoplastic GCs as the source of increased serum AMH in GCT-uOE, in accordance with others [31,34]. However, a statistical correlation between high serum AMH concentrations and immunohistochemical AMH expression could not be found in GCT-uOE. Increased

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serum AMH in mares with GCTs might therefore be caused by the abnormally high number of proliferative cells forming tumors of advanced size, similar to inhibin [22]. This fact could further explain the low serum AMH despite the pathohistological presence of ENCs in Case 10 of bOE, as the total number of neoplastic GCs was still low.

Serum testosterone measurements also resulted in significantly higher concentrations in GCT-uOE compared with bOE, although partly tested (bOE 55%, GCT-uOE 70%). The only case in bOE with increased serum testosterone revealed additional high serum AMH (Case 29), which could be explained by pathohistologically detected ENCs with LLCs present on both ovaries (Figure 3(B1,B2)). Elevation of serum testosterone is commonly associated with stallion-like behavior and/or the presence of a substantial theca cell component and LLCs in GCTs [8,22,24,25,56,60,61]. This was observed in all mares with increased testosterone concentrations of GCT-uOE (n = 2), as well as in Case 29 of bOE. Although the number of LLCs between neoplastic GC nests was still low (Figure 3(B1,B2)), we assume an association between the presence of LLCs and elevated serum testosterone in Case 29. Increased serum AMH and testosterone concentrations were suspicious for ovarian dysfunction or GCT existence in Case 29, but clinical examination revealed reproductive functionality in this mare due to CL presence on one ovary. This case therefore represents a diagnostic challenge, as serum AMH and testosterone concentrations might be unpredictable or even vary over time [58]. A transvaginal ovarian biopsy sample [62] could be helpful in determining which ovary is affected in such unclear cases or in mares where breeding is still desired. However, this invasive method might result in false negative outcomes in the case of widely distributed ENCs. Bilateral ovariectomy was successful in Case 29 regarding behavioral improvement and, moreover, efficient in the removal of pathological ovaries with bilaterally occurring ENCs, which were clinically unremarkable. Conclusively, routine diagnosis by means of clinical examination and serum hormone analysis might be unclear in some cases [58], especially in early tumor growth [17].

Due to the high success rate of bOE in our study (85%) and others [5,14–18], ovaries are suspected as the main cause of behavioral problems, although a detailed diagnostic workup to rule out other, abnormal behavior-causing issues [4,6] was mostly lacking in our study and others [5,14–18]. Therefore, other clinical causes for behavioral problems remained unknown before surgery, and a direct relation to estrus could not be determined by clinical history in our study. However, the positive response to preoperative conservative treatment was indicative of an association with estrus, which is moreover supported by the highly successful outcome of BO. Accordingly, Melgaard et al. (2020) [5] and Kamm and Hendrickson (2007) [16] found an improvement in behavior after BO not obviously related to the estrous cycle. Comparable to our results, Collar et al. (2021) reported a resolution of the initially presenting problems in 90% of 41 elective cases, whereas Melgaard et al. (2020) revealed an improvement of behavior of 40% and in rideability of 80% in 10 mares with pathohistologically normal ovaries. Furthermore, others reported similar high success rates of BO in mares with behavioral problems (83% [16], 89% [17]) but also included cases with other medical problems or pathohistologically diagnosed GCTs. Our overall success rate of ovariectomy in bOE and GCT-uOE was in a range of 85–95%, which is even higher but difficult to compare with others [16,17], as mares with pathohistologically diagnosed GCTs were only unilaterally ovariectomized in our study. All mares of GCT-uOE showed normal behavior after removal of the affected ovary, resulting in complete owner satisfaction and confirming the proven common cause for behavioral problems by endocrinologically active GCTs [8,20,60]. Owners were not satisfied with BO in only 15% (3/20) of bOE. This included two cases with recurrent colic signs (Case 39, 49), which might have been misattributed to the ovaries [4] and represented other, not-ovarian-related reasons for behavioral problems in those mares [6]. The third mare (Case 40) showed recurring behavioral problems simultaneously with persistent estrous signs. Persistent estrous signs after BO are described in 20–35% of cases [13–15]. In our study, the remaining estrous signs were with an incidence of 10% low and reported as mild by the owners. However, owners should be aware that BO might not be successful in all mares, especially if abnormal clinical

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findings and increased hormonal concentrations are absent [58]. Complications after BO were limited to 16.7% of all cases in our study with fevers (up to 39.7 °C) and seroma formation, as well as mild-to-moderate emphysema, suggesting this surgical procedure as a safe method [12].

Macroscopical evaluation of the removed ovaries in bOE was in accordance with rectal palpation and ultrasonographical examination regarding structure and size. However, Case 29 of bOE presented a suspicious pale area near the ovulation fossa on gross examination (Figure 3A, red circle), which was nondetectable on ultrasonographical examination. This area revealed nest-forming GCs with polyhedral, foamy cells resembling LLCs (Figure 3(B1,B2)) on pathohistological examination and were determined as ENCs. Such ENCs were histomorphologically also present as spindle-shaped GCs with LLCs in the theca interna cell layer of bOE (Case 10, Figure 3(C1–C6)). Early neoplastic changes are reported as early-stage GCTs by others [17], but a detailed histomorphological description is lacking. In bOE, ENCs were detected in functional ovaries with additional CL on the same (Case 29) or the contralateral ovary (Case 10) or with large follicular structures present (Case 36). Mares with developing GCTs are reported to continue normal estrous cyclicity or even to maintain pregnancy with the presence of a functional CL [63–66]. Consequently, a transitional period between the clinical manifestation of neoplastic GCs initially and loss of normal estrous cycle is assumed and explained by a lack of inhibitory effect of inhibin on the contralateral ovary [35,67]. Mares in this transitional period might already present abnormal behavior with no abnormalities on clinical examination and variable serum AMH and testosterone concentrations (Case 10, 29). Therefore, clinically nondetectable ENCs could be one explanation for the high success rate of BO by causing behavioral abnormalities with no clear detection by routine diagnosis. Anovulatory-like follicles were determined in 30% of the mares in bOE according to their histomorphological similarity to anovulatory follicles (Figure 5D, [50]). Anovulatory follicles are classified as persistent anovulatory follicles with limited clinical significance or luteinized hemorrhagic anovulatory follicles with an assumed responsibility for infertility in mares [68–71]. Clinical evaluation of persistence of anovulatory-like follicles was lacking in our study, but their histomorphological presence in 30% of the mares in bOE was regarded as pathological due to their high incidence compared with a reported 8.2% in clinically confirmed, persistent anovulatory follicles [68]. As 75% of ENCs were growing from anovulatory-like follicles, we further suggest these structures as possible precursor stages for ENCs and advanced GCTs [58,65]. Two mares with detected anovulatory-like follicles presented colic signs (33%), which were therefore considered "painful ovary syndrome" [3,10,19], whereas others showed mixed patterns of behavioral problems with the conclusion of no direct association to a special behavioral problem. However, anovulatory-like follicles might be a further explanation for successful BO, as 87% of the mares in bOE with those defined structures showed behavioral improvement after BO (Table S2). Histomorphological analysis revealed no abnormalities in 65% of the mares in bOE (13/20). Fossa cysts were frequently detected in bOE (35%) but not regarded as pathological due to their common occurrence in equine ovaries [47,70]. Those nonfollicular cysts are unlikely to cause behavioral problems but might trigger the development of anovulatory hemorrhagic follicles [70,71].

The typical macroscopical and histomorphological presence of GCTs in all mares of GCT-uOE with large size, ultrasonographically multicystic, honeycomb-like appearance (Figure 2(B1–B3)), and histological patterns were comparable to the results of others [25,54,60]. A similar presence of LLCs and SLCs in GCT-uOE at 70% was also found in other studies [22,25,54,56,60]. Leydig-like cells were located between neoplastic GCs or in the theca cell layer (Figure 5B), and some of them presented an invasive growing character (Case 24, Figure 5B, asterisks), which was not described by others before. Early neoplastic changes in bOE presented LLCs either between GC nests (Figure 3(B1,B2)) or in the theca cell layer (Figure 3(C1–C6)) and were hardly distinguishable from polyhedral cells in the theca interna of preovulatory follicles (Figure 4(B1–B7), asterisks) by means of immunohistochemical analysis (Table S3). In general, ENCs revealed similar immuno-

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histochemical patterns to adjacent healthy ovarian tissue of bOE and neoplastic GCs of GCT-uOE and could only be determined by their histomorphological appearance.

Immunohistochemical comparison between ovaries of bOE and GCT-uOE revealed no evident difference in the expression of Ki67, AMH, AR, EGFR, CAL, and E-Cad. Immunohistochemical expression patterns of GCs of large follicular structures in bOE resembled those of neoplastic, cyst-like structures of GCT-uOE (Figure 5). This was in accordance with others, where further markers like inhibin were tested [22,25,45]. In contrast, Dolin et al. (2023) [21] reported expression patterns of AMH, AR, CAL, and E-Cad in neoplastic tissue that differed from normal ovarian tissue but were evaluated in only a single GCT. We evaluated 40 clinically unremarkable ovaries and compared them with 10 pathohistologically confirmed GCTs but revealed neither an immunohistologically visible (Figure 5) nor statistical difference between the two groups, with the exception of Ki67. The significantly higher Ki67 PI in large follicles of bOE (median 51.0%) compared with cyst-like structures in GCT-uOE (median 4.5%) emphasizes the high proliferation activity in these large, non-neoplastic follicular structures. Granulosa cell tumors are regarded as predominantly benign and well differentiated [7,22,25]. Therefore, the mild expression of Ki67 in GCT-uOE (Figure 5B,C, Ki67) indicates a low proliferation rate [21,48]. Immunolabeling of AMH in both groups was similar to other reports [21,31]. The high AMH expression in GCs of preantral and early atretic follicles in bOE (Table S3) could explain the positive correlation ($p = 0.02$) between AMH expression detected by immunohistochemistry and serum AMH in bOE. Aromatase was highly expressed in LLCs (Figure 5B, AR) but showed a heterogeneous pattern in neoplastic GCs of GCT-uOE (Figure 5B,C, AR), according to Dolin et al. (2023) [21]. A previously reported high CAL expression in neoplastic GCs and LLCs [21] was also present in GCT-uOE, with additional high expression of EGFR, which was not evaluated before. The adhesion molecule E-Cad was restrictively expressed in the zona pellucida of oocytes (Figure 4A6, arrow) and in epithelial cells of fossa cysts of bOE and therefore indicative of those structures. However, we doubt the reported diagnostic value of E-Cad for GCT detection [21], as this marker was not present in any GCTs of GCT-uOE. According to recent studies regarding the prognosis for human GCTs, immunohistochemical markers like CD56, GATA-4, and FOXL2 were associated with reduced prognosis [40]. These markers might also be useful in veterinary medicine but have to be validated first.

The main limitations of this study include a low caseload of 20 mares and a total of 40 examined ovaries in bOE, but these numbers are comparable to similar studies [15–18]. A control group of healthy mares without behavioral problems and normal serum hormone concentrations, as well as physiological ovaries, was not included. The main objective of our study was to compare clinically unremarkable ovaries of mares with behavioral problems with GCTs. Furthermore, we concentrated on serum AMH and testosterone concentrations, as these hormones, together with inhibin, are reported as main indicators for behavioral changes [41]. However, testosterone concentrations were evaluated only in some mares. A complete hormonal panel, including progesterone and estradiol with repeatable measurements throughout the cycle, would help to gain more information regarding the mare's reproductive health. In the pathohistological evaluation, large follicular structures were determined in 58% of the examined ovaries and resulted in a smaller caseload to compare immunohistochemical expression patterns of bOE with GCT-uOE. As the clinical study was conducted independently of the pathohistological study, 24% of the ovaries ($n = 8$ in bOE, $n = 3$ in GCT-uOE) were buffered in formaldehyde up to 18 months before histological preparation was started and therefore resulted in a lack of detectable Ki67 expression. The assessment of behavioral problems by means of detailed clinical history and evaluation of behavioral improvement after 6 to 12 months by a retrospective telephone survey are commonly used for evaluating the owner's opinion [5,14,16–18] but might reflect only a subjective opinion of the owner. This could be reinforced by the fact that scoring of behavioral problems before and after surgery [5,18] was missing in our study. The lack of information on GnRH vaccination timepoints further complicates the interpretation of clinical examination and pathohistological findings in mares conservatively treated before

surgery. Moreover, information regarding the duration of ownership or assessment of other body systems [6] before referral was not available. Although not the aim of this study, this additional information could be helpful in determining non-ovarian-related issues and explaining why BO might fail in some mares (15% of bOE).

Comprehensive studies with a focus on behavioral problems regarding endocrine profiles or pathohistological evaluation revealed no significant association between specific behavior and elevated serum hormones [41] or absent luteal tissue [17]. Moreover, behavioral problems were associated with pathohistologically detected GCTs in 45% of examined mares [18]. However, the focus of these studies was set on the diagnosis of GCTs without a histomorphological and immunohistochemical evaluation of clinically unremarkable ovaries. Dolin et al. (2023) [21] reported a detailed immunohistochemical analysis in a single GCT and emphasized the need for a complete clinical data set for proper analysis. Our study involved a clinical evaluation of all mares, including the initially presenting problem, conservative treatments, clinical examination and analysis of serum AMH and testosterone concentrations before surgery, surgical outcome, and an owner´s survey of each case, with a following pathohistological evaluation of the removed ovaries. We could determine ENCs and/or anovulatory-like follicles in 35% of bOE as ovarian abnormalities and suggest them as a pathohistological explanation for the evaluated postoperatively high success rate in bOE. The remaining 65% of mares in bOE revealed no pathohistological findings, but 85% of them (11/13) improved after BO. We therefore conclude an individual hormonal influence, which might not be evaluable [41], or a detection failure of pathological ovarian changes by routine diagnosis, as demonstrated in the three cases with ENCs. The detection of microscopically small ENCs by pathohistology could, therefore, be missed in other cases, which might result in an even higher incidence of these ovarian abnormalities in mares with behavioral problems. This fact would strengthen the justification for BO due to medical necessity [11]. However, BO can also be regarded as medically necessary in the case of severe clinical problems like colic symptoms. In the case of aggressive behavior, animal welfare reasons (i.e., housing) justify a BO. In conclusion, further investigations into ENCs, their impact on serum hormone concentrations, and their association with behavioral problems are warranted. Moreover, improvements of routine diagnostic methods are necessary to detect early ovarian abnormalities and further decide which ovary to remove, especially if breeding is not excluded. Diagnostic accuracy might be achieved by means of other diagnostic markers like CD56, GATA-4, or FOXL2, which are indicative of the prognosis of human GCTs [40].

5. Conclusions

Bilateral ovariectomy resulted in a high owner satisfaction in 85% of mares with clinically unremarkable ovaries but behavioral problems and therefore confirmed previous reports. Serum AMH and testosterone were indicative of advanced GCTs but variable in mares of bOE. Immunohistochemical analysis of Ki67, AMH, AR, EGFR, CAL, and E-Cad was not helpful in differentiating between clinically unremarkable and GCT-affected ovaries and needs further investigations. However, pathohistological evaluation of bilaterally removed ovaries revealed clinically nondetectable ENCs in 15% and anovulatory-like follicles in 30% of mares with behavioral problems in bOE, which were regarded as precursor stages of neoplasia. These structures might be a pathohistological explanation for behavioral problems of ovarian origin in mares and a reason why BO may result in behavioral improvement.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/ani14192899/s1. Figure S1. Western blotting of antibodies (aromatase, anti-Müllerian hormone, epidermal growth factor receptor, and epithelial cadherin) to test for horse cross-reactivity. Blocking Reagent: Roche 11921673001. Table S1. Summary of findings in bilaterally ovariectomized mares with behavioral problems (bOE) and in mares with granulosa cell tumors (GCT-uOE). Clinical history with behavioral patterns, duration of behavioral problems, and conservative treatment (Altrenogest/GnRH vaccination). Table S2. Summary of findings in

bilaterally ovariectomized mares with behavioral problems (bOE) and in mares with granulosa cell tumors (GCT-uOE). Clinical examination before surgery with serum hormone concentrations, rectal and ultrasonographical examination, cyclic stage, time of surgery, outcome of surgery, and pathohistological findings in bOE and GCT-uOE. Table S3. Summary of immunohistochemical evaluation in ovaries of bilaterally ovariectomized mares (bOE) and in ovaries of mares with unilaterally removed granulosa cell tumors (GCT-uOE) including Ki-67 (Ki67), anti-Müllerian hormone (AMH), aromatase (AR), epidermal growth factor receptor (EGFR), and calretinin (CAL). Questionnaire SQ1. Owner questionnaire before surgery regarding behavioral patterns, duration of behavioral problems, and conservative treatments. Questionnaire SQ2. Telephone questionnaire 6–12 months after surgery regarding behavioral improvement, reoccurrence of behavioral problems, and presence of estrous signs post surgery.

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Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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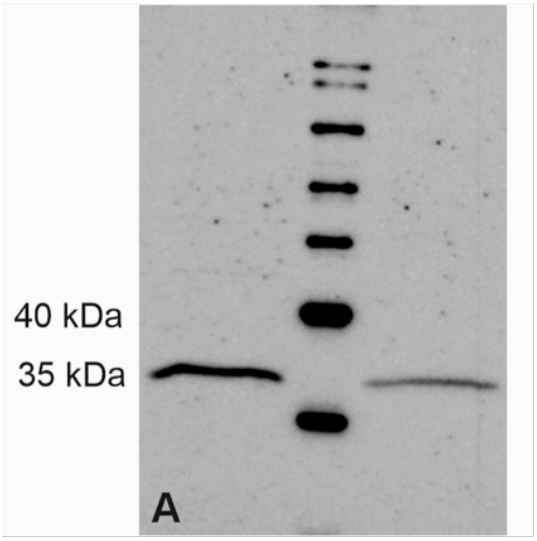
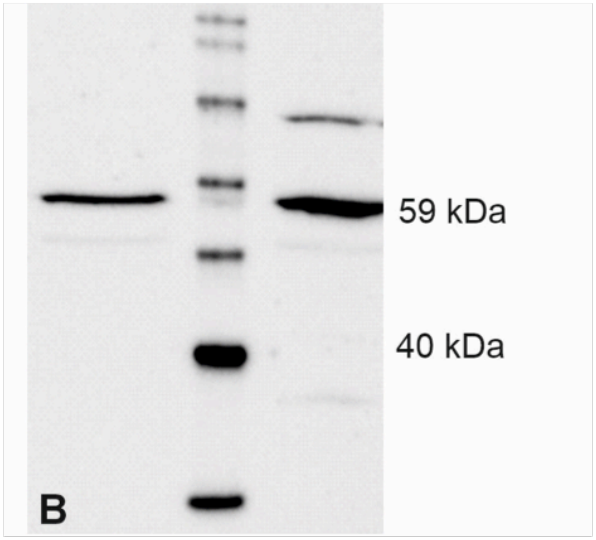
Supplementary data

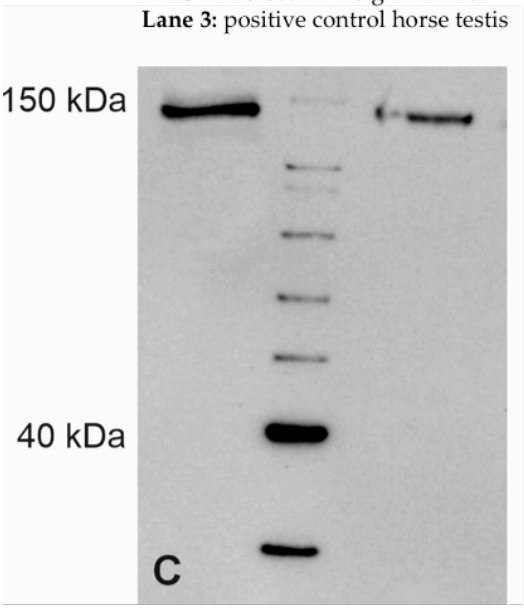
Figure S1. Western blotting of antibodies (Aromatase, anti-Müllerian hormone, epidermal growth factor receptor and epithelial cadherin) to test for horse cross-reactivity. Blocking Reagent: Roche 11921673001.



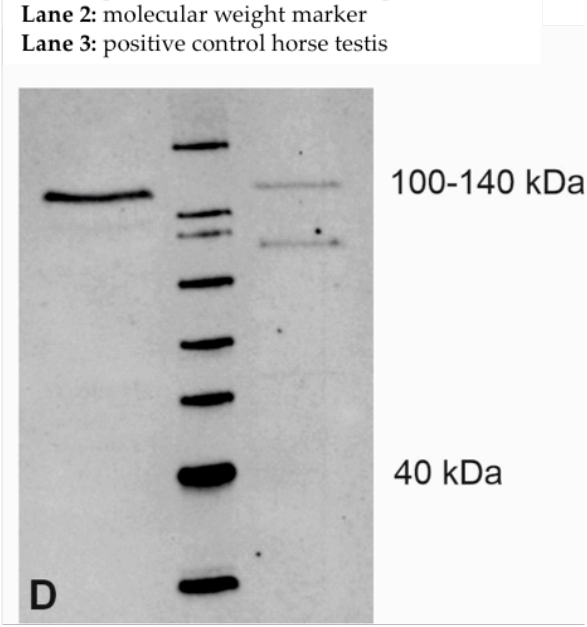
A=aromatase 1: 2000
Gel 10% , Lysis buffer RIPA
Lane 1: positive control human HepG2 cells
Lane 2: molecular weight marker
Lane 3: positive control horse testis



B=anti-Müllerian hormone 1:1000
Gel 10%, Lysis buffer RIPA
Lane 1: positive control human HepG2 cells
Lane 2: molecular weight marker
Lane 3: positive control horse testis



C=epidermal growth factor receptor 1:400
Gel 10% , Lysis buffer RIPA
Lane 1: positive control human A172 cells
Lane 2: molecular weight marker
Lane 3: positive control horse kidney



D=epithelial cadherin 1:1000
Gel 12.5% , Lysis buffer Tris Triton
Lane 1: positive control human HepG2 cells
Lane 2: molecular weight marker
Lane 3: positive control horse kidney

Table S1. Summary of findings in bilaterally ovariectomized mares with behavioral problems (bOE) and in mares with granulosa cell tumors (GCT-uOE). Clinical history with behavioral patterns, duration of behavioral problems, and conservative treatment (Altrenogest/GnRH vaccination).

	Case	Age	Clinical History											
			Behavioral Problem from Owner's Perspective										Duration of behavioral Problems (<12 or > 12 months)	Conservative Treatment	
			Moody	Stressed	Unwilling to be ridden	Aggressive towards People	Aggressive towards other Horses	Stallion-like Behavior	Increased Flank Sensitivity	Colic Symptoms	Prolonged or constant estrous Signs	No estrous Signs		GnRH vaccination **(V)**/ oral Altrenogest **(A)**	**Improvement** yes= + no= -
Bilaterally ovariectomized Mares (**bOE**)	**7**	14	+	+	+	-	-	-	+	+	-	-	<12	**A**	+
	8	12	-	+	+	-	-	-	-	-	-	-	<12	**A+V**	+
	9	16	+	-	+	-	+	-	-	-	-	-	<12	/	/
	10	4	-	-	-	-	-	+	-	-	-	-	>12	/	/
	15	12	+	+	+	-	-	-	+	-	-	-	>12	/	/
	16	10	-	-	-	-	-	-	-	-	-	-	(Coincidental finding)	/	/
	23	9	-	-	-	-	-	-	-	+	+	-	<12	**A**	+
	25	11	+	-	-	+	+	-	+	-	-	-	<12	**V**	+
	29	16	+	-	-	+	+	-	+	-	+	-	<12	/	/
	30	20	-	-	+	+	+	-	-	+	-	-	>12	/	/
	34	11	+	+	-	-	-	-	-	-	-	-	<12	/	/
	35	12	-	-	+	-	-	-	-	+	-	-	>12	**A+V**	+
	36	11	+	-	+	-	-	-	-	-	-	-	<12	/	/
	38	14	-	+	-	-	-	-	-	-	+	-	>12	**A**	+
	39	16	-	-	-	-	-	-	-	+	-	-	>12	**A**	+
	40	8	-	-	+	-	+	-	+	+	-	-	<12	**V**	+
	46	16	+	-	-	-	-	-	-	-	+	-	<12	**V**	+
	47	14	+	-	-	-	+	-	-	+	-	-	>12	**A**	-
	48	15	+	+	+	-	-	-	-	-	-	+	<12	/	/
	49	18	-	-	-	-	-	-	-	+	-	-	<12	**A+V**	+

Table S1. Continued.

	Case	Age	Clinical History											
			Behavioral Problem from Owner's Perspective										Duration of behavioral Problems (<12 or > 12 months)	Conservative Treatment	
			Moody	Stressed	Unwilling to be ridden	Aggressive towards People	Aggressive towards other Horses	Stallion-like Behavior	Increased Flank Sensitivity	Colic Symptoms	Prolonged or constant estrous Signs	No estrous Signs		GnRH vaccination **(V)**/ oral Altrenogest **(A)**	**Improvement** yes= + no= -
Unilaterally ovariectomized Mares (**GCT-uOE**)	**4**	8	-	+	+	-	-	+	-	-	-	+	>12	/	/
	6	7	+	-	+	-	-	-	-	-	-	-	<12	/	/
	12	10	-	-	-	-	-	-	-	-	-	-	(Coincidental Finding)	/	/
	24	18	+	-	-	-	-	+	-	+	-	-	<12	/	/
	27	9	-	-	-	-	-	+	-	-	-	+	<12	/	/
	28	16	-	-	+	-	-	+	-	-	-	+	<12	/	/
	32	16	-	-	-	-	-	+	-	-	+	-	<12	/	/
	33	10	-	-	-	-	-	-	-	-	-	+	>12	/	/
	37	22	+	-	-	-	-	-	-	-	-	-	<12	/	/
	44	16	+	+	-	-	-	+	-	-	-	-	>12	/	/

Table S2. Summary of findings in bilaterally ovariectomized mares with behavioral problems (bOE) and in mares with granulosa cell tumors (GCT-uOE). Clinical examination before surgery with serum hormone concentrations, rectal and ultrasonographical examination, cyclic stage, time of surgery, outcome of surgery, and pathohistological findings in bOE and GCT-uOE.

	Case	Clinical Examination					Time of Surgery (Month)	Outcome of surgery			Pathohistological Evaluation
		Serum Hormone Concentrations		Size[2] (Rectal palpation)	Structures[3] (Sonography)	Cyclic State[4]		Reoccurrence of behavioral Problems	Persistent estrous Signs after Surgery	Owner's Satisfaction[5]	Histomorphological Abnormalities in bOE[6]/ Special Cells in GCT-uOE[7]
		AMH[1] (pmol/L)	Testosterone[1] (nmol/L)								
Bilaterally ovariectomized Mares (**bOE**)	7	9.6	n.m.	Normal	L: mF, R: mF	I	9	-	-	Y	R: Fossa Cyst
	8	8.9	n.m.	Normal	L: LF, R: mF	D	10	-	-	Y	No Abnormalities
	9	11.6	n.m.	Small	L: mF, R: mF	I	11	-	-	Y	L: Fossa Cyst
	10	0.4	n.m.	L: small, R: normal	L: CL, R: mF	D	10	-	-	Y	R: ENCs
	15	5.1	n.m.	Normal	L: LF, R: CL	D	9	-	-	Y	L: AnovF
	16	43.5	n.m.	Normal	L: mF, R: mF	D	9	-	-	Y	No Abnormalities
	23	14.3	n.m.	Normal	L: mF, R: LF	E	3	-	-	Y	R: AnovF
	25	30.3	<0.14	Normal	L: LF, R: mF	E	3	-	-	Y	R: Fossa Cyst
	29	38.6	0.45	Small	L: mF, R: mF	D	7	-	-	Y	L: ENCs + AnovF, R: ENCs
	30	14.3	<0.14	L: small, R: normal	L: CL, R: mF	D	7	-	-	Y	L: Fossa Cyst
	34	15.7	<0.14	L: normal, R: small	L: CL, R: mF	D	10	-	-	Y	L: AnovF
	35	0.8	<0.14	Normal	L: mF, R: mF	I	11	-	-	Y	L+R: Fossa Cyst
	36	23.9	<0.14	Normal	L: LF, R: mF	E	10	-	-	Y	R: ENCs + AnovF
	38	11.9	n.m.	Normal	L: mF, R: mF	E	12	-	-	Y	No Abnormalities
	39	14.5	<0.14	Normal	L: mF, R: mF	I	12	+	-	N	R: AnovF
	40	8.8	<0.14	L: normal, R: small	L: mF, R: mF	I	1	+	+	N	R: Fossa Cyst
	46	1.9	<0.14	Normal	L: LF, R: CL	D	6	-	-	Y	R: Fossa Cyst
	47	4.5	<0.14	Normal	L: CL, R: mF	D	6	-	-	Y	No Abnormalities
	48	13.2	<0.14	Normal	L: CL, R: CL	D	6	-	-	Y	No Abnormalities
	49	5.4.	n.m.	Small	L: mF, R: mF	I	6	+	-	N	No Abnormalities

Table S2. Continued.

	Case	Clinical Examination					Time of Surgery (Month)	Outcome of surgery			Pathohistological Evaluation
		Serum Hormone Concentrations		Size[2] (Rectal palpation)	Structures[3] (Sonography)	Cyclic State[4]		Reoccurrence of behavioral Problems	Persistent estrous Signs after Surgery	Owner's Satisfaction[5]	Histomorphological Abnormalities in bOE[6]/ Special Cells in GCT-uOE[7]
		AMH[1] (pmol/L)	Testosterone[1] (nmol/L)								
Unilaterally ovariectomized Mares (GCT-uOE)	4	142	0.35	L: extra large*, R: small	L: hc, R: inac	I	7	-	-	Y	GCT
	6	127	0.76	L: large, R: small	L: hc, R: inac	I	9	-	-	Y	GCT; LLC, SLC
	12	143	n.m.	L: extra large*, R: small	L: hc, R: inac	I	6	-	-	Y	GCT; SLC
	24	150	n.m.	L: large, R: small	L: hc, R: inac	I	4	-	-	Y	GCT; LLC (*invasive)
	27	123	0.14	L: large, R: very small	L: hc, R: inac	I	6	-	-	Y	GCT; LLC
	28	142	n.m.	L: small, R: extra large*	L: inac, R: hc	I	6	-	-	Y	GCT; LLC, SLC
	32	150	1.25	L: small, R: large	L: inac, R: hc	I	8	-	-	Y	GCT; LLC, SLC
	33	127	<0.14	L: large, R: very small	L: hc, R: inac	I	9	-	-	Y	GCT; LLC, SLC
	37	150	<0.14	R: large, L: not present	R: hc	I	12	-	+	Y	GCT; SLC
	44	150	0.21	L: small, R: large*	L: inac, R: hc	I	6	-	-	Y	GCT; LLC, SLC

[1] Red labeled values= increased serum concentrations; n.m.=not measured; [2] Size: normal sized=50-80 mm length x 20-40 mm width [43], small sized= < 50 mm length x <20 mm width, large sized= >80 mm length x >40 mm width; extra large*= extra large ovary removed by a two-step-procedure; L=left ovary, R=right ovary; [3] Structures: mF=multiple follicles, follicles <30 mm diameter; LF=large follicles, follicles >30 mm diameter; CL=corpus luteum; hc=honeycomb-like structure, inac=inactive ovary; L=left ovary, R=right ovary; [4] Cyclic state: E=Estrus stage, D=Diestrus stage, I=Intermediate estrous stage; [5] Owner satisfaction: Y=Yes, N=No; [6] Histomorphological abnormalities in bOE: ENCs=early neoplastic changes, AnovF=anovulatory-like follicle; L=left ovary, R=right ovary; [7] Special cells in diagnosed granulosa cell tumors (GCT) of GCT-uOE: LLC=Leydig-like cells, *invasive= invasive growing LLC; SLC=Sertoli-like cells;

Table S3. Summary of immunohistochemical evaluation in ovaries of bilaterally ovariectomized mares (bOE) and in ovaries of mares with unilaterally removed granulosa cell tumors (GCT-uOE) including Ki-67 (Ki67), anti-Müllerian hormone (AMH), aromatase (AR), epidermal growth factor receptor (EGFR), and calretinin (CAL).

Immunohistochemical Expression of Cell Populations in different Ovarian Structures of bOE and GCT-uOE						
Structures of bOE	**Cell Population**	*PI grade*[1]	*Intensity of Expression*[2]			
		Ki67	**AMH**	**AR**	**EGFR**	**CAL**
Primordial Follicle[3]	Granulosa Cells	0	-	+	++	+
Primary Follicle[3]		0	+	++	++	+
Secondary Follicle[3]		0	+++	++	++	+
Tertiary Follicle[4]	Granulosa Cells	1-3	+++	++	+++	++
	Theca Cells	1	+	+	+	+
Preovulatory Follicle[4]	Granulosa Cells	2-3	+++	+++	+++	++
	Theca interna Cells	1	+	++	++	++
	Theca externa Cells	0	-	+	++	++
Early atretic Follicle[4]	Granulosa Cells	2-3	+++	++	+++	+++
	Theca interna Cells	1	+	++	++	++
	Theca externa Cells	0	-	+	+	+
Late atretic Follicle	Granulosa Cells	0	++	++	+	++
Anovulatory-like Follicle[4]	Granulosa Cells	0-2	+	+	++	++
Early neoplastic Changes	Granulosa Cells	0	+	+++	+++	++
	Leydig-like Cells	0	+	+++	+++	++
Corpus Luteum	Lutein Cells	0	-	+++	+++	+
Structures of GCT-uOE						
Granulosa Cell Tumor	Granulosa Cells	0-1	++	+/++	+++	++
	Leydig-like Cells	0	+	+++	+++	+++
	Sertoli-like Cells	0	++	+/++	+++	++
	Theca Cells	0	-	-	+	+

[1] The proliferation index (PI) of Ki67 is graded in 0-3: grade 0 for 0-25%, grade 1 for 26-50%, grade 2 for 51-75%, grade 3 for 76-100% stained cells [27]; grade 1-3 means a high PI of >25% stained cells [48]; [2] Intensity of expression: - for negative, + for mild, ++ for moderate and +++ for high expression [31]; [3] Primordial, primary and secondary follicles were summarized as preantral follicles; [4] Tertiary and preovulatory follicles were summarized as antral follicles and together with early atretic and anovulatory-like follicles summarized as large follicles;

Questionnaire Q1: Owner questionnaire before surgery regarding behavioral patterns, duration of behavioral problems, and conservative treatments.

Owner Questionnaire *before* Ovariectomy

TIERKLINIK STARNBERG
PFERDEZENTRUM

Name of the owner:
Name of the mare:
Age:
Breed:
Ovariectomy: O Unilateral / O Bilateral

1. **Behavioral pattern: does your mare present any of the following behavioral problems?**
- o Unwillingness to be ridden
- o Aggressive behavior towards people
- o Aggressive behavior towards other horses
- o Stallion-like behavior (mounting attempts)
- o Increased flank sensitivity
- o Colic symptoms (regularly / repeated)
- o Constant or prolonged estrous signs
- o No estrous signs
- o Further behavioral disorders:
  _____
- o My mare does not present any behavioral problem mentioned above.

2. **Duration of behavioral problems: when did you observe behavioral problems in your mare the first time?**
- o < 12 months ago
- o > 12 months ago

3. **Did your mare receive conservative treatment with oral Regumate Equine® before surgery?**
- o Yes
- o No

3.1. If yes, how was the effect of Regumate Equine® in your mare?
- o Good effect – symptoms resolved or were markedly improved.
- o No effect
- o Negative effect – symptoms worsened.

4. **Did your mare receive GnRH vaccine before surgery?**
- o Yes
- o No

4.1. If yes, how was the effect of Regumate Equine® in your mare?
- o Good effect – symptoms resolved or were markedly improved.
- o No effect
- o Negative effect – symptoms worsened.

Questionnaire 21: Telephone questionnaire 6-12 months after surgery regarding behavioral improvement, reoccurrence of behavioral problems, and presence of estrous signs post surgery.

Telephone Questionnaire *after* Ovariectomy

1. **Did your mare show improvement of behavior after ovariectomy?**
   - o Yes, completely (all behavioral problems resolved after the surgery)
   - o Yes, partly (the following behavioral problems are still present)
     _____
   - o No effect was observed after surgery

2. **Did your mare show reoccurrence of behavioral problems?**
   - o No
   - o Yes, after 1-4 weeks
   - o Yes, after 1-6 months
   - o Yes, after 6-12 months

3. **Does your mare present any estrous signs post surgery?**
   - o Yes, the same as before surgery
   - o Yes, but milder than before surgery
   - o Yes, but more strongly than before surgery
   - o Yes, estrous signs are persistent
   - o No, she does not express any estrous signs

4. **If your mare shows estrous signs post surgery, when did they reoccur?**
   - o Immediately after surgery (<1 month)
   - o After 1-6 months
   - o After 6-12 months

5. **Would you recommend the surgery to others?**
   - o Yes
   - o No

3. Extended Results

This study included a retrospectively evaluated clinical part, which was implemented as basis for pathohistological evaluation of removed ovaries in both groups. The clinical part was already provided in WOLF et al. (2024) and involved a complete data set of clinical history, clinical examination, serum AMH (measured in all mares) and testosterone concentrations (measured in 60% of the mares), and outcome of surgery. The pathohistological part of the study included a histomorphological and immunohistochemical examination of all removed ovaries in both groups (n=50). All tested immunohistochemical markers (Ki67, AMH, AR, EGFR, CAL, E-Cad) were published in WOLF et al. (III. 2. **Table 1**) and described in detail in the literature review (II. 3.3.2.). A detailed documentation of clinical and pathohistological findings of each mare is provided in **Table S1** and **S2** (III. 2. Supplementary data).

Evaluated parameters showed a wide variety in mares with bilaterally removed, clinically unremarkable ovaries (bOE) and special features in mares with unilaterally removed GCTs (GCT-uOE). Therefore, following selected special clinical cases (3.1.) are mentioned in detail due to remarkable outcomes of clinical presentation, serum hormone concentrations, or pathohistological findings. Moreover, a detailed description of special pathohistological findings in ovaries of both groups is provided in the following (3.2.) to emphasize their clinical relevance. As the publication (III. 2.) only involved immunohistochemical results of large follicular structures of bOE and cyst-like structures of GCT-uOE in order to compare those two groups, a further description of all examined structures is provided in the following (3.3.).

3.1. Selected special clinical cases

3.1.1. Bilaterally ovariectomized mares with behavioral problems and pathohistologically unremarkable ovaries

A ten-year-old Haflinger mare (Case 16) was randomly checked for serum AMH, which revealed an increased concentration of 43.5 pmol/L. The owner insisted on surgical removal of both ovaries, although the mare presented no behavioral problems and no abnormalities on clinical examination. Pathohistological evaluation revealed CL tissue on the left and several early and late atretic follicles on the right ovary declaring normal functional ovaries with no pathological abnormalities. Bilateral ovariectomy went well with no complications afterwards. The owner was satisfied with the outcome of BO and reevaluated serum AMH was within normal limits two months post-surgery.

An eleven-year-old Frisian mare (Case 25) showed aggressive behavior towards people and other horses since the beginning of ownership of two years. Serum testosterone was not measured, but serum AMH was increased (30.3 pmol/L). The mare responded to conservative treatment with GnRH-vaccination before surgery with minimal behavioral improvement. Rectal and ultrasonographical

examination revealed no abnormalities on both ovaries. Standing surgical ovarian removal by routine sedation protocol was not successful at first attempt due to extremely aggressive and uncooperative behavior of the mare in the stocks. Therefore, a repeated procedure with adapted sedation protocol (additional sedation with 50 mg/h Romifidinhydrochloride as constant rate infusion, Boehringer Ingelheim Vetmedica GmbH, Ingelheim, Germany) was necessary and went well without any complications. On pathohistological examination, antral follicles on the left and fossa cysts on the right ovary were determined. Aggressive behavior towards people already improved while still hospitalized and completely resolved after a few weeks post-surgery. The owner was highly satisfied with the outcome of BO in his mare.

An eight-year-old Polish Warmblood mare (Case 40) presented aggressive behavior towards other horses and unwillingness to be ridden since the existing ownership of one year. The mare additionally presented recurrent mild colic symptoms during estrus, which were caused by several ovarian hematomas and diagnosed by the referring veterinarian. Serum AMH and testosterone concentrations were within normal limits. The mare responded well to conservative treatment with GnRH-vaccination. Preoperative rectal palpation and ultrasonographical examination revealed bilaterally small and inactive ovaries. After removal, the ovaries presented tertiary follicles on the left and fossa cysts on the right ovary on pathohistological examination. Bilateral ovariectomy resulted in complete resolution of aggressiveness, buckling, and colic symptoms for several months. However, the mare showed persistent estrous signs 6 months after surgery with simultaneous reoccurrence of the initially presented behavioral problems. Repetitive measurements of serum AMH and ACTH concentrations were within normal limits. Reevaluation of the reproductive tract by rectal und ultrasonographical examination revealed no abnormalities. Daily treatment with oral Altrenogest led to behavioral improvement and absent estrous signs again. This was the only mare in our study with no success regarding behavior after bilateral ovariectomy.

A sixteen-year-old Hannoverian mare (Case 39) was presented due to estrous-related, recurrent colic signs. Serum testosterone was not measured, but serum AMH was with 14.5 pmol/L in the suspicious range. The mare responded well to oral Altrenogest treatment before surgery. Rectal and ultrasonographical examination revealed normal sized ovaries with bilaterally small follicles. On pathohistological examination, we could determine preantral follicles on the right and anovulatory-like follicles on the left. The mare showed recurrent colic symptoms two months after surgery and was euthanized due to a nephrospleenic entrapment of the large colon four months after surgery.

An eighteen-year-old Württemberg Warmblood mare (Case 49) showed estrous-related colic signs for one year and responded well to conservative treatment with Altrenogest and GnRH-vaccination before surgery. Serum AMH concentration was within normal limits, but serum testosterone was not measured. The mare presented bilaterally small ovaries on clinical examination. Pathohistological examination revealed early and late atretic follicles on both

ovaries with no abnormalities. Unfortunately, the mare was euthanized four weeks after surgery due to severe colic signs, which were reported as independent of the surgical procedure.

3.1.2. Bilaterally ovariectomized mares with behavioral problems and pathohistologically detected early neoplastic changes

A sixteen-year-old Polish Warmblood mare (Case 29) showed aggressive behavior and constant estrous signs since the beginning of ownership of one year. Serum AMH and testosterone concentrations were increased with 38.6 pmol/L and 0.45 nmol/L, respectively. We could determine bilaterally small ovaries with multiple follicles on rectal and ultrasonographical examination. On gross section after bilateral removal, a small pale area was detected near the ovulation fossa on the right ovary. Pathohistological examination revealed CL tissue on the left and antral follicles on the right ovary, declaring functional ovaries. Moreover, early neoplastic changes (ENCs) in form of GC nests were determined on both ovaries with allocation to the pale area in the right ovary (**Figure 5A, B**). An additional anovulatory-like follicle was further detected on the left ovary, which was in contact to additional occurring ENCs in this ovary. Bilateral ovariectomy resulted in a complete resolution of aggressiveness and constant estrous signs according to the owner.

An eleven-year-old Oldenburger mare (Case 36) with a history of moody behavior and unwillingness to be ridden for six months presented an increased serum AMH concentration of 23.9 pmol/L. Serum testosterone was within normal limits. Clinical examination revealed normal sized ovaries with large follicles on the left and multiple follicles on the right side. Pathohistological examination confirmed antral follicles on the left and antral follicles on the right. Moreover, we could find an additional anovulatory-like follicle and ENCs presented as GC nests on the right ovary. According to the owner, the mare responded well to BO with improvement in rideability and moody behavior. Estrous signs were not observed anymore after surgery.

A four-year-old Pura Rasa Española mare (Case 10) was introduced with stallion-like behavior for 1.5 years. Serum testosterone was not measured, but serum AMH concentration was normal with 0.4 pmol/L. Rectal examination revealed a small left and a large right ovary with no abnormalities on ultrasound. Pathohistological examination showed early atretic follicles and a CL on the left and ENCs presented as proliferative GC nests on the right side (**Figure 5C**). The owner reported a complete resolution of stallion-like behavior after BO.

3.1.3. Mares with unilaterally removed granulosa cell tumors

An eighteen-year-old Welsh Cop Pony mare (Case 24) was suspicious for GCT presence due to a rectally palpated ovarian enlargement during colic examination and a just beginning stallion-like, moody behavior. Serum AMH was significantly increased (>150 pmol/L) and ultrasonographical examination of the left ovary revealed a typical honeycomb-like structure. The GCT-affected

ovary presented a multicystic appearance on gross section and macrofollicular patterns on pathohistology. We could further determine numerous LLCs, of which some presented an invasive growing character by penetrating the basement membrane into the GC layer (**Figure 6**). The GCT was removed by a two-step procedure due to large tumor size and resulted in a fast return to normal behavior with high owner's satisfaction.

A nine-year-old Connemara mare (Case 27) had a history of sudden stallion-like behavior during estrus and markedly increased serum AMH (123 pmol/L) but normal serum testosterone concentration. Rectal and ultrasonographical examination revealed a goose-egg-sized left ovary with honeycomb-like structure and a small-sized and inactive ovary on the right side. The enlarged ovary accordingly presented a honeycomb-like appearance on gross section. On pathohistological examination, we could determine macrofollicular patterns with several LLCs and clusters of Ki67-positive cells in the GC layer (**Figure 7A**). Further evaluation of those accumulated cells by means of transmission electron microscopical imaging conducted by histological experts confirmed the presence of immature erythrocytes with an existing nucleus and were determined as erythroblasts (**Figure 7C**). Surgical removal of the GCT-affected ovary resulted in normal behavior and high owner's satisfaction.

A sixteen-year-old Trakehner mare (Case 44) was referred for unilateral ovariectomy due to moodiness and stallion-like behavior since more than one year. The mare showed a markedly increased serum AMH (>150 pmol/L) but normal serum testosterone concentration. We could find a small inactive ovary on the right and a significantly enlarged ovary on the left. The GCT-affected ovary presented a typical honeycomb-like structure on ultrasound. A two-step procedure was necessary for GCT removal due to the large tumor size in this case. Gross section revealed a multicystic appearance and pathohistological evaluation resulted in a mixture of macrofollicular, insular and diffuse patterns. Numerous LLCs, SLCs and further cluster formations of Ki67 positive cells were determined, similar to Case 27. The mare benefited from unilateral removal of the GCT and showed normal behavior afterwards.

3.2. Special pathohistological findings

Pathohistological evaluation by means of the diagnostic markers Ki67, AMH, AR, EGFR, CAL, and E-Cad revealed no remarkable outcome in a differentiation between clinically unremarkable ovaries of bOE and GCT of GCT-uOE (WOLF et al., 2024; III. 2.). However, remarkable ovarian structures were detected in some of the examined ovaries, which were mentioned in "Selected special clinical cases" (see 3.1.). Some of those structures were not reported by other authors before. We could determine ENCs and anovulatory-like follicles in ovaries of 35% of the mares in bOE and LLCs with invasive growing character in 10% and immature erythrocytes in 20% of GCTs in GCT-uOE. Routine pathohistological evaluation by board certified pathologists of an external laboratory (ExP) revealed further results in bOE, which were partly unequal to our findings and summarized in **Table A1** (VIII. Appendix). In the following these findings are

described in more detail.

3.2.1. Cyst-like structures and early neoplastic changes in mares with bilaterally removed ovaries

Pathohistological examinations by ExP revealed "follicular cysts" in 19/40 ovaries (48%), "ovarial cysts" in 6/40 ovaries (15%), "anovulatory cysts" in 3/40 ovaries (8%), and unclear cases in 3/40 ovaries (8%). No abnormalities were determined in 20% of all examined ovaries by ExP. Our pathohistological examinations revealed fossa cysts in 25%, anovulatory-like follicles in 15%, and ENCs in 10% of the same ovaries in bOE. Whenever the term "follicular cyst" was used by ExP (n=19), we could determine fossa cysts (26%), anovulatory-like follicles (11%), or no abnormalities (63%). Whenever the term "ovarial cyst" was used by ExP (n=6), we could determine fossa cysts (33%) or no abnormalities (67%). Whenever the term "anovulatory cyst" was used by ExP (n=3), we could either find anovulatory-like follicles, fossa cysts, or no abnormalities. No abnormalities were determined in eight ovaries of bOE by ExP (20%), where we could detect fossa cysts and anovulatory-like follicles in one ovary, respectively, and accordingly no abnormalities in 6/8 ovaries.

Anovulatory-like follicles were determined in 30% of the mares in bOE and characterized as large follicular structures with the size of preovulatory follicles and multiple GC layers but a poorly developed theca cell layer (**Figure 3**). This theca cell layer was mainly formed by theca cells with a retained fibroblast-type appearance in contrast to preovulatory follicles, which contained polyhedral cells in a well-developed and vascularized theca interna cell layer. Those large follicles were defined as anovulatory-like follicles according to the histomorphological definition of anovulatory follicles (WATSON and AL-ZI'ABI, 2002) but without clinical examination of persistence.

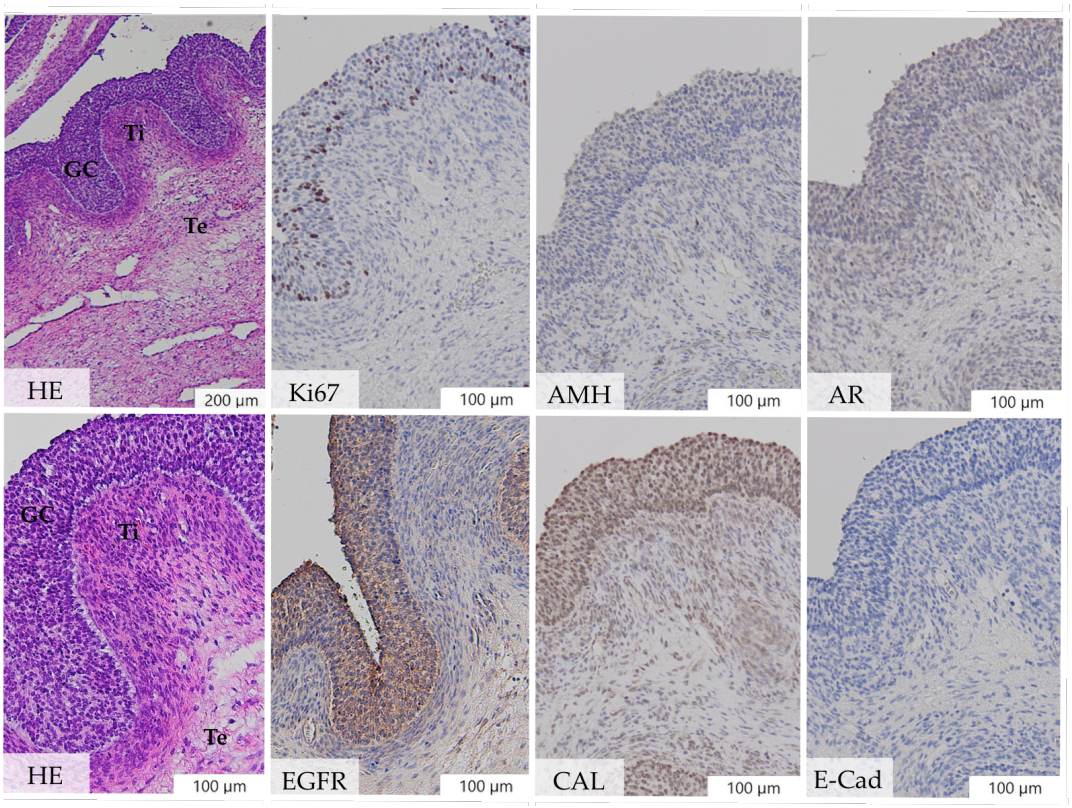


Figure 3. Anovulatory-like follicle in one ovary of a bilaterally ovariectomized mare (bOE) in hematoxylin and eosin (HE) and different immunohistochemical staining with Ki-67 (Ki67), anti-Müllerian hormone (AMH), aromatase (AR), epidermal growth factor receptor (EGFR), calretinin (CAL), and epithelial cadherin (E-Cad). Note the well-organized, multiple layers of granulosa cells (GC) contacted to the basement membrane and the poorly developed theca interna cell layer (Ti), which is mainly formed by theca cells with a retained fibroblast-type appearance. Te=theca externa cell layer; bars 200 µm, 100 µm.

Fossa cysts were determined in 25% of the ovaries and in 35% of the mares in bOE and characterized as cyst-like structures with a squamous, columnar, or cuboidal epithelium with additional cilia present (**Figure 4**).

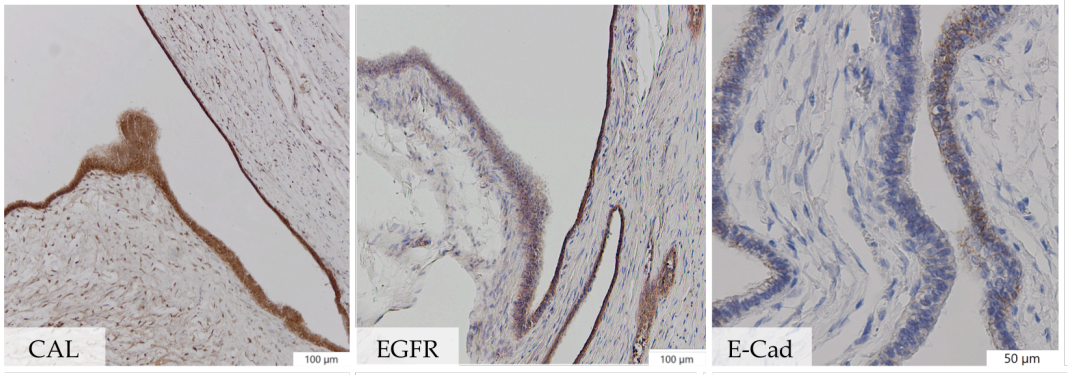


Figure 4. Fossa cysts with squamous, cuboidal, and columnar epithelium in ovaries of bilaterally ovariectomized mare (bOE) in immunohistochemical staining with calretinin (CAL), epidermal growth factor receptor (EGFR), and epithelial cadherin (E-Cad); bars 100 µm, bars 50 µm.

Pathohistological determination of ENCs in three cases of bOE (Case 10, 29 and

36) initially revealed unclear findings by ExP. In a joint reevaluation, however, ExP confirmed the presence of neoplastic GCs as precursor stages for GCTs in those cases. Early neoplastic changes were characterized by clinically nondetectable, small areas of GC nests or spindle-shaped GCs in the GC layer. They additionally showed polyhedral cells with foamy cytoplasm similar to LLCs in GCTs in the theca interna cell layer (**Figure 5C**) or within GC nests (**Figure 5B**). Seventy-five percent of ENCs were growing from anovulatory-like follicles.

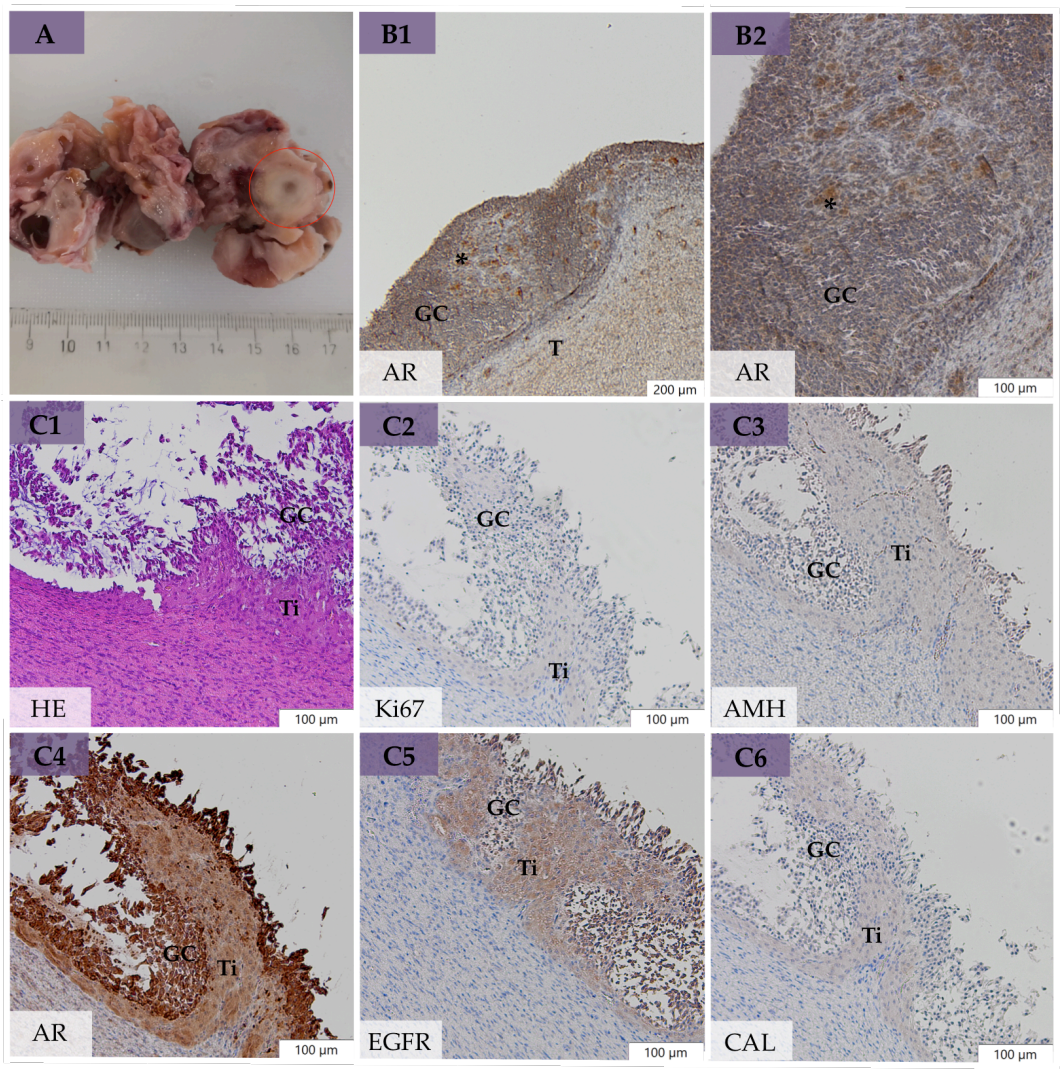


Figure 5. Early neoplastic changes (ENCs) in different ovaries of bilaterally ovariectomized mares (bOE, Case 29 and Case 10): (**A**) Gross section of a clinically unremarkable ovary with a pale area (red circle) near the ovulation fossa suspicious for ENCs (Case 29). (**B**) Immunohistochemical evaluation of this area in aromatase (AR) staining in different magnifications (**B1, B2**); note the granulosa cell (GC) nests with AR-positive cells resembling Leydig-like cells (LLCs) in between (asterisk), defined as ENC; Bars 200 µm, 100 µm. (**C**) Pathohistological findings in a clinically unremarkable ovary with detected ENCs (Case 10): spindle-shaped, neoplastic GCs with polyhedral, foamy cells in the theca interna cell layer resembling LLC. Figures are presented in hematoxylin and eosin (HE, (**C1**)) and different immunohistochemical staining with Ki-67 (Ki67, (**C2**)), anti-Müllerian hormone (AMH, (**C3**)), AR (**C4**), epidermal growth factor receptor (EGFR, (**C5**)), and calretinin (CAL, (**C6**)); bars 100 µm; GC = granulosa cells, T = theca cell layer, Ti = theca interna cells. (Figure out of WOLF et al., 2024; III. 2. Figure 3)

3.2.2. Invasive growing Leydig-like cells and erythroblasts in granulosa cell tumors of unilaterally ovariectomized mares

Invasive growing LLCs were found in one GCT of GCT-uOE (Case 24) and characterized by penetrating the basement membrane into the GC layer (**Figure 6**).

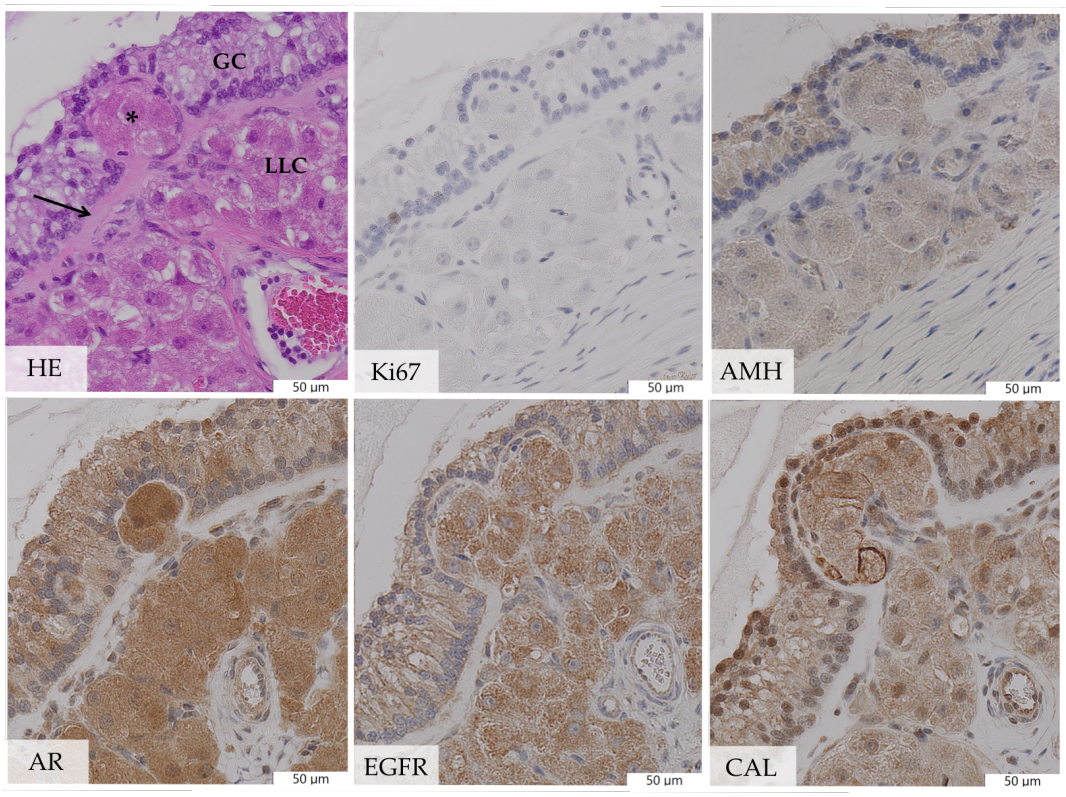


Figure 6: Invasive growing Leydig-like cells (LLC) in one granulosa cell tumor (Case 29) in hematoxylin and eosin (HE) and different immunohistochemical staining with Ki-67 (Ki67), anti-Müllerian hormone (AMH), aromatase (AR), epidermal growth factor receptor (EGFR), and calretinin (CAL); bars 50µm; GC=granulosa cells, LLC=Leydig-like cells, asterisk=invasive growing LLC, arrow=basement membrane.

Cell aggregations in the GC layer were detected by high Ki67 expression in two GCTs of GCT-uOE (20%, Case 27 and 44), as previously mentioned in "Selected special clinical cases" (3.1.). Those cells were determined as erythroblasts by means of additional electron microscopical evaluation. They contained a round nucleus with prominent nucleoli and only few cytoplasm and were located between mature erythrocytes and neoplastic GCs (**Figure 7**).

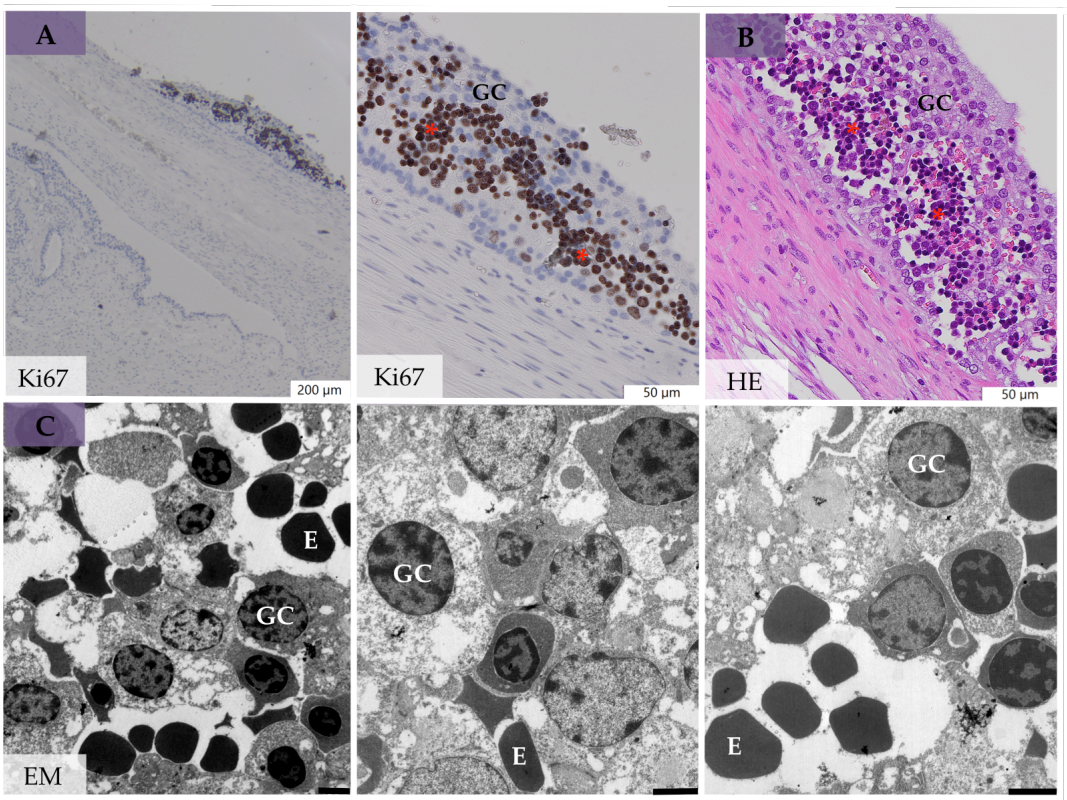


Figure 7: Immature erythrocytes (erythroblasts) in a granulosa cell tumor (Case 27). (**A**) Cluster formation of Ki-67 (Ki67) positive cells (red asterisk) in between neoplastic granulosa cells (GC) in overview and detail; bars 200 µm, 50 µm. (**B**) Cluster formation of those cells with a dark nucleus (asterisk) next to mature erythrocytes and neoplastic granulosa cells (GC) in hematoxylin and eosin (HE) staining; bars 50 µm. (**C**) Electron microscopic images (EM) of those cluster formations revealed erythroblasts (E) in between neoplastic GC; bars (black) 2500 nm.

3.3. Immunohistochemical findings

Immunohistochemical results of Ki67, AMH, AR, EGFR, CAL and E-Cad in large follicular structures of bOE and GCTs of GCT-uOE were mentioned (III. 2. **Table 1**) and discussed in the publication (WOLF et al., 2024). Further immunohistochemically evaluated structures of bOE included different cells of preantral follicles, late atretic follicles, and CLs. All immunohistochemical results of bOE and GCT-uOE are summarized in **Table A2** (VIII. Appendix). In the following, immunohistochemical findings of all structures in both groups are described in detail.

Preantral follicles were in general difficult to detect and located in the stromal tissue (**Figure 8**). Granulosa cells of primordial, primary, and secondary follicles were characterized by coexpression of AR, EGFR, and CAL. Anti-Müllerian hormone was expressed in GCs of primary follicles and increased in intensity in the follicular development. Expression of Ki67 was low in GCs of preantral follicles (grade 0) with rarely stained nuclei in GCs of secondary follicles (**Figure 8C**, arrow).

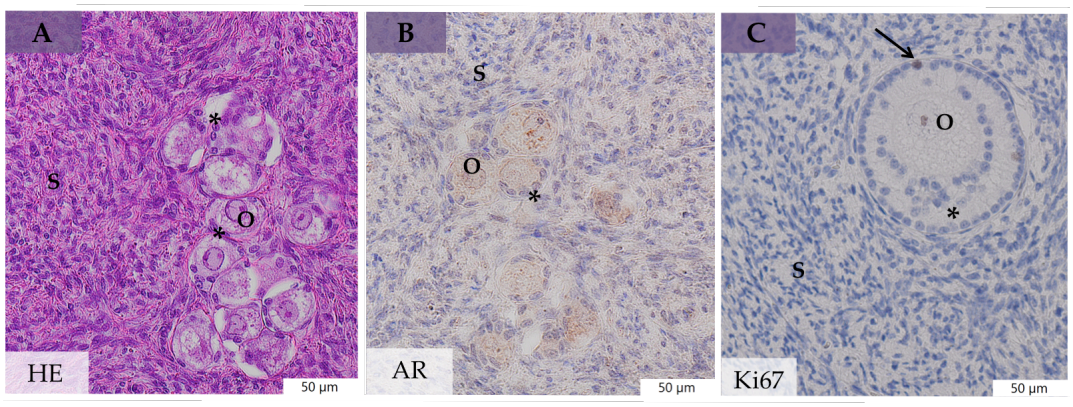


Figure 8. Preantral follicles in ovaries of bilaterally ovariectomized mares with behavioral problems (bOE). (**A**) Primordial follicle nest with several oocytes (O) surrounded by flattened granulosa cells (GC, asterisks) in hematoxylin and eosin (HE) staining. (**B**) Primary follicles with oocytes and a cuboidal GC layer (asterisks) with moderate aromatase (AR) expression. (**C**) Secondary follicle with two GC layers (asterisk). Granulosa cells (asterisk) rarely express Ki-67 (Ki67, arrow); S=stroma, bars 50 µm.

Granulosa cells of tertiary and preovulatory follicles showed a high Ki67 PI (grade 2-3), moderate CAL, and intense expression of AMH, AR, and EGFR (**Figure 9**). Theca cells of tertiary follicles presented mild expression of AMH, AR, EGFR, and CAL and a high Ki67 PI (grade 1). Theca interna cells of preovulatory follicles showed similar AMH and Ki67 expression and higher expression of AR, EGFR, and CAL compared to those of tertiary follicles. Polyhedral cells of the well-developed theca interna layer of preovulatory follicles showed high expression of AR and CAL and mild AMH and EGFR expression (**Figure 9B**, asterisks). Theca externa cells of preovulatory follicles were characterized by moderate expression of EGFR and CAL, mild AR expression, and no AMH expression. Epithelial cadherin was restrictively expressed in the zona pellucida of oocytes in follicular structures of bOE (**Figure 9A6**, arrow).

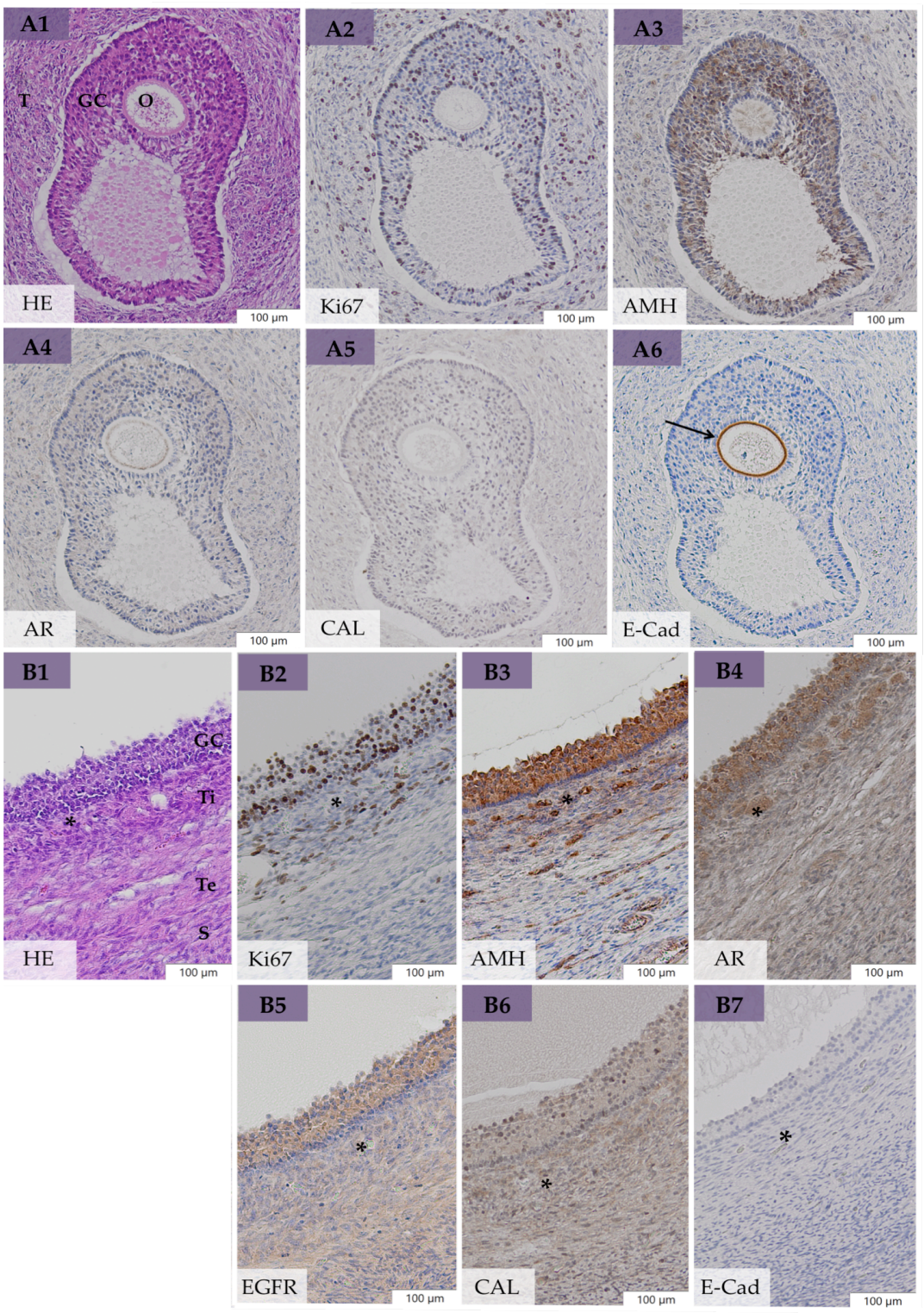


Figure 9: Antral follicles in ovaries of bilaterally ovariectomized mares (bOE) in different immunohistochemical staining with hematoxylin and eosin (HE (**A1, B1**)), Ki-67 (Ki67 (**A2, B2**)), anti-Müllerian hormone (AMH (**A3, B3**)), aromatase (AR (**A4, B4**)), epidermal growth factor receptor (EGFR (**B5**)), calretinin (CAL (**A5, B6**)), and epithelial cadherin (E-Cad (**A6, B7**)): (**A**) Tertiary follicle with oocyte (O), granulosa cell (GC) layer, and theca cell layer (T); note the positive E-Cad staining of the zona pellucida (**A6**, arrow). (**B**) Preovulatory follicle with GCs, theca interna (Ti), and theca externa cell layer (Te) and stroma (S); note the polyhedral cells with foamy cytoplasm in the theca interna cell layer (asterisks); bars 100 µm; GC = granulosa cells, O = oocyte, S = stroma. (Out of WOLF et. al., 2024; III.2. Figure 4)

Granulosa cells of early atretic follicles (**Figure 10**) showed almost similar expression patterns to GCs of preovulatory follicles (**Figure 9B**), as do theca interna and externa cells, but in a lower intensity. As mentioned before, E-Cad was restrictively expressed in the zona pellucida of oocytes (**Figure 9A6, Figure 10,** E-Cad) and in epithelial cells of fossa cysts (**Figure 4**, E-Cad). This marker was not expressed in GCs and theca cells of any follicular structures in bOE, or in any neoplastic structure in GCTs of GCT-uOE.

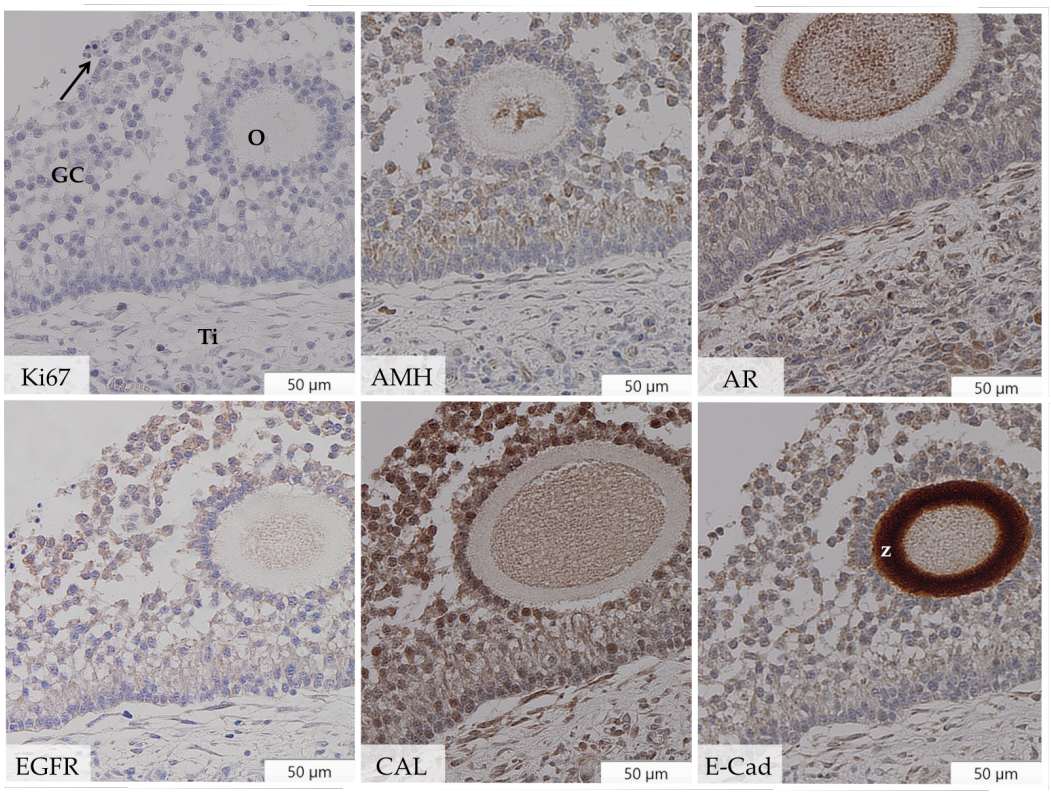


Figure 10. Early atretic follicle with a present oocyte (O) in one ovary of a bilaterally ovariectomized mare (bOE) in different immunohistochemical staining with Ki-67 (Ki67), anti-Müllerian hormone (AMH), aromatase (AR), epidermal growth factor receptor (EGFR), calretinin (CAL), and epithelial cadherin (E-Cad). Note the apoptotic granulosa cells (GC, arrow) and the intense E-Cad staining in the zona pellucida of the oocyte (z); Ti=Theca interna cell layer, bars 50 µm.

Granulosa cells of late atretic follicles showed moderate expression of AMH, AR, and CAL with mild EGFR expression and a low Ki67 PI and revealed in general less intensity of all markers compared to early atretic follicles.

Granulosa cells of anovulatory-like follicles (**Figure 3**) presented a varying Ki67 PI (grade 0-2) and AMH, AR, EGFR, and CAL were less intense expressed compared to GCs of preovulatory follicles. Theca cells of anovulatory-like follicles showed no Ki67 and AMH expression and mild AR, EGFR, and CAL expression, similar to theca externa cells of preovulatory follicles.

The squamous, columnar, or cuboidal epithelium of fossa cysts was immunohistochemically characterized by intense expression of EGFR and CAL and moderate expression of E-Cad (**Figure 4**). Epithelial cadherin showed delicate cell membrane-associated staining. Anti-Müllerian hormone and Ki67

were not expressed in fossa cysts.

Lutein cells of CLs were characterized by high AR and EGFR expression and mild CAL expression. Anti-Müllerian hormone and Ki67 were not expressed in CLs (**Figure 11**).

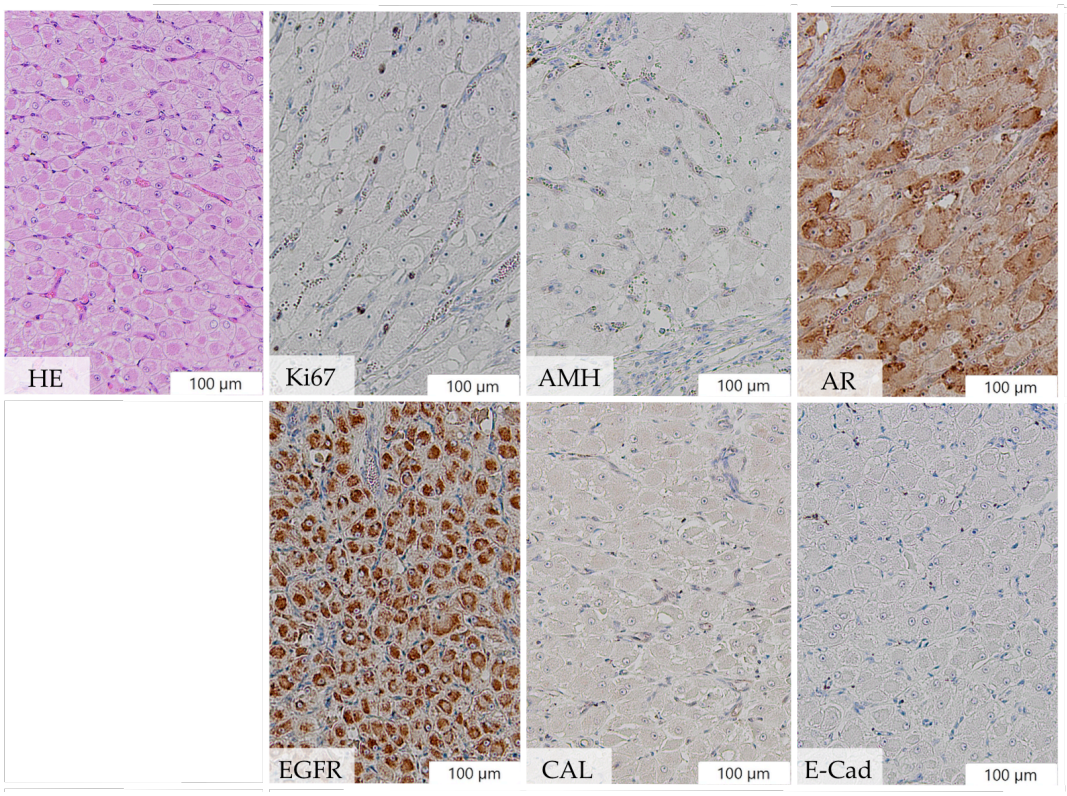


Figure 11. Lutein cells of a corpus luteum in one ovary of a bilaterally ovariectomized mare (bOE) in hematoxylin and eosin (HE) and different immunohistochemical staining with Ki-67 (Ki67), anti-Müllerian hormone (AMH), aromatase (AR), epidermal growth factor receptor (EGFR), calretinin (CAL), and epithelial cadherin (E-Cad). Note the intense expression of AR and EGFR; bars 100 µm.

Immunohistochemical expression of neoplastic GCs and LLCs in ENCs revealed high CAL and EGFR, moderate CAL, and mild AMH expression (**Figure 5**). Early neoplastic changes were therefore not distinguishable from normal large follicular structures in bOE, similar to neoplastic GCs of GCTs in GCT-uOE.

Comparative immunohistochemical evaluation of large follicular structures in bOE and cyst-like structures in GCT-uOE is demonstrated in **Figure 12**. Pathohistological findings revealed no differences in both groups, as published in WOLF et al. (III. 2.).

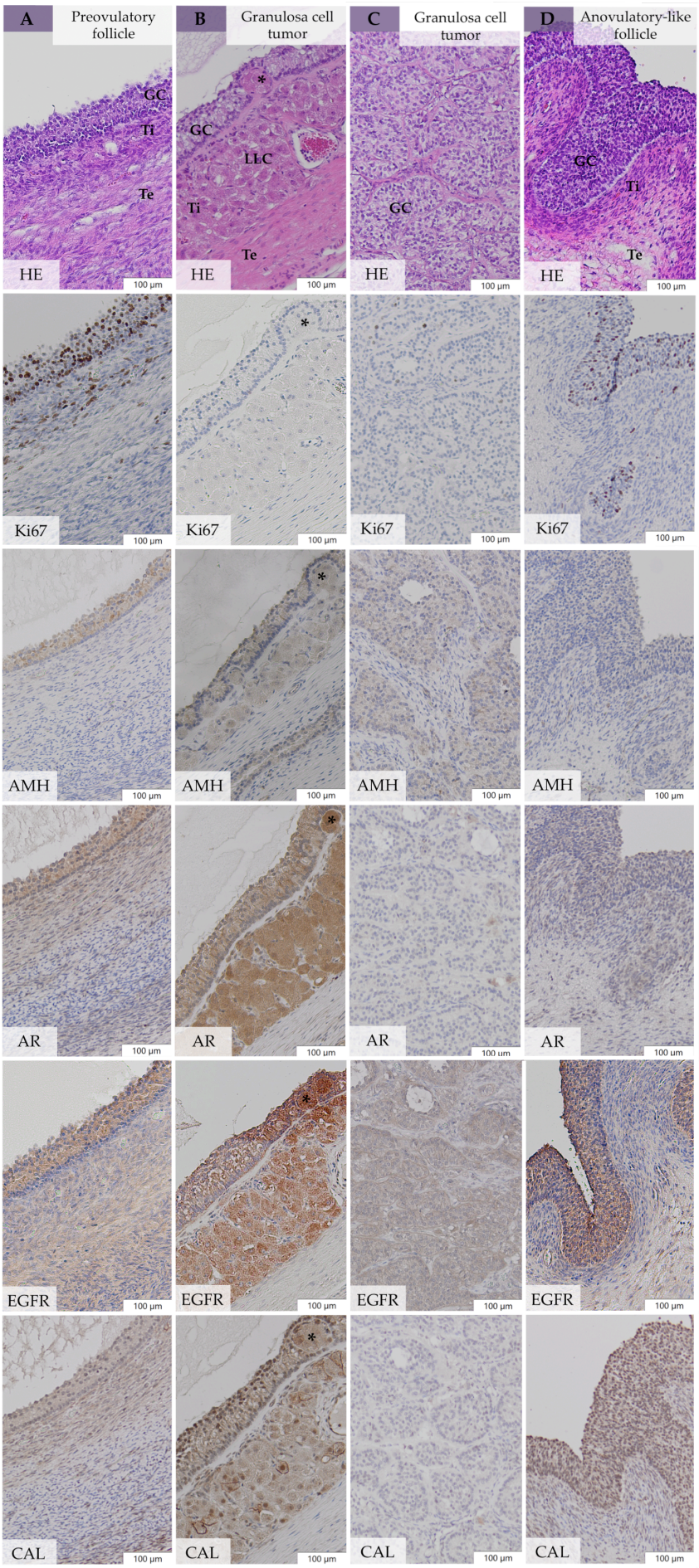

A
Preovulatory follicle
B
Granulosa cell tumor
C
Granulosa cell tumor
D
Anovulatory-like follicle
GC
Ti
Te
LLC
*
HE
Ki67
AMH
AR
EGFR
CAL
100 µm

Figure 12: Large follicular structures in ovaries of bilaterally ovariectomized mares (bOE; (**A**, **D**)) compared with cyst-like structures in two different granulosa cell tumors (GCTs) of unilaterally ovariectomized mares (GCT-uOE; (**B**, **C**)) in hematoxylin and eosin (HE) and different immunohistochemical staining with Ki-67 (Ki67), anti-Müllerian hormone (AMH), aromatase (AR), epidermal growth factor receptor (EGFR), and calretinin (CAL). (**A**) Preovulatory follicle with granulosa cell (GC) layer, theca interna, and theca externa cell layer. (**B**) GCT with a multiple GC layer and numerous Leydig-like cells in the theca interna cell layer, some show an invasive growing character (asterisk). (**C**) GCT with microfollicular pattern of the GC layer. (**D**) Anovulatory-like follicle with a multiple GC layer, poorly developed theca interna, and theca externa cell layer; bars 100 μm; GCs = granulosa cells, Ti = theca interna cell layer, Te = theca externa cell layer, LLCs = Leydig-like cells. (Out of WOLF et al., 2024; III.2. Figure 5)

IV. EXTENDED DISCUSSION

The objective of this study was to find a pathohistological explanation for the high success rate of BO in mares with behavioral problems but clinically unremarkable ovaries. To our knowledge, this is the first pathohistological study characterizing bilaterally removed, clinically unremarkable ovaries of mares with behavioral problems before surgery in comparison with pathohistologically confirmed GCTs by means of histomorphology and immunohistochemistry with additional regard to clinical parameters. We therefore aimed a detailed clinical history with preoperative presented behavior patterns and conservative treatment options, clinical examination, preoperative analysis of serum AMH and testosterone concentrations, and retrospectively evaluated behavioral improvement after surgery by means of owner's questionnaires via telephone. Analysis of a complete clinical data set was implemented as basis for the pathohistological examination and further revealed behavioral improvement after BO in 85% of mares in bOE, which is in accordance to recent studies (KAMM and HENDRICKSON, 2007; DEVICK et al., 2020; MELGAARD et al., 2020; COLLAR et al., 2021; STRATICÓ et al., 2023).

We hypothesized, that GCTs are not the only ovarian source of behavioral problems in mares and that other pathohistologically detectable changes might explain the success of BO. A detailed documentation of clinical and pathohistological findings in all cases is provided in **Table S1** and **S2** (III. 2. Supplementary data) and shows a wide variety and individual outcomes in bOE and GCT-uOE. Some cases, which were mentioned in detail in "Selected special clinical cases" (III. 3.1.), revealed special characteristics in clinical but also in pathohistological findings. Therefore, we emphasize the need of a complete data set to find a possible association to high success rates of BO in mares with behavioral problems.

We moreover hypothesized, that clinically unremarkable ovaries of bOE are differentiated from neoplastic changes of GCT-uOE by means of the immunohistochemical markers Ki67, AMH, AR, EGFR, CAL and E-Cad. However, immunohistochemical analysis between clinically unremarkable ovaries and GCT-affected ovaries did not result in a difference between the groups by means of the six diagnostic markers (III. 2. and 3.3. **Figure 12**). In bOE and GCT-uOE, expression patterns of all tested diagnostic markers were equal in all examined ovaries and distinctive differences between different follicular structures were hardly detectable. Although not the aim of this study, we pathohistologically evaluated all occurring structures in all examined ovaries of both groups and could find meaningful results. We could determine some new findings in histomorphological evaluation and immunohistochemical analysis of the tested diagnostic markers compared to previously published results by other authors.

1. New immunohistochemical findings in clinically healthy and granulosa cell tumor-affected ovaries

In follicular structures of bOE, immunohistochemical expression of AMH increased with the number of GC layers and resulted in a high expression in antral and early atretic follicles in bOE (III. 3.3. **Figure 9, 10**). This finding was contrary to results of BALL et al. (2008), who found reduced expression in those follicles of healthy ovaries. Multilayered GCs of anovulatory-like follicles in bOE showed faint expression of AMH (III. 3.2.1. **Figure 3**) and were therefore distinguishable from antral follicles (III. 3.3. **Figure 9**), beside their poorly developed theca cell layer. Additional AR expression was determined in GCs of preantral follicles and theca cells of all follicular structures (VIII. **Table A2**), which was in contrast to the reported absent expression in those structures (WATSON and THOMSON, 1996; MLODAWSKA and SLOMCZYNSKA, 2010).

Regarding GCTs, some authors concluded a conversation failure of testosterone into estradiol, as AR expression was absent in neoplastic GCs and LLCs in their study (HOQUE et al., 2003; IBRAHIM, 2019). However, according to DOLIN et al. (2023), we could find intense AR expression in LLCs (III. 3.2.2. **Figure 6**) and heterogeneous patterns in neoplastic GCs and SLCs of GCTs in GCT-uOE (III. 3.3. **Figure 12B, C**, AR). This remarkable intense AR expression in LLCs supports the idea of a potential androgen synthesis in LLCs (BAILEY et al., 2000; ELLENBERGER et al., 2007; NETO et al., 2010). We therefore assume an association between high serum testosterone concentrations in mares with GCTs and the capability of androgen synthesis by LLCs.

Expression of CAL and EGFR was markedly present in all evaluated structures in bOE and GCT-uOE, with no difference in GCs of large follicular structures in bOE and neoplastic GCs in GCT-uOE (III. 3.3. **Figure 12**). High CAL expression in neoplastic GCs and LLCs in GCT-uOE was according to DOLIN et al. (2023), but high EGFR expression in those cells and in equine GCTs was in general not reported before.

Epithelial cadherin was published as valuable diagnostic marker for LLCs in GCTs, as LLCs showed intense expression compared to healthy GCs (DOLIN et al., 2023). In our study, however, E-Cad was restrictively expressed in the zona pellucida of oocytes (III. 3.3. **Figure 9 A6, Figure 10**, E-Cad) and in epithelial cells of fossa cysts in bOE (III. 3.2.1 **Figure 4**, E-Cad). Neoplastic GCs of all examined GCTs in GCT-uOE did not express E-Cad at all. Therefore, we doubt the diagnostic value of E-Cad for detection of LLCs and neoplastic GCs in GCTs but would suggest this diagnostic marker as indicator for epithelial structures like fossa cysts. Moreover, E-Cad might be valuable to pathohistologically clarify the controversial debate of existing cystic ovaries in mares (MCCUE and SQUIRES, 2002; Förster, 2003) and needs further investigations.

2. Invasive growing Leydig-like cells and extramedullary hematopoiesis in granulosa cell tumors

Pathohistological evaluations of GCTs in unilaterally ovariectomized mares (GCT-uOE) of our study revealed two incidental findings, which, to our knowledge, had not been reported before. The pathohistological presence of invasive growing LLCs and erythroblasts in GCTs was mentioned in "Selected special clinical cases" (III. 3.1.) and further described in detail in "Special pathohistological findings" (III. 3.2.2.).

Invasive growing LLCs were present in one mare of GCT-uOE (Case 24) and characterized by penetrating the basement membrane into the GC layer (III. 3.2.2. **Figure 6**). This special feature might indicate fast tumor growth, as the mare presented behavioral problems just recently, but the ovary was already enlarged. The presence of invasive growing LLCs could further substitute the exceedingly rare phenomenon of malignancy and metastasis of equine GCTs (MEAGHER et al., 1977; PATRICK et al., 2003; ABBOTT et al., 2004; ELLENBERGER et al., 2007). ELLENBERGER et al. (2007) reported a single malignant GCT, which metastasized in the abdominal cavity and to several organs. The authors could not differentiate between tumor cells of the metastasizing neoplasms and tumor cells of the benign neoplasm, neither with HE staining nor with other used immunohistochemical markers including vimentin, glutathione S-transferase α, and c-erbB-2 oncoprotein. The GCT of Case 24 showed intense expression of AR, EGFR, and CAL in all LLCs and was accordingly immunohistochemically not distinguishable from other LLCs (III. 3.2.2. **Figure 6**). However, invasive growing LLCs might be a sign of initial phases of malignancy.

Immature erythrocytes (erythroblasts) were determined in 20% of GCTs in GCT-uOE (Case 27 and 44) and indicated by a high Ki67 PI as cluster formations (III. 3.2.2. **Figure 7**). Erythroblasts were located between mature erythrocytes and neoplastic GCs in the GC layer (III. 3.2.2. **Figure 7B**) but restricted to a small area of the examined tissue (III. 3.2.2. **Figure 7A**). Extramedullary hematopoiesis (EHM) is described as formation and development of blood cells outside the medullary spaces of the bone marrow and could be found in neoplasms involving hemic and non-hemic tissue (JOHNS and CHRISTOPHER, 2012). In hemangiosarcomas, EHM might be related to their stem cell origin of endothelial and blood cells and to development of a beneficial vascular niche environment (JOHNS and CHRISTOPHER, 2012). In other neoplasms, development of EHM might be caused by local hypoxia, tissue injury, or repair and production of growth factors by endothelial cells due to neovascularization (JOHNS and CHRISTOPHER, 2012). Nevertheless, the exact pathomechanism of EHM is not fully understood yet (JOHNS and CHRISTOPHER, 2012) and the presence of EHM in equine GCTs or GCTs of other species was not described before.

Conclusively, both detected structures, invasive LLCs and erythroblasts, might indicate a higher potential of tumor metastasis in equine GCTs as previously assumed (MEAGHER et al., 1977; PATRICK et al., 2003; ABBOTT et al., 2004; ELLENBERGER et al., 2007). However, further investigations on EMH and

invasive growing LLCs in equine GCTs are necessary to find a plausible explanation for their presence.

3. Cyst-like ovarian structures

Follicular cysts or ovarial cysts are commonly reported in bovine ovaries and represent an economic problem due to infertility caused by blockage of the estrous cycle (ZERBE et al., 1999). In equine medicine, however, the existence of real cysts in equine ovaries is controversially discussed (HUGHES et al., 1980; BOSU et al., 1982; ENGLAND,1996; MCCUE, 1998; MCCUE and SQUIRES, 2002; FÖRSTER, 2003). Some authors described cyst-like, persistent anovulatory follicles in equine ovaries that are comparable to the cystic ovarian syndrome in cattle (HUGHES et al., 1980; BOSU et al., 1982; KESLER and GARVERICK, 1982; MCCUE, 1998; MCCUE and SQUIRES, 2002). Moreover, MCCUE (1998) published a report with bilaterally occurring polycystic ovaries. However, detailed pathohistological descriptions are missing to evidence the presence of "real" cysts in equine ovaries.

Pathologists from the external laboratory in our study used different terms for cyst-like structures in ovaries of bOE, i.e. "follicular cyst", "ovarial cyst", and "anovulatory cyst". Those terms were incongruent to our pathohistological findings (VIII. **Table A1**). Nevertheless, the terms were associated to the presence of fossa cysts, anovulatory-like follicles, and early atretic follicles of our pathohistological evaluation. We therefore suggest that fossa cysts, anovulatory-like follicles, and early atretic follicles with multiple GC layers are often regarded as cysts due to their cyst-like histomorphological appearance.

Anovulatory-like cysts were present as large, cyst-like follicular structures in ovaries of bOE. We termed them as "anovulatory-like" follicles based on their histomorphological similarity to anovulatory follicles (WATSON and AL-ZI'ABI, 2002), but without information of clinical persistence. They consisted of multiple GC layers and a poorly developed theca cell layer, which was mainly formed by theca cells with a retained fibroblast-type appearance (III. 3.2.1. **Figure 3**). Anovulatory-like follicles showed a cyst-like appearance in ovaries of bOE, but a blood filled central cavity and polygonal luteinized cells were missing (ELLENBERGER et al., 2009). In literature, anovulatory follicles are classified into two types: they are present as persistent anovulatory follicles (15%) or hemorrhagic anovulatory follicles (HAFs, 85%) (MCCUE and SQUIRES, 2002; CRABTREE, 2020). Persistent anovulatory follicles have a limited clinical significance, as they regress spontaneously after several cycles (CRABTREE, 2020). Hemorrhagic anovulatory follicles, however, are regarded as pathological ovarian abnormalities due to their capability of progesterone synthesis and possible cause of estrous problems with reproductive infertility (CHOPIN et al., 2001; MCCUE and SQUIRES, 2002). According to WATSON and AL-ZI'ABI (2002), we did not distinguish between persistent anovulatory follicles and HAFs, as a long-term follow-up by ultrasonographical examination was missing in our study. However, they were regarded as ovarian abnormality due to their high incidence of 30% in mares of bOE compared to the reported occurrence of 8.2% in

clinically confirmed, persistent anovulatory follicles in cycling mares (MCCUE and SQUIRES, 2002). As five of six mares with pathohistologically detected anovulatory-like follicles in bOE improved after BO, we suspect that these structures could be related to behavioral problems in mares. Moreover, 75% of ENCs were growing from anovulatory-like follicles (WOLF et al., 2024). Therefore, we further suggest these structures as possible precursor stages for ENCs and advanced GCTs (RENAUDIN et al., 2021; CRABTREE et al., 2013).

Other cyst-like structures in our study included fossa cysts, which were present in 35% of the mare's ovaries in bOE. Fossa cysts are reported as non-follicular cyst formations within the region of the ovulation fossa (BOSU et al., 1982). They were histomorphologically present as cyst-like structures with a squamous, columnar, or cuboidal epithelium with additional cilia in our study (III. 3.2.1. **Figure 4**), according to a previous report (O'SHEA, 1968). Fossa cysts are hormonally inactive and not directly involved in behavioral problems in mares, but can cause infertility through ovulation blockage due to their localization next to the ovulation fossa (KENNEDY et al., 1998; ELLENBERGER et al., 2009). However, they were not regarded as pathological in our study, as they are commonly occurring in equine ovaries without clinical relevance (ELLENBERGER et al., 2009).

In conclusion, we assume an association of anovulatory-like follicles to behavioral problems in mares and suggest them as one pathohistological explanation for successful BO due to their high incidence in this study. However, more research on anovulatory-like follicles and other cyst-like structures in equine ovaries is necessary to determine, if their presence is comparable to follicular cysts or ovarial cysts in cattle, and moreover to evaluate their pathological background.

4. Early neoplastic changes

A further possible pathohistological explanation for behavioral improvement after BO could be ENCs. Early neoplastic changes were pathohistologically determined in 15% of bOE, including one ovary of Case 10 and 36, and both ovaries of Case 29. All three mares presented aggressive or stallion-like behavior and showed no abnormalities with normal sized ovaries on rectal and sonographical examination. Macroscopical analysis of ovarian gross sections with ENCs revealed a suspicious area in only one ovary (Case 29, III. 3.2.2. **Figure 5A, B**), but ovarian abnormalities were macroscopically not detectable in others. Pathohistological evaluation revealed ether CL tissue (Case 29 and 10) or antral follicles (Case 36) on the same and/or the contralateral ovary, which would indicate functional ovaries. Mares with developing GCTs are reported to continue normal estrous cycle with the presence of functional CL tissue (MCCUE et al., 1991). This is explained by the fact that the inhibitory effect of inhibin might not be present on the contralateral ovary yet (HINRICHS et al., 1990; MCCUE et al., 1991). Furthermore, cases with bilaterally occurring GCTs, elevated serum AMH, stallion-like behavior, but normal cyclicity, and sonographically detectable CLs are published (CASTILLO et al., 2019). Even

pregnant mares with diagnosed unilateral GCTs and subsequent foaling without complications are reported (VAN DER ZAAG et al., 1996; CHOPIN et al., 2002; CRABTREE et al., 2013). Therefore, a transitional period between the initial formation of neoplastic GCs and the loss of normal estrous cycle is assumed (BALL et al., 2013).

Early neoplastic changes were defined as clinically nondetectable, microscopically small nests of neoplastic GCs and polyhedral LLCs in this study and regarded as precursor stages for GCTs (DEVICK et al., 2020). An assumed transitional period might have contributed to varying outcomes of serum hormone concentrations, clinical examinations, and pathohistological analysis in those three cases with ENCs. Clinical and pathohistological examination by means of routine diagnostic could have failed to detect ENCs in other cases with successful outcome of BO, but no detectable pathohistological abnormalities.

In conclusion, ENCs could be a further pathohistological explanation for the high success rate of BO in mares with behavioral problems. However, clinical findings of this study underline, that their existence might be difficult to prove by routine diagnostics and therefore needs further investigation.

5. Anti-Müllerian hormone in mares with behavioral problems

In case of mares with behavioral problems and clinically and pathohistologically healthy ovaries, we assume an association to serum hormones, as removal of hormone-producing follicles resulted in rapid improvement of behavior after BO in some cases. Bilateral ovariectomy in Case 25 and other mares with no pathohistological ovarian abnormalities could have had a positive effect on behavioral improvement due to elimination of hormone-producing cells of ovarian follicles (CHRISTENSEN, 2011). Serum hormones are sensitive markers for ovarian pathologies and especially AMH represents a highly sensitive marker for GCT presence (BALL et al., 2008; ALMEIDA et al., 2011; CRABTREE, 2011). This hormone is reported as independent of daily fluctuations, cyclic stage, and pregnancy compared to the other hormones testosterone, estrogen, and progesterone (ALMEIDA et al., 2011; BALL et al., 2013, 2014; CRABTREE et al., 2013; CONLEY and BALL, 2017). However, 30% of the mares in bOE showed increased levels, although pathohistologically detectable ENCs were only found in two of the six mares.

Serum AMH is further regarded as reflection of the mare's reproductive age, as it depends on the mare's number of antral follicles (CLAES et al., 2015). This fact could explain the increased serum AMH of 43.5 pmol/L in Case 16 with otherwise no detectable ovarian abnormalities or behavioral changes. However, the reported mean AMH concentration of 3.4 pmol/L in middle-aged mares (CLAES et al., 2015) was much lower compared to 43.5 pmol/L of the ten-year-old mare (Case 16) and antral follicles were neither detected on ultrasonographical examination nor on microscopical analysis of the removed ovaries. Nevertheless, serum AMH of Case 16 decreased to normal levels after BO, which declares the ovaries as source of AMH (ALMEIDA et al., 2011; CLAES

et al., 2015), although a direct association to a pathohistological and behavioral abnormality was missing. In such mares with increased serum hormone levels, but otherwise no clinical evidence for an ovarian pathology, a transvaginal ovarian biopsy sample (HAAG et al., 2013a, 2013b) could provide additional information. However, this invasive method might result in false negative outcomes in case of ENCs or GCTs that involve only a small part of the ovary. Moreover, an endometrial biopsy might be only informative in mares with advanced GCTs (BARTMANN et al., 2001). Unclear cases like mare 16 therefore represent a diagnostic challenge and repeated measurements of serum hormones (CLAES et al., 2014) might be helpful to decide for BO. However, we should not rely on serum AMH, as wide fluctuations in individual mares are reported (RENAUDIN et al., 2021).

Regarding behavioral abnormalities, increased serum hormone concentrations were rarely associated to behavioral problems in one study (HUGGINS et al., 2023) and normal concentrations of serum hormones might also induce misbehavior (STRATICÓ et al., 2023). However, this assumed individual association might be difficult to prove by measurement of serum hormones. Therefore the influence of hormones on behavior should not be underestimated, especially in mares, where no pathohistological findings can be found, but improve with behavior after BO.

6. Other impacts on behavior

Behavior is a complex trait and influenced by many factors. Some behavioral problems might be unrelated to ovarian pathology and are therefore misattributed to the ovaries (MCDONNELL, 2017; HUGGINS et al., 2023). Consequently, BO might not be effective in all mares with behavioral problems, especially in case of learned behavior or other non-ovarian-related causes like gastrointestinal problems (NOUT-LOMAS and BEACOM, 2015; MCDONNELL, 2017).

Two mares (Case 39, 49) presented recurrent, estrous-related colic signs responsive to conservative treatment before surgery, but clinical examination revealed no abnormalities in both mares. Further information regarding other body systems (NOUT-LOMAS and BEACOM, 2015) was not available in most of the cases. Nevertheless, the ovaries were considered as source of pain in both mares, according to the “painful ovary syndrome” (PRYOR and TIBARY, 2005; CRABTREE, 2016, 2022), as colic signs were repeatable related to estrus. The failure of BO in those mares, however, emphasized the likelihood of non-ovarian related causes for behavioral problems (NOUT-LOMAS and BEACOM, 2015; MCDONNELL, 2017; HUGGINS et al., 2023), even if conservative treatment with oral Altrenogest or GnRH vaccination showed positive effects before BO. The owner of Case 40 was initially highly satisfied with the outcome of BO in his mare, until she showed persistent estrous signs with reoccurring behavioral problems after 6 months post surgery. Persistent estrous signs were with 10% of all bilaterally ovariectomized mares lower in this study compared to the reported occurrence of 20-35% (HOOPER et al., 1993; ROESSNER et al., 2015; COLLAR et

al., 2021). An extragonadal production of sex steroids, most likely from the adrenal cortex, is presumably responsible for persistent estrous signs (ASA et al., 1980; SILBERZAHN et al., 1984; HEDBERG et al., 2007a, 2007b). The fact of absent luteal tissue as primary source of progesterone production is moreover discussed, as progesterone has a more important role as inhibitory effect on estrous behavior than the presence of estrogens itself (NETT et al., 1976; WATSON and HINRICHS, 1989). We therefore assume, that persistent estrous signs after BO might have triggered the initially presented behavioral problems in Case 40, as both features occurred simultaneously after six months and further improved after daily Altrenogest treatment.

Consequently, as BO does not thoroughly eliminate behavioral problems in all mares, we highly recommend the advice of all possible outcomes before surgery, so that expectations of owners remain reasonable (ROESSNER et al., 2015).

7. Outcome of bilateral ovariectomy

Bilateral ovariectomy revealed successful outcomes in 85% of the mares in bOE regarding behavioral improvement from the owner's perspective. Our results were therefore comparable with other reported success rates (40%, MELGAARD et al., 2020; 83%, KAMM and HENDRICKSON, 2007; 89%, DEVICK et al., 2020; 90%, COLLAR et al., 2021; 91%, STRATICÓ et al., 2023). Owners were retrospectively asked 6-12 months after BO by means of a telephone survey, as commonly conducted for evaluating the mare's behavior (KAMM and HENDRICKSON, 2007; MELGAARD et al., 2020; COLLAR et al., 2021; STRATICÓ et al., 2023). In our study, a total of 90% of the owners in bOE and GCT-uOE reported behavioral improvement in their mares. However, additional scoring of behavioral problems (MELGAARD et al., 2020; STRATICÓ et al., 2023) was missing in owner's questioning in our study and might therefore reinforce a subjective owner's opinion.

Interestingly, the extremely difficult and aggressive behavior of Case 25 towards people and other horses significantly improved after BO within a few days while the mare was still hospitalized. A possible placebo effect from owner's perspective could therefore be ruled out. The mare presented an increased serum AMH concentration of 30.3 pmol/L with normal serum testosterone before surgery, but pathohistological examination of both ovaries revealed no abnormalities. As GnRH vaccination has a significant impact on ovarian activity and ovarian size (IMBODEN et al. 2006; ELHAY et al., 2007; BEHRENDT et al., 2021), possible ovarian abnormalities might have been suppressed by GnRH vaccination in Case 25. This finding was according to five other mares in bOE with GnRH vaccination before surgery. As mentioned before, detection of ovarian pathologies like ENCs by routine diagnostics might also have failed in some of those cases.

8. Practical relevance

A high success rate of BO in mares with behavioral problems has been reported

and could be confirmed by results of the present study. This could partly be explained by pathohistologically detected early neoplastic changes (ENCs) and anovulatory-like follicles in some mares with bilaterally removed, clinically unremarkable ovaries. In cases of mares with behavioral improvement after BO without routine pathological ovarian findings, we conclude an individual hormonal influence, which might not be measurable, or a detection failure of microscopically small ovarian changes by routine diagnostics. Early neoplastic changes were detectable on pathohistological evaluation, but clinical routine diagnosis by means of ultrasonographical examination and rectal palpation or evaluation of serum AMH or testosterone failed to clearly indicate their presence. Moreover, immunohistochemical analysis of Ki67, AMH, AR, EGFR, CAL, and E-Cad did neither detect ENCs nor advanced GCTs and failed to differentiate those tumorous structures from healthy ovarian tissue. Those findings therefore underline, that routine diagnostic is sufficient in distinct ovarian pathologies. However, especially in unclear cases or early changes, it is not completely reliable, when a decision for BO or unilateral removal of one ovary should be made. This detection failure by routine diagnostics would consequently mean a possibly higher incidence of ovarian abnormalities like ENCs and thus strengthens the justification for BO due to medical indication. We therefore suggest further studies on clinical and pathohistological detection of abnormal ovarian structures besides GCTs in order to justify BO for mares with behavioral problems in equine practice.

V. SUMMARY

Mares with behavioral problems but clinically unremarkable ovaries are a common presenting issue for owners, trainers, and veterinarians. Such mares are mostly successfully treated with bilateral ovariectomy (BO) with a reported high owner satisfaction due to behavioral improvement. In case of a clinically not detectable ovarian pathology, however, BO is discussed due to animal welfare aspects regarding removal of healthy organs. In general, there is a lack of a pathohistological explanation for behavioral improvement after BO.

We therefore conducted a pathohistological study on clinically unremarkable ovaries of mares referred from veterinarians for BO due to behavioral problems and hypothesized that ovaries of those mares demonstrate other, non-neoplastic abnormalities that could explain the fact of successful BO. The objective was to compare bilaterally removed, clinically unremarkable ovaries with pathohistologically confirmed ovarian granulosa cell tumors (GCTs) by means of immunohistology. We further aimed to assess a complete data set regarding clinical history, clinical examination, serum hormone concentrations, and surgical outcome. This clinical part was implemented as basis for the pathohistological study of the removed ovaries of each mare.

Case selection was based on uni- or bilateral ovariectomy. One group included twenty mares with bilaterally removed, clinically unremarkable ovaries (n=40) and was termed "bOE". The other group included ten mares with unilaterally removed ovaries, which were clinically suspected and histopathologically confirmed GCTs (n=10), and was termed "GCT-uOE". The clinical part involved a detailed clinical history by means of owner's questionnaire regarding behavioral patterns and conservative pre-treatments with Gestagen-Analoga or GnRH vaccination, clinical examination including rectal palpation and ultrasonographical evaluation of the ovaries, and determination of cycle stage, serum hormone measurement before surgery, and surgical outcome regarding behavioral improvement by means of postoperative owner's questionnaire. Serum hormone analysis included anti-Müllerian hormone (AMH) measurement of all mares and serum testosterone measurement of 55% in bOE and 70% in GCT-uOE. The pathohistological part started with a macroscopical evaluation of gross sections and size measurement of the ovaries immediately after surgical removal. One representative tissue sample of each ovary of both groups was additionally sent to an external commercial laboratory to confirm or exclude the presence of GCT. Further pathohistological preparation and evaluation of the ovaries was conducted in cooperation with the Institute of Morphology of the University of Veterinary medicine in Vienna (Austria). Ovarian structures were histomorphologically evaluated in bOE and GCT-uOE. Different diagnostic markers were used for immunohistochemical analysis in both groups including Ki-67 (Ki67), AMH, aromatase (AR), epidermal growth factor receptor (EGFR), calretinin (CAL), and epithelial cadherin (E-Cad). Immunohistochemical expression was assessed according to others and statistically compared between

the two groups.

Bilateral ovariectomy resulted in a high owner satisfaction with behavioral improvement in 85% of the mares in bOE. In the GCT-uOE group, all mares showed an improvement in behavior after removal of the neoplastic ovary according to the owners, resulting in an overall success rate of 90% for the mares in both groups, which is comparable to previously published success rates. Preoperatively measured serum AMH and testosterone concentrations were significantly higher in GCT-uOE compared to bOE. Serum AMH was reliably high in case of advanced tumor stage, which was present in all mares in GCT-uOE. However, serum AMH was variable in bOE with increased concentrations in 30% of the mares and led to adjustment of the reference concentrations to higher cut-off limits by the external laboratory. Immunohistochemical evaluation revealed no clear differentiation between large follicular structures of clinically unremarkable ovaries in bOE and cyst-like structures of GCTs in GCT-uOE by means of the diagnostic markers and is according to other reports. However, pathohistological evaluation of bilaterally removed ovaries in bOE revealed clinically nondetectable early neoplastic changes (ENCs) in 15% and a high incidence of anovulatory-like follicles in 30% of the mares with behavioral problems. Early neoplastic changes were regarded as precursor stages of neoplasia and anovulatory-like follicles were found more frequently than reported before. Therefore, surgical removal of ovaries with these two structures might be one explanation for the high success rate of BO regarding behavioral improvement. Furthermore, some ENCs (75%) were growing from anovulatory-like follicles in bOE and might be involved in GCT development. Frequently detected fossa cysts in 35% of bOE were not regarded as pathological due to their reported common occurrence without clinical relevance. Invasive growing Leydig-like cells (LLCs) in 10% and erythroblasts in form of extramedullary hematopoiesis in 20% of GCTs in GCT-uOE were observed. Both special characteristics were to our knowledge not reported in GCTs of equines or other species before.

In conclusion, BO resulted in a high success rate in mares with clinically unremarkable ovaries regarding behavioral improvement from the owner's perspective in this study. This benefit could partly be explained by the pathohistological presence of anovulatory-like follicles or ENCs, which could be indicative for the development of ovarian GCTs. The immunohistochemical analysis of Ki67, AMH, AR, EGFR, CAL, and E-Cad did not show significant differences between clinically unremarkable and GCT-affected ovaries and needs further investigations.

VI. ZUSAMMENFASSUNG

Stuten mit Verhaltensproblemen aber klinisch unauffälligen Ovarien stellen ein häufiges Problem für Besitzer und Trainer und eine Herausforderung für Tierärzte dar. Diese Stuten werden mit bilateraler Ovariektomie (BO) hinsichtlich Verhaltensverbesserung und Besitzerzufriedenheit meist erfolgreich behandelt. Im Falle einer klinisch nicht nachweisbaren Ovarpathologie wird die BO jedoch in Bezug auf die Entfernung gesunder Organe aus tierschutzrechtlicher Sicht kontrovers diskutiert. Es ist zudem weitgehend ungeklärt, wie eine Verhaltensproblematik bei Stuten auf pathohistologischer Ebene zu erklären ist, um damit eine BO bei klinisch unauffälligen Ovarien rechtfertigen zu können.

Aufgrund dieser Tatsache wurde eine pathohistologische Studie an klinisch unauffälligen Ovarien von Stuten durchgeführt, die aufgrund von Verhaltensproblemen zur BO überwiesen wurden. Wir stellten die Hypothese auf, dass die Ovarien dieser Stuten andere, nicht-neoplastische Veränderungen aufweisen, welche den Erfolg der BO hinsichtlich Verhaltensverbesserung erklären könnten. Ziel dieser Studie war es zudem, beidseitig entfernte, klinisch unauffällige Ovarien mit pathohistologisch bestätigten ovariellen Granulosazelltumoren (GCT) bei einseitig ovariektomierten Stuten mittels Immunhistologie zu vergleichen. Darüber hinaus wurde eine vollständige Datenanalyse aller untersuchten Stuten durchgeführt. Diese umfasste eine ausführliche Anamnese, klinische Untersuchungen des Reproduktionstraktes, Serumhormonanalysen von Anti-Müller-Hormon (AMH) und Testosteron sowie postoperative Besitzerbefragungen bezüglich Verhaltensverbesserungen. Die klinische Studie wurde retrospektiv als Basis für die pathohistologische Evaluierung der entnommenen Ovarien durchgeführt.

Die Stuten wurden basierend auf ein- oder beidseitiger Ovariektomie in zwei Gruppen unterteilt. Eine Gruppe umfasste zwanzig Stuten mit bilateral entfernten, klinisch unauffälligen Ovarien (n=40) und wurde als „bOE“-Gruppe bezeichnet. Die andere Gruppe wurde als „GCT-uOE“ bezeichnet und inkludierte zehn Stuten mit einseitig entfernten Ovarien mit GCT (n=10), die bereits klinisch verdächtig und pathohistologisch bestätigt wurden. Der klinische Teil umfasste eine ausführliche klinische Anamnese bezüglich Verhaltensmuster und konservativer Vorbehandlungen mit Gestagen-Analoga oder GnRH-Impfung, Ergebnisse von rektaler Palpation und Ultraschalluntersuchung, die Bestimmung des Sexualzyklusstandes, präoperative Serumhormonanalysen und Resultate der BO hinsichtlich Verhaltensverbesserung laut Besitzerbefragung. Serum-AMH wurde bei allen Stuten gemessen, Serum-Testosteron bei 55% in bOE- und bei 70% in GCT-uOE-Stuten. Der pathohistologische Teil beinhaltete eine makroskopische Untersuchung und Größenbestimmung der Ovarien unmittelbar nach chirurgischer Entfernung. Um das Vorhandensein eines GCT zu bestätigen oder auszuschließen, wurde jeweils eine repräsentative Gewebeprobe pro Ovar pathohistologisch durch ein externes Labor untersucht. Die weitere pathohistologische Aufbereitung und Beurteilung der Ovarien

wurde in Kooperation mit dem Institut für Morphologie an der Veterinärmedizinischen Universität Wien durchgeführt. Dabei wurden alle Ovarien beider Gruppen histomorphologisch und immunhistochemisch anhand der diagnostischen Marker Ki-67 (Ki67), AMH, Aromatase (AR), Epidermal-Growth-Factor-Receptor (EGFR), Calretinin (CAL) und Epithelial-Cadherin (E-Cad) untersucht. Die immunhistochemischen Ergebnisse wurden nach etablierten Methoden ausgewertet und beide Gruppen statistisch untereinander verglichen.

Die bilaterale Ovariektomie resultierte in einer Besitzerzufriedenheit von 85% hinsichtlich Verhaltensverbesserung der Stuten in bOE. In der GCT-uOE-Gruppe zeigten alle Stuten nach Entfernung des neoplastischen Ovars eine Verhaltensverbesserung laut Besitzerbefragungen, was für die Stuten beider Gruppen insgesamt zu einer Erfolgsrate von 90% führte und somit mit den Erfolgsraten früherer Studien vergleichbar ist. Stuten in GCT-uOE wiesen präoperativ signifikant höhere AMH- und Testosteron-Werte im Vergleich zu Stuten in bOE auf. Serum-AMH war somit zuverlässig hoch bei fortgeschrittenem Tumorstadium, welches bei allen Stuten in GCT-uOE vorhanden war. Bei Stuten in bOE wurden variable Serum-AMH-Werte mit erhöhten Konzentrationen bei 30 % nachgewiesen, was zu einer Anpassung an höhere Referenzwerte durch das externe Labor führte. Die immunhistochemische Auswertung anhand der diagnostischen Marker ergab keine eindeutige Differenzierung zwischen großen follikulären Strukturen klinisch unauffälliger Ovarien in bOE und zystenähnlichen Strukturen von GCT in GCT-uOE. Diese Ergebnisse sind mit Publikationen anderer Autoren vergleichbar. Die pathohistologische Evaluierung ergab jedoch klinisch nicht nachweisbare, früh-neoplastische Veränderungen (ENC) bei 15% der Stuten in bOE, welche als GCT-Vorstufen angesehen wurden. Des Weiteren konnten „anovulatorisch-ähnliche“ Follikel bei 30% der Stuten in bOE festgestellt werden, deren Inzidenz in der Literatur als deutlich niedriger angegeben wird. Daher kann davon ausgegangen werden, dass die operative Entfernung von Ovarien mit diesen beiden Strukturen an den hohen Erfolgsraten der BO bezüglich der Verhaltensveränderung beteiligt sein könnten. Zudem wurde in den pathohistologischen Untersuchungen festgestellt, dass einige ENC (75%) aus „anovulatorisch-ähnlichen“ Follikeln hervorgingen und diese daher an der Entwicklung von GCT involviert sein könnten. Fossazysten konnten in 35 % der Stuten in bOE evaluiert werden, wurden aber aufgrund ihres generell gehäuften Auftretens als klinisch irrelevant angesehen. Invasiv wachsende Leydig-like Zellen (LLCs) wurden in 10 % und Erythroblasten in Form von extramedullärer Hämatopoese in 20 % der GCT in GCT-uOE beobachtet. Nach unserem Wissensstand wurden diese beiden Merkmale noch nie in GCT von Stuten oder anderen Spezies beschrieben.

Zusammenfassend lässt sich sagen, dass auch in dieser Studie die BO bei verhaltensauffälligen Stuten mit klinisch unauffälligen Ovarien zu einer hohen Erfolgsrate hinsichtlich Verhaltensverbesserung aus Sicht des Besitzers führte. Diese Verhaltensverbesserung könnte auf pathohistologischer Ebene durch das Vorhandensein von „anovulatorisch-ähnlichen“ Follikeln oder ENCs erklärt

werden, die ein Hinweis auf die Entwicklung von ovariellen GCT sein könnten. Anhand der immunhistochemischen Analyse von Ki67, AMH, AR, EGFR, CAL und E-Cad konnte keine Differenzierung zwischen klinisch unauffälligen und GCT-betroffenen Ovarien ermittelt werden und bedarf weiterer Untersuchungen.

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VIII. APPENDIX

Table A1. Results of external and own pathohistological evaluation of ovaries from bilaterally ovariectomized mares with behavioral problems (bOE).

Microscopical Evaluation of removed Ovaries in bOE														
Case	**Ovary**[1]	**External pathohistological Evaluation**					**Own pathohistological Evaluation**							
							Normal Findings [2]					Abnormal Findings [3]		
		Normal	Follicular Cyst	Ovarial Cyst	An-ovulatory Cyst	Unclear	PF	AF	EAF	LAF	CL	Fossa Cyst	AnovF	ENCs
7	L		x						x					
	R		x					x		x		x		
8	L	x							x		x			
	R		x						x					
9	L		x				x					x		
	R		x							x				
10	L	x							x		x			
	R					x	x			x				x
15	L		x										x	
	R		x				x			x	x			
16	L			x						x	x			
	R			x					x	x				
23	L	x						x						
	R	x							x	x			x	
25	L			x			x	x						
	R			x			x			x		x		
29	L	x					x		x		x		x	x
	R					x		x						x
30	L		x									x		
	R		x								x			
34	L				x			x					x	
	R				x					x				
35	L		x				x					x		
	R				x					x		x		
36	L	x						x	x					
	R					x	x	x					x	x
38	L		x				x	x						
	R	x								x				
39	L		x				x							
	R		x				x			x			x	
40	L			x			x	x		x				
	R			x								x		
46	L	x						x						
	R		x				x				x	x		
47	L		x								x			
	R		x								x			
48	L						x	x		x	x			
	R		x				x	x	x	x				
49	L		x				x		x	x				
	R		x						x					

[1] L=left ovary, R=right ovary; [2] Normal findings: PF=preantral follicles including primordial, primary, and secondary follicles; AF=antral follicles including tertiary and preovulatory follicles; EAF=early atretic follicles; LAF=late atretic follicles; CL=Corpus luteum; [3] Abnormal findings: AnovF=anovulatory-like follicles; ENCs=early neoplastic changes;

Table A2. Summary of immunohistochemical evaluation in ovaries of bilaterally ovariectomized mares (bOE) and in ovaries of mares with unilaterally removed granulosa cell tumors (GCT-uOE) including Ki-67 (Ki67), anti-Müllerian hormone (AMH), aromatase (AR), epidermal growth factor receptor (EGFR), calretinin (CAL), and epithelial cadherin (E-Cad).

Immunohistochemical Expression of all determined Ovarian Structures in bOE and GCT-uOE							
Structures of bOE	**Cell Population**	*PI grade*[1]	*Intensity of Expression*[2]				
		Ki67	**AMH**	**AR**	**EGFR**	**CAL**	**E-Cad**
Primordial Follicle[3]	Granulosa Cells	0	-	+	++	+	-
Primary Follicle[3]		0	+	++	++	+	
Secondary Follicle[3]		0	+++	++	++	+	
Tertiary Follicle[4]	Granulosa Cells	1-3	+++	++	+++	++	-
	Theca Cells	1	+	+	+	+	
Preovulatory Follicle[4]	Granulosa Cells	2-3	+++	+++	+++	++	
	Theca interna Cells	1	+	++	++	++	
	Theca externa Cells	0	-	+	++	++	
Early atretic Follicle[4]	Granulosa Cells	2-3	+++	++	+++	+++	-
	Theca interna Cells	1	+	++	++	++	
	Theca externa Cells	0	-	+	+	+	
Late atretic Follicle	Granulosa Cells	0	++	++	+	++	-
Anovulatory-like Follicle[4]	Granulosa Cells	0-2	+	+	++	++	-
Fossa Cyst	Epithelial Cells	0	-	+	+++	+++	++
Corpus Luteum	Lutein Cells	0	-	+++	+++	+	-
Early neoplastic Changes	Granulosa Cells	0	+	+++	+++	++	-
	Leydig-like Cells	0	+	+++	+++	++	
Structures of GCT-uOE							
Granulosa Cell Tumor	Granulosa Cells	0-1	++	+/++	+++	++	-
	Leydig-like Cells	0	+	+++	+++	+++	
	Sertoli-like Cells	0	++	+/++	+++	++	
	Theca Cells	0	-	-	+	+	
	Invasive growing Leydig-like Cells	0	+	+++	+++	+++	-
	Erythroblasts	3	-	-	-	-	-

[1] The proliferation index (PI) of Ki67 is graded in 0-3: grade 0 for 0-25%, grade 1 for 26-50%, grade 2 for 51-75%, grade 3 for 76-100% stained cells; grade 1-3 means a high PI of >25% stained cells; [2] Intensity of expression: - for negative, + for mild, ++ for moderate and +++ for high expression; [3] Primordial, primary, and secondary follicles were summarized as preantral follicles; [4] Tertiary and preovulatory follicles were summarized as antral follicles and together with early atretic and anovulatory-like follicles summarized as large follicles;

IX. INDEX OF PUBLICATIONS

1. Research article

Wolf N, Hahn JA , Walter I, Zablotski Y, Zerbe H, Witte TS. **2024**.

Pathohistological findings in bilateral removed ovaries of mares with behavioral problems.

Animals 14(19), 2899

2. Poster

Witte TS, Wolf N, Walter I, Hahn JA, Zerbe H. **2023**.

Pathohistological findings in bilateral removed ovaries of mares with behavioral problems.

Journal of Equine Veterinary Science 125, 104754

ISER conference 2023

Witte TS, Wolf N, Walter I, Hahn JA, Zerbe H. **2025**.

Pathohistological findings in bilateral removed ovaries of mares with behavioral problems.

Journal of Reproductive Medicine and Endocrinology 22(1), 23-24

58th Annual Conference Physiology and Pathology of Reproduction and 50th Joint Conference of Veterinary and Human Reproductive Medicine at the Faculty of Veterinary Medicine of the University of Leipzig, February 26th-28th 2025

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