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Immunohistochemical markers for equine granulosa cell tumors: a pilot study

Aurelia DOLIN[1], Peter SCHWEIGER[1], Martin WASELAU[2], Monika EGERBACHER[3]* and Ingrid WALTER[4]

[1]Veterinary Practice Tierdoc, 6233 Kramsach, Austria
[2]Equine Hospital Aschheim, Equine Diagnostic Center Munich, 85609 Munich, Germany
[3]Administrative Unit Veterinary Medicine, UMIT TIROL-Private University for Health Sciences, Medical Informatics and Technology GmbH, 6060 Hall in Tirol, Austria
[4]Department of Pathobiology, Institute of Morphology, Vetmeduni Vienna, 1210 Vienna, Austria

Sex cord–stromal tumors (SCSTs), generally referred to as granulosa cell tumors (GCTs) or granulosa-theca cell tumors (GTCTs) in equids, show complex compositions and variable numbers of hormone-producing cells. These tumors can be difficult to diagnose, especially in early stages. Therefore, we tested a panel of antibodies for vimentin, smooth muscle actin, laminin, Ki-67, E-cadherin, calretinin, moesin, p-ezrin, AMH, and aromatase, markers used for tumor composition and classification, progression, and prognosis in human SCSTs, on an exemplary grapefruit-size equine GCT within the left ovary of a 13-year-old mare with stallion-like behavior and elevated testosterone levels in comparison with normal ovarian tissue. The tumor showed a low proliferation rate and prominent moesin and p-ezrin staining in granulosa cells. E-cadherin, calretinin, aromatase, and AMH are suggested to be potential markers for different cell components of equine SCSTs that can support tumor diagnosis and classification.

Key words: *diagnostic marker, horse, ovary, tumor*

J. Equine Sci.
Vol. 34, No. 2
pp. 37–46, 2023

Granulosa cell tumors (GCTs) are the most common type of tumors of the equine ovary, followed by teratomas, cystadenomas, and dysgerminomas [8, 33], and are also prevalent in other species [21, 30, 35]. Mares suffering from GCTs often present with persistent anestrus, stallion-like behavior with aggression towards handlers or other horses, prolonged estrus, or nymphomania [33, 39]. While the diagnosis equine GCTs is straightforward in many cases, a number of studies have reported unclear abnormal ovarian conditions, inconclusive hormonal statuses in relation to behavioral changes, and atypical ultrasonographic results [9, 22, 23, 33, 37, 40]. Therefore, we tested a panel of antibodies on an equine GCT case and normal ovarian tissue as a pilot study to establish potential markers for tumor diagnosis, composition, progression, and prognosis in equine patients.

A 13-year-old Wielkopolski mare was presented with severe behavioral changes. The mare was exhibiting increasing aggression towards other horses in the stable, was difficult and fractious under saddle, and had injured a handler. It was nulliparous and had a regular but prolonged cycle of 27 d. Transrectal palpation revealed a normal-sized, unremarkable right ovary, along with an enlarged, approximately grapefruit-sized left ovary (Ø15 cm) that felt irregular and dense to the touch. Transrectal ultrasonography revealed a functional right ovary (Fig. 1A) but exposed an enlarged left ovary with multiple honeycomb-like, fluid-filled structures (Fig. 1B). Blood was collected and sent to a reference laboratory (IDEXX Laboratories, Kornwestheim, Germany) for determination of testosterone levels. The results showed highly elevated levels of 0.34 ng/m*l*.

As all the diagnostics strongly suggested a granulosa cell tumor, the mare was referred to an equine clinic for ovariectomy of the left ovary. Surgery was performed in two steps. During phase one, the mare was kept sedated (0.02 mg/

Received: May 6, 2022
Accepted: January 18, 2023
*Corresponding author. e-mail: monika.egerbacher@gmail.com

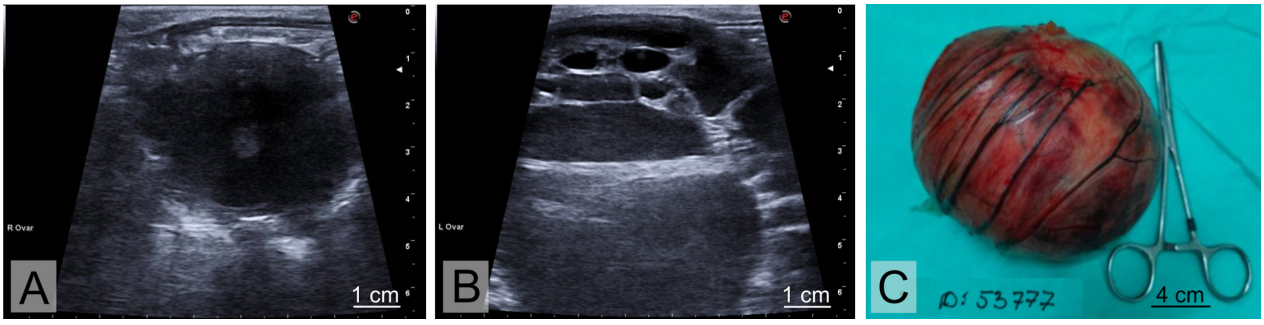


Fig. 1. Transrectal ultrasound of the contralateral right ovary with a normal follicular structure (A), ultrasonographic "honeycomb" appearance of the enlarged left ovary showing multiple cystic structures (B), and photograph of the GCT-affected ovary after surgical removal (C).

kg detomidine i.v., Cepesedan RP 10 mg/m*l*, CP-Pharma, Burgdorf, Germany, and 0.04 mg/kg butorphanol i.v., Butorgesic® 10 mg/m*l*, CP-Pharma) and standing in order to perform laparoscopy. The left flank was blocked with a local anaesthetic (lidocaine hydrochloride 2%, 20 mg/m*l*, Bela-Pharm, Vechta, Germany), and two incisions were made for insertion of instruments. The enlarged left ovary and mesovarium were located, the latter was injected with a local anaesthetic (lidocaine hydrochloride 2%), and the ovary was detached using LigaSure™ (Medtronic, Meerbusch, Germany) and dropped into the abdominal cavity. The two small laparoscopic incisions were closed in two layers in a routine fashion. In phase two, the mare was initially placed under general anaesthesia and sedated with xylazine 1.1 mg/kg body weight (bw) i.v. (XYLARIEM® 20 mg/m*l*, Ecuphar, Greifswald, Germany). Anesthesia was induced using a combination of diazepam 0.1 mg/kg bw i.v. (Solupam 5 mg/m*l*, Dechra Veterinary Products, Aulendorf, Germany) and ketamine 2.2 mg/kg bw i.v. (Ketamin 100 mg/m*l*, CP-Pharma,), followed by isoflurane 1.3% p.i. (Isofluran CP® 1 m*l*/m*l*, CP-Pharma), and the mare was placed in dorsal recumbency. A mini-laparotomy was performed to retrieve the detached ovary (Fig. 1C), and the wound was closed in three layers.

Postoperatively, a broad-spectrum antibiotic (benzylpenicillin-natrium 22,000 IU/kg i.v., Selectavet, Weyarn, Germany; gentamicin 6.6 mg/kg i.v., Genta 100 mg/m*l*, CP-Pharma,), nonsteroidal anti-inflammatory drug (flunixin 1.1 mg/kg i.v., Flunidol® RPS, 50 mg/m*l*, CP-Pharma), and gastric protective agent (omeprazole 4 mg/kg p.o., Gastro-Gard® 37%, Böhringer, Ingelheim, Germany) were administered for 6 days. Three months after removal of the GCT, testosterone levels normalized at 0.01 ng/m*l*, and the mare resumed a normal estrous cycle, confirming the tumor as the source of hormone production. After obtaining informed client consent, four representative parts of the tumor (Fig. 1C) were fixed in 4% buffered formaldehyde and embedded in paraffin for subsequent analysis of histopathology and immunohistochemistry. Sections were stained with hematoxylin and eosin, and consecutive serial sections were used for immunohistochemical detection (indirect method, horseradish peroxidase based) of anti-Müllerian hormone (AMH/MIS), aromatase, calretinin, E-cadherin, Ki-67, laminin, moesin, p-ezrin, smooth muscle actin (SMA), and vimentin (Table 1).

Archived tissue from horse ovary, testis, kidney, urinary bladder, fetal gonad, lymph node, cerebrum, and cerebellum were used as positive controls. For negative control experiments, the primary antibody was omitted, and respective isotype controls (mouse, rabbit, goat) were applied (Fig. 2). For quantification of proliferation, the percentage of Ki-67-positive stained nuclei in tumor cells among a total of 5×10^3 tumor cells were manually counted in 3 tissue blocks and 5 high-power fields each at a magnification of 40x (Olympus BX53).

The tumor parts showed variable structural compositions with follicles of different sizes (Fig. 3A and 3B), areas of accumulations of granulosa cells organized in strands and nests (Fig. 3B and 3C), as well as areas of densely packed stromal cells (Fig. 3D). Cystic structures showed intact multilayered granulosa cell linings and signs of initial atresia starting with loss of epithelial cohesion (Fig. 3E) to almost total loss of granulosa cells (Fig. 3A, 3B). Granulosa cells of basal layers quite frequently showed a Sertoli-like appearance (Fig. 3F). Numerous Leydig-like cells were found in groups in a highly vascularized theca interna or within GC nests (Fig. 3G).

In GCT and control tissue, granulosa cells were positive for vimentin, while theca cells were positive for vimentin, SMA, and laminin. Fibroblastic stroma cells were strongly positive for vimentin and SMA in control tissue (Fig. 4A-F). Ki-67-positive staining was identified by the presence of

Table 1. Source, pretreatment, and dilution of the antibodies and results of immunostaining

Antibody	Granulosa cells	Theca cells	Fibroblastic stroma cells	Sertoli-like cells	Leydig-like cells
Vimentin (V9; mouse 1:500, Code no. M0725, Agilent Technologies, Santa Clara, CA, USA; 30 min 0.01 M citrate buffer, pH 6.0, steamer)	+	+	+/−	+/−	−
Smooth muscle actin (SMA 1A4; mouse 1:1,000, Code no. M0851, Agilent Technologies, Santa Clara, CA, USA; w/o)	−	+	+	−	−
Laminin (Rabbit 1:10,000, code no. Z0097, Agilent Technologies, Santa Clara, CA, USA; 0.1% protease/PBS 20 min, RT)	−	+/−	+/−	−	−
Ki-67 (MIB-1; mouse 1:1,000, Code no. M7240, Agilent Technologies, Santa Clara, CA, USA; 30 min 0.01 M citrate buffer, pH 6.0, steamer)	+	−	−	−	−
E-cadherin (Rabbit 1:500, Cat. no. sc-7870, Santa Cruz Biotechnology, Dallas, TX, USA; 30 min Tris-EDTA, pH 9.0, steamer)	+/−	−	−	+/−	+
Calretinin (Rabbit 1:5,000, Product no. AB149, Chemicon, Temecula, CA, USA; 30 min 0.01 M citrate buffer, pH 6.0, steamer)	+	+	+/−	+	+
Moesin (EP1863Y; rabbit 1:800, Product code ab52490, Abcam, Cambridge, UK; 30 min Tris-EDTA, pH 9.0, steamer)	+	+	+/−	+	−
p-Ezrin (48G2; rabbit 1:800, Product no. 3726, Cell Signaling Technology, Danvers, MA, USA; 30 min 0.01 M citrate buffer, pH 6.0, steamer)	+	+/−	−	+	−
Anti-Müllerian Hormone (AMH/MIS; goat 1:200, Cat no. sc-6886, Santa Cruz Biotechnology, Dallas, TX, USA; 30 min Tris-EDTA, pH 9.0, steamer)	+	−	−	+	+
Aromatase (Rabbit 1:1,000, Cat no. 3599-100, BioVision, Milpitas, CA, USA; 30 min Tris-EDTA, pH 9.0, steamer)	+/−	+/−	+/−	+/−	+

Secondary antibodies: BrightVision Poly-HRP-anti-rabbit, anti-goat, or anti-mouse (Immunologic, Duiven, The Netherlands). Signal detection: diaminobenzidine (DAB; Quanto Chromogen TA-125-QHDX, Thermo Fisher Scientific, Waltham, MA, USA). Symbols: +, positive staining; +/−, weak staining; −, negative staining.

nuclear staining in granulosa tumor cells and control follicles (Fig. 4G and 4H). The mean Ki-67 index obtained as a percentage of 1,000 counted cells was 3.87 (standard deviation ± 1.90) in the tumor. The median Ki-67 index was 3.73 (range 1.37–8.07). Leydig-like cells were negative for cytoskeletal marker proteins vimentin and SMA but were prominently visible through staining for E-cadherin and calretinin (Fig. 4I and 4K).

Granulosa cells within the GCT showed delicate but very distinct cell membrane-associated staining for moesin and p-ezrin, which was not the case in the developing antral follicle of the control ovary. Both markers showed the endothelial lining of the blood vessels and capillaries in the theca interna and stroma. Theca externa cells were strongly positive for moesin but showed only weak immunoreactivity to anti-p-ezrin (Fig. 4M–P). AMH immunostaining revealed distinct staining of the GCT specifically highlighting the Sertoli-like cells and control follicle granulosa cells. In the GCT, Leydig-like cells within the theca were also clearly detected by AMH, whereas the theca in the control ovary was not stained (Fig. 4Q and 4R). Leydig-like cells were also identified by aromatase immunostaining in the GCT. The aromatase staining was much weaker in the tumor granulosa cells and the control follicle (Fig. 4S and 4T).

The present case of a GCT showed the typical clinical, diagnostic, and morphological pattern of a GCT with a multicystic appearance due to follicular structures of various size as well as areas composed of granulosa cell and stromal cell accumulation [32, 39]. The presence of Sertoli- and Leydig-like cells, however, pointed to a heterogeneous tumor composition, which allowed us to test potential immunohistochemical marker proteins for a more thorough diagnostic evaluation of equine GCTs/granulosa-theca cell tumors (GTCTs) that could be helpful in early unclear tumor stages.

In human ovarian cancer classification, sex cord-stromal tumors (SCSTs) originating from granulosa, theca, or other stromal cells are classified as different entities, namely

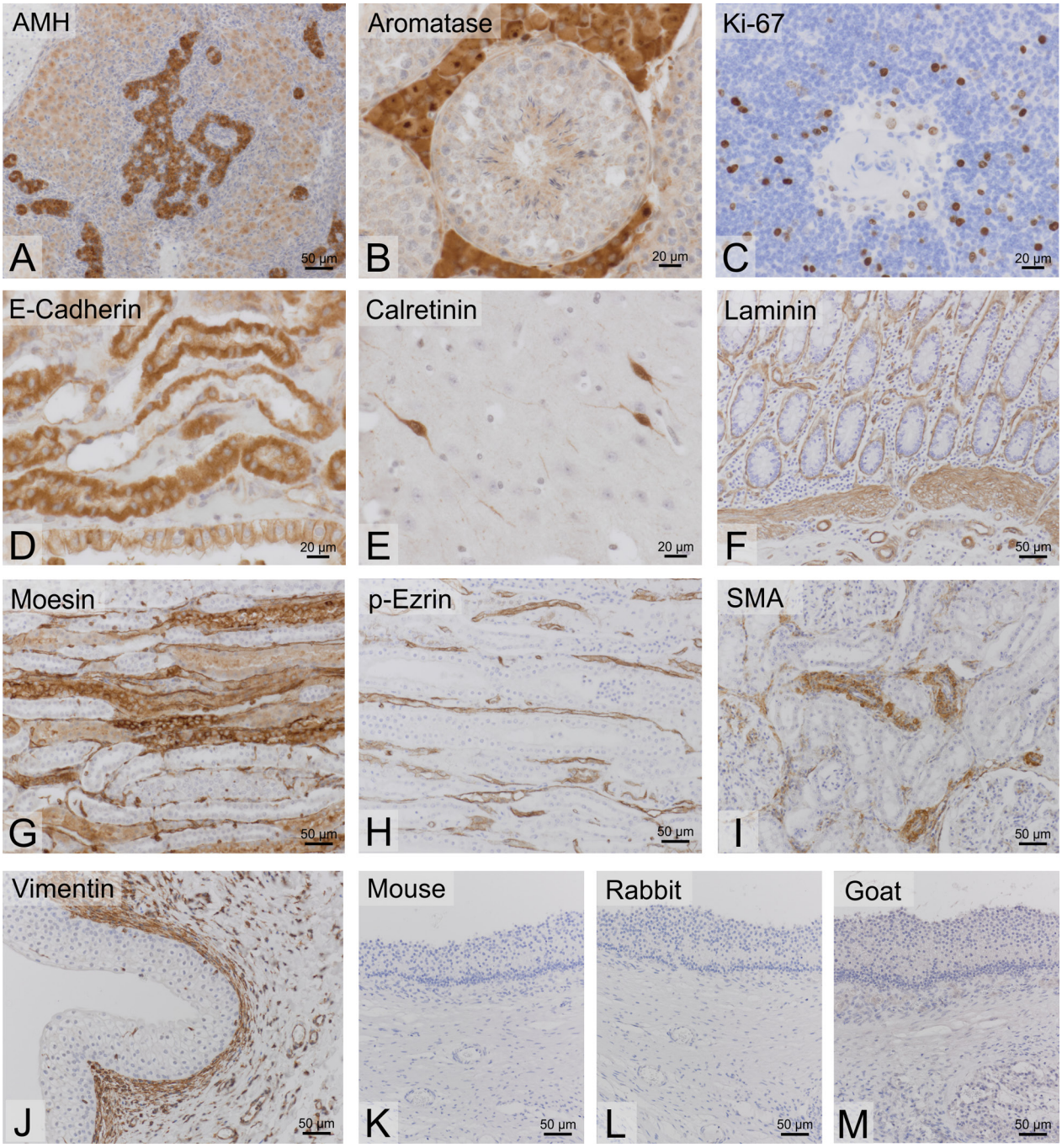


Fig. 2. Positive control staining of equine tissue including the fetal gonad (A), adult testis (B), lymph node (C), kidney (D), cerebrum (E), colon (F), kidney (G–I), and urinary bladder (J). Isotype controls for the respective secondary antibodies were applied on GCT sections: mouse (K), rabbit (L), and goat (M).

GCTs (adult or juvenile form), thecomas, fibromas, Sertoli cell tumors, and Sertoli-Leydig cell tumors [12]. While GCTs are associated with high estrogen levels and the presence of hormonally active theca cells, elevated testosterone levels with signs of virilization are rare in humans with GCTs [14, 27]. Sertoli-Leydig cell tumors coincide with high testosterone levels and consequent signs of virilization [12]. In contrast, no explicit distinction seems to be made among sex cord-stromal tumors of the equine ovary other than the adjunct of theca cells (GTCT) in cases where a

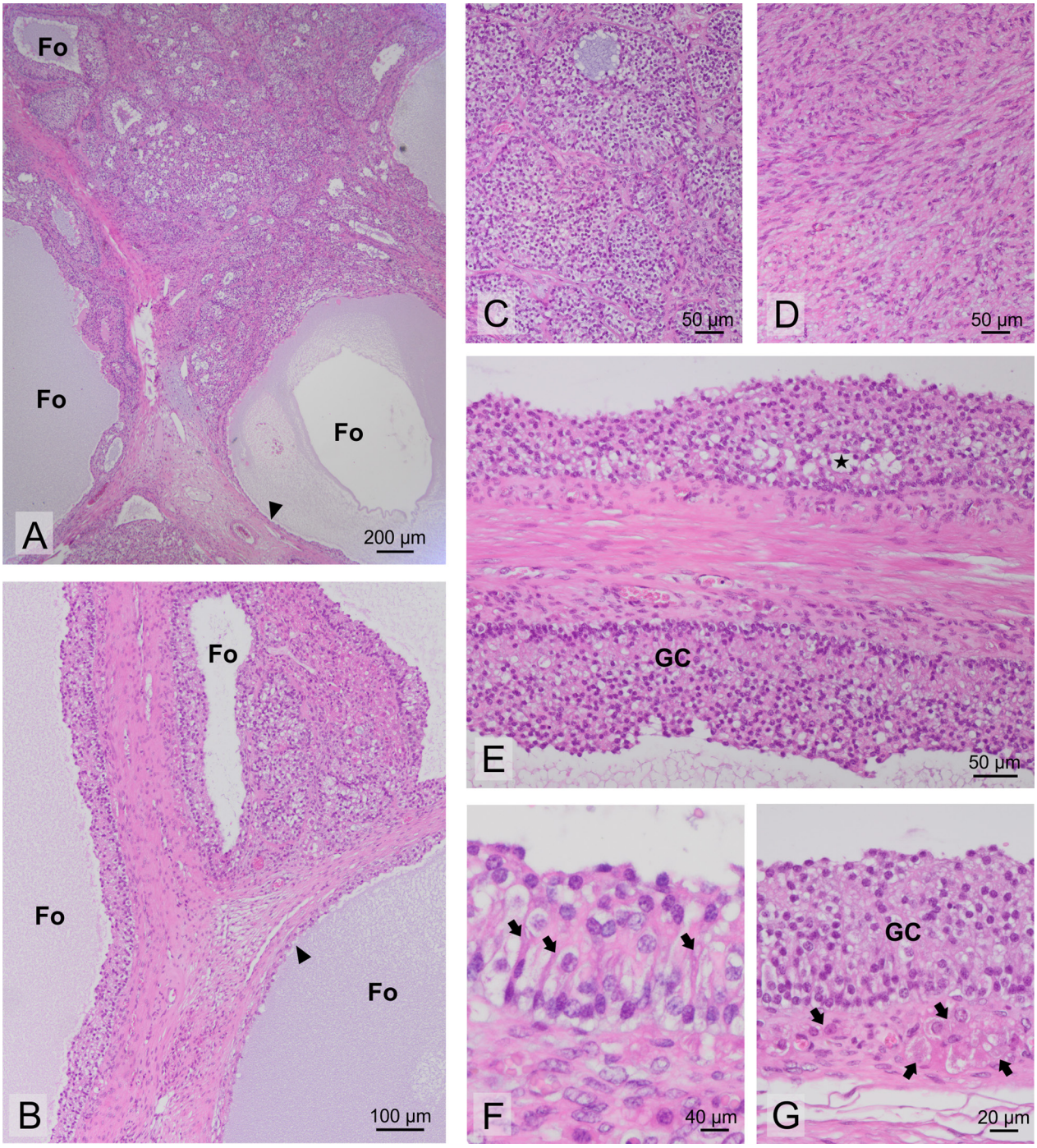
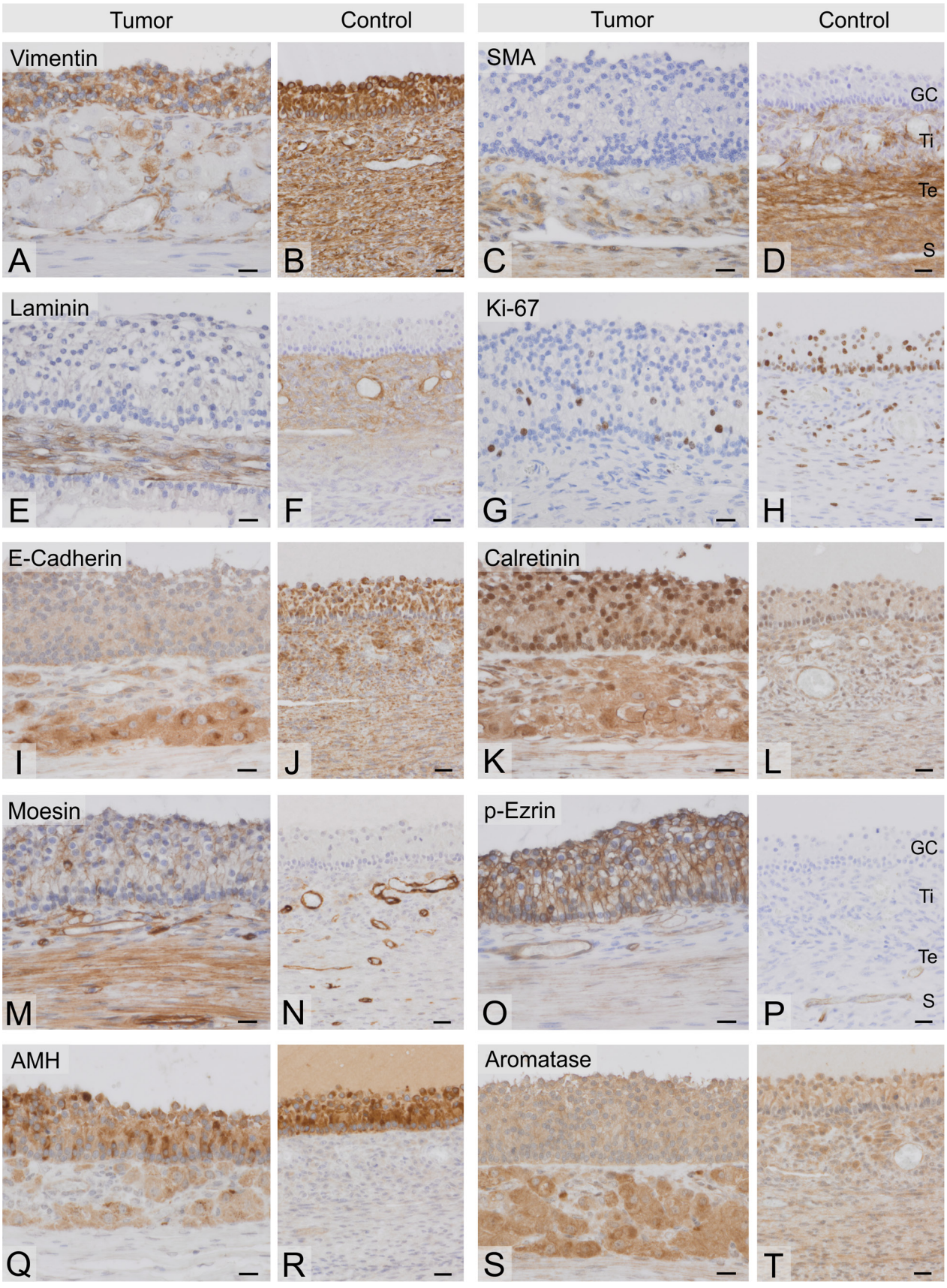


Fig. 3. Hematoxylin and eosin staining of the GCT. The tumor parts showed variable structural compositions with follicles (Fo) of different sizes (A and B), areas of accumulations of granulosa cells organized in strands and nests (B and C), as well as areas of densely packed stromal cells (D). Follicles with intact multilayered granulosa cell linings (GC), some with initial signs of atresia with loss of epithelial cohesion (asterisk in E) to almost total loss of granulosa cells (arrowheads in A and B) were seen. Granulosa cells of basal layers with Sertoli-like appearances (arrows in F) and numerous Leydig-like cells were found in groups in the highly vascularized theca interna (arrows in G).

substantial part of the tumor is composed of theca cells [22, 33]. This may contribute to the structural variability as well as to the inconsistency of diagnostic parameters for tumors classified as equine GCT or GTCT. As sex cord-stromal tumors, GCTs have the potential to produce hormones, so diagnosis also heavily relies on testing of serum hormone


Tumor
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Tumor
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Ki-67
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H
E-Cadherin
Calretinin
I
J
K
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Moesin
p-Ezrin
GC
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AMH
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Fig. 4.

Fig. 4. Representative micrographs of the GCT and control ovary. Granulosa cells (GC) were positive for vimentin (A), while theca cells (Ti, Te) were positive for vimentin (A), SMA (C), and laminin (E) in the GCT and control (B, D, F). Fibroblastic stroma cells (S) were strongly positive for vimentin and SMA in control tissue (B, D). Ki-67-positive staining identified proliferating cells in granulosa tumor cells and the control follicle (G, H). Leydig-like cells were negative for cytoskeletal marker proteins (A, C) but were prominently visible by E-cadherin and calretinin staining (I, K). Granulosa cells (GCs) in the tumor showed very distinct cell membrane-associated staining for moesin (M) and p-ezrin (O) which was absent in the developing antral follicle of the control ovary (N, P). Both markers showed the endothelial lining of the blood vessels and capillaries in the theca (Ti, Te) and stroma (S). Theca externa cells were strongly positive for moesin but showed only weak immunoreactivity to anti-p-ezrin (M, O). AMH was positive in granulosa cells in the tumor and control follicle, specifically highlighting the Sertoli-like cells of the GCT (Q, R). In the GCT, Leydig-like cells within the theca were also clearly marked by AMH (Q), whereas the theca in the control ovary was negative. Leydig-like cells showed aromatase immunostaining in the GCT (S); the staining was much weaker in the tumor granulosa cells and the control follicle (T). Bars (A–T), 20 μm.

levels. Previous studies have linked the endocrine differences in mares with different compositions of the tumors [32, 42]. A high proportion of theca cells is associated with high serum testosterone levels, and Leydig-like cells are suspected to be the source of the hormone production, as they are positive for glutathione S-transferase α (GSTα) and P450c17, respectively [2, 4, 22]. Abnormally high levels of testosterone (>0.04 ng/m*l* [7]; >0.06 ng/m*l*, Idexx reference), anti-Müllerian hormone (≥4 ng/m*l* [7]; >8 ng/m*l*, UC Davis Clinical Endocrinology Laboratory [19]), and inhibin (>0.7 ng/m*l* [7]) are considered indicative of GCT. While high testosterone levels have historically been seen as a diagnostic marker, recent studies reported increased concentrations only in 50 to 60% [33], 38% [22], 35% [40], and 56% [19] of mares with histologically confirmed GCTs. In addition, high testosterone does not always correlate with behavioral changes and vice versa [9, 37, 40]. Similar to human GCTs, the sensitivity of AMH for detection of GCTs has been described to be 98%, and therefore, it is significantly greater than that of inhibin (80%), testosterone (48%), or both of them combined (84%) [7]. In contrast, Devick *et al*. [19] found an increase of AMH only in 44% of mares with a confirmed GCT and suggested that the tumors were most likely at an early stage of growth. Thus, while clinical signs and serum endocrinology might be indicative of equine GCT, not all cases present themselves with classical signs. Therefore, early stages of GCT may be missed [37].

The intermediate filament proteins vimentin and SMA have previously been immunolocalized to GCTs [22, 36, 44], and the markers for these proteins allowed us to separate granulosa, theca, and stromal cell components within the tumor in the present study. Laminin, as a major component of the basement membrane, highlighted immunohistochemically contractile theca interna cells in the GCT and normal follicle. Differences between normal follicles and the GCT were seen in theca externa and stromal cells, and therefore, further investigations are necessary to determine stromal alterations of GCTs. E-cadherin belongs to a family of calcium-dependent cell-cell adhesion molecules that have been observed in healthy and tumorous ovaries in various species [38, 43]. Reduction of E-cadherin has been linked with tumor progression and metastasis in many types of tumors in multiple species [28]. In our study, E-cadherin was weakly positive in granulosa and theca externa cells but stained Leydig-like cells prominently in the GCT and might therefore be a useful marker to detect this cell population in equine GCTs. Recently, calretinin, a calcium-binding protein, has been promoted as a useful and sensitive marker for sex cord-stromal neoplasms in humans [5, 18, 34]. In normal human ovaries and testes, calretinin has been detected primarily in theca interna cells and Leydig cells, respectively. In human ovarian SCSTs, the Leydig cell component of Sertoli-Leydig cell tumors is strongly positive, with weaker staining in the granulosa cells of GCTs [10]. In the present GCT, this distribution was confirmed, with calretinin seen predominantly in the theca interna and Leydig-like cells as well as in the granulosa cells. The expression of calretinin in the aforementioned cells has been interpreted as a functional link with androgen production [10], which would also match the high serum testosterone level in our case. In the ovary, AMH is produced by granulosa cells of growing follicles and is thought to have a role in regulating follicular recruitment and development [14, 44]. In the equine and bovine GCT, the granulosa, Sertoli-like, and Leydig-like cells of the tumor have been shown to stain positively for AMH, albeit with weaker staining in the latter cells [6, 30, 44, 45], which was confirmed by our results. Aromatase is an enzyme responsible for converting testosterone to estrogen [3]. Faint staining of granulosa cells in GCTs as determined in our investigation has been observed before by Watson and Thomson [46]. Other authors reported that aromatase expression was restricted to the granulosa cells of normal follicular walls but not detected in any of their GCT samples or tumor categories [2, 26]. Similar to Leydig cells in the stallion testis [3], we found positive

immunostaining of the Leydig-like cell component in the equine GCT and thus suggest that testosterone was possibly converted to estrogen.

In horses, GCTs are usually unilateral and benign. Bilateral, malignant, and metastasizing tumors are rare but do occur [11, 19, 22–24, 36, 40]; however, potentially relevant markers are lacking. The proliferation marker Ki-67 is widely used in tumor assessment, and the proliferation rate has generally been shown to correlate with tumor progression, metastasis, and prognosis [1, 5]. As expected, the proliferation rate was low in our GCT, supporting the benignity of these types of tumors. In a previous study, the mean Ki-67 index was 1.69 ± 3.00% in benign human ovarian tumors (with granulosa cell tumors among them) compared with 38.75 ± 23.61% in malignant tumors [1], and in another, it was 3.2 ± 3.7% compared with 33.1 ± 16.7% [15], which is in accord with bovine [44] and canine [17] GCTs as well as with our results.

In many tumors, such as breast, lung, prostate, and osteosarcomas, as well as human ovarian carcinomas (OVCAs), malignancy, rate of metastasis, and poor prognosis are linked to the expression of members of the ERM protein family, including ezrin, radixin, and moesin [16, 25]. Ezrin (49%) and moesin (48%) were found to be expressed in human ovarian carcinomas and were correlated with overall survival [31], and ezrin was highly expressed in OVCA compared with normal tissues, with the highest values in the metastatic tissues and cells [13]. These proteins in their active, phosphorylated form function as links between the actin cytoskeleton and the cellular membrane and are therefore considered to regulate cell morphology, motility, and cell signaling [16] as well as angiogenesis [29]. Our results show strong membranous immunostaining for moesin and the activated p-ezrin in granulosa cells of the equine GCT. During our study, p-ezrin expression was observed in the corpus luteum but not in granulosa cells of developing antral follicles. We speculate that p-ezrin is associated with processes such as proliferation, migration, and angiogenesis during the remodeling of the ovary that occurs during follicle development, ovulation, and formation of the corpus luteum. Song *et al*. [41] found an estrogen-dependent effect on ezrin expression and increased invasive behavior in ovarian cancer cells. Predominantly high testosterone levels and normal to low estrogen levels in mares with GCT/GTCT [33, 47] might have potential protective effects and prevent recurrence and metastasis. The functional background for the activation of these effects in the equine GCT, metastatic progression of which is rarely observed, needs to be elucidated by further experiments.

Based on this preliminary study, we point out the relevance of complete sets of data including history, clinical documentation (pretreatment, surgery, outcome etc.), diagnostics (ultrasound and levels of testosterone, estrogen, inhibin, and AMH), histopathology, and immunohistochemistry for tumor classification. E-cadherin, calretinin, aromatase, and AMH are suggested to be potential markers for different cell components of equine SCSTs that can support tumor diagnosis and classification. The results of this study may be of specific relevance in cases where transvaginal ovarian biopsies (TVOBs) are obtained for diagnosis of unclear ovarian conditions [20].

Ethical statement: The authors declare that national guidelines for humane animal treatment were followed. The case management was in compliance with local laws and best practices of veterinary care. The animal owner gave express consent for the analysis of the materials and for the publication of the respective data. Archived tissue was collected post-mortem after euthanasia due to unrelated medical indications according to the guidelines of the Ethics and Animal Welfare Committee of the University of Veterinary Medicine Vienna.

Acknowledgements

The authors would like to thank Claudia Hoechsmann for her technical support. Financial support was received from the Provincial Veterinary Authority Tirol as a Young Veterinary Investigator Grant for A. Dolin.

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