

# Immunohistochemical significance of ER alpha, inhibin A, calretinin, and Ki67 expression in granulosa cell ovarian tumors

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## Abstract

Adult granulosa cell tumors (AGCTs) have a heterogeneous morphology and an unpredictable behavior, which can lead to a misinterpreted diagnosis. The aim of our study was to assess the immunoexpression of estrogen receptor (ER) alpha, Ki67, calretinin, and inhibin A in AGCTs, in order to evaluate their value in diagnosis and prognosis of this type of tumor. Immunohistochemical stainings for these markers were performed in 21 cases of AGCTs. The immunopositivity evaluation of calretinin and inhibin A was scored according to the percentage of staining intensity and the extent of positive cells, of ER alpha was scored based on the percentage of positive cells, and Ki67 score was recorded as the percentage of positively stained nuclei across the tumor, without taking in consideration the staining intensity. ER was positive in nine cases, Ki67 was expressed in 12 cases, calretinin showed positive immunoreactivity in 16 cases, and inhibin A was positive in 14 cases. Stromal cells presented also immunopositivity for inhibin A and calretinin in the negative cases. ER alpha and calretinin immunoexpression can help in identification of cell components of AGCT. Our results regarding Ki67 expression emphasize the potential utility of this marker in tumor behavior prediction. Inhibin A immunopositivity has an important value in AGCT diagnosis, in association to the other evaluated markers. Additional studies are needed to identify new specific and sensitive markers for AGCT or, at least, of a panel of markers which might contribute to a more accurate characterization of these tumors.

**Keywords:** granulosa cell tumor, ER, inhibin A, calretinin, Ki67.

## Introduction

Among ovarian sex cord-stromal neoplasms, granulosa cell tumors (GCTs) have the highest frequency of clinically malignant tumors within this category [1]. There are two distinct subtypes, with different clinical and histopathological parameters, juvenile and adult GCT, the former diagnosed around puberty (44% of patients being 10 years old or younger), and the latter being commonly diagnosed (95% from all the GCTs), with a higher prevalence in premenopausal and young postmenopausal women (with a median age of 50–54 years old) [2, 3]. Rarely, juvenile GCT can be encountered in the median interval of age characteristic for adult type, and similarly, adult GCT can be found in children [4].

Despite they share some biomolecular features, recent studies revealed that FOXL2 mutation status represents the key element responsible for the different molecular pathology feature of juvenile and adult GCT [2].

Considering the higher incidence of the adult type, the present study is focused on this type of GCT. These tumors are originating in hormonally active granulosa cells, responsible for estradiol production, but the clear etiology of GCT remains obscure, as no specific risk factors have been yet identified [5]. Due to their common indolent course, with late recurrences and low metastatic

rate, the long-term follow-up of patients is currently advised [6, 7].

The proportion of adult granulosa cell tumors (AGCTs) which secrete estrogens is not precisely established, because of difficulty in acquiring an endometrial specimen for the evaluation of the estrogenic stimulation effect [1]. Moreover, AGCTs histopathological diagnostic is often difficult due to the variable morphological phenotypes exhibited by this type of tumors [8]. Thus, adequate immunohistochemical panels may prove useful in the assessment of the diagnosis and may also efficiently orientate the management and prognosis of these malignancies.

The literature contains reports of independent or panels of markers used by different research teams for the characterization of these ovarian tumors. It is accepted that inhibins, estradiol, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are mostly useful in postmenopausal and post-ablation period follow-up [7]. During the reproductive life, the major source of estradiol is represented by granulosa cells, due to their production of aromatase, which irreversibly converts androstendione. In GCTs, the tumor cells generally secrete high levels of estradiol, because of the unregulated aromatase expression [9], thus making it a reliable marker for this type of tumors [2].

Inhibin represents a heterodimeric 32-kDa glycoprotein produced by normal ovarian granulosa cells and granulosa cell tumors, with two subunits, alpha and beta, the latter divided in A and B type, which form inhibin A and inhibin B, considered to have the same biological activity [6, 10]. Numerous studies revealed that inhibin A is very useful in the assessment of granulosa cell tumors [10–14]. Although inhibin, together with steroid hormones, characterizes the hormonal activity of the most AGCTs, it may not be expressed in some of these tumors [8].

Calretinin, a 29-kDa calcium binding protein expressed in ovarian thecal and isolated stromal cells, was found positive in some AGCTs, being also compatible with sarcomatoid areas [5, 10, 15–17]. Other studies revealed that both adult and juvenile GCT staining positive with inhibin and calretinin, making them reliable markers for GCT diagnostic and follow-up [7, 8, 18].

The proliferative marker Ki67 may be correlated to prognosis. The literature data concerning Ki67 value are controversial, as Ki67 index demonstrated a good correlation with GCT clinical behavior and stage in one study [19], while another research team demonstrated no consistent correlation between Ki67 expression in primary GCT and recurrent neoplasms, with no association between Ki67 and the metastasis-free interval [20]. Although numerous studies evaluated the Ki67 proliferative index in GCT, there are no straightforward conclusions regarding Ki67 index value in tumor behavior prediction [21, 22].

In this context, the aim of our study was to assess the immunoeexpression of estrogen receptor (ER alpha), Ki67, calretinin, and inhibin A in AGCTs, in order to evaluate their significance in diagnosis and prognosis of this type of tumors.

## Patients, Materials and Methods

### Patients

The retrospective study group comprised 21 pre- and postmenopausal women (range between 47–77 years), previously diagnosed with AGCTs, between 2001 and 2013, in the Department of Pathology, “Elena Doamna” Clinical Hospital of Obstetrics and Gynecology, Iași, Romania. The surgical treatment for all cases was total abdominal hysterectomy with bilateral salpingo-oophorectomy, associated with partial omentectomy in six cases. The conventional histopathological (HP) examination was performed on paraffin-embedded sections, stained with Hematoxylin–Eosin (HE), following the identification of the characteristic morphological elements of adult granulosa cell tumors: growth pattern, nuclear and cytoplasmic features, mitotic rate, differentiation, and nature of the stromal component. According to the pTNM staging, 19 cases with pT1A, one case with pT1B, and one case with pT1C have been categorized.

### Immunohistochemical (IHC) staining

IHC stainings for ER alpha, calretinin, inhibin A, and Ki67 were performed in all 21 cases of AGCTs. These were carried out on paraffin sections of 4 µm thickness, displayed on silanized slides (coated with poly-L-lysine). Deparaffinization and hydration were performed, in two successive baths with xylene, 15 minutes each, in five successive baths with decreased alcohol concentrations,

5 minutes each (100%, 90%, 80%, 70%, and 50%, respectively), followed by distilled water. Antigen unmasking was performed by immersion of the slides into citrate buffer retrieval (pH 6), in water bath, at 98°C, for 30 minutes, followed by slow cooling at room temperature. After endogenous peroxidase blocking, the sections were incubated with primary antibodies at an optimal dilution (Table 1), for 60 minutes, at room temperature.

**Table 1 – The antibodies clones and dilutions used for the immunohistochemical technique**

Antibody	Clone	Dilution
ER	NCL-L-ER-6F11	1/100
Ki67	NCL-L-Ki67-MM1	1/200
Inhibin A	NCL-L-Inhibin A	1/100
Calretinin	NCL-L-Calret-566	1/100

ER: Estrogen receptor.

The reaction was amplified using the Novolink™ (Novocastra) detection system kit. The following steps were performed: incubation of the sections with secondary antibody, 30 minutes at room temperature and incubation with the enzyme, 30 minutes at room temperature, with three successive washes with phosphate-buffered saline (PBS), after each incubation step (primary antibody, secondary antibody, and enzyme). The slides were developed in 3,3'-diaminobenzidine (DAB) medium, 5 minutes at room temperature. After counterstaining with Hematoxylin, the immunohistochemical reaction was interpreted using a standard light microscope. Negative controls, where the primary antibody was omitted and replaced with distilled water, were included. Positive controls for each antibody were also used. These comprised testis with Leydig and Sertoli cells, for anti-calretinin and anti-inhibin A, normal breast tissue for anti-ER, and breast carcinoma with known immunoreactivity for Ki67.

### Immunohistochemical assessment

Positive IHC reaction for calretinin and inhibin A, exhibited as granular cytoplasmic staining, was assessed by a semi-quantitative score with the following values: 0 – no positive cells, 1 – <10% positive cells, 2 – 11% to 50% positive cells, and 3 – 51% to 100% positive cells [23]. Five to 10 positive high-power fields (HPFs) were examined for each section.

Ki67 scores were recorded as the percentage of positively stained nuclei across the tumor, without consideration of staining intensity, calculated within three HPFs, arbitrarily selected [24]. The median score of 40% immunopositive cells has been considered the cutoff, to separate the lower expression (Ki67 score less than 40%) from higher expression (Ki67 score more than 40%). The extent of immunoeexpression for ER alpha was scored based on the percentage of positive cells, as follows: 0 – ≤5% positive cells, 1 – 6–25% positive cells, 2 – 26–50% positive cells, 3 – 51–75% positive cells, and 4 – 76–100% positive cells [25].

## Results

### Clinicopathological features of the study group

The cases included in this study were individually diagnosed and reviewed by two pathologists. The histopathological diagnosis was of AGCT for all 21 cases

and, according to *World Health Organization* (WHO) classification of tumors of the female reproductive organs [26], 17 cases had pT1a stage, one case pT2a, one case

pT1c2, with microscopic tumor invasion of the capsules of both ovaries, and two with pT2b because of the invasive peritoneal implants (Table 2).

**Table 2 – Histopathology, tumor stage and the clinical diagnosis of the studied cases**

Histopathological diagnosis	pTNM/grade	Associated microscopic findings	Clinical diagnosis
AGCT	pT1aNxG1	Diffuse and microfollicular pattern LVI	Ovarian cyst
Bilateral AGCT	pT2bNxG1	Diffuse and microfollicular pattern LVI Leiomyoma Invasive peritoneal tumor implants	Uterine fibroma Ovarian tumor
AGCT	pT1aNxG1	Predominantly diffuse pattern Leiomyoma Simple endometrial hyperplasia	Hemorrhagic uterine fibroma
Bilateral AGCT	pT2aNxG1	Diffuse and microfollicular pattern Fallopian tube invasion, uterine invasion, peri- and intratumoral lymphocytic infiltrate	Pelvic tumor
AGCT	pT1aNxG1	Predominantly cystic tumor, compact areas with macro- and microfollicular patterns Leiomyoma	Pelvic tumor
AGCT	pT2bNxG2	Predominantly trabecular pattern, with focal micro- and macrofollicular areas, mixoid and luteinized areas Adenocarcinoma <i>in situ</i> of the uterine cervix Simple endometrial hyperplasia Invasive peritoneal tumor implants	Ovarian tumor
AGCT	pT1aNxG1	Predominantly diffuse pattern, with macrofollicular areas Leiomyoma Simple endometrial hyperplasia	Ovarian cyst
AGCT	pT1aNxG1	Diffuse, micro- and macrofollicular pattern Endometrioid carcinoma with areas of EIN Leiomyomata Intraglandular cervical neoplasia	Uterine neoplasia
Bilateral AGCT	pT1c2NxG1	Solid pattern, focal microfollicular Microscopic tumor invasion of both ovarian capsules Villoglandular endometrioid endometrial carcinoma	Metrorrhagia
AGCT	pT1aNxG1	Diffuse, trabecular, microfollicular patterns Leiomyoma	Hemorrhagic uterine fibroma
AGCT	pT1aNxG1	Predominantly trabecular and solid patterns, and focally cystic Leiomyoma. Simple endometrial hyperplasia	Hemorrhagic uterine fibroma
AGCT	pT1aNxG1	Predominantly diffuse pattern Leiomyoma	Ovarian tumor
AGCT	pT1aNxG1	Trabecular and solid pattern LVI Leiomyoma Simple endometrial hyperplasia	Hemorrhagic uterine fibroma
AGCT	pT1aNxG1	Predominantly trabecular and solid pattern, and focally cystic Leiomyoma	Ovarian tumor
AGCT	pT1aNxG1	Diffuse and microfollicular pattern Leiomyoma Simple endometrial hyperplasia	Ovarian cyst
AGCT	pT1aNxG1	Predominantly diffuse pattern Leiomyoma	Metrorrhagia
AGCT	pT1aNxG1	Diffuse, trabecular, microfollicular pattern Leiomyoma	Pelvic tumor
AGCT	pT1aNxG1	Diffuse and microfollicular pattern	Ovarian cyst
AGCT	pT1aNxG1	Diffuse, trabecular, microfollicular pattern Leiomyoma	Ovarian tumor
AGCT	pT1aNxG1	LVI Leiomyoma Simple endometrial hyperplasia	Hemorrhagic uterine fibroma
AGCT	pT1aNxG1	Diffuse and microfollicular pattern Leiomyoma Simple endometrial hyperplasia	Pelvic tumor

AGCT: Adult granulosa cell tumor; LVI: Lymphovascular invasion; EIN: Endometrial intraepithelial neoplasia.

The growth pattern of the tumor granulosa cells was predominantly diffuse, combined with cords and trabeculae. In decreasing order of frequency, tumors exhibited microfollicular type characterized by small cavities filled with an eosinophilic fluid and bordered by well-differentiated

granulosa cells with pale, scanty cytoplasm and angular, often grooved nuclei, and macrofollicular type, with cystic cavities lined by neoplastic granulosa cells, focally surrounded by theca cells. The mitotic rate was generally low, less than two mitotic figures per 10 HPFs in 16



(76.19%) cases, and more than two mitotic figures per 10 HPFs in five (23.8%) cases, which mainly corresponded to the higher stage tumors.

The tumoral stroma was nearly absent in the diffuse areas or represented by evident fibroblasts and theca cells around cavities and trabeculae (Table 2).

Two cases had been diagnosed with synchronous endometrioid endometrial adenocarcinoma (pT1bNxG1 and pT1aNxG1), one case with cervical adenocarcinoma *in situ*, and three tumors were bilateral, one of which had synchronous endometrioid endometrial carcinoma (Table 2).

### ER alpha expression

Regarding steroid receptors, ER alpha was positive in nine (44.44%) tumors. Among these positive cases, ER exhibited a moderate extent, irrespective of staining intensity, which was also moderate (Figure 1) (Table 3).

### Calretinin and inhibin A expression

Calretinin showed positive immunoreactivity in 16 (77.77%) granulosa cell tumors, for which the cytoplasmic staining was mostly strong and consistently diffuse (Figure 2). Inhibin A was positive in 14 (66.66%) cases,

the cytoplasmic staining being predominantly moderate to severe and diffuse (Figures 3 and 4) (Table 3).

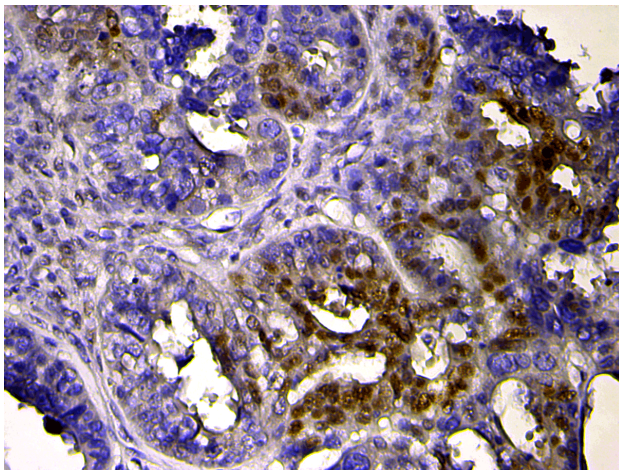
**Table 3 – The immunostaining pattern for ER alpha, calretinin and inhibin A**

Score	ER	Calretinin	Inhibin A
	No. of cases (percent)		
0	12 (57.14%)	5 (23.8%)	7 (33.33%)
1	–	2 (9.52%)	3 (14.28%)
2	3 (14.28%)	4 (19.04%)	6 (28.57%)
3	5 (23.8%)	10 (47.61%)	5 (23.8%)
4	1 (4.76%)	–	–

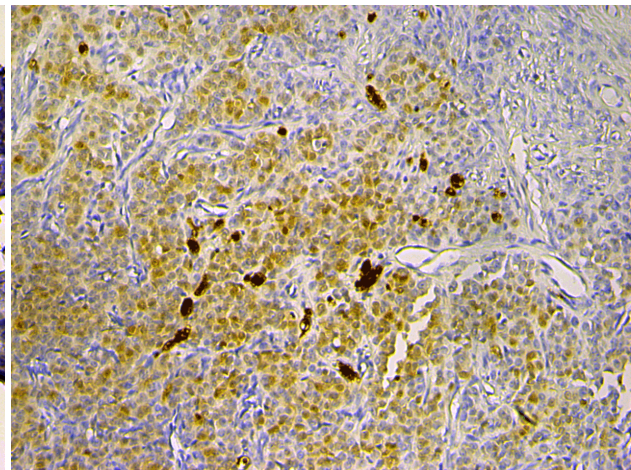
ER: Estrogen receptor.

### Ki67 expression

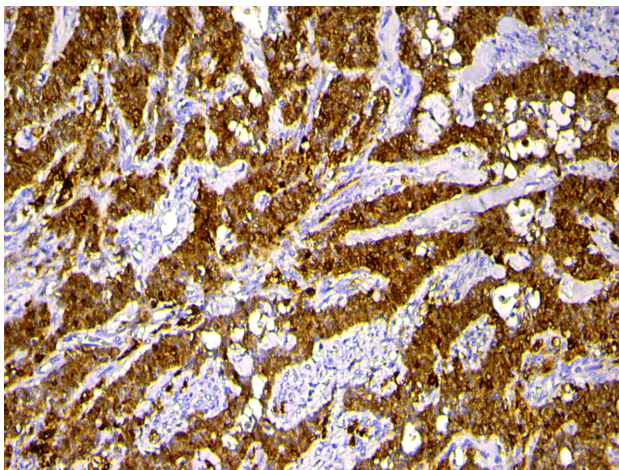
Ki67 was expressed in 12 (57.14%) cases. Ki67 positive cells showed a heterogeneous immunoreactivity, ranging from weak and focal (Figure 5) to strong and diffuse (Figure 6). Ki67 index was high in five (23.8%) cases, being correlated with the higher stages of the tumors (pT1c2, pT2a, and pT2b), and one with a case with lymphovascular invasion, nuclear atypia, and more than two mitotic figures per 10 HPFs. In seven (33.33%) cases, Ki67 index was low and in nine cases, Ki67 was negative, with no nuclear expression.



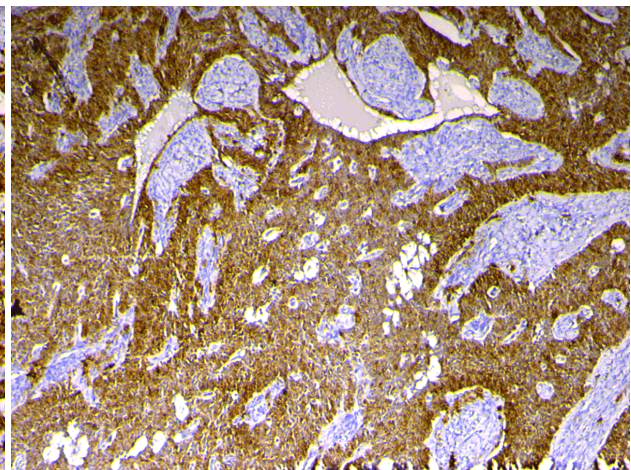
**Figure 1 – Moderate ER expression, nuclear staining in tumor cells. IHC staining, anti-ER alpha, ×400.**



**Figure 2 – Strong and diffuse calretinin expression, cytoplasmic staining in tumor cells. IHC staining, anti-calretinin, ×200.**

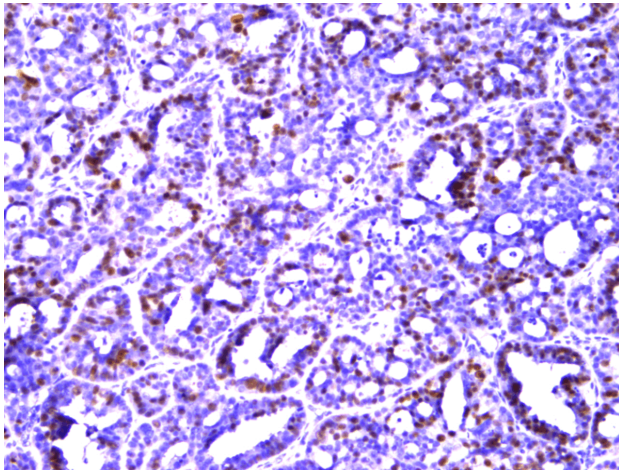


**Figure 3 – Strong and diffuse inhibin A expression, cytoplasmic staining in tumor cells. IHC staining, anti-inhibin A, ×200.**



**Figure 4 – Moderate to strong and diffuse inhibin A expression, cytoplasmic staining in tumor cells. IHC staining, anti-inhibin A, ×100.**





**Figure 5 – Weak and focal Ki67 expression, nuclear staining in tumor cells. IHC staining, anti-Ki67, ×200.**

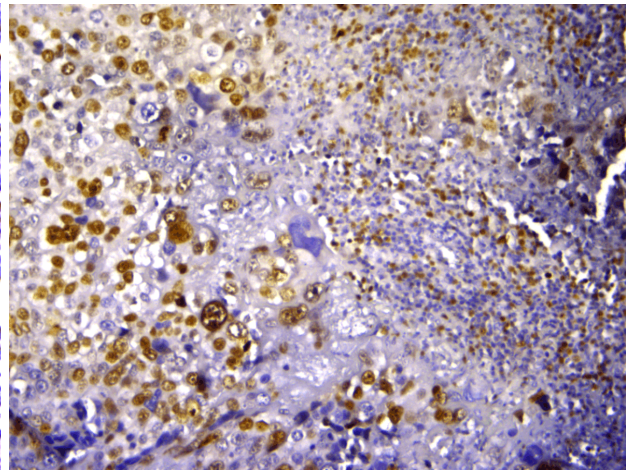
## Discussion

Among the two GCTs histological subtypes, adult form has a frequency of 95%, being characteristic in peri- and postmenopausal women [27]. Usually, the most GCTs (80–90%) are diagnosed in stage I [28]. These clinico-pathological features are in agreement with our findings, where 18 (85.71%) cases were diagnosed in stage I (17 cases with pT1a and one case with pT1c).

GCTs represent a rare category of malignant ovarian tumors, being the most common estrogen secreting ovarian tumor, responsible for patients' hyperestrogenism [28–30]. Therefore, the hormonal spectrum of manifestations and the frequent association of these tumors with leiomyoma, endometrial hyperplasia, and endometrial adenocarcinoma are directly correlated to permanent and unbalanced ovarian estrogen secretion [31–33]. Our study confirms these literature data, with the hormonal spectrum of diagnoses confirmed by multiple histopathological diagnoses association, as following: 16 (76.19%) patients with AGCTs presenting also leiomyoma, eight (23.81%) patients had also endometrial hyperplasia, two (9.52%) cases had been diagnosed with synchronous endometrioid endometrial adenocarcinoma, and one case (4.76%) with cervical adenocarcinoma *in situ*.

Although estradiol is the hormone responsible for the clinical manifestations of this type of tumor and can be used as a reliable tumor marker for GCTs, its fluctuating levels in patients cannot predict the tumor activity [30]. A possible mechanism responsible for estradiol variability is that theca cells are sometimes absent in GCTs stroma and consequently there is a shortage of androstendione production, as the source of estradiol due to enzyme cytochrome P450 aromatase action in granulosa cells [30]. While these aspects are conclusive for some cases, other studies found no correlation between hormonal levels and the tumor progression or disease recurrence [34, 35]. In this regard, estradiol is considered useful for postoperative follow-up of selected patients, but not constant enough to become a confident GCT marker [2].

Despite their importance in the normal development and ovary biology, the role of estrogen and ER in the functionality of the granulosa cells is not completely understood [2]. Some studies emphasize that among the



**Figure 6 – Strong and diffuse Ki67 expression, nuclear staining in tumor cells. IHC staining, anti-Ki67, ×400.**

two ER, ER alpha and ER beta, the latter represents the principal ovarian type [36], and also the most expressed type in GCTs [37], while others consider that ER alpha is mainly expressed in the gonads, including ovary, and at lower levels in other tissues and ER beta is especially secreted in colon, bone marrow, vascular endothelium, lung, bladder, and brain [38].

Genetically engineered mice have been used to demonstrate that the granulosa tumor cells in ER beta-/- mice produce estrogen and have also a significant expression of ER alpha [39]. Other studies with knockout mice found an important correlation between the distribution and functions of ER alpha and ER beta, ER alpha being responsible for proliferation and ER beta for differentiation [40]. However, ER alpha exhibits a crucial role in the neuroendocrine and reproductive systems, and is the only receptor mandatory for negative feedback regulation of gonadotropin-releasing hormone cells [41, 42]. Despite these findings, both ER alpha and ER beta are necessary for normal ovarian function [38].

It was also demonstrated that the transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B) inhibits ER signaling, which has impact on ER-mediated transcription pathway, thus E2 is not functional even though the receptors are expressed in GCTs [43, 44]. On the contrary, other data support the idea that ER signaling pathways provide a protective mechanism by ER alpha mutations, associated with the loss of both ERs [45].

These data are in agreement with our study results, which revealed ER alpha positivity in only nine (44.44%) cases, exhibiting a moderate extent of immunostaining. This feature could be related to the lack of thecal cells [2] in nine (44.44%) tumors from our study group.

The direct contribution of estrogen and ERs in GCT oncogenesis is controversial. *In vitro* studies using granulosa-like tumor cell lines suggested that ER beta is likely to have an anti-proliferative role in GCT [44]. Moreover, it was demonstrated that ER beta represents an anti-proliferative factor in colon, breast, and prostate cancers [46], and if estrogen action is significant for tumorigenesis, it is possible to involve the alpha subtype, which is known to be expressed at lower levels in GCT [37]. This speculation has led to the hypothesis that estrogen is likely to target stromal tissue and angio-

genesis, rather than granulosa tumor cells, leading to huge implications in GCT treatment [18, 47].

Inhibin represents a dimeric glycoprotein formed of an alpha subunit, covalently bound with a beta A and a beta B subunit, forming two heterodimers named inhibin A and inhibin B, respectively [2, 48]. In the ovary, inhibins are mainly secreted by granulosa cells, participating in the functional dynamics of the ovarian cycle, due to the particularities of their secretion. Therefore, inhibin A is considered the predominant type secreted during the late follicular and luteal phases of the ovarian cycle, while inhibin B is mainly produced in the early and mid-follicular phases [48, 49]. It is also stated that beta subunits are primarily characteristic for granulosa cells, with beta A positive in all follicle stages and in the theca cells of the dominant follicle as well as corpus luteum, while beta B expression is limited to primary follicles [2, 49]. Inhibin has two main biological roles, suppressing pituitary FSH secretion and representing a potent growth factor for granulosa cells [2].

In postmenopause, due to the exhaustion of the ovarian follicles, inhibin levels decrease dramatically [50]. This aspect has a clinical relevance in patients with GCT, where the inhibin levels are high [2]. Numerous studies have revealed that granulosa tumor cell secrete elevated levels of inhibin, the role of potential marker for GCT in premenopausal and postmenopausal women being attributed to this hormone [18], as an indicator of its biologically active phenotype [35, 51, 52]. Although a wide range of studies demonstrated that mainly inhibin A is an important immunohistochemical marker for granulosa cell tumors, recent data showed that inhibin B is the predominant form secreted in GCT, a feature which supports its value as an accurate marker for the detection of this tumor type [18, 53]. Other studies revealed that not all GCT express inhibin and thus, loss of inhibin expression can be correlated with higher tumor grade [54].

In our study, inhibin A was positive in 14 (66.66%) cases, the cytoplasmic staining being predominantly moderate to severe and diffuse. These results are in agreement with other studies, reporting a variable expression and possible correlation of lack of expression with the poorly differentiated GCT [54]. This pattern of expression can be also be related to the predominant type B of inhibin secreted by granulosa tumor cells [18, 53].

Considering that other types of ovarian tumors, especially mucinous variant of epithelial tumors, secrete inhibin, this hormone is not specific for GCT [18]. However, this marker is useful to differentiate GCTs and most sex cord stromal tumors from other epithelial neoplasms with similar histology. Usually, in these cases, the immunostaining is limited to non-tumoral stromal theca cells, and the intensity is weaker than the strong expression found in ovarian sex cord stromal tumors [6].

Calretinin immunoexpression can be useful in the diagnosis of sex cord cell tumors, including granulosa cell tumors, according to numerous studies [8, 55–58]. Calretinin represents a 29-kDa calcium binding protein, which was initially discovered in neuronal tissue and afterwards within ovary in theca lutein and theca interna cells, and in mesothelial cells [59–61].

The finding that inhibin is not expressed in all GCTs lead to the recommendation of other markers evaluation,

*e.g.*, calretinin, which has even a higher sensitivity than inhibin in AGCTs [8, 62].

Although specific and sensitive for granulosa cell tumors [56, 57], calretinin was found to have a stronger immunoexpression in fibromas and fibrothecomas compared with GCTs [55, 59]. This feature has a useful impact for the differential diagnosis between this tumor group and endometrial stromal sarcoma with fibroma-like phenotype [59, 63].

In our study, calretinin presented positive immuno-reactivity in 16 (77.77%) GCTs, for which the cytoplasmic staining of the tumor cells was mostly strong and diffuse. Our results are consistent with the literature data, which revealed that calretinin sensitivity is superior to its specificity in GCTs [10, 64].

As compared with inhibin, we found that calretinin demonstrated a slightly higher specificity and sensitivity than inhibin in AGCTs. This is in agreement with one report, which showed that calretinin was expressed in 100% of GCT cases whereas inhibin was identified in 73.9% of these tumors [8], while another survey demonstrated that calretinin and inhibin exhibited a comparable immunophenotype in GCTs, regarding the percentage of positive tumor cells and the degree of expression [59].

Because other tumors, which can be part of differential diagnosis for GCTs, occasionally express inhibin and calretinin, it is recommended to be used within a larger panel of immunomarkers in difficult cases [31, 59].

In agreement with other studies, we emphasize that calretinin appears to be useful in the differential diagnosis, because of its higher sensitivity compared with inhibin in adult granulosa cell tumors [10, 64].

The assessment of the rate of cell growth using Ki67 nuclear protein has long been studied in different types of tumors and its prognostic value has been demonstrated along with other clinicopathological factors [19, 65]. In ovarian tumors, Ki67 represents a useful proliferation parameter, which can predict the clinical course of GCTs [19].

In our study, Ki67 was expressed in 12 out of 21 (57.14%) cases. The immunopositive cells showed a heterogeneous immunoreactivity, ranging from weak and focal to strong and diffuse. Ki67 index was high in five cases, being correlated with the higher stages of the tumors (pT1c2, pT2a, and pT2b), and one case with lymphovascular invasion, nuclear atypia, and more than two mitotic figures per 10 HPFs. These results are in agreement with the literature data, where Ki67 index was found to be higher in adult granulosa cell tumors with an aggressive phenotype [16, 19, 66], and less than 40% in typical GCTs [58, 67]. In nine cases, Ki67 was negative, with no nuclear expression.

According to our findings, we can emphasize, in agreement with other studies, that in ovarian GCTs, Ki67 index is correlated with the clinical behavior and the stage of the tumor, the higher level being associated with higher tumor stage, and thus with a worse prognosis, and the lower Ki67 index with a better tumor prognosis [16, 19].

Although most GCTs have a favorable prognosis, their behavior is unpredictable, with late recurrences and thus the patients need a thorough long-term follow-up. The histopathological assessment itself is not enough for a complete prediction of the clinical course. Together with other prognostic factors, like age, tumor size and capsule integrity, bilaterality, mitotic activity, atypia, Ki67 repre-

sents a useful parameter for the assessment of the tumor clinical outcome. The finding of a recent study coordinated by Rajagopal & Ramesh [19] of a superior Ki67 index value correlation with the clinical tumor stage compared to the differentiation grade demonstrates its value as a predictive marker of tumor behavior.

The immunohistochemical markers used in our study have proved to be reliable tools in the diagnosis and assessment of the prognostic of ovarian GCTs, along with other clinicopathological parameters.

In agreement with other studies [8, 59], we concluded that calretinin and inhibin represent accurate markers for the diagnosis of GCTs. However, due to their different immunopatterns, their association is mandatory, as calretinin has proved to be more sensitive than inhibin A, whose specificity is higher in this tumor category.

However, because of still unexplained features of the ovarian tumors behavior, sometimes reflected in the controversial immunoexpression of these markers, additional studies with a higher number of cases and a larger IHC panel are necessary to better characterize these tumors and to build an appropriate algorithm for a better short- and long-term GCTs follow-up.

## Conclusions

ER and calretinin immunoexpression can help in identification of the cell components of AGCT. Our results regarding Ki67 expression emphasize the potential utility of this marker in tumor behavior prediction. Although non-specific, inhibin A immunopositivity has an important value in AGCT diagnosis in association to the other evaluated markers. Additional studies are needed to identify new specific and sensitive markers for AGCT or, at least, a panel of markers which may contribute to a more accurate characterization of these tumors.

## Conflict of interests

The authors declare no conflict of interests.

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