Precision medicine for granulosa cell tumors of the ovary

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Gerichte behandeling voor granulosaceltumoren van het ovarium

(met een samenvatting in het Nederlands)

Proefschrift

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Dr. J.W. Groeneweg

Dedicated to every woman facing this disease

Contents

CHAPTER 1

General Introduction

Ovarian cancer affects 295,400 women worldwide per year and is the leading cause of death from gynecological malignancies in developed countries.[1] Ovarian cancer represents a heterogeneous disease with distinct histologic subtypes (Figure 1). The majority of tumors arise from the lining of the fallopian tube[2], ovary or peritoneum, and are classified as **epithelial tumors** (90%). **Sex cord-stromal tumors** (5-10%) include tumors derived from granulosa cells, theca cells, Sertoli and Leydig cells, and fibroblasts of the ovarian stroma. **Germ cell tumors** (2-3%) arise from the primitive germ cells of the embryonic gonad.[3] The different histologic ovarian cancer subtypes also harbor different genomic alterations. A mutation in the tumor suppressor gene *TP53* characterize high grade epithelial tumors (>96%)[4], the *DICER1* gene is frequently affected in Sertoli-Leydig cell tumors (~97%)[5], *FOXL2* mutations are found in adult granulosa cell tumors (94-97%)[6,7] and germ cell tumors can harbor alterations in *KRAS*, *CDKN1B*, *CCND2*, *ETV6*, and *RAD52*.[8,9]

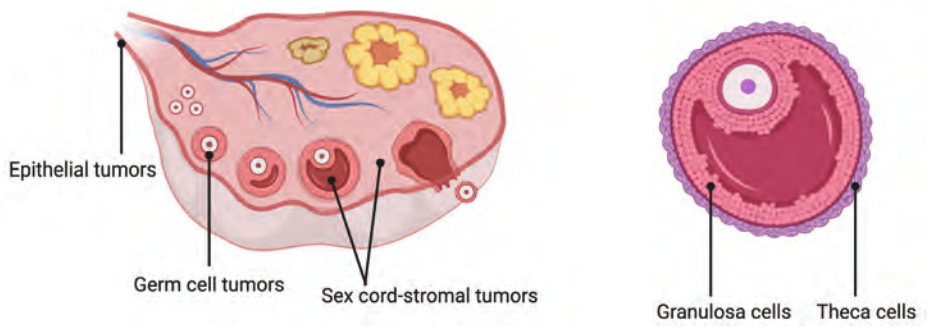

Figure 1. Left: Subtypes of ovarian cancer according to their cells of origin. Right: Follicle with an oocyte surrounded by granulosa cells and a layer of theca cells.

Research on ovarian cancer has focused predominantly on the high grade epithelial tumors, as they constitute the most common and most lethal type of ovarian cancer. As a result, treatment strategies in all ovarian cancer subtypes are mainly based upon studies performed in high grade epithelial ovarian cancer even though each subtype has a distinct origin and biological behavior.

Granulosa cell tumors, the most common type of sex cord-stromal tumors, will be the subject of this thesis. Our research aims to unravel the molecular characteristics of granulosa cell tumors to better understand its background and behavior, and to ultimately improve treatment.

Granulosa cell tumors have different histologic patterns

The granulosa cell tumor is a malignancy arising from the hormonally active granulosa cells of the ovary (Figure 1). The malignancy accounts for 70% of the sex cord-stromal tumors and represent 2-3% of all ovarian cancers.[10] The tumor is divided into an adult

(95%) and a juvenile (5%) subtype. Its microscopic features closely resemble those seen in the embryologic and post-fetal precursors of the Graafian follicle. These small follicle-like structures were later referred to as Call-Exner bodies, which are pathognomonic for adult-type granulosa cell tumors.[11] Within a tumor, different histologic patterns can be seen. Adult granulosa cell tumors can harbor diffuse, trabecular and corded, insular, microfollicular or macrofollicular patterns.[12] Juvenile tumors harbor a diffuse or macrofollicular pattern with irregular sized and shaped follicles lined by granulosa cells and luteinized theca cells. In general, granulosa cell tumors stain positive for inhibin, the estrogen receptor and calretinin and show modest mitotic activity. The tumor can contain both cystic and solid components and is encapsulated with a smooth lobulated surface. Its size ranges from microscopic growth embedded in an otherwise normal-looking ovary, to a large mass filling the pelvic cavity (Figures 2 and 3).

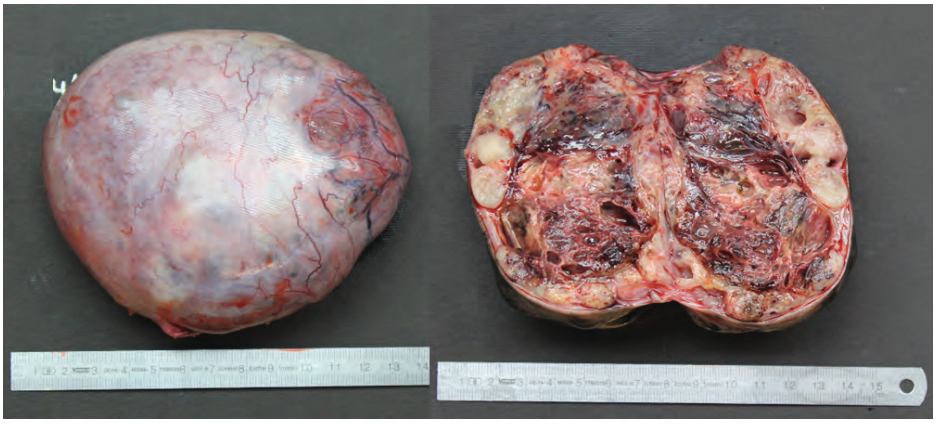

Figure 2. Granulosa cell tumor, the outer surface (left) and cross section (right). The tumor (13 cm) shows increased vascularization and contains cystic and yellow, fatty-like solid components.

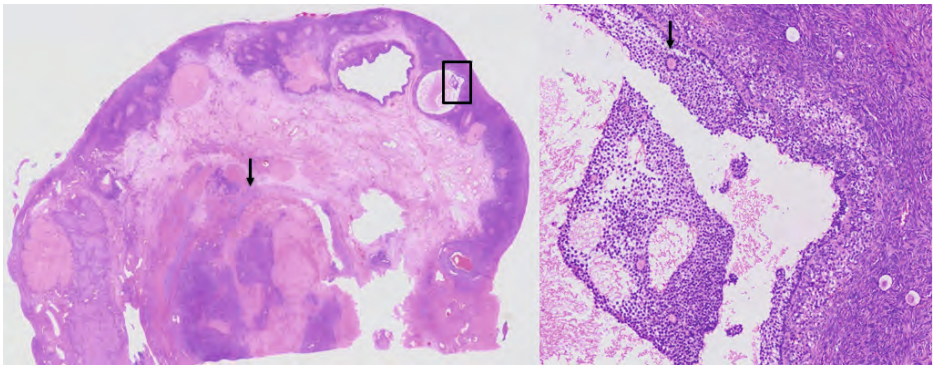

Figure 3. Microscopic section of granulosa cell tumor. Left: The tumor (12 mm diameter) is centrally embedded in the ovary (arrow). Follicles at different stages are present in the ovarian cortex (square). Right: magnification of the follicle, showing Call-Exner bodies in the granulosa cell layer (arrow).

A granulosa cell tumor should be suspected in patients with an adnexal mass, elevated inhibin and estrogen levels and endometrial proliferation

Adult granulosa cell tumors are most frequently seen in perimenopausal or postmenopausal women, with a peak incidence between 50-55 years.[10] Juvenile tumors are usually detected in prepubertal girls and associated with sexual pseudoprecocity.[13,14] Granulosa cell tumors secrete the female sex hormones estradiol, inhibin and anti-Müllerian hormone, which can be detected in blood serum at diagnosis. Patients can present with symptoms of abdominal pain, caused by a large ovarian mass. Torsion or rupture of the tumor may produce acute abdominal pain, necessitating surgical removal in a (semi-)urgent situation. Prolonged exposure to tumor derived estrogen may cause endometrial proliferation, resulting in symptoms of irregular or postmenopausal vaginal bleeding.[15] The tumors are characterized by a predominantly solid mass with cystic components, unilateral in 78-91% of patients, often accompanied by thickened endometrium.[10] Granulosa cell tumors have a propensity to rupture and bleed and up to 15% may present with haemoperitoneum.[16]

Granulosa cell tumors are usually not preoperatively recognized

Although ultrasound findings and elevated serum levels of inhibin and estradiol can lead to the suspicion of a granulosa cell tumor at disease presentation, the diagnosis is often only made after histopathological examination and rarely preoperatively recognized. The surgical treatment of granulosa cell tumors consists of a unilateral or bilateral salpingo-oophorectomy with or without hysterectomy, depending on the patient's age and tumor extension. Although there is no consensus on the necessity for full surgical tumor staging, current guidelines propose it should generally be avoided.[17,18] Surgical staging would usually require an extra surgery as the diagnosis is not known pre-operatively. Conservative surgery can be justified, especially in young women who wish to preserve fertility. In that case, endometrial biopsy is indicated to assess the presence of endometrial hyperplasia (26%) or concomitant endometrial cancer (6%), as a result of prolonged estrogen stimulation.[15] Prior or during surgery, cystic parts of these tumors can easily rupture which allows tumor cells to spread through the abdominal cavity. Tumor rupture increases the risk of recurrence and is the most important prognostic factor in granulosa cell tumors.[10,19]

Serum hormone levels may insufficiently reflect disease activity

Due to the endocrine activity of granulosa cell tumors, different hormones were suggested to be used as markers of disease at postsurgical surveillance. Normally, granulosa and theca cells produce estradiol according to the two-cell–two-gonadotropin theory (Figure 4). First, luteinizing hormone (LH) stimulates thecal cells to convert cholesterol into androgens. Second, follicle stimulating hormone (FSH) stimulates granulosa cells to convert androgens into estrogens via aromatase CYP19A1.[20] In turn, excess estradiol decreases the production of gonadotropin-releasing hormone (GnRH) by the hypothalamus and, as a consequence, of FSH and LH by the anterior pituitary.[21]

- **Estradiol** was the first suggested granulosa cell tumor marker.[22] Although levels may be high at the time of diagnosis, its serum levels do not correlate well with disease activity.[23] Furthermore, absence of estradiol secretion is observed in approximately 30% of patients, potentially due to a relative lack of theca cells in the tumor stroma producing the estradiol precursor androstenedione.[23]
- **Inhibin** is a hormone produced by granulosa cells during the follicular phase of the menstrual cycle and is a member of the transforming growth factor beta (TGFβ) family. It contains an α-subunit which is linked to either a βA or a βB subunit, forming inhibin A and B. The secretion of inhibin by granulosa cell tumors can lead to a raise in serum inhibin A or, more often, inhibin B. Inhibin is a more accurate marker of disease activity than estradiol, with a sensitivity for the detection of recurrence of 58% for inhibin A and 85% for inhibin B.[23–25] Levels may rise 1-3 years prior to detectable recurrence. However, limitations of inhibin B include its fluctuating levels in premenopausal women and its possible elevation in other ovarian cancers. In addition, up to 15% of granulosa cell tumors remain inhibin B negative.[25,26]
- More recently the **anti-Müllerian hormone** (AMH), also produced by the follicular granulosa cells and member of the TGFβ growth factor family, was proposed as a tumor marker[27]. Its sensitivity and specificity is comparable with inhibin B and serum levels may rise up to 3.5 years prior to the detection of a recurrence. Therefore, the use of either inhibin B or AMH is advised for disease monitoring and radiological evaluation follows when serum levels are consistently increased.[26]

Detection of a marker increase can be stressful for patients, even though it does not always indicate disease recurrence. Moreover, if tumor recurrence would be visible (i.e. on imaging), this would usually not require immediate treatment. Timing of treatment of recurrence is difficult and will be discussed in the next paragraph. In general, low levels of estrogen, inhibin and AMH are a good indicator of disease absence. The current tumor markers are usually high at diagnosis, have a moderate sensitivity for detection of recurrence and may be difficult to interpret in premenopausal women who still have a hormonally active ovary.

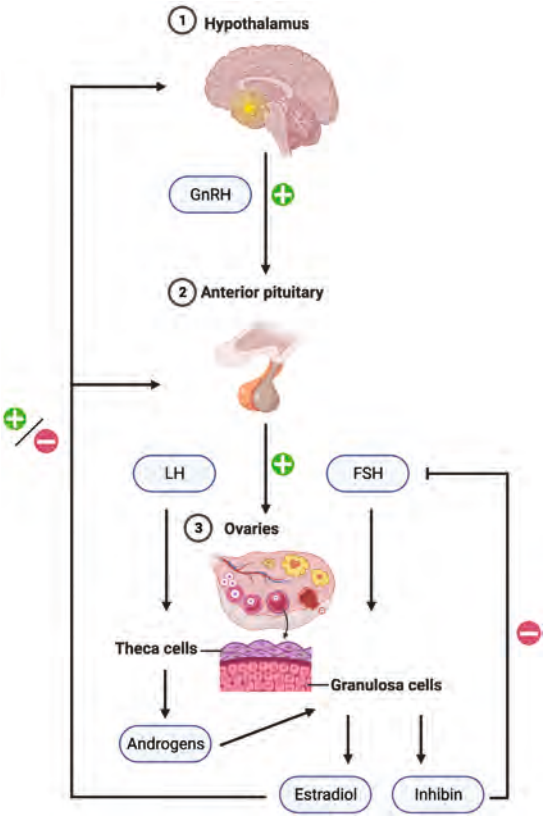

Figure 4. The hypothalamic–pituitary–gonadal axis.

A sensitive test for disease activity is more important than early detection of recurrence

In granulosa cell tumors, early detection of recurrence does not necessarily affect treatment. As risks of surgery increase with subsequent debulking procedures and tumors may slowly progress, watchful waiting with frequent disease monitoring can be applied. However, surgery should be performed when all tumor deposits can still be completely removed. Therefore, the correct timing of surgical resection is a clinical challenge. For this reason, a more sensitive measure of the tumor growth rate could help, such as levels of circulating cell free DNA fragments that are excreted by tumor cells.[28]

Surgery is the mainstay of treatment as effective systemic treatment options are lacking

Generally, surgical treatment for primary granulosa cell tumors is effective and patients have a good prognosis with a 10-year survival rate of over 90%.[10] However, approximately 50% of patients develop disease recurrence, often requiring repeated surgery. Up to 80% of patients who develop a recurrence ultimately die of their disease. Upon disease relapse, adjuvant treatment strategies are needed to target residual (microscopic) tumor and to increase the disease free interval.

- **Anti-hormonal treatment** was thought to be an effective treatment strategy, as granulosa cell tumors highly express estrogen receptors. Due to their success in hormone receptor positive breast cancer, anti-hormonal drugs such as the selective estrogen receptor modulator tamoxifen, aromatase inhibitor letrozole and estrogen receptor antagonist fulvestrant have been used in recurrent granulosa cell tumors. However, limited data is available on the efficacy of these strategies. In a retrospective analysis, anti-estrogen treatment decreased tumor load in 4 out of 22 (18%) tumors.[29]

- **Chemotherapy** targets rapidly dividing cells and is effective in many different cancer types. Systemic treatment for granulosa cell tumors is based on regimens that have shown to be successful in the treatment of high grade epithelial ovarian cancer treatment and primarily consists of carboplatin and paclitaxel or the combination of bleomycine, etoposide and cisplatinum (BEP). However, chemotherapy does not yield better results than anti-hormonal treatment, with an observed partial response in 11-25% of patients and a complete response in 11-26% of patients.[30] Also, patients need to be fit to undergo chemotherapy and significant side effects frequently result in early termination of treatment. Moreover, several studies found no survival benefit after treatment with either chemotherapy or endocrine therapy in patients with a granulosa cell tumor.[31–36] Hence, similar and low response rates have been found for both chemotherapy and anti-hormonal treatment, stressing the need for novel treatment options.

- Some studies also propose abdominal or pelvic **radiotherapy** as adjuvant treatment after incomplete surgical resection, as these tumors are extremely radiosensitive.[37] However, the morbidity of abdominal radiation is high and the survival benefit is unclear.[10,38,39]

- **Radiofrequency ablation**, a technique using heat to target specific tumor sites, can be successful in selected patients with inoperable liver metastases.[40]

The *FOXL2* mutation is a characteristic, but potentially non-targetable alteration

Granulosa cell tumors are characterized by a specific *FOXL2* c.402C>G mutation that was discovered by Shah et al. in 2009.[41] *FOXL2* is a transcription factor that regulates the development of ovarian granulosa cells and follicles. This specific *FOXL2* point mutation is found in over 90% of adult granulosa cell tumors and results in posttranslational modifications that may contribute to tumor growth.[6,42] Even though *FOXL2* is thought to be a major tumor driver, the absence of a *FOXL2* mutation in a small subset of patients suggests potential alternative mechanisms for tumorigenesis. Moreover, tumors that do not harbor the mutation do not show any difference in immunophenotype, tumor stage, recurrence rate and prognosis.[43] Thus far, efforts to target *FOXL2* as a novel therapeutic strategy have not been successful. Adult granulosa cell tumors may also harbor mutations in genes known

for their involvement in other cancers, such as *TERT*, *KMT2D*, *PIK3CA*, *AKT1*, *CTNNB1* and *NR1D1*.[44–48] As compared to high grade epithelial ovarian cancer, granulosa cell tumors have relatively few copy number alterations, including trisomy 12 and 14 and monosomy 22. Juvenile granulosa cell tumors rarely contain the *FOXL2* mutation, but frequently harbor an activating mutation in *AKT1* (60%) or the *GSP* oncogene (30%).[9,49]

Germline loss-of-function mutations in *FOXL2* can lead to blepharophimosis, ptosis and epicanthus inversus syndrome (BPES), which is an autosomal dominant disorder associated with premature ovarian failure.[50] Overexpression of wildtype *FOXL2* enhances GnRH-induced apoptosis. The molecular mechanism by which a mutation in *FOXL2* results in ovarian failure or ovarian tumor growth, remains to be elucidated.[51]

Treatment of recurrence requires a personalized approach

Table 1 summarizes the clinical challenges in the diagnosis and treatment of granulosa cell tumors. Currently, treatment of recurrence remains particularly challenging as the majority of patients with a granulosa cell tumor relapse will ultimately succumb to their disease. An accurate marker of disease activity may help to determine the optimal timing of treatment. With repeated surgery being the most effective treatment so far, novel and less invasive therapeutic strategies should be investigated. Due to the rarity of the disease, the effectiveness of various systemic treatment regimens is difficult to evaluate in the clinical setting and reliable preclinical models are needed. Identification of tumor characteristics that can be linked to treatment effects, may personalize treatment strategies and increase their effectiveness.

Table 1. What is needed in granulosa cell tumor management

Clinical Challenge	Current issues
Unraveling tumor characteristics	Besides a mutation in *FOXL2* and the *TERT* promoter region, little is known about the molecular mechanisms involved in tumor development and progression.
Disease recognition	This rare type of ovarian cancer is not always preoperatively considered.
Disease monitoring	Current tumor markers have a moderate sensitivity for detection of recurrence and can be difficult to interpret in premenopausal women with naturally fluctuating hormone levels.
Treatment for recurrence	Surgery is very effective, but should be postponed as long as possible as the number of laparotomies that can be performed is limited. Current systemic treatment options are only effective in a subset of patients.
Timing of treatment	A measure of disease activity could help to determine the optimal timing of treatment for recurrence.

Active patient engagement in research may benefit both patients and researchers

Although patients often only contribute to research by undergoing study procedures, they can be involved in research on many more levels. Patients could help to identify knowledge gaps and clinical needs from a patient's perspective, they could review the study design and procedures and/or work together with researchers, for example in writing a grant proposal or creating awareness of a (rare) disease. Furthermore, they could create patient platforms and initiate funding opportunities for research on their disease. The research group, on the other hand, could be a reliable and accessible source of information to patients. Different initiatives have been established to empower patient groups and propagate active patient involvement in research. This has led to an increase in active patient engagement in research over the past decade. However, patient engagement is not standard in research and little is reported on patient engagement in the field of gynecological oncology. This thesis illustrates the result of a collaboration between patients and researchers.

Thesis outline and aims

The aim of the research presented in this thesis is a step towards precision medicine for granulosa cell tumors of the ovary.

Our specific research objectives were to:

- Collaborate with patients in research and create disease awareness (chapter 2 and 7)
- Characterize granulosa cell tumors on a molecular level to identify new targets for treatment (chapter 3)
- Evaluate current and novel treatment strategies (chapter 4, 5 and 8)
- Improve disease monitoring by imaging and detection of circulating tumor DNA (chapter 5 and 6)
- Identify patient subgroups sensitive for specific treatment (chapter 4 and 5)

CHAPTER 2

Patient engagement in research on rare gynecological tumors

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Introduction

Although patient engagement has become increasingly relevant in science, researchers find it difficult to involve patients, specifically in translational research. Particularly in rare disease research, patient engagement is crucial. First, since grant donors focus on common diseases, patients can support funding applications or may even initiate private funding for research on their disease. Second, patients can help setting up collaborations in order to collect data.[52] Patients themselves can make their clinical data available for research or motivate their physicians to do so. Also, active patient engagement can identify relevant research questions from a patient's perspective. Different initiatives such as the European Network of Gynaecological Cancer Advocacy Groups (ENGAGE)[53] and the advisory group linked to the National Health Service of the United Kingdom (INVOLVE)[54], have been established to empower patient groups and propagate active patient involvement in research.

It can be challenging for patients to find appropriate information on their rare disease and their physicians may also not have state of the art knowledge of a rare tumor. Together with patients, researchers can establish centers of excellence as a valuable and reliable source of information. Currently, little has been reported on patient engagement in research in gynaecological oncology. Here, we aim to illustrate how we actively involved patients in our research on granulosa cell tumors (GCTs), a rare and well defined ovarian cancer subtype responsible for 2-5% of all ovarian malignancies.

Methods

Our approach to patient engagement

When discussing and having to explain the knowledge gaps to one of our patients, she and her family decided to initiate a fund to investigate granulosa cell tumors. A national multi-center study was started to collect clinical data, tissue and blood samples for research. As part of this initiative, we aimed to actively involve patients in our research in various ways and in different phases of research (Table 1). During the study, we strived to adequately inform patients about the progress of the projects through newsletters and group meetings with the patient organization and in parallel with the donors. During these meetings, patients and their family can ask questions regarding results and new insights of the research and the focus for future projects is discussed. In order to evaluate and improve our efforts to involve patients, we invite the members of the patient organization at one of their meetings to share their experience about involvement through a questionnaire.[55]

Table 1. Opportunities for patient engagement in different phases of research

Research issue	Research phase	Method	Putative Result
Identify knowledge gaps and clinical needs	Define research question	Direct contact with patients; Consultation of patient support groups	Define most urgent and relevant research questions
Explore grant opportunities	Research funding	Collaborate with a patient organization; Mobilize 'influencers' among the patients	Apply for funding sources likely to fund your (rare disease) research
Review of study design and study procedures by patients	Design study	Collaborate with a patient organization; Test if the patient information folder is clear and if patients are willing to undergo the procedures	If patients approve study procedures and information, more patients are likely to participate
Update patients on the research progress and share novel insights	During the study	Meetings, newsletters	Help patients to better understand their disease; They can ask their physicians about novel treatment or diagnostic strategies
Receive feedback on study results	During the study	Meetings, questionnaires	Patients can drive the implementation of research findings into the clinic
Show results of research on a group level and brainstorm on future directions	Publication of results	Confer with private sponsors and patient organizations	Patients can help to prioritize the research agenda

Patient driven research

Active patient engagement has guided our research directions and raised novel questions. At a group meeting, members found out that they were distant relatives (their grandfathers were brothers), which initiated a project on hereditary factors in granulosa cell tumors. We subsequently used the patients' international social media platform to circulate a call for additional patients with family members also diagnosed with a granulosa cell tumor. This resulted in the identification of four affected families. Furthermore, we learned about patient concerns, e.g. that having a six week time waiting interval between blood sampling and tumor marker results can be very stressful. Patients also appeared to be concerned about the radiation exposure resulting from repeated follow-up computed tomography scans, even though risks of radiation-induced cancer may be low.

Due to successful patient engagement, in particular through the patient organization[56], more than 100 patients were enrolled in our national study within two years. This represents five times the annual incidence (17-20 cases) of granulosa cell tumors in our country. We experienced that patients understandably have a great sense of urgency. These patients do not want research on their disease to be a low priority and find it hard to accept that clinical breakthroughs for rare tumors take a long time. Patients appreciate being informed on all the steps in the research process, both the successes and failures, to understand the time it takes to answer research questions. To further support research, three fundraising initiatives have been undertaken by patients with the aim to set up a sustainable funding source for granulosa cell tumor research. Frequent contact between researchers and the patient organizations also helped patients to better understand and manage their disease. Our research group provided the patients with evidence-based information on granulosa cell tumors and updated them on the latest developments in the field.

Evaluation of patient engagement

Sixteen patients responded anonymously through our questionnaire. The results are shown in Table 2. The majority felt they could always approach the research team. The interaction with the research group was adequate and helped to better understand their disease. Most patients would have liked to be involved even more, particularly in the implementation phase of the research and helping to translate findings to current practice. They varied in the role they would like to play, from being informed or giving their opinion or advice, to being a full partner or initiator. In general, patients felt more empowered and reassured.

Table 2. Results of the questionnaire to evaluate patient engagement

Question	Completely disagree	Disagree	Neutral	Agree	Completely agree
I feel part of the GCT research	2*	0	2	8	4
I would like to be more involved in the GCT research	0	1	1	7	7
The interaction with the research team helps me to better understand my disease	0	0	2	7	7
When I have a specific question about the research or GCT in general, I feel I can always approach the research team	0	1	4	9	1
The current GCT research encompasses the most relevant research questions	0	0	4	9	1

	Preparation	Execution	Implementation	None/not applicable
In which phase of research would you like to be involved (more answers may apply)	4	4	9	5

	Auditor	Think along	Advisor	Partner	Initiator
Which role** would you like to fulfill in the GCT research (select one answer)	7	5	1	2	1

* The two respondents that do not feel part of the research, commented that their material is not (yet) used for study purposes.

** Auditor: someone is being informed, e.g. reading the research proposal.
Think along: a patient is asked for her opinion, e.g. on study procedures or the patient information letter.
Advisor. Advisory role, e.g. to give feedback on study questionnaires.
Partner: the patient works together with the researchers, e.g. as co-writer of a research proposal.
Initiator: the patient takes the initiative and is the driving force, e.g. when creating a website to share information on the research projects.

Summary

The interactions with donors and the collaborations with patient groups have guided our research, led to novel patient-initiated research projects and facilitated patient recruitment and data collection (Figure 1). The evaluation of active patient engagement in our study showed that patients felt strengthened by our research and the interaction with the research team helped them to better understand their disease.

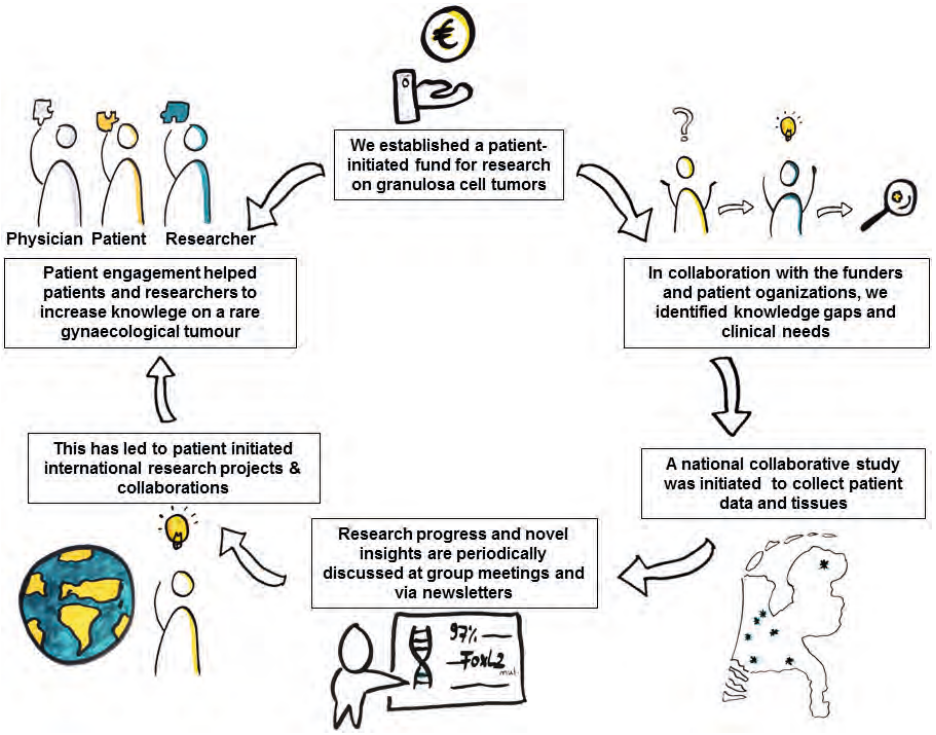

Figure 1. The concept of patient engagement

Challenges in patient engagement

The implementation of patient engagement seems to be hampered by concerns about time and costs. In our experience, patient engagement speeds up decisions and progress in research with moderate time investment. We attended meetings organized by the patient organization, answered questions from the patient group via e-mail and sent newsletters regularly. Also, it may be difficult to translate research findings into lay terms. However, this helps to communicate results to a broader audience and to implement research findings into practice and policy. Finally, it proved to be a challenge to find a balance between our role as researcher and as (treating) physician, especially when patients asked clinical questions on their specific situation at group meetings. Both patients and researchers have to acknowledge these separate roles in different settings.

Support in rare disease research

Receiving direct private financial support can be crucial to initiate research on a rare disease,. Apart from our patient's fund, another example of a private initiative is the granulosa cell tumor research foundation (GCTRF) which has raised more than $300,000 for research since 2004.[57] This fund started an online community of granulosa cell tumor patients and after the founder passed away, her husband returned to University to study oncology and started working in the laboratory at the age of 60, dedicating the rest of his life to study his wife's disease.

It can be frustrating to patients with a rare disease that knowledge on their disease is lacking, so they start investigating their own disease. A third-year medical student cultured his own tumor cells, recognizing his rare disease was unlikely to be investigated by anyone else.[58] To date, most scientific focus and financial resources are on common cancers. It is often assumed that advances made in these tumors would directly or indirectly benefit all cancer patients. However, rare tumors can have distinct features, such as a specific *FOXL2* mutation in ~97% of the tumors.[41] Therefore it is essential that as many different disease pathways and potential targets for treatment are studied, we advance drug discovery for all cancers.

Actions following patient engagement

We reviewed the concerns of our patients. First, improvements in the laboratory procedures reduced the waiting time for test results from 6 to 3 weeks. We are also exploring other imaging modalities without radiation, such as magnetic resonance imaging (MRI), that could potentially replace repeated computed tomography scanning in the future and reduce radiation exposure.

Tools to increase patient engagement in research

Tools and platforms are now available to guide patient engagement. In collaboration with the European Network of Gynaecological Oncological Trial groups (ESGO-ENGOT), ENGAGe started to involve and train patients for active involvement in various stages of clinical trials. Another successful example is the Angiosarcoma Project, which enables patients to share their clinical information and allow for the acquisition of tumor tissue samples for research by giving online consent.[52] Patients were empowered to actively help stimulate research progress by overcoming barriers in data and tissue collection.

Despite great initiatives, patient engagement it is still not common practice. Cooperation between investigators and patients can be mutually beneficial, particularly in studies on rare diseases. Education on how to effectively engage patients is needed. Here we aim to provide tools for patient engagement and to motivate physicians and researchers to actively engage patients in their research.

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Competing interests: RZ and RV were the treating physicians of the main sponsor of this study.

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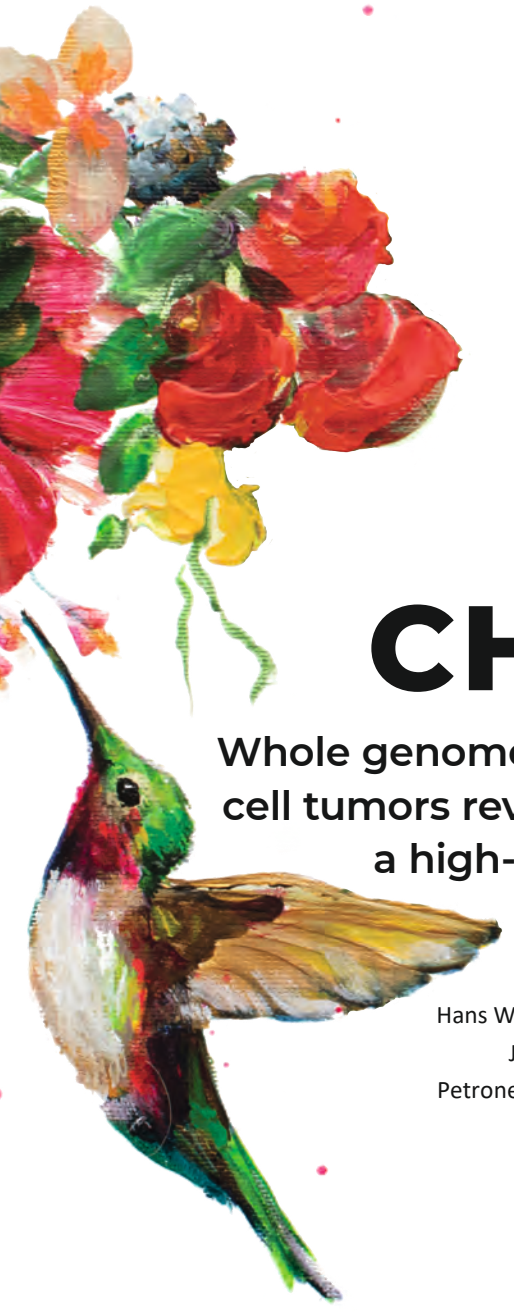

CHAPTER 3

Whole genome analysis of ovarian granulosa cell tumors reveals tumor heterogeneity and a high-grade TP53-specific subgroup

Joline F. Roze, Glen R. Monroe, Joachim Kutzera, Jolijn W. Groeneweg, Ellen Stelloo, Sterre T. Paijens, Hans W. Nijman, Hannah S. van Meurs, Luc R.C.W. van Lonkhuijzen, Jurgen M.J. Piek, Christianne A.R. Lok, Geertruida N. Jonges, Petronella O. Witteveen, René H.M. Verheijen, Gijs W. van Haaften, Ronald P. Zweemer

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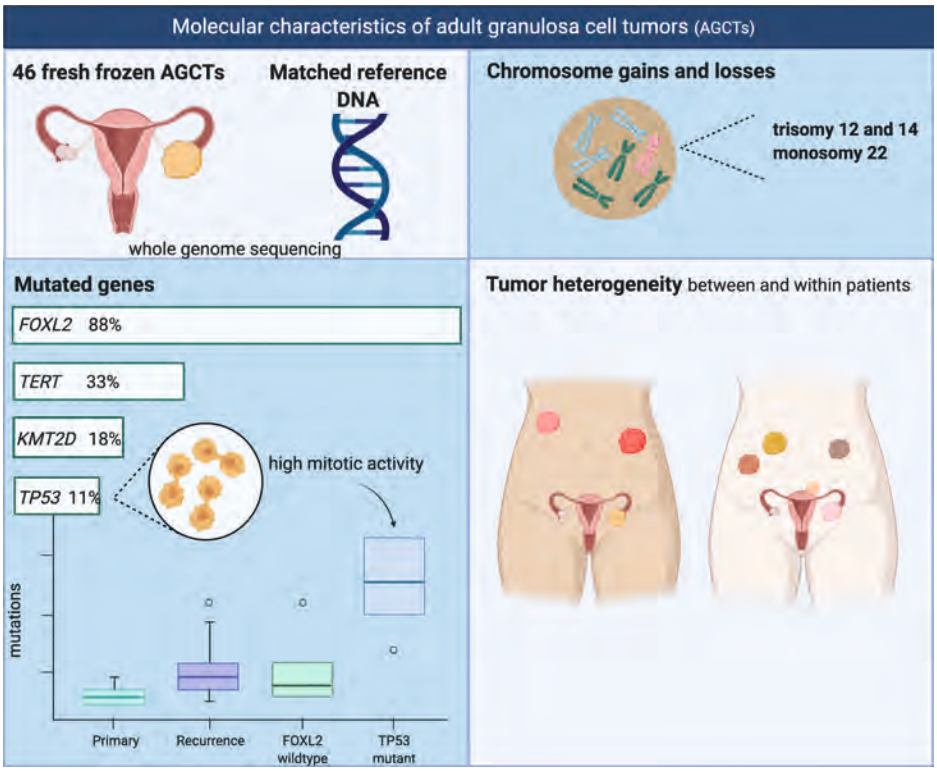
Abstract

Adult granulosa cell tumors (AGCTs) harbor a somatic *FOXL2* c.402C>G mutation in ~95% of cases and are mainly surgically removed due to limited systemic treatment effect. In this study, potentially targetable genomic alterations in AGCTs were investigated by whole genome sequencing on 46 tumor samples and matched normal DNA. Copy number variant (CNV) analysis confirmed gain of chromosome 12 and 14, and loss of 22. Pathogenic *TP53* mutations were identified in three patients with highest tumor mutational burden and mitotic activity, defining a high-grade AGCT subgroup. Within-patient tumor comparisons showed 29–80% unique somatic mutations per sample, suggesting tumor heterogeneity. A higher mutational burden was found in recurrent tumors, as compared to primary AGCTs. *FOXL2*-wildtype AGCTs harbored *DICER1*, *TERT*(C228T) and *TP53* mutations and similar CNV profiles as *FOXL2*-mutant tumors. Our study confirms that absence of the *FOXL2* c.402C>G mutation does not exclude AGCT diagnosis. The lack of overlapping variants in targetable cancer genes indicates the need for personalized treatment for AGCT patients.



Introduction

Adult granulosa cell tumors (AGCTs) belong to the sex cord-stromal tumors of the ovary and account for 2-5% of ovarian malignancies with an estimated incidence of 0.6-1.0 per 100.000 women per year worldwide.[10,15,38,59] Patients may develop symptoms, such as vaginal bleeding, caused by prolonged exposure to tumor-derived estrogen which may result in early detection of the disease. However, AGCTs are usually not preoperatively suspected and a histopathological diagnosis is made after surgical resection of an ovarian mass. Granulosa cell tumors also exist as a juvenile subtype, which generally occurs in young women and represents only 5% of this tumor type.[10] AGCTs are microscopically defined by grooved, uniform and pale nuclei and a variety and mixture of histologic patterns can be found including microfollicular, trabecular, insular and diffuse patterns.[10] Although the disease is frequently described as indolent in behavior, recurrences occur in approximately 50% of the patients of whom 50-80% ultimately die of their disease.[6,60,61] Recurrences often require repeated debulking surgeries since alternative treatment options including chemotherapy, radiotherapy and hormone therapy have thus far shown only limited effect.[7] The current lack of effective systemic treatment emphasizes the need for novel therapeutic strategies. Molecular characterization of AGCTs could help to identify potential targets for treatment.

In contrast with other ovarian malignancies, AGCTs harbor a specific *FOXL2* c.402C>G point mutation (C134W), which has been reported in 94-97% of patients.[6,41] FOXL2 is a transcription factor involved in ovarian function and granulosa cell differentiation.[62,63] Until now, efforts to target this gene have not been successful. Previous studies on genomic alterations in AGCTs identified copy number gains in chromosome 12 and 14 and loss of chromosome 22.[44,45,64–68] Furthermore, mutations were detected in genes that are known for their involvement in other cancer types, such as *TERT, KMT2D, PIK3CA, AKT1, CTNNB1* and *NR1D1*.[44–48,69] These studies included a subset of AGCTs within a larger ovarian cancer cohort and/or performed targeted or whole exome sequencing. Importantly, most studies did not analyze the corresponding normal reference DNA, essential for identifying true somatic variants.

We present the largest molecularly characterized cohort of AGCTs to date, in which we perform whole genome sequencing (WGS) on fresh frozen tumor material with matched normal reference DNA. We use this comprehensive method to investigate copy number changes, frequently mutated genes, mutational signatures and tumor heterogeneity. We define a subgroup of patients with high-grade AGCTs, harboring a pathogenic *TP53* mutation. In addition, we identify potential driver mutations in AGCTs without the *FOXL2* c.402C>G mutation, and find more variants in recurrent AGCTs as compared to primary tumors. Finally, we detect a high degree of intra-patient tumor heterogeneity.

Results

Description of WGS cohort

WGS was performed on 46 fresh frozen tumor samples from 33 patients (Table 1). We analyzed 11 patients with a primary tumor and 22 patients with recurrent disease. Microscopically assessed average tumor purity was 80% (range 40-90%). The whole genome was sequenced to an average read depth of 35X per sample (range: 26X–107X), with 97.5% of bases covered >10-fold (range: 93.9%-98.3%, Supplementary File 1). Matched normal reference DNA was obtained for all AGCTs. The median age at diagnosis was 53 years (range 29-75) and at the time of study, 21 patients had no evidence of disease, 11 were alive with disease and one patient had died of her disease. From five patients, tumors at multiple time points and/or multiple tumor locations were analyzed.

In our cohort, we detected a median number of somatic single nucleotide variants (SNVs) and small insertions/deletions (INDELs) of 3,579 variants per genome (range 1,346-21,452), of which 29 (range 13-238) were exonic, resulting in a tumor mutational burden (TMB) of approximately 1.25 per megabase (Mb, range 0.47-7.5). We identified 20 structural variants (SVs; excluding copy number variants (CNVs)) per sample (median, range 0-314). This low number of SVs indicates that DNA repair mechanisms are still intact in AGCTs. The median number of mutations (SNVs and INDELs) detected in primary tumors was 1.5x higher than reported in a previous WGS study including 10 primary AGCTs which identified 1,578 variants per tumor (range 630-2,706).[47] The TMB in our AGCT cohort was comparable to the TMB reported in a previous whole exome sequencing based study (1.2 mutations per Mb in primary AGCTs and 2.1 mutations per Mb in recurrences).[44] The number of variants detected in AGCTs falls within the same range of variants described in low grade serous ovarian cancer (median: 3,064, range 1,641-7,398).[70]

Previous studies reported conflicting results on the difference in mutational burden between primary and recurrent tumors.[44,45] In our study, primary tumors harbored 2,199 SNVs (median, range 1,346-4,120) and recurrent tumors 4,279 SNVs per sample (median, range 2,114-21,452; $p<0.001$). In addition, primary tumors carried fewer SVs (median 10, range 2-34) as compared to recurrent tumors (median 22, range 0-314; $p=0.018$). It is known that platinum based chemotherapy can increase the number of variants.[71] However, when we compared the mutations in primary tumors with recurrences that had not been treated with chemotherapy, the number of variants was still significantly different (median 2199 SNVs versus 3634 SNVs, t-test p-value = 0.0009). These results suggest that recurrences, also when stratified for prior chemotherapy treatment, harbor significantly more variants than primary tumors.

Common copy number alterations in chromosome 12, 14 and 22

Copy number analysis was performed on WGS data of 27 patients fulfilling the CNV caller pipeline requirements (see Methods). The majority of copy number alterations were duplications or losses of entire chromosome arms or chromosomes. In most patients, copy number loss of chromosome 22(q) (15/27, 56%) or gain of chromosome 14 (15/27, 56%) was found (Figure 1). Concurrent gain of chromosome 14 and loss of 22 was seen in 11/27 patients (41%). Loss of chromosome 16(q) was seen in 4/27 patients (15%) and gain of chromosome 1, 7, 8, 9, 12, 13, 14, 15, 18 or 20 were identified in at least 15% of patients. Concurrent copy number gain of chromosomes 8, 9 and 12 was detected in 7/27 patients (26%) and gain of both chromosome 18 and 20 was seen in 5/27 patients (19%). Within patients, copy number profiles remained stable between different time points and tumor locations (patient 8 and 13, Figure 1), or differed slightly between time points (patient 11 T1 versus T2 and T3). CNVs were detected in 7/9 (78%) patients with a primary tumor and in 16/18 (89%) patients with a recurrence. This study confirmed previously reported CNVs in either chromosome 12, 14 or 22 in 22/27 patients (81%).[44,45,64–68] Trisomy 12 is also often detected in other sex cord-stromal tumors and is usually the single copy number alteration in benign sex cord-stromal tumors such as fibromas and thecomas.[72] Monosomy 22 was identified as the sole anomaly in a mixed germ cell sex cord-stromal tumor of the ovary and in a fibrothecoma[73,74], and trisomy 8 as the single copy number variant in a Sertoli-Leydig cell tumor.[75] The effect of concomitant gain of chromosome 14 and loss of 22 in AGCTs is unknown and requires further investigation. In our cohort, copy number alterations are equally present in primary tumors and recurrences. It therefore remains unclear whether chromosome gains and losses are a cause or a consequence of tumor evolution.

Table 1. Clinical parameters and sample details of AGCT cohort.

Patient ID	Sample ID	Primary/ Recurrence	Sample location	Tumor purity	Reference DNA	Age at diagnosis	Initial tumor stage	Disease status
GCPA002		Recurrence	abdominal wall	90%	Saliva	35	IC	NED
GCPA005		Recurrence	mesentery	80%	Blood	66	IC	DOD
GCPA006		Recurrence	pelvic wall, left	90%	Blood	37	IC	NED
GCPA008	T1.1	Recurrence	small pelvis	90%	Blood	53	IA	NED
	T2.1	Recurrence	lung	90%	Blood			
GCPA011	T1.1	Recurrence	diaphragm right side	90%	Blood	53	IC	AWD
	T1.2	Recurrence	diaphragm right side	90%	Blood			
	T2.1	Recurrence	small bowel meso	70%	Blood			
	T3.1	Recurrence	liver	80%	Blood			
	T3.2	Recurrence	pelvis right side	60%	Blood			
	T3.3	Recurrence	ligamentum triangulare left	70%	Blood			
	T3.4	Recurrence	greater curvature stomach	60%	Blood			
GCPA013	T1.1	Recurrence	paracolic right	90%	Blood	50	IC	AWD
	T2.1	Recurrence	bladder peritoneum	80%	Blood			
	T2.2	Recurrence	iliac left	60%	Blood			
	T2.3	Recurrence	promontory	70%	Blood			
	T2.4	Recurrence	iliac right	90%	Blood			
GCPA016		Recurrence	abdominal wall	60%	Blood	69	IIB	AWD
GCPA017		Recurrence	below liver	80%	Blood	36	IC	AWD
GCPA019		Recurrence	lateral of psoas muscle	80%	Blood	57	IA	AWD
GCPA021		Recurrence	bladder peritoneum	70%	Blood	61	IC	NED
GCPA022	T1.1	Primary	ovary (left side within tumor)	70%	Blood	57	IA	NED

Table 1. (continued)

Patient ID	Sample ID	Primary/ Recurrence	Sample location	Tumor purity	Reference DNA	Age at diagnosis	Initial tumor stage	Disease status
	T1.2	Primary	ovary (right side within tumor)	80%	Blood			
GCPA023	T1.1	Recurrence	peritoneal cavity	80%	Blood	48	IA-C*	AWD
	T2.1	Recurrence	mesentery rectosigmoid	80%	Blood			
GCPA024		Recurrence	rectosigmoid	60%	Blood	30	IA-C*	AWD
GCPA030		Recurrence	bladder peritoneum	90%	Blood	43	IA-C*	AWD
GCPA031		Primary	ovary left	80%	Blood	49	IA	NED
GCPA032		Recurrence	obturatorius loge right	40%	Blood	61	unknown	NED
GCPA044		Primary	ovary	50%	Blood	61	IC	NED
GCPA046		Recurrence	spleen	90%	Blood	54	IA-C*	AWD
GCPA048		Primary	left ovary	80%	Blood	35	IC	NED
GCPA049		Recurrence	omentum	90%	Blood	52	unknown	NED
GCPA050		Recurrence	pouch of Douglas	60%	Saliva	32	IA	NED
GCPA051		Recurrence	pouch of Douglas	90%	Blood	39	IA-C*	NED
GCPA052		Recurrence	suprarenal infrahepatic	90%	Blood	37	IC	NED
GCPA053		Recurrence	ileocecal	80%	Saliva	39	IA	NED
GCPA054		Primary	ovary	90%	Blood	75	IC	NED
GCPA055		Primary	ovary	40%	Blood	65	IA	NED
GCPA057		Primary	ovary	80%	Saliva	65	unknown	NED
GCPA058		Recurrence	liver	80%	Saliva	53	IA	AWD
GCPA059		Primary	ovary	90%	Saliva	66	IA	AWD
GCPA060		Recurrence	ileum	90%	Saliva	47	IA	NED
GCPA061		Primary	ovary	80%	Saliva	71	IA	NED
GCPA065		Primary	ovary	90%	Blood	61	IA	NED
GCPA066		Primary	ovary	70%	Saliva	29	IA	NED

*Tumor limited to one ovary, no information available on potential capsule rupture before or during surgery. Sample ID's: first number indicates time point, second number indicates location (e.g. T3.1 is the first location of the third time point). NED: No evidence of disease. DOD: Dead of disease. AWD: alive with disease.

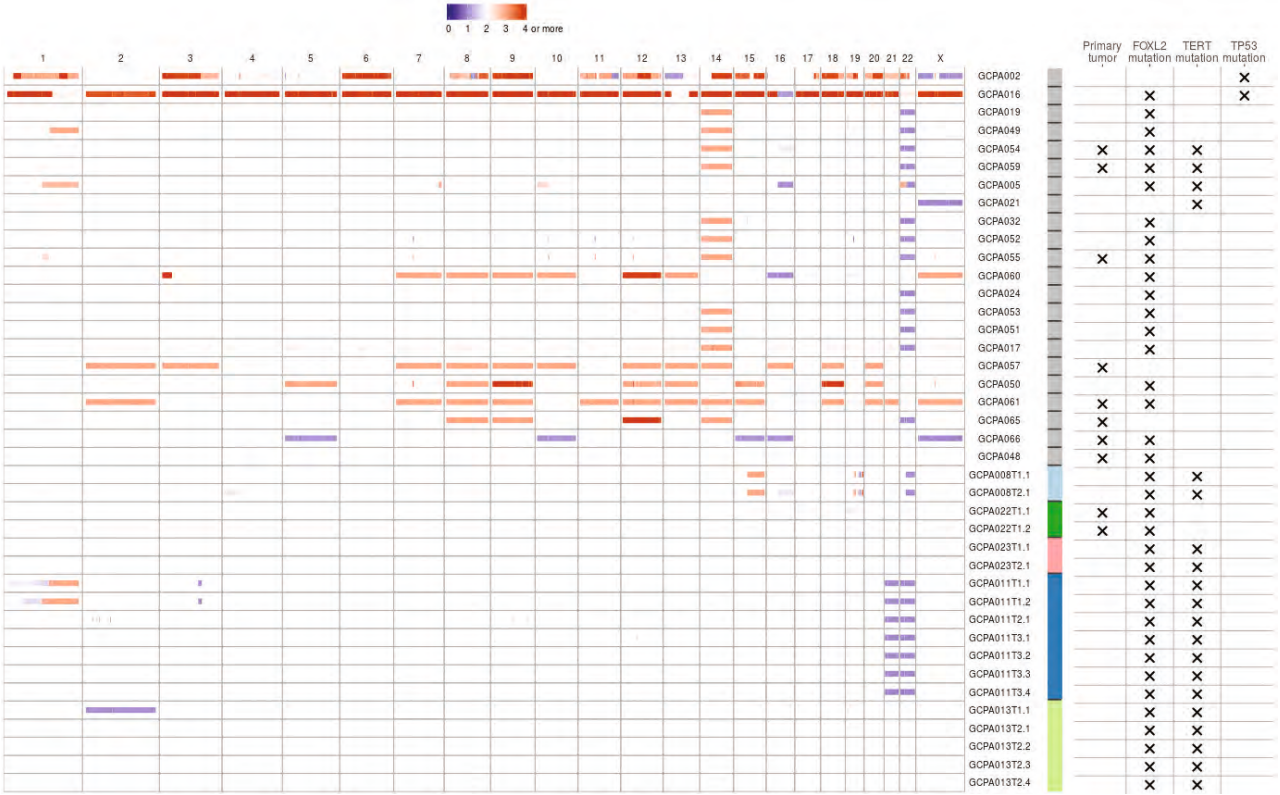


Figure 1. Copy number variants in AGCTs. The majority of copy number alterations are duplications or losses of entire chromosome arms or chromosomes. Blue/purple indicates copy number loss. Orange/red indicates copy number gain. Patients with a TP53 mutation had the most copy number alterations. One sample with a TP53 mutation did not fulfill CNV calling criteria and was therefore excluded from the CNV plot. We identified no differences in CNVs between primary tumors (X) and recurrent samples. Sample ID's: first number indicates time point, second number indicates location (e.g. T3.1 is the first location of the third time point). Sample ID's are ordered by TP53 mutation status, from highest to lowest total number of mutations and patients with multiple samples sequenced are clustered at the end. Color bar: gray indicates one sample per patient, the other colors represent patients with multiple samples.

Mutational signatures in AGCTs are related to ageing and platinum treatment

In addition to the number of mutations detected per sample, we investigated which mutational processes generated specific single base substitutions (C>A, C>G, C>T, T>A, T>C, and T>G). We applied de novo signature extraction and compared these signatures to COSMIC mutational signatures version 3 (Figure 2a,b). Four different major signatures were identified in the tumor samples. Two of the derived signatures were related to normal ageing processes being present in all cells (COSMIC 3, 5, 37 and COSMIC 3, 5, 40, respectively), one to platinum treatment (COSMIC 31, 35) and one signature with a yet unestablished cause (COSMIC 4, 20, 38, 45). No signatures related to microsatellite instability (MSI) or homologous recombination (HR) deficiency were detected (see Methods). Patients treated with chemotherapy demonstrated a significantly higher total number of variants (median 5463 SNVs) as compared to chemotherapy naïve patients (median 2861 SNVs, $p < 0.001$). Approximately 50% of the variants observed in these chemotherapy-exposed tumors can be explained by the platinum signature. In contrast, two patients (patient 30 and 49) treated with platinum-based chemotherapy did not show single base changes related to platinum treatment. These patients received only three out of six cycles of chemotherapy due to disease progression during treatment, whereas the patients that do show the platinum signature had a prolonged platinum exposure and received up to 2 × 6 cycles of chemotherapy. A recent study established that the contribution of platinum signature is dependent on the duration of treatment.[71] The absence of the platinum signature in these patients could possibly be explained by their resistance to platinum and the short duration of treatment.

Variants in known cancer genes were detected in FOXL2, TERT, KMT2D, PIK3CA and TP53

A total of 239 variants in known cancer genes from COSMIC[76] were detected in our AGCT cohort (Supplementary Table 2). Most recurrently mutated genes included *FOXL2*, *TERT*, *KMT2D*, *PIK3CA* and *TP53* (Figure 2d). We confirmed the *FOXL2* c.402C>G mutation in 29/33 patients (88%). Two patients had a second mutation in *FOXL2* consisting of a frameshift mutation and a variant in the UTR5 region, respectively (Supplementary Table 2). *TERT* C228T and C250T promoter mutations were present in respectively eight and three patients (together 33%), and were mutually exclusive. These variants were present in 2/11 (18%) patients with a primary tumor and in 9/22 (41%) of the patients with a recurrence. The majority of patients with active disease or who died of disease harbored a *TERT* promoter variant (7/12, 58%), while only 3/18 patients with no evidence of disease had a *TERT* promoter mutation (17%, Figure 2d,e). Although the two TERT promoter variants are known hotspots in cancer, previously only the C228T variant had been identified in AGCTs. However, our study and a recently published study have also detected the presence of C250T in AGCTs.[77] Our results corroborate the findings of a previous study that detected the TERT C228T variant more often in recurrences (41%) than in primary tumors (22%) and associated this mutation with impaired prognosis.[69]

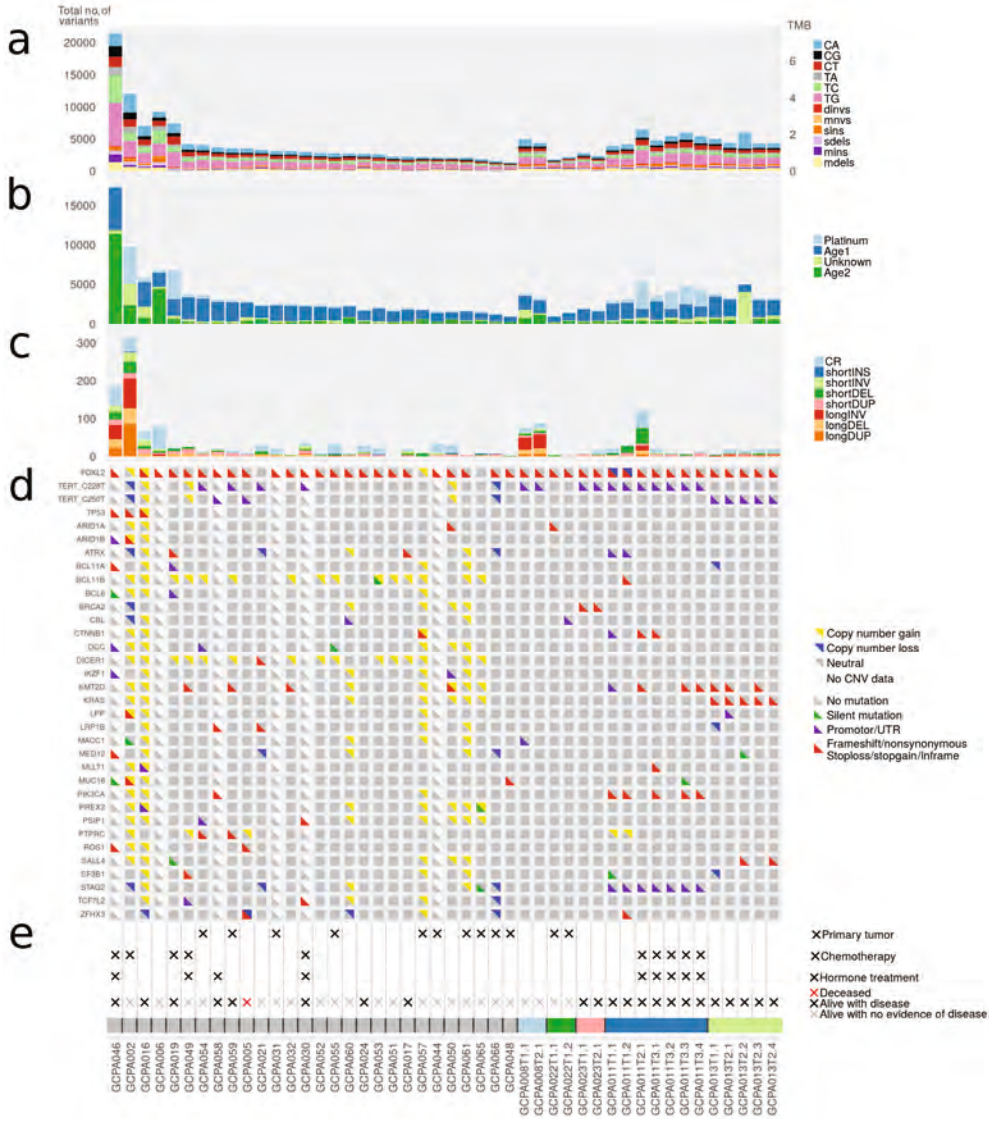


Figure 2. Number of somatic mutations, mutational signatures and variants in cancer genes.

(**a**) Total number of small somatic mutations in AGCTs. Dinvs=dinucleotide variants, mnvs=multi-nucleotide variants, sins=single insertions, sdels=single deletions, mins=multiple insertions, mdels=multiple deletions. (**b**) Mutational signatures. AGCTs show mutational signatures related to normal ageing processes (Age1: COSMIC 3,5,37 and Age2:COSMIC 3,5,40), platinum treatment (COSMIC 31, 35) and one signature with a yet unestablished cause according to COSMIC mutational signatures version 3 (COSMIC 4,20,38,45). (**c**) Total number of structural variants in AGCTs. CR =chromosomal rearrangements, INS=insertions, INV=inversions, DEL=deletions, DUP=duplications. All primary tumors harbor very few structural variants. (**d**) Mutations in genes linked to AGCTs and COSMIC Cancer Genes detected in ≥2 patients are shown. Silent variants include variants +/- 100bp into intron. (**e**) Clinical parameters and sample ID's. Primary tumors are indicated with (**X**), the remaining samples are recurrences.

Subgroup of patients with high-grade AGCT characterized by *TP53* mutation

Out of the five patients with the highest mutational load, three had a *TP53* mutation combined with loss of heterozygosity (R248G, H179R and C135Y, respectively. Figure 2). These tumors harbored numerous copy number alterations, and increased mitotic activity was seen on hematoxylin and eosin (H&E) slides from two patients (18 and 70 per $2mm^2$, respectively) as compared to *TP53*-wildtype patients (range 2-12 per $2mm^2$) (Figure 3). The *TP53* mutant tumors harbored a higher number of both SNVs (median 12,027, range 7,100-21,452) and SVs (median 188, range 66-314) as compared to the *TP53*-wildtype tumors (median 3,336 SNVs, range 1,346-9,211, median 20 SVs, range 0-120, Figure 2c, Figure 4 and Supplementary Figure 3a). Notably, none of the TP53 mutant samples harbored a TERT promoter mutation, which is detected in 41% of recurrent AGCTs. These mutually exclusive alterations may indicate a different molecular mechanism for disease progression.

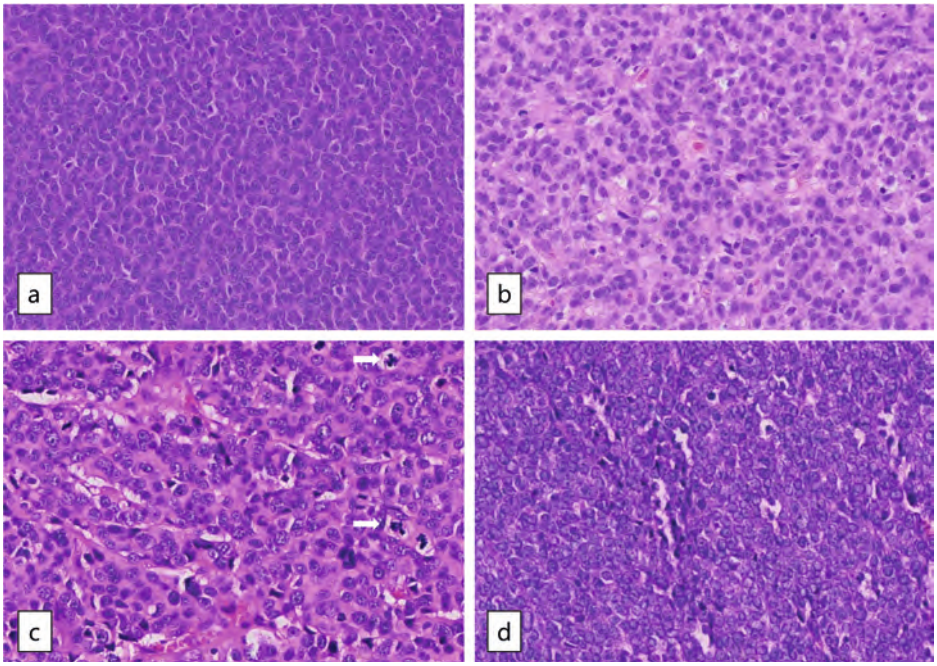


Figure 3. Microscopic examination of AGCTs. H&E staining of adult type granulosa cell tumors showing a homogenous cell population with typical grooved, coffee bean-like, nuclei. (a) Tumor GCPA011T1.1 with *FOXL2* mutation (20x magnification). (b) Tumor GCPA021 (with *TERT* C228T mutation, *DICER1* E1813D and *DICER1* stopgain variant Q1230*, 20x magnification). (c) and (d) Tumors GCPA016 and GCPA002 with pathogenic *TP53* mutation (H179R and C135Y, respectively, 20x magnification). Mitotic activity is indicated by arrows.

We investigated the clinical disease course in the patients harboring a *TP53* mutant tumor. The first patient (patient 46) was diagnosed with AGCT four years prior to study participation and was re-treated with chemotherapy after multiple surgeries and different hormonal and cytostatic treatment regimens (Supplementary File 1). The second patient (patient 2) had a total of four disease recurrences in eight years and is alive with no evidence of disease. The third patient (patient 16) presented with metastases at diagnosis (stage IIB disease), which is unusual since the vast majority of patients is diagnosed with a primary tumor confined to one ovary. This might indicate an early aggressive behavior of the tumor. The patient is being treated for her fifth recurrence and developed breast cancer as second primary tumor. In the literature, cases of AGCTs with areas of high-grade malignant morphology in either the primary tumor or recurrence have been reported. One study detected a *TP53* mutation in these high-grade components of 2/4 *FOXL2* mutant AGCTs and stated that *TP53* mutation likely plays a role in the high-grade transformation.[78] A recent case report describes an aggressive AGCT without a *FOXL2* mutation with strongly positive p53 immunohistochemistry and numerous mitoses[79], possibly similar to our finding of one *FOXL2*-wildtype *TP53*-mutant AGCT with high mitotic activity. *TP53* mutations have previously been found in 4-9% of AGCTs.[44,45,80] In conclusion, we found an association between the occurrence of a *TP53* mutation, high mutational burden, copy number alterations and mitotic activity. These characteristics define a subgroup of high-grade AGCTs with a potentially more aggressive tumor behavior.

FOXL2-wildtype AGCTs resemble FOXL2-mutant AGCTs

Four clinically and histopathologically confirmed AGCTs did not harbor the specific *FOXL2* variant (Figure 2d). As described above, we detected a damaging *TP53* variant in one *FOXL2* wildtype tumor. Interestingly, in one other tumor both the *TERT* C228T variant and two *DICER1* variants were found (E1813D and a stopgain variant Q1230* predicted to result in nonsense-mediated decay[81], Supplementary File 2). The *TERT* C228T variant has been previously detected in 26% of AGCTs[69] and similarly in 8/33 (24%) patients in our study. *DICER1* variants are detected in 2.38% of all cancers with non-small cell lung cancer, colorectal cancer, endometrial cancer, breast cancer, and melanoma having the greatest prevalence.[8] Somatic heterozygous mutations in *DICER1* are present in 29% of non-epithelial ovarian cancers, including Sertoli-Leydig cell tumors (60%, the majority harboring a E1705K or D1709N hotspot mutation), juvenile GCTs (7%), yolk sac tumors (13%) and mature teratomas (12%).[82] Specifically, *DICER1* mutations containing nonsense and missense variants in one of the four catalytic residues in the RNase IIIB domain (E1705, D1709, D1810, E1813), as we find, are the major known *DICER1* events in cancer.[83] No germline or somatic exonic truncating or missense *DICER1* variants were detected in the three remaining *FOXL2*-wildtype patients. *FOXL2*-wildtype patients also harbored somatic mutations in the cancer genes *ARID1B, STK11, TP53, PIK3R1, LRP1B, GATA1, NOTCH2, CTNNB1* and *PAX3*

(Supplementary File 2). However, none of the *FOXL2*-wildtype patients shared a variant in the same gene. Additionally, no SVs were detected in the vicinity of *FOXL2* (+/-10,000 base pairs) with a predicted effect on expression and/or regulation of *FOXL2*.

FOXL2 is thought to be a major tumor driver in AGCT, although the absence of a *FOXL2* mutation in a small subset of AGCTs suggests potential alternative mechanisms for tumorigenesis. In a recent study, granulosa cell tumorigenesis was associated with the combined inactivation of p53 and Rb pathways, with FOXL2 still present in newly developed AGCTs and *FOXL2* downregulation starting during AGCT growth.[84] Cluzet *et al.* suggest that *FOXL2* downregulation may be a late event in AGCTs and therefore possibly not a prerequisite for tumor development, but rather for tumor growth. This finding supports the hypothesis of alternative mechanisms for granulosa cell tumorigenesis.

The copy number profiles, number of SNVs and SVs of the *FOXL2*-wildtype tumors are similar to the tumors with *FOXL2* mutation (Figure 1 and Figure 4). *FOXL2*-wildtype tumors harbored 2,791 SNVs (median, range 1,919-12,027) and *FOXL2*-mutant 3,662 SNVs per tumor (median, range 1,346-21,452). Three out of four *FOXL2*-wildtype patients had CNVs in both chromosome 12 and 14 and one patient had additional loss of chromosome 22. One patient had monosomy X, which was previously detected in 1/17 (6%) AGCTs[65] and present in 3/28 (11%) of patients in our cohort. In all *FOXL2*-wildtype tumors, the diagnosis AGCT was reconfirmed by the pathologist. For example, the diagnosis Sertoli-Leydig cell tumor was considered and excluded in the *DICER1* mutated tumor because the tumor harbored the pathological characteristics of an AGCT (Figure 3b) and did not show signs of another ovarian tumor. In addition, this patient was postmenopausal when the ovarian mass was diagnosed, while the peak incidence of a Sertoli-Leydig cell tumor lies around the age of 25, and also had pre-operatively elevated circulating inhibin and estrogen levels. Our study confirms that the absence of the *FOXL2* c.402C>G mutation does not exclude the diagnosis AGCT. Moreover, in two patients we identified *TP53* and *DICER1*, respectively, as potential tumor drivers. These findings suggest there may be alternative mechanisms for AGCT development beside the *FOXL2* c.402C>G mutation.

Investigation of shared variants between AGCT patients confirms a limited number of recurrent mutations

Overlapping single nucleotide variants

Besides the specific missense variant in *FOXL2*, no overlapping somatic variants between ≥4 AGCT patients were detected in the coding regions of the genome. All variants detected by WGS with a CADD score >5 (Supplementary File 3a) were analyzed. Hereby, we identified 39 shared variants between ≥3 patients including the two *TERT* promoter variants (Supplementary File 3b). The sole detected coding variant shared by 3 patients, *TSPYL2* L34Pfs*28, was concluded to be a technical artefact upon Sanger sequencing validation.

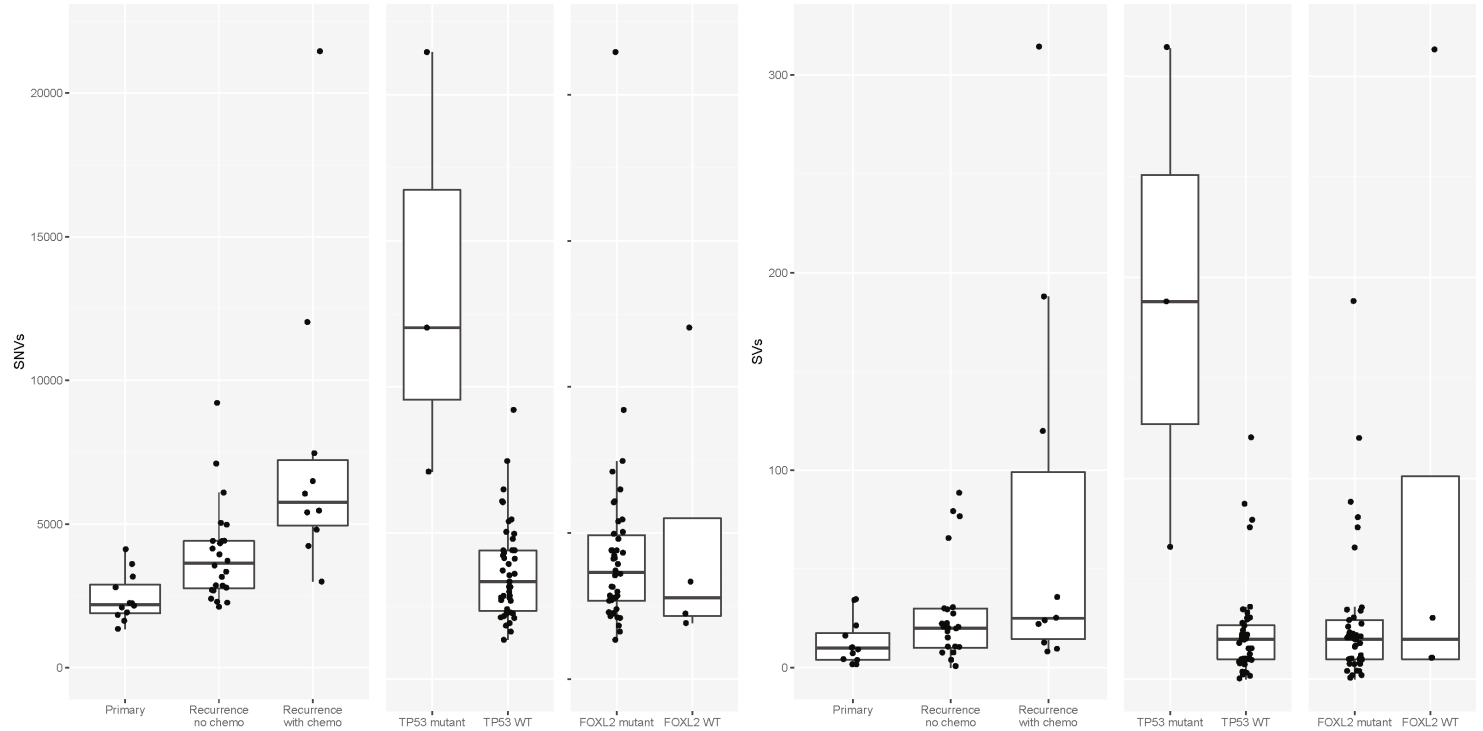


Figure 4. Differences in single-nucleotide variants (SNVs) and structural variants (SVs) between different subsets of AGCTs according to disease phase, prior chemotherapy treatment and mutational status. WT = wildtype.

We identified 305 non-coding variants present in only two patients. However, it remains challenging to interpret the effect of potential non-coding mutations on gene function. Variation in the non-coding areas of the genome is incompletely characterized and may be a result of sequencing and mapping artefacts as well as poorly understood localized hypermutation processes, and the functional effect that these variants may have on cis or trans regulatory elements is unknown.[85,86] Our study confirms the lack of overlap in mutations in AGCTs, as shown in previous studies.[44,45] These previous studies suggest that "second-hit" mutations leading to recurrence may be random.

Overlapping gene loss and structural variants

Additionally, we investigated if specific gene loss was recurrent across patients. We detected full loss of single or multiple genes in 7/33 (21%) of patients, although there was no overlap in deleted genes between patients. Finally, we investigated overlapping structural variants (other than CNVs) across patients and identified specific breakpoints on chromosome 1 in nine patients (Supplementary Figure 1, 2a-b).

Novel candidate gene analysis

Cohort analysis resulted in the identification of 13 genes with mutations in ≥3 patients that could possibly be deleterious, as predicted by the CADD PHRED score >5 (Supplementary File 3c). This list included two previously identified genes involved in AGCTs, *FOXL2* and *KMT2D*, and the newly described gene *TP53*. Our study confirms the previously reported limited number of recurrent mutations in individual genes in AGCTs.[45]

Intra-patient comparisons reveal tumor heterogeneity

To investigate tumor heterogeneity and tumor evolution over time, tumor tissue from multiple time points and/or multiple tumor locations of five different patients was sequenced and analyzed. In patients 8 and 23, we investigated tumor tissue obtained from two different recurrences and detected that 54-80% of the somatic variants (SNVs and INDELs) in these tumors were unique to a single time point (Supplementary Figure 3b). Surprisingly, two samples taken from the left and right side within a primary AGCT (patient 22) had the least overlap and carried 75-80% unique somatic variants (456 overlapping and 1,377-1,786 unique variants). The SVs also differed in this early stage of disease (1 and 2 SVs with no overlap, respectively, Supplementary Figure 3b). We investigated the variants in 7 and 5 different recurrences and tumor locations of patient 11 and 13, respectively (Figure 5). These patients showed a similar pattern of distribution between the proportion of overlapping (25-41% and 25-35%) and unique variants (29-73% and 31-66%). The detected larger structural variants also capture the heterogeneity of this tumor, with only 1 and 4 shared SVs between all samples of patient 11 and 13, respectively (Supplementary Figure

2c,d). Interestingly, all samples from patient 13 share the same SVs present at the initial time point and acquired additional SVs over time.

Patient 11 had active recurrent disease during the study period and required alternative treatment options after multiple surgeries and different chemotherapy and hormone treatment combinations (Figure 6). In this patient, recurrent *PIK3CA* mutations were found and this gene was identified as potential target for future treatment. However, during the course of her disease, different clonal variants in the *PIK3CA* pathway emerged and disappeared. Reads with the alternative allele, suggesting the presence of a subclonal mutation, were not detected in any of the other samples of this patient at these locations (sequencing depth mean = 32, range 19-43). The PI3K/AKT pathway was thought to be involved in granulosa cell tumorigenesis since PI3K activity within oocytes irreversibly transforms granulosa cells into AGCTs in mice.[87] Mutations in this pathway, however, have been detected in only a small proportion of patients (2/33, 6% in our study). Although this pathway was recurrently hit in this patient, it remains difficult to assess whether these mutations are drivers or passenger mutations. A previous study identified *KMT2D* inactivation as a driver event in AGCTs and suggest that mutation of this gene may increase the risk of disease recurrence.[44] In our study, mutations in *KMT2D* were present in 6/33 patients (18%). Patient 13 had different exonic inactivating mutations in *KMT2D* in 3/5 tumor samples (Figure 6). In this patient, *KRAS* was the only additional cancer gene besides *FOXL2* and *TERT* that was mutated in all samples. These examples illustrate both inter- and intra-patient heterogeneity in AGCTs.

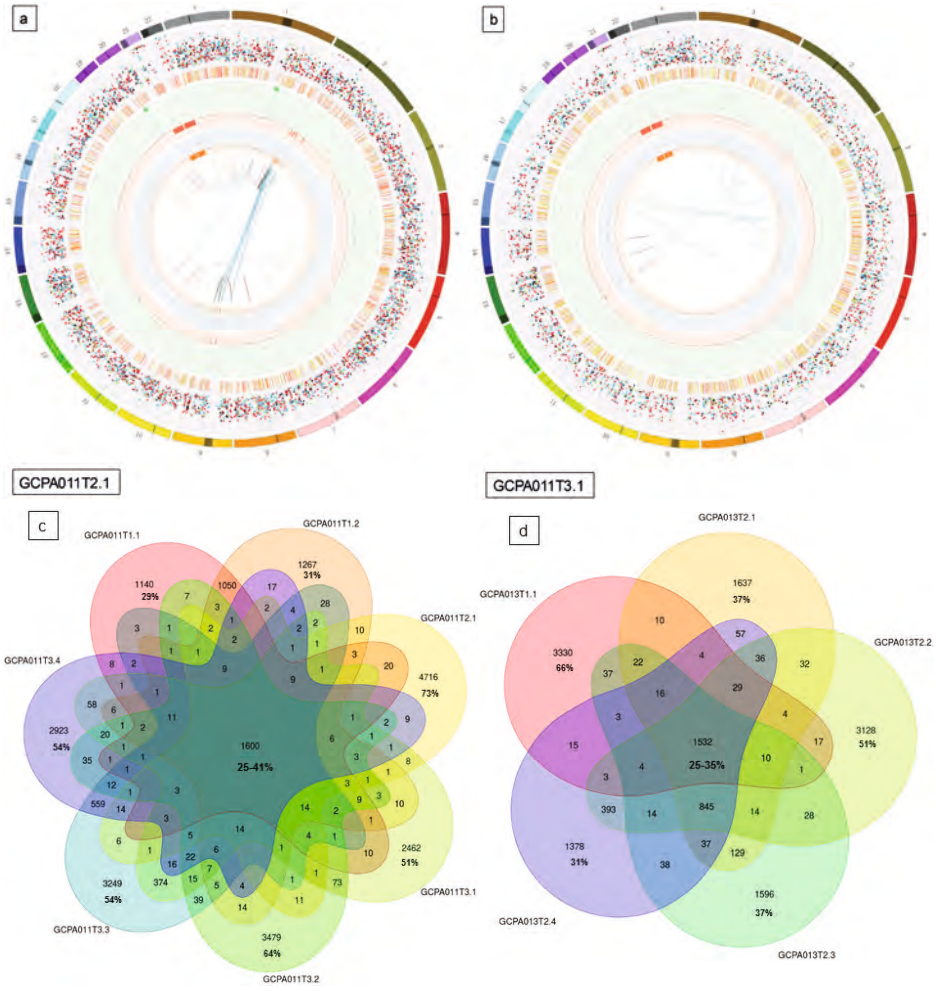


Figure 5. Circos plots and venn diagrams of intra-patient mutation comparisons. (**a**) and (**b**) Circos plots of samples GCPA011T2.1 and GCPA011T3.1 show differences in structural variants. The outer circle shows the chromosomes, the second circle shows the somatic single nucleotide variants, the third circle shows all observed tumor purity adjusted copy number changes, the fourth circle represents the observed 'minor allele copy numbers' across the chromosome, the innermost circle displays the observed structural variants within or between the chromosomes. Translocations are indicated in blue, deletions in red, insertions in yellow, tandem duplications in green and inversions in black. For detailed description of circos plots see Description of supplementary files. (**c**) and (**d**): Venn diagram from patient 11 and 13 showing the number of unique and overlapping variants between the tumor samples. Intra-patient comparison shows 29-73% unique and 25-41% overlapping variants. The samples GCPA011T2.1 and GCPA011T3.1 have 3117-4809 unique and 1683 overlapping variants.

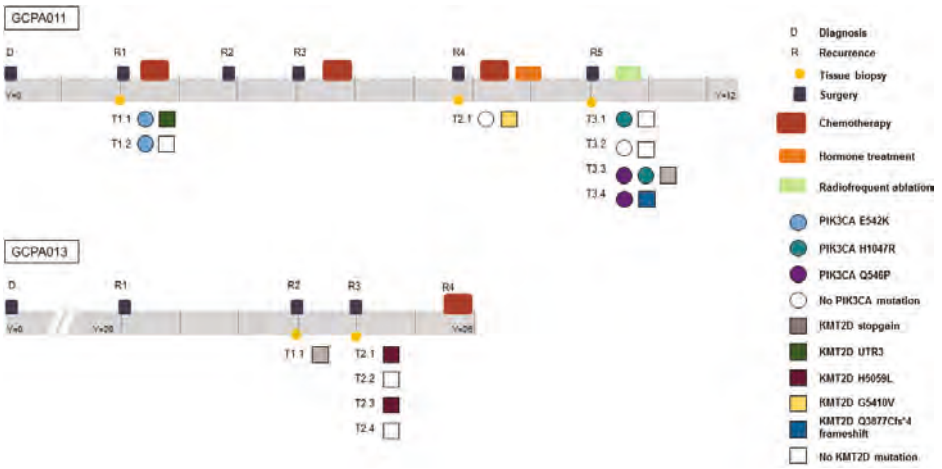


Figure 6. Timeline of patients 11 and 13 capturing the complexity of AGCT treatment and the intra-patient heterogeneity. Different time points and locations harbor different variants in the PIK3CA and KMT2D gene, respectively. Y= years since diagnosis. Both patients are alive with disease.

We also compared the dispersion of mutational signatures within the longitudinally obtained tumor samples from patient 11 and 13. Patient 11 was treated with chemotherapy after the first two tissue samples had been obtained (Figure 6). Only the tissue samples obtained at later recurrences showed the platinum signature (Figure 1b), while tissue obtained before treatment did not show the platinum signature. In line with these results, patient 13 had not been treated with chemotherapy before or during the study and none of her tumor samples harbored the platinum signature. This data confirms that chemotherapy can increase the number of variants, induces specific base changes and potentially plays a role in the heterogeneity between tumor metastases.[71] The lack of overlapping variants within and between patients provides evidence for tumor heterogeneity. This challenges the paradigm of AGCT as being a homogenous tumor. Our study shows that AGCTs, despite the microscopically homogenous cell population, are not homogenous in the genomic alterations they harbor. This can be a challenge for designing effective treatment strategies.

Discussion

This is the first study to investigate the whole genome of a large cohort of AGCTs by sequencing both tumor and matched reference DNA. AGCT patients did not share specific variants or affected genes except for the *FOXL2, KMT2D* and *TERT* promoter variants. A higher mutational burden was found in recurrent tumors, as compared to primary AGCTs. The molecular differences we detected between and within patients confirm tumor heterogeneity which is an established characteristic of cancer and rejects the view of AGCTs as being a homogenous tumor.[88] The few structural variants detected in AGCTs indicate that DNA repair mechanisms are still intact in these tumors. This study also illustrates the complexity of treating recurrent AGCTs, emerging with decreasing time intervals, and therefore suggests it should not be described as an indolent tumor. We confirm that absence of the *FOXL2* c.402C>G mutation does not exclude AGCT diagnosis and identified *TP53* and *DICER1* as potential drivers in these tumors. Furthermore, we define a subgroup of high-grade AGCTs characterized by a damaging *TP53* mutation, high tumor mutational burden and mitotic activity.

Whole genome sequencing has emerged as a comprehensive test over the past decade, enabling the detection of both exonic, intronic, and intergenic variation. Recent studies suggest that deeper sequencing (e.g. 90X) of the genome is needed to detect subclonal events with a low allele frequency in tumors and that multi-region sampling of the same tumor is necessary to capture the majority of variation present in a single sample [89,90]. In our study clonal tumor mutations could reliably be detected by 35X coverage of tumor samples with matched reference DNA. Moreover, the AGCTs in our study were high in tumor content, with the majority of tissues having a tumor percentage of 80-90% (31/46 samples). Variant allele frequency (VAF) calling at 30X in 80-90% tumor samples might be more accurate than VAF calling at 90X in a 30% tumor sample. As a result, this coverage is sufficient to detect clonal mutations in our cohort of high tumor purity samples and is currently used in large cancer studies.[91,92] Comprehensive deep- and multi-region sequencing of tumor samples will be necessary to detect the majority of mutations present in AGCTs and will most likely reduce intra-patient heterogeneity with the detection of all clonal and subclonal events within individual samples. However, the presence of heterogeneity amongst this tumor type, that has previously been defined as homogenous, is an important feature and has implications when searching for novel targets for treatment in these patients.

The lack of overlapping variants in targetable cancer genes in AGCTs indicates the need for personalized treatment. TERT is essential for the maintenance of telomere length and thus influences cellular immortality. TERT activity is an important mechanism for cancer to escape apoptosis and a promising therapeutic target for cancer, as it is highly expressed in most

tumor cells and hardly expressed in normal cells,. *TERT* mutations have frequently been detected in many cancers [93] and in our study a damaging promoter variant was consistently present in 33% of patients and in 41% of recurrences. Although it can be difficult to target a promoter variant, TERT silencing has been successful in NRAS mutant melanoma and might be applied to other tumors in the future.[94] In epithelial ovarian cancer, the presence of a *TP53* mutation determines high-grade disease. It can be hypothesized that AGCT patients harboring characteristics of a more aggressive tumor, such as a *TP53* mutation, might respond better to chemotherapy than patients without this variant since chemotherapy targets rapidly dividing cells. This requires further investigation in a larger cohort of *TP53*-mutant and wildtype tumors.

In conclusion, AGCTs harbor significant genetic heterogeneity between and within patients, and are not a homogenous and stable tumor type. This heterogeneity can be a challenge for future targeted treatment in AGCT patients, and suggests that a personalized, genotype-guided approach is required. Future personalized *in vitro* drug screens on patient-derived tumor tissue could facilitate the identification of potential patient-specific treatment and targeted sequencing of these tumors could identify actionable mutations.

Conclusions

This study shows that AGCTs harbor significant genetic heterogeneity and are not a homogenous and stable tumor type. This heterogeneity can be a challenge for future targeted treatment in AGCT patients, and suggests that a personalized, genotype-guided approach is required. We confirm that absence of the *FOXL2* c.402C>G mutation does not exclude AGCT diagnosis and identified *TP53* and *DICER1* as potential drivers in these tumors. Furthermore, we define a subgroup of high-grade AGCTs characterized by a damaging *TP53* mutation, high tumor mutational burden and mitotic activity.

Materials and Methods

Patient cohort and study inclusion criteria

A national prospective study was performed to obtain patient derived fresh frozen tumor tissue and corresponding germline DNA (blood or saliva). Patients were included consecutively during their hospital consultation in 5 hospitals between 2018-2019. In addition, patients with fresh frozen tumor material available in the hospital's pathology archive were asked for consent. Ethical approval was obtained by the Institutional Review Board of the University Medical Center Utrecht (UMCU METC 17-868) and by the board of directors of the participating centers. All participants provided written informed consent. Clinical data was acquired from patient reports.

Tissue acquisition

All tumor material was obtained directly from surgery, fresh frozen and transported in dry ice and stored in the -80 degrees freezer. From the tissue, 20x 5 µm slices were cut for DNA isolation. Hematoxylin and eosin (H&E) staining slides were made and reviewed by a pathologist (GNJ) to confirm AGCT diagnosis and assess tumor percentage. Minimal tumor percentage for study inclusion was 40%, with the majority of tissues having a tumor percentage of 80-90% (31/46 samples, Table 1). In parallel, fresh frozen tumor material was crushed in a liquid nitrogen cooled mortar using a pestle, and pulverized tissue was collected for DNA isolation. Material for normal reference DNA isolation was acquired by prospective blood draw during patient follow-up visit. In cases where patients no longer required follow up at the hospital, Oragene-DNA OG-500 saliva DNA isolation kits (DNAGenotek, Ottawa, Ontario, Canada) were mailed to the patient residence and taken by the patient after instructions, so that a hospital visit and blood draw were not required.

DNA isolation, quantification and qualification

DNA from the fresh frozen pulverized tumor tissue was isolated using Genomic-tip 100/G (Qiagen, Venlo, NL). Isolated DNA was quantified by Qubit 2.0 dsDNA broad assay kit (ThermoFischer Scientific, Waltham, MA, USA) and ensured to be of high molecular weight by visualization on a 1.5% agarose gel. Samples were then concentrated if needed to a minimal concentration of 15 ng/µl and a total of 1 µg was aliquoted for WGS. Normal reference DNA isolation from blood was performed according to the DNeasy blood isolation protocol (Qiagen, Venlo, NL) and normal reference DNA isolation from saliva samples performed according to the prepIT-L2P protocol (DNAGenotek, Ottawa, Ontario, Canada).

Whole-genome sequencing and variant calling

DNA was sent for 2X150bp paired-end sequencing on Illumina HiSeq X or NovaSeq 6000 instrument (Illumina, San Diego, CA, USA) to the Hartwig Medical Foundation (HMF, Amsterdam, NL, 23 tumor samples with normal reference DNA) or Novogene (Novogene, Beijing, China, 23 tumor samples with normal reference DNA). Mapping and variant calling from raw fastQ reads was performed using a pipeline (v4.8) from the Hartwig Medical Foundation (HMF) installed locally at the UMCU using GNU Guix (https://github.com/UMCUGenetics/guix-additions; https://github.com/hartwigmedical/pipeline[89]). Sequence reads were mapped with Burrows-Wheeler Alignment v0.75a[95] against human reference genome GRCh37. Realignment of insertions and deletions and base recalibration was performed with the Genome Analysis Toolkit (GATK, v3.8.1).[96] Somatic single nucleotide variants (SNVs) and small insertions and deletions (INDELS) were called with Strelka (v1.0.14).[97] All SNVs labelled as "PASS" were included in the analysis. The functional effect of the somatic SNVs and INDELS were predicted with SnpEff (v.4.3).[98] Somatic structural variants (SVs) were called using GRIDSS (v1.8.0) and copy number variation called by using PURPLE.[99]

WGS data analysis

The tumor mutational burden (TMB; mutations per Mb) for each sample was derived by dividing the sum of mutations across the entire genome (SNVs, MNVs (multiple nucleotide variants) and INDELS) by the total mappable sequence length of the GRCh37 FASTA file (2858674662) divided by 10^6, as has been described previously.[100] Wilcoxon rank-sum test was performed for between-group comparisons, two tailed, with a 0.05 significance level.

CNV analysis

The results obtained from PURPLE (CNVs per breakpoint and per gene) were processed and plotted using in-house R tools for inter- and intra-patient comparison of CNVs. Samples not fulfilling PURPLE quality control criteria for CNV calling (status: "FAIL_SEGMENT") were not plotted. PURPLE uses allele frequency data (bam reads) for breakpoint detection. In a subsequent quality control step, it checks the result from the SV caller (GRIDSS) to increase evidence for each breakpoint. It tolerates a number of breakpoints without proper SV evidence but flags a sample when that number exceeds a threshold. In samples with potential decreased CNV calling reliability, the SNV calling is unaffected.

Exonic variant analysis

The exonic and UTR variant analysis was performed using Alissa (Agilent Technologies Alissa Interpret v5.1.4). Variants in the exonic regions, ±100 base pairs into the intronic regions, and in the 5' and 3' UTR of Refseq transcripts were retained for analysis. Variants present in the COSMIC Cancer Gene Census (release v89) as somatic mutations in cancer were annotated (Supplementary File 2a,b).

To find novel candidate genes in which mutations could contribute to oncogenesis and/or recurrence, we performed a cohort analysis to identify genes with a somatic variant in multiple samples. As this study contained multiple samples from individual patients, this variant list was further filtered to remove any genes in which variation occurred solely within samples of a particular patient. Variants were annotated with the CADD PHRED (Combined Annotation Dependent Depletion)[101] score and genes with no or only one variant >5 were removed.

Whole genome variant analysis

Variation throughout the entire genome was assessed to identify recurrent variations and prioritized by CADD score. Briefly, somatic Variant Call Format (VCF) file of each sample was combined into one complete cohort specific VCF containing somatic variation across all samples. As the effect of non-coding variation is difficult to predict, we annotated all variant positions with the CADD PHRED score, a score that is suitable for assessing the effect of exonic, intronic, regulatory and intergenic variation. To exclude mutations with

minimal predicted deleteriousness, we selected the variants with a CADD PHRED score > 5 and identified mutations shared by multiple patients. Candidate variants shared by ≥3 patients were validated by Sanger sequencing, as recurrent unannotated variation can be a result of platform or library preparation technical artifacts.[102]

Mutational Signature analysis

Mutational signatures were created using the R-package mutationalPatterns.[103] We derived mutational signatures from the SNV data and selected the optimal number of signatures by inspecting the Non-negative Matrix Factorization (NMF) rank survey as described in the vignette of the MutationalPatterns R-package. In detail, We examined the "rss" (residual sum of squares) plot and the "cophronetic" (cophronetic correlation) plot. We checked for an elbow in the rss plot and aimed for a high cophronetic correlation between model and data. We created four signatures per mutation type and compared them to the signatures from COSMIC mutation signatures version 3 using the cosine-similarity measure. Derived signatures were named according to the proposed etiology of the closest COSMIC signatures.

Tumor heterogeneity assessment

SNV heterogeneity was assessed by comparing the number of overlapping SNVs for the patients with multiple tumor samples from different time points and/or tumor locations. SV heterogeneity was investigated using in house tools based on the R-package StructuralVariantAnnotation (Bioconductor version: Release 3.10).

Homologous Recombination

We investigated Homologous Recombination by the CHORD (https://github.com/UMCUGenetics/CHORD) method, which is a random forest model that predicts homologous recombination deficiency (HRD) using the relative counts of specific somatic mutation contexts. The main contexts used by CHORD are small deletions with flanking microhomology and 1-100kb structural duplications.[104]

Data availability

WGS Binary Alignment Map (BAM) files are available through controlled access at the European Genome-phenome Archive (EGA), hosted at the EBI and the CRG (https://ega-archive.org), with accession number EGAS00001004249. Requests for data access will be evaluated by the UMCU Department of Genetics Data Access Board (EGAC00001000432) and transferred on completion of a material transfer agreement and authorization by the medical ethical committee of the UMCU to ensure compliance with the Dutch ‘medical research involving human subjects’ act.

Supplementary Materials: The Supplementary Files and Figures are available online at https://www.mdpi.com/2072-6694/12/5/1308#supplementary .

Author Contributions: JR, JG, ES, RV and RZ designed the study and obtained ethical approval of the study protocol. JR, SP, HN, HM, LL, JP, CL, GJ, PW and RZ collected clinical data, tissue and reference DNA samples. JR, GM and JK performed data analysis and bioinformatics. GJ assessed tumor purity and reviewed all specimens for histological pathology. GM performed DNA isolations. JR, GM, JG, GH and RZ wrote the manuscript. All authors revised the manuscript and approved final version for publication.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

The Description of Supplementary Files

Supplementary File 1. Clinical characteristics and sequencing details of AGCT samples

Supplementary File 2. Variants in COSMIC somatic cancer genes

Filtering:

1. All somatic variants
2. Select all PASS variants
3. Select variants that are exonic and ±100 bases into intron, including all 5' and 3' UTR variants
4. Annotate all variants that reside in genes in which somatic variation is known to be involved in cancer (somatic, Cancer Gene Census)

Supplementary File 3. Shared variants and candidate genes.

a. Variants with CADD score > 5

b. Variants shared in ≥ 2 patients

c. Genes with variation in ≥ 3 patients.

See methods of manuscript.

Supplementary Figure 1. Full gene loss

Supplementary Figure 2. Structural Variants

a. Structural Variants in more than 1 sample

b. Structural Variants in chromosome 1*

c. Structural Variants in patient 11

d. Structural Variants in patient 13

In the labels, "|->" marks a front-to-back breakpoint (preserved direction), "|-|" a back-to-back breakpoint and "<->" a front-to front breakpoint. *The overlapping breakpoint shown in the figure are not exactly on the same position, but detected within a range of 50 base pairs.

Supplementary Figure 3. Circos plots and venn diagrams

a. Circos plots of TP53 mutant samples

b. Circos plots and venn diagrams from all intra-patient comparisons.

CHAPTER 4

In vitro systematic drug testing reveals carboplatin, paclitaxel and alpelisib as a potential novel combination treatment for adult granulosa cell tumors

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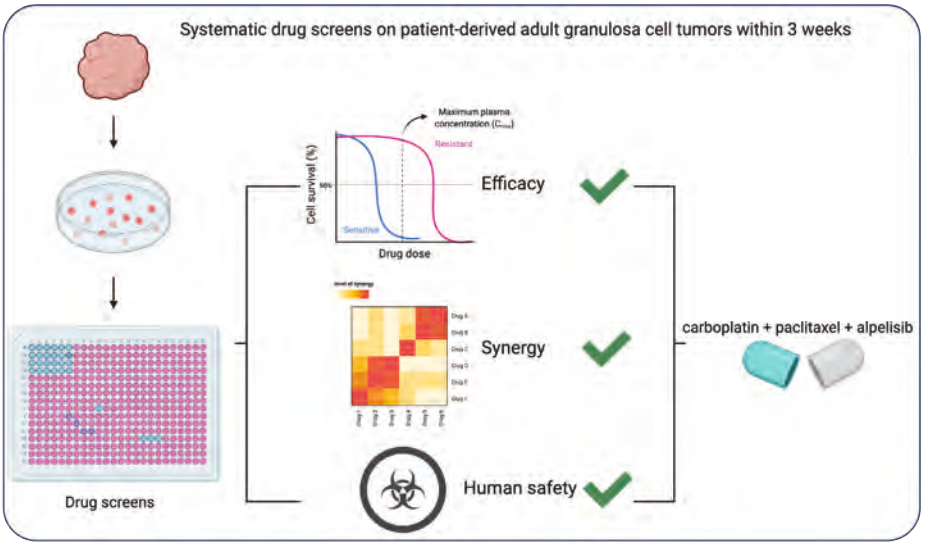
Abstract

Adult granulosa cell tumors (AGCTs), arise from the estrogen-producing granulosa cells. Treatment of recurrence remains a clinical challenge, as systemic anti-hormonal treatment or chemotherapy is only effective in selected patients. We established a method to rapidly screen for drug responses *in vitro* using direct patient-derived cell lines, in order to optimize treatment selection. The response to 11 monotherapies and 12 combination therapies, including chemotherapeutic, anti-hormonal, and targeted agents, were tested in 12 AGCT patient-derived cell lines and an AGCT cell line (KGN). Drug screens were performed within 3 weeks after tissue collection by measurement of cell viability 72h after drug application. Potential synergy of drug combinations was assessed. The human maximum plasma concentration thresholds (Cmax/Css), obtained from available phase I/II clinical trials, were used to predict potential toxicity in patients. Patient-derived AGCT cell lines demonstrated resistance to all monotherapies. All cell lines showed synergistic growth inhibition by combination treatment with carboplatin, paclitaxel and alpelisib at concentrations that are below the maximum achievable concentration in patients (IC50<Cmax). We show that AGCT cell lines can be rapidly established and used for patient-specific *in vitro* drug testing which may guide treatment decisions. Combination treatment with carboplatin, paclitaxel and alpelisib was consistently effective in AGCT cell lines and should be further studied as a potential effective combination for AGCT treatment in patients.



Introduction

Adult granulosa cell tumors (AGCTs) represent a hormonally active, rare subtype of ovarian cancer arising from stromal granulosa cells. The disease has an incidence of 0.6-1.0 per 100.000 women worldwide.[10,15,38,59] Patients are suspected to have an AGCT when presenting with postmenopausal or irregular vaginal bleeding, abdominal pain, high plasma estrogen and inhibin levels and/or ultrasound findings of a cystic and/or solid ovarian mass.[10] However, due to its rarity, AGCTs are often not preoperatively recognized and therefore mostly diagnosed at histopathological evaluation after surgery. Microscopically, AGCTs harbor granulosa cells with grooved nuclei, with or without other stromal cells. The classical Call-Exner bodies, areas of eosinophilic fluid surrounded by granulosa cells, are detected in 30-60% of cases.[105–107] Immunohistological staining is positive for inhibin and calretinin. When histopathology is inconclusive, *FOXL2* c.402C>G (C134W) mutation testing can be performed. This specific mutation is a hallmark of AGCTs and is present in 90-97% of patients.[6,41]

Primary tumors are confined to one ovary (stage I disease) in 78-91% of patients and surgically removed.[10] However, recurrences occur in approximately 50% of patients and often require repeated debulking surgeries. Ultimately 50-80% of patients with a recurrence will die of disease.[6,60,61] Due to its rarity, studies specifically designed for the treatment of AGCT are lacking resulting in a poor efficacy of treatment. Systemic treatment strategies for AGCTs are therefore based upon studies on more common ovarian cancer subtypes. First line systemic treatment is currently derived from high grade epithelial ovarian cancer treatment guidelines and consists of the combination carboplatin and paclitaxel, although treatment with bleomycine, etoposide and cisplatin (BEP) is also used. A prospective study showed that, compared to BEP, carboplatin and paclitaxel had a progression free survival of 27.7 months versus 19.7 months, and demonstrated a more favorable side effect profile (NCT01042522). A retrospective analysis investigating the efficacy of a chemotherapeutic treatment in AGCT demonstrated a partial response in 11-25% and complete response in a further 11-26% of patients.[30] These studies included only 5 - 39 patients for evaluation, with varying chemotherapy regimens, as large patient numbers in this rare tumor type are difficult to obtain.

Since AGCTs express hormone receptors, endocrine therapy was thought to be an effective treatment. Initially, the selective estrogen receptor modulator tamoxifen was utilized, and currently the aromatase inhibitor letrozole and the estrogen receptor antagonist fulvestrant are also given. A recent retrospective study showed that anti-estrogen treatment decreased tumor load in 4 out of 22 AGCT patients (18%).[29] The similar response rate and significantly fewer side effects of anti-hormonal treatment as compared to chemotherapy could warrant

consideration of endocrine therapy as first systemic treatment. However, response rates remain low and benefits are only expected in a subset of patients. Moreover, several recent studies found no survival benefit after treatment with either chemotherapy or endocrine therapy in AGCT patients, emphasizing the need for novel treatment options.[31–36]

Treatment of recurrent disease remains a clinical challenge since effective systemic therapies are lacking. Clinical drug trials are difficult to perform in rare diseases, such as AGCT. Therefore, critical evaluation of current systemic treatment options is needed to identify potential sensitive subgroups, as well as identification of promising novel targeted therapies. To date, only one study that performed a large scale drug screen on AGCT patient-derived cell lines has been published.[108] In this study, many individual drug compounds in seven AGCT cell lines and four drug combinations in the AGCT cell line KGN were tested. Paclitaxel combined with either the SRC tyrosine kinase inhibitor dasatinib or the mTOR inhibitor everolimus resulted in a synergistic response, and RNASeq established that the downstream targets of these drugs were abundantly expressed in AGCTs.[108] Previous studies have shown growth inhibition when these inhibitors are used as a monotherapy in KGN cells or in a GCT peritoneal carcinomatosis mouse model.[109,110] Further *in vitro* drug testing may help to identify effective drug combinations and personalize treatment for AGCT patients.

Here, we demonstrate a method to rapidly establish AGCT cell lines from patient-derived tumors and screen for drug responses. We confirmed the *FOXL2*.c402C>G mutation to verify tumor cell origin in the established cell lines and tested current and novel systemic therapies including drug combinations to assess potential synergy. We detect synergistic inhibitory effects on cell growth for the combination treatment of carboplatin and paclitaxel with the specific PIK3CA inhibitor alpelisib at concentrations that are clinically relevant for patients *in vivo*. We show that rapid, systematic patient-specific AGCT drug screens are feasible and could be used to personalize treatment selection.

Results

Rapid patient-derived AGCT cell line establishment and systematic drug screening
A multicenter prospective study was performed to obtain fresh patient-derived tumor tissue immediately after surgery. We were able to establish short term 2D cultures for 38 out of 48 tumors, resulting in a 79% success rate. The first established cell lines were used for optimizing culture conditions and the drug screen experimental set up. Subsequently, the growth inhibitory effects of 11 drug compounds were investigated *in vitro* in 12 AGCT patient-derived cell lines originating from five different patients. Drug screen results were obtained within an average of 3 weeks (median 20, range 12 – 30 days) after tissue collection. Of each cell line, three biological replicates containing two technical replicates

each were used for the drug screens. Drug compounds were selected based on current AGCT treatment, hormone-positive breast cancer treatment and novel targeted treatment in other cancers. AGCT cell line establishment and drug screen set up is summarized in Figure 1.

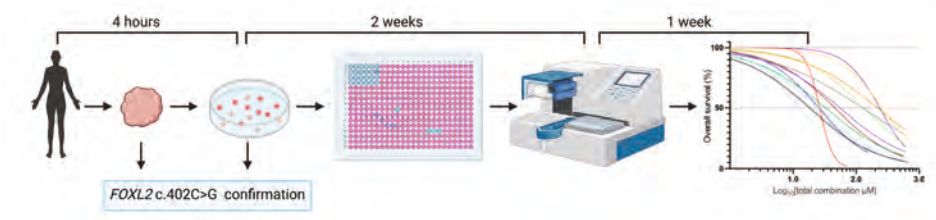


Figure 1. AGCT cell line establishment and drug screen work flow. Tumor samples were processed within 4 hours after collection. Confirmation of the *FOXL2* c.402C>G mutational status of the tumor and cell line was performed by Sanger Sequencing. Upon sufficient expansion, cells were seeded in a 384 well plate and drugs were applied 24 hours after cell seeding. The response to treatment was analyzed 72 hours after drug application by measuring cell viability.

Sanger sequencing confirmed the heterozygous *FOXL2* c.402C>G mutation in all tumors and in 9 of the 12 cell lines (Table 1). Tumor origin was confirmed by CytoSNP-850K snp array in the remaining two *FOXL2* wildtype cell lines (GCPA096T1.II and GCPA113T1.I, Supplemental Figure 1). Additionally, loss of the *FOXL2* wildtype allele was confirmed in GCPA113T1.II harboring a hemizygous *FOXL2* c.402C>G mutation. We used the breast cancer cell line MCF-7 and neuroblastoma cell line SH-SY5Y as positive controls for hormone treatment and chemotherapy, respectively. The immortalized human granulosa cell line SVOG-3e was used to test selective sensitivity for targeted drugs.

Treatment with chemotherapeutic, anti-hormonal or targeted monotherapy shows inefficacy at maximum plasma concentrations in all AGCT cell lines

Growth inhibitory effects of chemotherapeutic agents carboplatin and paclitaxel, anti-hormonal drugs tamoxifen, letrozole, anastrozole, fulvestrant and ulipristal, and the targeted drugs everolimus, alpelisib, dasatinib and 6-THIO-2dG, were tested as monotherapy in 12 direct patient-derived AGCT cell lines and the KGN cell line (Figure 2 and Supplemental Figure 2). The targeted drugs everolimus and dasatinib were previously shown to result in AGCT cell line growth inhibition in conjugation with paclitaxel.[108] Alpelisib was chosen to target the PI3K/AKT/mTOR pathway, which has been identified in AGCT pathogenesis.[108] 6-THIO-2dG is a telomerase blocker that results in telomeric DNA damage in cells expressing telomerase, as *TERT* promoter mutations and *TERT* activation are common in AGCTs.[69,111] Ulipristal is a progesterone receptor blocker, and was chosen to target progesterone, as a recent study identified a high progesterone receptor composite score associated with decreased recurrence-free and overall survival [112].For these drugs, we obtained the Cmax and Css values from phase I/II studies [113–119]. For 6-THIO-2dG, Cmax and Css values are not

available. The control cell lines SH-SYS5 and MCF-7 were sensitive for the chemotherapeutic agents and anti-hormonal drugs, respectively (IC50<Cmax, and IC10<Css, Supplemental Figure 3). Anastrozole did not decrease cell viability at tolerable concentrations in MCF7, in concordance with previous reports [120,121]. Furthermore, the SVOG-3e cell line showed potential sensitivity to alpelisib, as the IC50 was slightly above the Cmax (7.55 µM versus 6.9 µM, Supplemental Figure 3). Dose response curves of the individual drugs demonstrated similar response profiles for all AGCT cell lines including KGN. For all monotherapies the IC50 exceeded the Cmax and Css values, suggesting that monotherapy drug concentrations required for 50% cell death are unlikely to be achieved in vivo. Moreover, for all anti-hormonal drugs, concentrations higher than the Css were needed to obtain 10% cell death (tamoxifen: Css = 0.11 µM, range IC10 = 6.04-15.03 µM, Figure 2).

Combination treatment in KGN shows synergistic effects and therefore allows for drug dose reduction

In order to investigate combination therapies, we evaluated 12 drug combinations and tested for synergistic interactions in KGN cells. The drug dosages in combination were kept at a constant ratio throughout the experiment and based upon their monotherapy ranges (Table 3). KGN cells were most sensitive for the combination carboplatin, paclitaxel, alpelisib (10:1:2 ratio), carboplatin, paclitaxel, dasatinib (10:1:0.4 ratio), alpelisib with everolimus (2:1 ratio) and everolimus with tamoxifen (5:2 ratio) (Figure 3). Out of the twelve combinations tested, eight showed a certain degree of synergy (Combination Index (CI) < 1) (Supplemental Table 1). Therefore, significant dose reduction could be applied to the individual drugs when used in combination. Although multiple drug combinations demonstrated synergy, most of these combinations could not reach 50% cell death at maximum plasma concentrations for each individual drug (IC50>Cmax or Css). However, the single combination that was effective at concentrations below the Cmax of each individual drug was carboplatin, paclitaxel, and alpelisib (Figure 3). This combination was efficacious and showed strong synergy (CI = 0.14). These findings in the KGN cell line suggest that carboplatin, paclitaxel, and alpelisib, at 10:1:2 constant ratio, could be a safe, effective combination treatment.

Table 1. Patient-derived cell line characteristics

Cell line	Tumor origin	Tumor type	Previous systemic treatment	Tumor *FOXL2* c.402C>G mutational status	Cell line *FOXL2* c.402C>G mutational status
Direct patient-derived cell lines					
GCPA007	AGCT	Recurrence	Radiotherapy, chemotherapy[1]		
T2.I				+/-	+/-
T2.IV				+/-	+/-
GCPA028	AGCT	Recurrence	No		
T1.II				+/-	+/-
GCPA096	AGCT	Recurrence	No		
T1.I				+/-	+/-
T1.II				+/-	-/-[3]
T1.V				+/-	+/-
T1.XV				+/-	+/-
T1.XVI				+/-	+/-
GCPA108	AGCT	Recurrence	Anti-hormonal treatment, chemotherapy, RFA[2]		
T3.IV				+/-	+/-
GCPA113	AGCT	Recurrence	No		
T1.I				+/-	-/-[3]
T1.II				+/-	+/+[3]
T1.III				+/-	+/-
Control cell lines					
KGN	AGCT	Primary	No	+/-	+/-
SVOG-3e	Granulosa cells	N/A	No	N/A	N/A
MCF-7	Breast cancer	Recurrence	Radiotherapy, anti-hormonal treatment	N/A	N/A
SH-SY5Y	Neuroblastoma	Recurrence	Radiotherapy, chemotherapy	N/A	N/A

Sample IDs: first number indicates time point, second number indicates location (e.g., T2.I is the first location of the second time point) N/A: not applicable. AGCT: adult granulosa cell tumor.

[1]Four cycles bleomycine, etoposide and cisplatin.

[2]Anti-hormonal treatment: aromatase inhibitor anastrozole, progestogen megestrol and selective estrogen receptor modulator tamoxifen, four cycles carboplatin and paclitaxel, radiofrequency ablation (RFA) on liver metastases.

[3]In these cell lines, tumor origin was confirmed by CytoSNP-850K snp array.

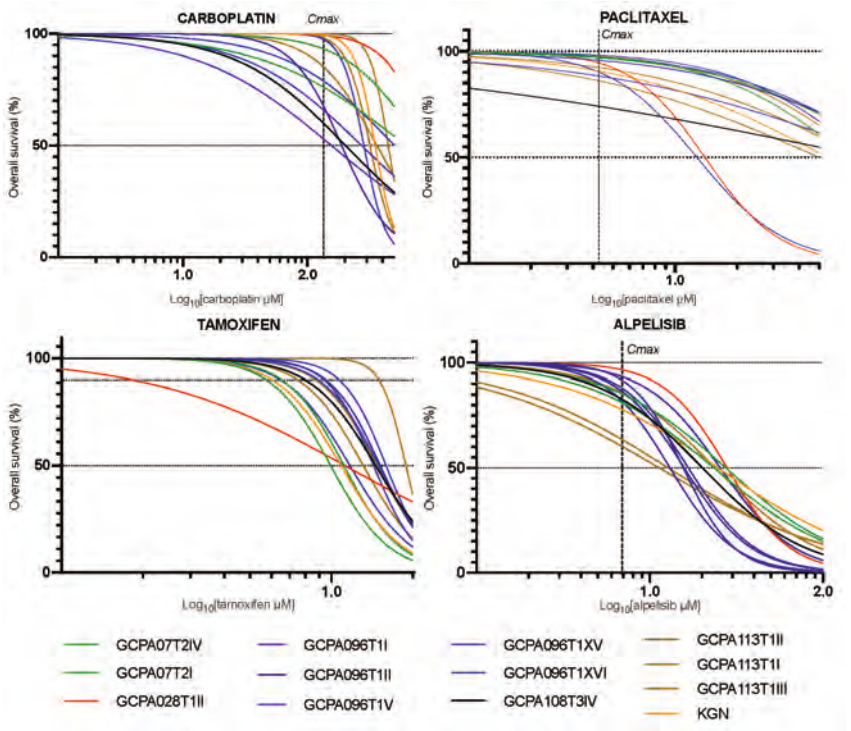


Figure 2. Representative drug screen results of four out of eleven tested monotherapies**.** For all monotherapies, the IC50 exceeded the Cmax and Css values. This suggests that monotherapy drug concentrations needed to achieve 50% cell death could not be achieved *in vivo*. Since the Log_{10}(Css) of tamoxifen is below 0, the Css is not displayed. Additionally, the concentration tamoxifen needed to achieve 10% cell death (IC10) also exceeded the Css. Each curve represents the average response of three biological replicates and two technical replicates. The Z-factor was ≥ 0.8 for all drug screens.

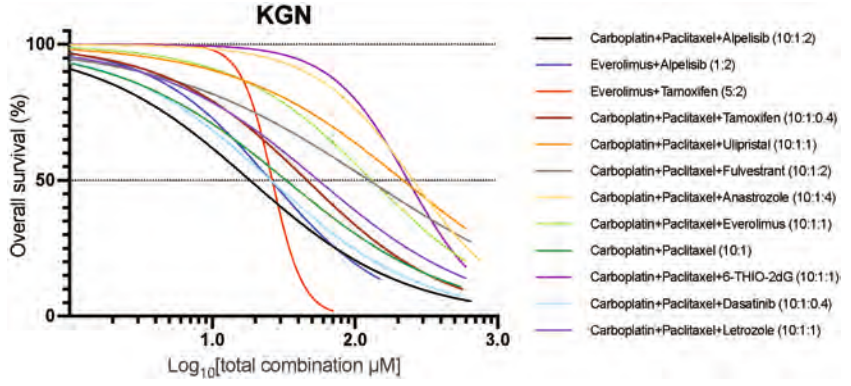


Figure 3. KGN response to drug combination treatment. Dose-response curves show differential sensitivity to drug combinations. Drug combinations were applied at a constant ratio to ensure a constant contribution of each drug in combination for all data points. Each curve represents the average response of three biological replicates and two technical replicates. Z-factor: 0.91.

The combination carboplatin, paclitaxel and alpelisib is also consistently effective in AGCT patient-derived cell lines

To investigate sensitivity of the patient-derived AGCT lines to combination therapies, we evaluated 12 drug combinations and tested for synergistic interactions. Overall, the dose-response curves for the drug combinations differed among cell lines, which indicates intra- and inter-patient drug sensitivity variation (Figure 4). In addition, the synergistic effects of similar drugs also varied among patients (Supplementary Table 1). Similar to KGN, the three most effective combinations in all cell lines included everolimus with alpelisib (at 1:2 ratio), carboplatin with paclitaxel and alpelisib (at 10:1:2 ratio), and everolimus with tamoxifen (at 5:2 ratio). However, the dose reduction of the combinations of either everolimus with alpelisib or everolimus with tamoxifen was not sufficient to enable the individual drugs to be below estimated tolerable plasma concentrations.

The combination of carboplatin, paclitaxel and alpelisib showed similar drug response profiles in AGCTs (Figure 5) and consistent synergy in 11 of 12 (92%) tested cell lines (Combination Index between 0.11-0.88, Supplemental Table 1). Although neither carboplatin, nor paclitaxel or alpelisib were effective as monotherapy (median IC50 values of 343.32, 93.31 and 14.99 µM, which exceeded the corresponding Cmax values 134.90, 4.27 and 6.92 µM, respectively), their synergistic interactions allowed for significant dose reduction when used in combination. This was the only combination with significant growth inhibition at values lower than the Cmax (in 10 of 12 cell lines, 83%), indicating potential antitumor activity at *in vivo* use. These findings are in corroboration to the findings in the KGN cell line and suggest that a combination of carboplatin, paclitaxel, and alpelisib, at 10:1:2 constant ratio, could be a safe, effective treatment for AGCT.

As alpelisib and everolimus specifically target the PI3K/Akt/mTOR pathway, we performed targeted next generation sequencing to test for mutations in 64 cancer genes including the PI3K pathway genes (Supplemental Tabel 2). We identified three variants in PI3K although they were either intronic (n=2) or synonymous (n=1) and not predicted to affect gene function. No other pathogenic mutations in these targeted genes were detected, particularly not in the PI3K pathway.

FOXL2 mutation status does not affect response to effective drug combinations

We compared the drug screen responses in three cell lines of patient GCPA113, which each have a different *FOXL2* mutation status (*FOXL2* wildtype, heterozygous mutant and hemizygous mutant, respectively). *FOXL2* mutation status was ascertained to verify tumor cell origin of the cell lines and additional typing by SNP array was performed in those lines in which the *FOXL2* mutation was not present (or present in hemizygous form). The hemizygous mutant cell line (GCPA113T1.II) was most resistant to alpelisib monotherapy (IC50 21.37µM, versus

9.59µM and 11.53µM, Supplemental Table 1). This cell line showed sensitivity to the three most effective combinations in general (everolimus with alpelisib, carboplatin with paclitaxel and alpelisib, everolimus with tamoxifen) and resistance to all other combination treatments (Figure 4). The combination of carboplatin, paclitaxel and alpelisib was consistently effective in AGCT cell lines, regardless of their mutational pattern or tumor location.

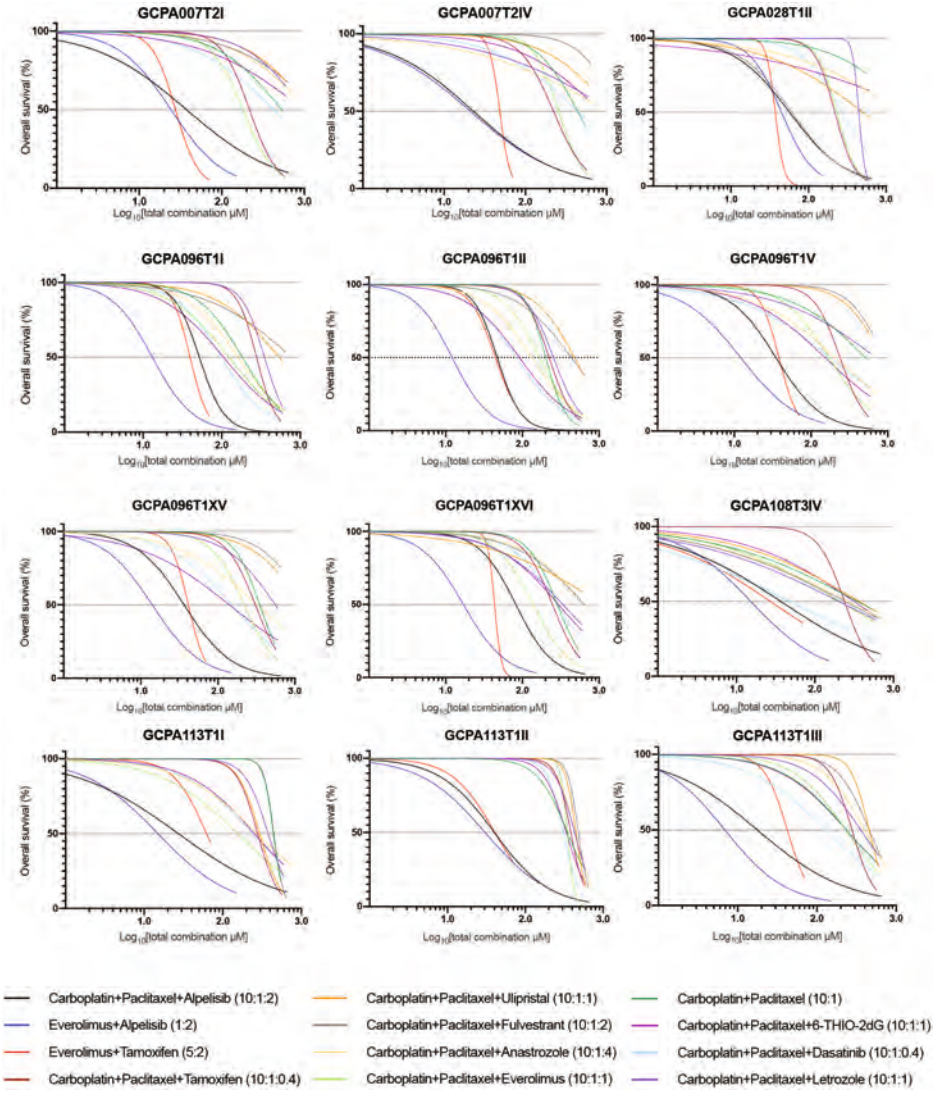


Figure 4. Patient-derived AGCT cell line response to drug combination treatment. Dose-response curves show differential sensitivity to drug combinations. Drug combinations were applied at a constant ratio to ensure a constant contribution of each drug in combination for all data points. Each curve represents the average response of three biological replicates and two technical replicates. The Z-factor was ≥ 0.8 for all drug screens.

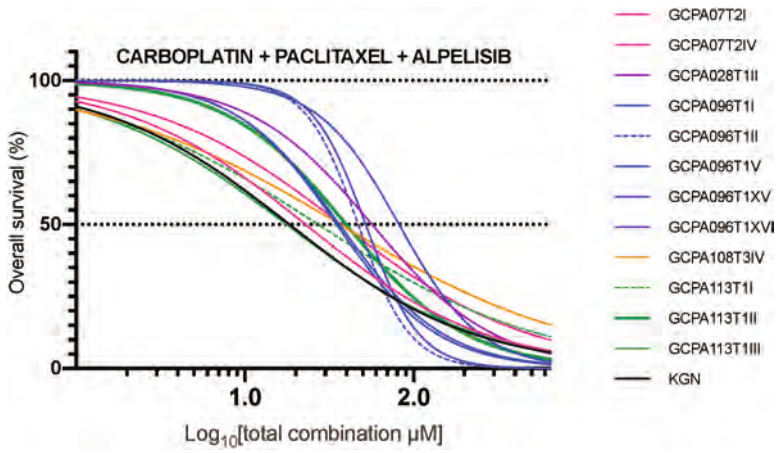


Figure 5. AGCT cell line response to carboplatin, paclitaxel and alpelisib. Pearson's correlation coefficient (r) was used to evaluate the association between two response profiles and ranged from 0.84 - 0.99 (mean: 0.95). In 10 of 12 cell lines, the IC50 was lower than the Cmax, indicating effectiveness at non-toxic levels *in vivo*. Each curve represents the average response of three biological replicates and two technical replicates. The Z-factor was ≥ 0.8 for all drug screens.

Discussion

This is the first study to evaluate drug monotherapies and combination treatment in short-term established patient-derived AGCT cell lines. With this approach, we were able to perform systematic drug screens in patient-derived AGCT cell lines and the AGCT model cell line KGN to test efficacy, synergy and potential human safety of current AGCT treatment, novel anti-cancer drugs and their combinations within three weeks of tissue collection. We found that the combination of carboplatin, paclitaxel and alpelisib was consistently effective and synergistic at individual drug concentrations deemed non-toxic for *in vivo* use in humans in 11 of 13 (85%) tested AGCT cell lines.

The PI3K inhibitor alpelisib

Alpelisib is an oral, selective phosphatidylinositol 3-kinase (PI3K) p110α inhibitor, which has been identified as a novel promising targeted treatment in different cancer types. The PI3K signaling pathway is one of the most frequently dysregulated pathways in human cancers, which controls key cellular processes involved in cancer cell proliferation and survival.[122] Activation of the PI3K pathway frequently occurs via mutations in *PIK3CA*, which encodes the PI3K p110α catalytic subunit. Although a somatic *PIK3CA* mutation is only present in a small proportion of AGCTs, dysregulation of the PI3K/AKT pathway plays a major role in the pathogenesis of AGCTs.[87,108,123] Approximately 15-17% of AGCTs harbor a mutation in genes involved in this pathway.[80,111]

PI3K inhibition in combination with chemotherapy

Our study demonstrates the effectiveness of PI3K inhibition when combined with chemotherapy in AGCTs *in vitro*. As our study did not include tumors with a PI3K pathway mutation, the effect of this combination therapy may be even more pronounced in tumors with a *PIK3CA* variant. The combination of alpelisib with paclitaxel is currently being studied in breast cancer patients and advanced solid tumors.[124,125] In gastric cancer, alpelisib and paclitaxel demonstrated synergistic anti-proliferative and anti-migratory effects in *PIK3CA*-mutant cells.[126] In a xenograft model utilizing gastric cancer cells, alpelisib and paclitaxel significantly increased apoptosis and tended to prolong the survival of tumor-bearing mice. Our study shows synergy for alpelisib in combination with chemotherapy, analogous to the previous observation in gastric cancer, and demonstrates consistent effectiveness in patient-derived AGCT cell lines.

Combined PI3K/mTOR inhibition

The efficacy of alpelisib with everolimus was also promising in our analysis, albeit not at presumed tolerable plasma concentrations. Preliminary results of a phase Ib trial show a manageable safety profile for this combination (NCT02077933). Trials testing alpelisib in combination with other targeted treatments such as monoclonal antibodies in breast cancer or MEK-inhibitors in meningiomas are ongoing (NCT04208178, NCT03631953).

PI3K-mTOR inhibition combined with anti-hormonal treatment

PI3K-mTOR pathway inhibition was also thought to be an effective strategy in combination with anti-hormonal treatment, since targeting the PI3K-mTOR pathway has the potential to restore sensitivity to estrogen receptor inhibition.[127] Therefore, studies are investigating this possibility using PI3K and mTOR inhibitors in combination with endocrine therapies. In hormone receptor positive breast cancer, adding alpelisib to the estrogen receptor blocker fulvestrant has shown to prolonge the progression-free survival in *PIK3CA* mutated tumors.[128] In addition, a randomized phase II trial in postmenopausal women with aromatase inhibitor resistant metastatic breast cancer showed that adding everolimus to tamoxifen increased the time to progression by 4.1 months and reduced the death risk by 55%.[129] These findings together with our encouraging drug screen results of everolimus and tamoxifen indicate that this strategy should be further studied in AGCTs.

Previous drug screen studies

Previous drug screen studies on KGN cells have described *in vitro* efficacy of mTOR inhibitors combined with paclitaxel, tyrosine kinase inhibitors, and PPARγ activation combined with XIAP inhibition.[108,109,130] In our study, everolimus showed some degree of synergy with chemotherapy in 9 of the 13 cell lines, but did not belong to the most effective drug combinations. Furthermore, we confirmed the effectiveness of the tyrosine kinase

inhibitor dasatinib combined with carboplatin and paclitaxel in KGN, although responses in our patient-derived AGCT cell lines varied widely. A previous study detected upregulation of the PI3K/AKT/mTOR pathway in AGCTs and found increased sensitivity to inhibitors of this pathway as compared to normal human granulosa-lutein (hGL) cells.[108] In contrast, we found increased sensitivity of the immortalized granulosa cell line (SVOG-3e) to alpelisib, as compared to the AGCT cell lines (IC50 7.55μM versus median IC50 14.99μM). This might be due to the fact that the PI3K/AKT/mTOR pathway also plays a crucial role in granulosa cell proliferation, or that the immortalization process may have increased sensitivity to PI3K inhibition in this specific cell line.[131] The upregulation of this pathway in AGCTs, together with our drug screen results suggest that PI3K-mTOR pathway inhibition may be an effective treatment strategy in AGCTs, particularly when utilizing synergistic drug combinations which allow for drug dose reduction.

The limited effects of monotherapies

Our study confirmed the limited effects of current AGCT treatment strategies, including carboplatin, paclitaxel and anti-hormonal treatment. All monotherapies were deemed ineffective as the IC50, and IC10 in case of the anti-hormonal drugs, exceeded the maximum and steady state plasma concentration. However, it may be difficult to simulate the effect of maintenance therapies, such as anti-hormonal treatment, with a single dose application drug screen set up. Our study does not include AGCT cell lines sensitive to either chemotherapy or anti-hormonal treatment as single therapy, due to the general resistance of AGCTs to these treatments. However, this drug screen approach could be used to identify individual patients that would respond to anti-hormonal treatment or chemotherapy in the future.

FOXL2 mutation status in patient derived cell lines

In this study, a hemizygous *FOXL2* mutation was detected in one cell line and two cell lines were *FOXL2* wildtype. The copy number profiles of the *FOXL2* hemizygous mutant or *FOXL2* wildtype cell lines corresponded with the initial tumor, confirming tumor origin. Although GCPA113T1.I presented a distinct tumor copy number profile, deviations with the initial tumor were seen. This could be caused by sampling bias or cultivation of a subclone of the tumor. All cell lines showed similar sensitivity for the three most effective combinations (carboplatin with paclitaxel and alpelisib, and everolimus with tamoxifen or alpelisib), regardless of their *FOXL2* mutation status.

Estimating efficacy of drug combinations

In order to test the efficacy of drug combinations, we assessed the IC50 concentrations on a linear distribution for each cell line and drug individually, and assessed drug ratios by their synergy. Hereby, we were able to test drug combinations in different concentrations while maintaining a constant ratio. In general, *in vitro* IC50 concentrations are usually an

accurate reflection of a drug's efficacy *in vivo*. However, these values are a proxy as IC50s may occasionally not reflect the achieved cytotoxicity *in vivo* [132]. A limitation of this study is that Cmax concentrations assessed for monotherapy use were applied whereas ideally Cmax concentrations should also be established for each drug in combination. However, Cmax values for drug combinations are usually not available as it requires large clinical trials to test specific drug combination dosages and ratios. Moreover, for some drugs used *in vivo*, dosages are calculated based on surface area (mg/mm^2) which is not possible *in vitro*. However, drug concentrations for future *in vivo* use can potentially be derived from current clinical trials testing these drug combinations in other cancer types.

A robust drug screen model

Finding an effective, targeted treatment for AGCTs has been subject of research over the past decade. As clinical trials for rare tumors will take many years, robust pre-clinical models are needed to screen for potential effective treatment strategies. This study illustrates an approach to establish patient-specific AGCT cell cultures directly from tumors, at high success rates, to rapidly screen AGCTs for potential effective treatment. Although we aimed to find personalized treatment options for some of the patients, similar response profiles were seen for the three most effective combinations, indicating that in general AGCTs could be sensitive to these combinations. When applying this method to an increased number of patient-derived AGCT cell lines or treatment options, we may find specific differential drug responses amongst patients. Future studies utilizing molecular and cellular markers confirming the specific pharmacodynamic response to these and other promising novel monotherapies and combinations may be necessary prior to clinical applicability. Furthermore, preclinical studies in PDX models can be used to replicate and validate the findings presented within this study in a more complex environment containing the appropriate hormonal milieu, vasculature, immune response and the tumor microenvironment, as has been performed previously in granulosa cell tumors.[133] The established drug screen model can constantly be adapted and therefore easily be used for novel drug combination testing and personalized drug selection in the future. The clinical use and effectiveness of these drugs in other cancer types will enable fast translation to the clinic.

Conclusions

This study shows that rapid, systematic patient-specific AGCT drug screens are feasible and can be used to test individual response to existing monotherapies and novel combinations. Findings on a set of 13 cell lines demonstrate synergistic growth inhibition of the PI3K inhibitor alpelisib combined with the current first line chemotherapeutic agents carboplatin and paclitaxel, at potential tolerable concentrations *in vivo*. Therefore, alpelisib, carboplatin and paclitaxel may be a promising novel combination for treatment of recurrent AGCTs.

Materials and Methods

Patient recruitment and tumor tissue acquisition

We conducted a national prospective study to obtain fresh patient-derived tumor tissue. Patients were included in five hospitals between 2018–2020. Ethical approval was obtained (UMCU METC 17-868) and all participants provided written informed consent. Tissue was obtained directly at the operating room or at the adjacent pathology department, and placed in Advanced DMEM/F12 (Thermo Fischer Scientific, Waltham, MA, USA), 1% glutamax (Thermo Fischer Scientific), 10 mM HEPES Buffer (Thermo Fischer Scientific), 100U/mL penicillin and 100 ug/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA) (resulting medium: ADMEM+++). Samples were transported at room temperature and processed within 4 hours.

Tumor tissue processing and 2D cell line establishment

Tumor tissue was mechanically sheared with scalpels to single-cells and very small tissue particles. Cells and tissue particules were collected by flushing the petri dish with ADMEM+++. Upon collection, the collection tube was inverted and large particulates allowed to settle for 30 seconds, while the upper layer of the cell suspension was collected for further processing. The upper layer of the cell suspension was centrifuged for 5 minutes at 800 rpm and the resulting supernatant removed via pipette and discarded. RBC lysis buffer (Sigma-Aldrich) was added as needed (0.5 ml – 2 ml) to the cell pellet, mixed for 3 minutes, and then centrifuged for 5 minutes at 800 rpm. Once single cells were isolated and RBC removed, single cells were seeded onto adherent cell-culture plates in ADMEM+++ supplemented with 10% Fetal Calf Serum (Thermo Fischer Scientific; FCS) and stored at 37°C / 5% CO2 in humidified incubators. Medium was replaced every 3-4 days and cell lines were passaged upon reaching ~80% confluency (approximately every 1-2 weeks). Passaging was performed by removing medium, washing with PBS and detachment from the plate with TryplE (1X, Thermo Fischer Scientific,). All utilized cell lines were mycoplasma negative. Presence of the AGCT-specific *FOXL2* c.402C>G mutation was verified with the primers 5'-CCGGCATCTACCAGTACATCA and 5'-GGAAGGGCCTCTTCATGC using the Acccuprime GC rich polymerase: 5' at 95°C followed by (30" at 95°C, 30" at 56°C, 30" at 72°C) for 35 cycles and ended by 5' at 72°C. In case of a homozygous *FOXL2* c.402C>G mutation or *FOXL2* wildtype genotype, a CytoSNP-850k array was performed on the cell line and corresponding tumor DNA to verify tumor origin and identify loss of heterozygosity.

Targeted pathway sequencing

DNA from the fresh frozen pulverized tumor tissue was isolated using DNEasy Blood and Tissue Kit (Qiagen, Venlo, NL). Targeted next generation sequencing with a mean coverage of 500X coverage was performed on the Ion Torrent S5 system (ThermoFischer Scientific)

using a custom NGS panel based upon the Ion Ampliseq™ Cancer Hotspot Panel targeting mutational hotspots of 64 cancer related genes. Variants with an allele frequency of at least 5% were reported. Variant call files were generated and analysis was performed using Alissa (Agilent Technologies Alissa Interpret v5.1.7).

Control cell models

Control cell lines were used as positive controls (MCF-7, SH-SY5Y) and to test differential response to treatment (SVOG-3e). MCF-7, an estrogen and progesterone receptor positive human breast cancer cell line, was used as positive control for the anti-hormonal therapies.[134] Additionally, SH-SY5Y, a human neuroblastoma cell line, was used as positive control for chemotherapeutic agents.[135] KGN, a human AGCT cell line derived from a 63 years old patient heterozygous for *FOXL2* c.402C>G, was used as an additional AGCT cell line, to evaluate potential AGCT response.[136] Finally, the immortalized human granulosa cell line SVOG-3e[137] was used to assess selective drug response in AGCT versus healthy granulosa cells. SH-SY5Y and MCF-7 were cultured in DMEM/F12 (Thermo Fischer Scientific) + 10% FBS and 1% Pen/Strep and RPMI 1640 (Thermo Fischer Scientific) + 10% FBS and 1% Pen/Strep, respectively. KGN was cultured in conditions identical to AGCT patient-derived cell lines. SVOG-3e was cultured in 1:1 Media 199:Media 105 (Sigma-Aldrich) with 5% FBS and 1% Pen/Strep.

AGCT viability assessment in response to monotherapy and combination treatment

Cell lines were seeded at a density of 500 cells/well in 40 ul in 384-well plates (Corning, NY, USA) with the Multidrop™ cell dispenser (Thermo Fischer Scientific, Waltham, MA, USA) and sealed with Breathe-Easy® membrane (Sigma-Aldrich, St. Louis, MO, USA) to prevent evaporation. The SVOG-3e line was seeded at 1000 cells/well due to contact-dependent growth requirements. Of each cell line, three biological repeats and two technical repeats each were performed in order to ensure the validity of the drug screens. Cells were grown for 24h prior to drug dispensing in 37°C, 5% CO2 and 95% relative humidity. Test substances were dissolved in 100% DMSO or MQ + 0.01% Tween-20 in the case of carboplatin, as DMSO inactivates platinum-based treatments (Table 2).[138] Subsequently, 11 drugs and 12 drug combinations were dispensed and all wells (excluding blanks and non-DMSO control wells) normalized to 1% DMSO using the HP Tecan D300e Digital Dispenser (Hewlett-Packard, Palo Alto, CA, USA). All drugs were applied in linear concentration ranges (Table 2) including 16 data points to enable combination drug testing at constant ratios. Next, the plates were covered with the Breathe-Easy membrane and incubated. After 72 hours, the Multidrop dispensed 15uL per well of Cell Titer Glo 2.0 (Thermo Fischer Scientific, Waltham, MA, USA) and plates were incubated in the dark for 10 minutes. After incubation, luminescence excitation was assessed in the Spectramax reader (Molecular Devices, San Jose, CA, USA) (top read, luminescence excitation at 500 nm) and raw data processed in Microsoft Excel

to subtract background luminescence. The Z-factor, a mathematical method to quantify the quality of a drug screen based on the calculation of the separation band between the values of the positive and negative controls, was utilized to assess the quality of drug screens.[139] Screens with a Z-factor value of >0.5 were used for further analysis. To optimize the systematic AGCT drug screens, we used 29 AGCT cell lines to assess the individual drug concentration ranges and drug ratios for combination drug testing. These cell lines were not included in the final drug screens, since all cells were utilized.

Table 2. Drug compounds

Drug	Mechanism	Concentration range (μM)	Solvent
Carboplatin	Intra- and inter-strand cross-linkage of DNA	500-0	MQ+0.01% Tween
Paclitaxel	Microtubule stabilizer, induces mitotic arrest	50-0	DMSO
Tamoxifen	Estrogen receptor blocker	20-0	DMSO
Letrozole	Aromatase Inhibitor	50-0	DMSO
Fulvestrant	Estrogen receptor blocker	100-0	DMSO
Ulipristal	Progesterone receptor blocker	50-0	DMSO
Anastrozole	Aromatase inhibitor	200-0	DMSO
Everolimus	mTOR inhibitor	50-0	DMSO
Alpelisib	PI3K inhibitor	100-0	DMSO
Dasatinib	Tyrosin kinase inhibitor	20-0	DMSO
6-THIO-2dG	Telomerase blocker	50-0	DMSO

**all drug compounds were obtained from Sigma-Aldrich.*

Efficacy and safety of monotherapies

Overall cell survival (in %) was plotted against the drug concentration range of each drug (in log10[μM]) using GraphPad Prism 8.3.1. Values that exceeded 100% survival (within +20% biological deviation) were normalized to 100% survival. Similarly, values below 0% cell survival (within -20% biological deviation) were normalized to 0% survival. Values exceeding this deviation were excluded. Dose-response curves were obtained using the model "log(inhibitor) vs. variable slope (normalized response)" to fit the dose-response curves and R^2 values were used to indicate how well the model fits the data (Supplemental Figure 4). We interpolated cell survival to 50% to obtain the corresponding IC50 values for each drug and their 95% confidence intervals (IC: inhibitory concentration). The maximum plasma concentration (Cmax) and the plasma concentration at steady state (Css) were used as toxicity thresholds. The Cmax was utilized for drugs in which short, high-dosage time-courses are required, while the Css was used for maintenance therapy regimes such as anti-hormonal treatment. Both the Cmax and Css are estimated during phase I and phase II clinical trials and provide an approximated threshold of drug toxicity in humans.[113–119] A drug was deemed effective when 50% cell death (IC50) was achieved at a concentration

below the maximum plasma concentration (Cmax) or steady state plasma concentration (Css) *in vivo*. The IC50 values were calculated based on Chou-Talalay method using Compusyn software [140]. For anti-hormonal treatment, mostly provided as maintenance therapy, we used IC10 values in addition to the IC50.

Combination treatment for AGCTs

In addition to the 11 monotherapies, 12 drug combinations were tested (Table 3) simultaneously with the individual drug treatments in the same 384 well plate. To ensure that the contribution of each drug in combination is similar for all data points, drugs in combination were applied in a constant ratio. Synergy was evaluated using the Mass-Action Law, a systematic analysis assessing dose-response dynamics in a cost-effective manner.[141,142] The Combination Index (CI) was used to indicate antagonistic (CI>1) additive (CI=1) and synergistic (CI<1) effects.[140] Similar to monotherapies, the safety for in vivo use of drug combinations were assessed by comparison to Cmax/Css values of the individual drugs (Supplemental Table 1). The Dose-Reduction Index (DRI) for each drug in combination was obtained from the Mass-Action Law[141,142], and indicates the dosage a drug can be reduced in combination to obtain the equivalent amount of cell death in monotherapy. A DRI value of >1 allows dose reduction. Effect calculations and data visualization was performed using both GraphPad Prism 8.3.1 and Compusyn software.[143] Finally, the IC50s of the drugs in combination was calculated and compared to the Cmax/Css values. A drug combination was deemed effective when 50% cell death (IC50) for all individual compounds was achieved at concentrations below Cmax/Css.

Table 3. Drug combinations and ratios

Drug combination	Combination ratio
Carboplatin + Paclitaxel	10:1
Carboplatin + Paclitaxel + Tamoxifen	10:1:0.4
Carboplatin + Paclitaxel + Letrozole	10:1:1
Carboplatin + Paclitaxel + Fulvestrant	10:1:2
Carboplatin + Paclitaxel + Ulipristal	10:1:1
Carboplatin + Paclitaxel + Anastrozole	10:1:4
Carboplatin + Paclitaxel + Everolimus	10:1:1
Carboplatin + Paclitaxel + Alpelisib	10:1:2
Carboplatin + Paclitaxel + Dasatinib	10:1:0.4
Carboplatin + Paclitaxel + 6-THIO-2dG	10:1:1
Everolimus + Tamoxifen	5:2
Everolimus + Alpelisib	1:2

Author Contributions: J.R., J.G., E.S., R.V., G.M. and R.Z. designed the study and obtained ethical approval of the study protocol. J.R., S.P., H.N., H.M., L.L., J.P., C.L., G.J., P.W., G.M. and R.Z. collected data and tissue. G.M., E.S.G., E.S., F.S. and C.S performed cell cultures, G.M., E.S.G., F.S. and J.R. performed drug screens. K.D. performed DNA isolations and *FOXL2* Sanger sequencing. G.M., E.S.G and J.R. performed data analysis. J.R., E.S.G., G.M., G.H. and R.Z. wrote the manuscript. All authors revised the manuscript and approved final version for publication.

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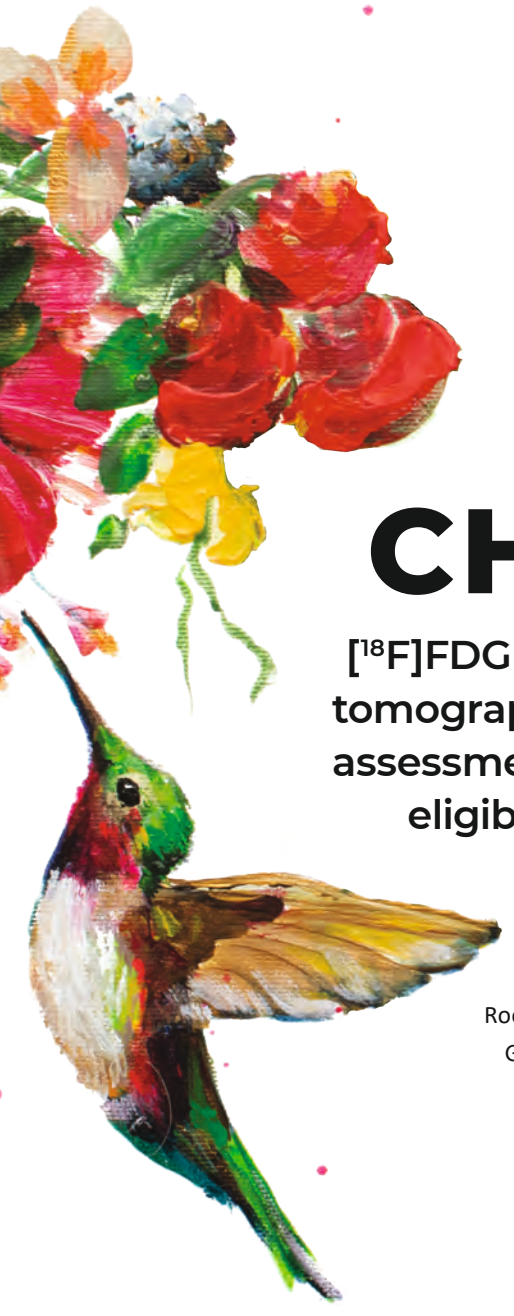
Conflicts of Interest: The authors declare no conflict of interest.



CHAPTER 5

[^{18}F]FDG and [^{18}F]FES positron emission tomography for disease monitoring and assessment of anti-hormonal treatment eligibility in granulosa cell tumors of the ovary

Joline F. Roze, Hannah S. van Meurs, Glen R. Monroe, Wouter B. Veldhuis, Luc R.C.W. van Lonkhuijzen, Roel J. Bennink, Jolijn W. Groeneweg, Petronella O. Witteveen, Geertruida N. Jonges, Ronald P. Zweemer, Arthur J.A.T. Braat

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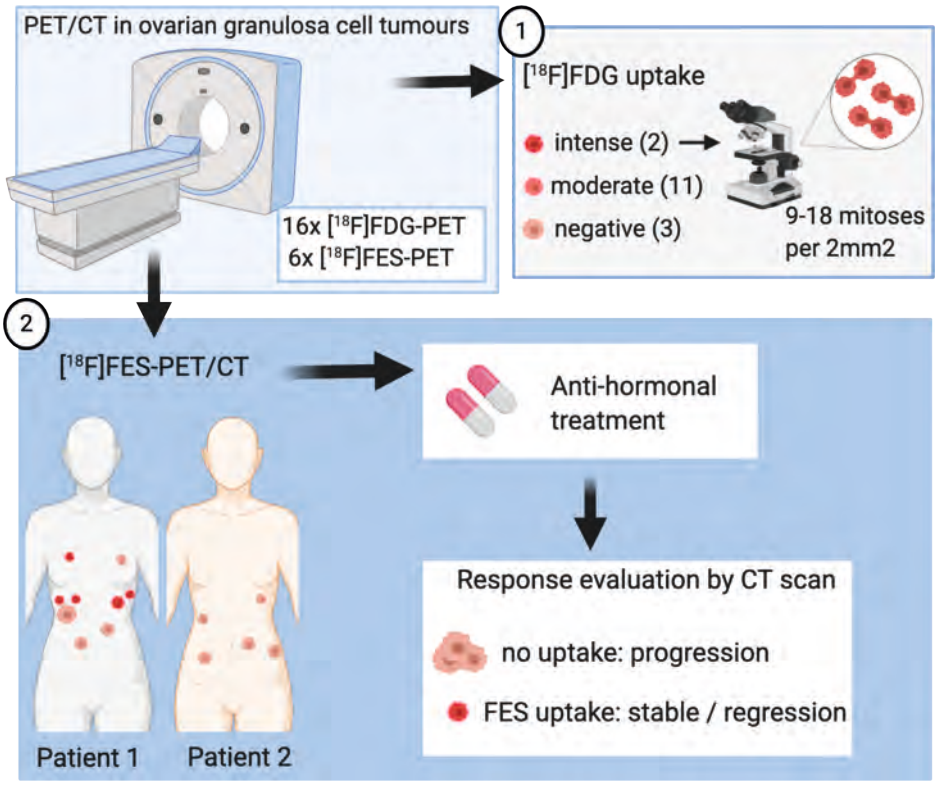
Abstract

Background: Adult granulosa cell tumors (AGCTs) of the ovary represent a rare malignancy that recurs in ~50% of patients. Due to its relatively slow growth, timing of treatment of disease relapse is a clinical challenge. Fluor-18-deoxyglucose positron emission tomography combined with computed tomography ([^{18}F]FDG-PET/CT) is a useful diagnostic tool for staging and detection of recurrence in most ovarian cancer subtypes. AGCTs highly express estrogen receptors (ER) and a subset of patients responds to anti-hormonal treatment. This study investigates the value of [^{18}F]FDG-PET/CT and 16α-18F-fluoro-17β-estradiol ([^{18}F]FES)-PET/CT in monitoring recurrent AGCTs and assessing eligibility for anti-hormonal treatment.

Methods: We evaluated 22 PET/CTs from 20 AGCT patients with recurrent disease to determine tumor [^{18}F]FDG and [^{18}F]FES uptake. We included all consecutive patients from two tertiary hospitals between June 2003 and May 2020. Sixteen [^{18}F]FDG-PET/CT and six [^{18}F]FES-PET/CT met quality criteria for qualitative analysis, of which 13 [^{18}F]FDG-PET/CT and six [^{18}F]FES-PET/CT could be used for quantitative analysis. Standardised uptake values (SUV) for [^{18}F]FDG and [^{18}F]FES were measured. Expression of ERα and ERβ and the number of mitoses per 2mm^2 were determined by immunohistochemistry and compared to [^{18}F]FES and [^{18}F]FDG uptake, respectively.

Results: Qualitative assessment showed low-to-moderate [^{18}F]FDG uptake in most patients (14/16), and intense uptake in 2/16. One patient with intense tumor [^{18}F]FDG uptake had a high mitotic rate (18 per 2mm^2), that could explain [^{18}F]FDG uptake. Compared to CT, [^{18}F]FDG-PET/CT detected one additional liver metastasis. Two out of six patients showed [^{18}F]FES uptake on PET/CT at qualitative analysis. Lesion-based quantitative assessment showed a mean SUV_{max} of 2.4 (±0.9) on [^{18}F]FDG-PET/CT and mean SUV_{max} of 1.7 (±0.5) on [^{18}F]FES-PET/CT. Within patients, differential expression of ERα and ERβ was detected and therefore did not correspond with [^{18}F]FES uptake. In one [^{18}F]FES positive patient, tumor locations with [^{18}F]FES uptake remained stable or decreased in size during anti-hormonal treatment, while all [^{18}F]FES negative locations progressed.

Conclusion: This study shows that in AGCTs, FDG uptake is limited and therefore [^{18}F]FDG-PET/CT is not routinely advised. [^{18}F]FES-PET/CT may be useful to non-invasively capture the estrogen receptor expression of separate tumor lesions and thus assess the potential eligibility for hormone treatment in AGCT patients.



Introduction

Granulosa cell tumors are a well-defined ovarian cancer subtype, responsible for 2-5% of ovarian malignancies with an annual incidence of 0.6-1.0 per 100.000 women worldwide.[10,15,38,59] The tumor arises from the estrogen producing granulosa cells present in the ovarian stroma. Adult (95%) and juvenile (5%) subtypes can be distinguished based on clinical and histopathological characteristics. The juvenile subtype generally occurs in prepubertal girls and young women, whereas the adult type has its peak incidence between 50-55 years.[10]

Adult granulosa cell tumors (AGCTs) harbour a specific missense mutation in *FOXL2* in approximately 95% of cases.[6,41] Continuous exposure to tumor-derived estrogen can cause endometrial proliferation. As a result, 6% of patients have concomitant endometrial cancer at diagnosis.[15] Common symptoms include abnormal vaginal bleeding and abdominal pain. Surgery is the mainstay of treatment throughout the disease course, due to generally limited effects of systemic treatments such as chemotherapy and hormone therapy.[6,29] Recurrences

occur in approximately 50% of patients and often require repeated debulking surgeries. Of women with recurrent disease, 50-80% ultimately succumb to their disease.[6,60,61]

Surgical treatment has its limitations, as risks of surgery increase with subsequent debulking procedures. Therefore, when a recurrence is detected, it can be justified to opt for watchful waiting with frequent disease monitoring while patients retain a good quality of life. Vice versa, surgery should be performed when all tumor deposits can still be completely removed. This results in a limited therapeutic window, making the timing of surgical resection a clinical challenge. Imaging tools that are able to measure disease activity, can potentially help to optimize the timing of surgery.

Fluor-18-deoxyglucose uptake on positron emission tomography combined with computed tomography ([^{18}F]FDG-PET/CT) defines the metabolic activity of cells and has proven to be useful for staging and detection of recurrence in many cancer types. To date, anecdotal case reports with a total of five patients describe the use of [^{18}F]FDG-PET in granulosa cell tumors, and show conflicting results.[144–147] These studies found no [^{18}F]FDG uptake in two patients, moderate uptake in two patients and intense [^{18}F]FDG uptake in one patient with a bone metastasis.

AGCTs commonly express the estrogen receptors alpha (ERα, 30-66%) and beta (ERβ, 94-100%).[148–151] Therefore, anti-hormonal treatment is thought to be a potentially effective treatment strategy. A previous study showed that anti-estrogen treatment can decrease tumor load in a subset (n=4; 18%) of 22 AGCT patients.[29] Anti-hormonal treatment is generally well tolerated and can be used continuously for many years. Nevertheless, it remains difficult to determine the treatment of choice and to identify patients that may benefit from this treatment. Recently, PET using the 16α-18F-fluoro-17β-estradiol [^{18}F] FES tracer became available to noninvasively assess ER expression. This novel technique is used in hormone receptor positive tumors, such as breast cancer, to identify candidates for anti-hormonal treatment.[152] In these cancers, tamoxifen (selective estrogen receptor modulator), anastrazole and letrozole (aromatase inhibitors) and fulvestrant (estrogen receptor antagonist), have been widely used and show comparable efficacy.[153,154] The use of [^{18}F]FES-PET/CT has not yet been evaluated in AGCTs.

This study investigates the value of [^{18}F]FDG-PET/CT for disease monitoring and [^{18}F]FES-PET/CT for indicating anti-hormonal treatment eligibility in AGCT patients.

Methods

Patients

To identify the available PET scans, all radiology and pathology reports of two academic hospitals from 2000-2020 including the term 'granulosa' were retrieved (Figure 1). Additionally, we searched for patients who underwent PET/CT evaluation in our AGCT research database. This database currently contains 102 patients with a pathologically confirmed AGCT, from six hospitals in the Netherlands, included between 2017 and 2020. All [^{18}F]FDG-PET scans with measurable disease from patients with a histologically confirmed AGCT were included. In addition, six [^{18}F]FES-PET/CTs were prospectively performed for clinical purposes and included in this study (Table 1). Ethical approval was obtained by the Institutional Review Board of the University Medical Center Utrecht. All participants provided written informed consent for the use of their clinical data. Statistical analyses were performed using SPSS v.25.0 (IBM Corp., Armonk, NY, USA). Patient characteristics are described as median and range, and all imaging parameters as mean and standard deviation (SD).

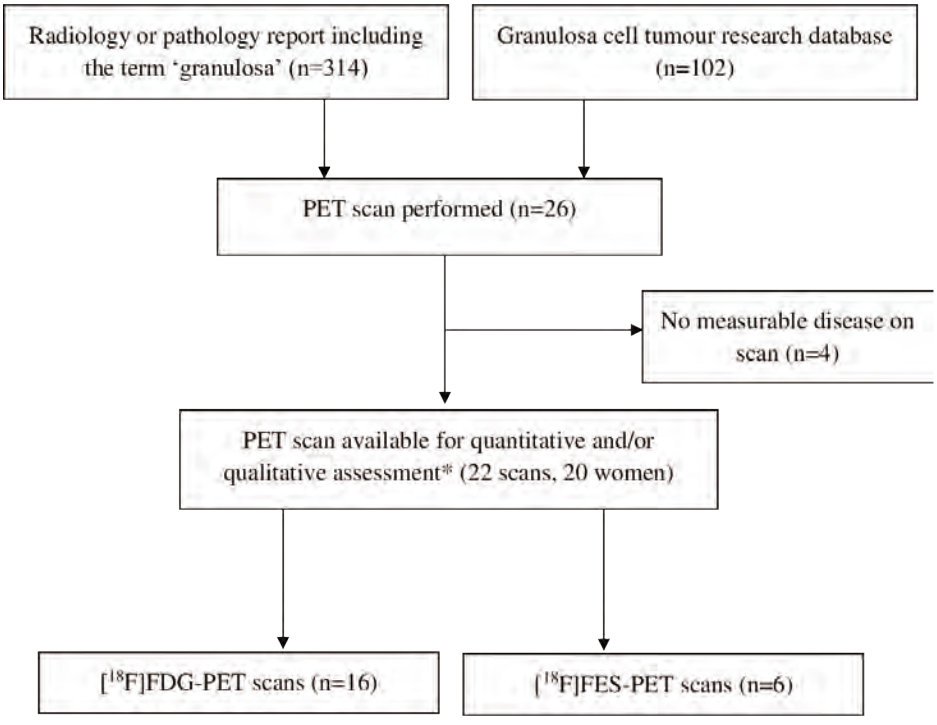


Figure 1. PET scan selection. *Three scans were eligible only for qualitative assessment

Table 1. Baseline characteristics and imaging parameters

		[18F]FDG	[18F]FES
Number of PET/CTs		16	6
		Median (range)	*Median (range)*
Age		67 (39-74)	69 (62-76)
Time since diagnosis (years)		11 (2-41)	12 (6-28)
Clinical indication	Detection of recurrence	15 (94%)	-
	Assess disease load prior to surgery	-	2 (33%)
	Assess FES uptake prior to anti-hormonal treatment	-	4 (67%)
	Assess response to chemotherapeutic treatment	1 (6%)	-
Serum glucose level in mmol/L		5.2 (4.0 – 5.9)	NA
IA in MBq		176 (79 – 325)	190 (129 – 202)
IA in MBq/kg		2.72 (1.46 – 5.2)	2.18 (1.55 – 3.39)
Acquisition time post-injection in minutes		60 (43-78)	63 (52-96)

Legend: IA = injected activity, NA = not applicable

[18F]FDG-PET/CT

Injected [18F]FDG activity was approximately 2 - 3 MBq/kg for all PET/CTs. Due to the multicentre retrospective nature of this study and the long time period of inclusion for this rare malignancy, no standardised methods for [18F]FDG-PET/CT reconstructions were available before 2010, limiting the reproducibility of standardised uptake value (SUV) measurements. From 2010 and onwards, after the introduction of the EARL-criteria in 2010, all [18F]FDG-PET/CTs were acquired and reconstructed according to these international guidelines.[155]

[18F]FES-PET/CT

Images were acquired from thighs to skull vertex on a single PET/CT scanner (Biograph mCT, Siemens, Erlangen, Germany) and approximately 60 minutes after intravenous injection of 200 MBq [18F]FES. A low dose CT was performed directly following PET acquisition. Images were acquired according to the European Association of Nuclear Medicine (EANM) criteria, a.k.a. EARL-reconstructions, with the following parameters: PET with time-of-flight and point spread function reconstruction, 4 iterations, 21 subsets, with a filter of 7.5 mm full width at half maximum.[155]

Image analysis

All retrospectively gathered and available [18F]FDG-PET/CT and prospective [18F]FES-PET/CT images were centrally reviewed and analysed, along with contrast enhanced CTs (CECT) of the thorax, abdomen and pelvis, by a nuclear medicine physician (AJATB, >5 years of experience). Besides assessment of the number and location of metastatic lesions and CECT,

all suspected metastatic lesions were divided between solid (predominantly enhancing) or cystic/necrotic (predominantly non-enhancing) lesions. Qualitative assessment of all available imaging included a blinded assessment of all PET/CT imaging, and comparison with a prior CECT. Additionally, all PET/CT imaging was scored (patient based) according to a visual scale, with a maximum of five lesions per patient (Supplementary Table 1).

Quantitative assessment of all [^{18}F]FDG- and [^{18}F]FES-PET/CTs was performed using software package Syngo.via (Siemens, Erlangen, Germany). As a reference, both liver uptake and blood pool measurements were acquired according to the PERCIST criteria [156]. All known lesions detected on contrast enhanced diagnostic CT and moderate or intense positive according to the qualitative assessment were measured on the PET/CTs (lesion based, with a maximum of five lesions per patient) (Supplementary Table 1). Standardised uptake values (SUV) were calculated according to the lean body mass method, in line with PERCIST recommendations. All measurements are reported as SUV_{max}, SUV_{peak} and SUV_{mean}.

Immunohistochemistry

Immunohistochemistry was performed in available surgical specimens, to assess expression of the estrogen receptors (ERα and ERβ rabbit antibody, Ventana RTU dilution, Roche), and ER expression was evaluated consistently by a single pathologist and scored as a percentage. The number of mitoses was counted per 2mm^2. The correlation between [^{18}F]FES uptake (SUV_{max}) and ER status and [^{18}F]FDG uptake (SUV_{max}) and number of mitoses, respectively, was assessed and visualised in a scatterplot.

Results

A total of 26 PET/CTs was performed, of which 19 were eligible for both qualitative and quantitative assessment, and three for qualitative assessment only (22 scans in 20 women, Figure 1). Four out of 26 scans were excluded due to a lack of measurable disease. All scans were performed between June 2003 and May 2020 in two academic hospitals (UMC Utrecht and Amsterdam UMC, location AMC). The studied PET/CTs included 16 [^{18}F]FDG-PET/CTs and six [^{18}F]FES-PET/CTs. [^{18}F]FDG-PET/CTs were performed for detection of a recurrence (15/16), or to assess response to chemotherapy (1/16). All [^{18}F]FES-PET/CTs were prospectively and consecutively performed in recurrent AGCTs to evaluate tumor [^{18}F]FES uptake prior to hormone treatment (4/6) or to assess disease load prior to surgery (2/6). Baseline characteristics and imaging parameters are presented in Table 1.

Qualitative assessment of [^{18}F]FDG-PET/CTs showed no uptake in three patients (19%), moderate uptake in eleven patients (69%), and intense uptake in two patients (13%), in previously known metastases found on CECT (Table 2). In one patient, an additional

moderate [^{18}F]FDG-avid liver metastasis was detected by [^{18}F]FDG-PET/CT after the initial CECT. In all other cases, [^{18}F]FDG-PET/CT did not detect any additional metastases compared to CECT. [^{18}F]FES-PET/CT imaging showed no uptake in one out of six patients (17%), low uptake in three out of six patients (50%) and moderate uptake in the remaining two patients (33%). No additional metastatic lesions were detected by [^{18}F]FES-PET/CT compared to those identified on the initial CECT.

Quantitative assessment of all visually detectable lesions (32) on [^{18}F]FDG-PET/CT showed a mean SUV_{max}, SUV_{mean} and SUV_{peak} of 2.4, 1.4 and 1.9, which was higher than the mean blood pool SUV_{mean} (1.2), however not markedly increased as compared to the mean liver SUV_{max} (2.2) or liver SUV_{mean} (1.6) (Table 3). Even though most patients were found to have solid metastases on CECT (24/32 lesions), no difference was noted in [^{18}F]FDG-avidity as compared to cystic lesions (8/32 lesions; Table 3). Quantitative assessment of all visually detectable lesions (12) on [^{18}F]FES-PET/CT showed a mean SUV_{max}, SUV_{mean} and SUV_{peak} of 1.7, 1.0 and 1.4, all within the range of the mean blood pool SUV_{mean} (1.4 ± 1.2 SD). In agreement with the [^{18}F]FDG-PET/CT findings, there was no difference in [^{18}F]FES uptake between suggested solid (7/12 lesions) and suggested cystic (5/12 lesions) metastases on CECT (Table 3).

Four patients received [^{18}F]FES-PET/CT to assess [^{18}F]FES tumor uptake prior to anti-hormonal treatment, of which one had multiple [^{18}F]FES positive lesions (Table 1). This patient underwent both [^{18}F]FDG and [^{18}F]FES-PET/CT imaging prior to anti-hormonal treatment. PET/CT showed low [^{18}F]FDG uptake and moderate[^{18}F]FES uptake of a peritoneal tumor lesion (Figure 2). After anti-hormonal treatment with letrozole for six months, a follow-up CECT showed progression of all [^{18}F]FES negative lesions, whereas all [^{18}F]FES positive lesions showed stable disease or regression (Figure 3).

The other three patients who received [^{18}F]FES-PET/CT prior to anti-hormonal treatment did not have [^{18}F]FES tumor uptake. Two of them were treated with tamoxifen and one patient refrained from anti-hormonal treatment. In these patients, the response to tamoxifen was evaluated on CT scan after four to six months treatment and showed disease progression and a newly developed peritoneal lesion in both patients.

Evaluation of ER expression and mitotic activity by immunohistochemistry was performed on available tissue of 15/16 (94%) patients in the [^{18}F]FDG-PET group and all patients in the [^{18}F]FES-PET group (Supplementary Table 2). The number of mitoses per 2mm^2 was <10 for all samples (median 5, range 1-18) except for one showing 18 mitoses per 2mm^2, which had intense uptake on [^{18}F]FDG-PET. We did not detect a correlation between ER expression and [^{18}F]FES uptake on PET/CT. ERα expression was positive (≥5%) in 5/6 patients (83%) and ERβ in all six patients. Most patients had either predominantly ERα or predominantly ERβ receptor expression.

Table 2. Qualitative assessment of [^{18}F]FDG-PET/CT and [^{18}F]FES-PET/CT

	[^{18}F]FDG-PET/CT	[^{18}F]FES-PET/CT
Patient based assessment		
Number of PET/CTs	16	6
Negative or low uptake	3	4
Moderate uptake	11	2
Intense uptake	2	0
Metastatic lesions		
Number of lesions	41	22
Suspected solid (enhancing) on CECT	31	8
Suspected cystic (non-enhancing) on CECT	10	14

Table 3. Quantitative assessment of [^{18}F]FDG-PET/CT and [^{18}F]FES-PET/CT

	[^{18}F]FDG-PET/CT			[^{18}F]FES-PET/CT		
General parameters (patient based)						
Number of scans	13			6		
Mean SUV_{mean} BP (±SD)	1.2 (±0.4)			1.4 (±1.2)		
Mean SUV_{max} BP (±SD)	1.5 (±0.5)			1.7 (±1.2)		
Mean SUV_{mean} liver(±SD)	1.6 (±0.4)			9.6 (±4.4)		
Mean SUV_{max} liver (±SD)	2.2 (±0.6)			18.3 (±16.1)		
Metastatic lesion measurements (lesion based)						
	All	*Solid*	*Cystic*	All	*Solid*	*Cystic*
Number of lesions	32	24	8	12	7	5
Mean SUV_{max} (±SD)	2.4 (±0.9)	2.5 (±0.9)	1.8 (±0.8)	1.7 (±0.5)	1.8 (±0.5)	1.6 (±0.4)
Mean SUV_{mean} (±SD)	1.4 (±0.6)	1.5 (±0.5)	1.2 (±0.5)	1.0 (±0.3)	1.0 (±0.3)	1.0 (±0.3)
Mean SUV_{peak} (±SD)	1.9 (±0.8)	2.0 (±0.8)	1.5 (±0.6)	1.4 (±0.4)	1.4 (±0.4)	1.5 (±0.4)
No. SUV_{mean} > BP SUV_{mean} (%)	18 (57%)	15 (63%)	3 (38%)	5 (38%)	3 (38%)	2 (40%)
No. SUV_{mean} > liver SUV_{mean} (%)	5 (16%)	4 (17%)	1 (12%)	*	*	*
No. SUV_{max} > liver SUV_{max} (%)	16 (50%)	13 (54%)	3 (38%)	*	*	*

Legend: SD = standard deviation, BP = blood pool

*for [^{18}F]FES-PET/CT this measurement is not applicable, because of high physiological biliary excretion of the tracer

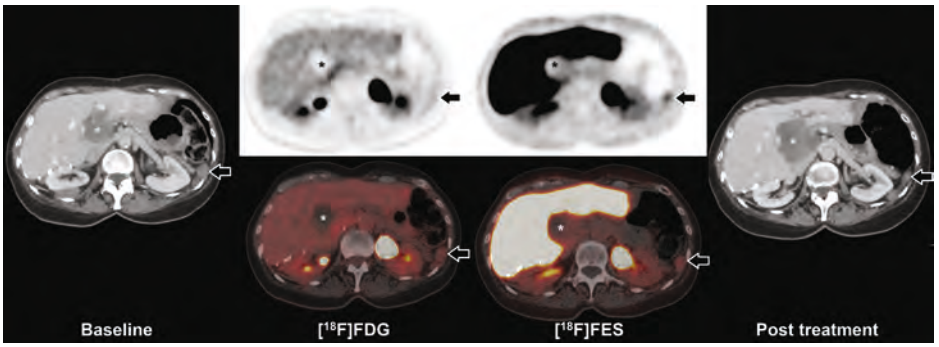


Figure 2. [^{18}F]FES-PET and response to hormone treatment. Example of [^{18}F]FDG and [^{18}F]FES in a 64 year old woman with peritoneal and hepatic metastases of AGCT. Baseline CECT (left) shows peritoneal disease (arrow) and hepatic disease (asterisk). Additional [^{18}F]FDG-PET/CT shows low uptake in the peritoneal disease, whilst [^{18}F]FES shows moderate uptake (arrows). The liver hilum lesion accumulates neither [^{18}F]FDG nor [^{18}F]FES (asterisks). After initiation of hormonal treatment with letrozole for six months, follow-up CECT (right) showed partial regression of the [^{18}F]FES positive peritoneal lesion (from 23 mm to 17 mm maximal diameter; arrows), whilst the [^{18}F]FES negative hepatic lesion showed progression (from 50 mm to 65 mm maximal diameter; asterisks).

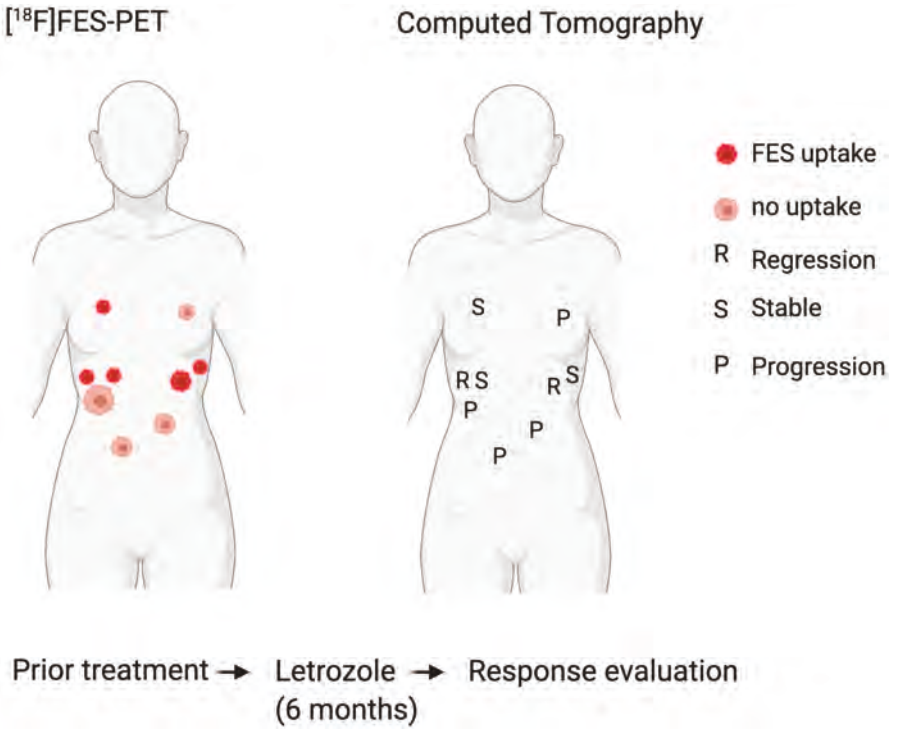


Figure 3. Correlation of [^{18}F]FES positive and negative tumor locations (left) with response to hormone treatment (right). All [^{18}F]FES negative lesions showed progression after six months hormone treatment, whereas all [^{18}F]FES positive lesions showed stable disease or regression.

Discussion

This is the first study to investigate the value of [^{18}F]FDG-PET/CT in monitoring recurrent AGCTs and [^{18}F]FES-PET/CT to assess eligibility for anti-hormonal treatment. [^{18}F]FDG-PET/CT showed low to moderate uptake in most patients (14/16 scans) and identified only one additional tumor location as compared to CT scan. [^{18}F]FES uptake on PET/CT was present in 33% (2/6) of the patients. One of these patients also had [^{18}F]FES negative lesions, which progressed after six months of anti-hormonal treatment, whereas all [^{18}F]FES positive lesions showed stable disease or regression. In this patient, PET/CT using the [^{18}F]FES tracer captured the intra-patient tumor heterogeneity and [^{18}F]FES uptake correlated with the individual tumor response to hormone treatment. Moreover, there was also a clear clinical correlation for the patients without [^{18}F]FES tumor uptake, as the CT scans showed progressive disease after anti-hormonal treatment with tamoxifen.

Although four out of five AGCT cases previously described in case reports showed low to moderate [^{18}F]FDG avidity, contradictory results and lack of [^{18}F]FDG-PET/CT series emphasised the need for further investigation. The current study shows that [^{18}F]FDG avidity of AGCTs is low or moderate and result in low detection rates of (metastatic) disease by [^{18}F]FDG-PET only. In particular peritoneal metastases may be difficult to distinguish from physiologic bowel uptake. Imaging by [^{18}F]FDG-PET may therefore not be helpful in monitoring AGCTs. However, one of two patients with intense [^{18}F]FDG tumor uptake and with the highest mitotic rate (18 per 2mm^2) harboured an AGCT with a *TP53* mutation, which has been described in a subset of AGCT patients (9-11%). This mutation may explain the [^{18}F]FDG uptake in this patient, since TP53 mutant AGCTs are associated with a higher tumor mutational burden, mitotic rate and metabolic activity [111]. If this finding can be confirmed in a larger subset of AGCT patients with TP53 mutations, [^{18}F]FDG-PET/CT may be of value in this small subpopulation.

Previous studies showed that [^{18}F]FES uptake corresponds with ER expression on immunohistochemistry in breast cancer and uterine cancer.[157,158] We could not confirm this correlation in AGCTs, potentially due to the small sample size of the study, the time interval between tissue withdrawal and the [^{18}F]FES-PET/CT, and varying ER expression levels between tumor lesions. Heterogeneous ER expression has also been detected in breast cancer and discordant expression between primary tumors and metastasis is seen in up to 40% of the patients.[152,159] Compared to immunohistochemistry, [^{18}F]FES-PET/CT is a noninvasive method that provides a comprehensive overview of all existing tumor locations and the ER expression of individual metastatic lesions. Intra-patient tumor heterogeneity is common in AGCTs[111], making the comparison between ER expression on older tissue samples and [^{18}F]FES uptake more difficult. Additionally, the affinity of [^{18}F]FES for the ERα is

6.3 times higher than that for the ERβ. [^{18}F]FES-PET/CT imaging may therefore better reflect ERα expression, while ERβ overexpression is more common in AGCTs (Supplemental Table 2).[152] Nevertheless, this study demonstrates that [^{18}F]FES-PET/CT can visualise estrogen receptor binding in AGCTs and that [^{18}F]FES tumor uptake correlates with the response to anti-hormonal treatment in a single case. Therefore, [^{18}F]FES-PET/CT could help to provide a rationale for anti-hormonal treatment. In addition, absent tumor [^{18}F]FES uptake in AGCT lesions may predict failure of hormonal therapy or resistant locations in patients, as illustrated by one of our cases.

The current study has a few limitations. Both in case reports and in case series, results may be influenced by selection bias. In this study, patients were not randomly selected to undergo PET scanning and most of them had a long history of AGCT recurrences. It is uncertain whether this may have influenced the outcome of the PET scans, as [^{18}F]FES uptake may potentially be higher in primary disease than in recurrent lesions.

To our knowledge, this study is the first to investigate the value of a targeted hormone tracer in AGCTs. Nuclear agents binding to other hormone receptors expressed by AGCTs, such as AMH and the progesterone receptor, could also be good candidates for targeted PET scanning. It remains a clinical challenge to establish the optimal timing of treatment for AGCT recurrences. Besides PET-CT, other diagnostics such as detection of circulating tumor DNA in plasma, are currently being investigated for disease monitoring and estimation of disease activity.[160,161] Given the low incidence of this disease, performing prospective trials in AGCT is difficult. Future prospective research on [^{18}F]FES-PET/CT could elucidate whether this imaging tool can be used to predict the response to hormonal treatment in AGCT patients.

Conclusions

This study shows that [^{18}F]FDG uptake by AGCTs is low-moderate and [^{18}F]FDG-PET/CT may not be helpful in monitoring AGCTs. Our [^{18}F]FDG-PET/CT findings are in corroboration with previous reports and provide a possible explanation for incidental patients with high [^{18}F]FDG tumor uptake (i.e. high mitotic index due to a *TP53* mutation). Furthermore, [^{18}F]FES uptake was seen in a subset of AGCT patients and correlated with the response to anti-hormonal treatment. [^{18}F]FES positive tumors remained stable or decreased in size as [^{18}F]FES negative tumors progressed after anti-hormonal treatment. Therefore, [^{18}F]FES-PET/CT may be useful to assess the potential eligibility for anti-hormonal treatment in AGCT patients by noninvasively capturing the estrogen receptor expression levels of all separate tumor lesions.

Informed consent: All patients provided written informed consent for the use of their clinical data and publication of results. Ethical approval was obtained by the Institutional Review Board of the University Medical Centre Utrecht. Clinical data were acquired from patient reports.

Conflict of interest: The authors declare no conflict of interest.

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Writing - original draft preparation: Joline F Roze, Arthur J Braat, Ronald P Zweemer
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Resources: Joline F Roze, Hannah S van Meurs, Luc RCW van Lonkhuijzen, Roel J Bennink, Arthur JA Braat, Ronald P Zweemer
Supervision: Ronald P Zweemer

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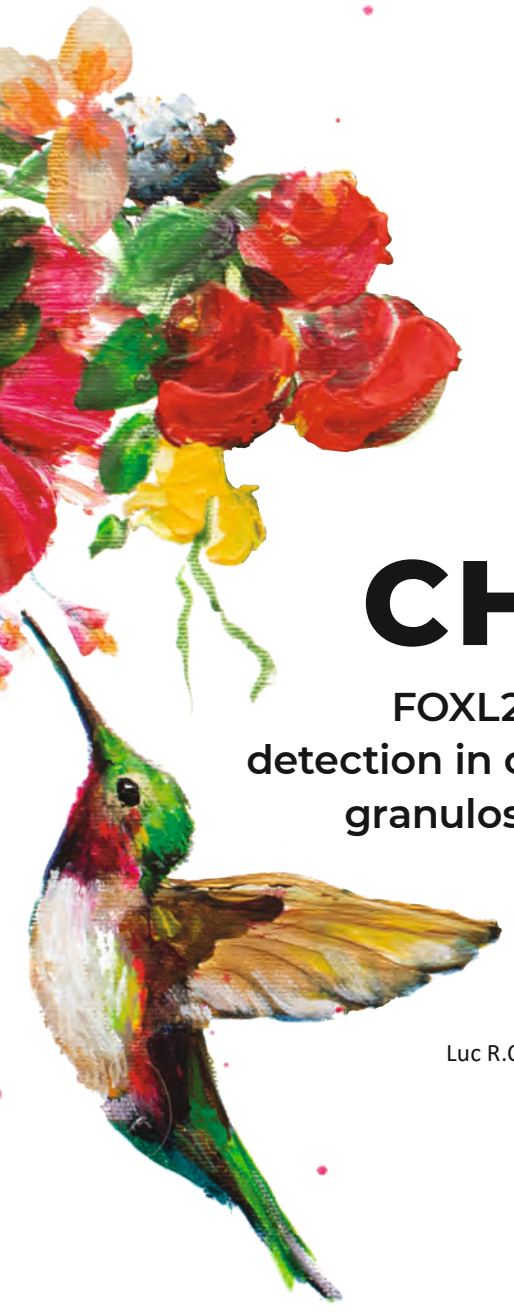

CHAPTER 6

FOXL2 and TERT promoter mutation detection in circulating tumor DNA of adult granulosa cell tumors as biomarker for disease monitoring

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Submitted

Abstract

Objective: Adult granulosa cell tumors (AGCT) represent a rare, hormonally active subtype of ovarian cancer that has a tendency to relapse late and repeatedly. Current serum hormone markers are inaccurate in reflecting tumor burden in a subset of AGCT patients, indicating the need for a novel biomarker. We investigated the presence of circulating tumor DNA (ctDNA) harboring a *FOXL2* or *TERT* promoter mutation in serial plasma samples of AGCT patients to determine its clinical value for monitoring disease.

Methods: In a national, multicenter study, plasma samples (*n* = 110) were prospectively collected from 21 patients with primary (*n* = 3) or recurrent (*n* = 18) AGCT harboring a *FOXL2* 402C>G and/or a *TERT (*C228T or C250T) promoter mutation. Circulating cell-free DNA was extracted and assessed for ctDNA containing one of either mutations using droplet digital PCR (ddPCR). Fractional abundance of *FOXL2* mutant and *TERT* mutant ctDNA was correlated with clinical parameters.

Results: *FOXL2* mutant ctDNA was found in plasma of 11 out of 14 patients (78.6%) with an aGCT with a confirmed *FOXL2* mutation. *TERT* C228T or *TERT* C250T mutant ctDNA was detected in plasma of 4 of 10 (40%) and 1 of 2 patients, respectively. Both *FOXL2* mutant ctDNA and *TERT* promoter mutant ctDNA levels correlated with disease progression and treatment response in the majority of patients.

Conclusion: *FOXL2* mutant ctDNA was present in the majority of aGCT patients and *TERT* promoter mutant ctDNA has been identified in a smaller subset of patients. Both *FOXL2* and *TERT* mutant ctDNA detection may have clinical value in disease monitoring.

Introduction

Adult granulosa cell tumors (AGCTs) belong to the subgroup of sex cord-stromal cell tumors of the ovary, accounting for 3 - 5% of ovarian cancers. Although a granulosa cell tumor can be suspected in patients presenting with abnormal vaginal bleeding or abdominal pain and an ovarian mass, the majority of AGCTs are diagnosed after surgical removal of the ovarian tumor.[162,163] Most AGCTs are found at an early stage with a 5-year survival rate > 90%, and characterized as indolent tumors. However, recurrent disease is found in at least one third of AGCT patients, typically occurring late with a time to relapse of 4-8 years.[19,164] Surgery remains the cornerstone of treatment for both primary and recurrent AGCTs, as alternative therapeutic options such as chemotherapy and anti-hormonal therapy have shown limited response.[7,29,30] Relapsed AGCTs tend to recur multiple times, eventually leading to death in 50-80% of patients.

The hormonal activity of granulosa cells permits the use of several serum hormones as biomarkers in the follow-up of AGCTs. Inhibin is a hormone produced by granulosa cells during follicular development and a member of the TGF-β family of growth factors.

It consists of an alpha and beta (β) subunit, the latter being either a βA subunit forming inhibin A or a βB subunit forming inhibin B. Inhibin levels fluctuate during the menstrual cycle, and decrease to undetectable or very low levels after menopause.[165,166] Inhibin B is superior to inhibin A as a marker of disease activity in AGCT, with reported sensitivities of 89-93% for inhibin B and 67% for inhibin A.[25,26] Anti-Müllerian hormone (AMH) is another member of the TGF-β growth factor family and is secreted by granulosa cells of developing follicles. Serum AMH has also been validated as a biomarker for AGCT and its accuracy was found to be similar to inhibin B, with a described sensitivity of 92%.[26,27] Although estrogen is a well-known hormone produced by granulosa cells and frequently secreted by AGCTs, no consistent correlation between serum estradiol levels and tumor activity has been reported.[23,27] Despite the high sensitivity of inhibin B and AMH, the use of serum hormone markers for AGCT has its limitations. Hormone levels are physiologically elevated and fluctuate over time in premenopausal women. In addition, up to 15% of AGCTs do not produce inhibin B, and normal or fluctuating levels of the described markers can be found in patients with recurrent disease. Also, elevated levels of hormone markers without evidence of disease are sometimes seen.[25,26] Therefore, currently utilized serum hormone markers do not always correlate with disease activity, hampering their use as a biomarker. This highlights the need for a novel method to monitor AGCT relapse and treatment response.

The identification of circulating tumor DNA (ctDNA) as a fraction of cell-free DNA (cfDNA) in plasma has emerged as a non-invasive "liquid biopsy" in a variety of cancers.[167,168] Small ctDNA fragments are released from tumor cells into the bloodstream following processes such as necrosis or apoptosis. As a consequence, ctDNA harbors tumor-specific genetic alterations.[169] With the detection of these tumor-specific mutations in patients' plasma samples, ctDNA has been investigated as a genomic biomarker for disease monitoring and the assessment of treatment response in many cancer types including non-small cell lung cancer, breast cancer, colorectal cancer, gastric cancer, bladder cancer and ovarian cancer.[170–175] Techniques used for the detection of ctDNA include polymerase chain reaction (PCR) to identify specific mutations in single genes, and targeted next-generation sequencing (NGS) to assess alterations in multiple genes at once.

The most defining molecular feature of AGCT is a mutation in the transcription factor *FOXL2*. This 402C>G (C134W) *FOXL2* mutation is present in approximately 95% of tumors.[41] One study has described the development of a specific digital droplet PCR (ddPCR) assay to analyze the *FOXL2* 402C>G mutation in plasma ctDNA of AGCT patients.[28] FOXL2 mutant ctDNA was identified in 36% of subjects and the authors suggested the use of this assay for the diagnosis and monitoring of AGCT. Recent studies have reported alterations in cancer-related genes in a subset of AGCTs, including *TERT, TP53, PIK3CA, KMT2D* and *CTNNB1*.[45,48,69,77,111] Most notably, the C228T *TERT* promoter mutation was found in 24-40% of AGCTs, with a higher prevalence in recurrent tumors as compared to primary tumors.[45,69,111] In addition, two studies also revealed the presence of the C250T *TERT* promoter mutation in a smaller percentage of AGCTs.[77,111] Both *TERT* promoter mutations have previously been detected in ctDNA of melanoma and hepatocellular carcinoma patients.[176–178]

In the current study, we evaluate the clinical potential of *FOXL2* mutant ctDNA as biomarker for monitoring disease activity and treatment response in a prospective cohort of AGCT patients in different disease stages. In addition, our investigation is the first to identify ctDNA harboring a *TERT* C228T or C250T mutation in plasma of AGCT patients by ddPCR and correlate these findings with disease status and *FOXL2* mutant ctDNA detection.

Methods

Patients and samples

A national multicenter prospective study was conducted, with approval of our institutional review board (UMCU METC 17-868) and the review boards of the participating centers. Patients diagnosed with a primary or recurrent granulosa cell tumor were included during a hospital visit between 2018 and 2020. All patients provided written informed consent. Tumor tissue was obtained at the time of surgery as previously described.[111] In addition, blood

samples were collected prospectively from participating AGCT patients: at diagnosis of primary or recurrent disease, prior to surgery or other treatment, during systemic treatment and at regular follow-up visits. Clinical data including patient characteristics, treatments, serum marker levels, histopathology and radiology reports and follow-up information were collected. Upon blood withdrawal for study purposes, 2x 10 ml of venous whole blood was collected into PAXgene® tubes (BD Biosciences, Eysins, Switzerland). Within 7 days after collection, samples were centrifuged for 10 minutes at 1900 *x g* (3000 rpm) at 4°C. The supernatant plasma was then transferred to 15 ml centrifuge tubes and centrifuged for 10 minutes at 16,000 x *g* (in fixed-angle rotor) to remove additional cellular nucleic acids attached to cell debris. The supernatant was then divided in 1 ml aliquots and stored at -80°C until further analysis.

Selection and preparation of plasma samples

We confirmed the presence of the *FOXL2* 402C>G mutation using whole genome sequencing (WGS) on DNA isolated from tumor tissue of all AGCT patients whose plasma samples were used for analysis of ctDNA harboring the *FOXL2* mutation.[111] In addition, we used the plasma samples of patients with a *TERT* promoter mutation in their tumor, identified by previous WGS analyses, for detection of ctDNA with the *TERT* C228T or *TERT* C250T mutation. In order to extend this number of samples, additional tumor specimens of patients with active disease and at least one plasma sample available were tested for the *TERT* C228T or *TERT* C250T mutation by PCR amplification followed by Sanger sequencing using the primers 5'-AGCACCTCGCGGTAGTG-3' and 5'-GGGCTCCCAGTGGATTC-3'. The thermal cycling conditions were 95°C for 5 minutes, followed by 32 cycles of 95°C for 30 seconds, 65°C for 30 seconds and 72°C for 30 seconds, followed by 72°C for 10 minutes and infinite hold at 4°C.

Plasma aliquots were thawed and cfDNA was isolated from 3 ml plasma per sample using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Isolated DNA samples were eluted in 30-40 µl elution buffer. The quantity of cfDNA was measured using a Qubit fluorometer with the dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Quality control of DNA was performed using the Agilent TapeStation system with D5000 SreenTape assay (Agilent, Santa Clara, California, USA).

Digital droplet PCR (ddPCR)

CfDNA extracted from plasma samples was analyzed for *FOXL2* or *TERT* mutations by ddPCR. For *FOXL2* mutation detection, validated ddPCR Mutation Assays for *FOXL2* wild-type (WT) and *FOXL2* p.C134W c.402C>G were used (Bio-Rad Laboratories, Hercules, California, USA). For *TERT* promoter mutation detection by ddPCR, the *TERT* C228T_88 Expert Design Assay and the *TERT* C250T_88 Expert Design Assay (Bio-Rad Laboratories) were utilized as previously described.[176] Reaction volumes of 22 µl per well of a 96-well plate were prepared. Each reaction

for *FOXL2* testing contained 11 µl supermix for probes (no dUTP) (Bio-Rad Laboratories), 1 µl primer-probe mix for both mutant (labeled with FAM) and WT (labeled with HEX) *FOXL2*, 4-8 µl cfDNA from patient plasma, and purified water to a total of 22 µl. Each reaction for *TERT* testing consisted of 11 µl supermix for probes (no dUTP), 1 µl primer-probe mix of either *TERT* C228T or C250T assays (mutation specific probe labeled with FAM and WT specific probe labeled with HEX), 2 µl of 5M betaine (Sigma-Aldrich, St. Louis, Missouri, USA), 1 µl of 20 mM EDTA (Thermo Fisher Scientific), 4-6 µl cfDNA from patient plasma, and purified water to a total of 22 µl. Reactions were subjected to ddPCR analysis using the QX200 system according to the manufacturer's protocol (Bio-Rad Laboratories). Each ddPCR experiment was optimized by performing a temperature gradient experiment to establish the best amplification temperature for separation of positive and negative droplets. The final thermal cycling conditions for the *FOXL2* assay were 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds and 55°C for 1 minute, followed by 98°C for 10 minutes and infinite hold at 12°C. For the *TERT* C228T assay, the PCR conditions were 95°C for 10 minutes, followed by 50 cycles of 96°C for 30 seconds and 60°C for 1 minute, followed by 98°C for 10 minutes and infinite hold at 12°C. For the *TERT* C250T assay, the PCR cycling conditions were 95°C for 10 minutes, followed by 50 cycles of 96°C for 30 seconds and 62°C for 1 minute, followed by 98°C for 10 minutes and infinite hold at 12°C. Positive and negative controls, consisting of AGCT tumor DNA samples with and without the *FOXL2* or *TERT* promoter mutation, as well as no-template controls were included in every run. Each cfDNA sample was analyzed at least in duplicate wells in each run, and in two separate ddPCR runs. Quantasoft software (Bio-Rad Laboratories) was utilized for data analysis. Only wells with total droplet counts greater than 10,000 droplets were analyzed. For each ddPCR assay, thresholds separating positive and negative droplets were set manually based on the droplet distribution in the positive and negative control sample. Double positive droplets were excluded. Based on the amount of false-positive droplets in negative controls, samples with ≥ 3 mutation positive droplets were considered true positive.

Results

Patient cohort

Twenty-one patients with histologically confirmed AGCT and active disease on CT or MRI imaging or elevation of serum markers were included in the current study. A total of 110 serial blood samples were prospectively obtained. Patient characteristics are shown in Table 1. Three of the included patients had a primary AGCT, while all other patients had recurrent disease. Plasma samples (n = 94) of 14 patients were used for ddPCR analysis of the *FOXL2* mutation. For *TERT* promoter mutation testing, 46 plasma samples of 12 patients were used; 36 samples from ten patients harboring a *TERT* C228T mutation and ten samples from two patients harboring a *TERT* C250T mutation, as confirmed by whole genome sequencing or Sanger sequencing of corresponding tumor tissue.

Table 1. Patient characteristics.

Patient	Age (years)	Primary / Recurrence	Age at diagnosis	Stage at diagnosis	Previous treatment	Disease status
P1	64	Recurrence	53	IC	Surgery, CT, HT	Progressive disease
P2	76	Recurrence	50	IC	Surgery	Progressive disease
P3	74	Recurrence	68	IIB	Surgery	Progressive disease
P4	44	Recurrence	35	IC	Surgery, CT	Stable disease
P5	74	Recurrence	57	IA	Surgery, CT	Died of disease
P6	63	Recurrence	61	IC	Surgery	No evidence of disease
P7	48	Primary	48	IA	n/a	No evidence of disease
P8	50	Recurrence	48	I	Surgery	No evidence of disease
P9	42	Recurrence	30	IA	Surgery	Stable disease
P10	59	Recurrence	52	IC	Surgery	No evidence of disease
P11	49	Recurrence	43	IC	Surgery, CT	Died of disease
P12	50	Primary	49	IA	Surgery	No evidence of disease
P13	56	Recurrence	50	IA/C	Surgery	Progressive disease
P14	57	Recurrence	53	IA	Surgery, CT, HT	Died of disease
P15	68	Recurrence	52	IIB	Surgery, CT, HT	No evidence of disease
P16	47	Recurrence	40	IA	Surgery, HT	Stable disease
P17	74	Recurrence	71	IC	Surgery	No evidence of disease
P18	45	Primary	44	IA	n/a	No evidence of disease
P19	84	Recurrence	60	I	Surgery	No evidence of disease
P20	68	Recurrence	52	I	Surgery, RT	Progressive disease
P21	64	Recurrence	49	IC	Surgery	No evidence of disease

CT = chemotherapy, HT = hormonal therapy, RT = radiotherapy, IP = intraperitoneal, n/a = not applicable.

Prevalence of *FOXL2* mutant ctDNA in plasma of AGCT patients

The isolated cfDNA from plasma of AGCT patients was analyzed for presence of the *FOXL2* 402C>G mutation by ddPCR. *FOXL2* mutation positive ctDNA was found in 11 out of 14 patients (78.6%). Fractional abundance, defined as the relative fraction of mutation positive ctDNA compared to the fraction of WT cfDNA, varied between patients and ranged from 0.49% to 48.56% (Table 2). In one of three patients (Patient (P) 7) without detectable plasma *FOXL2* mutant ctDNA, blood samples from before and after surgery for a primary AGCT (stage IA) were analyzed. The other two patients (P10 and P15) who were negative for *FOXL2* mutant ctDNA had their first and fifth recurrence of AGCT with unifocal and multifocal intraperitoneal disease, respectively, both requiring debulking surgery. Contrary to the patients with active disease but undetectable ctDNA, in one patient (P12) *FOXL2* mutant ctDNA was detected in plasma during follow-up after a surgically treated primary AGCT, although there was no evidence of disease. Two of her five plasma samples showed low fractions of ctDNA (0.33% and 0.49%, four and seven months after surgery) whereas no detectable ctDNA fraction was found in the two subsequent

follow-up samples, with inhibin A and AMH levels also slightly elevated followed by normal values and inhibin B remaining at normal levels. The remaining ten patients whose plasma contained *FOXL2* mutant ctDNA had active, measurable recurrent disease at the time of ctDNA detection.

Detection of *TERT* mutant ctDNA in plasma of AGCT patients

After optimizing the ddPCR assays for *TERT* mutation testing in tumor DNA of AGCT patients, cfDNA from patients with a confirmed *TERT* mutant tumor was analyzed for the presence of the *TERT* C228T or *TERT* C250T mutation by ddPCR. In plasma of four out of ten patients (40%) the *TERT* C228T mutation was found, with mutant ctDNA fractions ranging from 0.24% to 4.64% (Table 2, Figure 1). In addition, ctDNA with the *TERT* C250T mutation was detected in one of two studied patients with a fractional abundance of 0.59% (*Table 2, Figure 1*). Examples of ddPCR positive and negative control tissue samples for both *TERT* mutations are shown in Supplementary Figure 1.

Table 2. Presence of FOXL2 mutant ctDNA and TERT promoter mutant ctDNA in AGCT plasma samples.

Patient	Primary / Recurrence	FOXL2 mutant ctDNA detected	Fractional abundance (%)	*TERT* mutation in tumor	*TERT* mutant ctDNA detected	Fractional abundance (%)
P1	Recurrence	Yes	1.75	C228T	Yes	0.24
P2	Recurrence	Yes	2.03	C250T	Yes	0.59
P3	Recurrence	Yes	18.07	WT	n/a	
P4	Recurrence	Yes	1.86	WT	n/a	
P5	Recurrence	Yes	48.56	WT	n/a	
P6	Recurrence	WT		C228T	No	
P7	Primary	No		WT	n/a	
P8	Recurrence	NT		C228T	No	
P9	Recurrence	Yes	7.97	C228T	Yes	1.68
P10	Recurrence	No		C228T	No	
P11	Recurrence	Yes	1.25	C228T	Yes	0.29
P12	Primary	Yes	0.49	WT	n/a	
P13	Recurrence	Yes	0.82	WT	n/a	
P14	Recurrence	Yes	18.61	WT	n/a	
P15	Recurrence	No		WT	n/a	
P16	Recurrence	Yes	1.43	WT	n/a	
P17	Recurrence	NT		C228T	No	
P18	Primary	NT		C250T	No	
P19	Recurrence	NT		C228T	Yes	4.64
P20	Recurrence	NT		C228T	No	
P21	Recurrence	NT		C228T	No	

WT = wild-type, NT = not tested, n/a = not applicable

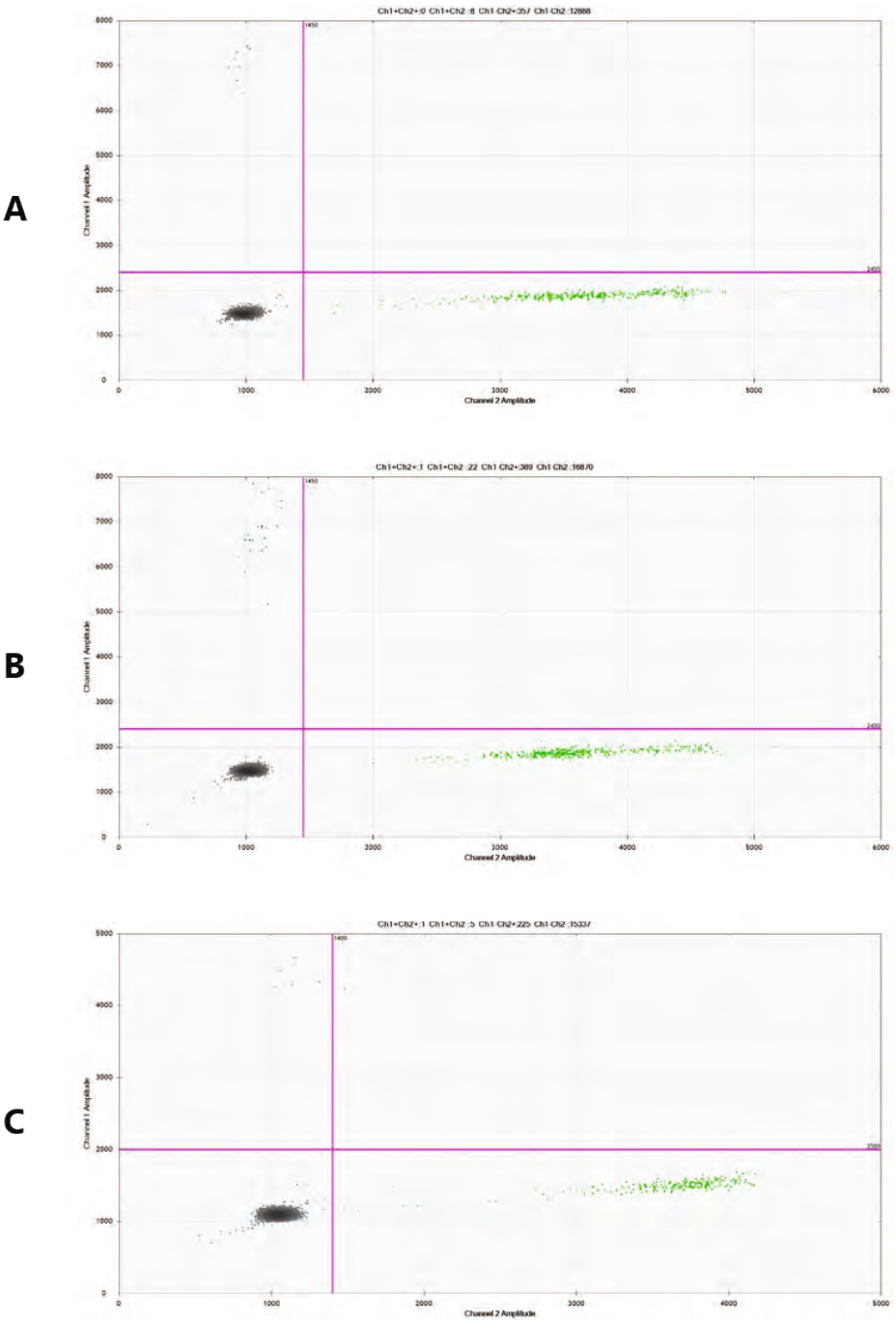

Figure 1. Presence of *TERT* mutant ctDNA in plasma of AGCT patients.
A = *TERT* C228T mutant ctDNA in patient P9. B = *TERT* C228T mutant ctDNA in patient P19. C = *TERT* C250T mutant ctDNA in patient P2.

FOXL2 mutant ctDNA testing for disease monitoring

From eight patients with recurrent AGCT whose plasma harbored *FOXL2* mutant ctDNA, 7-11 plasma samples per patient were collected at multiple time points during follow-up and treatment. These serial samples allowed for the evaluation of ctDNA levels related to disease progression and treatment response. Figure 2 depicts the changes in *FOXL2* mutant ctDNA fractions during the course of each patient's disease, with inhibin B levels shown as a comparison. In six of eight patients (75%), changes in ctDNA levels correlated with disease activity and response to treatment.

Case descriptions

Patient P1 was included in the study when she was being treated with carboplatin and paclitaxel for a fifth AGCT recurrence, followed by incomplete debulking surgery. She developed progression of liver metastases within three months after surgery, for which she underwent radiofrequency ablation (RFA). After a period of stable disease she again developed progressive disease and was treated with letrozole. In plasma samples collected during this course, *FOXL2* mutant ctDNA was found in low amounts. A peak at 1.8% was seen when her liver metastases increased in size, then a *FOXL2* mutant ctDNA decrease was observed after RFA treatment. No rise in *FOXL2* mutant ctDNA was seen alongside the subsequent disease progression. Patient P2 was monitored after a complete debulking surgery for a fourth AGCT recurrence when serial plasma collections were initiated. Inhibin and *FOXL2* mutant ctDNA levels were rising (ctDNA peak at 2.03%) and CT scans confirmed a multifocal fifth recurrence. Patient was treated with six cycles of carboplatin and paclitaxel, leading to a partial response on CT scan, decreased inhibin B and absence of *FOXL2* mutant ctDNA. The first blood sample of patient P3 was collected prior to a complete debulking surgery for a third recurrence. *FOXL2* mutant ctDNA was highly present (18.1%) which subsequently declined to 0.7% six weeks after surgery. Another relapse was confirmed four months later by CT imaging and accompanied by increased ctDNA and inhibin B levels. This recurrence was treated with carboplatin and paclitaxel, with a partial response and a sharp decline of ctDNA to undetectable levels. Despite anti-hormonal therapy that was initiated after completion of chemotherapy, disease progressed as was also shown by a rise of *FOXL2* mutant ctDNA fractions. Patient P4 was initially found to harbor low fractions of *FOXL2* mutant ctDNA during chemotherapeutic treatment (carboplatin and paclitaxel) for a third AGCT recurrence, resulting in a partial response with absent *FOXL2* mutant ctDNA after treatment. Complete cytoreductive surgery was performed, which was followed by a rise in *FOXL2* mutant ctDNA and suspicion of limited recurrent or residual disease on CT scan, then stable disease during the following year. Similar to patient P3, *FOXL2* mutant ctDNA levels in plasma of patient P5 with a fourth AGCT recurrence were high (up to 48.6%) and a clinical response to treatment with chemotherapy and debulking surgery was accompanied by a decrease in *FOXL2* mutant ctDNA levels.

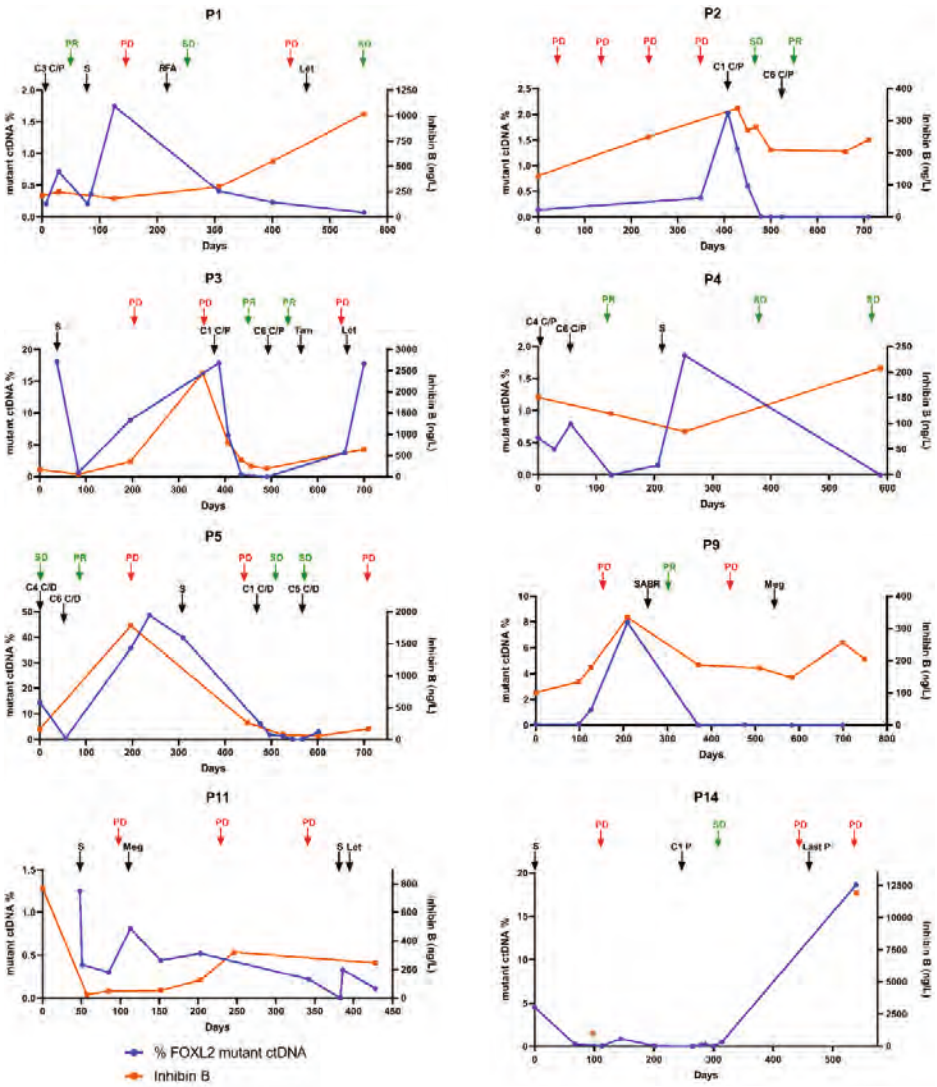

Figure 2. Clinical course of AGCT patients and their changes in *FOXL2* mutant ctDNA and inhibin B. Blue line = ctDNA, orange line = inhibin B. PR = partial response, PD = progressive disease, SD = stable disease, S = surgery, C = cycle, C/P = carboplatin/paclitaxel, RFA = radiofrequency ablation, Let = letrozole, Tam = tamoxifen, C/D = carboplatin/docetaxel, SABR = stereotactic ablative radiotherapy, Meg = megestrol, P = paclitaxel.

Patient P9 had a fifth recurrence located in the liver with rising *FOXL2* mutant ctDNA and inhibin B. She was treated with stereotactic ablative radiotherapy and had a partial response which was confirmed by decreasing ctDNA and inhibin B levels. Subsequently, disease progression occurred which was treated with megestrol leading to stable disease. Patient P11 harbored low amounts of *FOXL2* mutant ctDNA, with the highest fraction (1.2%) found

just prior to debulking surgery for a third AGCT recurrence and lower fractions thereafter, despite progressive disease requiring another debulking surgery and anti-hormonal therapy. Finally, patient P14 was also included just prior to surgical treatment for a third AGCT recurrence, starting with 4.5% *FOXL2* mutant ctDNA which declined to 0.2% after surgery. This *FOXL2* mutant ctDNA mildly increased upon disease progression, then was undetectable during treatment with paclitaxel and stable disease, and then strongly increased to 18.6% when disease progressed rapidly leading to the patient succumbing to her disease.

TERT promoter mutant ctDNA levels during disease monitoring

Similar to studying *FOXL2* mutant ctDNA as a biomarker in AGCT, we analyzed *TERT* promoter mutant ctDNA fractions during the course of the disease in three patients from whom serial plasma samples were available (Figure 3). In all three cases, *TERT* mutant ctDNA fractions were found to correlate with disease progression or response to treatment, resembling the *FOXL2* mutant ctDNA changes in the same patients. Patient P2 was found to harbor *TERT* C250T mutant ctDNA and although levels were low, a clear increase was seen during progression of disease and a ctDNA decline accompanying the clinical response to treatment with carboplatin and paclitaxel. In plasma samples of patient P9, an initial *TERT* C228T mutant ctDNA fraction of 1.7% during disease progression decreased to undetectable levels as a result of treatment with a partial response. Moreover, patient P11 had a low *TERT* C228T mutant ctDNA fraction that decreased to zero following surgery, and subsequently increased with progression of disease.

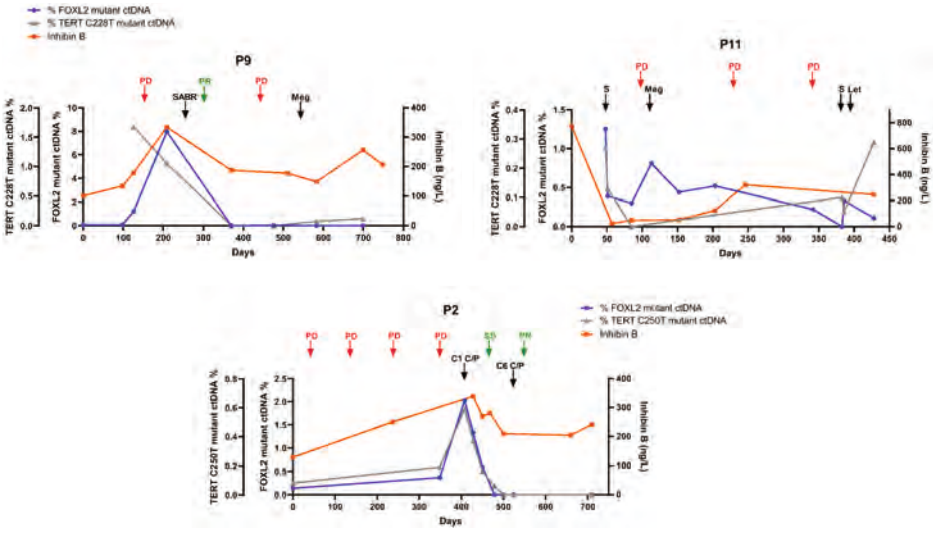

Figure 3. Clinical course of AGCT patients and their changes in *TERT* mutant ctDNA, *FOXL2* mutant ctDNA and inhibin B.

Discussion

In the current study, *FOXL2* mutant ctDNA was detected in 79% of analyzed patients with an AGCT harboring a *FOXL2* 402C>G mutation. We are the first to report the presence of *TERT* promoter mutant ctDNA in a subset of patients with AGCT harboring a C228T or a C250T *TERT* promoter mutation. In addition, ctDNA analysis of longitudinal samples showed a correlation between *FOXL2* mutant or *TERT* mutant ctDNA levels and disease activity in the majority of patients.

Current biomarkers in AGCT, most notably inhibin B and AMH, sufficiently measure disease activity in most AGCT patients. However, they can be inadequate in premenopausal patients due to hormonal fluctuations related to the menstrual cycle, or show false negative or false positive results for unexplained reasons. The course of relapsed AGCT commonly includes multiple recurrences requiring repeated surgery and other therapeutic strategies, underscoring the importance of monitoring disease reliably to determine the timing and effectiveness of treatment.

The potential application of ctDNA as a genomic biomarker for disease monitoring has been a main focus of investigation in a variety of cancers.[168,179] The presence of plasma ctDNA was found to reflect minimal residual disease and serve as a prognostic biomarker in several solid tumors including breast cancer, colorectal cancer, lung cancer and pancreatic cancer.[168,180] In addition, other studies have described the use of ctDNA for the assessment of treatment response. In breast cancer, a clear suppression of plasma *PIK3CA* mutant ctDNA within four weeks of treatment with paclitaxel with or without the AKT inhibitor capivasertib was seen in a subset of patients, in some cases followed by a rise in ctDNA that marked the development of resistance.[181] Several studies have described the use of *KRAS* mutant ctDNA as a marker of response to chemotherapy and disease progression in pancreatic cancer, with changes in ctDNA during treatment being more rapid and pronounced than changes in protein-based tumor markers.[182,183] A recent study in metastatic colorectal cancer showed similar results, comparing ctDNA with carcinoembryonic antigen (CEA) for the assessment of chemotherapy response and finding ctDNA to be a more sensitive and responsive marker of tumor burden than CEA.[172] In line with these studies, we observed a correlation between ctDNA levels and response to treatment or progressive disease in the majority of patients, with chemotherapy leading to a more pronounced decrease in ctDNA as compared to inhibin B.

In AGCT, plasma ctDNA containing the highly prevalent 402C>G (C134W) *FOXL2* mutation has been previously studied by other investigators, who detected *FOXL2* mutated ctDNA in 35% of patients with primary disease and in 19% of patients with recurrent disease.[28] A sensitivity of their ddPCR assay as low as 23% was described, which the authors attributed at least in part to the use of limited amounts of plasma (1 ml on average) resulting in small quantities of cfDNA being analyzed. Serial plasma samples of six AGCT patients showed limited correlation with clinical disease activity. In our study of mostly recurrent AGCT patients, larger volumes of plasma and consequently larger amounts of cfDNA were used for ddPCR analyses, showing a substantially higher frequency of *FOXL2* mutant ctDNA detection and a better reflection of the clinical course. Detection of ctDNA with the *TERT* promoter mutation has not yet been reported in AGCT, although previous studies have identified the C228T or C250T *TERT* promoter mutation in 24-49% of tumor specimens of AGCT patients.[45,69,77,111] A *TERT* promoter mutation was found more frequently in recurrent AGCT as compared to primary tumors, and was associated with a worse prognosis.[69,77,111] Others have detected *TERT* C228T mutant plasma ctDNA in 51% of patients with advanced hepatocellular carcinoma and *TERT* C228T or C250T mutant ctDNA in 78% of melanoma patients.[176,177] In our small pilot cohort of plasma samples from ten patients with AGCT harboring a C228T *TERT* promoter mutation and two AGCT patients harboring a C250T *TERT* promoter mutation, we detected *TERT* mutant ctDNA in four of ten (40%) and in one of two (50%) patients, respectively.

Consistent with available evidence on the detection of ctDNA in other malignancies, the observed fractions of plasma ctDNA in our cohort are highly variable and range from 0.24% to 48.56% of total plasma cfDNA in our study.[179] The presence of cfDNA, including ctDNA, is thought to be the result of its release from cells through apoptosis, necrosis and possibly active secretion.[169,184] The largest proportion of plasma cfDNA is released following hematopoietic cell death, and shedding of cfDNA may vary depending on processes such as infection, intense exercise or body mass index. In addition, the half-life of cfDNA is very short, estimated to be shorter than 2.5 hours, which is why ctDNA assessment is considered a 'real-time' measure of tumor burden.[167] These characteristics of cfDNA lead to the possibility of intrapatient and interpatient variability in plasma ctDNA levels. Also, the relatively low amounts of ctDNA in plasma samples can reduce the sensitivity of ctDNA detection assays. Low ctDNA fractions in small quantities of cfDNA can be difficult to assess and lead to false negative results. Despite recent advances in the development of technologies such as ddPCR or targeted sequencing for the detection of plasma ctDNA, it remains a challenge to quantify very low ctDNA fractions. We observed the best correlation of ctDNA levels with clinical disease status in patients whose plasma contained highest ctDNA fractions, suggesting that changes in ctDNA are measured more accurately when its abundance is higher.

Limitations of our study include the small sample size and the relatively short follow-up for this disease with a prolonged time to recurrence. As AGCT comprises a rare malignancy, studies including a sufficient number of patients are a challenge and require international collaboration. Due to the small size of our cohort, we were unable to make a comparison between primary and recurrent AGCT or determine the value of ctDNA detection as a diagnostic tool. In addition, the prognostic relevance of the presence and abundance of *FOXL2* mutant or *TERT* mutant ctDNA could not be established and remains to be investigated.

In conclusion, *FOXL2* mutant ctDNA is present in the majority of AGCT patients and *TERT* promoter mutant ctDNA has been identified in a smaller subset of patients. Both *FOXL2* and *TERT* mutant ctDNA levels were found to correlate with disease progression and response to treatment in most patients, suggesting the clinical potential of ctDNA as a biomarker in AGCT. Larger collaborative studies are warranted to validate our findings and establish the clinical value of ctDNA testing in AGCT.

CHAPTER 7

Familial occurrence of adult granulosa cell tumors: analysis of whole genome germline variants

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Submitted

Abstract

Objective. Adult granulosa cell tumor (AGCT) is a rare ovarian cancer subtype, with a peak incidence around 50-55 years. Although AGCT can occur in specific syndromes, a genetic predisposition for AGCT has not been identified. The aim of this study is to identify a genetic variant in families with AGCT patients, potentially contributing to tumor evolution.

Methods. We identified four families, each including two women diagnosed with AGCT. Whole genome sequencing was performed to identify overlapping germline variants or affected genes. Familial relationship was evaluated using genealogy and genomic analysis. Patient characteristics, medical (family) history and pedigrees were collected. Findings were compared to a reference group of 33 unrelated AGCT patients.

Results. Mean age at diagnosis was 38 years (range 17-60) versus 51 years in the reference group, and 7 of 8 patients were premenopausal. In two families, three first degree relatives were diagnosed with breast cancer. Furthermore, polycystic ovary syndrome (PCOS) and subfertility was reported in three families. Predicted deleterious variants in *PIK3C2G*, *BMP5* and *LRP2* were identified.

Conclusions. AGCTs occur in families and could potentially be hereditary. In these families, the age of AGCT diagnosis is lower and cases of breast cancer, PCOS and subfertility are present. We could not identify an overlapping genetic variant or affected locus that may explain a genetic predisposition for AGCT.

Background

Ovarian cancer is the fifth leading cause of cancer-related death among women and arises from epithelial, sex-cord stromal or germ cells.[185] Granulosa cell tumors belong to the sex-cord stromal tumors and represent 5% of ovarian cancers, with an estimated incidence of 0.6-1.0 in 100,000 women worldwide per year.[10,59] The tumor is derived from the hormonally active granulosa cells that produce estradiol. Patients may develop symptoms such as vaginal bleeding, caused by prolonged estrogen exposure, or abdominal pain. Occasionally, a granulosa cell tumor is diagnosed in patients presenting with subfertility, potentially as a result of unregulated inhibin secretion by the tumor.[23,186] Although granulosa cell tumors can occur at any age, they mostly present perimenopausal or early in postmenopause, with a median age of diagnosis between 50 and 54 years.[10] Granulosa cell tumors are subdivided into an adult type (95%) and juvenile type (5%) by their histological and molecular characteristics[187], the latter occurring mostly at a younger age.

Germline mutations are involved in the evolution of ovarian cancer. Approximately 10-15% of epithelial ovarian cancer is caused by a germline mutation in the *BRCA1* or *BRCA2* gene.[188,189] In addition, specific hereditary syndromes result in an increased risk for sex cord-stromal tumors. Peutz-Jeghers syndrome is a rare autosomal dominant disease caused by germ line mutations in *STK11/LKB1*. Mutations in this gene are associated with gastrointestinal polyps, pigmentation of lips and ovarian granulosa and Sertoli cell tumors.[190] Also, a germline mutation in *DICER1* can cause a hereditary syndrome that is associated with Sertoli-Leydig cell tumors.[191] Furthermore, Olliers disease and Maffucci syndrome are rare disorders caused by early post-zygotic mutations in *IDH1* and *IDH2* genes and are associated with juvenile granulosa cell tumors.[9]

Adult granulosa cell tumors (AGCTs) harbor a specific somatic *FOXL2* c.402C>G mutation in approximately 95% of cases.[6,41] The C134W protein change caused by this mutation leads to reduced apoptosis, although the mechanism of granulosa cell tumorigenesis has not yet been entirely unraveled.[63,192,193] *FOXL2* is preferentially expressed in the ovary, the eyelids and the pituitary gland. Inactivating germline mutations in this gene do not result in AGCTs but in the autosomal dominant blepharophimosis–ptosis–epicanthus inversus syndrome (BPES) type I.[194] This disease affects the eyelids and is associated with granulosa cell dysfunction and premature ovarian failure.

Despite the fact that rare autosomal disorders are associated with the development of granulosa cell tumors, there is no known genetic predisposition that is specific for AGCT. To date, there has been only one reported case of a family in which both mother and daughter were diagnosed with a granulosa cell tumor.[195] This was seen as a coincidental finding and

DNA analysis was not performed. We identified four different families in which two relatives were diagnosed with a granulosa cell tumor.

In this study, we performed whole genome sequencing on familial AGCT patients to investigate overlapping germline mutations or shared affected genes as a potential cause for AGCT development. Findings were compared to a reference group of 33 unrelated AGCT patients.[111] Identification of an overlapping germline variant or affected genetic locus in these families could help to unravel the pathological mechanism of tumor evolution in AGCT patients.

Methods

We identified four different families that each had two women previously diagnosed with an AGCT. Patients were identified through contact with the national and international granulosa cell tumor patient organization and gynaecological oncologists involved in our national research on granulosa cell tumors.[196] Ethical approval was obtained by the Institutional Review Board of the University Medical Center Utrecht (UMCU METC 17-868). All participants provided written informed consent. Peripheral blood samples (n=6) or saliva swabs (n=2) were collected for germline DNA analysis. DNA isolation and sequencing was performed according to previous described methods.[111] Clinical data was provided by the treating gynaecologists (n=6) or by the patients themselves (n=2) including the age at diagnosis, disease stage, medical history and family history. We retrieved information on the family relations and on the medical history of non-affected family members, in order to build pedigrees. A genealogist traced back family lineages to investigate whether a relationship between the four families exists. In addition, we used TRIBES to assess genetic relatedness within and between families. TRIBES is a pipeline for relatedness detection in genomic data, using the 1000 Genomes European cohort, which can accurately assess genetic relatedness up to 7th degree relatives.[197] Additionally, we used the whole genome sequencing results of germline DNA from 33 unrelated AGCT patients as reference group.[111] We tested for potential relatedness within and between the reference group and the families. Furthermore, we investigated whether clinical characteristics (i.e. age of onset and disease stage) differed between the related AGCT patients and the unrelated reference group and checked whether overlapping germline variants or affected genes present in the germline DNA of the families, were also present in the germline DNA of the reference group.

Whole genome sequencing and variant calling

Whole genome sequencing was performed with 30X coverage on Illumina HiSeq X or NovaSeq 6000 instrument (Illumina, San Diego, CA, USA) by the Hartwig Medical Foundation (HMF, Amsterdam, NL). Genome analysis was performed using the UMC IAP pipeline.[198] Sequence

reads were mapped with Burrows-Wheeler Alignment v0.75a[95] against human reference genome GRCh37. Single nucleotide variants and small insertions and deletions were called with GATK (v3.4.46).[199] The functional effect of these variants were predicted with SnpEff (v.4.1).[98] Structural variants were called using DELLY (0.7.2)[200] and Manta(v0.29.5).[201]

Genome analysis

First, all coding variants shared *within* families were analyzed. Variants with a high population frequency (>1:1000 according to population databases[202–205]) were filtered out, as AGCT constitutes a rare malignancy. We further removed frequent variants and sequencing artefacts using an in-house cancer reference database from the Hartwig Medical Foundation (HMF pool of normal variants V2.0, a resource with 78655034 unique variant calls from 1762 individuals).[206] The exonic (±10 base pairs into the intronic regions) variant analysis was performed using Alissa (Agilent Technologies Alissa Interpret v5.1.7). Furthermore, variants present in the COSMIC Cancer Gene Census (release v89) were annotated and reviewed (Supplemental Table 1). We assessed all variants present in cancer genes in the Human Gene Mutation Database (HGMD® Professional 2020.3) to identify potential inherited disease-causing mutations. Finally, variants genome wide were ranked on their potential pathogenicity by using the Combined Annotation Dependent Depletion (CADD PHRED) score.[101] Variants with a deleteriousness score ≥ 5 were annotated (Supplemental Table 1), and intra-family variants with a score ≥ 20 were assessed.

Secondly, we investigated overlapping genome wide variants and affected genes *between* families. To assess shared variants, we selected variants present in both individuals in at least one family, and all variants shared by ≥ 2 families were evaluated by predicted pathogenicity of the variant and gene function. Additionally, we searched for recurrently affected genes across different families and evaluated them on mutation effect and gene function.

Thirdly, we investigated larger structural variations across samples. Structural variants present in the GNOMAD catalogue[207] and the HMF SV pool of normals were removed prior to analysis. We used in-house scripts based on the R-package StructuralVariantAnnotation to analyze the samples.[208] Shared breakpoints were determined using the function "findBreakpointOverlaps" from StructuralVariantAnnotation with default parameters, and variants with overlapping breakpoints between samples were grouped together. Structural variants present in less than one family were removed. We annotated the remaining structural variants, the genes present within each structural variant region and investigated those genes for known involvement in granulosa cells or cancer development (Supplemental Table 1).

Results

Description of families

We identified four families with two women diagnosed with AGCT, three originating from the Netherlands and one American family. Pedigrees are shown in Figure 1. Family lineages were traced back for at least seven generations. All families (for 7-9 generations) came from different geographical areas and did not have any overlapping family names. Furthermore, their genomic relatedness correlated directly with their actual relatedness based on the pedigrees and there was no close genetic relationship between families (all predicted ≥ 9th degree relatedness according to TRIBES, see Supplemental Table 2). None of the families were linked, indicating families did not share a recent common ancestor.

Family A includes two women with an AGCT, who were fifth-degree relatives as their grandfathers were brothers. The patients were diagnosed with stage IIB and IA AGCT at 35 and 36 years of age, respectively. The medical history revealed congenital clubfoot in the first patient and subfertility associated with polycystic ovary syndrome in the other patient (Table 1). Family B contains two first-degree relatives, mother and daughter, both diagnosed with AGCT at the age of 39 and 46, respectively. The mother underwent surgery and radiotherapy for stage IC disease and the daughter was treated with surgery only for stage IC AGCT. The five sisters and three brothers of the affected mother, as well as their offspring (in total 19 children), were all unaffected by any cancer type. Family C includes two second-degree relatives (aunt and niece) diagnosed with stage IA AGCT at 35 and 60 years of age, respectively. The first patient was diagnosed after unsuccessful subfertility treatment, as she suffered from polycystic ovary syndrome. More cases of subfertility were reported in this family. The respective mother and sister of the affected patients was diagnosed with breast cancer when she was 56 years old. Family D consists of two fifth-degree relatives, related via their grandfathers, diagnosed with stage IC AGCT at the age of 17 and 39 years. The mothers of the affected patients had a history of breast cancer and were diagnosed at 38 and 61 years of age. Furthermore, one of the patient's sisters was unable to conceive. All patients were successfully treated with surgery (n=5), with surgery and radiotherapy (n=1) or with surgery and chemotherapy (n=2). None of the patients developed a recurrence during a median follow up time of 6 years (range 4-38 years). In summary, polycystic ovary syndrome (PCOS) and subfertility was reported in three families and three first degree relatives had a history of breast cancer.

Table 1. Patient characteristics

Patient	Age at diagnosis	Tumor stage	Treatment**	Medical history	Family history
A1	35	IIB*	Surgery	Clubfoot	
A2	36	IA	Surgery	Polycystic ovary syndrome, 2x vaginal delivery after in vitro fertilization	
B1	46	IC	Surgery		
B2	39	Unknown	Surgery and radiotherapy		
C1	35	IA	Surgery	Polycystic ovary syndrome, subfertility	Breast cancer, PCOS, subfertility
C2	60	IA	Surgery		
D1	17	IC	Surgery and chemotherapy		Breast cancer, subfertility
D2	39	IC	Surgery and chemotherapy		

* location dorsal from uterus
** no recurrences occurred and all patients are currently alive with no evidence of disease.

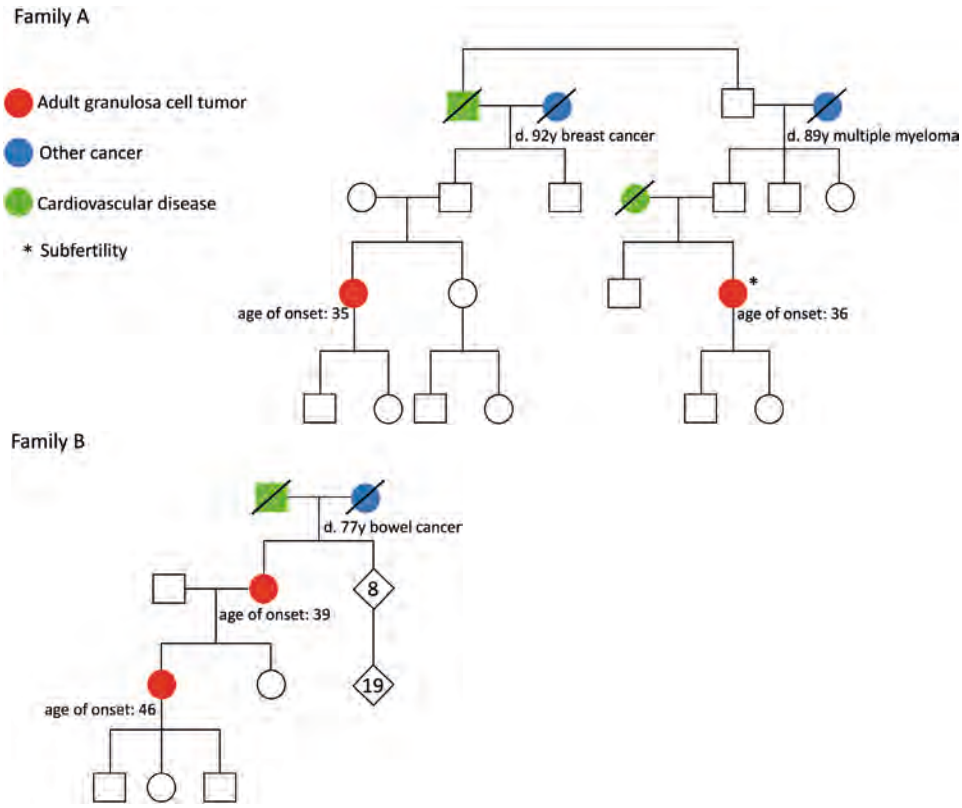

Figure 1. (continues next page)

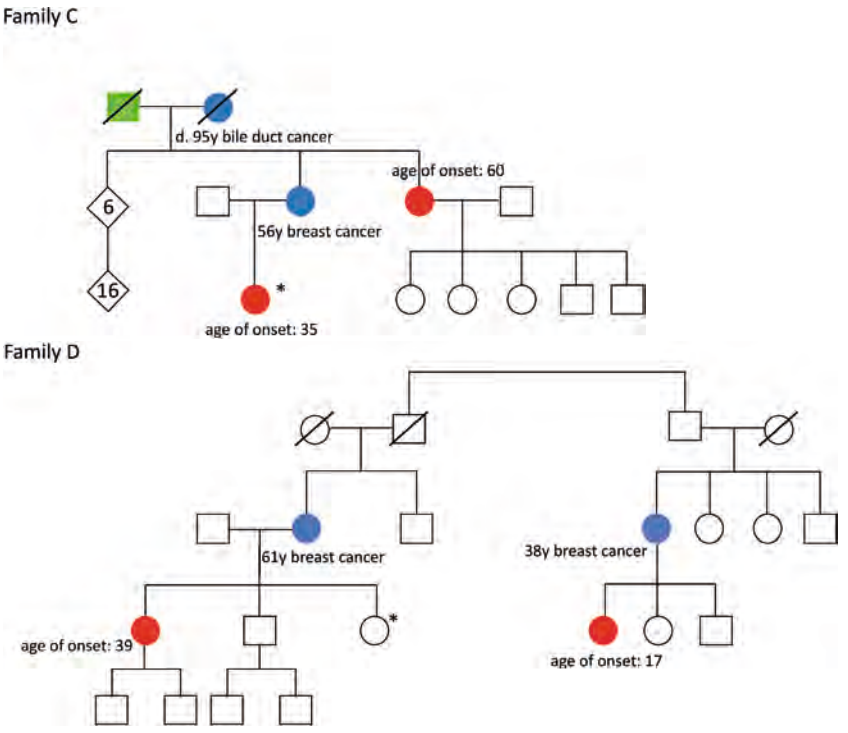

Figure 1. Family pedigrees. Colors represent diagnosis of AGCT, other cancer or cardiovascular disease. Deceased persons are indicated with a slash (/).Family A. Patients are related via their grandfathers. Family B. The AGCT patients represent mother and daughter. Family C. The AGCT patients represent niece and aunt. Their mother, respectively sister was diagnosed with breast cancer. Patient C2 is involuntarily childless because of subfertility and more cases of subfertility are reported in this family. Family D. The AGCT patients are related through their grandfathers, who were brothers. Both mothers of the patients were diagnosed with breast cancer. One of the patient's sister is also involuntarily childless due to subfertility.

Exome analysis

Within the families, 26 to 202 shared coding germline variants (exonic +/-10 base pairs) were detected (Figure 2). Exonic variants passing quality filters that resulted in a frameshift, stop/start loss, or a nonsynonymous variant predicted to be pathogenic from 3/5 pathogenicity algorithms were retained (n = 93) (Supplemental Table 1). Candidate genes affected by these variants in individual families are listed in Table 2. Furthermore, we detected 143 variants in 66 cancer associated genes, including *BMP5*, with the highest predicted deleteriousness score of 22.7, *MET* and *USP44* (Supplemental Table 1). None of the detected variants in cancer genes were officially classified as inherited germline disease-causing mutations (HGMD® Professional 2020.3).

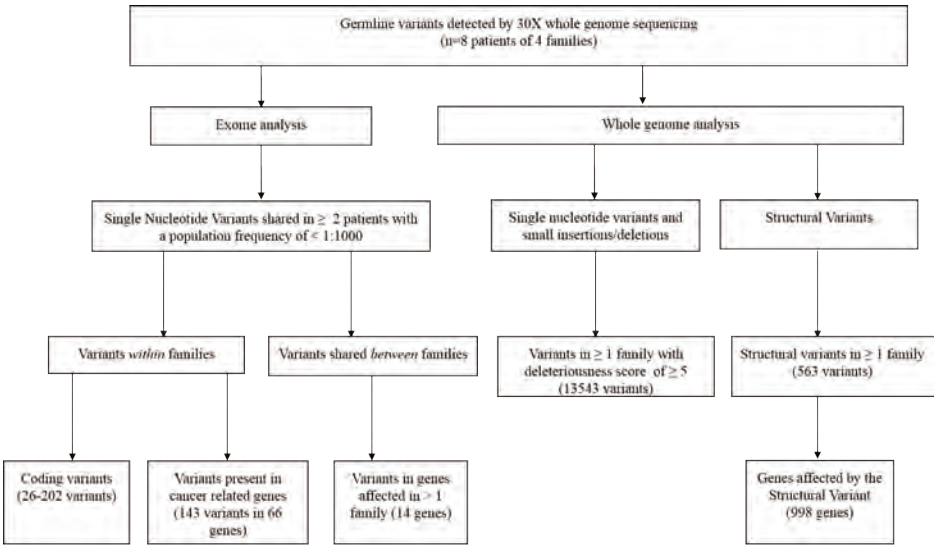

Figure 2. Filter approach.

Subsequently, we investigated genes that were recurrently hit in at least two families, which could point to a molecular mechanism of pathogenesis for AGCT. Across all coding genes, we identified fourteen genes that were affected by identical or different single nucleotide variants in the same gene amongst at least two families (Supplemental Table 1). No genes remained after filtering out variants present in low quality or intronic regions and genes that commonly have false-positive variant calls from next generation sequencing.[209]

Whole genome analysis

Genome wide, we found 13543 single nucleotide variants present in at least 1 family with a CADD PHRED deleteriousness score of ≥ 5 (Figure 2 and Supplemental Table 1). Variants with the highest deleteriousness score (CADD PHRED > 30) included frameshift variants predicted to result in loss of function of *PIK3C2G* (p.Asn1129Thrfs*27, rs1038560744), *KLHL33* (rs747485435) and *MYH1* (rs545765873), and three intergenic variants. Furthermore, 52 single nucleotide variants were shared by at least two families. Two single nucleotide variants were present in three families, of which one could not be validated by PCR and was classified as a sequencing artefact (rs1306359244, chr12:103223803, near *IGF-1*). The other variant (rs781644268, chr12:53833990) was an intergenic variant downstream of *AMHR2* and upstream of *PRR13* and positioned at a long T stretch.

Table 2. Detected overlapping germline variants per family.

Family	Gene	Effect	Variant (cDNA)	Variant (protein)
A	**HTRA4**	Nonsynonymous	c.1009G>C	p.V337L
	LRP2	Nonsynonymous	c.2688C>G	p.H896Q
	PCSK9	Nonsynonymous	c.479G>A	p.R160Q
B	**BMP5**	Nonsynonymous	c.1291T>C	p.Y431H
	CRLF2	Frameshift	c.496_497delGT	p.N166Yfs*111
	FSCN3	Nonsynonymous	c.212G>A	p.G71D
	HFM1	Nonsynonymous	c.4283T>C	p.L1428S
	MET	Nonsynonymous	c.3409G>A	p.G1137R
	NOX5	Nonsynonymous	c.1600G>C	p.G534R
	SPTBN5	Nonsynonymous	c.10672T>C	p.W3558R
	TEAD2	Frameshift	c.1286_1287delAT	p.Y429Cfs*55
C	**CBX8**	Nonsynonymous	c.916C>T	p.R306W
	HYDIN	Frameshift	c.6584_6585ins59	p.P2196Ifs*17
	IGSF1	Nonsynonymous	c.709C>G	p.P237A
	LAMA3	Nonsynonymous	c.701T>A	p.I234K
	PSMD5	Nonsynonymous	c.820G>A	p.V274M
	PXDN	Nonsynonymous	c.3464C>A	p.A1155E
	TBP	Frameshift	c.231_234delGCAGinsCAG	p.Q77Hfs*67
D	**USP44**	Nonsynonymous	c.1250G>A	p.R417H
	RASSF2*	Nonsynonymous	c.389T>A	p.L130Q

Reported variants include exonic variants passing quality filters that resulting in a frameshift, stop/start loss, or a nonsynonymous variant predicted to be pathogenic from 3/5 pathogenicity algorithms and candidate genes affected in two families. *Variant does not meet inclusion criteria but is reported as the gene is a tumor suppressor and the variant is rare and was predicted damaging in 2/5 algorithms.

A total of 563 structural variants were identified that were shared within at least one family, including 72 structural variants shared by two different families (Figure 2 and Supplemental Table 1). These structural variants collectively contained 998 individual genes that were affected by these variants. We assessed the genes that were affected in at least two families (n = 104; Supplemental Table 1). No gene was affected in more than two families.

AGCTs have characteristic somatic copy number gains and losses that are present in a large proportion of tumors, specifically gain of chromosome 14 and concurrent loss of chromosome 22.[111] It is unclear whether these structural variants facilitate tumor development, or if also germline copy number variation could contribute to AGCT evolution. We did not identify germline duplications on chromosome 14 or loss on chromosome 22 affecting multiple families.

Variants in genes associated with sex cord-stromal tumor or hereditary ovarian cancer
Genes associated with the development of sex cord-stromal tumors or hereditary ovarian cancer were specifically investigated. No rare (< 1:1000) germline single nucleotide or structural variants were identified within 75 kB of *FOXL2, STK11/LKB1, IDH1/2, DICER1* or for *BRCA2*. A noncoding mutation shared by 5 individuals across three families (rs12938971) was identified 22 kb downstream of the coding region of *BRCA1,* however this variant was present in a polyG stretch in a low complexity region and therefore likely to be a sequencing artefact.

Comparison with unrelated AGCT patients
We compared the clinical characteristics and germline variants of the patients from the four families to a reference group of 33 unrelated AGCT patients. The mean age of the familial patients at diagnosis was 38 years (range 17-60), significantly lower than the reference group (mean age at diagnosis 51 years, range 29-75, T-test p-value = 0.016), and 7 of the 8 women were premenopausal at time of diagnosis. Although one of the patients had metastatic disease at diagnosis (12.5% vs 3% in the reference group), none of the patients developed a recurrence. The genomic relatedness within the unrelated AGCT patient cohort was predicted to be at least 7th degree and between the families and the unrelated patients at least 8th degree (Supplemental Table 2), indicating no relationship between these patients.

Furthermore, we investigated if recurrent or predicted pathogenic single nucleotide variants and structural variants were present in a reference group of unrelated AGCT patients for whom whole genome sequencing had been performed on germline DNA derived from blood or saliva.[111] Of the 52 single nucleotide variants shared by at least two families in the present study, 26 variants were present in the reference group and eight variants present at least two times in the reference group. No variant was in a coding area of the genome, and manual inspection of the next generation sequencing reads of the one variant (rs1185417161) seen seven times in our reference AGCT group revealed reads with many mismatches and most likely mapped to the genome incorrectly. Finally, no structural variants present in this study were identified in the reference group. None of the patients in the reference group had a *PIK3C2G* variant and one patient had a *BMP5* variant, although it was not predicted to be pathogenic. A predicted damaging *LRP2* p. His896Gln variant was identified in family A. Homozygous pathogenic variants in *LRP2* result in Donnai-Barrow syndrome[210], and heterozygous loss-of-function variants are not well tolerated in the general population.[211] Splice variant *LRP2* somatic mutations have also been identified previously in AGCTs.[45] We identified thirteen exonic nonsynonymous *LRP2* variants in the reference group, of which two were predicted to be pathogenic by 3/5 prediction algorithms (Supplemental Table 1). One of these variants was identified only once (rs760331558) and the other (rs61995913) was present at an allele frequency of 0.004. Therefore, no specific mutations were shared, although predicted pathogenic variants in *LRP2* were found in both related patients and the reference group.

Discussion

This study describes the familial occurrence of adult granulosa cell tumors of the ovary. For the first time, the germline DNA of familial AGCT patients was investigated, with the aim to identify an overlapping genetic variant or affected gene that could have contributed to tumor evolvement. The genomic analyses covered both small single nucleotide variants, insertions and deletions, and larger structural variation. We focused on both variation that was shared between families as well as predicted damaging variants present in one family.

Although we did not find a genetic variant that was shared within all four families, we did identify variants in genes that were predicted to be damaging. Of these genes, *LRP2* (variant in family A) is involved in lysosomal regulation of lipid metabolism.[212–214] Additionally, a nonsynonymous coding variant in *LRP1* was detected in family C. Although this variant was not predicted to be damaging, *LRP1* is also involved in lipid metabolism.[214] Previous studies have shown that the lipid metabolism in granulosa cells plays a vital role during follicular development and is indispensable for oocyte maturation.[215–217] In addition, *LRP2* expression is induced by the peroxisome proliferator-activated receptor-gamma (PPARγ), a key transcriptional factor regulating lipid metabolism which is widely expressed in granulosa cells.[218] PPARγ activation, combined with inhibition of the X-linked inhibitor of apoptosis protein (XIAP), has been suggested as a novel therapeutic strategy in AGCTs.[130]

Furthermore, we found a predicted damaging *PIK3C2G* variant in family C (deleteriousness score: 33*,* predicted to result in nonsense mediated decay*). PIK3C2G* is involved in cell signaling pathways that regulate cell proliferation, survival and oncogenic transformation, and is altered in 0.58% of all cancers.[8] An analysis of 4,034 cases from The Cancer Genome Atlas identified germline truncating mutations in 34 genes, including *PIK3C2G.* Germline *PIK3C2G* truncating variants were associated with cancer predisposition, specifically with ovarian cancer.[219]

We also found a very rare predicted damaging heterozygous variant in *BMP5* in family B. Bone morphogenetic proteins (BMPs) play an important role in embryonic and postnatal development by regulating cell differentiation, proliferation and survival, thus maintaining homeostasis during organ and tissue development.[220] BMPs can serve as either oncogenes or tumor suppressors, leading to tumorigenesis and regulating cancer progression.[221] The BMPs are cytokines belonging to the Transforming Growth Factor (TGF)-β superfamily, which also include TGF-βs, activin, inhibin, nodal, and myostatin.[220] BMPs activate SMAD pathways, phosphatidylinositol 3-kinase (PI3K)/AKT, mitogen-activated protein kinase (MAPK), nuclear factor kappa B (NF-κB), and Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathways. A recent study found that TGF-β signaling enhances the

effect of mutant *FOXL2* (c.402C>G) and BMP stimulation (via signaling through *SMAD1*, *SMAD5* and *SMAD8*) seems to counteract this effect. Moreover, conditional double deletion of *SMAD1* and *SMAD5* or triple deletion of *SMAD1, SMAD5, SMAD8* led to infertility and granulosa cell tumor development in mice.[222] *BMP5* somatic missense mutations are present in 7.7% of colorectal cases and reduction of BMP5 through loss of function or damaging nonsynonymous variants has been linked to disease progression in cancer.[223–225] Targeting BMPs and their receptors were successful in preventing tumor growth and invasion in preclinical and clinical cancer studies.[226]

It is not yet known whether a genetic cause for AGCT exists in these patients, since we could not identify a shared variant or affected locus between the families that could be linked to granulosa cell tumor development. Additionally, the families included many unaffected females, indicating that if an autosomal dominant causal variant is present, it could be a variant with incomplete penetrance. Reduced penetrance may result from a combination of genetic, environmental, and lifestyle factors. This phenomenon can make it challenging for genetic professionals to interpret an individual's family medical history and predict the risk of passing on a genetic condition. However, affected family members from both family A and D are fifth-degree relatives and their environmental and lifestyle factors are therefore likely to differ. Another possibility is a familial detrimental variant in a tumor suppressor gene or a gene involved in granulosa cell proliferation, in which another somatically acquired variant is needed to knock out or reduce function of the gene and initiate tumorigenesis or facilitate tumor development.

However, the identification of four different families, of which three families originate from a country with only 15-20 new cases per year, including first-, second- or fifth-degree relatives with a very rare gynaecological tumor makes a genetic germline contribution plausible. The fact that the age of onset in the familial cases was significantly lower than in incidental AGCTs, with the youngest patient being diagnosed at the age of 17 years, strengthens this hypothesis as a disease caused by a germline variant usually manifests at a younger age.

Besides the occurrence of granulosa cell tumors, cases of breast cancer were found in first-degree relatives of the AGCT patients in two families. In these patients, the median age at diagnosis was lower (51.7 years) than the average age at breast cancer diagnosis in the general population (62 years).[227] A potential link between AGCTs and breast cancer has previously been suggested. A recent study including 1908 AGCT cases found a higher observed (n=79, 4.14%) than expected number of breast cancer cases (n=27, 1.41%).[228] Moreover, other studies report a breast cancer rate of 5-10% among AGCT patients.[229–231] Furthermore, two AGCT patients were subfertile and diagnosed with polycystic ovary syndrome. Several studies reported a possible connection of granulosa cell tumors to

subfertility, with improved fertility after surgical removal of the tumor.[232,233] Granulosa cell tumors at the fertile age were associated with nulliparity and with a clinical presentation of anovulatory infertility, while AGCTs later in life were associated with a normal average fertility pattern. In our study, breast cancer and subfertility were also present in non-affected family members. The co-occurrence of granulosa cell tumors, breast cancer and/or subfertility in these families may indicate a shared etiology, for example a genetic predisposition.

It is known that trying to resolve a familial disease can be a daunting task. For example, in familial high grade ovarian carcinoma, the hereditary basis of approximately 50% of cases is still unexplained.[234] A recent study on suggested familial BRCA1/2 wildtype high grade ovarian cancers found that only 6.6% of cases could potentially be explained by genes known or suggested to be linked with a higher risk of ovarian cancer.[235] This study reported a high number of individual rare loss of function variants, suggesting that this could be genuine predisposing variants, which is in agreement with our findings (rare loss of function variants in *PIK3C2G*, *KLHL33* and *MYH1*).

The ongoing advances in the field of whole genome sequencing data analysis may lead to novel insights and may resolve unexplained familial cancer cases. Nonetheless, the identification of a causal germline variant may not have direct clinical implications as, due to the rarity of the disease, genetic testing for AGCT patients or their family members in general may not be necessary. Moreover, the identification of four families and one previous reported family indicates that the vast majority of cases are sporadic rather than familial. However, identification of a genetic predisposing factor could help to unravel the pathological mechanism of AGCTs.

Conclusion

Adult granulosa cell tumors can occur in familial clusters and could potentially be hereditary. We did not identify an overlapping genetic variant or affected genetic locus that may explain a genetic predisposition for AGCT in the four investigated families. In these families, the age of AGCT diagnosis is lower than in unrelated cases and breast cancer, PCOS and subfertility are reported in these families, suggesting potential shared etiologic factors.

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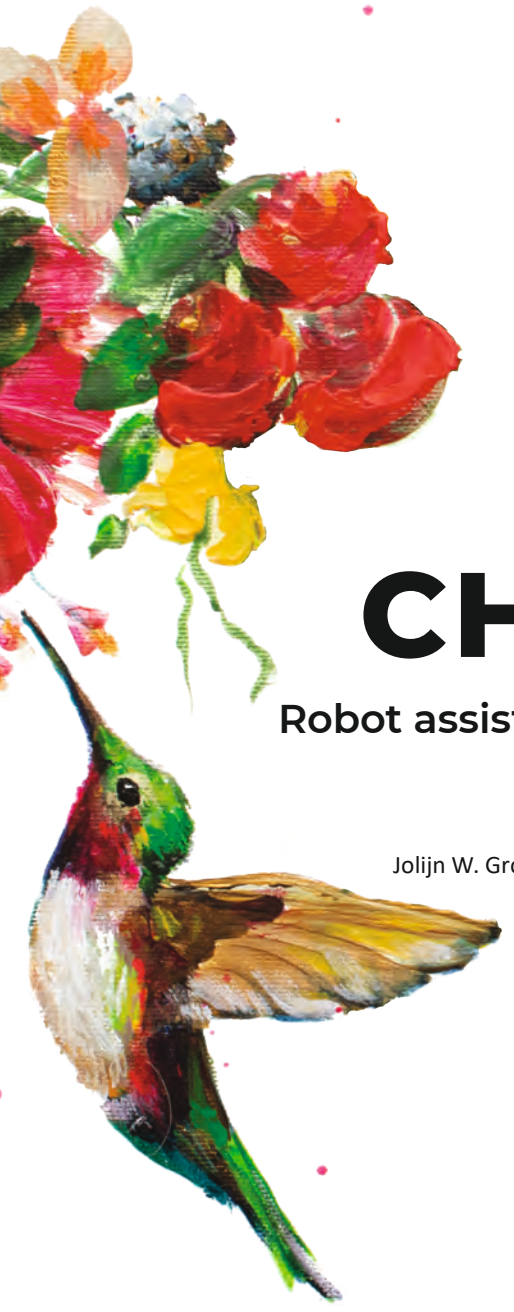

CHAPTER 8

Robot assisted debulking surgery in adult granulosa cell tumors

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Submitted

Abstract

Study Objective: Despite an often early diagnosis and effective initial surgical management, half of adult granulosa cell tumors (AGCTs) eventually, and often repeatedly, recurs. Debulking surgery remains the preferred treatment modality for recurrent AGCT, although the risk of intraoperative complications increases with repeated laparotomy. Minimally invasive surgery may limit the risk of complications. We aim to share our initial experience with robotic debulking surgery for recurrent AGCT.

Methods: Clinical and surgical data of patients with recurrent AGCT who underwent robotic cytoreductive surgery over a three year period at a tertiary referral center were retrospectively collected and analyzed.

Results: Between June 2017 and July 2020, three patients underwent robotic debulking surgery for recurrent AGCT at our institution. Complete cytoreduction was achieved in all patients. No intraoperative or postoperative complications were reported.

Conclusion: This small pilot series at a single academic institution suggests that robot-assisted laparoscopy may be feasible and safe in selected patients with recurrent AGCT. A minimally invasive approach could reduce the complexity of successive surgeries for AGCT relapse.

Introduction

Granulosa cell tumors (GCTs) of the ovary represent a rare subtype of ovarian cancer, belonging to the subgroup of sex cord-stromal cell tumors and accounting for approximately 3-5% of all ovarian malignancies. The vast majority of GCTs is of the adult type (AGCT), while 5% is of the juvenile type. Although AGCTs are generally described as tumors with an indolent behavior that are often diagnosed at an early stage with a favorable prognosis, up to 50% of AGCT patients eventually develops a disease relapse leading to death in 50-80% of recurrences.[7,19,163] Surgery is the mainstay of treatment for both primary and recurrent AGCT.[236] Only limited effectiveness of alternative treatment modalities such as chemotherapy and hormonal treatment has been described.[29,30] Despite optimal surgical debulking of relapsed AGCT, multiple recurrences are commonly seen. Repeated debulking surgeries are therefore often necessary in this setting, with increased risk of intraoperative complications with every laparotomy.

In recent years, minimally invasive surgery is increasingly used in the treatment of ovarian cancer. Advantages of this surgical approach over laparotomy have been widely described and include smaller incisions, reduced blood loss, improved intraoperative visualization, shorter hospitalization, faster recovery and a lower risk of formation of adhesions.[237,238] Laparoscopy and robot-assisted laparoscopy were found to be feasible and safe surgical routes in selected patients with primary or recurrent epithelial ovarian cancer, in terms of surgical and oncologic outcomes.[239–241]

The advantages of a minimally invasive surgical route could be particularly meaningful for the management of AGCT, when multiple surgeries may be needed for relapsed disease. In the primary treatment of AGCT, laparoscopy was shown to be an accurate approach for both initial surgery and re-staging, with comparable oncologic outcomes when compared with laparotomy.[242,243] In addition, a recent case report showed the use of laparoscopy for tertiary cytoreductive surgery in recurrent AGCT.[244] The use of robot-assisted laparoscopy for the surgical treatment of AGCT has not yet been reported. With this small series from a single institution, we aim to share our initial, positive experience with robotic surgery for the treatment of recurrent AGCT.

Methods

Patients who underwent robotic cytoreductive surgery for a recurrent AGCT in our institution between June 2017 and July 2020 were retrospectively analyzed. All patients had given written informed consent for their data to be used for study purposes, with approval of our institutional review board.

All surgeries were performed by two gynecologic oncologists (RZ and CG) experienced in robot-assisted laparoscopy, in some cases accompanied by a gastro-intestinal surgeon specialized in robotic surgery (JR). The da Vinci Surgical System (Intuitive Surgical Inc., Sunnyvale, CA, USA) was used, the da Vinci Si for the first case and the da Vinci Xi for the second and third case. For all procedures, patients were placed in lithotomy position. Following a small incision just below or just above the umbilicus, depending on the upper or lower intra-abdominal locations of the intended procedure, a pneumoperitoneum of 24 mmHg was created using a Veress needle. The camera port was placed, followed by placement of three robotic ports and one laparoscopic port for the assistant, all in one line at the level of the camera port. In one case, an additional suprapubic assistant port was placed. After lowering the intra-abdominal pressure to 14 mmHg and routine inspection of the peritoneal cavity, the da Vinci robot was docked and surgical instruments were introduced with the patient in 28° Trendelenburg position.

Patient characteristics and operative outcomes were collected from medical records. The clinical parameters collected for each patient included: age at time of surgery, body mass index (BMI), history of smoking, American Society of Anesthesiologists (ASA) classification, previous abdominal surgery, initial AGCT stage, years after initial diagnosis, previous treatment for AGCT, recurrence number, inhibin B level, tumor locations as seen on CT or MRI scan, number of lesions and size of largest lesion. The studied perioperative characteristics included the performed surgical procedure, operative (cutting) time, estimated blood loss, need for blood transfusion, conversion to laparotomy, cytoreduction status, length of hospital stay, complications and readmission. The length of hospital stay was counted from the day of surgery. Complications were registered using the Clavien-Dindo classification of postoperative complications.

Results

Between June 2017 and July 2020, ten patients underwent debulking surgery for recurrent AGCT at our institution, of which three patients were treated by robot-assisted laparoscopy. They were estimated to be good candidates for robotic debulking surgery based on preoperative CT or MRI findings. All three patients were treated for their first recurrence (Table 1). The age at time of surgery ranged from 51 to 74 years. The first patient had a unifocal recurrence in the pelvis, for which a robot-assisted laparoscopic resection of the tumor was performed (Table 2). The other two patients were found to have multifocal peritoneal disease. In the second patient, preoperative CT imaging showed two peritoneal deposits on the spleen and one deposit on the mesocolon. In the third patient, a preoperative MRI scan showed peritoneal deposits in Douglas and left paracolic gutter as well as in Morison´s pouch. Their robotic cytoreductive surgery included a hysterectomy with unilateral salpingo-oophorectomy and selective peritonectomy to remove the peritoneal deposits (Figure 1).

Table 1. Clinical characteristics of study patients.

	Patient 1	Patient 2	Patient 3
Age (years)	74	51	58
BMI (kg/m²)	36	23	25
Smoking	No	No	No
ASA classification	3	2	2
Previous abdominal surgery	Vaginal hysterectomy with BSO	Laparoscopic USO	Laparoscopic USO Two caesarean sections
Initial AGCT stage	IA	IC1	IC1
Years after initial diagnosis	6	5	7
Previous treatment for AGCT	Surgery	Surgery	Surgery
Recurrence #	1	1	1
Inhibin B level (ng/L)	86	129	144
Tumor locations	Pelvis left	Peritoneum of spleen, mesocolon	Peritoneal deposits
Number of lesions	1	>5	>5
Size of largest lesion	89 mm	43 mm	30 mm

ASA: American Society of Anesthesiologists; BSO: bilateral salpingo-oophorectomy; USO: unilateral salpingo-oophorectomy.

Table 2. Operative outcomes. USO: unilateral salpingo-oophorectomy.

	Patient 1	Patient 2	Patient 3
Procedure	Resection of pelvic tumor	Hysterectomy, USO, selective peritonectomy	Hysterectomy, USO, selective peritonectomy, partial omentectomy
Operative time	99 min	231 min	162 min
Estimated blood loss	100 ml	400 ml	50 ml
Blood transfusion	No	No	No
Conversion to laparotomy	No	No	No
Cytoreduction status	Complete	Complete	Complete
Length of hospital stay	9 days	3 days	3 days
Complications	None	None	None
Readmission	No	No	No

Macroscopically complete cytoreduction was achieved in all three patients. The operative time, defined as the time between the first incision and final closure, was 99 minutes for the first case, 231 minutes for the second case and 162 minutes for the third case. The first patient had a hospital stay of 9 days due to social circumstances unrelated to her surgery, and the second and third patient had a hospital stay of 3 days. No intraoperative or postoperative complications occurred. The first patient had no signs of disease at her most recent follow-up, three years after the robotic debulking surgery. The second patient had her most recent follow-up 8 months after her surgery, and was then found to be in good clinical condition but with a mildly increased inhibin B level (98 ng/L to 133 ng/L). The third patient has only had a three-month follow-up since her surgery, when there were no signs of disease despite a continued elevated inhibin B (144 ng/L before surgery and 133 ng/L after surgery).

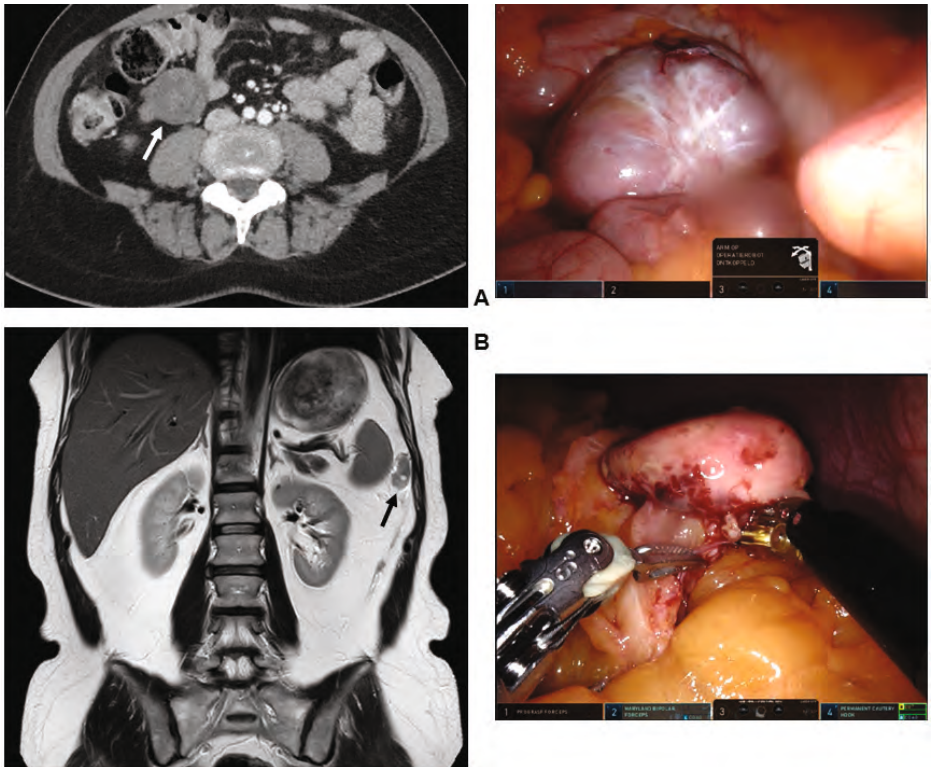

Figure 1. Imaging of recurrent AGCT lesions and correlating image of the robotic surgery. A: Patient 2. The deposit on the mesocolon is shown on CT imaging (left) and during surgery (right). B: Patient 3. A paracolic peritoneal deposit is shown on MRI imaging (left) and during surgery (right).

Discussion

With this small pilot series, we describe the use of robot-assisted laparoscopy in recurrent AGCT. In all three cases, a first unifocal or multifocal recurrence of AGCT could be completely removed by robotic cytoreductive surgery. No intraoperative or postoperative complications occurred. These findings suggest that in selected patients, robot-assisted laparoscopy may be a safe and effective surgical approach in recurrent AGCT.

Only limited evidence exists regarding the role of laparoscopy or robot-assisted laparoscopy in the surgical treatment of AGCT. One retrospective study found no differences in oncologic outcomes between laparoscopy and laparotomy in the initial treatment of patients with stage I AGCT, after a median follow-up of 81 months.[243] A second retrospective analysis demonstrated the feasibility of laparoscopic re-staging in patients with incompletely staged AGCT.[242] Finally, a recent video article showed the successful use of laparoscopy for cytoreductive surgery in AGCT recurrence.[244] In epithelial ovarian cancer, the role of

minimally invasive surgery has been assessed by multiple observational studies. Available evidence on the use of laparoscopic or robotic staging for early stage ovarian cancer suggests that minimally invasive staging procedures are feasible and do not compromise oncologic outcomes.[245,246] In patients with advanced stage epithelial ovarian cancer, interval cytoreductive surgery by laparoscopy or robot-assisted laparoscopy was found to be adequate in selected patients.[247–250] The MISSION trial, a phase II multicenter study, reported the feasibility and safety of minimally invasive interval debulking surgery in patients with a clinically complete response to neoadjuvant chemotherapy.[247] Furthermore, the International MISSION study demonstrated the benefits of a minimally invasive approach when interval surgery is limited to low-complexity standard cytoreductive procedures.[250] An international, randomized, multicenter phase III trial will be conducted to compare minimally invasive interval debulking surgery with laparotomy in patients who had a complete or partial response to neoadjuvant chemotherapy.[251] In addition to its use for primary treatment, other studies have shown favorable perioperative outcomes and similar survival rates when using a minimally invasive approach for secondary cytoreductive surgery in recurrent epithelial ovarian cancer.[241,252,253] In line with these previous studies in both AGCT and epithelial ovarian cancer, our series of three patients with recurrent AGCT surgically treated by robot-assisted laparoscopy suggests that this approach is safe and adequate in selected cases.

Our findings are particularly relevant in AGCT, where surgery represents the mainstay of treatment for both primary and recurrent disease. Minimally invasive surgery offers advantages such as less blood loss, shorter hospitalization and faster recovery when compared with open surgery. When repeated abdominal surgeries are needed, which is not uncommon in the setting of a recurrent AGCT, a minimally invasive surgical route could potentially reduce the risk of intraoperative complications with subsequent surgeries. Prior laparoscopy was previously shown to significantly reduce the formation of anterior abdominal wall adhesions when compared with prior laparotomy.[237,238,254] In addition, significantly fewer unfavorable incidents during subsequent laparoscopy were reported in patients who had previous laparoscopic colorectal cancer surgery, compared with patients who had previous open surgery.[255]

The present study is the first to report the use of robot-assisted laparoscopy for recurrent AGCT, as demonstrated by an experienced surgical team. The importance of experience in robotic surgery and its influence on oncologic outcomes in cervical cancer has recently been reported by our group.[256] However, important limitations of the current study include the small size of our series, its retrospective nature and relatively short follow-up. Further collaborative research is warranted to confirm our findings supporting the use of robot-assisted laparoscopy in selected patients with recurrent AGCT.

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CHAPTER 9

General Discussion

Over the past decade, the extensive genomic investigation of cancers has identified novel targets for treatment and has shown the remarkable complexity of cancer. Next-generation sequencing has transformed rapidly from a research tool to a diagnostic tool used in clinical practice and patient management. As a result, genotype-guided therapy is becoming a reality in cancers such as breast cancer where the treatment choice is now based on molecular diagnostic tests that can identify therapeutic targets. Due to the increased availability of whole genome sequencing and its decreasing costs, large pan-cancer studies have been performed. These studies have created valuable resources and identified common mutational patterns and driver mutations in cancer.[89,92]

The aim of the research presented in this thesis is to unravel the molecular features of granulosa cell tumors and to make a step towards precision medicine. Therefore, we attempted to link tumor characteristics to potentially effective treatment strategies and studied novel tools for monitoring disease. Table 1 shows the contribution of this research to granulosa cell tumor management. It will be further discussed whether and how these results may lead to a more personalized treatment for patients in the future.

Table 1. What is needed and what is new in granulosa cell tumor management

Clinical challenge	Findings and possible solutions proposed from this thesis
Unraveling tumor characteristics	This research has described frequent mutated genes and copy number changes, detected tumor heterogeneity, defined a high grade *TP53* specific subgroup, confirmed that the absence of the *FOXL2* mutation does not exclude granulosa cell tumor diagnosis and detected and described familial occurrence of granulosa cell tumors.
Disease recognition	Active patient engagement and national collaborative research have created awareness of the disease among gynecologists and have led to centralized knowledge and a center of expertise.
Disease monitoring	Circulating tumor DNA harboring *FOXL2* or *TERT* promoter mutations can be detected in plasma of granulosa cell tumor patients and can aid in monitoring disease.
Timing of treatment	FDG tumor uptake is limited and FDG-PET/CT is therefore not routinely advised in monitoring granulosa cell tumors.
Treatment of recurrence	Tumor characteristics can be linked to treatment effects (see Table 2). FES uptake on PET/CT may reflect estrogen receptor expression and thus assess the potential eligibility for hormone treatment. Robot-assisted laparoscopy can safely be performed as minimally invasive approach in selected patients with recurrent disease. Patient-specific granulosa cell tumor drug screens are feasible and can be used to test individual response to existing monotherapies and novel combinations. Alpelisib, carboplatin and paclitaxel may be a promising novel combination for treatment of recurrent disease.

Unraveling tumor characteristics

About 10-15% of ovarian cancers are caused by a hereditary germline mutation
An estimated 5–10% of all cancers are hereditary and more than 300 different inherited cancer syndromes exist.[257] One well-studied example includes hereditary breast cancer and ovarian cancer which is usually caused by a germline mutation in the *BRCA1* or *BRCA2* gene. Approximately 10-15% of epithelial ovarian cancer is caused by a *BRCA1*/2 mutation and the lifetime risk of developing ovarian cancer is 39% for *BRCA1* and 16% for *BRCA2* mutation carriers.[188,189,258] Patients with hereditary breast and ovarian cancer are characterized by an earlier age of onset of cancer, advanced disease and a family history of cancers, as compared to sporadic cases of breast or ovarian cancer.[257] *BRCA1/2* mutations are not associated with granulosa cell tumor development.

Granulosa cell tumors can occur in families and may be associated with breast cancer
Granulosa cell tumors can be part of a few rare hereditary syndromes. Peutz-Jeghers syndrome, an autosomal dominant disease caused by germline mutations in *STK11*/LKB1, is associated with gastrointestinal polyps, pigmentation of lips and ovarian granulosa and Sertoli cell tumors.[190] Furthermore, Olliers disease and Maffucci syndrome are characterized by multiple central cartilaginous tumors that are accompanied by soft tissue hemangiomas in Maffucci syndrome.[9] These syndromes are caused by mutations in *IDH1* or *IDH2* and are associated with juvenile granulosa cell tumors. One case has been reported of an adult granulosa cell tumor in two first-degree relatives, which was seen as a coincidental finding.[195] Although syndromes associated with sex cord-stromal tumors exist, a known inherited predisposition for the development of granulosa cell tumors has not yet been detected.

In a research project driven by patients, who noticed multiple cases of granulosa cell tumors in their family, we investigated the germline DNA of familial patients with a granulosa cell tumor. In agreement with the findings in hereditary cancers, we detected in these patients a significantly earlier age of onset. Furthermore, breast cancer, polycystic ovary syndrome (PCOS) and subfertility were seen in these families. Despite the fact that we could not identify an overlapping germline variant or affected gene that may have contributed to the evolution of granulosa cell tumors in these families, a hereditary low-penetrant predisposition may play a role in some cases. The role of the possible excess of breast cancer cases and occurrence of PCOS and subfertility in these families warrants further investigation. For patients, it is important to bear in mind that the vast majority of cases is clearly non-hereditary and the detection of familial granulosa cell tumors does not imply the need for genetic testing. However, we should be attentive to identify more cases as finding a genetic factor in familial patients could help us to understand an underlying mechanism of granulosa cell tumor development.

Also, an increased incidence of secondary malignancies has been observed in granulosa cell tumor patients. A large study including 1908 granulosa cell tumors found a higher observed (n=79, 4.14%) than expected (n=27, 1.41%) cases of breast cancer. [228] Several studies reported a breast cancer rate of 5-10% among granulosa cell tumor patients and an increased risk of thyroid and soft tissue cancers and leukemia has been detected.[38,230,231] The suggested increased incidence of secondary malignancies might strengthen the hypothesis of a genetic germline component in some cases of granulosa cell tumor development.

Mechanisms involved in granulosa cell tumor evolution

Although the mechanism of tumor evolution remains unknown, one well-accepted hypothesis is that the degeneration of follicular granulosa cells after oocyte loss and the consequent compensatory rise in the pituitary gonadotrophins LH and FSH may induce irregular granulosa cell proliferation and eventually a granulosa cell tumor.[236] This hypothesis is in agreement with the observation that most tumors occur rapidly after menopause, when oocyte depletion and high levels of gonadotrophins are observed. However, it does not explain situations of granulosa cell tumor development during the reproductive years or before menarche.

Several pathways were suggested to be involved in granulosa cell tumor development, such as TGF-β (including FOXL2, BMP, activin A, inhibin and AMH), Notch and PI3K/AKT.[87,259,260] We identified predicted damaging germline mutations in *BMP5* and *PIK3C2G*, genes involved in these pathways, in familial granulosa cell tumor patients. These pathways are part of a complex signaling network and their exact function in granulosa cell tumor pathogenesis is not fully elucidated. Efforts to clarify granulosa cell tumor evolution are ongoing and are mostly focused on defining the role of *FOXL2,* as approximately 95% of granulosa cell tumors harbor a specific *FOXL2* c.402C>G mutation. A recent study found that the *FOXL2* c.402C>G mutation can act as a gain of function mutation and acquires the ability to bind *SMAD*, forming an aberrant FOXL2/SMAD complex.[261] This complex associates with new sites in the genome, which leads to the expression of genes involved in epithelial-to-mesenchymal transition and induction of various cytokines, oncogenes, and factors involved in stemness. Future research should focus on tumor evolution in both *FOXL2* mutant and *FOXL2* wildtype tumors.

Absence of the FOXL2 mutation does not exclude granulosa cell tumor diagnosis

Although cancer diagnosis based on histopathology is still gold standard, the use of molecular tests is emerging. Several research groups have suggested the standard use of *FOXL2* mutation testing in granulosa cell tumor diagnosis. However, the absence of a *FOXL2* mutation should not prevent classification as a granulosa cell tumor if the clinicopathological features are classic.[43] Conversely, the *FOXL2* mutation has incidentally been found in fibromas (1.6%), thecomas (20%), juvenile granulosa cell tumors (20%), Sertoli-Leydig cell

tumors (13%), granulosa theca cell tumors (50%) and gynandroblastomas (8%).[43] Therefore, the *FOXL2* mutation is highly but not perfectly sensitive nor specific for adult granulosa cell tumors. When the diagnosis is uncertain, histopathological classification can be supported by *FOXL2* mutation testing.

Granulosa cell tumors do not share many targetable mutations

For patients, it is important to have effective treatment alternatives besides surgery as surgical risks increase with each successive surgery and systemic treatment is needed to target disseminating tumor cells. Current systemic treatment options consist of chemotherapy and anti-hormonal therapy, which have thus far shown only a limited effect.[7] In order to find potential novel treatment strategies, the first step is to identify targetable pathways. Efforts to target *FOXL2* have not yet been successful, as a transcription factor is usually difficult to target. Next to *FOXL2*, mutations in the *TERT* promoter region, *KMT2D*, *PIK3CA* and *TP53* have been found in patient subgroups. Of these genes, *PIK3CA* is currently the only targetable gene.[6,7,44,69,80,111] However, our research showed that tumors can gain and lose variants in this pathway over time and that mutations may not be consistently present in all tumor sites, which may influence the efficacy of a targeted treatment. As the tumor mutational status is usually based upon a single tumor biopsy, it remains unclear whether the observed variants are present as tumor drivers in the majority of tumor locations or whether they are passenger mutations.

In future clinical practice, targeted gene panel testing may help to stratify granulosa cell tumors based on their molecular characteristics. For example, detection of a *TP53* or *TERT* promoter mutation may indicate a more aggressive tumor, which could influence the management of tumor relapse. Moreover, gene panel testing may allow patients to receive a specific treatment when their tumor harbors an actionable variant.

Disease recognition

Active patient engagement facilitates research whereas rules and regulations can complicate rare disease research

It can be frightening for patients to be diagnosed with and suffer from a rare and not very well known disease. In our research, active patient engagement has helped to increase knowledge and has guided our research agenda.[56,262] Furthermore, our national collaborative research has created awareness of granulosa cell tumors amongst healthcare professionals which may lead to early disease recognition in the future. The collaboration between physicians of participating centers, researchers and patients has led to centralized knowledge and an established network of communication. The study initiating center was regularly consulted for second opinions and became a center of expertise.

These developments enable more standardized management and rapid implementation of knowledge and clinical breakthroughs.

Over the past decades, the number of requirements for medical research to be initiated and performed have increased due to ethical concerns. Currently, the medical ethical committee reviews research proposals before a study is allowed to start, which is needed to prevent patients from undergoing unethical study procedures. Although this is an important process, the extensiveness and redundancy of current regulations may extremely delay research which is not in the best interest of patients. In our study, for example, each participating center needed individual approval and a separate study contract for the enrollment of only a few patients. This has led to situations in which patients were very willing to make their clinical and tumor data available for research, but their data could not be used as they were not treated in a participating center. Therefore, ethical research committees could facilitate rare disease research, for example by enabling the establishment of (inter-) national collaborations with central approval and a single study contract. Recently, several research initiative were established which enabled patients to remotely share their clinical information and allow for the acquisition of tumor tissue samples for research by giving online consent.[52] These initiatives help to empower patients to actively stimulate progress in research and data collection.

Disease monitoring and timing of treatment

ctDNA and PET-CT to assess disease activity in granulosa cell tumor patients

Disease monitoring and the timing of treatment for recurrent disease are challenges in the management of granulosa cell tumors. Currently, the hormones inhibin, estradiol and/or AMH are used during follow up as markers of disease. Although low levels of these markers are usually reassuring, they have a moderate sensitivity for detection of recurrence and may be difficult to interpret in premenopausal women. We found that circulating tumor DNA (ctDNA) can reflect disease activity in patients with a granulosa cell tumor. By expanding our cohort we hope to be able to estimate whether ctDNA will be a helpful addition to current markers to detect a recurrence and assess disease activity. Notably, we hope to establish whether ctDNA can be used as an early predictor of response during systemic treatment and/or as a liquid biopsy to assess whether a targetable mutation is still present.

When tumor markers rise, patients may undergo a CT scan to visualize potential recurrent disease. We found that fluorodeoxyglucose (FDG) does not specifically bind to granulosa cell tumor cells and FDG-PET/CT is therefore not advised in monitoring disease. We found estradiol uptake by FES-PET/CT in some granulosa cell tumors, though this imaging modality did not identify more tumor lesions than CT scan.

Treatment of recurrence

Precision medicine

The morbidity caused by the treatment of repeated recurrences creates a significant burden for patients. Moreover, the toxicity caused by current and actually ineffective chemotherapeutic drugs, emphasizes the need for personalized treatment strategies. It is important to estimate the sensitivity of the tumor prior to treatment, based on tumor characteristics (Table 2).

With respect to current systemic treatment options, we found that FES uptake on PET/CT corresponds with the effectiveness of **anti-hormonal treatment**. Therefore, patients with FES positive tumors are likely to benefit from an estrogen receptor blocker. Secondly, **chemotherapy** targets rapidly dividing cells and is therefore a successful treatment in high grade tumors. We found a damaging TP53 mutation combined with a high tumor mutational burden and mitotic index in a small subgroup of patients. Therefore, further clinical research is warranted to evaluate if patients with a *TP53* mutation and/or high proliferation rate respond better to chemotherapy.

We have also investigated novel anticancer treatments. Treatment with **PI3K pathway inhibitors** has shown promising results in various cancer types, such as PIK3CA-mutated hormone receptor positive metastatic breast cancer.[128] PI3K pathway inhibitors alpelisib and everolimus showed *in vitro* efficacy in patient-derived granulosa cell tumor cell lines when used in combination with chemotherapy or anti-hormonal treatment. We expect the effects of these treatments to be more pronounced in tumors with a PI3K pathway mutation. The first patients with a *PIK3CA* mutated granulosa cell tumor who had received repeated surgeries, chemotherapy and anti-hormonal treatment, have received treatment with PI3K pathway inhibitors (alpelisib or everolimus) and results need to be evaluated.

Immunotherapy has emerged over the past decade with impressive effects in cancer types with a high tumor mutational burden, such as melanoma.[263] From our national cohort, one patient could enroll in a trial in which she received immunotherapy (the PD-1 checkpoint inhibitor pembrolizumab) due to her unusually high tumor mutational burden (>20.000 whole genome variants), although this treatment could not prevent further disease progression in her particular case. Since the tumor mutational burden in granulosa cell tumors is usually low and we did not find PD-1 expression, neither by immunohistochemistry nor by RNA sequencing (data not shown), this treatment may in general not be beneficial for this group of patients.

As successive surgeries are necessary in patients with recurrent disease, we explored minimally invasive options to remove tumor metastases. We found that **robot-assisted laparoscopy** is feasible and can safely be performed in selected patients with a recurrent granulosa cell tumor. Future research should investigate whether this approach may also reduce the operative risks associated with successive surgeries for recurrent disease. Finally, **radiofrequency ablation** (RFA) of liver deposits can be performed in patients with inoperable liver metastasis. RFA may increase the percentage of granulosa cell tumor patients considered surgically treatable.[40]

Other potential targeted treatments suggested in literature are (1) the peroxisome proliferator-activated receptor-gamma (PPARγ) activation combined with inhibition of the X-linked inhibitor of apoptosis protein (XIAP),[130] (2) procaspase activating compound-1 (PAC1) combined with the TNF-related apoptosis-inducing ligand (TRAIL) inhibition[264] and (3) angiogenesis inhibitors such as bevacizumab.[265]

Table 2. Tumor characteristics linked to a potentially effective treatment

Tumor characteristic	Potential treatment (with limited experience)
FES uptake on PET/CT	Anti-hormonal treatment
TP53 mutation	Chemotherapy
PI3K pathway mutation	PI3K pathway inhibitors
High tumor mutational burden	Immunotherapy
Multiple localized tumors	Robot-assisted laparoscopy
Inoperable liver metastases	Radiofrequency ablation

Future perspectives: clinical basket trials based on tumor specific molecular characteristics

Cancer treatment is to an increasing extent based on the specific molecular characteristics of a tumor, rather than solely the organ the tumor originates from. This development requires molecularly stratified clinical 'basket' trials, in which patients that share a particular tumor characteristic, usually a distinctive genomic alteration, may be treated with a specific therapeutic strategy. Each basket offers a specific drug or drug combination that is linked to specific tumor features. Basket trials test the hypothesis that it is the specific biomarker that defines the therapeutic response, rather than the specific tumor type.[266] The research presented in this thesis suggests treatment opportunities for specific granulosa cell tumor patient subgroups based on small pilots and first experiences (Table 2).

A clinical basket trial may allow granulosa cell tumor patients to receive novel anti-cancer drugs that have shown efficacy in different tumor types with similar molecular characteristics. Doherty et al., determined different layers of evidence for clinical efficacy of treatment.[266] Since granulosa cell tumors are usually excluded from general ovarian cancer research and randomized controlled trials in rare diseases may take many years to include a sufficient

number of patients, other approaches are necessary in order to translate novel findings into clinical care. Figure 1 shows different layers of evidence for the efficacy of targeted granulosa cell tumor treatments. These layers are derived from the methods used in this thesis, starting from DNA sequencing to identify a targetable alteration, to the use of imaging or biomarkers to predict treatment eligibility, to drug sensitivity testing on patient-specific tumor cell lines. In the future, a combination of these approaches will achieve the highest level of evidence to assess the efficacy of a treatment for a specific tumor characteristic in granulosa cell tumors.

Conclusion

In conclusion, the research presented in this thesis is a step towards precision medicine for granulosa cell tumor patients. Granulosa cell tumors are a complex and fascinating disease, whose molecular mechanisms are still not fully understood. In addition to current knowledge, we have investigated genomic alterations as potential targets, found that the absence of the *FOXL2* mutation does not exclude granulosa cell tumor diagnosis, described tumor heterogeneity and the familial occurrence of granulosa cell tumors. We explored novel imaging modalities, less invasive treatment and markers for disease monitoring. Furthermore, we established an *in vitro* model to screen patient-derived cell lines for treatment efficacy. Our research is the result of national collaborations and active patient engagement and has provided insights on diagnosis, management, disease monitoring and potential novel treatment options. Future research should focus on the application of novel therapeutic options, based on different layers of evidence and patient-specific tumor characteristics.

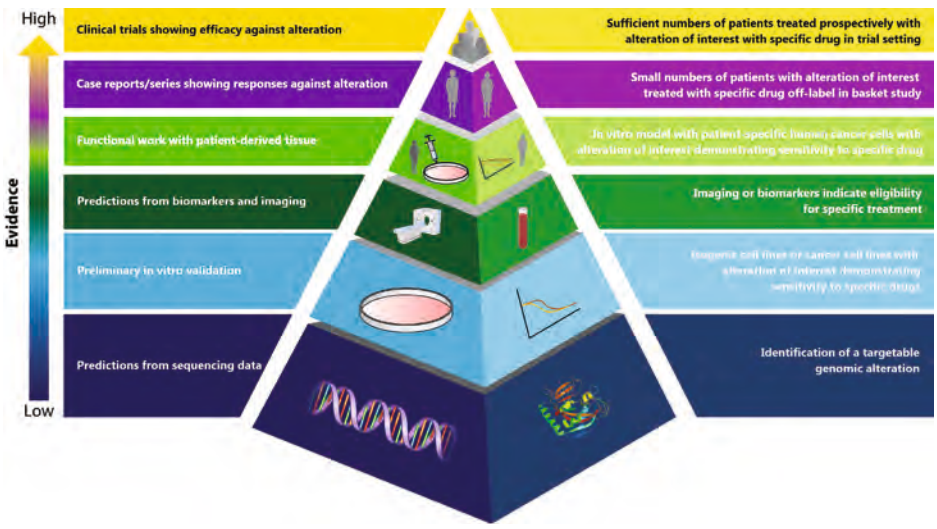

Figure 1. Hierarchy of evidence for determining putative clinical actionability. Adapted from Doherty GJ, et al. 2019.[266]

CHAPTER 10

Summary

Nederlandse samenvatting

Summary

Granulosa cell tumors are a well-defined and rare type of ovarian cancer, with approximately 15-20 new cases in the Netherlands per year. Patients can present with symptoms of abdominal pain caused by a large ovarian mass. Prolonged exposure to tumor derived estrogen may cause endometrial proliferation, resulting in symptoms of irregular or postmenopausal vaginal bleeding. At diagnosis, the tumor has usually not metastasized and is confined to one ovary in 78-91% of patients, in contrast with other ovarian cancers which are usually detected at an advanced stage. The surgical treatment consists of a unilateral or bilateral salpingo-oophorectomy with or without a hysterectomy. Up to 50% of the patients develop recurrences, often requiring repeated surgeries and adjuvant systemic treatment such as chemotherapy or anti-hormonal treatment. These systemic treatments target disseminated tumor cells, although they have significant side effects and are only effective in a subgroup of patients. The aim of this research is to personalize treatment for granulosa cell tumor patients, based on specific tumor characteristics.

It can be challenging to obtain funding for research, especially for research on a rare disease. The research described in this thesis is funded by the Granulosa Fund Philine van Esch. This fund was initiated specifically for granulosa cell tumor research. From the beginning, the patient and her family were actively involved in the research process. Her particular experiences helped to identify knowledge gaps and clinical needs from a patient's perspective. We have actively engaged patients and collaborated with patient organizations, which has guided our research directions, raised novel questions and has resulted in patient-initiated research projects. Along these lines, the research group was a reliable and accessible source of information for patients. We updated patients frequently on the research progress via newsletters and meetings. This active patient engagement and our national collaborative research have created awareness of the disease among gynecologists and have led to centralized knowledge and a center of expertise. **Chapter 2** describes how patients were actively involved in our research and how patient engagement benefits both patients and researchers.

Granulosa cell tumors are characterized by a specific *FOXL2* c.402C>G mutation, present in over 90% of tumors. This gene has an important role in granulosa cells, although its role in granulosa cell tumor development remains poorly understood. The results of our whole genome analysis of 46 adult granulosa cell tumors and matched normal DNA are shown in **chapter 3**. Copy number analysis showed frequent gains in chromosome 12, 14 and loss of chromosome 22. The total number of variants per tumor was low with an average of 3500 variants per tumor. We found that recurrent tumors harbored more variants than primary tumors. Frequent affected genes were *FOXL2* (88%), *TERT* promoter variants (33%),

KMT2D (18%), *TP53* (9%) and *PIK3CA* (6%). Pathogenic *TP53* mutations were identified in three patients with highest tumor mutational burden and mitotic activity, defining a high-grade granulosa cell tumor subgroup. These patients may respond better to chemotherapy, as it targets rapidly dividing cells. *FOXL2*-wildtype tumors harbored *DICER1*, *TERT*(C228T) and *TP53* mutations and similar copy number profiles and microscopic features as *FOXL2*-mutant tumors. Comparison of different tumor locations within patients revealed tumor heterogeneity, an established characteristic of cancer, and rejects the view of granulosa cell tumors as a homogeneous tumor.

An important step in granulosa cell tumor research is the identification of novel effective therapeutic options. In **chapter 4**, we established a method to rapidly screen for drug responses *in vitro* using direct patient-derived cell lines, in order to optimize treatment selection. Drug screen results were obtained within three weeks after tissue withdrawal. The response to 11 monotherapies and 12 combination therapies, including chemotherapeutic, anti-hormonal, and targeted agents, were tested in 13 granulosa cell tumor cell lines. All cell lines showed synergistic growth inhibition by combination treatment with carboplatin, paclitaxel and the PI3K inhibitor alpelisib at concentrations below the maximum achievable concentration in patients. The combination of everolimus with alpelisib or everolimus with tamoxifen were also effective, although these combinations could not reach 50% cell death at tolerable plasma concentrations. In agreement with clinical practice, patient-derived granulosa cell tumor cell lines demonstrated resistance to all monotherapies. Hereby, we show that granulosa cell tumor cell lines can be rapidly established and used for patient-specific *in vitro* drug testing which may guide treatment decisions.

Chapter 5 shows the value of PET imaging for disease monitoring. We found that FDG uptake in granulosa cell tumors is limited and therefore FDG-PET/CT is not routinely advised in monitoring this disease. We also investigated the value of FES, a specific estrogen tracer, in assessing the eligibility for anti-hormonal treatment. Three of the six patients that underwent FES-PET/CT scanning received anti-hormonal treatment. The two patients that did not show FES uptake developed disease progression during their treatment. The other patient had both tumor lesions with and without FES uptake. In her case, the tumors with FES uptake remained stable or decreased in size after anti-hormonal treatment and the tumors without FES uptake progressed. This research shows that FES-PET/CT may be useful to non-invasively capture the estrogen receptor expression of separate tumor lesions and thus assess the potential eligibility for anti-hormonal treatment in patients with a granulosa cell tumor.

Monitoring disease activity remains an important clinical challenge. Measuring disease activity could help to optimize the timing of treatment and may predict the response to treatment at an early stage. Previous studies in other cancer types showed that short DNA fragments excreted by the tumor and circulating in the blood, could be a good measure of disease activity. **Chapter 6** shows the results of our prospective study in which we investigated this circulating tumor DNA (ctDNA) in 110 longitudinally collected blood samples of 21 patients. We investigated whether ctDNA, either harboring the specific *FOXL2* variant or one of the two *TERT* promoter variants, could be a useful biomarker in disease monitoring. In most patients, ctDNA was a good marker for disease activity, especially in patients with high ctDNA levels, and ctDNA may therefore be a valuable addition to current methods for disease monitoring.

Chapter 7 describes the familial occurrence of granulosa cell tumors. Although granulosa cell tumors can occur in rare syndromes and one familial case of a granulosa cell tumor has been described, a genetic predisposition for granulosa cell tumors has not been identified. Through our collaborations with patients, we identified four families in which two women of each family were diagnosed with an adult granulosa cell tumor. Although predicted deleterious variants in *PIK3C2G*, *BMP5* and *LRP2* were found, we could not identify an overlapping genetic variant or affected locus that may explain a genetic predisposition for granulosa cell tumors. The age of onset in the familial patients was significantly lower (median 38 years, range 17-60) than in sporadic patients (median between 50-55 years). Furthermore, breast cancer, polycystic ovary syndrome and subfertility were seen in these families.

As patients with recurrences often need repeated surgeries to eliminate disease, we investigated in a pilot study whether less invasive treatment was possible. **Chapter 8** shows that debulking surgery via robot-assisted laparoscopy may be feasible and safe in selected patients with a recurrent granulosa cell tumor. Patients may benefit from this minimally invasive treatment as it could potentially reduce the operative risks associated with successive surgeries for recurrent disease.

In summary, the research described in this thesis provides additional knowledge and insights in the diagnosis, treatment and monitoring of granulosa cell tumors. Future research should extend our experiences and focus on the application of targeted therapeutic options, based on patient-specific tumor characteristics.

Nederlandse samenvatting

Granulosaceltumoren zijn een specifieke zeldzame vorm van eierstokkanker, met ongeveer 15-20 nieuwe gevallen in Nederland per jaar. Patiënten presenteren zich vaak met buikpijn of abnormaal vaginaal bloedverlies. Dit bloedverlies is het gevolg van oestrogeen, dat door de tumorcellen wordt geproduceerd en zorgt voor opbouw van baarmoederslijmvlies. De tumor wordt meestal ontdekt wanneer er nog geen uitzaaiingen zijn. Daardoor kan de tumor met een operatie worden verwijderd en is er na een operatie meestal geen aanvullende behandeling noodzakelijk. Dit is een groot verschil met andere vormen van eierstokkanker, die vaak pas in een vergevorderd stadium worden ontdekt. Bij ongeveer de helft van de vrouwen met een granulosaceltumor keert de ziekte terug, waarbij er opnieuw een operatie plaatsvindt en soms ook chemotherapie en/of anti-hormonale behandeling wordt gegeven. Deze aanvullende behandelingen zorgen voor bestrijding van tumorcellen door het hele lichaam, hoewel ze slechts bij een deel van de patiënten effectief zijn en vaak bijwerkingen geven. Het doel van dit onderzoek is om patiënten met een granulosaceltumor een gerichtere behandeling te kunnen bieden op basis van de specifieke kenmerken van de tumor.

Subsidies voor onderzoek zijn moeilijk te verkrijgen en dit geldt zeker voor onderzoek naar zeldzamere ziekten. Het onderzoek in dit proefschrift is mede mogelijk gemaakt door de financiering vanuit het Granulosafonds Philine van Esch. Dit fonds is speciaal door een patiënt en haar familie voor dit onderzoek opgericht. Zij waren vanaf het begin actief betrokken bij het onderzoek en haar ervaringen hebben geholpen om de belangrijkste kennishiaten en benodigde verbeteringen met betrekking tot diagnose, behandeling en ziekte monitoring vast te stellen. Daarnaast hebben we ook samengewerkt met patiëntenorganisaties, die mede de richting van het onderzoek hebben bepaald. Dit heeft onder andere geleid tot een onderzoeksproject opgezet door patiënten. Van onze kant hebben we patiënten regelmatig op de hoogte gehouden van het onderzoek door middel van nieuwsbrieven en bijeenkomsten. Deze samenwerking heeft geresulteerd in landelijke bekendheid van granulosaceltumoren. Die bekendheid heeft ertoe geleid dat we in relatief korte tijd veel klinische data en patiënten materiaal konden verzamelen waardoor onze kennis omtrent granulosaceltumoren is toegenomen en het UMC Utrecht nu als expertisecentrum op dit gebied functioneert. **Hoofdstuk 2** beschrijft hoe patiënten actief hebben deelgenomen in ons onderzoek en welke voordelen dit voor zowel de onderzoekers als de patiënten heeft opgeleverd.

Bijna alle granulosaceltumoren bevatten een specifieke fout (mutatie) in het *FOXL2* gen. Dit gen speelt een rol in granulosacellen, maar welke veranderingen er verder optreden is nog grotendeels onbekend. In **hoofdstuk 3** tonen we de resultaten van DNA onderzoek van het hele genoom van 46 granulosaceltumoren. Uit deze zogenaamde sequencing data bleek dat granulosaceltumoren vaak een verkeerd aantal chromosomen hebben: meer dan 2 kopieën van chromosoom 12 en 14 en minder dan 2 kopieën van chromosoom 22. Het aantal genetische fouten (mutaties) in granulosaceltumoren is echter laag in vergelijking met andere typen kanker, gemiddeld ligt dit aantal bij granulosaceltumoren rond de 3500 mutaties per tumor. Ook vonden we meer mutaties in een recidief tumor dan bij de eerste tumor. Genen die vaak een mutatie bevatten zijn *FOXL2* (88%), de *TERT* promotor regio (33%), *KMT2D* (18%), *TP53* (9%) en *PIK3CA* (6%). Bij tumoren met een *TP53* mutatie waren veel celdelingen zichtbaar onder de microscoop en deze tumoren bevatten ook in totaal de meeste mutaties en grote chromosomale veranderingen. Deze mutatie wijst daarom op een subgroep met een mogelijk slechtere afloop. Deze tumoren reageren mogelijk beter op chemotherapie, omdat chemotherapie aangrijpt op snel delende cellen. De tumoren die de specifieke *FOXL2* mutatie niet hadden, lieten mutaties zien in *DICER1*, *TERT* en/of *TP53*. Ze zagen er onder de microscoop uit als granulosaceltumoren en er was bij deze tumoren geen twijfel over de diagnose. Verder bleek uit dit onderzoek dat granulosaceltumoren op DNA niveau verschillen tussen patiënten laten zien maar ook dat er binnen één patiënt verschillen zijn in DNA afwijkingen tussen tumorlocaties: dit wordt tumor heterogeniteit genoemd.

Een belangrijke stap in onderzoek naar granulosaceltumoren is het vinden van nieuwe effectieve behandelingen. **Hoofdstuk 4** omschrijft hoe we een methode hebben opgezet om verschillende medicijnen te testen op tumorcellijnen die gekweekt zijn van individuele patiënten. Binnen 3 weken na de weefselafname bij een operatie konden we zien hoe de tumor op verschillende medicijnen reageert. We testten 11 afzonderlijke anti-kankermiddelen en 12 verschillende combinaties (chemotherapie, anti-hormonale behandeling en gerichte nieuwe kanker behandelingen) op 13 verschillende cellijnen. Alle tumorcellen reageerden het beste op de combinatie van carboplatin, paclitaxel en de PI3K remmer alpelisib. De interactie tussen deze medicijnen versterkte het effect, waardoor de individuele medicijnen effectief bleken in concentraties onder de maximaal in een patiënt haalbare dosering. De combinaties everolimus met alpelisib en everolimus met tamoxifen waren ook effectief, maar in concentraties boven de maximale dosering. De cellijnen reageerden op geen enkel kankermiddel als het afzonderlijk werd gegeven, in overeenstemming met wat in de praktijk wordt gezien. Op deze manier hebben we laten zien dat snel en betrouwbaar per patiënt een uitslag gekregen kan worden over de gevoeligheid van de tumorcellen voor een medicijn. Deze methode kan mogelijk in de toekomst gebruikt worden bij het bepalen van de behandeling voor een patiënt.

In **hoofdstuk 5** hebben we onderzocht of nieuwe beeldvormende technieken zoals PET-scans kunnen helpen om de ziekte activiteit weer te geven. Dit kan mogelijk helpen om, bijvoorbeeld als de ziekte terug komt, te besluiten wanneer en welke behandeling gestart moet worden. We hebben onderzocht of een standaard gebruikelijke fluor-18-deoxyglucose(FDG)-PET scan de ziekte activiteit van de tumor in beeld kan brengen. De meeste tumoren lieten geen tot matige FDG opname zien en daarom lijkt FDG-PET niet nuttig in het vervolgen van ziekte. Ook werd onderzocht of een PET scan gebruik makend van een specifieke oestrogeen (FES) tracer kan helpen om vast te stellen of een patiënt baat heeft bij anti-hormonale behandeling. Van de zes patiënten die een FES-PET ondergingen, kregen drie patiënten daarna anti-hormonale behandeling. Twee patiënten zonder FES opname in de tumor hadden progressie van ziekte onder anti-hormonale behandeling. In één patiënt, met zowel FES positieve als negatieve tumorlocaties, bleven de FES positieve tumoren stabiel of werden kleiner na anti-hormonale behandeling en de FES negatieve locaties namen toe in grootte. Dit onderzoek laat zien dat FES tumor opname overeen komt met de klinische respons op anti-hormonale behandeling en dat deze manier van beeldvorming mogelijk nuttig is om te voorspellen of anti-hormonale behandeling effect zal hebben.

Een belangrijke klinische uitdaging is het monitoren van ziekte activiteit. Het meten van ziekte activiteit kan bijvoorbeeld helpen om de timing van een operatie te bepalen of de respons op een behandeling vroegtijdig in kaart te brengen. Uit studies in andere typen kanker is gebleken dat korte stukjes DNA die in het bloed circuleren en afkomstig zijn van tumorcellen, zogenaamd ctDNA, mogelijk een goede maat zijn voor ziekte activiteit. **Hoofdstuk 6** laat de resultaten zien van onze prospectieve studie waarbij we ctDNA hebben onderzocht in 110 bloed samples van 21 patiënten. We onderzochten ctDNA dat ofwel de *FOXL2* mutatie ofwel een van de twee *TERT* promotor mutaties bevatten. In de meeste patiënten was ctDNA een goede marker voor het monitoren van ziekte activiteit, vooral in de patiënten met veel ctDNA in het bloed. CtDNA kan daarom een waardevolle aanvulling zijn in het monitoren van ziekte.

Hoofdstuk 7 beschrijft het voorkomen van granulosaceltumoren in families. Hoewel de tumoren kunnen voorkomen als onderdeel van zeldzame erfelijke syndromen en er een geval is beschreven van een moeder en een dochter met beiden een granulosaceltumor, is er geen bekende erfelijke oorzaak die zorgt voor een hogere kans op het ontwikkelen van granulosaceltumoren. In samenwerking met patiënten hebben we vier families geïdentificeerd waarin twee familieleden een granulosaceltumor hadden. Er kwamen mutaties voor in de genen *PIK3C2G*, *BMP5* en *LRP2*, maar geen van deze mutaties vonden we in meer dan één familie terug. Deze genetische veranderingen konden daardoor niet het ontstaan van de granulosaceltumor verklaren in deze families. De patiënten waren relatief

jong toen ze de ziekte kregen: gemiddeld op 38-jarige leeftijd (range 17-60 jaar), terwijl de ziekte normaal bij vrouwen rond de 50-55 jaar wordt gevonden. Verder werd in deze families borstkanker, polycysteus ovariumsyndroom en verminderde vruchtbaarheid gezien.

Bij patiënten die bij herhaling recidieven ontwikkelen, is vaak steeds opnieuw operaties nodig om de ziekte te verwijderen. In **hoofdstuk 8** hebben we onderzocht of er ook minder invasief geopereerd kan worden met behulp van een operatierobot. In deze pilot studie vonden we dat robot geassisteerde laparoscopie mogelijk en veilig is in geselecteerde patiënten met een recidief granulosaceltumor. In patiënten met herhaalde recidieven kan deze minimaal invasieve methode de operatierisico's en complexiteit van telkens weer een operatie mogelijk verminderen.

Samenvattend heeft het onderzoek beschreven in dit proefschrift nieuwe kennis en inzichten geleverd in de diagnose, behandeling en monitoring van granulosaceltumoren. Toekomstig onderzoek zal zich moeten richten op het uitbreiden van onze ervaring met het toepassen van specifieke behandelopties, op basis van patiënt-specifieke en tumor kenmerken.

CHAPTER 11

List of publications

About the author

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About the author

Joline Frederique Roze was born on June 25th 1991 and grew up in the picturesque village IJsselmuiden with her parents and sisters Loes and Elise. In her free time, she enjoyed performing on stage in musicals and participated in jazzdance competitions at national level. After graduating from secondary school (Ichthus College Kampen) in 2008, she moved to Groningen to study medicine at the Rijksuniversiteit Groningen. During her bachelor, Joline joined the liver transplant team which enabled her to assist at complex surgeries and participate in related research. In 2012, she completed a research internship on the role of advanced glycation end-products in patients with hearth failure at the department of Cardiology of the University Medical Center Groningen, leading to her first publication as a co-author. This experience sparked her enthusiasm for research.

Amazed by her experiences at the delivery rooms, Joline discovered her interest in Obstetrics and Gynecology and completed multiple chosen internships in this field. To broaden her horizon and to learn from a different healthcare system, she went to Surinam for an internship Emergency Healthcare and Public Health at the Academic Hospital of Paramaribo in 2014. After completing her medical degree in 2015, Joline worked as a clinical resident in Obstetrics and Gynecology at the Haaglanden Medical Center the Hague under supervision of dr. M.J. Kagie, which confirmed her passion for this specialization.

In 2016 she started as a PhD candidate at the University Medical Center Utrecht under supervision of prof. R.H.M. Verheijen and prof. R.P. Zweemer, studying granulosa cell tumors of the ovary. During her research, Joline enjoyed conducting translational research and working in a complementary research team with different fields of expertise. In addition to present and share her research with colleagues in the field, she finds it important to communicate her findings to non-scientists as well. Therefore, Joline participated in the “Breaking Science” pitch competition and initiatives from the “Wetenschapsknooppunt” to learn how to communicate her research to a broader audience. Furthermore, she actively involved patients in her research which has led to novel insights and joint projects, of which this thesis is the final product.

To further develop her research skills, Joline combined her PhD trajectory with a Postgraduate Master in Epidemiology at Utrecht University, specializing in both Clinical Epidemiology and Medical Statistics. She participated in teaching activities for medical students and obtained the University Teaching Qualification (BKO) in 2019. As a PhD candidate, she was the representative of the PhD program “Clinical and Translational Oncology” in the PhD council of the Graduate School of Life Sciences. This council represents the interests of PhD candidates, aims to increase the quality of the PhD programs and organizes events. Currently, Joline is a board member of “Dokters in Debat”, an organization that aims to encourage societal and political engagement among medical doctors. In December 2020, she has started working at the Meander Medical Center Amersfoort under supervision of dr. I.M. Evers and dr. M.V.E. Veenendaal and she aims to pursue a career in the field of Obstetrics and Gynaecology.

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In dit onderzoek zijn we samen met patiënten op zoek gegaan naar de belangrijkste vraagstukken omtrent granulosaceltumoren en daaruit zijn weer nieuwe onderzoeksprojecten ontstaan. Lieve **Ien**, wat een geweldig werk doe je voor de GCT lotgenoten groep (of hoe jullie jezelf noemen: de GCT sisters). Dank je wel voor je betrokkenheid en enthousiasme. Ook bedank ik graag alle andere **GCT patiënten** die ik heb mogen ontmoeten. Blijf vooral al die goede en kritische vragen stellen! Het is bijzonder om zo dicht bij de patiënten te staan en te zien voor wie je het onderzoek doet.

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Promoveren is als een Luikse processie, 3 stappen vooruit, 2 stappen terug – RV
Mooi de voortgang te zien. Was al enthousiast, maar wordt echt een goed stuk zo!- RZ
Vanaf nu is alles een ei'tje! – RS
Deze review voelt als een niet vorderende uitdrijving - JH
What is the phenotype of this genetic disease? " Uhh… dead." – GH
Mijn timeline is "alles zsm af" – NH
Are you going to win the nobel prize? – FB
Tumoren lezen geen boekjes - TJ
When we find this novel candidate gene, we're going to pop the champagne - GM
Het ziet er indrukwekkend uit. Ik kan het niet lezen, te technisch. Top gedaan – Papa
De toetsingscommissie is roomser dan de paus – FT
Het besef dat er een heuse onderzoeksgroep aan t werk is voor je, maakt dat je je opgetild voelt als patiënt en dat heeft een enorme positieve werking op je welbevinden. - IF
Heb je mijn naam wel eens opgezocht op PubMed – PvD
I got into a coding frenzy yesterday and I am almost done with a tool for that job! - JK
Als je elke dag een stapje zet in de richting waar je naartoe wil, dan kom je er vanzelf - DV

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