Hunting a Silent Killer. Biomolecular Approaches in Ovarian Cancer

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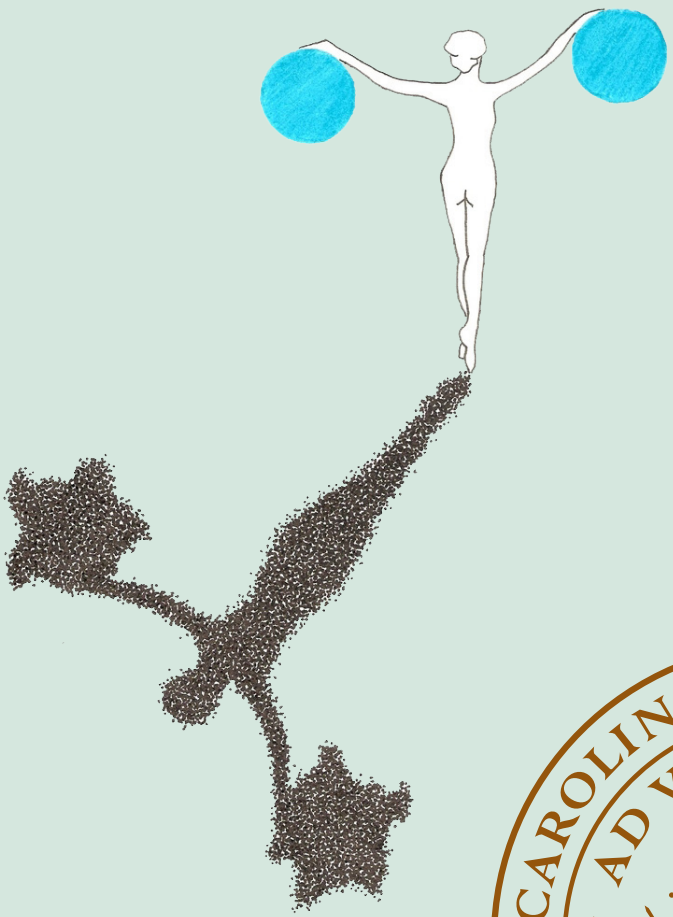

Hunting a Silent Killer

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Biomolecular Approaches in Ovarian Cancer

Nicolai Skovbjerg Arildsen

LUND
UNIVERSITY

DOCTORAL DISSERTATION

by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended in the lecture hall in the Radiotherapy Building, Skåne Oncology Clinic, Lund, Sweden on Thursday the 28th of March at 13.00.

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Title and subtitle: Hunting a Silent Killer – Biomolecular Approaches in Ovarian cancer

Abstract: Ovarian cancer is a heterogeneous disease and recent advances in improving patient outcome have been limited. It is estimated that a woman's risk of developing ovarian cancer during her lifetime is about 1 in 70, making it a frequently occurring cancer type in women.

This thesis investigated biological events in ovarian cancer, which were translated into clinically relevant observations using different biomolecular approaches.

Study I investigated the use of sex steroid hormone receptor expression as a prognostic marker in ovarian cancer. We evaluated the expression of estrogen receptor (ER)α, ERβ, progesterone receptor (PR) and androgen receptor (AR) in a cohort of serous and endometrioid cancers. We found that expression of PR and AR was associated with favorable outcome, and co-expression of AR and PR granted an additional prognostic effect. Although we were unable to detect any association between mRNA expression and a favorable outcome in an independent data set, molecular subtypes in the data set differentially expressed *PGR* and *ESR1*. Whether this effect accounted for the reported improved outcome in some of the subgroups remains to be investigated.

Study II characterized ovarian clear cell carcinomas (OCCC) with the purpose of identifying potential treatment candidates. OCCC presents a distinct molecular subtype of ovarian cancer, with high chemoresistance. Through integrative bioinformatics we evaluated combined gene expression data, DNA sequencing data and protein expression data from OCCC tumors. The collective data suggested Rho GTPases as a potential treatment candidate in OCCC.

Study III evaluated the effect of targeting Rho GTPases in OCCC using simvastatin and CID-1067700 in OCCC cell lines. All OCCC cell lines were more sensitive to simvastatin as compared to conventional platinum-based chemotherapy. Both of the drugs we evaluated were found to disorganize the cytoskeleton and inhibitit migration. The cellular response mechanisms differed between cell lines, however a potential effect on both the PI3K/AKT/mTOR and RAS/ERK pathways was suggested.

Study IV aimed at evaluating the effects of screening prediagnostic liquid based vaginal samples for *TP53* mutations, an approach applied for early detetion of ovarian cancer. We identified 8 women with somatic *TP53* mutations in high-grade serous ovarian cancer (HGSOC) and analyzed both prediagnostic (presymptomatic) and diagnostic vaginal samples. We used ultrasensitive droplet digital PCR (ddPCR) (IBSAFE™) and found mutations in diagnostic samples from 75% (6/8) of the patients; however, no mutaitons were detected in the prediagnostic samples. Despite this ddPCR was able to analyze samples with very limited DNA, where other methods would fail. This provides a basis for the further evaluation of IBSAFE™ in a larger cohort of patients.

In conclusion, these studies further characterized ovarian cancer biology and heterogeniety, and have provided the basis for future studies in ovarian cancer with the potential of improving outcome.

Key words: Ovarian cancer, early detection, droplet digital PCR, IBSAFE, high-grade serous ovarian cancer, ovarian clear cell cancer, simvastatin, Rho GTPases, *TP53*, sex hormone receptors, integrative bioinformatics

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To Dennie Skovbjerg Lohse

”Kein Operationsplan reicht mit einiger Sicherheit über das erste Zusammentreffen mit der feindlichen Hauptmacht hinaus”

Helmuth von Moltke the Elder (1800-1891)

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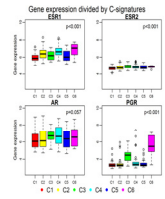
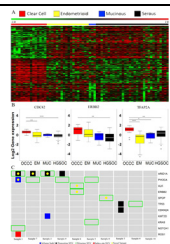
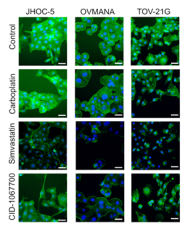
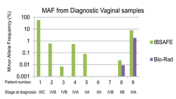
Thesis at a glance

Study	Question	Methods		Results and implications
I	Does sex hormone receptor expression have a prognostic value in ovarian cancer? And can sex hormone receptors be used to stratify molecular subtypes of ovarian cancer?	• Immunohistochemical (IHC) analysis of ERα/β, AR and PR in 118 ovarian cancers. • Analysis of correspondng mRNA levels in molecular subtypes of ovarian cancer.		AR+ and PR+ were prognostically favorable both alone and in combination. *ESR1* and *PGR* were differentially expressed beween molecular subtypes.
II	Can an integrative bioinformatic analysis of ovarian clear cell cancer (OCCC) reveal potential treatment candidates? Is OCCC a distinct molecular subtype?	• Gene expression profiling of 67 tumors, (15 OCCC). • Next generation sequencing of 10 OCCC tumors. • IHC of 43 OCCC tumors.		OCCC is a distinct molecular subtype in ovarian cancer. Rho GTPases are a potential treatment candidate in OCCC, while HER2 seems not to be overexpressed despite *ERBB2* overexpression.
III	Are Rho GTPases a potential target for OCCC treatment based on the integrative analsyis in study II and are OCCCs more sensitive to the Rho GTPase inhibitors simvastatin and CID-1067700?	• Cell culture assays. • Dose response assays. • Cytoskeletal staining. • Cellular response assays. • FACS. • Western blotting.		Simvastatin is more potent that carboplatin in OCCC. Both simvastatin and CID-1067700 interefere with the cytoskeleton. Cell line specific cellular responses to treatment indicate targeting of Rho GTPases and the RAS/ERK, PI3K/AKT/mTOR pathways.
IV	Can we detect *TP53* mutations in presymptomatic liquid based vaginal samples from women with somatic *TP53* mutations in HGSOC tumors?	• Ultrasensitive droplet digital PCR (ddPCR); IBSAFE™. • Next generation sequencing.		IBSAFE achieved a sensitivity of 75% in the diagnostic samples, while no mutations were detected in presymptomatic samples. IBSAFE proved to work in low abundance DNA samples.

Populærvidenskabelig sammenfatning

Æggestokskræft er en af de største dræbere for kvinder. Omkring 1 ud af 70 kvinder vil på et tidspunkt få kræft i æggestokkene, og med øgenavnet "Den stille dræber" er der god grund til at udfordre det behandlingsparadigme som æggestokskræft lider under, nemlig at alle typer af æggestokskræft behandles ens. Dette betyder, at dødeligheden ikke har sænket sig mærkbart i de seneste 20 år.

Vi ved nu, at æggestokskræft i hvert fald er 5 histologisk forskellige sygdomme, og specielt de serøse adenokarcinomer, som udgør 70% af alle tilfælde, har en dårlig prognose.

Denne afhandling har forsøgt at undersøge æggestokskræft ved hjælp af biomolekylære metoder og tankegange. Resultaterne er efterfølgende sat ind i en klinisk relevant kontekst, så forskningen kan give det størst mulige udbytte for patienterne.

Det første studie undersøgte de prognostiske værdier, som hænger sammen med kønshormonreceptorerne: østrogen receptor α/β, progesteron receptor, androgen receptor og overlevelse. Vi fandt, at progesteron og androgen receptor positivitet hang sammen med en bedre prognose, både hver for sig og tilsammen. Vi forsøgte også at teste dette i et uafhængigt datasæt. Datasættet var baseret på genudtryk, men desværre lykkedes det ikke at vise sammenhængen i dette datasæt. Grundene herfor kan være mange, dog er det sandsynligt, at der er store forskelle mellem datasættene. Det uafhængige datasæt var inddelt i undergrupper baseret på tumorernes genudtryk, og i disse undergrupper fandt vi dog en potentiel positiv effekt af østrogen α og progesterongenerne. Det vil kræve en yderlige undersøgelse af en større gruppe kvinder at klarlægge effekterne af genudtrykkene, men vores indledende forsøg viste en potentiel klinisk fordel.

I studie to undersøgte vi, hvordan man ved hjælp af flere forskellige datasæt med både genetisk og cellulær information, samt bioinformatiske metoder, kunne generere en hypotese for behandling af klarcellet æggestokskræft. Klarcellet æggestokkræft er særlig interessant, idet den er kemoresistent. Vi opdagede, at Rho GTPaser spillede en stor rolle igennem hele vores analyse, og derfor var det oplagt at teste om man kunne behandle klarcellet æggestokskræft med medikamenter, der hæmmer Rho GTPasers aktivitet.

I studie tre testede vi simvastatin og CID-1067700, to stoffer der rammer Rho GTPasers aktivitet, og fandt at begge stoffer kunne hæmme væksten af klarcellede æggestokskræftcellelinjer. Dog var simvastatin meget mere effektivt sammenlignet med CID-1067700, men også sammenlignet med den almindelige chemobehandling. Vi kunne observere, at cellens cytoskelet blev forstyrret, og at forskellige cellulære signaleringsveje blev påvirket. Vi konkluderede derfor, at

simvastatin var et potentielt nyt middel mod klarcellet æggestokskræft, og da det allerede bruges i behandlingen af forhøjet kolesteroltal, kan en behandling være tæt på. Dog kræves flere studier.

Studie IV evaluerede en metode til at diagnosticere æggestokskræft tidligt. De fleste kvinder som bliver diagnosticeret med æggestokskræft, har allerede en kraftigt spredt sygdom, hvorfor behandlingen er svær. Vi undersøgte derfor celleforandringsprøver taget hos ni kvinder for livmoderhalskræft for mutationer i genet *TP53*. Dette gen er ofte muteret i æggestokskræft, og er derfor en oplagt kandidat at kigge efter i sådanne prøver. Vi brugte en yderst fintfølende metode som bruger bitte små dråber af prøver til at undersøge mutationer. Vi fandt ingen mutationer i prøverne taget hos kvinderne før deres diagnoser, men for 75% af kvinderne fandt vi mutationer i *TP53* i deres prøver taget i forbindelse med diagnosen. Derudover virkede vores metode med stor sikkerhed, selv i prøver med meget lidt DNA. Vi forsøger nu at udvide forsøget fra de oprindelige ni kvinder til omkring 30 kvinder fra hele Sverige.

Arbejdet i denne afhandling afspejler 4 års arbejde med æggestokskræft, og igennem disse år er vores viden om denne kræfttype blevet bedre. Vi er nu klar over at undersøgelser, som dem i studie 2 og 3 er nødvendige for de mere sjældne typer af æggestokskræft, mens at forsøg som studie 4 kan virke for den primære serøse æggestokskræft, da tidlig diagnose her giver en rigtig god prognose.

De næste år vil de nye teknologier indenfor sekvensering have kortlagt æggestokskræft så effektivt, at vi kan begynde at se hvilke grupper af kvinder, der skal have hvilke behandlinger, så som det gøres i brystkræft i dag. Derudover vil immunterapi og PARP-inhibitorer også komme til at gøre en stor forskel for behandlingen indenfor de næste år.

Det er min tro, at æggestokskræft indenfor en overskuelig årrække er en sygdom man dør med og ikke af.

List of original studies

This thesis if based on the following studies, which are referred to throughout the text with their roman numerals:

I. Jönsson JM, **Arildsen NS**, Malander S, Måsbäck A, Hartman L, Nilbert M, Hedenfalk I. Sex Steroid Hormone Receptor Expression Affects Ovarian Cancer Survival. Transl Oncol. 2015;8(5):424-33.

II. **Arildsen NS**, Jönsson JM, Bartuma K, Ebbesson A, Westbom-Fremer S, Måsbäck A, Malander S, Nilbert M, Hedenfalk IA. Involvement of Chromatin Remodeling Genes and the Rho GTPases RhoB and CDC42 in Ovarian Clear Cell Carcinoma. Front Oncol. 2017;7:109.

III. **Arildsen NS**, Hedenfalk IA. Simvastatin is a potential candidate drug in ovarian clear cell carcinomas. *In manuscript*.

IV. **Arildsen NS**, Martin de la Fuente L, Måsbäck A, Malander S, Forslund O, Kannisto P, Hedenfalk IA. Detecting *TP53* variants in diagnostic and archival liquid vaginal samples from ovarian cancer patients using an ultra-sensitive ddPCR method. *Under revision at Scientific Reports*.

Contributions

Study I

I evaluated two of the immunohistochemical markers, and I was responsible for the processing and statistical analysis of the external validation data set. I aided in the writing and revision of the manuscript.

Study II

I was involved in the study design and performed all the bioinformatic and statistical analyses. I was responsible for the quality control and evaluation of the sequencing data and I evaluated immunohistochemical markers. I was responsible for writing and revision of the manuscript.

Study III

I was involved in the study design and performed all the experiments. I was responsible for writing and revision of the manuscript.

Study IV

I was involved in the study design and methodological considerations. I conducted DNA extractions, analyzed sequencing data and performed validation of the IBSAFE™ data. I was responsible for writing and revision of the manuscript.

Abbreviations

AKT	AKT Serine/Threonine Kinase 1
AR/AR	Androgen receptor - Protein/Gene
ARID1A	AT-rich interactive domain-containing protein 1A
BLAST	Basic Local Alignment Search Tool
BRCA1/2	Breast cancer type 1/2 susceptibility gene
CA-125	Cancer antigen 125
CCNE1	Cyclin E1
CDC42	Cell Division Cycle 42
cDNA	complementary deoxyribonucleic acid
CI	Combination index
c-MET/*MET*	Mesenchymal epithelial transition receptor tyrosine kinase - Protein/Gene
ddPCR	Droplet digital polymerase chain reaction
DNA	Deoxyribonucleic acid
dPCR	Digital polymerase chain reaction
EOC	Epithelial ovarian cancer
ERBB2	Erb-B2 Receptor Tyrosine Kinase 2
ERK	Mitogen-Activated Protein Kinase 1
ERα/*ESR1*	Estrogen receptor alpha - Protein/Gene
ERβ/*ESR2*	Estrogen receptor beta - Protein/Gene
FACS	Fluorescence-activated cell sorting
FFPE	Formalin fixed paraffin embded
FIGO	International Federation of Gynecology and Obstetrics
FTE	Fallopian tube epithelium
FTSEC	Fallopian tube secretory epithelial cell
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GGPP	Geranylgeranyl pyrophosphate
HER2	Human Epidermal Growth Factor Receptor 2
HGSOC	High-grade serous ovarian cancer
HMG-CoA	3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase
HNF1B	Hepatocyte Nuclear Factor 1-Beta - Protein/Gene
HR	Hazard ratio
HRD	Homologous recombination deficiency
IC50	The half maximal inhibitory concentration
IHC	Immunohistochemistry
KRAS	KRAS Proto-Oncogene, GTPase
LGSOC	Low-grade serous ovarian cancer
MAF	Mutation/Minor allele frequency
MAPK	Mitogen activated protein kinase

MEV	Multiple experiment viewer
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NCBI	National Center for Biotechnology Information
NGS	Next generation sequencing
OCCC	Ovarian clear cell cancer
OS	Overall survival
OSE	Ovarian surface epithelium
p16	Cyclin Dependent Kinase Inhibitor 2A
p53/*TP53*	Tumor protein 53 - Protein/Gene
PARP	Poly (ADP-ribose) polymerase
PAX8	Paired Box 8
PCP	Planar cell polarity
PCR	Polymerase chain reaction
PFS	Progression free survival
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PR/*PGR*	Progesterone receptor - Protein/Gene
PTEN	Phosphatase And Tensin Homolog
qPCR	Quantitative PCR
RhoB	Ras Homolog Family Member B
RNA	Ribonucleic acid
SAM	Significance analysis of microarrays
SRB	Sulforhodamine B
STIC	Serous tubal intraepithelial carcinoma
SWI/SNF	SWItch/Sucrose Non-Fermentable
TCGA	The Cancer Genome Atlas
TFAP2A	Transcription Factor AP-2 Alpha
TMA	Tissue microarray
WG-DASL	Whole genome cDNA-mediated Annealing, Selection, extension and Ligation
WHO	World Health Organization
WT1	Wilms tumor protein 1

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Preface

It is estimated that one in three humans will develop cancer during their lifetime, which leads to the conclusion that every human being on earth will at some point encounter cancer, either as a patient or as a relative.

Though cancer is no longer considered just one disease, rather multiple different diseases, the paradigm in ovarian cancer is still to treat it as one disease. Although much research has been done in e.g. breast cancer, resulting in an a more favorable prognosis, ovarian cancer has been at a stalemate for the past 20 years. Its highly heterogeneous nature is probably what has made it able to earn its nick-name: The Silent Killer.

The most powerful weapon in the battle against ovarian cancer is knowledge. Knowledge through research. And research is just what this thesis is about. This thesis is the completion of five years of research in ovarian cancer, and it stands as a testimony to my belief that ovarian cancer will become a manageable disease.

I believe that the use of high throughput methods holds the key to improve ovarian cancer prognosis. As we have powerful biomolecular methods at our disposal, it is now more than ever a matter of interdisciplinary collaborations in order to gain the most benefit of these methods.

This thesis explores some of the biomolecular approaches available and emphasizes the synergy which can be obtained through interdisciplinarity in research. I came from a background in molecular biology and drug formulation, and into the world of preclinical research. The benefit for me has been that the research in this thesis is not only biological in nature, as the individual studies all hold a relevant clinical perspective.

Whether we assessed the prognostic effect of hormone receptors, used multilayered ‘omics data sets to generate a hypothesis, or evaluated a method for early detection, the clinical backbone has been invaluable. Research is all about asking the right questions, and in this thesis these questions were those of clinical relevance.

In my opinion the integrative holistic approach will be taking cancer research into the future and provide even better treatments than we have now.

A future who among others belong to my nephew, who was only possible because of the advances in cancer research as my brother is a cancer survivor.

Frederiksberg, February 2019

Introduction

A historic perspective on cancer

Cancer has been termed a disease of the old, and although it mainly affects elderly people, cancer itself is old. Cancer has existed alongside evolution since the dawn of DNA, and these two phenomena are tightly intertwined: One could not exist without the other [1, 2]. One of the oldest records of cancer dates to ancient Egypt (3,000 BC) and the Edwin Smith Papyrus in which cases of breast cancer are described although the word "cancer" is not used. The word "cancer" came into existence in ancient Greece, where the "Father of Medicine" Hippocrates (460-370 BC) described tumors with the word: "carcinoma". This was later translated by the Roman physician, Celsus (28-50 BC) into the modern-day word "cancer".

Even though cancer has been recognized for almost 5,000 years, we have only just begun to understand the complex dynamics evolving around cancer. The latest review by Douglas Hanahan and Robert A. Weinberg on the hallmarks of cancer highlighted the current understanding of cancer and expanded their original six hallmarks as defined in their paper from 2000, into the ten hallmarks seen in Figure 1 [3], all evidence of the complex nature of cancer.

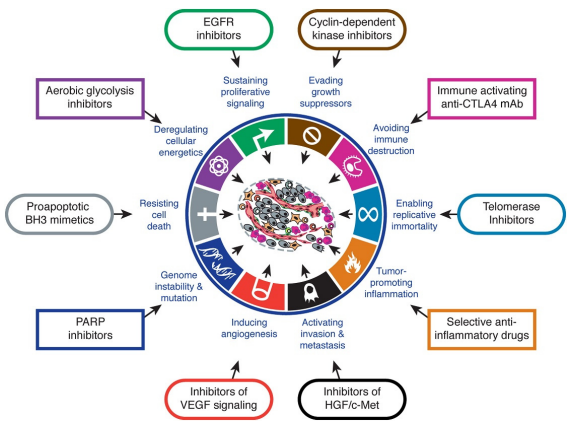

Figure 1: Therapeutic Targeting of the Hallmarks of Cancer
Illustration of the ten hallmarks of cancer and suggested treatment options. Reprinted from Cell, Volume 144 Issue 5, Douglas Hanahan and Robert A. Weinberg, Hallmarks of cancer: the next generation, 646-674, 2011, modified with permission from Elsevier.

Cancer is the second leading cause of death, accounting for 9.6 million deaths worldwide in 2018 [4], and the western world has the highest incidence of cancer as seen in Figure 2 [5]. However, cancer is not a single disease with one definite cause, and through history attempts have been made to identify such underlying causes. Bernardino Ramazzini (1633-1714), an Italian physician, observed in 1713 that nuns had a higher incidence of breast cancer which he attributed to their life of celibacy [6]. Interestingly, one of the risk factors of breast cancer is in fact nulliparity [7].

Despite the grim perspective, we now more than ever, have the possibility to fight cancer. With the emergence of chemotherapy in the 1940's up until the modern-day immunotherapy [8], we are taking the battle to the frontline and in fact the latest reports are that we see a steady decline in incidence and deaths of cancer [9].

Ovarian cancer

Incidence and mortality

It is estimated that 1 in 70 women will develop ovarian cancer in her lifetime [9], and ovarian cancer is the 7th most common female cancer in the world. There were more than 250,000 estimated new cases, and an estimated 143,180 deaths among women in 2018 [4]. The median age of onset is 63 years, and the incidence peaks in the late 70's [10]. The incidence of ovarian cancer varies in the world, with the highest incidence in Europe and the lowest in Africa [4], which is probably due to a significant difference in life-style and environmental factors (Figure 2).

Ovarian cancer is diagnosed in more than 2,000 women annually in the Nordic countries, with 700 and 500 cases in Sweden and Denmark, respectively. Denmark has the worst relative 5-year survival rate of 40% [11]. Due to unspecific symptoms, or even lack of symptoms, the majority of ovarian cancers are diagnosed in stage III or higher [10], with a significantly worse prognosis compared to cancers in stage <II. Ovarian cancer is staged by the staging system of the Fédération Internationale de Gynécologie Obstétrique (FIGO) [12]. The FIGO staging system evaluates ovarian tumors according to their spread, where stage I cancer is localized to the ovaries, while stage IV cancer has widespread metastases. Stage I cancer confers a 5-year survival rate of >90% compared to <30% for FIGO stage III tumors, while the overall 5-year survival rate of ovarian cancer is 47% [13].

Several attempts have been made to improve diagnostics, e.g. through population-based screening like in breast cancer. However, two large studies (Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO, US) and UK Collaborative

Trial of Ovarian Cancer Screening (UKCTOCS, UK)) have found no evidence that population based screenings using currently available methods would increase the survival of ovarian cancer patients [14, 15].

Epithelial ovarian cancer

Ovarian cancer constitutes two tumor types: Epithelial and non-epithelial ovarian cancer. Epithelial ovarian cancer (EOC) accounts for 90% of all ovarian cancer cases while non-epithelial ovarian cancer, mainly germ cell and sex cord-stromal cell cancers account for 10%. EOC is further divided by histological appearance into five main subgroups: High-grade serous ovarian cancer (HGSOC) (70%), ovarian clear cell cancer (OCCC) (10%), endometrioid cancer (10%), low-grade serous ovarian cancer (LGSOC) (<5%) and mucinous cancer (3%) [16, 17]. These cancer subtypes comprise 95% of EOC, while the rest are of other or mixed subtypes, e.g. carcinosarcomas and undifferentiated cancers. Henceforth, the term "ovarian cancer" refers to EOC unless otherwise specified.

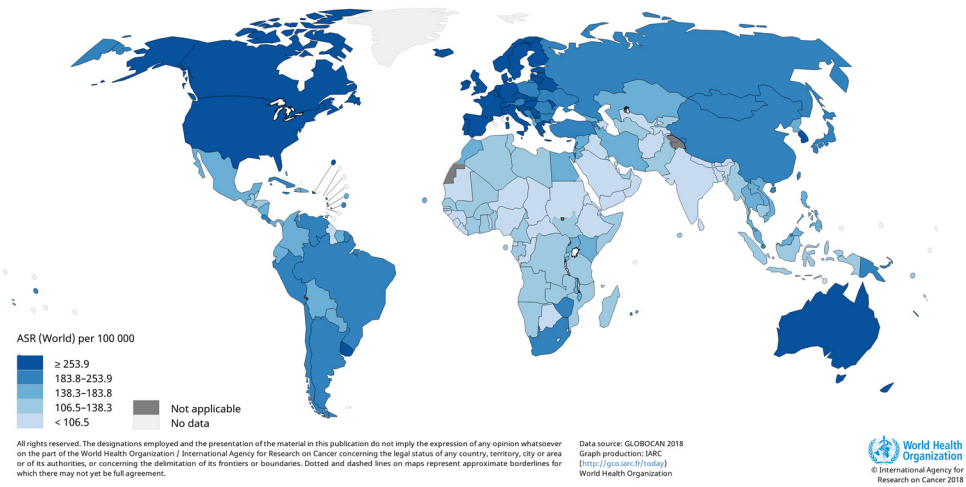

Figure 2: World wide cancer incidence
Estimated age-standardized global cancer incidence rates in 2018 [5]. Lighter blue represents lower incidence compared to darker blue.

The dualistic model

Up until 2004, the absence of malignant precursors and the theory of incessant ovulation had led to the belief that ovarian cancer developed *de novo* [18, 19]. This paradigm was challenged in 2004 by Ie-Ming Shih and Robert J. Kurman and their theory through which ovarian cancer was divided into type I and type II tumors,

each with different extra-ovarian origins [20]. The tumor type characteristics are outlined in Table 1 following the updated theory by Kurman *et al.* (2016) [21].

Table 1: Characteristics of Type I and Type II tumors

	Type I	Type II
Behaviour	Indolent	Aggressive
Genetic stability	Stable	Unstable
***TP53*-mutation frequency**	Low	High
***BRCA1/2* mutation frequency**	Low	High
Proliferativ	No	Yes
Histological subtypes	OCCC	HGSOC
	Low-grade endometrioid cancer	High-grade endometrioid cancer
	Mucinous cancer	
	LGSOC	
Precursor lesions	Borderline tumors	Serous tubal intraepithelial carcinoma (STIC)
	Endometriosis	

Evidence for the dualistic theory of ovarian cancer has been provided by several studies in the past decade [16, 22]. The emergence of next generation sequencing (NGS) provided the platform for an in-depth analysis of the fallopian tubes as the site of origin for HGSOC through the common clonality of serous tubal intraepithelial carcinomas (STIC)s and HGSOC [23]. Furthermore, GEX analysis including normal tubal epithelium also found evidence supporting the dualistic theory, with tumors correlating with extra-ovarian tissue of e.g. the fallopian tube [24-26].

The theory has proved robust, both genetically and molecularly, and explains the difference in proliferation and aggressiveness between the tumor types in the two groups. Type I tumors present with a better prognosis following radical surgery, but respond less well to chemotherapy, which is probably due to their low proliferation (Table 1) [27].

The dualistic model could also help explain why early detection methods for ovarian cancers have so far proved insufficient [15, 28], since current screening methods (transvaginal ultrasound and cancer antigen 125 (CA-125)) are not targeting the sites of ovarian cancer initiation. Furthermore, the dualistic model may support that prophylactic surgeries can be restricted to salpingectomies, rather than salpingo-oophorectomies, in order to reduce ovarian cancer risk [29].

Molecular subtypes in ovarian cancer

Gene expression (GEX) data for molecular subtypes

Given the importance of GEX based molecular subtypes in e.g. breast cancer, efforts have been made in ovarian cancer to identify clinically relevant subtypes [30, 31]. The first attempt to identify such molecular ovarian cancer subtypes was published by Tothill *et al.* (2008). In a cohort if primarily HGSOC and endometrioid ovarian cancer they identified 6 subgroups: C1: high stroma, C2: high immune signature, C3: low-malignant potential, C4: low stromal response, C5: mesenchymal, low immune signature and C6: low-grade endometrioid. The names were associated to the function of the majority of the genes found in the respective subgroups' molecular classifiers, and the subgroups were associated with clinical outcome.

The Cancer Genome Atlas (TCGA), analyzing only HGSOC, replicated the subgroups of C1, C2, C4 and C5 in their cohort and renamed them into C1: mesenchymal, C2: immunoreactive, C4: differentiated and C5: proliferative; however they failed to link the subgroups to clinical outcome, while the refined nomenclature stayed on [32]. Further attempts have been made to further expand and refine the molecular classifiers for the HGSOC subgroups [33-36] or even expand the subgroups outside that of HGSOC [37].

A recent study by Chen *et al.* (2018) evaluated the robustness of different molecular classifiers for ovarian cancer and found that they rarely performed satisfactorily outside of their test data sets [38]. The reasons for the poor performance are potentially many, however differences in clinicopathological characteristics, correct assessment of histological subtypes and heterogeneity in ovarian cancer are probable significant factors [39, 40]. Interestingly, a molecular classifier has not yet made it into a clinical setting.

Integrative approach to subtypes

Recent methodology for creating classifiers for molecular subtypes has seen the need for a more integrative approach as suggested by Bowtell *et al*. (2015) [41]. The concept is to combine data from several different platforms such as genomics, transcriptomics and proteomics, to create a multilayered data set which could capture the complex biology of ovarian cancer.

Currently both mutation and copy number signatures are now being explored in the predominant HGSOC subtype [42-45], however, some also outside of the HGSOC subtype [46, 47]. This could potentially lead to the discovery of complex, but clinically relevant subgroups in ovarian cancer.

Histopathology and molecular characteristics

Ovarian cancer is a highly heterogeneous disease, which is evident when looking at the tumors through a microscope (Figure 3). The five major subtypes of ovarian cancer: HGSOC, OCCC, endometrioid cancer, LGSOC and mucinous cancer display clear morphological differences and molecular differences.

This thesis mainly focuses on HGSOC and OCCC, and therefore these subtypes are described in greater detail below.

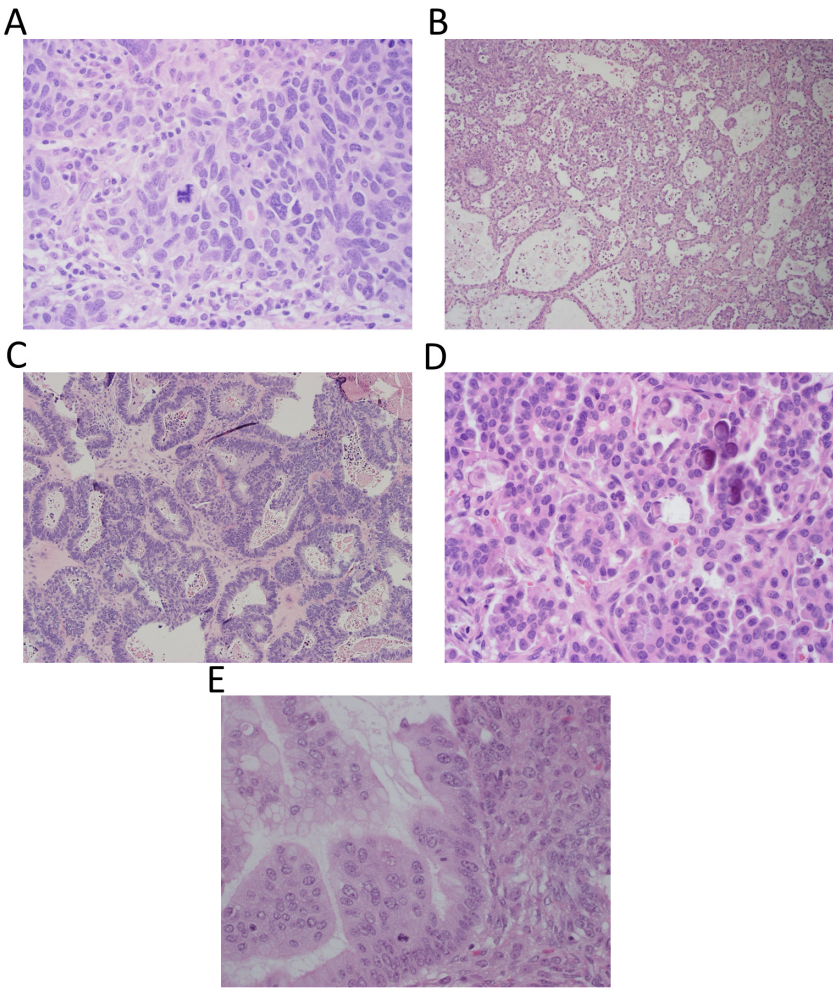

Figure 3: Photopmicrographs of histological subtypes in ovarian cancer.
A: High-grade serous ovarian cancer, B: Ovarian clear cell cancer, C: Endometrioid cancer, D: Low-grade serous ovarian cancer, E: Mucinous cancer. Published with permission from Anna Måsbäck, Department of Clinical Pathology, Skåne University Hospital, Lund.

High-grade serous ovarian cancer

HGSOC and the STIC

HGSOC is a type II tumor and the most common histological subtype of the ovarian cancers (Table 2). It is highly aggressive and is thought to arise from a STIC precursor lesion in the fallopian tube (Figure 4) [48]. STIC lesions may initiate malignant tumors in either the fallopian tubes or, if cells are shed to the ovaries, in the ovaries, and are collectively termed high-grade serous cancers. This is supported by the transcriptional resemblance of HGSOC cells with those of the fallopian tube epithelium (FTE) [26]. This has been supported by several studies providing a genetic link between STICs and HGSOC based on shared *TP53* mutations [23, 49-51]. The hypothesis of premalignant transformation of STICs as a precursor for HGSOC remains debated as studies in mice suggested that ovarian surface epithelium (OSE) can initiate HGSOC, even if the fallopian tubes are removed [52]. Even though HGSOC can develop after removal of the fallopian tubes, this might reflect endosalpingiosis, where normal oviductal tissue is displaced to the omentum or peritoneum [53]. The tissue can then hypothetically initiate the same malignant transformation and subsequently develop into HGSOC.

Table 2: Differences between the major subtypes of ovarian cancer

*: Prognosis in other subtypes are compared to HGSOC

	HGSOC	OCCC	Endometrioid	Mucinous	LGSOC
Cases	70%	10%	10%	5%	<5%
Stage at diagnosis	Advanced (>III)	Early (I or II)	Early (I or II)	Early (I or II)	Early (I or II)
Suggested precursor	Fallopian tube STIC	Endometriois	Endometriois	Adenoma, teratoma	Serous borderline tumor
Genetic risk	*BRCA1/2*	Lynch syndrome	Lynch syndrome	Unknown	Unknown
Genetic alterations	*TP53* *BRCA1/2* Genomic instability	*HNF-1β* *ARID1A* *PTEN* *PIK3CA*	*PTEN* *CTNNB1* *ARID1A* *PIKC3CA* *K-RAS*	*K-RAS* *ERRB2*	*B-RAF* or *K-RAS*
Response to chemotherapy	Good	Poor	Poor	Poor	Poor
Prognosis*	Poor	Moderate	Good	Good	Good
Ovarian cancer type	II	I	I	I	I

One of the earliest events preceding STICS is the occurrence of a benign "p53-signature" in the fallopian tube secretory epithelium cells (FTSEC) (Figure 4) [54]. The p53-signature is defined as non-proliferative but shows evidence of DNA damage. However, additional genetic mutations can ultimately drive the p53-signature from its benign state to a malignant one, eventually leading to HGSOC

[39, 48, 51]. These events result in a high frequency of *TP53* mutations in HGSOC (>95%). Recent studies have shown that PAX8, a transcription factor which is expressed in FTSEC, might also be involved in HGSOC carcinogenesis. A study by Adler *et al*. (2017) reported that knockdown of *PAX8* significantly reduced tumorigenicity both *in vitro* and *in vivo* [55]; however the role of PAX8 is to be further evaluated [41].

Half of HGSOCs show homologous recombination deficiency (HRD)

Approximately 50% of HGSOC tumors have been found to have aberrations in HRD associated genes, including *BRCA1/2* mutations (15%) [32, 41, 56]. This has led to the use of Poly (ADP-ribose) polymerase (PARP)-inhibitors for the treatment of HGSOC [57].

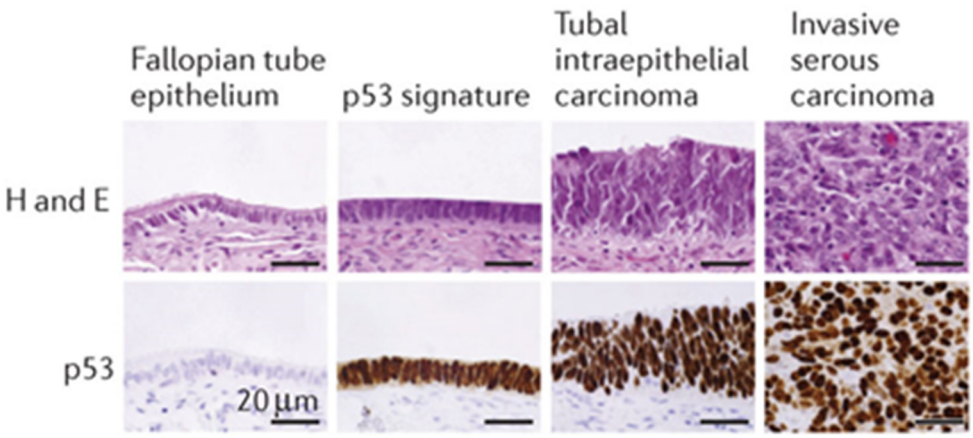

Figure 4: Development of STIC lesions in FTSEC
The development of STIC lesions in the fallopian tube secretory cells from left to right. The genomic abberations incease as the p53-signature transform into STICs. Top panels are hematoxylin and eosin stains. Bottom panels are p53 stains. Notice the loss of single cell layers in the last two panels. Adapted by permission from Springer Nature: Nature, Nature Reviews Cancer, Rethinking ovarian cancer II: reducing mortality from high-grade serous ovarian cancer, David D. Bowtell, Steffen Böhm,[…]Frances R. Balkwill), 2015. [41].

The remaining half do not show evidence of apparent HRD defects, but amplifications of *CCNE1* (Cyclin E1), *MYC*, *PIK3CA* and *MECOM* are frequent (>20%), and Cyclin E1 overexpression in FTSEC with a present p53-signature has been suggested to drive the transformation of the signature towards STIC [56, 58].

HGSOC is often diagnosed in advanced stages, and although initial response to platinum-based chemotherapy is often good, 70% of the patients experience relapse and development of platinum resistance is common. There are many mechanisms responsible for platinum resistance, however, the initial clonal diversity of HGSOC might be a contributing factor. The analyses of recurrent tumors and their drivers are currently insufficient, but a study by Patch *et al.* (2018) showed *CCNE1*

amplification and *BRCA1/2* reversions in platinum resistant recurrent tumors [59]. Other evidence points towards the inhibition of AKT signaling as a potential treatment and a phase IB dose-escalation study of Afuresertib (AKT inhibitor) in recurrent platinum resistant ovarian cancer is underway [60].

Microscopically, HGSOCs have a heterogeneous papillary growth with intermediate to highly atypical cells. Immunohistochemical stains for PAX8, Wilms tumor protein 1 (WT1), and p16 are positive, while a high nuclear expression of Ki67, indicating high proliferation, is also seen [16]. Furthermore, p53 staining is generally aberrant (not wildtype).

Ovarian clear cell cancer

OCCC is a rare type I tumor accounting for 5-10% of all ovarian cancer cases in Europe and North America, while the incidence in Asia is 15-20% [61, 62]. The reasons for this remain unknown. Although OCCC is considered chemo resistant [63], it often presents as stage I disease and the overall prognosis is generally good, with a 5-year survival rate of >85% [64]. Approximately 30% of OCCC patients experience a relapse from a primary stage I disease, and following relapse the prognosis is even worse than that for HGSOC [65].

ARID1A, PIK3CA and endometriosis drives OCCC

Several studies have linked OCCC carcinogenesis to endometriosis (See Risk factors), and OCCC shares similarities in its mutational profile with endometrioid ovarian cancer, another endometriosis associated subtype [22]. *ARID1A*, a gene in the SWItch/Sucrose Non-Fermentable (SWI/SNF) complex, and *PIK3CA*, a gene for one of the subunits of phosphatidylinositol 3-kinase of the PI3K/AKT/mTOR pathway, are found mutated in 40-50% of all OCCCs [66-68]. Mutations in the tumor suppressor gene *PTEN* and the oncogene *KRAS* are also frequent [67, 69]. Co-occurrence of mutations in *ARID1A* and *PIK3CA* are common and are thought to drive OCCC carcinogenesis [66, 70, 71]. Interestingly, OCCC patients with endometriosis have been associated with improved outcome compared to OCCC patients with no endometriosis [72]. The transcription factor Hepatocyte Nuclear Factor 1-Beta (HNF-1β) is upregulated in OCCC, which has been associated with the unique methylation profile of OCCC compared to the other subtypes of ovarian cancer. HNF-1β has been found to methylate several promotors in the estrogen receptor α (ERα) pathway [73].

Lynch syndrome, a disease characterized by mutations in DNA mismatch repair genes and microsatellite instability, is associated with an increased risk of OCCC [74]. Lynch syndrome is also associated with better survival [75]. A report by Jönsson *et al*. (2014) found that Lynch syndrome associated endometrioid, but not

OCCC, cancers had a distinct GEX profile [76]. This indicates that OCCCs may have a strong histology-related GEX profile, regardless of Lynch syndrome status. This is supported by other studies which found that the GEX profile of OCCC is unique compared to other ovarian cancer subtypes [77, 78]. A recent integrative study of the kinome from tumors from 124 patients with OCCC revealed inhibitors of the PI3K/AKT/mTOR and RAS/ERK pathways in combination as potential drugs for OCCC [79].

OCCCs display large round cells with a clear cytoplasm, hence the name, or hobnail cells containing abundant glycogen (Figure 3B) [80, 81]. Immunohistochemical stains are positive for Napsin A and HNF-1β and negative for WT1, ER and progesterone receptor (PR).

Endometrioid cancer

Endometrioid ovarian cancers account for 10% of all ovarian cancers and are often associated with endometriosis (Table 3) [16, 22]. Endometrioid cancers usually present in early stage, correlating with a good prognosis [82]. The relative 5-year survival rate of early stage endometrioid cancer is >80% [64].

Endometrioid cancer can be both low- and high-grade, with low-grade endometrioid cancer being the most common [16]. Low-grade endometrioid cancer frequently harbors mutations in *ARID1A* and *PTEN* and these mutations are thought to drive carcinogenesis. Evidence suggests that through a common precursor, endometriosis, [22, 83] and either a co-occurring *PTEN* or *PIK3CA* mutation, either endometrioid (*PTEN*) or OCCC (*PIK3CA*) cancers can arise [71]. Furthermore, *CTNNB1* is frequently mutated (40-50%) [16]. High-grade endometrioid cancer resembles HGSOC, with frequent *TP53* mutations [66].

Like OCCC, endometrioid cancers are also linked to Lynch syndrome [74], however these cancers express distinct GEX profiles compared to sporadic endometrioid cancer [76].

Immunohistochemical stainings of endometrioid cancers are positive for PAX8, ER, PR and they are p53-wildtype.

Low-grade serous ovarian cancer

LGSOC accounts for <5% of all ovarian cancers and corresponds to serous ovarian cancers previously classified as grade 1 [17]. LGSOCs are generally diagnosed at earlier stages compared to HGSOCs [17], with a good prognosis to follow and a relative 5-year survival of >80% [64]. Mutations in *KRAS*, *BRAF*, or *ERBB2* are

mutually exclusive and are collectively detected in approximately 60% of LGSOC tumors [16]. As opposed to HGSOC, *TP53* mutations are rare.

Immunohistochemical staining is positive for PAX8, ER, PR and WT1, while wild-type p53. The Ki67 nuclear expression is low.

Mucinous ovarian cancer

Mucinous cancers constitute 5% of all ovarian cancers [16]. These tumors often present at stage I but can be composed of large adnexal masses. The overall prognosis is favorable, with a relative 5-year survival of >80% [64]. Microscopically, mucinous cancers are highly heterogeneous, with the presence of both borderline, benign-appearing and invasive components in one tumor. *KRAS* mutations and *ERBB2* amplification occur in 40% and 20% of mucinous ovarian cancers respectively and are mutually exclusive [84]. Immunohistochemical staining is positive for CK7, while other markers such as CK20 and CDX2 can be either positive or negative, possibly owing to the heterogenous appearance of the tumors.

Risk factors and prevention

Risk factors for ovarian cancer are well established [10, 85] and Table 3 lists some of them together with their associated relative risk. The relative risk compares the risk of disease for people with exposure to the factor to the risk for people with no exposure.

Table 3: Risk factors for ovarian cancer

*: The relative risk is compared between subjects exposed to the risk compared to people not exposed to the risk

	Factor	Relative risk*
Increased Risk		
	Familial history	
	- First degree relative	4.3
	- Second-degree relative	2.1
	Genetic predisposition (*BRCA1/2*)	11.8 / 5.3
	Hormone replacement therapy	1.2
	Excess bodyweight	1.1
	Endometriois (OCCC and endomtrioid)	1.5
	Smoking (Mucinous)	1.8
	Lynch syndrome	1.1
Decreased Risk		
	Tubal ligation	0.7
	Pregnancy (first birth)	0.6
	Use of oral contraceptive	0.6-0.8

As seen in Table 3 the strongest factors for increased ovarian cancer risk are familial history and genetic predisposition. Genetic counselling with regards to risk reducing surgery is therefore important for ovarian cancer prevention [86]. In contrast, oral contraceptives have been shown to decrease ovarian cancer risk but increase breast cancer risk [87]. Furthermore, endometriois and Lynch syndrome are associated with OCCC and endometrioid cancers [22].

Steps should be taken to reduce the risk of ovarian cancer as survival is associated with stage at diagnosis for all histological subtypes [64]. Early detection or prevention (e.g. through risk reducing surgery) of ovarian cancer are the most cost-effective opportunities for ovarian cancer patients especially in high risk groups such as *BRCA1/2* mutation carriers [88]. However, as current screening methods fall short [15, 28], new methods are being evaluated. Most of these methods rely on NGS and one of the most promising is the PapSEEK method which is based on mutational analysis of vaginal samples to detect ovarian cancer [89]. Circulating tumor DNA has also been proposed as a means to detect ovarian cancer [90, 91].

Ovarian cancer in the clinic

The treatment modalities for ovarian cancer have not changed much during the last 20 years. Radical surgery followed by platinum-based chemotherapy treatment combined with paclitaxel is the current treatment regardless of histological subtype [27, 92]. The ongoing TRUST study [93] aims to evaluate the use of neo-adjuvant therapy before surgery as opposed to upfront surgery, while the DESKTOP study will evaluate the effect of surgery following relapse [94].

CA-125 has been the topic of controversy for its effect in the screening setting with a sensitivity of 80% and specificity of 75% [15, 28]. Even though elevated CA-125 levels are associated with ovarian cancer, the prognostic and predictive value is low at best [95-97].

Targeted therapies

Recent years have seen an increase in targeted therapies, some of which have shown promise in ovarian cancer. Bevacizumab, an angiogenesis inhibitor targeting the VEGF receptor, has proved useful in post-operative treatment after macroscopically non-radical surgery and in the case of platinum resistant disease [27]. Its effect on overall survival benefit seems modest, rather it may improve the disease-free interval [98-100].

Olaparib, a PARP-inhibitor, was approved for treatment in *BRCA1/2* advanced ovarian cancer patients in 2014 in the US and further accepted as a maintenance therapy for all chemo-sensitive ovarian cancers in 2018 [101]. Initial results from a

Swedish registry study, where olaparib was approved for platinum sensitive recurrent *BRCA1/2* ovarian cancer patients in 2015, suggests that olaparib is well tolerated and initial overall survival is good [102].

While the most dominant prognostic markers in ovarian cancer are FIGO stage and residual tumor following surgery [12, 64], only one predictive factor currently exists in the clinic, the *BRCA1/2* status for the use of PARP-inhibitors [50, 103]. The reason as to why so few treatment predictive factors have been identified is probably the same as why molecular subtyping has failed in ovarian cancer, namely the high degree of heterogeneity (See Molecular Subtypes in ovarian cancer). Recent studies suggest that integrative analysis of the genomic, transcriptomic and proteomic landscape might be able to identify ovarian cancer subgroups that can benefit from different treatments. The success of immunotherapy in other tumor types has initiated studies into the use of immunotherapy also in ovarian cancer [104, 105].

Despite evidence of high ER, PR and androgen receptor (AR) expression in ovarian cancer, the use of endocrine therapy has not been proven effective [106-108]. A large meta-analysis of endocrine therapy in ovarian cancer by Paleari *et al.* (2017) reported a potential benefit for endocrine therapy in ovarian cancer [109]. However, the true effect of endocrine therapy might be obscured by the fact that patients in clinical trials are heavily pretreated and sex hormone receptor expression changes following treatment are not assessed [110].

Aim

The aim of this thesis was to improve the understanding of biological events driving ovarian cancer, and to translate these events into clinically relevant observations using different biomolecular approaches.

The specific aims of the studies were to:

Study I

Investigate the prognostic effect of sex steroid receptor hormone expression and co-expression in ovarian cancer and their prognostic and potential predictive value.

Study II

Find potential treatment candidates in OCCC using integrative bioinformatics based on multilevel 'omics data from OCCC tumors.

Study III

Evaluate the potential treatable candidate of study II in OCCC cell lines, to assess whether integrative analyses for the discovery of potential treatment candidates would be of benefit in OCCC.

Study IV

Evaluate the use of high sensitivity ddPCR for screening of *TP53* mutations in a small cohort of women with vaginal samples collected *pre-symptomatically*, for the potential early detection of ovarian cancer. To our knowledge, this is may be one of the first studies of its kind.

Experimental and methodological considerations

An overview of the materials and methods used in this thesis is found in Table 4. The following section provides a brief outline of the main methods and the experimental and methodological considerations of the studies. For a detailed report on methods and experimental setups please refer to the appended papers.

Table 4: Overview of the materials and methods used in studies I-IV

Study	Design	Materials	Methods
I	Cohort study	Tissue micro array (TMA) with 87 serous and 31 endometrioid tumors	- Immunohistochemical staining and evaluation of ERα/β, PR and AR expression
		GEX data from an external independent dataset of 246 serous malignant, 20 endometrioid, 18 low-malignant potential serous and 1 adenocarcinoma	- Analysis of corresponding mRNA expression profiles in the independent dataset - Survival analyses
II	Integrated multilayered *In silico* study	Cohort 1: GEX data from 31 HGSOC, 18 endomtrioid cancer, 15 OCCC and 3 mucinous cancers	- Significance analysis of microarray (SAM) - Targeted DNA sequencing of a 60 gene panel
		Cohort 2: DNA from 10 Formalin-fixed paraffin embeded (FFPE) samples from OCCC patients	- Integrative bioinformatics analyses of GEX data and sequencing data -Immunohistochemistry (IHC)
		Cohort 3: TMA with 43 OCCC tumors	
III	*In vitro* study	Three OCCC cell lines: JHOC-5, OVMANA and TOV-21G	- Dose-response assays for single and combination treatments - Fluorescent imaging
		One HGSOC cell line: Caov3	- Cell response analysis by FACS, immuno-blotting
IV	Cohort study	Cohort of 9 ovarian cancer patients with prediagnostic (presymptomatic) and diagnostic vaginal samples	- Targeted sequencing using the INVIEW OncoPanel - Droplet digital polymerase chain reaction (ddPCR)

Patient cohorts

Biological and clinical composition

One of the most crucial steps applying to all experimental designs is the selection of cohorts or models to best represent the problem. It can be difficult to design an optimal study, especially when working with cancers with a distinct biological distribution of subtypes, such as in ovarian cancer. For ovarian cancer, the general guidelines for classification of tumors are issued by the World Health Organization (WHO) and the classifications are under constant revision [17]. Reclassifications have the potential to alter conclusions significantly. Therefore, when evaluating findings from other and older studies, such considerations should be corrected for. This follows for studies using old data sets as well.

For comparisons between groups one should strive for an equal sample size of each group. But biology can complicate matters. There can be biological or clinicopathological differences for which it is impossible to correct for when designing a study, e.g. age distributions between subgroups. Such factors must be corrected for in later analyses.

The cohorts used in study I, II and IV are outlined in Table 5.

Table 5: Overview of the cohorts included in study I, II and IV
GEX: Gene expression, TMA: Tissue microarray, FFPE: Formalin-fixed paraffin embedded.

	Study					
	I	I	II	II	II	IV
	Cohort 1	**Tothill *et al.* [25]**	**Cohort 1**	**Cohort 2**	**Cohort 3**	**Cohort 1**
Origin of data	TMA - Protein	GEX – mRNA (GSE9899)	GEX – mRNA (GSE37394)	FFPE tumor - DNA	TMA - Protein	Fresh frozen tumor - DNA
Number of patients	118	285	67	10	43	9
Median age (years)	58	59	51	48	63	57
Range	26-83	22-80	27-78	34-60	41-90	50-70
Histology (%)						
Serous	87 (74)	246 (86)	31 (46)			9 (100)
Endometrioid	31 (26)	20 (7)	18 (27)			
Clear cell			15 (22)	10 (100)	43 (100)	
Mucinous			3 (5)			
Serous, low-malignant potential		18 (6)				
Adenocarcinoma		1 (1)				
Stage (%)						
I	15 (13)	24 (8)	28 (46)	7 (78)	27 (63)	
II	16 (14)	18 (6)	9 (15)	1(11)	6 (14)	3 (33)
III	70 (59)	217 (76)	20 (33)	1(11)	9 (21)	2 (22)
IV	17 (14)	22 (8)	4 (7)		1 (2)	4 (44)
Missing		4 (1)		1 (11)		
Blood samples				0		8

The tissue microarray (TMA) from study I lacked information on residual tumor following surgery, which is a strong prognostic factor in ovarian cancer [111]. Information regarding chemotherapy was also missing for a subset of patients (21%). The missing information was subsequently handled in the analyses of the study when possible. Furthermore, a GEX data set from a study by Tothill *et al*. (2008) was available online (GSE9899, Table 5) [25].

The three cohorts used in study II were a result of optimizing the number of samples for the integrative approach. GEX data from patients from cohort one were available online (GSE37394, Table 5) [76]. DNA from tumors in cohort 2 of study II was derived from formalin-fixed paraffin embedded (FFPE) tissue. DNA from FFPE samples has a lower quality and care should be taken when analyzing data from such samples. The TMA of 43 OCCC patients in cohort 3 is interesting because of the low occurrence of OCCC and provides a basis for further studies in OCCC. There was an overlap of two patients between cohort 1 and 2. The lack of overlap between samples is a limitation to the study; however, with so few OCCC cases such limitations are common. One way of overcoming such a problem is through collaborations, but problems with data and sample sharing can complicate things.

Tissue Microarray

In study I and II we used TMAs to analyze several protein expression levels in tumors from patients (Figure 5). The history of the TMA dates to 1986 when H. Battifora developed a multi-tumor tissue block with more than 100 tissue samples [112]. This was later refined by Kononen *et al*. (1998) and named the Tissue microarray [113]. The TMA can contain hundreds of 0.6-2 mm cylindrical cores taken from FFPE tumor blocks. This allows for analyses of DNA and RNA levels using *in situ* hybridization and protein expression using conventional immunohistochemistry (IHC) across multiple samples simultaneously. Besides the advantage of analyzing multiple samples, the TMA allows for a series of advantageous methodological considerations, such as experimental uniformity, decreased assay volume and preservation of the original FFPE blocks from which the TMA is constructed.

However, a limitation of the TMA is the limited size of the cylindrical core. Especially for highly heterogenous tissues such as ovarian cancer this provides a challenge [114]. Therefore, careful consideration should be taken when constructing a TMA such that the scientific question asked can be answered. Furthermore, when designing a study using a pre-existing TMA, the quality of the associated patient information should be carefully examined as this will determine the usefulness of the TMA for the study in question. However, if such problems are considered, a TMA can provide information which is in concordance with corresponding studies of full size sections [115].

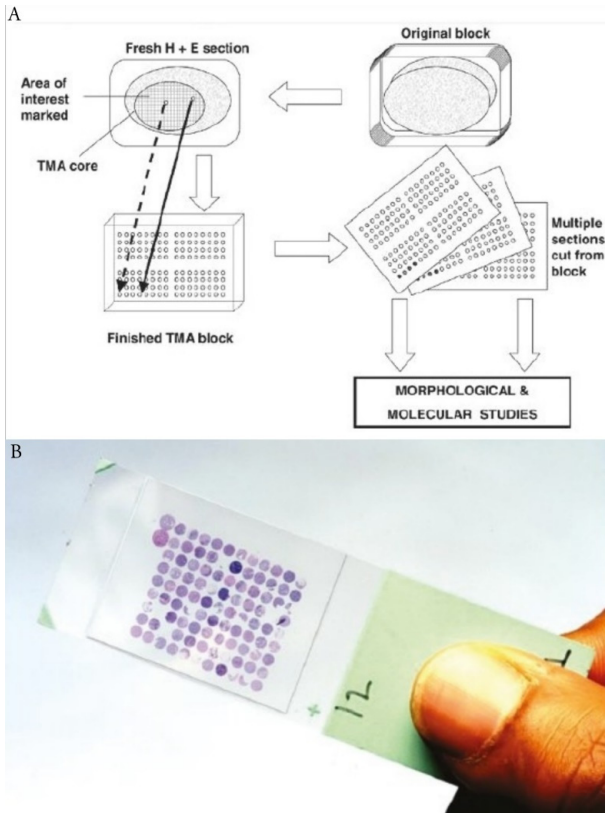

Figure 5: Illustration of the principle of a TMA.
A: The construction of a TMA starts with a series of FFPE blocks with the tissues of interest. These are marked for area of interest, and a cylindrical core (TMA core) is taken from the marked area and transferred to the TMA. B: A tissue microarray slide. Figure is adapted from the work of Nazar M.T. Jawhar (2009) [114].

Immunohistochemistry

The history and basis of IHC

In 1876, Wissowzky (1876) described the use of hematoxylin and eosin to visualize blood cells from mammals [116] and in 1941 Coons *et al.* (1941) reported the first use of an antibody conjugated with a fluorescent probe [117]. This provided the basis for IHC and its use in everything from classification of tissues to evaluation of protein expression. This changed the morphologists into pathologists and the use of IHC is a cornerstone in modern day medical science [118].

IHC is a technique often used for the evaluation of protein expression in FFPE tumor sections of around 3-5 μm in thickness. TMAs are often used for IHC staining, while cell lines can also be used (immunocytochemistry).

The most commonly used technique (indirect) evolves around the binding of an antibody to the protein of interest. After the binding, a secondary antibody is then added which binds to the first antibody. The secondary antibody is conjugated with an enzyme, usually horseradish peroxidase. Horseradish peroxidase can catalyze the oxidation of substrates such as 3,3'-diaminobenzidine, which then turns brown. The brown color can then be analyzed as a readout of protein expression.

Several factors affect the results from IHC, such as antibody clonality, affinity, stability and specificity, while the tissue itself is also a factor [119, 120].

The use of IHC in study I and II

We used indirect IHC in the TMAs of study I and II to assess different protein expression levels. To assess the protein expression, the scores from blinded assessments by at least two readers were averaged. We used predefined cut-off values for positivity in study I. Samples were positive if $\geq$ 10% of the cells were positive for protein expression of ERα/β, PR or AR. This approach dichotomizes the response variable. For study II dichotomizing cut-offs were also used. This approach ensures the least bias from subjectivity, however also tends to overestimate effect size [121]. Overestimation can be countered by several readers evaluating the expression independently, and subsequently averaging the scores. Ideally, samples should be blinded to ensure the least bias when evaluating the expression of the target protein. Differences between cut-off values and sample preparation between studies are a major factor for discrepancies in studies evaluating such effects. Efforts should therefore be made to use standard cut-off values or take these factors into consideration when interpreting results.

High throughput methods

Next Generation Sequencing

A brief overview and basis of NGS

Since the sequencing of the first protein coding gene, that of the coat protein of bacteriophage MS2 by Walter Fiers' laboratory in 1972, DNA sequencing has evolved [122]. With the introduction of the chain-termination sequencing by Sanger *et al*. (1977) [123], sequencing speeds increased significantly. First generation sequencers followed, which allowed for a fully automated approach. The publication of the polymerase chain reaction (PCR) method by Mullis *et al*. (1987) by which DNA could be multiplicated [124] paved the way for NGS by pyrosequencing in 1996 [125]. Pyrosequencing functions by emitting light when a

known DNA base is added by DNA polymerase during the sequencing. The principle of pyrosequencing is thus called sequencing-by-synthesis; if there is a flash of light, then a base has been added.

The 454 GS 20 was the first automated high throughput machine and was released by 454 Life Sciences in 2005 [126]. Subsequent systems followed, with Illumina systems currently dominating. Common for the next generation systems are that they monitor DNA-sequencing while it happens, through a combination of PCR and variants of pyrosequencing. Illumina systems use different colored fluorescently labelled DNA bases with unique colors for each DNA base. The color of the light emitted when the base is added during sequencing translates into a base.

NGS now allows for a precise (1/1000 error) sequencing of thousands of DNA samples in parallel, but the method does have its limitations. The importance of a study design has never been more important as the amount of information obtained through NGS is astronomical. The choice of method, whether whole genome sequencing, whole exome sequencing or a subset of genes through a targeted panel, requires careful consideration depending on the question to be investigated. Study designs for cancer research should strive to always include paired samples of normal and tumor from patients, in order to evaluate somatic and germline mutations.

Moreover, the NGS platform has evolved and more specialized applications are developed each year, and now not only DNA but also RNA can be analyzed by sequencing. Yet another method of great potential is the single cell sequencing method [127], by which we in the future can expect our knowledge of e.g. the effects of immunotherapy to greatly increase [128].

The use of NGS in studies II and IV

In studies II and IV we employed NGS to analyze tumor DNA from either FFPE tumors or fresh frozen tumors (Table 5). For study II we wanted to search for somatic mutations in DNA from FFPE OCCC tumors. This approach has its limitations due to the degraded state of the DNA, while our samples also lacked a paired blood sample to act as a control. We therefore chose to sequence the samples using the SureSeq™ Solid Tumour Panel (Oxford Gene Technology, UK) with reported success using DNA from FFPE tissue. Subsequently the results were screened with various parameters such as minor allele frequencies (MAF) to decrease the possibility of detecting germline mutations. Also comparing results to various online databases decreases such a risk. Furthermore, our group recently discovered significant differences in variance calls related to the combination of aligners and mutation callers (unpublished data), thus such combinations should be accounted for as well.

In study IV we chose the INVIEW Oncopanel All-in-one from (GATC, Germany), as we were primarily interested in sequencing *TP53*. The choice of panel in this case

was economical; however, we had paired blood samples from 8 of 9 patients, which greatly increased the value of the sequencing results.

Gene expression (GEX) analysis

A brief overview and basis of GEX arrays

The understanding of RNA was forever changed in 1954 with the introduction of the central dogma of molecular biology: DNA turns into RNA, and RNA into Protein [129]. The importance of RNA in the process led to a new platform of research called GEX studies. The GEX studies were used to detect and quantify messenger RNA (mRNA) levels of a specific gene. In 1977 the further development of the RNA-based GEX methods resulted in the Northern Blot by Alwine *et al.* (1977) [130]. The GEX microarrays which allow for the simultaneous evaluation of multiple genes under multiple conditions was published in 1996 by Shalon *et al*. [131]. However, currently with the emergence of NGS, RNA sequencing has become the golden standard for transcriptome analysis [132].

The principle of the GEX microarray assays is to extract mRNA, convert it into complimentary DNA (cDNA) and label the cDNA with biotin. The cDNA is then hybridized to the microarray, on which probe sets with known sequences (genes) hybridize to the cDNA. The subsequent binding of a readable conjugate to biotin then allows for the assessment of GEX by imaging of the array [132].

There are some concerns regarding the use of GEX microarrays that should be considered when analyzing data. Signal saturation and high background signal limits the detection rates, and it is difficult to compare results between arrays (batch effect). Furthermore cross-hybridization between probe sets of similar sequences also presents a significant problem. This is due to different probe sets hybridizing to different positions in the cDNA and hence can have sequences that identify multiple genes.

The use of GEX arrays in studies I and II

In study I we used an external GEX dataset from an online repository (Table 5). We followed the methods described in the original study for preparation of the data set, and subsequently identified the probe set with the highest possible consensus sequence with the target gene of interest.

In study II we used GEX data from the whole genome cDNA-mediated Annealing, Selection, extension, and Ligation (WG-DASL, Illumina) arrays, which make it possible to analyze 24,000 targets (genes) in 8 samples simultaneously. As we retrieved the collected data from an online repository (Table 5) most of the quality

control parameters had been evaluated. Therefore, the combined set of collected batch corrected raw data could be evaluated in our pipeline.

The raw data were quantile normalized, log2 transformed and subjected to a presence filter of 80% across probes, with a detection *p*-value ≤0.01, leaving 12,747 genes. We then analyzed genes for which multiple probe sets were present and chose the probes with the highest variance. This resulted in precisely 10,000 genes to be analyzed out of the starting pool of 24,000 (42%). We made sure that the data set was still normally distributed, and the subsequent analysis could continue.

As evident we chose two different approaches when analyzing the two GEX data sets. This represents one of the key concerns when evaluating results and comparing between studies. One approach cannot be ruled superior to the other, and as evident in the Results and discussion section, the choice of probe set can greatly impact a study, such as study I in our case. Ultimately, considerations for study design such as those for NGS are also appropriate in the GEX setting.

In silico studies, bioinformatics and statistics

A brief overview and basis of GEX and NGS analysis

Computers have existed in biology since the 1940's and bioinformatics as a term appeared in a Dutch article in 1970 [133]. However, the need for bioinformaticians increased after the completion of the human genome. An interesting notion was raised by Vincent *et al.* (2015) in their paper: "Who qualifies to be a bioinformatician?" [134]. They state that two branches exist in bioinformatics: computational biology and analytical bioinformatics. They continue to state that there exist both users of bioinformatics and experts in bioinformatics, and that most researchers (within the biological field) are users of bioinformatics and hence not bioinformaticians.

As high throughput methods generate large amounts of data, the downstream analyses of such data sets can be predefined by a good study design. Thus, the prime importance of bioinformatics for a biologist is to reduce huge amounts of data loosely associated to biology into smaller data sets that can form a stronger association with biology.

The availability and usability of bioinformatic tools for analyzing data is high and the online tools such as those presented by the Broad Institute (MA, US) are a prime example. They offer tools for the analysis of external data sets; however, they also provide online portals in which a scientist can explore pre-analyzed datasets. The TCGA recently launched the online platform: "Pan Cancer Atlas" (2018) (the conclusion of TCGA project by 27 papers in April 2018) also provides an example of how multilayered information becomes available through online platforms [135].

The online portals and databases can give a researcher a reliable overview of the current data in the field, and furthermore provide the basis for using bioinformatics to create initial hypotheses. One such data base was used in study II with great success. The Ovarian cancer database of Cancer Science Institute Singapore, is a transcriptomic microarray database of 3,431 human ovarian cancers with a simple online set up [136].

One of the strengths of bioinformatics is also one of its flaws. The data sets are often created to be explorative. Thus, the analysis of data is explorative, and therefore not testing a specific hypothesis rather than generating it. Furthermore, the quality of the sample input influences the results, and considerations of the ratio between tumor vs normal/stromal tissue should be taken into account [137].

The use of SAM in study II

In study II we used a commonly used method for comparing GEX data: the significance analysis of microarrays (SAM) developed by Tusher *et al*. (2001) [138]. Very simplified it is a modification of a t-test, accounting for the multiple testing by using permutation tests. SAM can be used to explore up- and downregulated genes between two or more groups.

In study II we used the free software Multiple Experiment Viewer (MEV, http://mev.tm4.org) tool box for the GEX SAM. The advantage of MEV is that it integrates multiple analyses alongside the SAM, e.g. subsequent hierarchical clustering. The resulting gene list of ~500 up and downregulated genes was analyzed by both online databases and by locally installed R software packages. Multiple analyses of a gene list can yield different results due to differences in algorithms. A comparison of the results from such analyses for overlap between two or more outputs will give indications of the robustness and functionality of the genes in the list.

However, the results should always be compared to other studies and their reported findings if available, so as to evaluate correlations between findings.

Premade pipelines for NGS analysis in study II and IV

For study II and IV we also evaluated NGS data from various tumor settings (Table 2).

Several different pre-made analysis pipelines are available for NGS data, and we chose the bcbio-nextgen (https://github.com/bcbio/bcbio-nextgen) pipeline for analyses of the sequencing data.

The advantages of bcbio-nextgen is the simultaneous use of multiple variant callers in the data. The GEnome MINIng (GEMINI) is also integrated [139], and is used for evaluating the results from an NGS experiment with the use of several reference

databases. Furthermore, the overlapping mutation calling allows to screen multiple callers with multiple aligners, thus accounting the difference between their algorithms.

The use of statistics in study I

The use of survival analysis and Kaplan Meier plots in study I was to evaluate the prognostic effect of ERα/β, PR and AR. Endpoints were progression free survival (PFS) and overall survival (OS). Hazard ratios (HRs) were calculated with 95% confidence intervals (CI) using both univariable and multivariable Cox regression. Multivariable Cox regression is used to adjust for factors known to affect the endpoints. In our case such factors included e.g. age, histopathological subtype and the presence of *BRCA1/2* mutations. Such corrections are important for assessing the strength of a potential prognostic variable. However, lack of information, such as information on residual tumor burden in our case, needs to be considered when evaluating the results.

The discussion of statistics is like the discussion of bioinformatics. Are biologists also statisticians or are they users? One key problem concerning many studies is that of choosing the right statistical test [140], especially when sample groups are small. As most biologists are only users, collaborations with statisticians are pivotal and such a collaboration has greatly improved the results of the studies in this thesis.

In vitro 2D Experimental Models

Cell cultures and drug screens

A brief overview and basis of cell culture analysis and drug screens

In vitro modelling of complex biological problems using cell cultures was first reported by Harrison (1907) [141]. Since then, cell cultures have been a cornerstone for experimental models for all kinds of scientific questions. Whether an established cancer cell line or a primary cell culture, these experimental models provide a glimpse of the underlying biology driving that system. Thus, changes to the system can be monitored, evaluated and extrapolated to the *in vivo* setting. These thoughts are derived from the principle of the three Rs, replace, reduce and refine outlined by Russell and Burch (1959) in their book: "The Principles of Humane Experimental Technique" [142]. Replacement concerns the idea of always trying to replace an experimental model with something of lower status, such as tissue cultures. They conclude that tissue cultures are an inexpensive and potentially fast solution for e.g.

drug screens. However, there are limitations such as the loss of the complex relationship of biology *in vivo* [142].

When screening the effect of a drug using *in vitro* models, the most used assay is that of a dose response curve. Adding drug formulations in a serial dilution will allow for an assessment of the drug sensitivity. One such variable is termed the half maximal inhibitory concentration (IC50) and is often used for comparing sensitivities between drugs. The development of the colorimetric sulforhodamine B (SRB) assay [143] laid the foundation for the high throughput screening method using 60 cell lines described by Monks *et al*. (1991) [144].

Recent advances in cell culturing have included the development of 3D assays, in which cells can mimic the behavior of an *in vivo* system to a higher degree than in more conventional 2D cell culture assays (refinement) [145].

Further analysis of molecular responses can also be assessed in cell lines and the characteristics of most ovarian cancer cell lines have been studied thoroughly [146, 147]. The methods to evaluate cellular responses are many and diverse, and considerations should be done as to assess the best method for evaluating drug effect.

The use of cell cultures to evaluate drug response in study III

In study III we used three OCCC cell lines and one HGSOC cell line for the comparison of drugs targeting Rho GTPases. Carboplatin was used as a comparison to the effect of standard treatment. SRB assays were used for creating dose-response curves, both for single drug treatments and combinations. Although reproducibility is high, optimization is required for SRB assays to function optimally. We chose three-day assays to evaluate IC50 concentrations of single and combination treatments using a 96 well plate setup. Furthermore, we used the methods suggested by Chou (2010) for the design of the combination studies [148].

The methods to evaluate the effect of the drugs should also be considered with regards to the hypothesis. We decided to evaluate the integrity of the cytoskeleton and migration using fluorescent microscopy in a 96 well plate setting. This was done to mimic the dose response assay setting in the best possible way.

Immunoblotting (western blotting) and fluorescence activated cell sorting (FACS) are commonly used methods for evaluation cellular response following treatments. We used FACS to assess cell cycle phase distribution, and western blotting to evaluate the effects of intra cellular signaling on the protein level.

The use of such methods should be evaluated carefully as the choice of experimental setup and design affects the outcome of the experiment. We chose an endpoint method for both the western blot and FACS analyses. Serial methods using different time points could potentially be used to evaluate the effect through the cells' life

cycle and the underlying biology. However, for the testing of our hypothesis, end point analysis was enough. Furthermore, the underlying biology is important, as illustrated by the western blot experiments; the cell lines responded differently, and conventional loading controls for western blots such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were not useful.

Droplet Digital Polymerase Chain Reaction (ddPCR)

The PCR method was published in 1987 by Mullis *et al*. [124] and the method was further developed during the next decade. The concept of copying DNA by the aid of specially designed primers laid the foundation for digital PCR (dPCR), with the publication by Vogelstein and Kinzler (1999) [149]. The main difference between PCR and dPCR is the partitioning of the PCR sample into multiple wells of a 384 well plate. Subsequently, the use of a Poisson distribution allows for the calculation of an absolute concentration, without the use of a standard curve such as in quantitative PCR (qPCR).

The improvement of the dPCR assays led to the introduction of Droplet dPCR (ddPCR). Whereas dPCR requires the portioning of the sample into multiple wells, ddPCR splits the sample into multiple droplets, each one a unique PCR reaction. The principle is outlined in Figure 6. The use of primers and fluorescently labelled probes designed to detect mutations can then be used to quantify mutations in individual samples. The advantage is that the ddPCR method is highly sensitive as the individual droplets are analyzed separately.

Limitations include that of both quality and quantity of the DNA used. The sensitivity is directly linked to the amount of DNA, with 100 ng of genomic DNA being the equivalent of 28,000 DNA molecules. Thus, a sensitivity of up to 1 in 10,000 can be achieved with 100 ng input, due to mathematical and experimental (signal to noise) limitations.

The use of ddPCR in study IV

In study IV we evaluated the use presymptomatic liquid based vaginal samples obtained from women prior to their HGSOC diagnosis. We evaluated the detection of *TP53* mutations found in the patients' tumors, in the vaginal samples using IBSAFE™, an improved ddPCR method developed by SAGA Diagnostics.

IBSAFE has a greatly increased signal to noise ratio, allowing for an increased specificity for accurate estimation of mutant/minor allele frequencies (MAF) at exceedingly low concentrations (~0.001% MAF), whereas Bio-Rad ddPCR assays suffer from false-positive noise below 0.1% MAF. Confidence in low abundance quantification is critical for early detection, although the available amount of DNA

in our samples is still a limitation of the study. A sensitivity can never be higher than that of the corresponding number of DNA molecules detected.

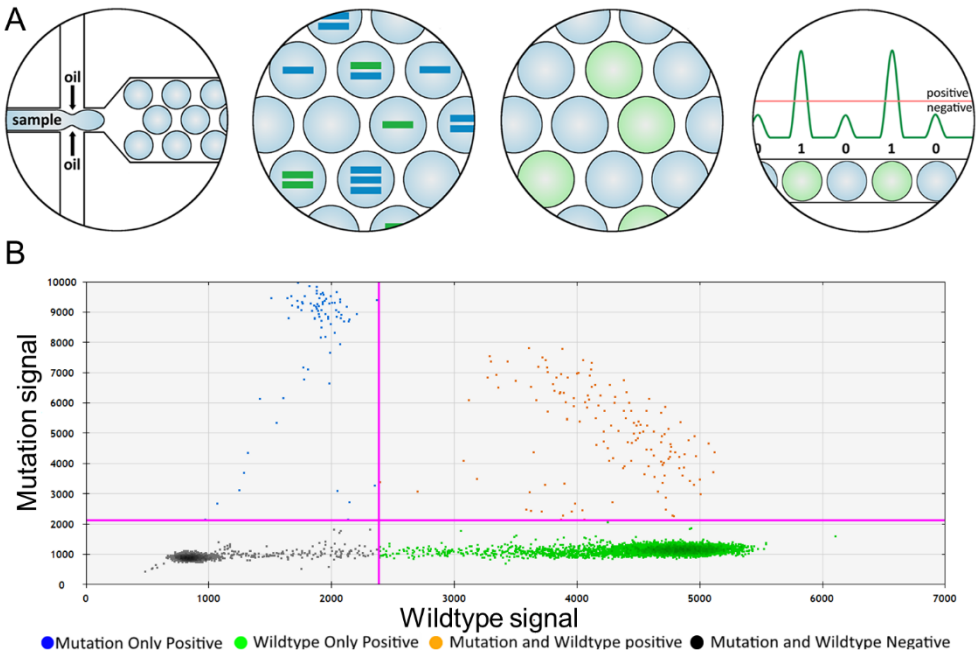

Figure 6: Principle of ddPCR
A: The sample is prepared as regular PCR, however with the addition of fluorescently labelled probes targeting the mutation of interest. The sample is then split into smaller droplets, amplified and each droplet is then measured. Figure is adapted from Verheul *et al.* (2016) [150]. B: The result of a ddPCR measurement of a vaginal smear sample from study IV. Each droplet is plotted according to both wildtype and mutation signal. Notice the smear in the bottom part of the figure. Blue: Mutation only droplets, Green: Wildtype only droplets, Orange: Mutation and wildtype positive droplets, Black: Empty droplets. The cutoff values for drop characterization are visualized by the pink lines.

Results and Discussion

Study I: Sex steroid hormone receptors and molecular subtypes in ovarian cancer

Sex hormones play a role in cancer – biomarker and targeted therapy

Sex steroid hormone receptors (hereafter referred to as hormone receptors) are expressed in the normal tissue of the female reproductive system [151]. Although hormone receptor functions remain a topic of discussion, their role in cancer progression within the reproductive organs of both males and females is established [152-155]. Hormone receptors have been studied extensively in breast cancer [156-158], and notably, the ERα expression displays distinct GEX patterns [159]. ERα expression also dictates the use of endocrine therapy treatment, as well as outcome in breast cancer [160-162]. The intrinsic molecular breast cancer subtypes demonstrated by Perou *et al.* (2000) [30], was elaborated by Sørlie *et al*. in 2001 with the finding that ERα positive tumors could be divided into two molecular subtypes with different clinical outcomes [31]. These findings have provided the basis for multigene tests such as Prosigna®, Oncotype DX® and MammaPrint®, which are used for clinical decision making with regards to adjuvant systemic therapies [163-167].

Six molecular subtypes in ovarian cancer

As previously stated in the introduction, the first attempt to identify molecular subtypes in HGSOC was reported by Tothill *et al*. (2008). They identified six molecular subtypes (C1-C6) based on GEX data in a cohort of mainly HGSOC and endometrioid cancers. The six molecular subtypes were named based on their distinct GEX profiles which led to the names: C1: High stroma, C2: High immune signature, C3: Low-malignant potential, C4: Low stromal response, C5: Mesenchymal, low immune signature and C6: Low-grade endometrioid. Tothill *et al*. found that HGSOC predominantly clustered into clusters C1, C2, C4 and C5, while endometrioid and low malignant tumors clustered into clusters C3 and C6 [25]. The reported subtypes were associated with outcome. Among the HGSOC clusters, C2 and C4 had a better prognosis than C1 and C5. The C1, C2, C4, and C5 subtypes were later supported by the TCGA project in their analysis of 489 HGSOCs. According to their classifications, the TCGA named the four subtypes:

Differentiated (C4), Immunoreactive (C2), Mesenchymal (C1) and Proliferative (C5) [32]; however the TCGA did not find any association between the subgroups and survival. A possible explanation could be the difference in tumor histology between data sets, with TCGA having only HGSOC.

Can hormone receptor expression improve prognostication?

Although similarities between breast and ovarian cancer have been reported [168-170], the prognostic effect of hormone receptors in ovarian cancer remains unclear. Therefore, we aimed to further characterize hormone receptor expression and their prognostic value in ovarian cancer in study I.

Hormone receptor positivity in study I

In a TMA cohort of 118 ovarian cancers (87 high-grade serous and 31 endometrioid ovarian cancers) the hormone receptor status of AR, ERα, ERβ and PR was evaluated using IHC. The prognostic value was then analyzed by survival analysis and by both uni- and multivariable Cox regression. Multivariate Cox regression was adjusted for clinicopathological factors such as grade, age and stage as these factors can predict prognosis independently [171]. We found AR positivity in 44% (52/118), ERα positivity in 44% (52/118), ERβ positivity in 82% (102/117) and PR positivity in 31% (36/118) tumors. While AR, ERβ and PR expression levels were comparable to previous findings, ERα expression was lower. However, ERα expression levels have been shown to vary greatly across studies [106, 108, 172, 173]. A review from 2016 by Voutsadakis reported positivity for ERα between 32% and 81% across seven studies [174].

AR and PR positivity are beneficial in ovarian cancer

We found that AR and PR positivity, either alone or in combination, were associated with lower grade, increased 5-year PFS and OS (Table 6), and these associations remained significant after adjusting for stage, grade, age at diagnosis, *BRCA* status and histology. The findings of PR positivity associated with improved outcome are in line with several studies of PR status in ovarian cancer [106, 107, 175]. The findings of AR positivity associated with improved outcome were in contrast to two previous studies [175, 176], while another study confirmed our findings [108]. Studies evaluating the prognostic effect of co-expression of hormone receptors are limited and have been centered around PR and ER expression [106, 177]. These studies show a positive effect on outcome. This was not found in our study, but instead a combination of AR and PR positivity was associated with better outcome. The interaction between hormone receptors is still unclear, but recent evidence suggests a complex interaction mechanism with feedback loops controlling each hormone receptor [152, 178].

Table 6. Key findings of study I.

Results from analyses of hormone receptor status and their association with clinical characteristics. *: Analyses were done using Fisher's exact test, **: Analyses were done using univariable Cox regression. HR: Hazard ratio, CI: Confidence interval.

Variable	Association	p-value	HR	95% CI	
AR+ vs AR-	Grade*	0.025			
	Age at diagnosis*	0.017			
	5 year progression free survival **	0.001	0.48	0.30	0.75
	5 year over-all survival **	<0.001	0.38	0.23	0.63
Erβ+ vs Erβ-	Grade*	0.016			
PR+ vs PR-	Stage*	0.006			
	Grade*	0.001			
	5 year progression free survival **	0.001	0.42	0.24	0.71
	5 year over-all survival **	<0.001	0.34	0.19	0.62
AR+/PR+ vs PR- and/or AR-	5 year progression free survival **	0.001	0.29	0.15	0.59
	5 year over-all survival **	<0.001	0.21	0.092	0.49

ERα and ERβ disagreements

ERβ positivity was associated with lower stage, which is supported by previous findings [179-181], however a recent study suggests that the specificity of ERβ antibodies is questionable. The interpretations of ERβ stains should therefore be considered carefully [119, 182]. We did not find any association between ERα levels and clinicopathological markers. The individual expression of ERα and its association to outcome are contradictory [173, 176, 177], while recent meta-analyses have reported that ERα does not correlate with outcome in ovarian cancer, supporting our findings [106, 183].

Sex hormone GEX is (not) prognostic in molecular subtypes

Next, we investigated the GEX levels of *AR*, *ESR1*, *ESR2* and *PGR* in the molecular subtypes in the Tothill dataset (Figure 7) [25].

We found that *PGR* and *ESR2* were upregulated in subtypes C3 - Low-malignant potential and C6 - Low-grade endometrioid, while *ESR1* was upregulated in subtypes C2 - Immunoreactive, C4 - Differentiated and C6 (Figure 7). These findings were consistent with the more favorable outcomes in the C2, C3, C4 and C6 subtypes as reported by Tothill *et al*. Using the median expression of the hormone receptors, we divided the subgroups into high and low expressing groups. We found no significant associations between 5-year outcome and hormone receptor high vs low GEX, either in in the full dataset or within the molecular subtypes. This might be explained by the already improved outcomes in subgroups with increased *ESR1* and *PGR* expression, thus removing the effect of further stratification, or it may be the relatively small size of the subgroups.

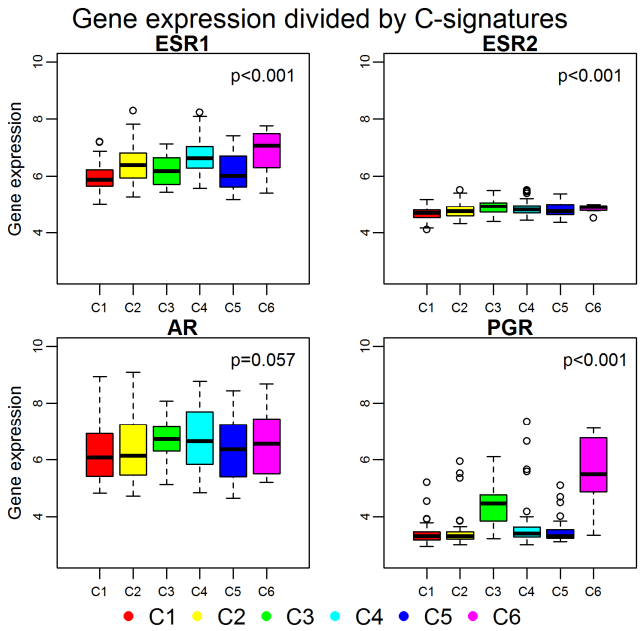

Figure 7: Gene expression of sex hormone receptors in C-signature subtypes.
GEX of sex hormone receptors in C-signature subtypes from Tothill *et al.* [15]. Red: C1 - Mesenchymal, Yellow: C2 - Immunoreactive, Green: C3 - Low-malignant potential, Light blue: C4 - Differentiated, Dark blue: C5 – Proliferative, Purple: C6 - Low-grade endometrioid. p-values were calculated using a Kruskal-Wallis test across all C-signatures.

Despite evidence of a strong effect of PR and AR positivity on improved PFS and OS in our TMA cohort, this was not captured in the independent external dataset when evaluating the entire cohort. Furthermore, individual GEX levels of the hormone receptors did not show any association with improved outcome either. This was surprising, as others have reported both *PGR* and *ESR1* GEX to have a prognostic effect, correlating with improved survival in ovarian cancer [184, 185]. Differences in the histological composition between studies might account for this, as prognosis is associated with histological subtype [106, 184, 185]. However, other factors such as residual tumor burden, grade and stage might affect the cohort as well. A study by Chan *et al.* (2006) of ovarian cancer survival associated with optimal surgery from 1988-2001 reported it to increase from 76.1% (1988-1992) to 87.9% (1998-2001) [186]. Although the residual tumor burden was evaluated in the Tothill dataset, 43% (125/285) of tumors lacked this information which is a critical prognostic factor.

Differences between probe sets provide an explanation

Although care should be taken when correlating mRNA and protein levels, several reports have indicated that there exists a high correlation between GEX and

corresponding protein abundance for the hormone receptors [187-190], and a study by Nagaraj *et al*. (2011) reported the correlations when using high throughput methods [191].

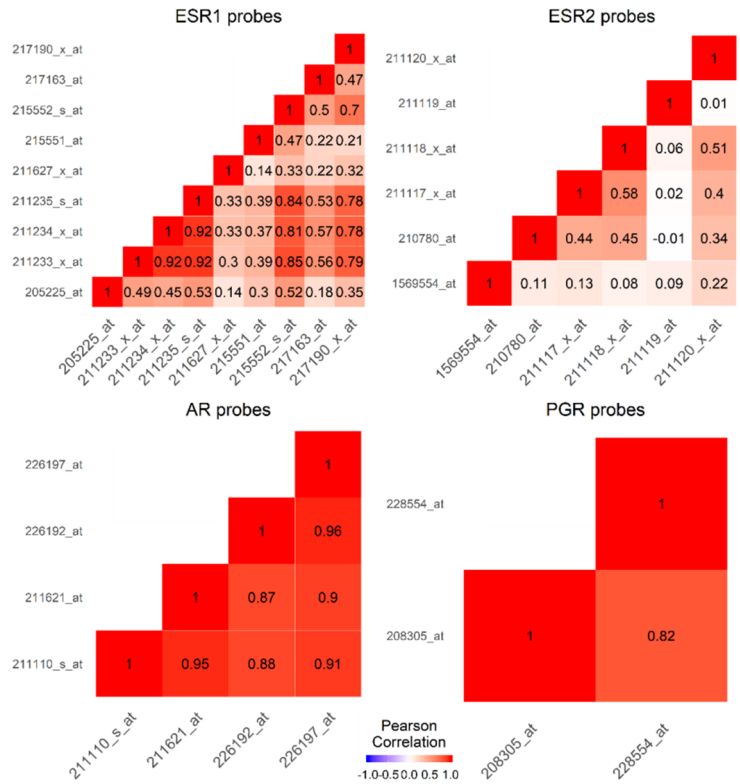

Figure 8: Correlation between probe sets for the hormone receptors.
Pearson correlation plots of probe sets from the GeneChip™ Human Genome U133 Plus 2.0 Array, identifying hormone receptor *ESR1, ESR2, PGR* and *AR* genes expression levels in an independent data set of 285 ovarian cancers [25].

The lack of association in the independent dataset could be due to the choice of probe set used for GEX. Probe sets are short DNA sequences targeting a short region of a transcript for a gene. They are used to detect the presence of nucleotide sequences through hybridization. The probe sets can therefore be used to evaluate GEX (See Experimental and methodological considerations). Several probe sets recognized the same gene and as seen in Figure 8, the correlation between probe sets is highly variable, in particular in relation to *ESR1*. The correlation between the nine probe sets ranged from 0.14 to 0.92. Furthermore, the resulting survival analysis was also affected, and the nine probe sets produced p-values ranging from

0.01-0.67. This variation underlines the importance of choosing the most appropriate probe set.

The choice of probe set significantly impacts variability across samples and steps are taken to select the most appropriate probe. In the present study, we chose the probe set with the highest degree of similarity to the target gene (*ESR1*) using the Basic Local Alignment Search Tool (BLAST). If we had established other criteria for choosing probe sets, e.g. the most variable across samples or the mean/median of all probe sets, then the interpretation of the results may have been different. In our case, the most variable probe set across samples was also the one with the lowest score in the BLAST analysis, and therefore the variance was probably due to noise from cross-hybridization, rather than true biological sample variance.

The robustness of molecular subtypes in ovarian cancer

The findings that the choice of probe set can greatly impact results was no surprise. However, if we continue the line of thought into the context of molecular subtypes, some interesting observations can be made. Although the choice of probe sets is critical for analysis of array-based GEX levels, the sampling method is just as crucial. The amount of tumor tissue can vary in samples, which should also be taken into consideration when evaluating assays. Tothill *et al*. (2011) made efforts to mitigate these challenges by evaluating the tumor percentages. By comparing the GEX of normal tissue to their samples, they concluded that the tumor percentages did not significantly affect the GEX analysis.

Several other groups have attempted to discover molecular subtypes or elaborate on existing classifiers. Verhaak *et al*. (2013) elaborated on the four subtypes from the TCGA. They further found that tumors might be classified into several subgroups and not just one [33]. Konecny *et al*. (2014) also identified four subtypes [34], while Way *et al*. (2016) reported only three subtypes [35].

A recent report by Chen *et al*. (2018) analyzed the above listed classifiers and found that they lacked robustness, and stated that most of the proposed classifiers performed well, if they were allowed to leave out ambiguous samples [38]. In fact, only 25% of HGSOC could readily fit into a single cluster. A comment by Waldron *et al*. (2014) on the work by Konecny *et al*. (2014), stated that although the subtypes were becoming increasingly significant, they lacked clinical relevance [192]. Since then the classifiers have been discussed and they remain debated.

One explanation may be found in a recent publication by Schwede *et al*. (2018). The report highlighted that most of the molecular subtypes identified can be explained by the extent of stromal tissue and other non-tumor tissue in the samples [137]. They further concluded that single cell analysis will be necessary in order to classify ovarian cancer into clinically relevant molecular subgroups.

Are molecular subtypes only a result of gene expression?

With the considerations that molecular subtypes derived from GEX of bulk tumor tissue might be biased due to normal tissue contamination, other attempts have been suggested to increase prognostication for ovarian cancer patients. Recent work from Dr. James Brenton's group by Macintyre *et al*. [42] proposed the use of DNA copy number alterations and suggested seven copy number subtypes that correlated with survival. A large collaboration (Zhang *et al*. 2016) added proteomic data to the HGSOC TCGA data set and correlated this to copy number alterations [193]. These results emphasize the need for integrated multilayered analyses for the discovery of clinically relevant subgroups in HGSOC. Tools for analysis are currently being developed [194, 195], and data is becoming available through online repositories such as the TCGA Genomics Data Commons Data Portal.

Hormone receptors in ovarian cancer should be targeted for further study

In conclusion, we found strong effects of PR and AR positivity on outcome in ovarian cancer patients, which has also previously been reported by others. We also investigated the effect of co-expression and found that patients with combined PR and AR positivity had a significantly better outcome. Our data suggests that hormone receptors do have a role, potentially for stratification of patients, and these findings provide a basis for further studies. A recent meta-analysis of 2,490 cases evaluating the effect of endocrine therapy in ovarian cancer reported an overall summarized benefit rate of 41% (34%-48%) for ovarian cancer patients [109]. Although promising, the studies in the meta-analysis suffered from the fact that most patients did not receive endocrine therapy as first-line treatment, rather as second-, third- or fourth-line treatment. However, before undertaking such a study the underlying mechanisms of the hormone receptors need to be better understood through e.g. multilayered analyses of 'omics data.

Study II: Identifying treatment candidates in OCCC

Introduction

With the emergence of computers in biology in the 1940's and the development of computational biology and bioinformatics in the 1960's, research prospects changed forever [196, 197]. In 1955, after ten years of work, Frederick Sanger sequenced insulin, and established the basis for sequencing and high-throughput sequencing [198]. In 2003, after 13 years of work, the human genome was sequenced [199]. In 2017 the National Institutes of Health reported the cost of a whole genome

sequencing to be $1,015[1], while the cost for whole exome or targeted sequencing is already below the $1,000 mark [200]. The speed at which we generate sequencing data has increased tremendously. In 2015 the output was one zetta-bases (10^{21}) annually, with storage requiring up to 40 exa-bytes (10^{18}) [201], hence the setup of the framework for doing computational research needs to be considered closely [202].

These huge amounts of data have made it possible to perform genome wide association studies, and with companies such as deCODE genetics (Reykjavik, Iceland) which are dedicated to analyzing sequencing data from the Icelandic population, many markers for different diseases have been discovered [203, 204].

We now have the power to discover correlations and markers in the genome, transcriptome, epigenome and proteome, though these methods are highly explorative. The combination of such data sets allows for the creation of hypotheses. However, generating a hypothesis is worthless if it cannot be adequately tested. Therefore, considerations should be given before undertaking a hypothesis generating experiment, as to the relevance of the question asked, e.g. does it contain any clinical perspective [205, 206].

Aim

The combination of study II and III describes two joined experimental setups that led to a potential treatment strategy for OCCC. The hypothesis was generated in study II, and it was tested in study III. In study II, we characterized OCCC using multilayered 'omics data from three patient cohorts. The motivation for a multilayered approach in ovarian cancer comes from the discussion regarding molecular subtypes. One type of data is not enough in ovarian cancer to fully evaluate its biology. By exploring OCCC on multiple levels for potential treatment candidates, a potential overlap between approaches could be discovered. Such an overlap could lead to a hypothesis for a treatment candidate in OCCC.

OCCC background

OCCC has been shown to have distinct clinical and morphologic features compared to other ovarian cancer subtypes. OCCCs share similarities with renal clear cell cancer [24, 77, 78, 81, 207], and the tumors are often resistant to chemotherapy [63]. The genomic landscape of OCCC is dominated by co-occurrence of mutations in *ARID1A* and *PIK3CA*, activation of the PIK3CA/AKT/mTOR pathway and *MET* and *HNF1B* amplification and *PTEN* loss [62, 68, 70, 208-210]; however treatment options are limited if patients experience chemoresistance [211]. A study from Jönsson *et al*. (2014) investigated Lynch syndrome associated ovarian cancer and

[1] https://www.genome.gov/27565109/the-cost-of-sequencing-a-human-genome/ (accessed January 2019)

reported that OCCC clustered together in an unsupervised hierarchical clustering regardless of Lynch syndrome status.

Methods

We characterized OCCC through integrative analysis of genomic alterations, and gene and protein expression using three different cohorts. For a detailed description of the cohorts, refer to the appended study or the Experimental and methodological considerations section.

We analyzed the transcriptome of 67 ovarian cancer cases (Cohort 1: HGSOC: 31, Endometrioid: 18, OCCC: 15 and Mucinous: 3) (WG-DASL assay), the genomic landscapes in a cohort of ten tumors (Cohort 2: targeted sequencing), and the proteome using a cohort of 43 tumors (Cohort 3: IHC).

OCCCs have a distinct gene expression profile

A supervised hierarchical clustering of the histological subtypes based on the GEX data from a microarray of cohort 1 is shown in Figure 9A. The gene clusters were based on 505 differentially expressed genes and revealed that OCCCs have a distinct GEX profile compared to the other histological subtypes. To test whether the effect was due to supervision of samples, i.e. having fixed groups for the samples based on histology, we also performed an unsupervised test. The unsupervised test revealed that OCCC samples do have a distinct GEX profile when compared to endometrioid and HGSOC tumors, as they clustered tightly together (data not shown). There was an overlap of 217 genes between the two analyses. Gene set enrichment analysis revealed pathways controlling the extra-cellular matrix, MAPK cascades and TFAP2A transcription pathways to be enriched in OCCC compared to the other subtypes as seen in Table 7. This lines with previous findings [24, 77].

Biological relevance of ERBB2 in OCCC

In depth analysis of the data revealed both *ERBB2* and the transcription factor *TFAP2A* to be differentially expressed (Figure 9B). Both *ERBB2* and *TFAP2A* have been linked to OCCC in previous studies [77, 207].

As *ERBB2* is a treatable target in breast cancer, we further investigated the relevance of *ERBB2* in OCCC. We analyzed the protein expression of HER2 (corresponding protein of *ERBB2*) and found only one of 43 tumors overexpressing HER2 in correlation with amplification of *ERBB2*. The lack of HER2 protein expression in OCCC has been reported in other studies [212, 213]. The result from a GEX module score evaluating the activity of *ERBB2* signaling [214], revealed an *ERBB2* score not significantly different from zero indicating no *ERBB2* pathway activity. A recent study by Koopman *et al.* (2018) reported wide spread inconsistencies in HER2/*ERBB2* evaluation by IHC and *in situ* hybridization in OCCC. Their conclusion was that the discordance between reports of HER2/*ERBB2*

overexpression in OCCC can be explained by the difference in IHC antibodies for HER2 assessment [215].

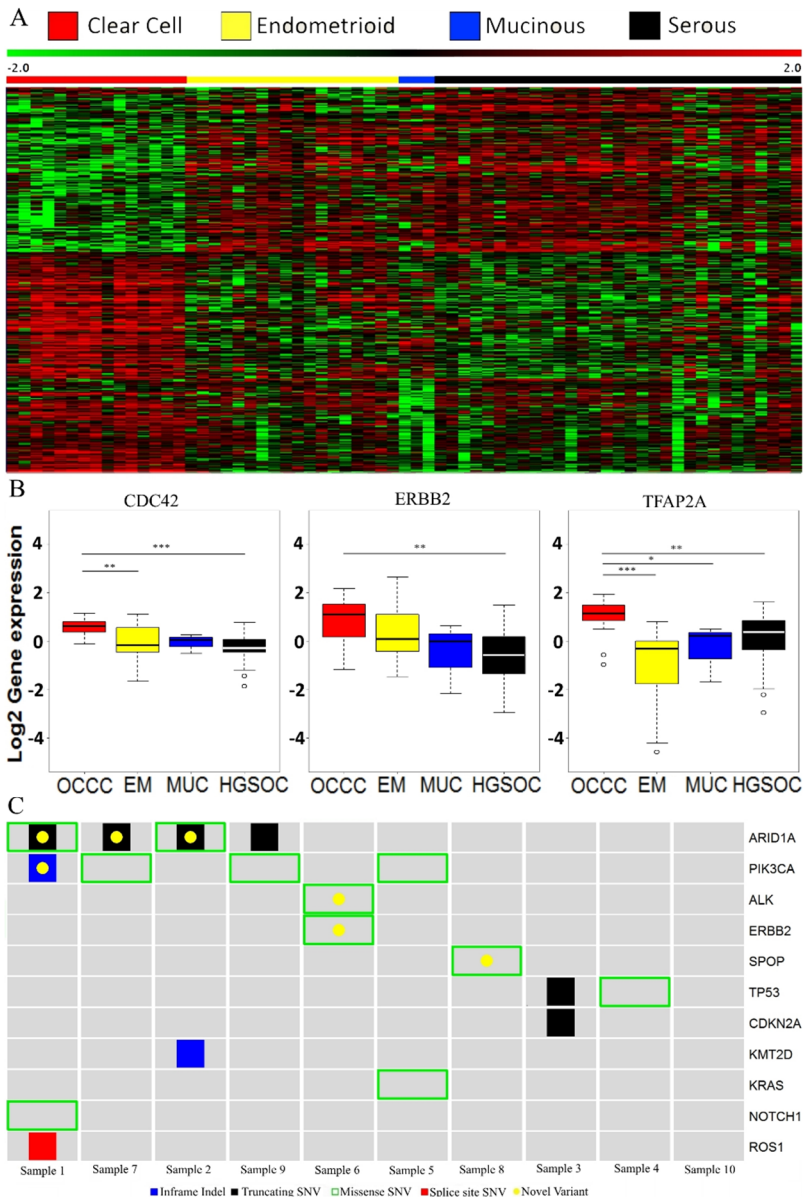

Figure 9: Key findings of study II.
A: Gene expression heatmap of the 505 most significant differentially expressed genes between four ovarian cancer subtypes. Red: Clear cell (OCCC), Yellow: Endometrioid (EM), Blue: Mucinous (MUC), Black: High-grade serous (HGSOC) (n=67). B: Differentially expressed genes between subtypes (n=67). C: Overview of genomic alterations (mutations) in ten OCCC tumors. Blue: Inframe Indel, Black: Truncating mutation, Green: Missense mutation, Red: Splice site mutations, Yellow: Previously unreported variant. Modifed from [216].

Table 7: Differentially expressed pathways between in OCCC
Select results from GEX and genomic analyses from study II

Gene expression	Pathway	q-value
No variance filter		
	Extracellular matrix organization	0.026
	Axon Guidance	0.045
Variance filter		
	Transcriptional regulation by the AP-2 (TFAP2) family of transcription factors	0.017
	Extracellular matrix organization	0.017
	Axon guidance	0.039
	Signaling by GPCR	0.039
	RAF/MAP kinase cascade	0.039
	MAPK family signaling cascades	0.039
	Signalling to RAS	0.045
Mutations		
	Wnt signaling	
	P53 associated signaling	
	EGR receptor signaling	
	PIK3CA/KRAS signaling	
	Angiogenesis	

The lack of HER2 protein in our study suggests that HER2 does not play a significant role in OCCC as a treatment candidate. Contradicting this, two meta-analyses of HER2 in ovarian cancer suggested that increased levels of HER2 correlated with worse survival [107, 217], although the lack of sufficient histological stratification limits these studies. However, a recent integrative study by Caumanns *et al*. (2017) found that although the *ERBB2* pathway was not directly active, the family of ERBB signaling seemed active to some extent [79]. A thorough evaluation of HER2 in ovarian cancer histological subtypes is needed to fully understand its role in OCCC.

OCCC and Rho GTPases

Further analysis revealed that Rho GTPases such as *CDC42* and *RhoB* were differentially expressed between subtypes, and they were found to be involved in many of the pathways listed in Table 7.

Rho GTPases are a part of the Ras small GTPase superfamily (Rho, Ras, Rab, Ran, Arf). Together they link extracellular signals to intracellular signaling networks, thereby exerting their roles as both mediators and regulators in the cell [218, 219]. Rho GTPases have been studied as targets for cancer treatment due to their role in regulating key cellular functions including the maintenance of cytoskeletal integrity, axon guidance, cell migration, proliferation, metastasis and progressive disease in many cancer types [220-225]. Furthermore, Rho GTPases have been implicated in carboplatin resistance in HGSOC cell lines [226].

Although GEX analyses of OCCC compared to other histological subtypes are limited, two reports support our findings. Although not identifying Rho GTPases directly, Zorn *et al*. (2008) reported *ARAP3* and *SRGAP2*, genes of the Rho GTPases signaling pathways, as part of a gene set of 171 genes able to distinguish OCCC from other ovarian cancer histological subtypes. Schaner *et al*. (2003) identified *ARHGAP8* and *RAP1GA1*, two other genes of the Rho GTPases signaling pathway, but also *RHOB* as genes able to distinguish between ovarian cancer histological subtypes [77].

ARID1A and PIK3CA mutations dominate OCCC and activate the PI3K/AKT/mTOR pathway

Further studies of the genomic alterations of OCCC were performed in cohort 2. Ten FFPE OCCC tumors were sequenced using a gene panel of 60 genes commonly mutated in solid tumors. We found inactivating *ARID1A* and activating *PIK3CA* co-occurring mutations in three of ten tumors, in line with both previous and more recent findings [67, 68, 80, 227, 228]. The loss of *ARID1A* and activation of the PI3K/AKT/mTOR pathway has been shown as a key driver element in OCCC [70].

The immunohistochemical evaluation in cohort 3 supported this, with AKT expression in 53% (23/43) of the tumors. Analysis of mTOR and PTEN was previously done in a subset of tumors of cohort 3. mTOR was reported to be overexpressed in 58% (7/12) of the tumors, while PTEN was lost in 66% (8/12) [76]. Inhibitors of the PIK3CA/AKT/mTOR pathway have been suggested in OCCC [79, 229, 230], while drugs such as Dasatinib (a SRC kinase inhibitor) have been shown to be synthetic lethal in *ARID1A* mutated OCCC cell lines [231, 232]. There are currently two ongoing phase 2 clinical trials investigating, respectively Dasatinib [233] and TAK228 (a dual TORC1/2 inhibitor) [234] for the treatment of patients with recurrent OCCC.

A potential non-canonical pathway for Wnt signaling in OCCC

Through pathway analyses of the detected mutations we found that Wnt signaling was affected. Studies by Bodnar *et al*. (2014) and Gamallo *et al*. (1999) suggested that Wnt-signaling in ovarian cancer might be through non-canonical pathways because β-catenin was absent in the cell nuclei of the tumors [235, 236]. The canonical pathway for Wnt-signaling is through the activation of β-catenin (*CTNNB1*). Although we found no mutations in *CTNNB1*, the planar cell polarity (PCP) pathway is one of the non-canonical Wnt-pathways acting through Rho GTPases, activating JNK and subsequently AP-1 meditated transcription, leading to cytoskeletal rearrangements [237-239] (Figure 10). These findings indicate that Rho GTPases could play a pivotal role throughout the OCCC cells, linking both genomic alterations with GEXs and protein functions.

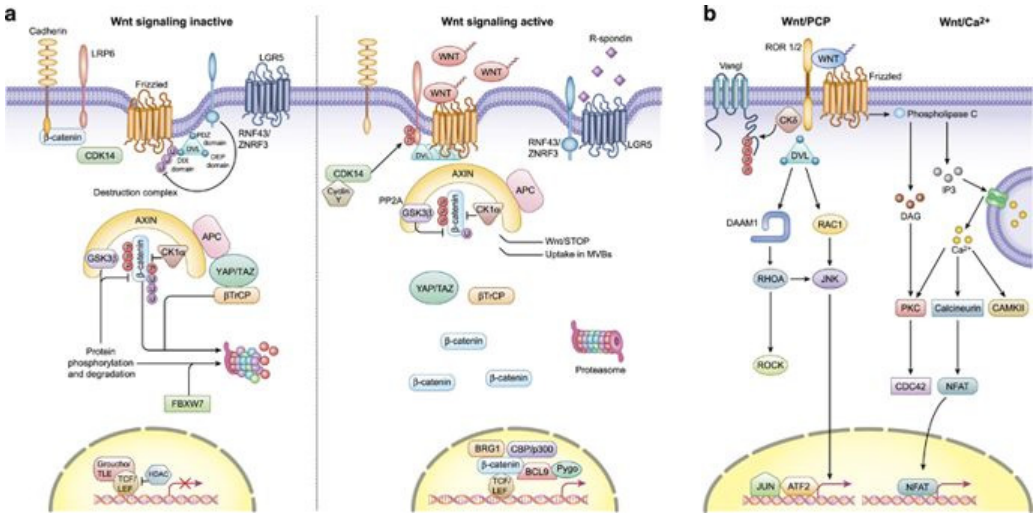

Figure 10: Overview of canonical and non-canonical Wnt signaling.
A: Canonical Wnt-signaling with binding of Wnt to Fzd receptors and LRP co-receptors leading to activation of β-catenin. B: Non-canonical pathways for Wnt signaling, Wnt/PCP and Wnt/Ca^{2+} where Wnt binds to the ROR-Frizzled receptor complex which leads to cytoskeletal rearrangements through RHOA and JNK or CDC42 and NFAT. Figure is adapted from Zahn *et al.* (2017) [239].

Limitations and considerations

Although study II suffered from the use of different cohorts and a small number of samples in each of the cohorts, we were able to utilize a multilayered 'omics approach and to combine results into reasonable interpretations. Furthermore, our findings were in line with those of other groups, while also providing new insight into the pathogenesis of OCCC. Our findings support Rho GTPases as potential targets of treatment in OCCC. We therefore proposed the hypothesis that drugs targeting Rho GTPase activity or intracellular levels may be a promising strategy for treating OCCC. This was further investigated in study III.

Study III: Simvastatin in OCCC

Introduction

In study III, we tested the hypothesis generated in study II, that drugs targeting Rho GTPases would be a promising strategy for the treatment of OCCC. However, targeting Rho GTPases directly can be difficult and methods as those outlined in Figure 11 are potential strategies [240-243].

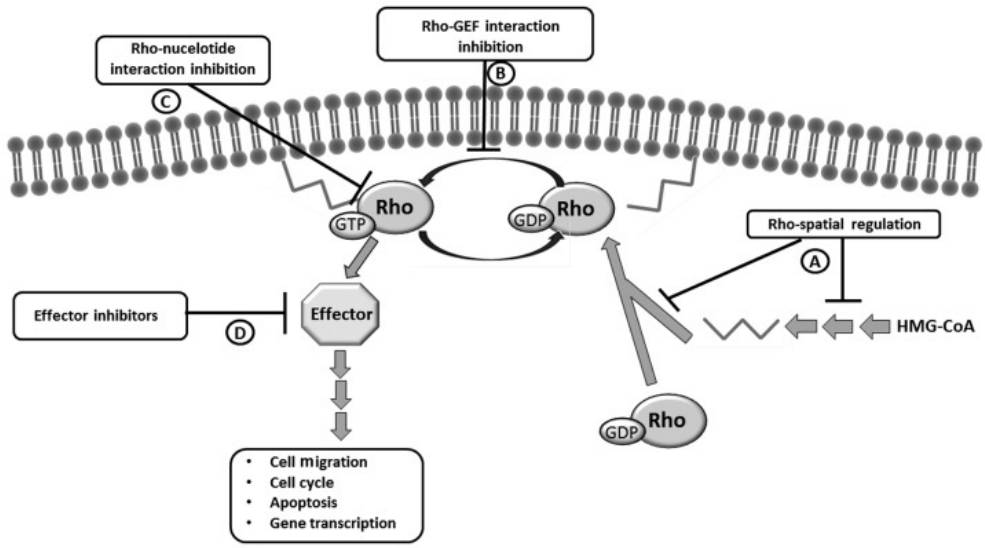

Figure 11: Different strategies to interfere with the Rho-GTPase signaling pathway.
A: Reduce isoprenoid precursors using statins or inhibition of farnesyl-transferases and geranylgeranyl transferases interfering with the prenylation of Rho GTPases, regulating spatial localization of the GTPase proteins. B: Activation cycle inhibition, by interfering Rho-GEF interaction. C: Interfering Rho-nucleotide binding. D: Direct inhibition of Rho GTPase effectors. Figure is adapted from Cardama *et al.* (2017) [241].

We chose two different drugs (CID-1067700 and simvastatin), each with a different targeting strategy, for the *in vitro* studies. CID-1067700 is a pan-GTPase designed as a molecular probe on which compounds for targeting Rho GTPases could be attached; however, the probe itself also inhibits the activity of Rho GTPases (and other small GTPases). The mechanism of action is not yet understood but it is suggested to bind either directly to or near the GTP binding site thus preventing Rho GTPase activation [244].

The other drug chosen was simvastatin, an FDA approved cholesterol lowering drug targeting HMG-CoA reductase in the mevalonate pathway [245]. By inhibiting HMG-CoA simvastatin prevents the synthesis of the isoprenoid intermediates farnesylpyrophosphate and geranylgeranyl pyrophosphate (GGPP) (Figure 11, section A) [246]. GGPP is used for the translocation of Rho GTPases to the membrane for them to elicit their effect, and thus Rho GTPase activity is prevented by the depletion of GGPP by simvastatin [240]. Simvastatin exhibits antitumorigenic properties in many cancer cell lines [247-252], and has been evaluated in clinical trials either alone or in combination therapies with varying success, despite promising preclinical studies [253-255]. Simvastatin has also been evaluated in *in vitro* studies in ovarian cancer, although mostly in the HGSOC subtype [247, 256, 257]. A study by Matsuura *et al*. (2011) reported anticancer effects of simvastatin in two OCCC cells lines and showed increased survival in

mice with xenografted OCCC tumors following simvastatin treatment [258]. The rationale for their study was the overexpression of osteopontin in OCCC and its role in metastasis and invasiveness, which simvastatin was shown to inhibit [259]. Although the use of statins as an anticancer agent in ovarian cancer is debated, some recent evidence suggests a correlation with increased survival in humans [260, 261]. A study by Habis *et al.* (2014) even reported that patients with ovarian cancer other than HGSOC, had improved survival with statin use [262].

The following section describes results from the appended manuscript (Study III), however it also presents new data not yet described in the manuscript.

Methods

We evaluated the sensitivity to simvastatin and CID-1067700 in three OCCC cell lines (JHOC-5 [263], OVMANA [264] and TOV-21G [265]) and one HGSOC cell line (Caov-3). We compared the sensitivity to that of carboplatin, a heavily used chemotherapy in ovarian cancer treatment [27, 266]. We also evaluated the effect of the treatments on the cytoskeletal integrity and the migration of the cells using fluorescent microscopy. Lastly, we evaluated the cellular responses to the treatments using western blotting and cell cycle analysis by FACS.

OCCC cell lines are most sensitive to simvastatin

We first evaluated the sensitivity of the three drugs individually. The findings are shown in Figure 12. Simvastatin was the most potent of the drugs in OCCC cells, with similar potency in all the OCCC cell lines, while the potency was lower in the HGSOC cell line Caov3 (Figure 12A). However, the sensitivity to CID-1067700 was lower than to simvastatin in all cell lines, possibly reflecting the fact that CID-1067700 is a pan-GTPase inhibitor and not specific for Rho GTPases, and therefore targets small GTPases in a dose-dependent competitive manner (Figure 12B) [244]. Both patients from whom the JHOC-5 and OVMANA cell lines were derived had received platinum based chemotherapy, which could explain their increased resistance to carboplatin compared to the more sensitive HGSOC cell line Caov3 and the untreated OCCC cell line TOV-21G (Figure 12C) [263-265, 267]. Still, chemotherapy resistance is common in OCCC, with 30-50% of patients lacking a response to first-line platinum-based treatments [65, 268, 269].

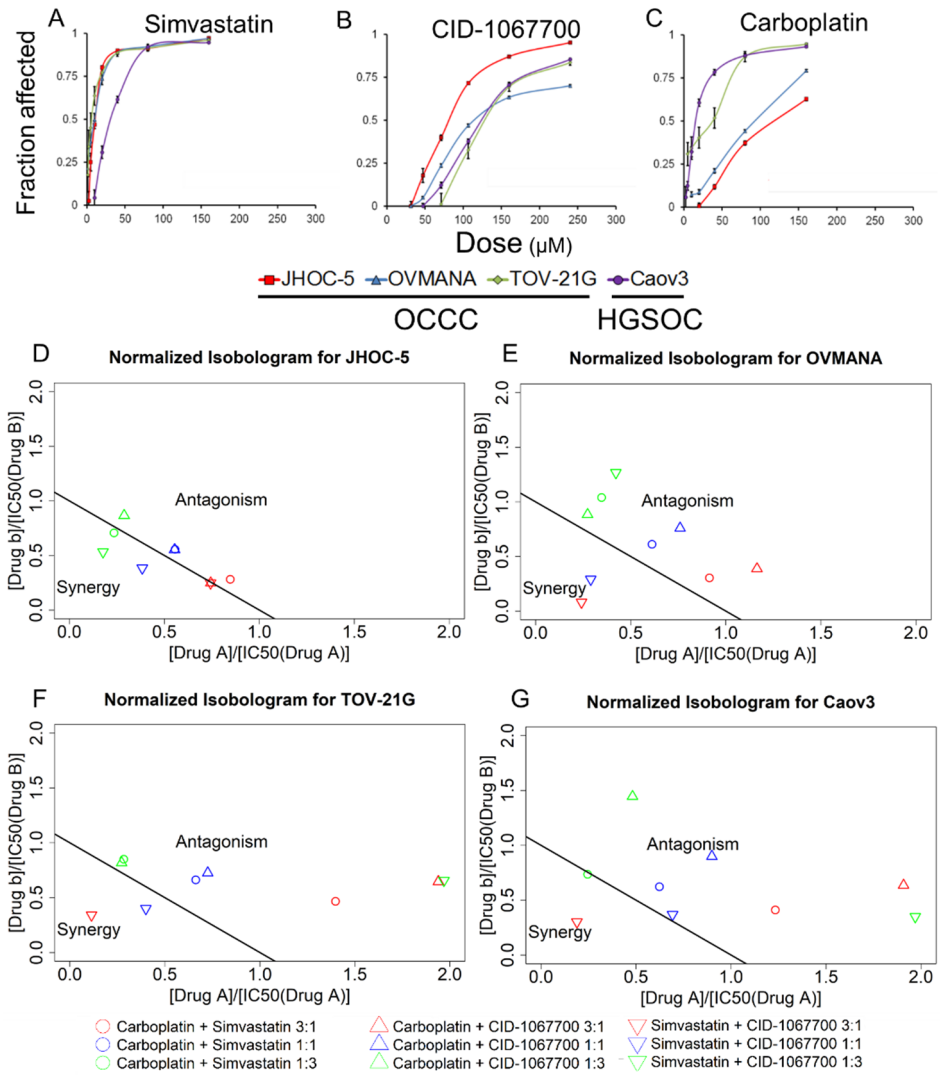

Figure 12: Dose response curves and isobolograms.
Top panels: Dose response curves for single treatment with A: Simvastatin, B: CID-1067700, C: Carboplatin. Lower panels: Normalized isobolgrams for combination therapies in D: JHOC-5, E: OVMANA, F: TOV-21G and G: Caov3. Red: 3:1 ratio, Blue: 1:1 ratio, Green: 1:3 ratio. Ratios are linked to the prewritten combination therapy. Circles: Carboplatin + Simvastatin, Upward triangles: Carboplatin + CID-1067700 and Downward triangles: Simvastatin + CID-167700.

Table 8: Drug combinations and their effects in Study III
CI: Combination index

	Ratio	IC50 (μM)	Individual drug dose (μM)			CI-value
			Carboplatin	Simvastatin	CID-1067700	
JHOC5						
Carboplatin + Simvastatin	1:3	34.1 ± 2.7	27.04	7.06		0.94±0.05
Carboplatin + CID-1067700	3:1	105.6 ± 8.7	85.41		20.19	0.99±0.01
Simvastatin + CID-1067700	3:1	27.6 ± 1.2		20.17	7.43	0.99±-0.08
OVMANA						
Carboplatin + Simvastatin	1:3	36.8 ± 2.3	29.31	7.49		1.39±0.06
Carboplatin + CID-1067700	1:3	137.3 ± 9.8	23.09		114.21	1.15±-0.03
Simvastatin + CID-1067700	3:1	31.8 ± 2.5		0.58	31.22	0.32±0.01
TOV21G						
Carboplatin + Simvastatin	1:3	9.8 ± 0.8	5.04	4.76		1.13±0.05
Carboplatin + CID-1067700	1:3	114.2 ± 11.2	4.86		109.34	1.09±-5.32
Simvastatin + CID-1067700	3:1	46.3 ± 3.0		0.64	45.66	0.46±0.01

Synergy is limited in combination treatments

We also evaluated the effect of dual combination of the drugs and found cell line specific differences (Table 8, Figure 6D-G). Notably, only combinations between simvastatin and CID-1067700 were synergistic (Figure 12D-G, Downward triangles). A perspective report by Chou (2010) highlighted the importance of evaluating CI values across the entire dose-range [148]. We observed a synergistic effect with increasing dose (and effect) between carboplatin and simvastatin in JHOC-5 and OVMANA cells for treatments with a high simvastatin ratio, while an increase in sensitivity with increase in simvastatin ratio in treatments with simvastatin (see appended study).

Both simvastatin and CID-106770 affect cytoskeletal integrity

Further investigation revealed that both simvastatin and CID-1067700 affected the cytoskeleton. After 72 hours of treatment we observed a significant loss of cytoskeletal integrity with disorganized actin filaments at the cell borders (Figure 13), which correlated with an inhibition of migration (see appended study). Despite the lack of synergy, almost all treatments with simvastatin and/or CID-1067700 were able to disorganize the cytoskeleton, as well as inhibit migration in all cell lines. This is in line with several other studies of statins in different cancer cell lines [256, 257, 270-273]. The effect, however, is much less studied in OCCC and the pathways in which statins work are not yet fully understood. In fact, Robinson *et al*. (2013) raised the question of several mechanisms at large, as they reported increased

levels of both LC3A/B and P62 following simvastatin treatment [256]. LC3A/B is a marker for autophagy, while P62 is an inhibitor. In support of this, increased levels of Rho GTPases have been shown after treatment with statins [274], while others have reported decreased p-AKT levels [275]. The similar effects of simvastatin and CID-1067700 in our study suggests that Rho GTPase activity is in fact being targeted; this was confirmed by the addition GGPP, which inhibited cell death in simvastatin containing treatments, but not in CID-1067700 treatments (data not shown).

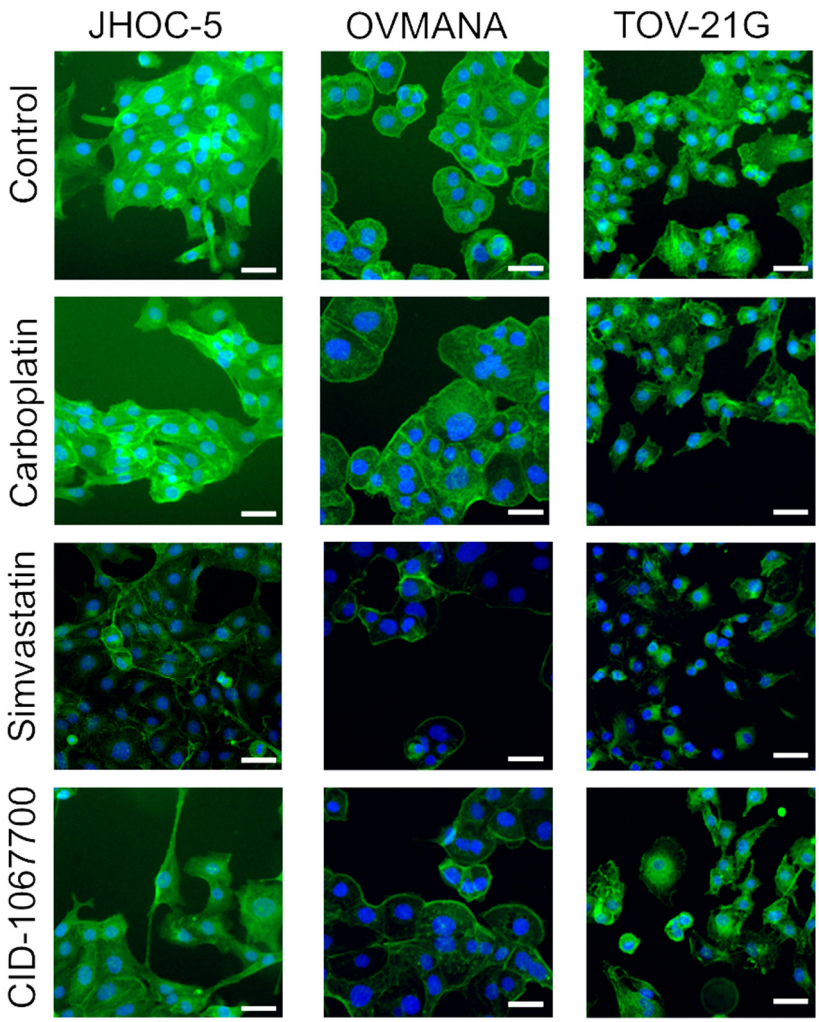

Figure 13: Effects of single treatment on the cytoskeleton.
Cells were treated for 72 hours with single treatments of IC50 doses. Actin was then stained with phalloidin and cell nuclei with DAPI and fluorescenct signals were captured. Images have been background corrected to the controls to better illustrate the loss of actin staining. White scale bar is 50 µm.

Cell cycle arrest is cell line specific

Next, we analyzed the effects of treatments on cell cycle phase distribution and found that all treatments caused a G1 arrest in JHOC-5 and TOV-21G cells, while this was not the case for OVMANA cells (see appended study). The differences between the cell lines became more profound when we analyzed protein expression based on the reported effects of simvastatin (Figure 14).

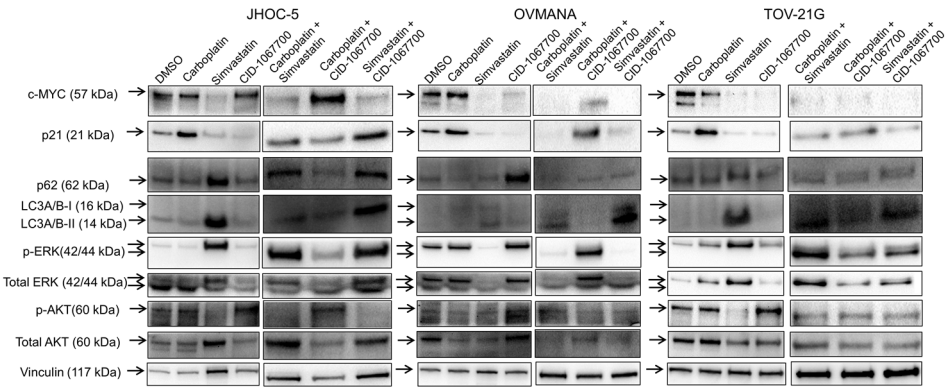

Figure 14: Cellular response to treatments for all cell lines
Immunoblots of key proteins reported to be targets of simvastatin, as well as key regulators in OCCC.

Simvastatin reduces c-MYC expression

We found an increased expression of p21, a marker for cell cycle arrest, following single treatment with carboplatin (Figure 14). A modest increase in p21 levels was observed when carboplatin was used in combination with CID-1067700. Interestingly JHOC-5 cells showed a significant increase in p21 for the combination of simvastatin and CID-1067700. Another common denominator was the decreased levels of c-MYC after simvastatin treatments (Figure 14). CID-1067700 also decreased c-MYC expression in OVMANA and TOV-21G cells. c-MYC is frequently overexpressed in OCCC and has been described as a potent oncogene and therefore a promising target [276]. Furthermore c-MYC has been reported to cross-talk with RhoA and regulate the cytoskeleton, which the lack of c-MYC in OVMANA and TOV-21G cells after treatment with CID-1067700 might reflect [277], and c-MYC expression is linked to the Wnt-pathway [278].

The roles of p-ERK and p-AKT are cell line specific

The reported effects of a dual mechanism of both autophagy initiation and inhibition was evident in JHOC-5 and TOV-21G, while P62 expression was increased in OVMANA following single treatment with CID-1067700 (Figure 14).

Combinations of carboplatin/simvastatin and simvastatin/CID-1067700 increased expression of the autophagy markers LC3A/B.

Expression of p-AKT was decreased in all cell lines following simvastatin treatment. For JHOC-5 and TOV-21G cells this was due to loss of phosphorylation, whereas in OVMANA cells it was caused by a decrease in total AKT. An inverse correlation was seen in p-ERK levels between OVMANA and both JHOC-5 and TOV-21G cells. Simvastatin increased phosphorylation of ERK in JHOC-5 and TOV-21G, while a decrease in OVMANA cells followed a decrease in total ERK. The distinct expression profiles of p-ERK and p-AKT suggest different dependencies in the cell lines. A possible role for the increase in p-ERK could reflect the huge number of cells arrested in G1, as p-ERK activity is required for the transition to S-phase [279]. However, the lack of p-ERK in OVMANA cells in response to simvastatin suggests an alternate pathway here, reflecting the absence of G1 arrest in these cells (data not shown). Also, cross-talk between the Ras-ERK and PIK3CA/AKT/mTOR pathways is established, although our study did not evaluate this [280, 281].

The contradicting effects in the cell lines are probably due to the differences in geno- and phenotypes, highlighting the notion of tumor heterogeneity which may exist in OCCC depending on the co-existence of *ARID1A* and *PIK3CA* mutations [70, 71]. Despite the contradictions we have shown that drugs targeting Rho GTPases are potent in OCCC, and while their effects are cell line dependent, they are effective, nonetheless. Simvastatin emerged as a promising candidate and our study has warranted further investigations into the use of statins in OCCC.

Conclusion

We successfully integrated multiple datasets into a hypothesis and subsequently tested the hypothesis using cell cultures as experimental models. A larger but similarly designed study published by Caumanns *et al.* (2017), concluded that drugs targeting both the Ras-ERK and PIK3CA/AKT/mTOR pathways could be a promising strategy for OCCC treatment [79]. They evaluated the kinome from 17 OCCC cell lines and 70 tumors from patients, and their results correlate well with our findings, although the initial hypothesis was different [79]. Our results correlated with the previous studies of simvastatin as an anticancer agent. However, it was evident that although simvastatin and CID-1067700 both targeted the cytoskeleton, their roles on a cellular level were different, thus supporting the idea of multiple mechanisms of action by simvastatin.

Study IV: Detection of *TP53* mutations for early diagnosis in HGSOC

Introduction

Ovarian cancer is termed "The Silent Killer", and while the incidence and mortality of cervical cancer have decreased radically since the introduction of the Papanicolaou test [282-284], overall survival from ovarian cancer has not changed substantially over the past 20 years [285]. Despite approaches aimed at early detection, including the use of serum CA-125 and transvaginal ultrasound, neither has been successfully applied in a screening setting due to limited specificity and sensitivity [28, 286, 287]. Although several prognostic factors have been identified in ovarian cancer, one of the strongest being stage [288-290], the majority of ovarian cancers are diagnosed in late stages (FIGO stage ≥III) with poor prognosis [291, 292]. Early detection therefore presents as a critical option for impacting survival in ovarian cancer, especially HGSOC.

TP53 as a potential biomarker

Through sequencing of HGSOC, *TP53* has emerged as a biomarker candidate for early detection [32, 293] as it is mutated in more than 95% of HGSOC cases. A recent study showed a shared *TP53* mutation in matched pre-cancerous and cancerous lesions (p53 signatures, Serous Tubal Intraepithelial Carcinoma lesions (STICs) and invasive carcinomas) from nine patients with HGSOC. These findings provide support for the possibility of discovering tumor driving mutations in early stages of the disease [23].

NGS represents a powerful tool for mutation detection and several techniques based upon NGS have emerged in recent years. These can detect mutations of low abundance in many types of samples such as blood, urine, cyst fluid and vaginal samples [90, 91, 294-297]. The most promising development in early detection of ovarian cancer to date occurred in 2013, when Kinde *et al.* showed that somatic mutations in DNA shed from endometrial and ovarian cancers could be detected in standard liquid-based Pap test specimens by NGS [298]. The PapSEEK method assays 18 genes for alterations commonly found in endometrial and ovarian cancers. PapSEEK is highly sensitive for endometrial cancer, however less sensitive for ovarian cancer. A recent study reported a sensitivity for PapSEEK alone of 33%, however with a specificity of 99%, although the addition of plasma samples increased the sensitivity to 63% [89].

ddPCR, an alternative to NGS

While NGS is becoming less expensive each year, there is still a long way until NGS can be applied in a population based diagnostic screening setting, although it could

be applicable for high risk patient groups such as *BRCA* mutation carriers [299, 300]. An at least as sensitive alternative to NGS is ddPCR, which has a fast turnover and ease of use [301]. Analysis of circulating tumor DNA using ddPCR has shown great potential for prognostication and monitoring of treatment response in several tumor types including gynecological cancers [302, 303].

Most of the previous studies evaluating the sensitivity of detection or early markers of disease originate in samples collected at the time of diagnosis. This may be too late for ovarian cancer as described above. The MaNiLa study (Mats, Nicolai, Laura, the original PhD students in the study) at Lund University aims to evaluate ddPCR as a platform for HGSOC screening and has enrolled 187 women with suspected ovarian cancer (Figure 15). A subset of these patients was found to have archival vaginal samples available from the cervical cancer screening program (n=9). These archival samples were taken four to seven years prior to the diagnosis of HGSOC and allow for an initial evaluation of the window of opportunity proposed by Labidi-Galy *et al.* (2017) [23].

Aim

In study IV we therefore aimed to evaluate the potential window of opportunity using the presymptomatic archival liquid based vaginal samples from 9 patients with HGSOC. The goal was to discover whether *TP53* mutations present in tumors could be detected in archival vaginal samples.

Methods

We analyzed both the archival vaginal samples as well as vaginal samples collected at the time of diagnosis from nine women diagnosed with HGSOC, using the ultra-sensitive ddPCR IBSAFE™ technology developed at Lund University by Saga Diagnostics. To our knowledge the analysis of liquid vaginal samples from pre-symptomatic women has, not been done before.

We used targeted sequencing of matched tumor and blood samples from each patient to detect possible *TP53* mutations, and then assessed the archival and diagnostic vaginal samples using ddPCR.

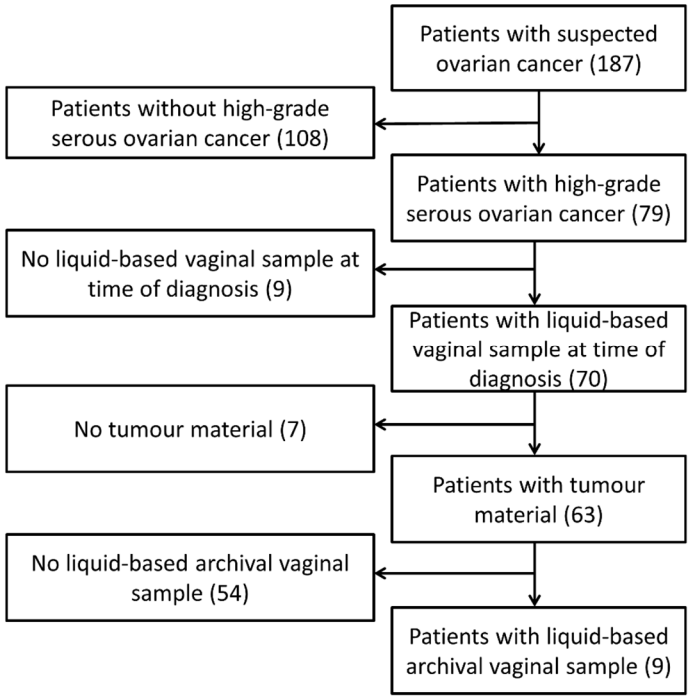

Figure 15: Flowchart of patients included in study IV.
A total of 187 patients were enrolled in the MaNiLa study. A total of 9 patients had both presymptomatic liquidbased vaginal samples and diagnostic vaginal samples, while also tumor tissue.

Results

IBSAFE™ identified 75% of the somatic *TP53* (6/8) mutations found in the patients' tumors in the corresponding diagnostic sample, including 66% (2/3) of patients with low stage tumors (FIGO stage <IIB). Early diagnosis of low stage tumors is a desirable feature for screening methods and PapSEEK currently holds a sensitivity of 50% for both early and late stage ovarian cancer [89].

Unfortunately, we were not able to detect any of the tumor occurring *TP53* mutations in the archival samples. One patient (patient 1) had a germline mutation that was not filtered out (Figure 16). Despite this, we found that IBSAFE™ was able to perform in samples with very limited DNA (0.17 ng). Other ddPCR assays, such as the Bio-Rad assays, are optimized for 100 ng input (detection rate of 1:10,000), while less input can be used at the cost of sensitivity [301].

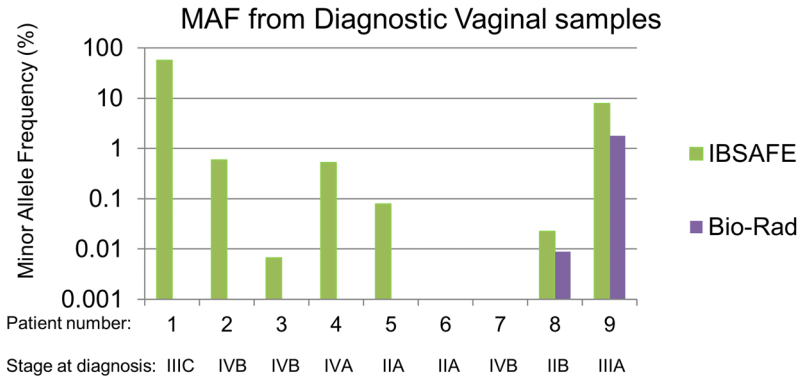

Figure 16: Tumor minor allele frequencies (MAF) for diagnostic vaginal samples from study IV
MAFs (%) listed for diagnostic vaginal samples for IBSAFE™ (Green) and Bio-Rad (Purple). FIGO stage at time of diagnosis is listed below each patient number. Note the logarithmic scale bar.

Conclusion

Even though we failed to detect any *TP53* mutations in the archival samples, we are currently investigating the possibility of evaluating ultra-sensitive ddPCR in a larger cohort, with serial archival vaginal samples obtained closer to the time of diagnosis, e.g. 1-3 years prior.

Study IV highlights the before mentioned effects of collaborations between clinical and preclinical researchers. With clinicians following and collaborating actively in a study, the road from the laboratory to the clinic is much shorter. I, as a non-clinician have learned to appreciate the knowledge clinicians hold, and the studies in this thesis have been driven by an ever-present clinical perspective.

Conclusions and Future Perspectives

The studies in this thesis explore different biomolecular approaches, which can ultimately be used for improving patient outcome. The secondary effect of these studies is a deeper understanding of the pathogenesis of ovarian cancer. The strong synergy which can be obtained when combining biology, pre-clinical and clinical research was pivotal for the creation of this thesis. This is supported by the study conclusions, which hence are presented here as a context.

Sex hormone receptors

Study I was an explorative study aiming to evaluate the effect of co-expression of hormone receptors. Although limited in size the association of AR/PR expression with improved outcome is promising, but it needs further evaluation. We failed to show the effect in an independent data set, albeit through mRNA expression. The need for a better understanding of hormone receptor function in the ovaries and in ovarian cancer is warranted. Furthermore, the study highlighted the need for better stratification of patients. This is both in general terms, however, also to assess the effect of hormone receptors, both as predictive and prognostic markers. Recent results suggest that integrative analysis of multilayered data could provide a future use for endocrine therapy in ovarian cancer based on hormone receptor status. This could lead to clinical studies of the effect of endocrine treatment in less heavily pretreated patient groups.

Finding a way aided by computers

Study II was also an explorative study, although in OCCC, a rare histological subtype of ovarian cancer. The study demonstrated that integrative analysis of data from multiple platforms could provide information that led to the discovery of potentially actionable treatment candidates. Although a limited study, where three different cohorts were used due to the rarity of the tumor subtype, the results correlated well with previous findings on all levels. The findings also suggest that, although OCCC might not be as homogenous as suggested by subtype comparisons, common treatment candidates can be discovered. Rho GTPases have been extensively researched for their roles in cancer and in this study, Rho GTPases are suggested as potential treatment candidates in OCCC. Essentially, we used a hypothesis generating method to create a hypothesis of treatment candidates in OCCC. The use of integrated analyses in patient samples and cell lines is becoming

the standard exploratory tool in this context, and information about rare tumor types such as OCCC should be made readily available to enhance sample size across borders.

OCCC can be targeted with simvastatin

Study III elaborated on the hypothesis generated in study II, that Rho GTPases would be treatment candidates in OCCC.

We assessed the sensitivity to simvastatin and CID-1067700 in three OCCC cell lines and found that OCCC cell lines were more sensitive to simvastatin than to carboplatin. The cytoskeleton became disorganized and migration was inhibited. Further analysis of the molecular events suggested involvement of multiple pathways leading to cell death, however there was also evidence of differences between cell lines. Our findings indicated involvement of both the PI3K/AKT/mTOR and RAS/ERK pathways, but this remains to be further investigated.

Despite the limitations of an *in vitro* 2D model assay for potential *in vivo* 3D effects, we found evidence to support further evaluation of statins in OCCC. The future perspective for clinical trials, however, is limited as it is a rare subtype, with only a few cases a year. One approach could be to use registry research and correlate statin use to the incidence of OCCC in a retrospective study. But with only a limited number of cases a year in a small country the study would have low power. Hence the need for collaborations across borders. E.g. a study similar to our approach was recently published by Caumanns *et al*. (2017), in which an impressive 124 tumors were collaboratively analyzed using integrated analyses.

Ultrasensitive ddPCR holds promise for vaginal samples

Study IV evaluated the use of ultrasensitive ddPCR (IBSAFE™) for *TP53* testing of presymptomatic liquid based vaginal samples from women with HGSOC. Despite the success of IBSAFE™ in samples with low abundance of DNA, we did not detect any of the tumor associated *TP53* mutations in the presymptomatic vaginal samples. However, we did detect the tumor associated mutations in the diagnostic samples, with a sensitivity of 75%, and also providing support for further investigation of IBSAFE™ in vaginal samples, which often contain low amounts of DNA.

Although we were only able to test a total of 9 patients, the evaluation of presymptomatic samples has not been shown previously. The identification of these patients was made possible only due to the unique registries and biobanks in Sweden and through an extensive collaboration with both clinicians and pathologists, and thus represents a unique opportunity for further studies.

This approach is as close as we can come to designing a prospective study, without the need to account for the timeframe for cancer initiation. The use of the IBSAFE™ method warrants further investigation and we are currently trying to identify additional patients nationwide in Sweden who have the combination of a diagnostic and a presymptomatic vaginal sample(s) as well as tumor tissue available. Initial investigations are currently promising, and we expect to test more patients within the next two years.

The ease of use and fast turnover of IBSAFE™ and the potential of multiplexing for evaluating multiple mutations in the same sample, is in favor of this method, as opposed to the NGS approach, which, although able to detect multiple mutations in a sample, lacks the sensitivity of the IBSAFE™ method.

Concluding remarks

There is no question in my opinion that clinically relevant molecular subtypes will be discovered in HGSOC, and it is not a matter of time more than it is a matter of collaboration. As more advanced methods become readily available so does the need for specialists but also mediators. In my opinion the scientific community will see an increase in the demand for these mediators i.e. interdisciplinary researchers. We have gained so much knowledge of the cancer biology, that the time has now come to integrate that knowledge and take a proactive role in the fight against cancer.

Collaboration, integration and interdisciplinarity will be the keywords that will mediate the next breakthrough in cancer research.

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Hunting a Silent Killer

About the thesis

Epithelial ovarian cancer affects 1 in 70 women worldwide and is very aggressive in its nature. However, epithelial ovarian cancer is also not just one cancer, it is five distinct cancers each with their own morphological and molecular fingerprint. Ovarian cancer has been nick named: The Silent Killer.

This thesis explores epithelial ovarian cancer using different biomolecular approaches aimed at characterizing the cancer further. The combination of gene expression analyses, genome sequencing, bioinformatics and in vitro models provide a platform from which we can gain further knowledge of this heterogeneous disease.

By enhancing our knowledge, we find new targets to treat, or new biomarkers with the promise of early detection. The combination of multiple aspects of research brings us closer to the ultimate goal. To improve the prognosis of a cancer that has not seen improvement for the past 20 years.

About the author:

Nicolai Skovbjerg Arildsen is a molecular biologist who has ventured into the field of bioinformatics in a clinical setting, and he has managed to combine his part time hobby of computers with his professional interest of cancer research. In his spare time he enjoys board games and whiskey, preferably together.

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