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Intrinsic subtypes and prognostic implications in epithelial ovarian cancer

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Intrinsic subtypes and prognostic implications in epithelial ovarian cancer

Jenny-Maria Jönsson

DOCTORAL DISSERTATION

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Title and subtitle: Intrinsic subtypes and prognostic implications in epithelial ovarian cancer

Abstract: Ovarian cancer is the seventh most common cancer in women globally, with approximately 240,000 new cases annually. Although a rare disease, it is the most lethal gynecologic malignancy. Unspecific symptoms result in late diagnosis and a generally poor prognosis. However, ovarian cancer is a heterogeneous disease comprising different disease entities, which is of importance in clinical decision-making as well as in research. This thesis describes explorative approaches to investigate the ovarian cancer heterogeneity in a hereditary ovarian cancer subset and in histopathological and molecular subtypes of ovarian cancer.

In study I, a gene expression profile differentiating the rare subgroup of Lynch syndrome-associated ovarian cancer from a matched sporadic cohort was identified. The Lynch syndrome-related expression profile was associated with proliferation and cell death processes. An external dataset was used to refine the gene expression profile, but validation with immunohistochemical staining of key proteins did not reveal any differences between the hereditary and sporadic cases. A distinct cluster of hereditary serous and endometrioid cancers was seen, whereas clear cell carcinomas (OCCCs) clustered together, whether hereditary or sporadic. In study II, gene expression profiling of OCCCs revealed extensive inter-tumor heterogeneity. Targeted deep sequencing of 60 cancer-related genes in an OCCC cohort revealed frequent mutations of chromatin remodeling genes, including mutations not previously reported in ovarian cancer. These results remain to be validated.

Study III outlined gene expression profiles in malignant, borderline, and benign serous ovarian tumors. Pre-defined molecular subtypes of ovarian cancer as well as intrinsic breast cancer subtypes were applied to our cohort. Associations between the most aggressive ovarian cancer subtypes and the basal-like breast cancer subtype were identified. The results were validated using a large, external dataset. Furthermore, associations between borderline ovarian tumors and the luminal A breast cancer subtype were discovered. The luminal A breast cancer subtype characterizes hormone receptor positive breast cancer. In study IV, we therefore outlined the protein expression of estrogen receptor (ER) α, ERβ, the progesterone receptor (PR), and the androgen receptor (AR) as well as the prognostic effect of receptor expression in serous and endometrioid ovarian cancer. Expression of PR and AR was associated with a favorable prognosis, and co-expression of PR and AR conferred an additional prognostic benefit. The mRNA levels of the encoding genes were investigated in the molecular subtypes of ovarian cancer using an external dataset. The expression varied between the different subtypes, but no prognostic benefit of dual high *PGR* and *AR* levels were revealed.

In conclusion these studies further characterize the ovarian cancer heterogeneity, and support that future ovarian cancer studies need to be stratified for both histopathologic subtypes and molecular features

Key words: ovarian cancer, gene expression profiling, Lynch syndrome, targeted deep sequencing, chromatin remodeling, molecular subtypes, endocrine receptors, prognostic factors, tumor heterogeneity

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Intrinsic subtypes and prognostic implications in epithelial ovarian cancer

Jenny-Maria Jönsson

Contents

Thesis at a glance

Study	Question	Methods		Results and implications
I	Does gene expression profiling differentiate between Lynch syndrome-associated and sporadic ovarian cancer?	Expression profiling of 48 matched heredi-tary and sporadic ovarian cancers using FFPE tissue. Immunostaining.		Lynch syndrome-associated ovarian cancer conferred a distinct expression profile, but immunostainings were inconclusive. The results may be useful for future studies.
II	Which molecular events characterize ovarian clear cell carcinomas (OCCCs)?	Expression profiling of 67 ovarian cancers, including 15 OCCCs. DNA deep sequen-cing of 10 OCCCs.		OCCCs harbored inter-tumor heterogeneity. Deep sequencing. revealed involvement of chromatin remodeling genes. Validation is ongoing.
III	Do clinically relevant similarities between gene expression-based subtypes of ovarian and breast cancer subtypes exist?	Gene expression profiling of 59 fresh-frozen serous ovarian tumors. Application of ovarian and breast cancer molecular subtypes. Validation in an external dataset.		Ovarian and breast cancer subtypes correlated well, including associations between ovarian borderline tumors and luminal A breast cancers. This may potentially indicate endocrine treatment response in subsets of ovarian cancers.
IV	Does hormone receptor expression, including two or more receptors, have a prognostic role in ovarian cancer?	Immunohistochemi-cal analysis of ERα, ERβ, PR, and AR in 118 serous and endometrioid ovarian cancers. Analysis of corresponding mRNA values in an external dataset.		PR+ and AR+ expression was associated with a favorable prognosis. Dual PR+/AR+ expression conferred an additional prognostic benefit. mRNA values of the *PGR* and *AR* genes varied between the molecular subtypes, without clear prognostic implications.

List of original studies

This thesis is based on the following studies, which will be referred to in the text by their Roman numerals.

I. **Jönsson JM***, Bartuma K*, Dominguez-Valentin M, Harbst K, Ketabi Z, Malander S, Jönsson M, Carneiro A, Måsbäck A, Jönsson G, Nilbert M.
Distinct gene expression profiles in ovarian cancer linked to Lynch syndrome. *Fam Cancer.* 2014 Dec;13(4):537-545
*shared first authorship

II. Skovbjerg Arildsen N, **Jönsson JM**, Bartuma K, Sichmanova Z, Ebbesson A, Westbom-Fremer S, Måsbäck A, Malander S, Nilbert M, Hedenfalk I.
Targeted sequencing of ovarian clear cell carcinomas reveals frequent involvement of chromatin remodeling genes. *In manuscript*

III. **Jönsson JM**, Johansson I, Dominguez-Valentin M, Kimbung S, Jönsson M, Hansen Bonde J, Kannisto P, Måsbäck A, Malander S, Nilbert M, Hedenfalk I.
Molecular subtyping of serous ovarian tumors reveals multiple connections to intrinsic breast cancer subtypes. *PLoS One*. 2014;16;9(9)

IV. **Jönsson JM**, Skovbjerg Arildsen N, Malander S, Måsbäck A, Hartman L, Nilbert M, Hedenfalk I.
Sex steroid hormone receptor expression affects ovarian cancer survival. *Transl Oncol* 2015 8(5):424-433

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Study contribution

Study I

I participated in data collection and analysis, performed the immunohistochemical evaluations together with Katarina Bartuma, and was responsible for writing and revision of the manuscript.

Study II

I was involved in study design and applying for ethical approval for the study. I participated in RNA and DNA extractions, data analyses and drafting of the manuscript.

Study III

I was involved in study design, collection of tumor material, participated in the data analyses and was responsible for writing and revision of the manuscript.

Study IV

I participated in study design and evaluation of two of the immunohistochemical markers. I was responsible for data collection and analysis as well as writing and revision of the manuscript.

Abbreviations

AR	androgen receptor
BRCA1/2	breast cancer 1 and breast cancer 2, early onset; genes predisposing to hereditary breast and ovarian cancer
CA125	cancer associated antigen 125, ovarian cancer tumor marker
C1-C6	serous and endometrioid molecular ovarian cancer subtypes
CI	confidence interval
Ct	cycle threshold
DAB	3'3´diaminobenzidine, color used in immunohistochemical staining
DASL	cDNA mediated Annealing, Selection, extension and Ligation
ER	estrogen receptor
FIGO	International Federation of Gynecologic Oncology
FFPE	formalin fixed, paraffin-embedded
GnRH	gonadotropin releasing hormone
GO	gene ontology
HBOC	hereditary breast and ovarian cancer
HE4	human epididymis protein 4, ovarian cancer tumor marker
HGSOC	high-grade serous ovarian carcinoma
HNPCC	hereditary non-polyposis colorectal cancer (Lynch syndrome)
HR	hazard ratio
HRD	homologous recombination repair defect
HRP	horse radish peroxidase, enzyme for immunohistochemical staining
IHC	immunohistochemistry
Indel	insertion or deletion of DNA bases
IP	intraperitoneal

IV	intravenous
LGSOC	low-grade serous ovarian carcinoma
LINE-1	long interspersed nuclear element-1
MLH1	mutL homolog 1, gene predisposing to cancers related to Lynch syndrome
MMR	mismatch repair
MSH2/6	mutS homologs 2 and 6, genes predisposing to cancers related to Lynch syndrome
MSI	microsatellite instability
NGS	next generation sequencing
OCCC	ovarian clear cell carcinoma
OS	overall survival
PARP	poly-(ADP-ribose)-polymerase
PCR	polymerase chain reaction
PFS	progression-free survival
PMS2	post meiotic segregation increased 2, gene predisposing to cancers related to Lynch syndrome
PR	progesterone receptor
RIN	RNA integrity number
RMI	risk of malignancy index
RNAseq	RNA-based, massive parallel sequencing
SAM	significance analysis of microarrays
SNP	single nucleotide polymorphism
SNV	single nucleotide variation
SOEB	bilateral salpingo-oophorectomy, removal of the fallopian tubes and the ovaries
STIC	serous tubal intraepithelial carcinoma
TMA	tissue microarray
TP53	tumor protein 53, tumor suppressor gene
VEGF	vascular endothelial growth factor
WG-DASL	whole genome-DASL

6

Summary in Swedish - populärvetenskaplig sammanfattning på svenska

Varje år drabbas cirka 700 kvinnor i Sverige av äggstockscancer. Det motsvarar två fulla X2000-tåg. Bara ett av dessa tåg med äggstockspatienter kommer att nå sitt mål - bot. Äggstockscancer är en ovanlig sjukdom och risken att drabbas är liten (jämfört med t.ex. bröstcancer, som fyller hela 22 tåg i Sverige), men prognosen är dyster. Färre än hälften av dem som får diagnosen äggstockscancer är i livet fem år senare. Få eller diffusa symptom, som inte uppenbart signalerar allvarlig sjukdom, leder ofta till sen diagnos då sjukdomen redan hunnit sprida sig. Operation och cytostatika (cellgifter) botar en del, men majoriteten drabbas av återfall i sjukdomen inom två år.

Trots detta är bilden inte nattsvart. Mycket talar för att äggstockscancer är flera liknande sjukdomar snarare än en enda. Det kan förklara varför vissa patienter botas, medan andra får återfall. En del skillnader mellan de olika äggstockscancerformerna är välkända, som histologiska subtyper (cellutseendet i mikroskop), muterade (förändrade) gener samt ärftliga former av äggstockscancer. Mutationer orsakar cancertumörer, men gör också tumörerna sårbara så att de kan behandlas med olika läkemedel. Forskningen kring äggstockscancer tar alltmer hänsyn till skillnaderna som beskrivs här, t.ex. i studier på nya läkemedel, men många frågor återstår att lösa. I den här avhandlingen ingår fyra delarbeten som studerar olika former av äggstockscancer och undersöker hur de kan behandlas.

I studie I användes metoden genexpressionsprofilering för att analysera aktiva (påslagna) gener i ärftlig äggstockscancer kopplad till Lynch syndrom. Lynch syndrom medför en ökad risk att drabbas av flera olika cancersjukdomar. Genaktiviteten i 24 ärftliga äggstockstumörer skilde sig markant från genaktiviteten i 24 icke-ärftliga tumörer. När vi studerade genaktiviteten i de olika histologiska subtyperna fann vi att genaktiviteten i ärftlig äggstockscancer av serös och endometrioid histologisk typ skilde sig mycket från genaktiviteten i icke-ärftlig serös och endometrioid äggstockcancer. Äggstockscancer av klarcellstyp hade däremot liknande genaktivitet vare sig den var ärftlig eller icke-ärftlig. Vi analyserade också tre viktiga proteiner, p-mTOR, EGFR och PTEN, som kunde kopplas till de ärftliga tumörerna. Proteinerna studerades med immunhistokemi

(antikroppsfärgning av proteinerna) i mikroskop. Proteinuttrycket skilde sig dock inte mellan de ärftliga och de icke-ärftliga tumörerna.

Äggstockscancer av klarcellshistologi är ovanlig och cytostatika har ofta dålig effekt. I studie II genomförde vi genexpressionsprofilering av 15 klarcellstumörer och fann mycket varierande genaktivitet i tumörerna, trots att de föreföll väldigt lika i studie I. Tio klarcellstumörer analyserades därför med en mutationsanalys som kallas djupsekvensering. Vi noterade att flera gener som är inblandade i hur cellernas arvsmassa (DNA) kontrolleras och läses av till proteiner var muterade. Generna kallas kromatinreglerande och flera av dem verkar vara känsliga för olika läkemedel. Dessa resultat behöver dock analyseras ytterligare.

Den vanligaste formen av äggstockscancer är av serös histologi och har likheter - bl.a. speciella genmutationer - med aggressiv bröstcancer som kallas basal-lik. Indelning av cancerformer baserad på bl.a. genmutationer kallas molekylär subtypning. Sådan indelning används ofta för bröstcancer, men än så länge sällan för äggstockscancer. I studie III analyserades 59 serösa äggstockstumörer med genexpressionsprofilering och delades in i olika molekylära subtyper, både för bröstcancer och för äggstockscancer. Vi jämförde subtyperna och noterade likheter mellan de aggressiva äggstockscancerformerna och den basal-lika bröstcancergruppen. Vi fann också likheter mellan en form av äggstockstumör som kallas borderline (som kan omvandlas till aggressiv äggstockscancer) och hormonberoende bröstcancer. Detta bekräftades i ett annat, större material bestående av 285 tumörer.

I studie IV studerade vi hormonreceptorer med immunhistokemi i 118 serösa och endometrioida äggstockstumörer. Hormonreceptorer är proteiner på tumör-cellernas yta och de styr bl.a. vilka gener som översätts till vilka proteiner i cellen. Receptorerna kan blockeras med s.k. antihormonella läkemedel. Vi studerade två olika östrogenreceptorer (ERα och ERβ), progesteronreceptorn (PR) samt androgenreceptorn (AR). Fler av patienterna vars tumörer hade många PR eller AR levde efter fem år jämfört med dem som hade få PR eller AR. De vars tumörer som hade både många PR och många AR hade ännu bättre prognos, vilket är ett helt nytt fynd. Därefter undersöktes hormonreceptorgenerna i de olika molekylära subtyperna av äggstockscancer i materialet med 285 tumörer. Genaktiviteten varierade mellan subtyperna, men vi fann inga tydliga bevis för att hög aktivitet av PR- och AR-generna kunde kopplas till en bättre prognos. Resultaten i studie III-IV kan tyda på att antihormonella läkemedel möjligen kan vara effektiva mot vissa former av äggstockscancer, men det behöver studeras vidare.

Sammantaget visar de här fyra studierna att det är nödvändigt att gruppera äggstockscancer för att kunna ta hänsyn till alla skillnader, även mellan tillsynes liknande tumörer inom samma histologiska subtyp. Det kan göra det enklare att identifiera vilka tumörtyper som bör få vilka behandlingar och öka sannolikheten att båda tågen når sitt mål – att fler kvinnor med äggstockscancer blir botade.

8

Preface

Research is all about systematically asking the right questions in order to increase knowledge. Or, as Mark Twain phrased it: "Supposing is good, but finding out is better". This is as valid in the lab, as in the clinic, as in life in general. When gardening, *e.g.*, one of my main interests outside work, you do not settle with knowing where a plant grows to decide how to treat it. And you definitely do not treat all flowers the same way just because they are all red. Historically, though, that is how we have handled ovarian cancer (and many other cancers as well) in research and in clinical practice.

It has been known for a long time that ovarian cancer indeed comprises several disease entities. Recent evidence depicts the majority of ovarian cancers as non-ovarian originating and challenges old truths. Apart from the histopathologic features of ovarian cancers, molecular analyses are now routinely performed and clinical trials stratify patients with the aim of identifying subgroups of patients responding to new targeted treatments. Still, though, tumor heterogeneity is a major challenge. Hence, in order to manage the great heterogeneity among ovarian cancers, ovarian cancer research needs to focus on distinct questions.

This thesis describes an explorative approach to some of those questions. The included studies investigate a rare cause of hereditary ovarian cancer, distinct histologic subsets, and prognostic markers. We assess gene expression profiles in Lynch syndrome-associated ovarian cancer, study genetic alterations and molecular subtypes in clear cell and serous ovarian cancer, and determine the prognostic role of sex steroid hormone receptor expression in serous and endometrioid ovarian cancer.

With an integrated view on ovarian cancer research, considering origin, histopathologic, molecular, and clinical features, and predisposing factors, the chance of classifying the different ovarian cancers correctly increases and most certainly will lead to better therapeutics and identification of treatment predictive markers. Hence, resembling the botanic approach where you assess the *whole* plant and its environment before you may decide what you have in front of you.

> *"He who thus considers things in their first growth and origin…will obtain the clearest view of them." (Aristotle)*

Lund, December 2015

9

Background

General introduction

Ovarian cancer comprises two distinct tumor groups; epithelial ovarian cancer that arises in epithelial cells and accounts for approximately 90% of all ovarian cancer cases, and non-epithelial ovarian cancer that arises in germinal cells and in sex chord stromal cells and accounts for approximately 10% of all ovarian cancer. Approximately 240,000 women globally, including some 750 in Sweden, are diagnosed with ovarian cancer annually, with an incidence peak in the age group 65-69 years [1, 2]. This makes ovarian cancer the seventh most common female cancer type around the world [1].

The lifetime risk of developing ovarian cancer is only 1.3%, but it is the most lethal gynecologic malignancy [3]. Unspecific symptoms and the lack of specific biomarkers result in late diagnosis, rendering ovarian cancer the unflattering reputation of being a silent killer. In Europe, ovarian and fallopian tube cancer account for 4.1% of all female cancer diagnoses but 5.5% of all female cancer deaths. This is to be compared with *e.g.* breast cancer that in Europe accounts for 28.6% of female cancers and 16.9% of the female cancer deaths. However, the age-adjusted incidence of developing ovarian cancer varies greatly around the world, with the highest incidence seen in Europe, where ovarian cancer is the 6th most common female cancer, compared with eastern Asia where it is the 10th most common female cancer [1]. These differences are probably related to environmental and lifestyle factors as well as varying genetic predisposition.

The relative 5-year survival of epithelial ovarian cancer is 46%. However, long-term survival is closely associated with tumor stage and ranges from >80% in stage I (tumor localized to the ovaries/fallopian tubes) to 35% in stage III (tumor spread to the peritoneum outside of the pelvis) [3].

To date there is no evidence that systematic screening of women without family history or without hereditary, disease-predisposing mutations leads to earlier diagnosis or reduces the overall or ovarian cancer specific mortality [4-6]. Thus, population-based screening is not recommended. Results of the by far largest trial, the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS), including >200,000 women are however awaited late 2015.

11

Women with an increased risk of developing ovarian cancer (*i.e.* family history and/or verified disease-predisposing mutations) are recommended regular gynecological examinations and/or prophylactic surgery, but whether screening with transvaginal ultrasound and CA125 for this high-risk group is useful needs to be further explored [7, 8].

Histopathology

This thesis specifically studies epithelial ovarian cancer. From here on the terms ovarian cancer and epithelial ovarian cancer are used interchangeably and refer to epithelial ovarian cancer.

Epithelial ovarian cancer is a heterogeneous disease, consisting of five major histopathologic subtypes according to the WHO 2014 classification; high-grade serous ovarian carcinomas (HGSOCs), endometrioid carcinomas, ovarian clear cell carcinomas (OCCCs), mucinous carcinomas, and low-grade serous ovarian carcinomas (LGSOCs) [9]. Other histopathologic subtypes are present at lower incidences but are not outlined in this thesis. Examples of histological subtypes of ovarian cancer are shown in Figure 1.
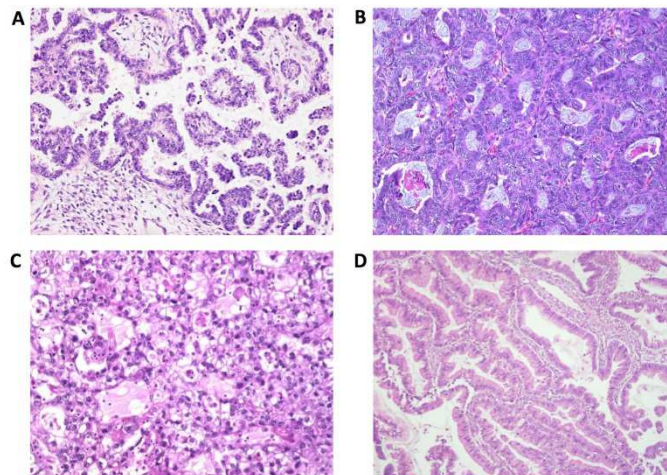

Figure 1. Photomicrographs of histological subtypes of ovarian cancer. A. High-grade serous ovarian cancer. B. Endometrioid ovarian cancer. C. Clear cell ovarian cancer. D. Mucinous ovarian cancer. Published with permission from Anna Måsbäck, Department of Clinical Pathology, Skåne University Hospital, Lund

High-grade serous carcinomas

HGSOC is the most common ovarian cancer subtype, accounting for 70% of all epithelial ovarian cancers [10]. The mean age at diagnosis is 63 years [9]. These tumors are characterized by a high degree of genomic instability and >95% harbor mutations in the tumor suppressor gene Tumor protein 53 (*TP53*). Amplifications of the oncogenes *PIK3CA*, *AKT*, *NOTCH3*, and *CCNE1* are also common [11]. Clinically, HGSOCs are often advanced at diagnosis, with bilateral tumors with both solid and papillary growth patterns. Microscopically, necrosis and numerous mitoses are frequently seen. Immunohistochemical (IHC) nuclear expression of WT1 and p53 can be used for diagnosis [9]. This tumor type is highly proliferating, making it aggressive but also generally sensitive to chemotherapy. Despite the often remarkable initial response to chemotherapy, the prognosis for advanced HGSOCs is generally poor, with <50% of the patients alive 5 years after diagnosis.

HGSOC is the most common ovarian cancer subtype among women with hereditary mutations in the breast cancer genes, early onset 1 and 2 (*BRCA1* and *BRCA2*), which are part of the homologous recombination repair system needed for repair of DNA double strand breaks. Several other possible homologous recombination repair defects (HRDs) can occur, and there are indications that as many as 50% of all HGSOCs harbor HRDs in some way. This is outlined in Figure 2.

As outlined in the Other targeted treatments section, HRDs and especially *BRCA1* and *BRCA2* mutations are important for a relatively new targeted therapeutic regimen for ovarian cancer inhibiting the poly-(ADP-ribose)-polymerase (PARP).

According to the WHO 2014 classification, HGSOC and LGSOC should be viewed as different tumor types and not just tumors with varying differentiation grade which the previous three-tiered grading system indicated. HGSOCs correspond to serous carcinomas classified as grade 2 or 3 in previous WHO classifications [9, 12, 13].

Low-grade serous carcinomas

LGSOCs account for <5% of all ovarian cancers and have only recently been identified as a distinct subtype of ovarian cancer [9, 10]. LGSOCs are diagnosed at a significantly lower age, mean 42 years, compared with HGSOCs [14]. Like HGSOCs, the LGSOCs often present with bilateral tumors with a papillary growth pattern. Calcifications and psammoma bodies are frequent. Borderline tumors are often found adjacent to LGSOCs. Approximately two-thirds of all LGSOCs harbor mutations in *KRAS*, *BRAF*, or *ERBB2*, whereas *TP53* mutations are rare [15]. In

general, long-term survival for LGSOCs even in advanced stages is favorable, with a median survival of >6 years compared with <2 years for HGSOCs [15, 16].

LGSOCs correspond to serous carcinomas previously classified as grade 1 [9, 12, 13].
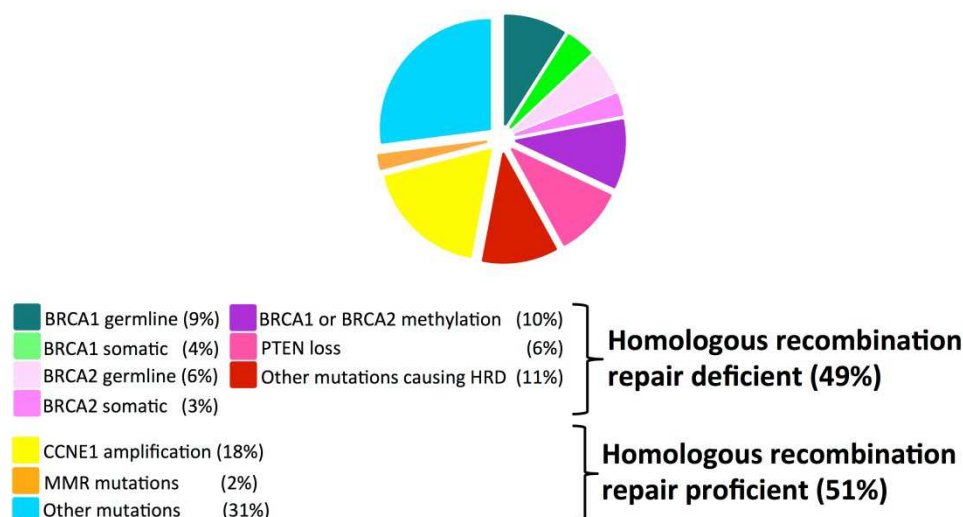
Figure 2. Overview of homologous recombination repair defects in HGSOCs. Figure adapted from [17].

Endometrioid carcinomas

Tumors of endometrioid histology account for approximately 10% of all ovarian cancers [10]. Endometrioid tumors are often associated with endometriosis (see Other risk factors). Some 15-20% of the patients also present with synchronous endometrial cancer. The mean age at diagnosis is 58 years. Endometrioid ovarian cancers are more often than HGSOCs confined to the ovaries at diagnosis. Macroscopically, the tumors appear smooth on the outside with a solid, hemorrhagic, or necrotic inside. The microscopic growth pattern is glandular and 30-50% of endometrioid ovarian cancers display squamous differentiation [9]. The mutation spectra differ between low- and high-grade endometrioid ovarian cancers. The former group may harbor mutations in *CTNNB1*, *PIK3CA*, *PTEN* and *ARID1A*, whereas the latter group resembles HGSOCs with frequent mutations in *TP53* [18, 19]. Hereditary defects in mismatch repair (*MMR*) genes, associated with Lynch syndrome (see the Hereditary ovarian cancer section) are

14

overrepresented. The prognosis for advanced, high-grade endometrioid carcinomas resembles that of HGSOCs with a poor 5-year survival, whereas low- and intermediate-grade endometrioid carcinomas generally have a favorable prognosis.

According to the International Federation of Gynecologic Oncology (FIGO) recommendations, histological grading of endometrioid ovarian cancers corresponds to the grading of endometrioid uterine cancers with grade 1 (well differentiated), grade 2 (moderately differentiated), and grade 3 (poorly differentiated) [9, 20]. Due to the worse prognosis, FIGO grade 3 is considered "high-grade" and FIGO grade 1-2 is considered "low-grade".

Ovarian clear cell carcinomas

OCCCs share many molecular features with well differentiated endometrioid ovarian cancers and account for 5-10% of all ovarian cancer in Europe and Northern America and as many as 25% in Asia, especially in Japan [10, 18, 21]. OCCCs are associated with *MMR* defects and this ovarian cancer subset is overrepresented in Lynch syndrome. OCCCs are often unilateral and confined to the ovary at diagnosis. Microscopically, these tumors show a clear, glycogen rich cytoplasm, from which the name clear cell is derived. The nucleus is central and often hyperchromatic (so called hob nail cells) [9]. More than 70% of OCCCs have been reported to be associated with endometriosis. Activating mutations in *PIK3CA* and inactivating mutations in *PTEN* and especially *ARID1A* are frequent [18, 22, 23]. Napsin A and HNF1β may be helpful diagnostic IHC markers [24, 25]. Although the majority of OCCCs are diagnosed in early stages the overall prognosis is worse than for the other subtypes [26, 27]. Apart from the tumors being low proliferative and thus *per se* less responsive to chemotherapy, the poor prognosis may also be due to a higher degree of primary chemotherapy resistance [28]. OCCCs are not histologically graded.

Mucinous carcinomas

Mucinous carcinomas make up 3-4% of all ovarian cancers [10]. These tumors often present as large, unilateral adnexal masses associated with borderline tumors. Microscopically, mucinous carcinomas show a complex glandular growth pattern, sometimes cribriform, and may appear labyrinth like with many mitoses. Mucinous metastases from the gastrointestinal tract are relatively common and represent a differential diagnosis in cases with bilateral mucinous ovarian tumors [9]. *KRAS* and *ERBB2* mutations occur in 40% and 20% of mucinous ovarian carcinomas respectively. Mucinous ovarian carcinomas are not histologically graded.

15

Borderline tumors

Borderline tumors constitute a subgroup of epithelial ovarian cancer, comprising ovarian tumors that morphologically appear to be in between benign cystadenomas and malignant adenocarcinomas (hence the name "borderline"). The morphological features display an atypical, proliferative epithelium. The majority of borderline tumors are serous (55%), followed by mucinous (30-50% of all borderline tumors). Endometrioid and clear cell borderline tumors are rare (4% and 1% respectively). There is little evidence that borderline tumors other than the serous subset actually spread or affect survival [9]. The prognosis of a metastasized serous borderline ovarian tumor is far better than the prognosis of a metastasized serous ovarian adenocarcinoma, and the serous borderline extra-ovarian extensions are therefore called implants rather than metastases [29]. The implants can be sub-divided into non-invasive and invasive implants, and this classification impacts prognosis [29, 30]. Nowadays serous invasive implants are classified as LGSOCs [9]. There is no resemblance between serous borderline tumors and HGSOCs [12].

Carcinomas of the fallopian tube and the peritoneum

Epithelial tumors of the fallopian tube and the peritoneum are predominantly of serous histology and resemble their ovarian counterpart HGSOC. Both fallopian tube and primary peritoneal carcinomas frequently harbor *TP53* mutations and are associated with germline *BRCA1* and *BRCA2* mutations. Diagnosis of a primary peritoneal cancer requires minimal or no tumor involvement of the fallopian tubes or the ovaries. The diagnosis fallopian tube carcinoma is more complex. Historically, the diagnosis ovarian cancer has been used when tumor was present in the ovary regardless of the amount of tumor in the fallopian tube, but a change in praxis is under way. When serous tubal intraepithelial carcinomas (STICs), *i.e.* small, early carcinomas found in the fallopian tube and fimbriae, are detected, the diagnosis fallopian tube cancer is often preferred. Both fallopian tube and primary peritoneal carcinomas are staged, graded and treated like ovarian cancers [9].

Ovarian cancer carcinogenesis

Despite numerous efforts to outline the molecular events that drive ovarian carcinogenesis, no linear progression model such as that described in colorectal cancer has been defined [31]. It has, logically, been thought that the origin was ovarian. Tumors within the ovary have been thought to arise from inclusion cysts with ovarian surface epithelium that have undergone metaplasia. Thereby, the

tumors have developed as müllerian like serous, endometrioid, or clear cell ovarian tumors, rather than expressing mesothelial features like the ovary itself [32].

In the absence of defined precursors, ovarian cancers have been said to arise *de novo* [33]. This theory was challenged in 2004 when Ie Ming Shih and Robert J. Kurman proposed a dualistic model of ovarian cancer development, dividing ovarian cancers into type I and type II tumors. Type I tumors were proposed to be slowly proliferating from benign adenomas and adenofibromas via borderline tumors to low-grade tumors, and type II tumors rapidly evolving to high-grade, aggressive tumors. LGSOC represents the prototypic type I tumor and HGSOC the prototypic type II tumor [32]. The theory was molecularly and genetically robust, and was supported by several reports during the following years. STICs, for example, were detected and could be associated with both serous peritoneal cancers and as many as 70% of all sporadic ovarian cancers [34-36]. This was in line with previous findings of hyperplastic changes in tubal epithelium from *BRCA1* and *BRCA2* mutation carriers who had undergone prophylactic salpingo-oophorectomies [37]. Furthermore, a limited gene expression study revealed that the expression profile of serous ovarian cancers (mainly HGSOCs) resembled that of the fallopian tube rather than that of the ovarian surface epithelium. Likewise, endometrioid and clear cell cancers displayed gene expression profiles resembling profiles derived from the endometrium, and expression profiles of mucinous cancers resembled the normal colon epithelium [38]. A paradigm shift had occurred and the fallopian tube was proposed to represent the origin of serous cancers [39]. The anatomical proximity of the fimbriae of the fallopian tube and the ovary allows for cell drop (at *e.g.* ovulation) or simply overgrowth from the fimbriae to the ovary, leading back to the theory of ovarian inclusion cysts but proposing another cell origin. This is further supported by the fallopian tube being of müllerian embryological origin.

The dualistic model has been refined during the recent years [12, 40]. Based on the histologic presentation and the genetic changes in different ovarian cancers LGSOCs, low-grade endometrioid cancers, OCCCs, and mucinous cancers are referred to as type I, all with a proposed successive progression from benign via intermediate precursors. The LGSOC precursor probably arises in the ovary, whereas endometrioid ovarian cancers and OCCCs are thought to arise from endometriosis. The *KRAS* and *BRAF* mutations found in LGSOCs and mucinous cancers as well as the *PTEN* and *CTNNB1* mutations found in endometrioid cancers have also been revealed in their respective precursors [32]. The exact origin of mucinous ovarian cancer remains to be elucidated.

HGSOCs and high-grade endometrioid ovarian cancers are referred to as type II tumors. These tumors rapidly progress into highly aggressive cancers [40]. HGSOCs are thought to originate in the fallopian tube. Type I tumors account for

17

25% and type II tumors for 75% of all ovarian cancers [12]. An overview of the dualistic type I and type II model is outlined in Figure 3.
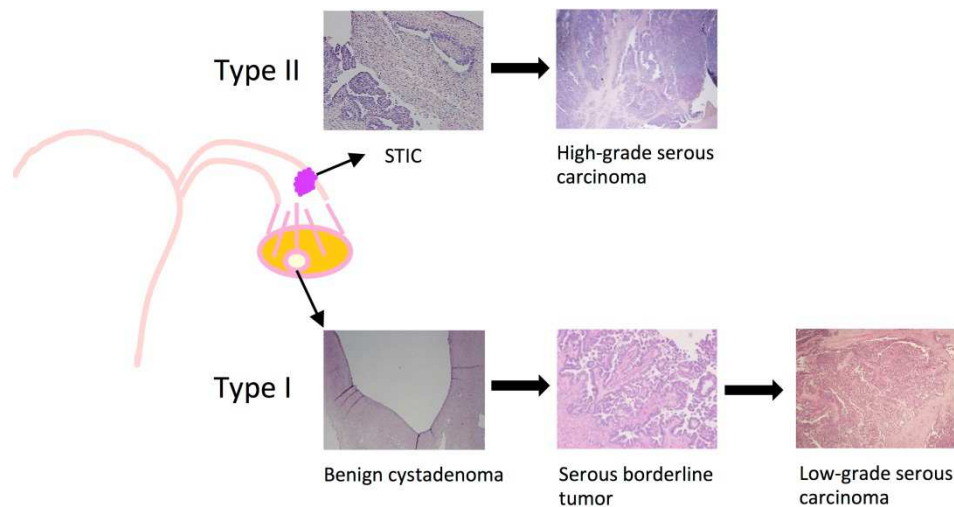
Figure 3. Schematic view of the ovarian cancer pathogenesis model. The top row outlines the so called type II tumors, originating from STICs in the fallopian tube. The left photomicrograph shows proliferative, irregular intraepithelial carcinoma cells in the left corner. The right picture shows an infiltrating mass of high-grade carcinoma cells (dark purple).

The bottom row outlines the typ I tumor propagation. The left picture shows an inclusion cyst, the borderline (middle) picture shows a papillary growth pattern with atypical, but not invasive, cells (dark purple). The right picture shows infiltrating, low-grade carcinoma cells (dark pink). Photomicrographs are published with permission from Anna Måsbäck, Department of Clinical Pathology, Skåne University Hospital, Lund.

Although the dualistic model referred to here is a theoretical, but thoroughly reinforced, explanation model it is important in that it may provide part of the explanation for why screening for ovarian cancer has not been successful. If the precursor is not ovarian, *e.g.* HGSOCs will be hard to prevent or detect early when focusing on the ovaries. Furthermore, this model underlines that ovarian cancer should be regarded as different diseases or at least as different disease entities, which is of importance when deciding which tissue type to use as reference tissue for comparison and also for stratification in clinical trials.

18

Molecular subtypes of ovarian cancer

The purpose with tumor subtyping is to create stratification for comparison, *i.e.* simply to compare like with like. This is routinely done using histopathological subtyping, which has evident clinical implications for treatment decisions and prognosis. During the latest 15 years gene expression-based molecular subtyping of tumors has evolved, starting off with the intrinsic breast cancer subtypes being proposed in the year 2000 and being followed by molecular subtyping of other tumor types such as bladder cancer and melanoma [41-43]. The Cancer Genome Atlas Research Network (TCGA) has published several studies attempting to decipher the molecular basis for cancer [44-46]. Likewise, the large amount of publicly available data within the TCGA, with tumor samples and normal tissue from 11,000 patients with >30 different cancer types, help facilitate the analysis of tumors and validation of results, especially when working with relatively rare cancer forms like ovarian cancer (http://cancergenome.nih.gov.).

Gene expression analyses have been used to assess differences between the histopathological subtypes of ovarian cancer, to identify similarities with proposed tissues of origin, and to characterize differences between ovarian tumors with different malignant potential [38, 47-51]. These studies support the dualistic type I and type II pathogenesis model [32]. Efforts have also been made to use gene expression analyses for prognostication, outlining profiles of early stage vs. advanced stage disease, short-term vs. long-term survivors as well as chemotherapy responders [52-57].

In 2008, David Bowtell's group outlined molecular subtypes of ovarian cancer using 285 tumors, primarily HGSOCs, fallopian tube, and primary peritoneal cancers, but also LGSOCs, endometrioid cancers, and serous borderline ovarian tumors [58]. Six novel subtypes, called C1-C6, each with a specific gene expression profile, were reported. C1, C2, C4, and C5 represented high-grade serous and some high-grade endometrioid cancers. The C3 and C6 profiles were even more distinct, in line with these subtypes consisting of borderline and some LGSOCs (C3) and low-grade endometrioid cancers (C6). The subtypes were further characterized and the C1 subtype was associated with a high expression of stroma genes and desmoplasia, whereas C5 was associated with high expression of proliferation genes and upregulation of Wnt signaling. C2 and C4 both showed high expression of immune response genes, but differed in terms of stroma gene expression. In terms of survival, the C1 expression profile had the worst prognosis, followed by C5, whereas C3 and C6, logically, conferred a favorable prognosis [58].

In 2011 the TCGA revised these molecular profiles using only HGSOCs and established refined signature names referred to as "mesenchymal" (C1), "proliferative" (C5), "immunoreactive" (C2), and "differentiated" (C4).

Surprisingly though, the prognostic effect of the subtypes could not be replicated in the TCGA dataset [11]. Despite this, molecular profiling of ovarian cancer, primarily HGSOC, has continued to gain interest. Competing subtypes based on different histopathologic subtypes of ovarian cancer have also been proposed [59]. The HGSOC gene expression profiles have been further revised, but kept their TCGA-assigned names. The prognostic differences have been successfully replicated, and the use of molecular subtyping for treatment prediction is suggested although not proven [54, 60, 61]. It should also be pointed out, that so far an overlap has been noted between the suggested profiles within the different studies. Thus, no ideal "classifier" exists to date. The use of molecular ovarian cancer subtypes in the clinical practice certainly needs further investigations, but underlines the heterogeneity of ovarian cancer, even within an already defined subgroup like HGSOCs, and is relevant to keep in mind for future studies.

Hereditary ovarian cancer

Genetic susceptibility represents the strongest risk factor for ovarian cancer. Of all ovarian cancers, 10-15% are estimated to be hereditary [62, 63]. The contributions of inheritable mutations to ovarian cancer are outlined in Figure 4. The major contributors to hereditary ovarian cancer are *BRCA1* and *BRCA2* mutations and mutations in the *MMR* genes associated with Lynch syndrome [64-66]. Mutations in the *BRCA1*, *BRCA2*, and *MMR* genes have a high penetrance, *i.e.* confer a high risk of developing cancer. Other hereditary mutations associated with an increased risk of ovarian cancer are *TP53* (Li Fraumeni syndrome), *CHEK2*, and *RAD51* [67, 68]. Low penetrance mutations may be due to single nucleotide polymorphisms (SNPs); common amino acid changes in the coding sequence which may be disease-predisposing and which may also influence the penetrance of cancers associated with *e.g. BRCA1* and *BRCA2* mutations [62, 69-71]. So called site-specific ovarian cancer also exists and is represented by an accumulation of ovarian cancer but no other cancer cases in the family. No defined mutations have been linked to this syndrome.

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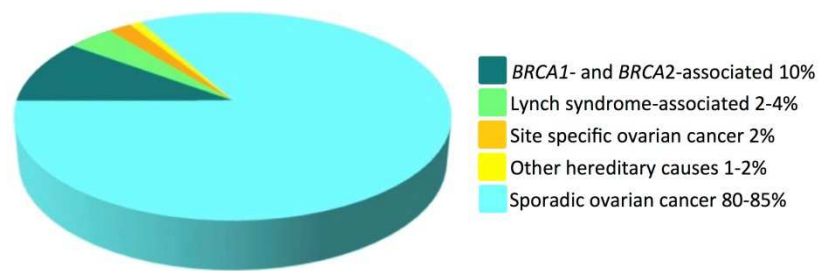
Figure 4. Overview of hereditary causes and their contributions to ovarian cancer.

BRCA1-, *BRCA2*-, and Lynch syndrome-associated ovarian cancers are outlined in this section. Mutations in *BRCA1* and *BRCA2* cause approximately 65-85% of all hereditary ovarian cancers whereas 10-15% of the hereditary cases are linked to Lynch syndrome [66, 72, 73]. Hereditary causes should be suspected if

- the age at diagnosis is <40 years

- the patient or a relative has been diagnosed with both ovarian and breast cancer

- there are several cases of ovarian and breast cancer (especially breast cancer diagnosed <40 years of age and/or bilateral breast cancer) in the family

- there are cases of male breast cancer in the family

- the patient or a relative has been diagnosed with both ovarian and/or endometrial or colorectal cancer

- there are several cases of colorectal and endometrial cancer in the family

Then the patient should be referred to oncogenetic counseling. In Sweden this means risk assessment and possibly mutation testing. If a mutation is verified, the patient is offered further education and advice regarding prophylactic surgery.

On average, the lifetime risk for a woman to develop ovarian cancer when she has one first degree relative (parent or sibling) with ovarian cancer is approximately 5% and when two first degree relatives are affected the risk is approximately 7%. The risk is generally higher before 50 years of age and thereafter gradually declines [62].

BRCA1- and *BRCA2*-associated ovarian cancer

The *BRCA1* and *BRCA2* genes were identified in the early 1990's and were associated with hereditary breast and ovarian cancer (HBOC). *BRCA1* was mapped to chromosome 17q21 and *BRCA2* to chromosome 13q12 [74-76]. The presence of families with an accumulation of breast cancer had, however, been recognized for a long time and was first described by the French physician Paul Broca in the mid 1800's, though it took more than 100 years before the precise molecular events were discovered [62].

The *BRCA1* and *BRCA2* genes are large, complex tumor suppressor genes. Mutations in these genes are inherited in an autosomal dominant way. As previously described, *BRCA1-* and *BRCA2*-mutated cells have a defective homologous recombination repair system, resulting in an impaired ability to repair double strand DNA breaks. This increases the susceptibility to other DNA damaging events, resulting in an increased cancer frequency. It does however also make them sensitive to PARP inhibitors (see Other targeted therapies).

Hereditary ovarian cancer associated with *BRCA1* is more common than *BRCA2*, and the clinical presentation also differs. The lifetime risk of developing ovarian cancer for a *BRCA1* mutation carrier is 40-50% (70-80% risk of breast cancer) and the mean age at diagnosis is lower than for sporadic ovarian cancer; 50-55 years [77]. There is also an increased risk of fallopian tube and primary peritoneal cancer [37]. For a *BRCA2* mutation carrier the lifetime risk of developing ovarian cancer is 15-25% (40-50% risk of breast cancer) and the mean age at diagnosis is somewhat higher, 55-56 years. *BRCA2* mutations are also associated with pancreatic cancer, as well as male breast cancer and prostate cancer [76-79].

Histologically, *BRCA1-* and *BRCA2*-associated ovarian cancer cannot be distinguished from sporadic ovarian cancer. HGSOC is the most common subtype and approximately 20% of all HGSOCs have been reported to harbor germline mutations in *BRCA1* and *BRCA2*. However, *BRCA1-* and *BRCA2*-associated ovarian cancers tend to respond better to platinum-based chemotherapy both as first line treatment and as repeated treatment at relapse compared with sporadic ovarian cancers [80].

Hundreds of different mutations in the *BRCA1* and *BRCA2* genes have been identified, but a few mutations account for the majority of all germline variants identified [62]. Among them are several founder mutations, *i.e.* specific mutations with a high frequency in a defined population group. Three specific founder mutations (185delAG and 5382insC in *BRCA1* and 6174delT in *BRCA2*) are identified in 2% of Ashkenazi Jews, and strong founder mutations have been identified also in the Nordic countries [62, 81]. This is important for identifying mutation carriers. The most common *BRCA1* and *BRCA2* mutations are nonsense (resulting in truncated, non-functioning proteins) and frameshift deletions

22

(resulting in changed proteins with altered or no functions). Among the other mutations are *e.g.* missense mutations, which may not necessarily be disease-predisposing.

Some 40% of all patients with germline *BRCA1* or *BRCA2* mutations have no family history of breast and/or ovarian cancer, and up to 25% of all HGSOCs may harbor either germline or somatic mutations in the *BRCA1* and *BRCA2* genes [11, 80, 82]. These latter mutations are not inherited, but *e.g.* somatic inactivation of *BRCA1* or *BRCA2* identifies ovarian cancers that respond to PARP inhibitors. This may also be valid for non-*BRCA1/2* HRDs. Hence the guidelines for identifying patients who should be offered genetic counseling need to be modified. In a near future we will hopefully be able to offer genetic testing for *BRCA1* and *BRCA2* mutations to all women diagnosed with HGSOC, regardless of family history.

Lynch syndrome-associated ovarian cancer

Lynch syndrome, formerly referred to as hereditary non-polyposis colorectal cancer (HNPCC) was first described in the early 1900's by the American physician Aldred Warthin, who identified a link between hereditable gastrointestinal cancer and endometrial cancer [83]. However, just like for *BRCA1*- and *BRCA2*-associated cancers it took several decades until the presence of this cancer syndrome was confirmed and thoroughly described [84, 85]. In the early 1990' Lynch syndrome-associated cancers were linked to mutations in the DNA *MMR* genes mutL homolog 1 (*MLH1*, chromosome 3p21-23), mutS homolog 2 (*MSH2*, chromosome 2p21-22), mutS homolog 6 (*MSH6*, chromosome 2p16), and postmeiotic segregation increase 2 (*PMS2*, chromosome 7p22) [86-89]. The term homolog refers to the genes as being homologous to the genes first discovered in *E.Coli* [90].

MMR proteins encoded by the above mentioned genes are necessary for repair of single strand DNA breaks induced during replication [91]. Gene complexes formed by *MSH2/MSH3* (MutSα) and *MSH2/MSH6* (MutSβ) interact with a MutL complex, among which the one constituted by *MLH1/PMS2* (MutLα) is the most common. Together these complexes scan the DNA for replication errors, excise the nucleotides that are incorrect (mismatched) and resynthesize the DNA [92, 93].

Across the genome there are several so called microsatellites, short DNA sequences (1-6 base pairs) that are frequently repeated. The microsatellites are involved in regulation of gene transcription. They are prone to errors in MMR since the polymerase involved in replacement of incorrect nucleotides has a tendency to "slip" on these repetitive sequences. This results in variable lengths of the microsatellites, referred to as microsatellite instability (MSI), which constitutes a hallmark of Lynch syndrome [67, 92].

23

Heterozygous *MMR* mutations are inherited in an autosomal, dominant pattern and are associated with a high risk of colorectal cancers, hence the previous term HNPCC. Lynch syndrome is responsible for approximately 5% of all colorectal cancers, but the lifetime risk of developing colorectal cancer for *MMR* mutation carriers is 80% [94]. A range of tumor types are associated with Lynch syndrome, such as ovarian cancer, endometrial cancer, urothelial cancer, brain tumors, and skin tumors, and nowadays the name Lynch syndrome is preferred [95]. More than half of the women with *MMR* gene mutations will present with a gynecologic cancer as the first ("sentinel") cancer [96]. There are also indications that the risk of developing endometrial cancer is higher than the risk of colorectal cancer for female *MMR* mutation carriers, with a cumulative endometrial cancer risk of 40-60% [97, 98].

For female *MMR* gene mutation carriers the lifetime risk of developing ovarian cancer is approximately 6-12% [95]. The age at diagnosis is substantially younger than for sporadic ovarian cancer, <50 years of age, and the majority of tumors are low stage and low-grade [99-102]. Furthermore, Lynch syndrome-associated ovarian cancers are often endometrioid, clear cell, or mucinous, compared to the most common serous histology among sporadic and *BRCA1*- and *BRCA2*-associated ovarian cancers [103, 104]. The prognosis is generally favorable; a retrospective multi-center study with longtime follow up data has reported approximately 65% 10-year survival for serous ovarian cancers linked to Lynch syndrome and >80% 10-year survival for non-serous ovarian cancers [104]. More than 20% of Lynch syndrome-associated ovarian cancers also present with synchronous endometrial cancers [99]

MLH1 and *MSH2* mutations make up >90% and *MSH6* and *PMS2* mutations account for 7-10% of all mutations associated with Lynch syndrome. The clinical presentation of the tumors associated with the respective genes differs somewhat, with *MSH2* and *MSH6* mutations conferring a higher risk of ovarian cancer than mutations in *MLH1*. The age at diagnosis is also lower for ovarian cancers associated with mutations in *MSH2* than *MLH1* [66].

The Amsterdam II criteria and the Bethesda guidelines can be used as guides to select patients for whom MMR testing is indicated, apart from the general guidelines regarding identification of patients with potential hereditary ovarian cancer outlined at the introduction of this section [105, 106]. However, no guidelines are perfect. Therefore, routine *MMR* gene testing for all colorectal cancers and endometrial cancers diagnosed before 70 years of age would probably be beneficial [107]. For identification of Lynch syndrome carriers either an MSI analysis (performed on tumor tissue or blood samples) and/or MMR IHC staining can be performed. In Sweden, the method of choice is MMR IHC, which has a sensitivity of 90-95% and a specificity of 98-100% in colon cancer tissue [108]. The MMR proteins function as heterodimers (as in the previously described gene

24

complexes), with *MLH1* mutated tumors mostly showing concomitant loss of MLH1 and PMS2 whereas *MSH2* mutated tumors show loss of MSH2 and MSH6.

Surveillance and prophylactic intervention in hereditary cases

The lack of efficient screening methods for ovarian cancer implies that there is no truly effective way of identifying early stage ovarian cancers in mutation carriers. Two Dutch studies including women at high risk of hereditary ovarian cancer have reported advanced stage ovarian cancers despite regular screening visits as well as a disappointingly low sensitivity for both pelvic examinations and transvaginal ultrasound [109, 110]. In the absence of better surveillance, the International Gynecologic Cancer Society recommends that healthy *BRCA1* and *BRCA2* mutation carriers should undergo regular gynecological examinations (www.igcs.org/). As in the general population, the use of oral contraceptives reduces the risk of ovarian cancer (see Other risk factors) [111-113]. Epidemiological studies have reported a slight increase in breast cancer risk among oral contraceptive users, but the risk is very modest and the *BRCA1* and *BRCA2* mutations *per se* confer a considerably higher risk [114-116]. However, the best intervention to prevent ovarian cancer is bilateral salpingo-oophorectomy (SOEB) once childbearing is completed (but preferably before 40 years of age), which decreases the ovarian cancer specific mortality with approximately 80% and has also been reported to decrease the risk of breast cancer with as much as 50% [117-119].

Taking into account the pathogenesis model with type I and type II tumors, prophylactic salpingectomy (*i.e.* leaving the ovaries) would potentially be sufficient to prevent fallopian tube/ovarian cancer in *BRCA1* and *BRCA2* mutation carriers [120]. Risk-reducing salpingectomy, though, does not reduce the risk of breast cancer since the ovaries are not removed. The evidence of a reduced breast cancer risk after prophylactic SOEB has however recently been questioned and may support that salpingectomy is sufficient, at least for *BRCA1* mutation carriers, but the data are insufficient to recommend only salpingectomy before the proven SOEB [121-124].

For women with *MMR* gene mutations, annual gynecological examinations, including transvaginal ultrasound to assess the thickness of the endometrium and endometrial biopsies if indicated, are recommended, but the scientific evidence for these yearly follow-up procedures is scarce [107]. Prophylactic hysterectomy, though, is recommended after completed childbearing [125].

Other risk factors

Apart from hereditary causes, other risk factors for developing ovarian cancer include nulliparity, endometriosis, and anthropometric factors.

Obesity, body height, and smoking are general risk factors that confer an increased risk of ovarian cancer. In epidemiological studies obesity (body mass index ≥30) has been associated with LGSOCs, endometrioid, and mucinous carcinomas among premenopausal women, whereas height ≥170 cm has been associated with a general increase in ovarian cancer, although the biological relationship between height and ovarian cancer is not clear [126]. A prospective study of >145,000 women in the USA has not revealed any associations between the level of physical activity and risk of ovarian cancer [127]. Smoking, though, is associated with an increased risk of mucinous ovarian cancer [128].

Parity

Nulliparity is a risk factor for developing ovarian cancer. In line with this observation, pregnancy and the use of oral contraceptives containing estrogens and gestagen (synthetic progesterone) are protective factors [112, 129]. In fact, the more pregnancies, the higher the protection rate. This implies that ovulation has a role in ovarian cancer carcinogenesis and has been referred to as the "incessant ovulation hypothesis" due to the repeated ovulations taking place during the fertile period of a woman's life. During ovulation, a wound in the ovary is inflicted. The "break" from ovulation during pregnancy or the use of oral contraceptives decreases the risk of DNA lesions inflicted by repeated inflammatory and wound healing mechanisms initiated during ovulation [130, 131]. The protective effect from oral contraceptive use is evident up to 30 years after the last intake [129].

In the normal ovary, estrogens are growth and differentiation regulating, and also seem to harbor mutagenic effects [132]. Progesterone is growth inhibiting and induces apoptosis, although this effect is likely evident at higher concentrations. At low concentrations, progesterone may in fact be growth stimulating [132, 133]. In line with this, hormone replacement therapy, especially such only including estrogen, is associated with a slightly increased risk of ovarian cancer. The risk is highest for the serous subtypes [134-137]. Hormone replacement therapy for menopausal symptoms after ovarian cancer treatment does however seem to be safe [138]. There is conflicting evidence regarding the potential risk of fertility stimulating drugs. An increased risk of borderline tumors has been observed, but no evident increase in ovarian carcinomas [139, 140].

Endometriosis

Endometriosis is a well-known risk factor of endometrioid and clear cell ovarian cancers [141, 142]. Endometriosis is the presence of endometrial tissue outside of the uterus, probably due to retrograde menstruation. It is a common feature, though the prevalence varies depending on age and other associated factors such as infertility. Overall, the incidence in the female population is estimated to be approximately 5% [141]. Endometriosis is associated with 30-40% of endometrioid ovarian cancers and 45-70% of OCCCs [22, 143]. The high estrogen and low progesterone concentrations as well as oxidative stress related to the high iron concentrations associated with endometriotic cysts are hypothesized to be the link between endometriosis and these specific histopathologic subtypes [144]. Although endometriosis is often found adjacent to endometrioid ovarian cancers and OCCCs, the majority of women with endometriosis will *not* develop ovarian cancer. The risk seems to be associated with atypical endometriosis, differing from the average benign endometriosis in terms of hyperplasia and cellular atypia, and occurring in <4% of all endometriosis cases. Atypical endometriosis, however, shares the same molecular changes as endometrioid and clear cell ovarian cancer in terms of frequent *ARID1A*, *PTEN*, *KRAS* and *CTNNB1* mutations [18, 145, 146]. The risk of developing ovarian cancer may also be associated with the anatomical location of endometriosis.

Ligation of the fallopian tubes as a sterilization method has been associated with a decreased risk of ovarian cancer [147]. The reasons for this are not fully elucidated, but are in line with the theory that prevention of retrograde menstruation, which contributes to endometriosis spread, would potentially reduce the risk of ovarian cancer. Other mechanisms clearly must be present as well, though.

Ovarian cancer in clinical practice

Early diagnostics is challenging in ovarian cancer due to unspecific symptoms and the lack of reliable screening methods [148]. The majority of patients with ovarian cancer are diagnosed with advanced stage disease with involvement of lymph nodes and tumor spread within the abdomen outside of the pelvis. This is due to the anatomical location of the ovaries and the lack of disease-specific symptoms. Many patients suffer from early satiety, bloating/abdominal swelling, and diffuse abdominal pain. A woman presenting with symptoms that may indicate ovarian cancer should undergo a clinical gynecological examination, including abdominal and transvaginal ultrasound for visualization of ovarian/adnexal cysts, and blood tests for the tumor marker Cancer associated antigen 125 (CA125). If the suspicion

of ovarian cancer is confirmed a computed tomography scan is advised to reveal potential tumor spread. Enlarged lymph nodes, ascites, or pleural effusions should be assessed using fine needle aspirations. The Risk of Malignancy Index (RMI) considers CA125, menopause status and grading of ultrasound findings. The RMI I by Jacobs *et al.* has a sensitivity of 85% and a specificity of 97% at the cut-off level 200 [149]. In practice this means that an RMI >200 strongly indicates a potential malignant adnexal mass that requires further investigation. Fine needle aspiration of an ovarian cyst that may represent early stage ovarian cancer should however not be performed due to the risk of tumor cell spread. Patients with ovarian cancer should be evaluated pre-operatively at multidisciplinary team conferences with participation from gynecologic oncology surgeons, gynecologic oncologists, radiologists, gynecologic pathologists, and contact nurses or coordinators [150].

Tumor staging

Clinically, epithelial ovarian cancer, fallopian tube cancer and primary peritoneal cancer are managed in a similar way. Tumor staging for these tumor types is uniform and follows the FIGO classification [151]. Adequate staging requires histological/cytological verification of the tumor and of the tumor spread. The FIGO classification system and the proportion of ovarian cancer diagnosed in the respective stages are outlined in Figure 5.
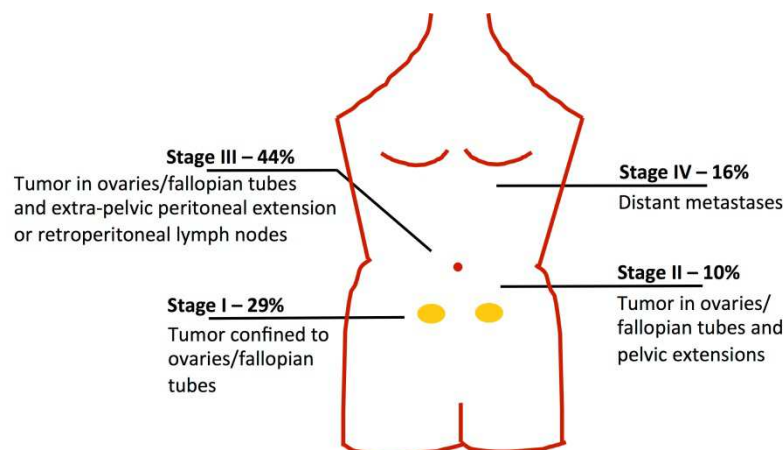

Figure 5. Schematic overview of the FIGO staging system with the proportion of ovarian cancer diagnosed in the respecive stages. The numbers are obtained from [150] and based on Swedish incidence numbers 2008 through 2012 (1% of the ovarian cancers were unstaged/stage X).

28

As outlined here, stage I comprises tumors that are confined to the ovaries or fallopian tubes. However, there is great variability in the prognosis between stage IA (tumor confined to one ovary with an intact capsule and without tumor cells on the surface) and stage IC (tumors confined to one or both ovaries/fallopian tubes but with either capsule rupture or surgical spill, tumor cells on the surface or malignant cells in ascites/abdominal washings) [151]. This impacts the treatment of stage I disease, which is outlined in the Post-operative chemotherapy section.

Ovarian cancer surgery

Surgery is the single most important treatment modality for patients with ovarian cancer. The principles for ovarian cancer surgery have evolved during recent years and require tumor-specific gynecological surgical competence [152]. The surgical procedure depends on the tumor stage, but overall the aim with up-front surgery is to confirm the diagnosis, allow for a proper tumor staging and, optimally, to remove all macroscopic tumor tissue (radical surgery). If radical surgery is not possible, removal of as much macroscopic tumor as possible, so called debulking or cytoreductive surgery, is performed. For advanced stage ovarian cancer (stages II-IV) radical surgery is extensive and apart from SOEB, hysterectomy, and omental resection also includes resection of peritoneal carcinomatosis, extirpation of enlarged lymph nodes, and, if necessary, *e.g.* bowel resection and peritoneal stripping. Macroscopically radical surgery increases the overall survival (OS), thus justifying this extensive surgical procedure [153, 154]. The benefit of total lymph node resection in advanced stage ovarian cancer surgery is under evaluation in clinical trials.

Post-operative chemotherapy

The purpose of post-operative (adjuvant) chemotherapy is to eradicate remaining tumor cells, increase the chance of cure, and postpone disease relapse. Platinum agents (cisplatin and carboplatin) were introduced during the 1980's and still constitute the chemotherapeutic backbone of ovarian cancer chemotherapy.

Stage IA-IB LGSOC, grade 1-2 endometrioid tumors, and mucinous tumors have an excellent prognosis with almost 95% 5-year OS after surgery and are not recommended adjuvant treatment [155, 156]. Post-operative chemotherapy is reserved for high-risk stage I disease and advanced disease (stages II-IV). High-risk stage I disease includes OCCCs, any stage I grade 3 and/or any stage IC tumors. Based on the ICON1 and ACTION trials, which showed an 8-10% increase in recurrence-free survival (both studies) and OS (ICON1), six cycles of platinum-based chemotherapy is recommended [157, 158]. Although no significant survival differences between carboplatin and cisplatin have been

shown, carboplatin is the most commonly used agent due its preferable toxicity profile.

Based on the GOG 111 and the OV10 trials, the addition of a taxane to the platinum agent is recommended for any grade 3 tumor as well as for stage II-IV ovarian cancer regardless of histopathologic subtype [159, 160]. The use of cisplatin/paclitaxel increased the median OS with approximately 10 months compared with cisplatin/cyclophosphamide. The current golden standard treatment consists of six cycles of a combination of carboplatin and paclitaxel administered intravenously (*IV*).

Neoadjuvant chemotherapy

The role of neoadjuvant chemotherapy, *i.e.* chemotherapy treatment up-front with the aim of reducing the tumor burden before surgery, has been debated in ovarian cancer during the past 10-15 years. There is no clear benefit in terms of OS or progression-free survival (PFS), and outcome seems to be similar for patients who receive neoadjuvant chemotherapy compared to those who receive adjuvant treatment [161, 162]. Another controversial issue is whether neoadjuvant platinum treatment may induce platinum resistance, and thereby a risk of missing the chance of radical surgery in platinum resistant cases [163, 164]. Neoadjuvant chemotherapy therefor remains an option for carefully selected patients in stage IIIC-IV where up-front radical surgery is not possible. When neoadjuvant chemotherapy is applied, three cycles are administered followed by interval surgery and post-operative chemotherapy to a total of at least six cycles [165].

Intraperitoneal chemotherapy

Considering that ovarian cancer primarily spreads loco-regionally within the abdomen, administration of intraperitoneal (*IP*) chemotherapy is a logical approach. Several clinical trials have been conducted, and one of the most cited is the GOG 172 study published in 2006, which compared *IV* cisplatin + paclitaxel to *IV* cisplatin + *IP* cisplatin and paclitaxel in first line treatment of optimally debulked ovarian cancer stage III [166]. This study was in favor of the *IP* trial arm for both PFS and OS, and was one of the reasons why the National Cancer Institute the same year announced the superiority of *IP* chemotherapy in ovarian cancer treatment. However, *IP* chemotherapy has not really been adopted into clinical practice due to increased side effects (nausea, vomiting, abdominal pain) which decreased the number of patients fulfilling the chemotherapy treatment, and the risk of catheter-related infections [166-168]. *IP* chemotherapy remains an option, but is currently not used as a standard treatment for ovarian cancer in Sweden.

30

Chemotherapy for recurrent disease

Although the majority of ovarian cancer patients that receive post-operative chemotherapy initially respond well, the median time to recurrence is less than two years [159]. Overall, the prognosis in this situation is poor, with a median survival of only 10 months [169].

A relapse ≤6 months from the end of a platinum containing chemotherapy regimen is defined as platinum resistant disease and is associated with a worse outcome compared with a platinum sensitive relapse (>6 months from the end of a platinum containing chemotherapy). In the case of platinum sensitive relapse, platinum-based chemotherapy is repeated if possible.

Several regimens are available for platinum resistant disease, *e.g.* pegylated liposomal doxorubicin and single agent paclitaxel, but the response is often limited to approximately 20% and the response duration only around four months [170]. Apart from chemotherapy, various targeted treatments, such as anti-angiogenic drugs and PARP inhibitors, may be used. Clinical trials evaluating the effect of surgery for recurrent disease are ongoing.

Endocrine treatment

Despite the high estrogen receptor (ER), progesterone receptor (PR), and androgen receptor (AR) expression in the normal ovary and in serous and endometrioid ovarian cancers, the response to anti-hormonal (endocrine) treatment is very modest [171-174]. The overall objective response to the partial ER antagonist tamoxifen is <20% in recurrent ovarian cancer, with a somewhat better response seen in patients who are not platinum resistant [175-178]. This was also ruled out in a Cochrane database analysis in 2001, which similarly concluded that ER expression is insufficient to predict response to tamoxifen in ovarian cancer [179]. The selective ER antagonist fulvestrant seems somewhat more efficient, as does the aromatase inhibitor letrozole, the latter with an objective response of 20-25% in recurrent ovarian cancer [180-184]. The PR antagonist mifepristone has not proven to be effective in recurrent or persistent ovarian cancer [185]. Androgen-targeting agents have also been investigated, with as variable and disappointing results as the anti-estrogens [186-188]. Most clinical studies are carried out on heavily pre-treated patients. Studies on adjuvant treatment with tamoxifen and gonadotropin releasing hormone (GnRH) analogs have also been performed, without revealing any clear benefits from endocrine treatment, but the study designs have been suboptimal [189].

In conclusion, endocrine treatment has no role in standard treatment for ovarian cancer to date. However, endocrine treatment is generally well-tolerated compared to chemotherapy. In selected patients, for whom chemotherapy is no longer an

option or who have intolerable chemotherapy side effects, endocrine treatment remains an option although the benefit is uncertain.

Other targeted treatments

Anti-angiogenic treatment with the vascular endothelial growth factor (VEGF) inhibitor bevacizumab has proven effective in advanced stage ovarian cancer with remaining tumor burden. Bevacizumab is added to adjuvant chemotherapy for non-radically operated ovarian cancer stage IIIC and all stage IV. The recommendations are based on two clinical trials; the GOG218 trial and the ICON7 trial [190, 191]. The benefit from bevacizumab in ovarian cancer in general is modest, but for non-radically operated patients the median PFS increased with 4-8 months. Recently, the OS data for the ICON7 trial were published, confirming the initial results and showing a five month OS benefit for the selected high-risk group compared with chemotherapy alone [192]. Side effects include hypertension, venous thromboembolism, impaired wound healing and the rare but severe intestinal perforations, that occur in 1-3% of the patients [190, 191]. The effect of bevacizumab in patients with recurrent disease has been evaluated in clinical trials, revealing a PFS increase of approximately four months [193, 194]

Multi-kinase inhibitors antagonizing different targets involved in angiogenesis are also being evaluated. Pazopanib is one such drug. Maintenance therapy with pazopanib for up to 2 years after completed standard chemotherapy compared to placebo yielded a median of 5 months increased PFS in favor of the pazopanib group [195]. A recent comparable study of nintedanib, however, revealed a very modest increase in PFS (in median <3 weeks) for the nintedanib group, but a more evident effect in the optimally debulked subgroup [196].

Tumors that harbor germline or somatic mutations in *BRCA1* or *BRCA2* have a defective double strand DNA breakage repair. These cells rely on the enzyme PARP and the base excision repair mechanism to repair single strand DNA breaks. PARP inhibitors prevent single strand DNA break repair, which leads to an accumulation of single strand breaks. Eventually, double strand breaks occur and when these cannot be repaired, *e.g.* in *BRCA1* or *BRCA2* mutant cells, the cells will die. To date, there are no data from PARP inhibition in first line treatment of ovarian cancer, but a phase II study of maintenance treatment with the PARP inhibitor olaparib (vs. placebo) in platinum sensitive relapse of ovarian cancer has shown an increased PFS for *BRCA1* and *BRCA2* mutation carriers [197]. In Sweden, olaparib was approved for this indication in 2015. Response to olaparib has also recently been described in prostate cancer harboring germline or somatic *BRCA2* mutations or other HRDs [79].

32

Tumor markers

CA125 is a glycoprotein expressed by coelomic derived cells such as the peritoneum, the fallopian tube, and the endometrium, but normally not on the ovarian surface epithelium. Serum CA125 levels are increased in the majority (>80%) of non-mucinous ovarian cancers. It is not ovarian cancer specific, as it is also elevated in inflammatory conditions in the abdomen. CA125 is used in the primary diagnostic setting when ovarian cancer is suspected, preferably as part of the RMI. The sensitivity of an elevated CA125 (>35 IU/ml) alone is approximately 80% and the specificity 75% (higher for advanced stage and lower for early stage ovarian cancer) [198]. CA125 is also used to monitor treatment response, where a successive decrease in CA125 levels correlates well with the response to treatment.

CA125 levels often rise several months before a relapse can be detected clinically or radiologically. The role of serial CA125 tests after completed chemotherapy has, however, been questioned. In 2010 Rustin *et al.* published the results of a large, British trial, including >1,400 women with complete response after first line platinum-containing chemotherapy. No differences in OS were revealed between women starting second-line chemotherapy when CA125 started to increase (>70 IU/ml) compared with women who started chemotherapy upon clinical and/or radiological signs of relapse [199]. Hence, there is no evidence supporting the use of CA125 in follow up of ovarian cancer patients after completed chemotherapy. CA125 should, however, be assessed if there is suspicion of a relapse. This strategy may be modified in the future following the introduction of new biomarkers.

Human epididymis protein 4 (HE4) is a promising serum marker which has shown a superior sensitivity and specificity compared with CA125, especially in early stage cancer [200]. Both CA125 and HE4 are approved by the American Food and Drug Administration for routine use in ovarian cancer, but HE4 is not implemented in clinical practice in Sweden since confirmatory studies are needed [201].

Prognostic and predictive factors

There are several clinically useful prognostic factors for advanced stage ovarian cancer, but the treatment predictive factors are few. A large, retrospective study of >1,800 platinum-treated ovarian cancer patients has shown that endometrioid histology is associated with a favorable outcome, whereas residual tumor after surgery, clear cell or mucinous histology, increasing age (a 6% increasing risk for every 10 year increase in age), and inferior performance status at diagnosis is associated with a worse outcome [202]. Furthermore, advanced FIGO stage and

low serum-albumin is associated with an inferior prognosis [203, 204]. The only reliable markers predicting treatment response are *BRCA1* and *BRCA2* mutations, which, as previously mentioned, indicate response to PARP inhibitors. Germline *BRCA1* and *BRCA2* mutation carriers also display a generally better prognosis than non-mutation carriers [205]. Likewise, it has been reported that *BRCA2* mutation carriers respond better to platinum-based chemotherapy than *BRCA1* and non-mutation carriers, but this should be considered as a favorable prognostic marker rather than a true predictive one, and the study methodology has also been questioned [206].

Aims

The general aims of this thesis were to investigate gene expression profiles and molecular subtypes in epithelial ovarian cancer in relation to diagnostic and prognostic subsets. In study I, hereditary ovarian cancer was investigated, studies II-III investigated molecular features in distinct histopathological subtypes, and study IV focused on prognostic implications of sex steroid hormone receptor expression

The detailed aims were to:

- Outline a gene expression profile of Lynch syndrome-associated ovarian cancer compared to sporadic ovarian cancer and to identify discriminating targets of potential relevance for targeted therapies (**study I**).

- Perform a detailed investigation of the inter-tumor heterogeneity and to assess the mutation spectrum in ovarian clear cell carcinomas (**study II**).

- Study whether gene expression profiles in serous ovarian tumors, including malignant, borderline, and benign tumors may identify key markers of tumor progression and to assess whether similarities can be discerned between the molecular subtypes of ovarian cancer and the intrinsic breast cancer subtypes (**study III**).

- Investigate the prognostic impact of sex steroid hormone receptor expression, with a specific focus on co-expression of two or more receptors, in epithelial ovarian cancer and within its molecular subtypes (**study IV**).

Materials and Methods

Summary

Table 1. Overview of materials and methods in studies I-IV

Study	Design	Materials	Methods
I	Case-control study	24 Lynch syndrome-associated and 24 matched sporadic epithelial ovarian cancers	WG-DASL, immunohistochemical staining. Refinement of gene signature using a public dataset. Statistical analyses aimed at class comparison.
II	Case series	67 epithelial ovarian cancers (for WG-DASL), and 10 clear cell ovarian cancers (for deep sequencing)	WG-DASL, targeted deep sequencing.
III	Case series	Serous ovarian tumors: 37 malignant, 5 borderline, and 17 benign	Gene expression profiling. Application of subtype signatures (ovarian cancer and breast cancer). Statistical analyses aimed at class comparisons.
IV	Cohort study	87 serous and 31 endometrioid ovarian cancers	Immunohistochemical staining. Analysis of corresponding mRNA values in a public dataset. Survival analyses.

Patients

Study I

This study cohort consisted of patients with Lynch syndrome-associated cancers. The tumors were derived from individuals with verified disease-predisposing *MMR* gene mutations and loss of the corresponding MMR protein expression. MMR stainings were performed in a study by Ketabi *et al.* [100]. Formalin-fixed, paraffin embedded (FFPE) tumor tissue was obtained from Swedish and Danish

Pathology departments, the latter in collaboration with the Danish HNPCC register. Sporadic ovarian cancers retrieved from a prospective, population-based study were used as controls [65]. From an initial series of 28 Lynch syndrome-associated ovarian tumors and 44 sporadic ovarian tumors, high quality expression profiles were obtained from 25 (89%) hereditary and 42 (95%) sporadic tumors. From these 67 tumors, 24 hereditary and 24 sporadic controls were matched according to histology, tumor stage and age at diagnosis and were used for further analysis. In the sporadic controls, absence of MMR protein loss and *BRCA* mutations was confirmed.

Studies II and III

Patients who underwent surgery for cancer suspected ovarian masses at the department of Gynecology and Obstetrics, Skåne University Hospital, Lund, September 2004 through July 2013 were invited to a biobank study and provided informed consent to store fresh-frozen tumor tissue and accompanying, pre-operative blood samples. During this time period, 807 patients had been recruited, of whom 390 were diagnosed with malignant disease (including borderline ovarian tumors) and 417 with benign tumors. Patients from this biobank were included in studies II and III.

In study II, two patient cohorts were used. *Cohort 1* consisted of the 67 tumors recruited for study I. *Cohort 2* consisted of all nine clear cell ovarian carcinomas in the local biobank. Since frozen tumor tissue was only available for three of these cases (blood samples from 8/9), we retrieved accompanying FFPE tissue blocks from the department of Pathology, Skåne University Hospital, Lund. Two FFPE blocks from sporadic controls from *cohort 1* were also included. High quality DNA was obtained from 10 tumors, which were used for further analyses.

In study III, all patients with serous tumors included in the biobank 2004 through 2011 were eligible for the study. Tumor tissue was available from 40/94 (43%) serous adenocarcinomas stage ≥IC, from 5/11 (45%) serous borderline tumors and from 17/54 (31%) serous adenomas/adenofibromas. Three adenocarcinomas were excluded due to poor RNA quality. Thus in total 37 malignant, 5 borderline and 17 benign serous tumors were used for further analyses in study III.

Study IV

All patients diagnosed with ovarian cancer in the southern Swedish healthcare region June 1998 through June 2000 were invited to participate in a prospective study. Ascertainment of the tumor material is described in detail by Malander *et al.* [65]. In total, 161 patients were included in the prospective study. FFPE blocks

from 128 (79.5%) of these tumors, together with FFPE blocks from 18 patients recruited at the oncogenetic counseling service at Lund University Hospital 1981 through 1997 were assembled in a tissue microarray (TMA). This TMA consisted of 146 ovarian tumors of varying histopathological subtypes, of which 118 serous and endometrioid samples were used in study IV. Since this study aimed to examine the prognostic relevance of sex steroid hormone receptor expression in ovarian cancer, the clear cell and mucinous tumors, which are normally not expected to express hormone receptors, were excluded [132, 171]. Furthermore, all cancers of unknown primary or mixed or undifferentiated histologies were also excluded.

A summary of the patients included in studies I-IV is provided in Table 2.

Table 2. Summary of the patients included in studies I-IV.

	Study				
	I	**II**	**II**	**III**	**IV**
		Cohort 1	*Cohort 2*		
Number of patients	48	67	10	59	118
Median age (years)	50.5	51	48	65	58
range	30-78	27-78	34-60	40-91	26-83
Histology (%)					
Serous	20 (42)	31 (46)	-	59 (100)	87 (74)
Endometrioid	14 (29)	18 (27)	-	-	31 (26)
Clear cell	14 (29)	15 (22)	10 (100)	-	-
Mucinous	-	3 (5)	-	-	-
Heredity					
BRCA1/2 mutations	-		-	-	30 (25)
MMR gene mutations	24 (50)	25 (37)	-	-	-

Methods

Immunohistochemistry

IHC staining is a well-established method used to assess the distribution and localization of proteins in tissue and is widely used for tumor diagnosis. The indirect IHC technique was used in the studies in this thesis. To visualize the protein of interest, the tissue is stained with a primary antibody that binds to an

antigen, in this case a protein. A secondary antibody conjugated to a polymer and an enzyme, *e.g.* horse radish peroxidase (HRP), and binding to the primary antibody is added. The enzyme in turn is labeled with a color, *e.g.* 3,3'-diaminobenzidine (DAB), which makes it possible to identify the protein in the cells; both the number of stained cells and where the protein is localized (*e.g.* in the nucleus or cytoplasm). A hematoxylin staining is often added to identify morphologic structures. The hematoxylin staining colors the cell nuclei of unstained cells blue and thus make them easy to detect and contrasts to the antibody staining. An overview of the indirect IHC method is shown in Figure 6.
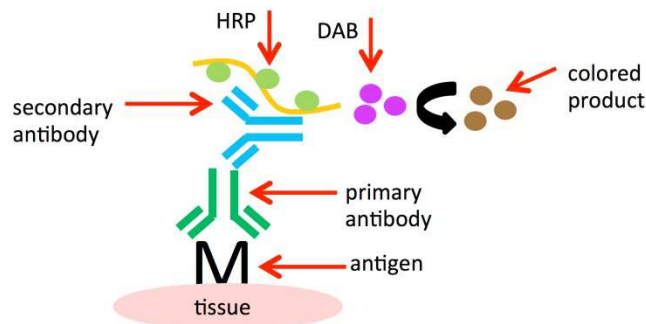
Figure 6. Schematic presentation of the indirect antibody staining technique.

Tissue samples are stored frozen or as FFPE blocks to preserve tissue morphology as well as the genetic material within the tissue. Both FFPE and frozen tissue can be used for IHC staining, but require different preparations in order to visualize the proteins. In the studies in this thesis only FFPE tissue was used for IHC staining. In FFPE tissue, the epitopes of the proteins, to which the primary antibodies bind, are "hidden" in the formalin. The tissue is therefore deparaffinized and rehydrated and then pre-treated with heat or microwaves to retrieve the epitopes before the primary antibody is added. Regardless of tissue preservation method, the goal with IHC is to achieve a specific staining with as little non-specific background staining as possible. This requires optimal tissue sections, suitable antibody dilution, incubation time and temperature, adjusted pH as well as careful handling of the reagents. Apart from these requirements, antibody affinity (the binding between antibody and epitope), antibody cross-reactivity (when an antibody binds to more than one antigen), and antibody stability may impact the quality of the staining.

The antibodies used for IHC stainings are either *monoclonal*, *i.e.* are immunochemically identical, derived from one clone of plasma cells and react only with one epitope on the specific antigen (protein), or *polyclonal*, *i.e.* are

40

immunochemically dissimilar, derived from different cell clones and react with several epitopes. Monoclonal antibodies are more sensitive to changes in temperature and pH than polyclonal antibodies, but have a higher specificity. Polyclonal antibodies, on the other hand, normally have a high affinity, but may produce an unspecific background staining due to cross-reactivity. Examples of IHC stainings are shown in Figure 7.
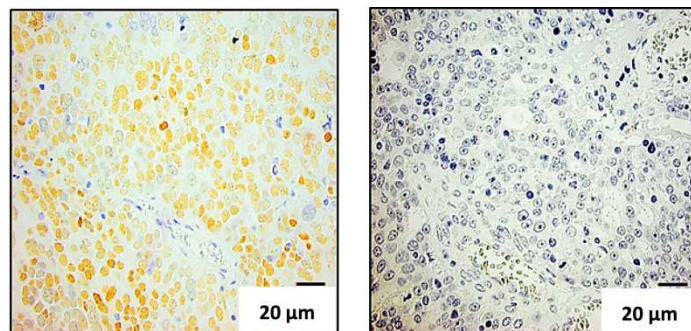
Figure 7. Examples of positive (left) and negative (right) nuclear ERα IHC staining of ovarian cancer tissue. 40x magnification.

IHC was used in studies I and IV. In study I, whole tissue sections (3 μm) were stained with p-mTOR, PTEN, and EGFR antibodies. The tissue sections were pre-treated according to the manufacturers' instructions (details are outlined in the appended study), stained in an automated immunostainer (TechMate 500 plus, DAKO) and the DAKO EnVision™ Systems (DAKO) was applied for visualization. Non-small cell lung cancer tissue (p-mTOR), colon tumor tissue (PTEN), and placental tissue (EGFR) were used as positive controls. Evaluations were blinded to hereditary status and gene expression data and independently performed by Katarina Bartuma and Jenny-Maria Jönsson. Details regarding antibodies used in studies I and IV are outlined in Table 3.

Tissue microarray

TMAs were used in study IV. A TMA consists of individual tissue cores assembled in a recipient paraffin block in an array pattern. Normally, several cores from the same tissue are retrieved, and the core diameter varies from 0.6-2.0 mm [207]. In general, two core biopsies of 0.6 mm have been shown to give a representative view of the staining pattern [208]. Sections from a TMA block are mounted on microscope slides and stained as previously described.

41

Table 3. Overview of antibodies used in the studies in this thesis

Antibody	Vendor	Type	Clone	Evaluation
p-mTOR (study I)	Cell Signaling Technology	Rabbit monoclonal	49F	1=any cytoplasmatic staining 0=no cytoplasmatic staining
PTEN (study I)	Dako A/S	Mouse monoclonal	6H2.1	1=equal or stronger staining than surrounding tissues 0=no or weaker staining than surrounding tissues
EGFR (study I)	Dako A/S	Mouse monoclonal	E30	1=>25% tumor cells moderately-intensely stained 0=weak staining and/or $\leq$25% tumor cells stained
ERα (study IV)	Dako A/S	Mouse monoclonal	1D5	1=$\geq$10% stained cells 0=<10% stained cells
ERβ (study IV)	Dako A/S	Mouse monoclonal	M7292	1=$\geq$10% stained cells 0=<10% stained cells
PR (study IV)	Dako A/S	Rabbit polyclonal	#A0098	1=$\geq$10% stained cells 0=<10% stained cells
AR (study IV)	Dako A/S	Mouse monoclonal	M3562	1=$\geq$10% stained cells 0=<10% stained cells

*1=positive scoring; 0=negative scoring

The advantage of TMAs comprises the possibility to easily and cost-effectively evaluate multiple markers and samples using only a limited amount of tissue, and with minimal differences in staining conditions. The disadvantages include representability and heterogeneity, *i.e.* the risk of not capturing the region(s) or the staining pattern(s) of interest in the tissue, especially when using small cores. We used TMA cores 0.6 mm in diameter, and at least three cores/tumor sample were evaluated. The construction of the TMA is outlined by Malander *et al.* [65].

The TMA slides were stained with ERα, ERβ, PR, and AR antibodies. The slides were pre-treated according the manufacturers' instructions (details outlined in the appended study), and all stainings were performed using an automated immunostainer (TechMate 500 plus, DAKO) and the DAKO EnVision™ Systems (DAKO) was applied for visualization. In cases where heterogeneity was observed, the staining pattern representing the majority of the tumor cells was used. Breast cancer tissue, known to be positive for the respective proteins, was used as positive controls. The ERα and PR stainings were independently evaluated

by Susanne Malander and Mef Nilbert, whereas the ERβ and AR stainings were independently evaluated by Nicolai Skovbjerg Arildsen and Jenny-Maria Jönsson. A comparison between a whole-tissue section and a TMA slide is shown in Figure 8.
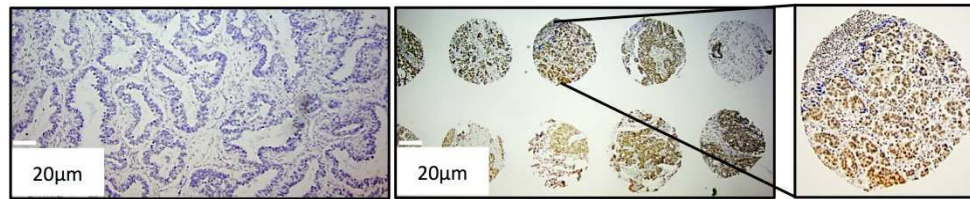
Figure 8. Examples of a whole tissue section (left), a TMA slide (middle) and an individual TMA core (right). Photmicrographs of slides in 4x magnification and the single TMA core in 10x magnification.

IHC staining, mainly on FFPE tissue, is one of the most commonly used diagnostic, prognostic, and predictive methods used in pathology. Advantages include ease, low costs, and high efficiency. Disadvantages include staining quality, which depends on tissue fixation, uncertainty whether the staining reliably reflects the morphology in question, and evaluator dependent results. These uncertainties underscore the need for defined and validated cut-offs for interpretation and the use of established antibodies with stable performance [209].

Polymerase chain reaction

Polymerase chain reaction (PCR), first described in the 1980's, is a method for amplification of specific DNA sequences used in laboratory work around the world. It is well-established in clinical work for identification of mutations in known gene sequences. Briefly, the DNA is denatured at high temperature, thus forming two complementary strands. DNA oligonucleotides (short DNA fragments), called primers, that are complementary to the DNA regions of interest are added together with a DNA polymerase and nucleotides and the temperature is lowered. The separated DNA strands become templates for the formation of new double stranded DNA, and the process is catalyzed by the DNA polymerase. The initial DNA separation and the latter formation of new double stranded DNA fragments (referred to as annealing and elongation) are performed at different temperatures and the process is referred to as thermal cycling. The PCR cycles are repeated in a chain reaction as long as there is active polymerase present and/or until no more single stranded DNA is available. Detection of mutations in the *KRAS* and *BRAF* genes using PCR was performed in study III, using Roche's

43

cobas K-RAS Mutation Kit, detecting mutations in codons 12, 13 and 61 of the *KRAS* gene, and the cobas BRAF V600 mutational analysis, detecting mutant or wildtype V600 at the *BRAF* V600 site in exon 15, respectively (Roche, Pleasanton, CA, USA). Both the *KRAS* and *BRAF* assays were run on the z480 Lightcycler (Roche) and the analyses were performed at the Department of Pathology, Clinical Research Center, Hvidovre Hospital, Denmark.

Gene expression profiling

When IHC gives a picture of the expression, but not the function, of proteins, other methods are needed to measure the activity of the encoding genes. DNA-based microarray technology allows measurement of the expression levels of several genes and in several samples at the same time, which can thus be compared and more general patterns discovered (*e.g.* genes with a high expression level in a subgroup of tumors) [210]. Since the start in the mid 1990's, the microarray technology field has exploded and all kinds of cells can now be analyzed using whole transcriptome analysis. From the initial projects studying just a few tumors, microarray technology can now be used for efficient analysis of thousands of tumors. In the field of ovarian cancer this has been used to discover and verify prognostic markers as well as inter-tumor heterogeneity and molecular subtypes [58, 211, 212]. Since the technological advances are rapidly evolving, the preferred approach today would be to use next generation sequencing (NGS) for massive parallel sequencing of genes. Limitations and strengths regarding gene expression profiling and gene sequencing are outlined in the Targeted deep sequencing section.

Global gene expression profiling is one microarray technology, together with *e.g.* array comparative genomic hybridization. Gene expression profiling was used in studies I, II, and III. Gene expression analysis requires RNA, which is optimally obtained from fresh frozen tissue. Most tumor samples are, however, stored as FFPE blocks, since this is a cheap and easy method to preserve tissue and its morphology. The formalin fixation, though, causes cross links between nucleic acids and between proteins and modifies the RNA. This can make the cDNA synthesis, a major step in gene expression analysis preparation, difficult [213-215]. Likewise, the nucleic acids in old FFPE blocks may be at least partially degraded [216]. Although the superiority of RNA quality extracted from fresh frozen or acetone fixed tissue over formalin fixed has been outlined, RNA has been reported to be successfully extracted from formalin fixed tissue using pre-treatment with proteinase K and incubation in a formalin free buffer at >45°C. RNA extraction protocols using FFPE tissue are available and FFPE tissue has been demonstrated to be suitable for microarray analyses such as *e.g.* gene expression profiling in the majority of cases [214, 215, 217, 218].

In studies I and II, based on the same cohort, we performed global gene expression profiling using FFPE blocks of varying ages (range 3-54 years). In total, 72 tumor samples and 26 technical duplicates (the same sample analyzed twice) were included in the expression analysis. We used 10-μm hematoxylin and eosin stained tissue slides to identify areas with >70% tumor cells and no tumor necrosis, and extracted RNA from these areas after macro dissection. The samples were pre-treated with proteinase K and incubated in a formalin-free (Tris) buffer at 55°C. RNA extraction was performed using the High Pure Paraffin RNA Kit (Roche), and RNA concentration was determined using a Nanodrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Intact RNA will produce a light peak at a wavelength of 260 nm, whereas degraded RNA has a peak at 280 nm. The 260/280 ratio is thus a measurement of the amount intact RNA in relation to the amount of degraded RNA. For gene expression profiling in study I/II, 200 ng RNA with an RNA 260/280 ratio of >1.8 for each sample was required. 68/72 (95%) of the samples fulfilled these criteria.

The Whole Genome cDNA mediated Annealing, Selection, extension and Ligation (WG-DASL) assay (Illumina Inc., San Diego, CA, USA) was used for gene expression analysis. The DASL assay allows for expression profiling based on a small amount and partially degraded RNA which, as previously described, is normally the case with RNA extracted from FFPE tissue. The DASL assay consists of Bead Chips, *i.e.* microscopic silica beads assembled in microwells, and hundreds of thousands of oligonucleotide copies are attached to each bead, thereby creating an array pattern. Each oligonucleotide acts as a capture sequence with an address tag, and the HumanRef-8 v3 that was used on the DASL assay contains >24,000 probes representing >18,000 unique genes and known alternative splice variants from the RefSeq database (release 22). The oligonucleotide addresses are used for mapping and decoding the array, whereas the probes are used for quantification of expression levels.

The first step in the DASL assay is conversion of RNA to complementary DNA (cDNA) using primers, both biotinylated oligo (dT), hybridizing to the 3' poly A-region of the RNA, and random nonamer primers. The biotinylated cDNA is annealed to the probes and each probe consists of a 5' and 3' oligonucleotide with a specific gene sequence and a universal PCR primer sequence. The 5' oligonucleotide hybridizes to the cDNA, which is extended and ligated until it reaches the complementary oligonucleotide at the 3' end and forms a PCR template, which in turn is amplified. A fluorochrome is attached to one of the PCR strands, followed by hybridization to a BeadChip target. Thereafter the BeadChips are scanned and the intensity data are analyzed. A schematic overview of gene expression profiling is outlined in Figure 9.
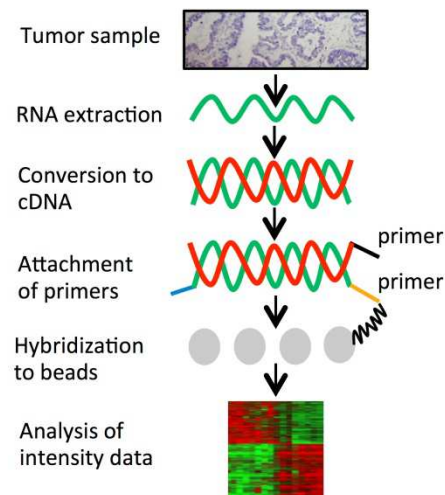
Figure 9. Overview of the main steps in gene expression profiling using bead arrays.

In study III, we performed whole-genome expression profiling using fresh frozen tumor tissue from the biobank (see the Patients section, studies II and III, above). The samples (approximately 2 cm^3) were collected from macroscopic tumors at surgery. Where pathology reports verified that the whole tissue section removed consisted of tumor cells, the biobank sample was used for further analysis. When questions arose regarding tumor cell content, an imprint was used for verification of tumor cells in the current sample and analyzed by a senior gynecologic pathologist (Anna Måsbäck).

In total, 40 malignant, 5 borderline and 17 benign tumors were used for expression profiling, together with 13 biological replicates (*i.e.* a second tissue sample from either the contralateral ovary, omentum, or the pelvic wall; 9 malignant, 1 borderline, 3 benign). RNA was extracted using the Allprep kit (Qiagen, Heidelberg, Germany). As in Study I, the Nanodrop Spectrophotometer (Nanondrop Technologies) was used to assess the RNA concentration, with 200 ng RNA with 260/280 ratios $\geq$1.8 regarded sufficient. The RNA quality was further analyzed using RNA Integrity Numbers (RINs), calculated using a Bioanalyzer (Agilent technologies, Santa Clara, CA, USA). In comparison with the 260/280 ratios, RINs use the total electrophoretic trace of the sample RNA, *i.e.* take into account both intact and degraded RNA simultaneously and facilitate comparison between samples using numerals. RINs >6 were regarded sufficient. 37/40 (92.5%) malignant tumors met the RNA concentration and quality criteria. Illumina's cDNA synthesis, labeling and subsequent hybridization to the Human HT12 v4 expression BeadChips (Illumina Inc.) were used for gene expression analysis of the remaining 59 samples and 13 replicates. This assay is similar to the DASL assay in terms of cDNA conversion and PCR amplification, but consists of

46

>47,000 probes representing >28,000 genes and splice variants from the Refseq database (release 38).

The gene expression profiling analyses in studies I, II, and III were performed at the SCIBLU Genomics Centre, Lund University, Sweden.

The raw data from study I/II are freely available in the National Center for Biotechnology and Information's Gene Expression Omnibus under the accession number GSE37394 and the raw data from study III under the accession number GSE57477 [219].

Data analysis

The larger the dataset used for gene expression analysis, the greater the chance of detecting small differences in gene expression levels. The aim with gene expression profiling, however, is of course to detect reliable and biologically relevant differences. Data handling is therefore central for the validity of the results and the interpretation thereof [220]. Probes with low signal intensity, *i.e.* probe signals that may be unspecific, are normally removed from the dataset. The remaining probes are *normalized*, aiming at making arrays comparable by eliminating differences in sample preparation (*e.g.* different signal intensity due to different amount of staining when labeling the cDNA, differences in hybridization time or temperature, different amount of samples on the individual arrays). Briefly, to eliminate variations that have nothing to do with variations in gene expression levels.

Different normalization methods may be used. In studies I, II, and III we used quantile normalization and in study I we also used cubic spline normalization. Quantile normalization is a rank-based method aiming at making the distribution of probe intensities equal across arrays [221]. In general, each gene expression value in each sample and each array is assigned a rank. The highest rank in each gene and array is changed to the value of the corresponding rank (or more exactly the value of the rank for the corresponding quantile) across arrays, which makes the gene expression values across arrays comparable. Cubic spline normalization is another quantile method, where a curve is adjusted to the quantiles and gives a smoother distribution of gene expression values. In general though, the results of quantile and cubic spline normalization are comparable [220].

After normalization, a *presence filter* may be added, with the goal of only keeping probes with a sufficient (statistically significant) expression in a defined number of samples. An 80% presence filter implies that probes with expression in fewer than 80% of the samples and p-values above a specific cut-off are removed. Without a presence filter, a probe that is just present in some of the arrays may be interpreted as highly expressed when it in fact has a relative low expression. The p-value for

each probe is calculated using the Mann-Whitney test. Having defined which probes to analyze, the data are *log$_2$-transformed*. This procedure makes each step on the scale comparable, regardless of direction. Without log$_2$-transformation, an increase in intensity from 0 to 1 appears stronger/bigger than a decrease from 0 to -1. Thus the log$_2$-transformation allows for a more true identification of intensity changes. To decide whether a specific gene has a high or low expression (*i.e.* is up- or down-regulated), a center is defined and changes are deemed up or down in relation to this value. This procedure is called *centering*, and the data can be centered on a mean or a median value. A *variance filter* can also be added, selecting a specified number of probes or genes with the greatest expression variation across samples.

Hierarchical cluster analysis visualizes samples and groups of samples through ordering them in a hierarchical cluster tree. A cluster analysis can be *unsupervised* or *supervised.* In unsupervised clustering, the smallest distance from the first to the second sample is calculated, followed by the next smallest distance and so forth, leading to groups – clusters – of samples with similar expression values. In supervised clustering the samples are already assigned a group and this cluster analysis aims at discovering differences between the pre-defined groups. A *linkage method* is used to measure the distance between clusters and a *distance metric test* to measure similarities in patterns of over- and under-expression.

The difference in gene expression between two groups of genes can be described using a fold change, where a fold change of 1.5 means a 1.5 times upregulation of the one group compared with the other and a fold change <1.0 means a relative downregulation. *Significance Analysis of Microarrays (SAM)* is a statistical method determining whether differences in gene expression between groups are statistically significant, performing a gene specific T-test with each gene assigned a score based on its change in gene expression in relation to the standard deviation of repeated measurements for that gene [222]. Genes assigned scores greater than a defined cut-off are considered significantly changed. The percentage of genes assigned such scores by chance is called the *False Discovery Rate* (FDR), and is adjusted for large amounts of data. Likewise, instead of a standard p-value defining statistical significance, a *q-value* (an FDR adjusted p-value) is used. SAM is a non-parametric test, *i.e.* it does not assume that the data follow the normal distribution. A hierarchical tree is shown in Figure 10.
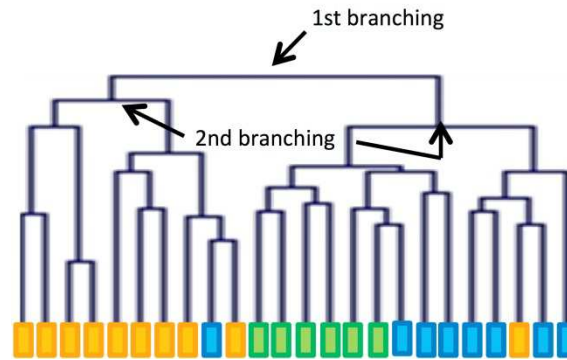
Figure 10. Hierarchical cluster tree. Colored boxes represent individual samples. Green and blue samples are similar in terms of gene expression (1st branching) compared with the yellow ones, which form a separate cluster. Subclusters (2nd branching) reveal that also the green and blue samples express some differences in gene expression. The "missplaced" blue and yellow samples seem to share features with samples of another color in an unexpected way.

In studies I and II the arrays were scanned in a BeadArray™ Reader using BeadScan software (v 4.2). An average signal intensity value >250 for each probe and >8,000 detected genes/sample were required for further analyses of the samples. Using the GenomeStudio software (Illumina Inc.) the data were quantile normalized and an 80% presence filter with a probe detection p-value <0.01 was applied, leaving 12,897 probes for analysis. 1/68 (1.5%) sample was removed in this analysis step. Thereafter the remaining data were $\log_2$-transformed and mean centered across samples. In Study I this was performed in the Mev 4.6.02 software and in study II using R version 3.1.0 [223]. The technical reproducibility was granted through inclusion of duplicate samples. The mean correlation of duplicate samples was 0.98 (range 0.90-0.99) and the mean r^2 value 0.96 (range 0.81-0.99). Comparison of gene expression data in the duplicate samples was performed using Pearson distance metric. In study II, multiple probes corresponding to the same gene were removed, leaving 10,000 probes for further analyses, prior to $\log_2$-transformation.

In study III, arrays were scanned using an i-Scan (Illumina inc.) and uploaded to the GenomeStudio software (Illumina inc.). The data were quantile normalized, background corrected, and $\log_2$-transformed. Probes with a mean intensity <2.5 and a variance (in signal intensity between samples) <0.1 were removed, leaving approximately 16,000 probes corresponding to >12,000 genes. The data were then uploaded to the MeV 4.6.02 software, mean centered and a 20% variance filter was applied.

In studies I, II, and III, unsupervised hierarchical cluster analyses using the complete linkage method and Pearson correlation metrics were performed; in study I on the 48 matched samples (see Patients section above) and in study II

49

using the whole dataset (67 tumors) from study I as well as a smaller subset (15 tumors) encompassing only clear cell cancers. SAM analyses including 100 permutations were then performed to identify differentially expressed genes between Lynch syndrome-associated and sporadic tumors (study I), between histopathological subtypes (study II) and between tumors of different malignant potential (study III). Permutations are used to test the extremes of the dataset and estimate their potential impact on the results.

To ensure data robustness, we reanalyzed the data from the 48 matched samples in study I using alternative parameters. This procedure included cubic spline normalization and a 70% presence filter with probe detection p-value ≤ 0.01, leaving 3,380 probes for further analyses. As described above, hierarchical cluster analysis and SAM analysis were applied to this smaller dataset. *Leave-one-out analysis,* which estimates how well a predictive model works in a new setting, was performed to control whether the data could be accurately divided into hereditary and sporadic tumors. In the 3,380 probe dataset, unsupervised hierarchical clustering was also performed in the separate histologic subtypes and SAM analyses (FDR <0.01) were performed in the serous and endometrioid subtypes.

An independent, publicly available dataset consisting of 285 ovarian, fallopian tube and primary peritoneal tumors (high-grade and low-grade serous and endometrioid tumors and borderline serous tumors) and 4,732 probes corresponding to 2,844 unique genes were applied to the cohorts in study I (matched sample set of 48 tumors) and study III [58]. In study I, this was done with the aim of further testing the data robustness and the robustness of our results. The independent dataset was downloaded, $\log_2$-transformed and mean centered, and the 1,346 genes overlapping between the datasets were used to study differences between the Lynch syndrome-associated and sporadic tumors as described above.

In study III, the aim was to validate the molecular ovarian cancer subtypes. The independent dataset was normalized, $\log_2$-transformed, and low quality probes (intensity <4, variance <0.15) were removed, leaving 4,099 probes corresponding to 2,725 genes. Of these, 1,295 genes overlapped with our dataset. Firstly, the molecular classifier was validated using the overlapping genes and thus the tumors in the independent dataset were re-assigned subtypes. Secondly, the classifier was applied to our cohort, assigning each of our tumors a molecular subtype. This was also performed using a gene signature for molecular subtyping of breast cancer [224]. For validation purposes, the breast cancer signature was applied to the independent ovarian cancer dataset. The method used for application of the ovarian and breast cancer subtypes is called *nearest centroid classification*, and identifies gene subsets within each specific subgroup that best classifies the subgroup [225]. The centroid of a subgroup is equal to its mean expression profile. Likewise, the mean expression profile of each sample in our cohort was computed and compared with the centroids of the independent datasets using a correlation

50

test, and assigned the subgroup whose centroid it was closest to. A similar method using computed module scores was used for application of breast cancer gene modules to our cohort [226].

Gene ontology

One strategy to reduce the risk of exaggerating the expression of individual genes detected among large number of genes in small cohorts is to apply gene ontology (GO) analyses. GO sorts genes with similar functions or involved in the same processes and makes it easier to discover patterns among groups of genes up- or down-regulated in a dataset. Likewise, tools identifying signaling pathways in which many of the genes are involved can be used. In study I we used the Ingenuity Pathway Analysis (www.ingenuity.com), in study II the PANTHER tool, and in study III we used the GOrilla and ToppGene tools to analyze GO terms focusing on functional and biological differences between subgroups [227-229].

Targeted deep sequencing

While gene expression profiling allows for detection and investigation of numerous genes and their relative expression across samples, a major drawback is the limitation of the arrays. Gene sequences not covered by the probes cannot be investigated. Likewise, the data processing filters out low intensity probes, with the risk of losing information about minor gene expression changes, and, conversely, equalizes high intensity probes with the risk of underestimating major changes. The method of choice today is RNA-based massive parallel sequencing (RNAseq), an effective method to uncover the whole transcriptome, *i.e.* all forms of RNA in the cell. The term RNAseq covers various NGS techniques, with NGS simply meaning the ability to sequence millions of genes in parallel, compared with the initial chromatography and electrophoresis-based methods developed in the 1970's. RNAseq was first described in 2008, and proved to be at least as accurate as cDNA and oligonucleotide microarrays, but without the limitations of requiring predefined gene sequences, and also covering the non-coding part of the RNA where the activity of a gene is often regulated [230, 231]. The procedure used for actual sequencing in different analyses is the same regardless of whether RNA or DNA is analyzed, (but the sample preparation differs) [232]. Thus, both DNA and RNA sequencing may be referred to as efficient high through-put techniques.

One high through-put sequencing method is *targeted deep sequencing*, and this technique was used in study II. This method *targets* sequences in predefined genes of potential interest for the material that is being studied. The term *deep* refers to

51

the high number of repetitive gene sequencing procedures performed (for cancer cells sequencing is normally performed up to thousands of times). The number of sequences thus read is defined as the reading depth, and outlined "x", with 300x meaning that the sequencing is performed 300 times. *Variant,* or *mutation, calling* refers to the percentage of alleles with detected changes needed to define a mutated sequence. Identification of 100% mutated alleles would infer that the mutation most certainly is a germline SNP, present in all cells in the body (and thus heritable, regardless of whether the SNP is functional or not). A basic model of gene sequencing is outlined in Figure 11.
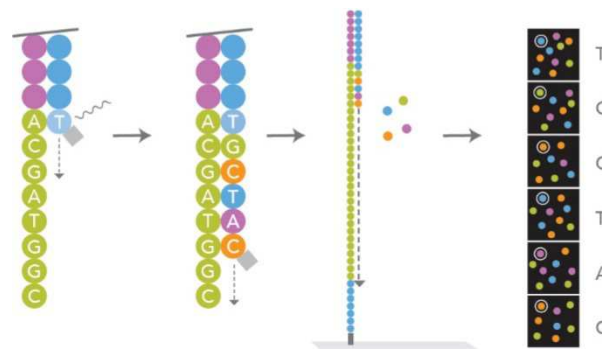
Figure 11. Schematic overview of the principle of gene sequencing. DNA is sequenced by the addition of labeled nucleotides (colored balls, 2nd figure from the left). The individual labeled nucleotides together form a copy of the specific gene sequence and sequencing is performed in a massive parallel fashion. Courtesy by Illumina Inc.

In study II, we used targeted deep sequencing of DNA from FFPE tissue. DNA preservation in FFPE blocks harbors the same limitations as previously described for RNA, but is still feasible [233]. Prior to sequencing, the histopathologic subtype was reviewed by two gynecologic pathologists (Anna Måsbäck and Sofia Westbom-Fremer). 3 μm hematoxylin stained slides, retrieved before the sections used for DNA extraction, were also reviewed by Sofia Westbom-Fremer to ensure that tumor cells were present in the part of the tumor sample used and to assess the percentage of tumor cells in each section (median 90%, range 65-99%). DNA was extracted from 10μm whole-tissue sections using the Allprep DNA/RNA kit (Qiagen) and >1μg DNA was obtained from all samples.

DNA concentrations were measured using the Qubit Fluorometric Quantitation® (Life technologies, Thermo Fisher Scientific, Waltham, MA, USA), which quantifies the amount of intact DNA using fluorescence. The Trusight Tumor Sample Preparation Kit (Illumina Inc.) was used for assessing DNA quality. This is a quantitative PCR method, assessing delta (Δ) cycle threshold (Ct) values,

where Ct values indicate how many times the cycle (*i.e.* the PCR analysis) has to be repeated for the fluorescent signal to exceed the background (*i.e.* cross the threshold). The Ct number is inversely correlated to the amount of nucleic acid in the sample. Δ-Ct values <6.5 were considered sufficient, and 10/11 (91%) samples met this criterion. 600 ng genomic DNA/sample (50 ng/μl) was used for targeted single strand DNA sequencing. Sample preparation and sequencing were performed at Oxford Gene Technology™ (OGT, Oxford, Great Britain).

The NGS SureSeq™ solid tumor panel, consisting of 60 key cancer genes, was used for targeted sequencing. This panel is validated for research use on FFPE tissue, and is a hybridization-based tool targeting all codons of the exons in the included genes. Data analysis was performed by Nicolai Skovbjerg Arildsen. The public ENSEMBL Genome Browser was used for classification of the mutations according to severity across genes [234]. Only mutations classified as "serious" by ENSEMBL *and* concurrently defined as "deleterious" by the CONsensus DELeteriousness (CONDEL) score of non-synonymous single nucleotide variations (SNVs) were used for further analyses [235]. "Serious" mutations according to ENSEMBL are major non-conservative amino acid changes, splice or frameshift variants or changes resulting in stop codons, which affect SNVs or insertions or deletions (indels) of DNA bases. CONDEL classifies mutations based on an average of predictive scores derived from different tools. Synonymous mutations, affecting the DNA sequence but not the sequence of amino acids, were thus entirely removed. The reading depth cut-off was set to 300x. Details regarding technical equipment and software used for analyses are outlined in the appended study.

Statistical analyses

Study I

Immunohistochemical stainings in study I (p-mTOR, PTEN, EGFR) were dichotomized as previously described and thus treated as categorical, binary variables and were compared using Fisher's exact test. P-values <0.05 were considered statistically significant. Comparison of gene expression data in the duplicate samples was performed using the R software, and comparisons of IHC stainings were performed in SPSS statistics version 19.

Study III

In study III, our in-house cohort was stratified into different gene expression-based molecular subtypes; five molecular ovarian cancers signatures (C1-C5), five intrinsic breast cancer subtypes (luminal A, luminal B, normal-like, basal-like, HER2-enriched), and seven functional breast cancer gene modules ("invasion and metastasis", "immune response", "estrogen signaling", "angiogenesis", "proliferation", "apoptosis", "HER2 signaling"). Differences in gene expression between the subtypes were assessed in separate analyses.

The stratification into ovarian cancer C-signatures was assessed separately within each of the breast cancer modules. In each of the seven modules, one C-signature was chosen and the mRNA levels of the genes defining the breast cancer module were compared with the other C-signatures using the Mann-Whitney test. The Mann-Whitney test is a non-parametric test used for comparisons between independent groups. The natural stratification into malignant, borderline, and benign tumors was also assessed separately within each breast cancer gene module. The three tumor groups in each of these seven comparisons were compared using Kruskal Wallis test, which is an extended version of the Mann-Whitney test used for comparisons between >2 groups.

The external dataset used for validation was already stratified into six ovarian cancer C-signatures (C1-C6). We assigned these tumors the five gene expression-based intrinsic breast cancer subtypes as well as the seven functional breast cancer modules. Differences in gene expression between the six ovarian cancer C-signatures were separately investigated within each of the seven breast cancer modules. Like in our in-house cohort, one C-signature was compared with the other C-signatures using the Mann-Whitney test.

Comparisons between tumor group (malignant, borderline, benign) and assigned molecular subtype (either ovarian or breast cancer derived subtypes) as well as cross-comparisons between molecular ovarian and breast cancer subtypes were performed using Fisher's exact test. P-values <0.05 were considered statistically significant. Analyses were performed using SPSS statistics version 19.

Study IV

In study IV, the prognostic value of ERα, ERβ, PR, and AR as well as the prognostic effect of co-expression of PR and AR was assessed using PFS and OS time as clinical endpoints. PFS time was defined as the time interval between date of diagnosis and time to recurrence (clinical or radiological) or death of any cause, which ever came first. OS time was defined as the time interval between date of diagnosis and death of any cause. PFS and OS analyses were censored after a follow-up time of five years. The censoring after five years was performed to

54

allow for a sufficient number of remaining patients in each group (≥4 patients/group) [236]. Within this time frame, all deaths were due to ovarian cancer and no patients were lost to follow-up. PFS and OS were also used for assessment of the prognostic value of high versus low mRNA levels of the *ESR1*, *ESR2*, *PGR,* and *AR* genes in an external dataset. Due to limited follow-up time in this cohort, these analyses were censored three years after diagnosis. PFS and OS were defined as previously described and patients lost to follow-up were censored at the date of last notification. PFS time and OS time for both datasets were estimated using the Kaplan-Meier method and compared between groups using the Log Rank test.

For protein expression of the respective receptors, Hazard Ratios (HRs) with 95% Confidence Intervals (CIs) were calculated using univariable Cox regression models as well as multivariable Cox regression models adjusted for clinical factors with known impact on prognosis (histopathologic subtype, stage, histological grade, age at diagnosis and presence of *BRCA1* or *BRCA2* mutation). In line with the REMARK criteria, all prognostic factors were included in the multivariable analyses, regardless of outcome in univariable analyses [237]. Histological grade was treated as a categorical factor on three levels with grade 3 as reference. All other factors were binary, *i.e.* categorical factors separated into two outcome groups (typically yes/no).

Since data on chemotherapy were missing for 24/118 (21%) of the patients, multivariable analyses without the chemotherapy variable were consistently performed throughout the study. As a stability analysis, though, we assessed the effect of chemotherapy by including it in the multivariable analysis, and verified that the observed prognostic effect of the markers studied remained approximately the same.

Fisher's exact test was used to assess associations between dichotomized protein receptor expression and the clinical factors mentioned above. For stage and histological grade, though, the Mann-Whitney test was used, since these factors are ordinal, *i.e.* categorical factors with an inherent natural order (*e.g.* grade 3 is worse than grade 2, which in turn is worse than grade 1). In the external dataset, mRNA levels of the hormone receptor genes were compared within the molecular ovarian cancer subtypes using Kruskal Wallis test. All tests were two-sided. Statistical analyses of protein expression were performed using SPSS statistics version 22, and of mRNA levels using the R software version 3.1.0.

As outlined here, Fisher's exact test was consistently used for comparison of binary variables throughout studies I, III, and IV. This test is valid regardless of the cohort size and was therefore preferred rather than the Chi2 test, which requires at least 5 expected (calculated) frequencies in each compared subgroup to obtain a valid p-value.

Studies I, II, and III

Pearson's distance metric (correlation test) and the use of FDR and fold change, which were used to analyze gene expression data in studies I-III as well as the use of nearest centroid classifications in study III, are described in detail in the section Gene expression. In studies I and III, FDRs <0.01 were used to determine which genes were regarded as significantly deregulated between groups. In study II, a different approach based on the 500 most differentially expressed genes between groups was used, and therefore the FDR was higher (FDR <0.05). For GO analyses in study I a p-value <0.001 was required and in study III FDR <0.05 and ≥3 genes/biological function were required to consider a function significantly affected.
Statistical analyses in study II were performed using the R software version 3.1.0.

Methodological considerations

Limitations and strengths

Limitations regarding RNA and DNA extraction from FFPE blocks as well as with array-based gene expression profiling in general are described in previous sections. The remaining methodological considerations are common for all studies included in this thesis, and are therefore discussed in general in this section.

All studies were retrospective, a study design that allows for efficient evaluation of risk factors established at study start, especially when studying rare events or risks. With follow-up data already available, this study design is also time-efficient. The risk of bias and confounding is however greater than with prospective study designs and thus implies potential limitations. One way of overcoming the risk of systematic bias in *e.g.* evaluation of IHC stainings is to blind the evaluators to data used for stratification. In study I, hereditary status was not known to the investigators when evaluating the antibody stainings. Likewise, in both studies I and IV, two investigators evaluated the stainings independently and thereafter the results were compared. Confounders are factors that either do not impact the outcome variable or, in fact, contribute to the outcome in such a way that evaluation of the variable of interest will not be reliable. Different methods may be used to overcome confounders. In study I, the Lynch syndrome cohort was matched to a sporadic cohort. In study II, the influence of FFPE block age, tumor cell content, and the number of genes with good gene expression quality was taken into consideration. In study III, the ability of the statistical classifiers to correctly group the tumors was assessed, and in study IV multivariable Cox regression analyses were used to adjust for known clinical risk factors.

56

The material size in all studies was numerically relatively small. Results from small cohorts should always be interpreted with caution, but similarly the limited size aggravates the probability of demonstrating results with a sufficient statistical strength. The material size must however be put in a context. Study I included 24 Lynch syndrome-associated tumors (and 24 sporadic controls), a relatively large cohort since approximately 15-20 new cases of Lynch syndrome-associated ovarian cancer are expected to be diagnosed every year in Sweden [238, 239]. The material size thus strengthens study I. However, the samples were collected during a very long time period, with the oldest FFPE block being 54 years old, which is a limitation since tissue handling and storage could not be influenced. Likewise, the 15 and 10 OCCCs in study II could be compared to the expected 35-50 OCCC cases diagnosed in Sweden each year, but where the experience from our local biobank reveals that it is difficult to gather this rare tumor type (see the Patients section above) [239]. Studies III and IV were restricted to serous and serous/endometrioid tumors respectively, which reduced the number of tumors available for analysis. Focus on defined subtypes is however a strength due to the heterogeneity of ovarian cancer. Furthermore, the findings in both study I and study III remained when investigated in larger, independent cohorts, which strengthens the results.

The associations between borderline ovarian tumors and the luminal A breast cancer subtype in study III and the prognostic benefit of tumors expressing both PR and AR in study IV are based on statistical computations. Since the material sizes are limited and several associations were evaluated in the respective studies (associations with different subtypes and outcome of different receptor combinations) there is a substantial risk of overfitting, *i.e.* minor findings may be exaggerated and the results must thus be interpreted with caution. The results are however reinforced by biological similarities and explanation models [132, 240, 241].

Results and Discussion

Lynch syndrome and its impact on ovarian cancer gene expression

Study I

In study I, we aimed to investigate gene expression profiles and to identify genetic discriminators in Lynch syndrome-associated ovarian cancer. Whereas Lynch syndrome-associated colorectal and endometrial cancers are relatively well characterized and gene expression changes have been delineated in MSI colorectal cancers, gene expression data from the rare MMR defective ovarian tumors are scarce [242-245].

Unsupervised as well as supervised hierarchical clustering of gene expression data revealed distinct clustering between the Lynch syndrome-associated and the sporadic cancers in study I, indicating that the ovarian tumors linked to Lynch syndrome share more genetic features with each other than with the sporadic tumors of the same histopathologic subtype. This was verified also in an independent analysis using different analytical methods. Furthermore, a leave-one-out analysis correctly classified 79% of the hereditary tumors and 62.5% of the sporadic tumors. The interpretation is that the dataset itself is relatively stable and that the predictive model that classifies tumors as hereditary or sporadic works favorably. Genes overlapping between our dataset and an independent, publicly available dataset containing serous and endometrioid adenocarcinomas as well as serous borderline tumors were also used for unsupervised hierarchical clustering in our cohort, thus refining the discriminative gene expression profile of Lynch syndrome-associated tumors [58].

A SAM analysis revealed 349 significantly deregulated genes between the Lynch syndrome-associated and sporadic tumors (FDR <0.01). Among the up-regulated genes in the hereditary subset were *PTPRH*, a protein tyrosine phosphatase with both tumor suppressor and oncogenic functions, *BIRC3* that affects NF-kappa (κ) B-signaling and is associated with both resistance to apoptosis and chemotherapy and *TNFRSF6B* (also known as *DCR3*), which is a member of the tumor necrosis factor super family protecting against apoptosis and associated with

adenocarcinomas of the colon [246] (www.genecards.org). In line with this, a GO analysis revealed involvement of processes related to cell growth and proliferation as well as cell death. This in turn may be associated with the fact that Lynch syndrome-associated ovarian tumors encompass a disproportionate enrichment of well-differentiated endometrioid as well as clear cell tumors, which are generally thought to proliferate slowly from benign and intermediate precursors and more often being confined to the ovary at diagnosis than serous tumors [12, 247].

The histological distribution and the shift toward tumors diagnosed at early stages (stages I-II) likely contribute to the generally favorable outcome for patients with Lynch syndrome-associated ovarian cancer [104, 247]. At the same time, well-differentiated endometrioid cancers and clear cell cancers respond less well to chemotherapy [40]. Therefore, the identification of molecular targets is crucial to improve survival in this patient group.

To further assess potential genetic targets in Lynch syndrome-associated ovarian cancer, IHC stainings were performed with antibodies directed at p-mTOR (*i.e.* the phosphorylated, active, form of the protein), PTEN and EGFR. The genes encoding these proteins are functionally linked to the genes discovered in the SAM analysis and involved in the processes derived from the GO analysis, which suggests that these genes represent potential key targets. *EGFR* is located upstream of many targets and pathways, among them the PAK pathway, in which *PTPRH* is involved. *EGFR* promotes cell proliferation, and is also associated with activation of NFκB-signaling (see *BIRC3* above). Moreover, *PTPRH* induces apoptosis via inhibition of PI3K mediated signaling and also affects *MAPK* activation. *TNFRSF6B* is also associated with the MAPK pathway, which is connected to the PI3K pathway as well as the mTOR pathway. The phospholipid PIP3, which is a major component of the PI3K pathway, activates the downstream protein target AKT, which is essential for cell growth and is in turn inhibited (dephosphorylated) by PTEN, a phosphatase crucial for regulation of the delicate balance between cell survival and cell death. Loss of PTEN will thus lead to enhanced cell survival. The pathways described and their connections are outlined in Figure 12.

Our hypothesis was that EGFR and p-mTOR would be overexpressed, whereas PTEN loss would be more frequent in the Lynch syndrome-associated cancers compared with the sporadic ovarian cancers. Despite the potential connections found in cluster analyses and in GO analyses, no significant differences in IHC staining patterns were discovered, but a rather similar distribution between the groups. This may have several causes; up-regulation defined in gene expression analyses is always relative to another group. Hence, up-regulation in one group may indeed rather signify down-regulation in the comparative group. Likewise, proteins function through signaling cascades and networks, which implies that other primary target proteins may be relevant. Due to the limited possibilities to obtain high-quality RNA from FFPE blocks, we refrained from validation using

60

PCR or Western blotting. The validation method itself, IHC, represents another source of bias since various protein modifications rather than expression may be relevant to regulate protein function. Epitope selection may also influence the staining results. For IHC validation we could also have chosen to assess some of the top upregulated genes. Several therapeutic options are available to achieve EGFR inhibition and mTOR inhibition, and although the results from IHC stainings in study I were negative the findings from the gene expression data remain. It would be interesting to further analyze the involvement of these targets in Lynch syndrome-associated ovarian tumorigenesis.
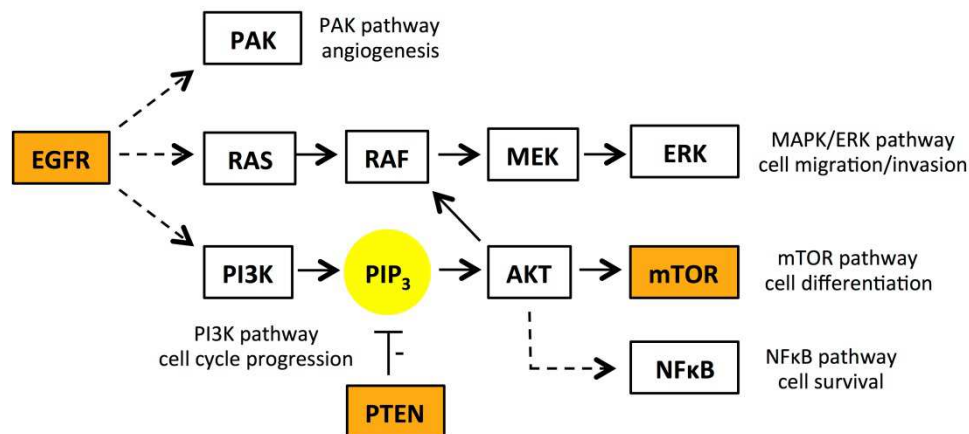
Figure 12. Oveview of signaling pathways and their intrinsic connections, in which the top up-regulated genes in Lynch syndrome-associated tumors are involved. Each box represents a protein. Colored boxes represent proteins assessed usig immunohistochemistry in the present study. The circle represent a key phospholipid. Arrows indicate the direction of signaling and represent stimulating actions.

During recent years, several reports and review articles have been published regarding the clinical features of ovarian cancer linked to Lynch syndrome as well as screening of mutation carriers [66, 83, 101, 248, 249]. A more biological approach has been taken on by Niskakoski *et al.*, who have assessed promotor methylation of tumor suppressor genes [102, 250]. Methylation of tumor suppressor genes normally results in gene silencing, and is thus important for the understanding of the underlying biologic mechanisms in tumor development. Promotor hypermethylation of tumor suppressor genes was more frequently observed among the Lynch syndrome-associated cancers compared with the sporadic cases, and was more frequent in non-serous than in serous ovarian cancer. Hypomethylation of LINE-1, which is part of a group of a self-amplifying genetic elements (Long Interspersed Nuclear Elements, LINEs) and a biomarker for global

DNA hypomethylation, was, however, more common among the sporadic tumors [102]. Hypomethylation of LINE-1 has been described as a poor prognostic marker in both ovarian and colorectal cancer and could potentially be related to the favorable prognosis in Lynch syndrome-associated compared with sporadic ovarian cancers [251, 252]. Among the most frequently methylated genes in Lynch syndrome-associated ovarian cancer reported by Niskakoski *et al.,* only two genes were affected in our study; *APC* was upregulated and *WT1* was downregulated in our Lynch syndrome cohort. Although our data as well as the observations reported by Niskakoski *et al.* need further validation, the results point in the same direction of a distinct molecular profile of ovarian cancer linked to Lynch syndrome. Likewise, a distinct gene expression clustering pattern has been described in colorectal cancers linked to Lynch syndrome by members of our research group [253]. The upregulated genes in that study did however not overlap with the ones in our study (a stricter cut-off for defining significantly upregulated genes was used in study I), thus precluding further comparisons, but still our result is in line with the studies discussed here.

Lynch syndrome is inherited in an autosomal dominant manner; the penetrance varies between the affected genes and the syndrome predisposes to a number of malignancies [65, 94, 95, 254]. Although Lynch syndrome-associated ovarian cancer is rare, Lynch syndrome-associated colorectal and endometrial cancers are not [95, 254-256]. Identification of mutation carriers thus may prevent other tumors in both the affected patient and her family members. Likewise, the preventive measures, *i.e.* screening for ovarian cancer in predisposed groups, are generally not very effective. To perform risk-reducing hysterectomy and SOEB is effective, but a brutal intervention if not absolutely necessary [249]. As previously mentioned, type I ovarian tumors, to which the Lynch syndrome-associated ovarian tumors in general belong, are also more resistant to chemotherapeutic agents due to the low proliferation rate thus desperately warranting refined therapeutic strategies and also research interest [40].

In study I, apart from the failure of the ICH validation, the data remained stable using different data analysis approaches. To further assess the homogeneity of Lynch syndrome-associated tumors, we analyzed the small subgroups of serous, endometrioid, and clear cell tumors independently. Unsupervised hierarchical clustering revealed a strong clustering of serous and endometrioid tumors linked to Lynch syndrome, whereas the clear cell tumors were intermingled. The observation that the clear cell histology seems to override the influence of heredity, led us to continue our investigation into this distinct ovarian cancer subset in study II.

Ovarian clear cell carcinomas and chromatin remodeling

Study II

In study II, we aimed to characterize OCCCs using targeted deep sequencing. This tumor type has distinct clinical features, but may be difficult to distinguish from other histopathological subtypes using immunohistochemistry. Transcriptional signatures of OCCCs have been described, but at study start only a few OCCC specific genes had been thoroughly characterized, and since OCCCs are rare most studies encompassed a limited number of cases [47, 212].

In *cohort 1*, a multiclass supervised clustering analysis based on the 5% (n=515) most differentially expressed genes across all tumors and using the histopathologic subtypes as groups revealed great transcriptional differences between the histologies analyzed. In line with the result in study I, the OCCCs were distinct and created a seemingly homogenous subcluster. Among the upregulated genes in the OCCC subcluster, *ERBB2*, which was identified also in a previous publication, was identified [257]. The observation of a homogenous OCCC cluster is also in line with a previous publication by Zorn *et al.*, who noted that clear cell tumors regardless of origin (ovarian, endometrial, or renal) displayed similar gene expression profiles. This contrasted to serous and endometrioid tumors which displayed different gene expression profiles in different organs [48]. The 515 differentially expressed genes identified in *cohort 1* were used in a GO analysis of biological processes, revealing involvement of lipid transport, morphogenesis, developmental processes, and the signaling pathway cytoskeletal regulation by rho GTPase.

A comparison of the deregulated genes identified in our cohort with the genes upregulated in the study by Zorn *et al.* revealed 14 overlapping genes corresponding to the GO enrichment terms metabolic processes and structure-specific DNA binding, among others. Many of the genes also revealed associations with inflammatory response and cytokine activation, of interest since inflammation is proposed to be pro-tumorigenic in OCCC development [258]. Among the overlapping genes, *BCL6* is of particular interest since it is involved in chromatin binding, which was to attract our interest during the work with this project [259].

Unsupervised cluster analysis of the 5% (n=500) most differentially expressed genes across the 15 OCCCs in *cohort 1* displayed a heterogeneous pattern that contrasted to the previously mentioned homogeneity compared with other histologies. This observation is in line with a previous publication that revealed distinct OCCC clusters in 50 OCCCs (in its context a relatively large cohort) using comparative genomic hybridization arrays [257]. No enrichment of GO terms was

discovered among the 500 differentially expressed genes or among the 55 genes overlapping between the whole dataset (n=67 tumors) and the 15 clear cell tumors in our study, indicating large inter-tumor variation among the OCCCs. We therefore performed targeted deep sequencing in order to further investigate the OCCCs. Due to lack of tissue availability, this analysis was performed using a partially overlapping cohort consisting of 10 OCCCs, *cohort 2*.

We sequenced 60 cancer-related genes with minimum 30x coverage for >99% of the target bases across all 10 tumor samples. The mean target coverage was 534x (range 314-698x), and approximately 3,000 variants (altered DNA sequences) were called, of which 1,590 were considered serious according to ENSEMBL and CONDEL as previously described (see Targeted deep sequencing in the Materials and Methods section). Serious gene variants change the coding sequence, *i.e.* may alter the protein structure or function, and are subsequently referred to as mutations. Since the dataset displayed a variant frequency peak around 50% for SNVs, possibly due to germline heterozygous mutations, this range was removed to improve the call for potential somatic mutations. Likewise, variance frequencies <5% (most likely not true mutations) or >95% (to remove possible germline SNPs creating a hit at 100%), were removed for both SNVs and indels. Hence, the mutation call cut-off was set to 5-45% or 55-95% for SNVs and 5-95% for indels, and left us with 114 mutations in 15/60 genes in the 10 tumors. Some genes harbored more than one mutation/individual tumor, and the median number of mutations/tumor was 12.5 (range 8-16).

BRCA1 was mutated in two tumors, a mutation not likely to occur in OCCCs. Therefore these mutations were further analyzed and identified as commonly occurring, non-disease causing germline variants, *i.e.* in the case of genetic testing they would not have been reported*. An *ERBB2* mutation was identified in one tumor, but this mutation was not activating and thus probably not of clinical relevance. Overall, the mutations identified in this study were not related to age off FFPE blocks, a relevant concern due to the difficulties regarding DNA extraction/quality from formalin-fixed tissue. Eleven previously unreported mutations were identified in 9 genes (*KMT2C*, *ARID1A*, *PIK3CA*, *TP53*, *ZFHX3*, *ERBB2*, *NF1*, *SMARCA4*, and *SPOP)*. The mutations identified in study II are outlined in Table 4.

*http://www.ncbi.nlm.nih.gov/clinvar/variation/41815/

Table 4. Overview of mutations identified in study II. The gene names of chromatin modifiers are highlighted in red. The colored boxes represent the mutation frequency in the individual tumors. Green=1 mutation, orange=2 mutations, yellow= ≥3 mutations (the exact number specified within each box).

Sample Id / Gene name	3	6	4	9	10	7	8	2	1	5
KMT2C	11	8	12	10	11	7	8	9	8	7
ARID1A				■		■		■		
CHD1	■	■				■				
PIK3CA						■			■	
BRCA1	■						■			
TP53	■									
ZFHX3	■	■								
ASXL1				■						
ATM							■			
CDKN2A				■						
ERBB2		■								
KRAS										■
NF1		■								
SMARCA4		■								
SPOP							■			

As expected, mutations in *ARID1A* (identified in 3/10 tumors) and in *PIK3CA* (identified in 2/10 tumors) were found in *cohort 2*, although in a somewhat lower frequency than expected [19, 23, 260]. Furthermore, *ARID1A* and *PIK3CA* mutations have been reported to co-exist in OCCCs, but only co-existed in one tumor in our study [261]. The result in our study probably reflects the limited cohort size rather than true differences. Using a mouse model, it has even been proposed that co-existence of *ARID1A* and *PIK3CA* mutations drive OCCC tumor development, although this is controversial and so far not replicated [258, 262].

Involvement of chromatin remodeling genes in OCCCs is, however, identified [23]. The tumor suppressor gene *ARID1A* is a member of the BAF (SWI/SNF) family, a complex ensuring proper chromatin segregation and thus essential for transcription of genes otherwise suppressed by dense chromatin. *ARID1A* affects the chromatin structure around the genes and facilitate transcription. Since chromatin remodeling is fundamental for regulation of transcription, remodeling may affect gene regulation without interference with DNA sequences [263]. It has been proposed that *ARID1A* inactivation is an early event in development of OCCC and endometrioid ovarian cancer, that *ARID1A* and *TP53* co-operate to maintain normal cell growth in healthy cells, and that a mutation in only one of the genes is required for carcinogenesis [18]. This suggests a plausible explanation for why these mutations seem to be mutually exclusive in OCCCs [261, 264]. So far, this remains a hypothesis and it is uncertain whether *ARID1A* mutations *per se* are sufficient for OCCC development or [258, 263, 264]. Interestingly, in our study

only two single nucleotide deletions in *TP53* were found in one single tumor, which was not *ARID1A*-mutated.

In line with the involvement of *ARID1A* in OCCCs, *KMT2C* was the most frequently mutated gene across all tumors in study II. *KMT2C*, also known as *MLL3*, is a methyltransferase gene in the Mixed-Lineage Leukemia (MLL) family involved in epigenetic modification of chromatin. The involvement of *KMT2C/MLL3* in leukemias is well characterized, and it is also affected in several solid tumor types [265, 266]. Truncating germline mutations in *KMT2C/MLL3* have also recently been described in HGSOC [267]. *MLL* genes are identified as part of fusion genes in leukemias. Interestingly, two truncating indels in *KMT2C/MLL3*, which may make the gene more prone to fuse with other genes, were revealed in our study [268]. *KMT2C/MLL3* accounted for 91/114 (80%) of all mutations called in this study, and the number of mutations in each tumor ranged from 7 through 12. Several mutation loci were common between tumors. Eighteen (20%) of the *KMT2C/MLL3* mutations were found in functional domains.

Apart from *KMT2C/MLL3* and *ARID1A,* three additional chromatin modifiers were identified among the 15 mutated genes in study II; *CHD1* (mutations in 3/10 tumors), *SMARCA4* (1/10 tumors) and *SPOP* (1/10 tumors). Just like *ARID1A*, *SMARCA4* (also known as *BRG1*) is part of the BAF family and together these two proteins are required for proper chromatin binding of topoisomerase IIα (TOP2A), which facilitates DNA breaking and rejoining [269]. TOP2A inhibitors such as etoposide and doxorubicin are widely used in clinical practice and thus further studies of the potential use of these drugs in OCCCs would be interesting [270]. A schematic diagram of the relationship between *ARID1A*, *SMARCA4*, *TP53* and *TOP2A* is shown in Figure 13.

To the best of our knowledge, mutations in *CHD1* and *SPOP* have not been described in ovarian cancer, but these genes are frequently mutated in prostate cancer [272, 273]. *SPOP* mutations have also been described in endometrial cancer, although the clinical utilization hereof is still uncertain [274]. Although our study is limited in size and validation is still to be performed, the results suggesting involvement of chromatin remodeling in OCCCs are in line with several previous studies discussed here. Chromatin remodeling is essential in cancer evolution, and the identification of several chromatin remodeling genes in OCCCs is of potential interest for refined therapeutic strategies. *In vitro* studies with small molecules targeting chromatin remodeling are ongoing (https://clinicaltrials.gov/).

66
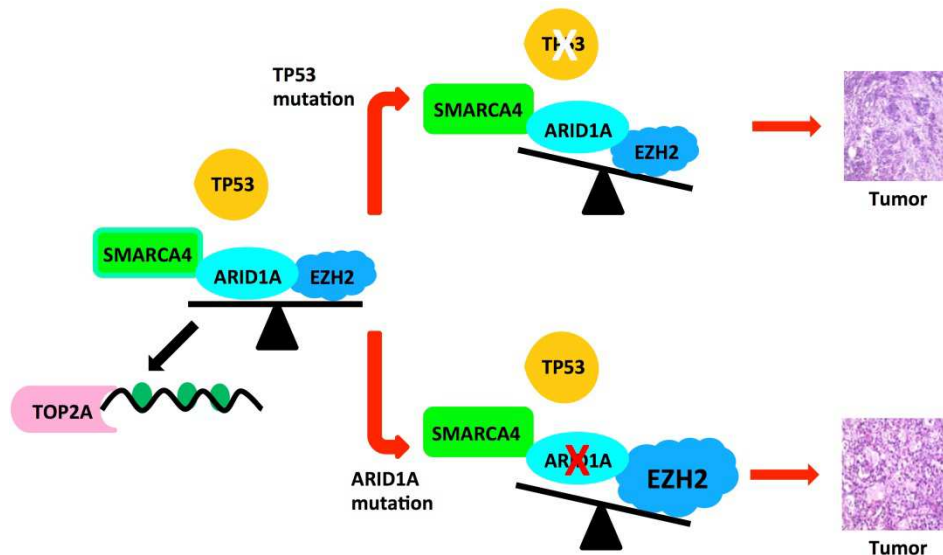
Figure 13. Overview of the BAF complex with *SMARCA4*, *ARID1A* and *EZH2*. In a healthy cell they co-operate to maintain a proper chromatin structure, allowing for *e.g.* *TOP2A* to bind to the chromatin. Mutations in *TP53* and *ARID1A* seem to be mutually exclusive, and mutations in either gene can enhance carcinogenesis. In the event of *ARID1A* mutation the significance of its catalytic subunit *EZH2* increases, which promotes proliferation. Adapted from [271].

Similarities between subtypes of serous ovarian cancer and breast cancer

Study III

The purpose of study III was to investigate gene expression profiles in malignant, borderline, and benign serous ovarian tumors and to assess potential similarities to the intrinsic subtypes of breast cancer. As outlined in the Background part of this thesis, gene expression analyses have been used for more than a decade to identify differences between histopathological subtypes of ovarian cancer and to explore the potential prognostic impact [38, 47, 53, 57, 61]. The motives for our study were the evolving evidence that the different histopathologic subtypes may indeed represent distinct diseases and that heterogeneity exists within the defined subtypes as well as between individual tumors [10, 275, 276].

67

Molecular subtypes of breast cancer were described more than a decade ago and are now partly used in clinical decision-making [41]. A similar effort to investigate subtypes of serous and endometrioid ovarian cancer was made by David Bowtell's group in 2008, and has been followed by several other projects aiming at delineating subtypes of mainly HGSOCs [54, 58, 60]. In 2012, the TCGA published a thorough work on breast cancer that revealed striking similarities between basal-like breast cancer (in the clinical setting often equalized with triple negative tumors, *i.e.* breast cancers not expressing hormone receptors or *HER2* amplification) and HGSOC in terms of tumor heterogeneity, genomic instability, frequent *TP53* mutations and an aggressive growth pattern [44]. Apart from studying gene expression in different serous ovarian tumors, we aimed to compare ovarian and breast cancer subtypes, with the general aim of identifying breast cancer markers that could be of potential interest also in the field of ovarian cancer.

Gene expression profiling was performed using 37 malignant, 5 borderline, and 17 benign ovarian tumors. Unsupervised hierarchical clustering of the malignant and benign cases using the 20% most differentially expressed probes revealed stable clusters related to malignant potential, *i.e.* the malignant tumors formed one cluster and the benign tumors formed another, with the exception of four malignant cases that were intermingled among the benign tumors. Of these, 3 were grade 1 or 2, though. Pairwise clustering of all but one of the 13 biological replicates was seen in study III, and the clusters remained stable after removal of the replicates.

A SAM analysis revealed 5,944 significantly deregulated genes in the malignant compared with the benign tumors. Several cell cycle kinases were among the upregulated genes, and a GO analysis revealed enrichment of cell cycle-associated biological processes. The few borderline tumors were investigated using a similar approach; first an unsupervised clustering of malignant, borderline and benign tumors was performed (using the 20% most differentially expressed genes) to explore the borderline affinity in an unbiased manner, and then a clustering analysis supervised by the 5,944 genes from the malignant-benign analysis was performed. As in previous analyses two clusters, one malignant and one benign, were seen, and the borderline tumors were intermingled between clusters. This stable clustering, implying robust transcriptional profiles of malignant and benign tumors, is in line with previous reports [49, 50].

Although our finding that the borderline tumors were divided between the benign and malignant clusters differs from a previous study by Bonome *et al.*, with a larger borderline cohort, it is in line with the results recently published by Curry *et al.*, revealing a heterogeneous pattern among serous borderline tumors [50, 277]. Our study with five borderline tumors and a total of 59 ovarian tumors and the study from Curry *et al.* with 13 borderline tumors and a total of 24 ovarian tumors both suffer from the limitation of small numbers of tumors, making it difficult to

68

draw any firm conclusions. Despite the limited sample sizes, though, the results are consistent and further studies of the transcriptional landscape of borderline ovarian tumors would be of interest. For instance, Curry *et al.* outlined a gene profile separating borderline tumors into malignant-like or benign-like tumors and validated this signature using the independent, publicly available dataset from Tothill *et al.*, which was first used to describe the molecular ovarian cancer subtypes; the same dataset as we used in study III [58]. Our borderline tumor subset was too small to allow meaningful classification using the gene profile by Curry *et al.*, but the result warrants further investigations as does their finding of an enrichment of claudin genes in the malignant-like borderline tumors. Claudins are described also in breast cancer, where a heterogeneous, poor-prognosis claudin-low intrinsic subtype has been proposed [278]. The proposal of a borderline heterogeneity does not necessarily imply that the tumors are evolutionarily different, but may reflect different steps of the malignant transformation.

We stratified our cohort into the six gene expression-based ovarian cancer molecular subtypes ("C-signatures") proposed by Tothill *et al.* [58]. A C-signature was assigned to each of the 59 tumors in study III. Group comparisons revealed significant correlations between the malignant tumors in our cohort and the C1 (nowadays termed "mesenchymal"), C2 ("immunoreactive"), and C4 ("differentiated") signatures as well as between our borderline and benign tumors and the C3 ("borderline and low-grade serous") signature. No significant correlations were, however, identified between our cohort and the C5 ("proliferative") signature. No tumors in our cohort were assigned to the C6 signature ("low-grade endometrioid"), which is in line with our study not encompassing any endometrioid tumors. For application of the ovarian cancer C-signatures, we used nearest centroid classification of the genes overlapping between the datasets. The C-signatures encompassed 2,725 good quality genes, of which 1,295 (47.5%) were present in our dataset. The classifier itself worked well; 239/251 (95.2%) of the tumors in the Tothill cohort were correctly re-assigned to their respective subtype.

To outline the similarities between ovarian and breast cancer subtypes, apart from those already established by the TCGA, we stratified the tumors in our cohort to the gene expression-based intrinsic breast cancer subtypes (luminal A, luminal B, Normal-like, Basal-like, HER2-enriched). Here we used the breast cancer signatures outlined by Hu *et al.*, that comprise 306 genes of which 208 (68%) were present in our dataset [224]. Other breast cancer classifiers are available, *e.g.* the commercially available 50-gene signature Pam50 (Prosigna®), but since members of our group already were acquainted with the Hu signature from previous work on male breast cancer we chose to work with that [279, 280].

As expected, the cross-comparisons identified significant correlations between our malignant ovarian tumors and the basal-like breast cancer subtype [44]. Outcome could not be fully evaluated due to few and unbalanced numbers of tumors in the different groups, but the five luminal-like malignant ovarian tumors seemed to harbor a favorable prognosis compared with the ovarian cancers that corresponded to the basal-like breast cancer subtype. Borderline and benign tumors did, as expected, correspond to the normal-like breast cancer subtype which was originally derived from normal breast epithelium [41]. Further analysis of the five borderline ovarian tumors revealed strong correlations to the luminal A breast cancer subtype. This correlation was not seen in the analysis of the whole cohort, probably due to the skewed numbers of the respective tumor types. The correlations between our ovarian tumors and both the ovarian cancer C-signatures and the intrinsic breast cancer subtypes are outlined in Table 5.

Table 5. The serous ovarian tumors in study III with corresponding ovarian cancer C-signatures and intrinsic breast cancer subtypes. The rows outline the tumor types, with the representation in each subtype in percent within paranthesis

	Ovarian cancer C-signatures					
	C1	C2	C3	C4	C5	
Tumor feature						**Total**
Malignant, n (%)	12 (32.4)	8 (21.6)	3 (8.1)	9 (24.3)	5 (13.5)	37
Borderline, n (%)	0 (0)	(0.0)	5 (100.0)	0 (0)	0 (0)	5
Benign, n (%)	1 (5.9)	0 (0)	15 (88.2)	0 (0)	1 (5.9)	17
	Intrinsic breast cancer subtypes					
	Luminal A	Luminal B	Basal	Normal	HER2	
Tumor feature						**Total**
Malignant, n (%)	2 (5.4)	3 (8.1)	21 (56.8)	6 (16.2)	5 (13.5)	37
Borderline, n (%)	4 (80.0)	0 (0)	0 (0)	1 (20.0)	0 (0)	5
Benign n (%)	1 (5.9)	0 (0)	0 (0)	16 (94.1)	0 (0)	17

p<0.001 (Fisher's exact test) for comparison between tumor features and C-signatures and comparison between

tumor features and breast cancer subtypes respectively

To further strengthen the potential correlations between the ovarian cancer and the breast cancer molecular signatures, the latter were applied to the tumors in the Tothill cohort, revealing a connection between the ovarian cancer C2 signature and the basal-like breast cancer subtype, as well as between the ovarian cancer C3 signature and the normal-like breast cancer subtype. Application of the breast cancer subtypes to our tumors with assigned C-signatures displayed the same results. In general, breast cancers corresponding to the normal-like breast cancer subtype have been shown to have a low percentage of tumor cells [281]. The

cellularity of the Tothill tumors was not available to us, but the connection between the ovarian cancer C3 (borderline) signature and the normal-like breast cancer subtype seems logical since borderline tumors *per se* have a lower malignant (semi-malignant) potential.

Although a limited-size cohort, the results from study III demonstrate similarities between the molecular classifiers of serous (and endometrioid) ovarian cancer and breast cancer, not only for HGSOC and basal-like breast cancer, but probably also for other subgroups of the respective tumor types. The findings observed in our study are outlined in Figure 14.
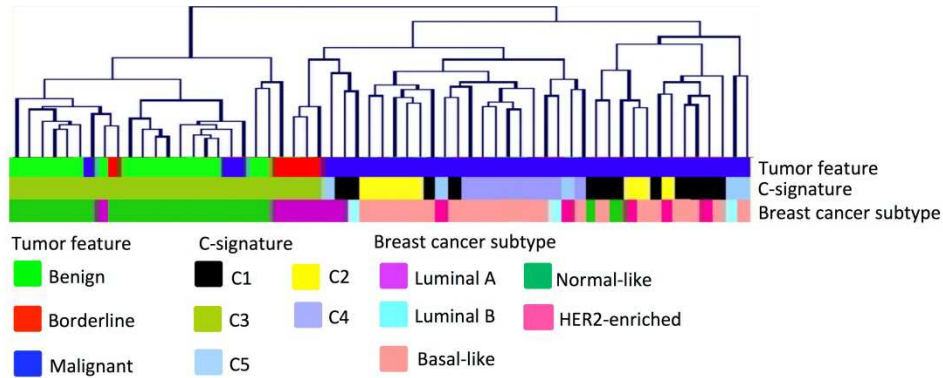
Figure 14. Clustering of the serous ovarian tumors in study III with corresponding, assigned ovarian cancer C-signatures according to [58] and intrinsic breast cancer subtypes according to [224].

It is tempting to rationalize gene expression profiles into specific features, which is done in clinical practice when the luminal breast cancer signature is translated into hormone receptor positive breast cancer, the basal-like signature into triple negative breast cancer, and the HER2-enriched signature into HER2-amplified breast cancer. This is motivated by clinical application of the intrinsic breast cancer subtypes [282, 283]. Although the molecular and clinical breast cancer subtypes show strong resemblance, overlap is present and the picture is not clear-cut. Most tumors in the basal-like subtype are triple negative, but not all triple negative tumors are basal-like, and even when they are basal-like they may be further sub-classified [281]

Another way of addressing this problem is to use sets of functionally related genes, gene modules, defined by gene expression profiling [226, 284]. We applied seven such gene modules corresponding to key tumor features such as "proliferation", "immune response", "apoptosis", "angiogenesis", "HER2 signaling", "ER(α) signaling", and "invasion and metastasis" to our study cohort

71

[226]. These gene modules are based on genes involved in key biological processes related to breast cancer development, though several features are relevant for multiple tumor types. Differences in mRNA levels between malignant, borderline, and benign tumors within each breast cancer gene module were assessed, and a strong association between the malignant ovarian tumors in our cohort and five of the seven modules, excluding the "ER(α) signaling" and "invasion and metastasis" modules, was revealed. The borderline and benign tumors in our cohort, however, corresponded to the "ER(α) signaling" module, in line with the previous findings in this study as well as the notion that the lower the malignant potential of the ovarian tumor the higher the hormone receptor expression [171, 240]. The lack of association between our malignant tumors and the "invasion and metastasis" module may be due to the wide distribution of mRNA values for the included genes among the malignant tumors. However, when our cohort was stratified into the ovarian cancer C-signatures and the mRNA levels of the separate breast cancer gene modules were compared between C-signatures, a strong association between the C1 tumors and the "invasion and metastasis" gene module was revealed, which is in line with the poor prognosis seen among tumors classified as C1 ("mesenchymal") [54, 58]. Furthermore, an association was detected between the C2/immune reactive ovarian cancer signature and the "immune response" module and between the C3/borderline-assigned tumors and the "ER(α) signaling" module.

The results were stable also when the tumors in the larger Tothill cohort were compared within the separate breast cancer gene modules. To assign gene modules to gene expression derived signatures with similar characteristics may seem self-fulfilling. Since the molecular breast cancer subtypes have shown a strong prognostic and predictive value and are wide-spread in a way that the molecular ovarian cancer subtypes are not, it is however of interest to investigate the robustness of the ovarian cancer subtypes as well as potential correlations to other subtypes. Thus the finding of a favorable resemblance between the ovarian cancer subtypes and the breast cancer classifiers (both the intrinsic subtypes and the gene modules) in this study supports the use of the molecular subtyping in ovarian cancer. Even better would of course be to investigate gene modules based on key features in ovarian cancer, and such gene modules (or molecular subtypes) should preferably consist of fewer genes than the breast cancer derived modules assessed in this study, in order to make the analysis easy and, hopefully, clinically useful.

One of the distinctive features between type I and type II ovarian tumors is the mutational spectra, with *KRAS*, *BRAF*, and *PTEN* mutations frequently occurring in type I tumors (such as LGSOCs), whereas *TP53* mutations are found in >95% of type II tumors (such as HGSOCs) [11, 12]. *KRAS* and *BRAF* mutations are considered early events in the stepwise development of type I tumors from benign precursors, via intermediate borderline tumors, to low-grade malignant tumors [285]. We were therefore interested in whether mutations in these genes

72

corresponded to the molecular subtypes and in their potential prognostic implications. Mutation analysis was performed in the malignant and borderline tumors, and *KRAS* mutations were detected in two malignant and two borderline tumors, and *BRAF* mutations were identified in two borderline tumors. The numbers are limited but broadly supporting an expected rate, since only 5/37 (13.5%) of the malignant tumors in our cohort were grade 1.

The two malignant *KRAS* mutated tumors were both grade 1 and corresponded to the C3 and normal-like subtypes and the C4 and luminal B subtypes respectively. This further supports the explorative results of this study revealing associations between the molecular subtypes of ovarian cancer and breast cancer. *KRAS* mutations have been identified in benign ovarian cancers, and mutation analysis of the benign tumors in our cohort had potentially been illustrative in that they may have contained information regarding possible malignant transformation [286]. The *KRAS* mutated borderline tumors all corresponded to the C3 and luminal A subtypes. Luminal breast cancers also frequently harbor mutations in the MAPK/ERK pathway, hence another feature in common between ovarian and breast cancers [44]. KRAS and BRAF are upstream targets of the MAPK/ERK (MEK) pathway, and MEK inhibitors are available and have been investigated in a phase II trial with recurrent LGSOCs, so far with promising results [287].

The associations revealed in this study may be explained by differences in proliferation and cell growth rate, since both HGSOCs and basal-like breast cancers are highly proliferative, in contrast to LGSOCs and luminal breast cancers [11, 44]. Other biological similarities should also be investigated, including the MAPK/ERK pathway and hormone receptor expression. As mentioned previously, benign and borderline ovarian tumors also generally express higher levels of hormone receptors than their malignant counterparts [174]. The association between borderline ovarian tumors and the luminal A breast cancer subtype is of interest not least in relation to our further observations in study IV.

Impact of endocrine receptors on prognosis and subtypes

Study IV

Although a number of prognostic factors have been identified in ovarian cancer, predictive markers are scarce [54, 61, 202, 288-291]. Motivated by the histologic, genetic, and clinical heterogeneity of ovarian cancer, studies of prognostic and predictive markers need to be better stratified [10, 11, 32, 54, 58, 172, 292]. In study IV, we aimed at further exploring the role of the sex steroid hormone receptors ERα, ERβ, PR, and AR (from here on referred to as hormone receptors)

in ovarian cancer in general and within the molecular subtypes described in study III. The studies were performed on both protein and mRNA levels. We hypothesized that hormone receptor expression could have prognostic impact similar to survival differences reported in ER positive/PR positive vs. ER positive/PR negative breast cancers [293]. Furthermore, we aimed to explore mRNA levels of the genes encoding the hormone receptors separately in the defined molecular subtypes of ovarian cancer.

A TMA consisting of in total 118 ovarian cancers (87 serous, 31 endometrioid) was used for analysis of protein expression of ERα, ERβ, PR, and AR. Regarding the clinical information, there was an overrepresentation of stages III-IV and grade 3 tumors in the serous subset compared with the endometrioid cases, which displayed a more even distribution of tumor stages and histological grades. These differences, however, resemble the scenario observed in the clinic [3, 294]. Among the 118 patients, 77 (65%) had received post-operative platinum containing chemotherapy, 17/118 (14%) were reported not to have received post-operative chemotherapy, and treatment data were missing for 24/118 (21%) of the patients. Verified mutations in *BRCA1* or *BRCA2* were reported in 30/118 (25%). Information on residual tumor burden after surgery, *i.e.* whether the patients underwent macroscopically radical surgery or not, as well as information regarding the use of post-operative hormonal treatment was not available. The lack of information on residual tumor burden is a limitation of this study, but relates to the collection of the cohort in study IV prior to 2001, after which this factor has received increased attention [295]. Thus, this information would still have been difficult to interpret. Hormonal treatment (also referred to as endocrine treatment) is not standard treatment for ovarian cancer and is only rarely used in the palliative setting. It is therefore unlikely that it was administered to the patients in this cohort to such an extent that it may have affected the results [172, 173].

PR protein expression was detected in 36/118 (31%) tumors and AR expression in 52/118 (44%) of the tumors, in line with the expression frequency of these receptors described in previous publications [172, 173, 296]. ERα staining was detected in 52/118 (44%) of the tumors, a lower frequency than in previous reports [173, 288, 297]. ERβ staining was detected in 102/117 (82%) of the tumors, a much higher frequency than expected [174, 288]. In general the receptor expression varies somewhat even between the previous studies referred to here. This can probably be explained by antibody specificity and differences in cut-off levels used, not to mention that tumor tissues are biological specimens and never exactly the same although the diagnosis and clinical features may be so. Likewise, validation of IHC stainings may suffer from limited reproducibility, which underscores the need for validated antibodies and defined cut-offs [209]. The unexpectedly high ERβ expression identified in study IV may reflect such limitations.

74

Although hormone receptor expression in different ovarian tumors has been assessed in several studies, with reports of higher receptor levels in benign and borderline tumors compared with malignant tumors, and also higher expression in endometrioid tumors than in serous tumors, limited data are available on receptor co-expression [132, 171, 172, 240, 298, 299]. Co-expression of ERα and AR as well as ERα and PR has been described, but without really assessing the functionality of such a co-expression [171, 297, 300]. The observation that PR+/ERα- expression may confer a better prognosis than other combinations of these receptors, mostly reported in older studies, may be a result caused by the favorable prognosis associated with PR+ tumors [171, 240, 301]. Although a recent publication reported an unfavorable prognosis associated with hormone receptor expression, the finding that PR expression is prognostically favorable seems well-established [299, 302, 303]. In line with this, the results in study IV revealed an association between PR negativity and advanced stage and grade. We also identified an association between AR negativity, grade, and older age (≥70 years at diagnosis) as well as between ERβ positivity and high grade. No associations were identified between ERα and clinical characteristics, or between histopathologic subtype, *BRCA1* or *BRCA2* mutation status, and clinical characteristics. A detailed table of the clinical data stratified for hormone receptor expression can be viewed in the appended study.

Positive PR expression and AR expression was independently associated with an improved 5-year PFS (52.8% vs. 18.3% for PR+ and PR- tumors respectively; 44.2% vs. 16.7% for AR+ and AR- tumors respectively, Log Rank p=0.001 for both analyses) and 5-year OS (63.9% vs. 24.4% for PR+ and PR- tumors respectively; 57.7% vs. 19.7% for AR+ and AR- tumors respectively, Log Rank p<0.001 for both analyses) whereas no such associations were seen for ERα or ERβ. As outlined in Table 6., patients whose tumors co-expressed PR and AR (PR+/AR+) had a significantly better PFS and OS compared with those whose tumors displayed discordant (PR+/AR- or PR-/AR+) or absent (PR-/AR-) protein expression. This is also visualized in Figure 15. The data remained stable after adjustment for stage, histological grade, age at diagnosis, *BRCA1* and *BRCA2* mutation status, and histopathological subtype. ERα and ERβ were not included in the multivariable analyses since their expression did not correlate with survival.

Table 6. A multivariable Cox regression analysis of 5-year PFS and OS adjusted for stage, grade, age at diagnosis, *BRCA1* and *BRCA2* mutation status, and histology.

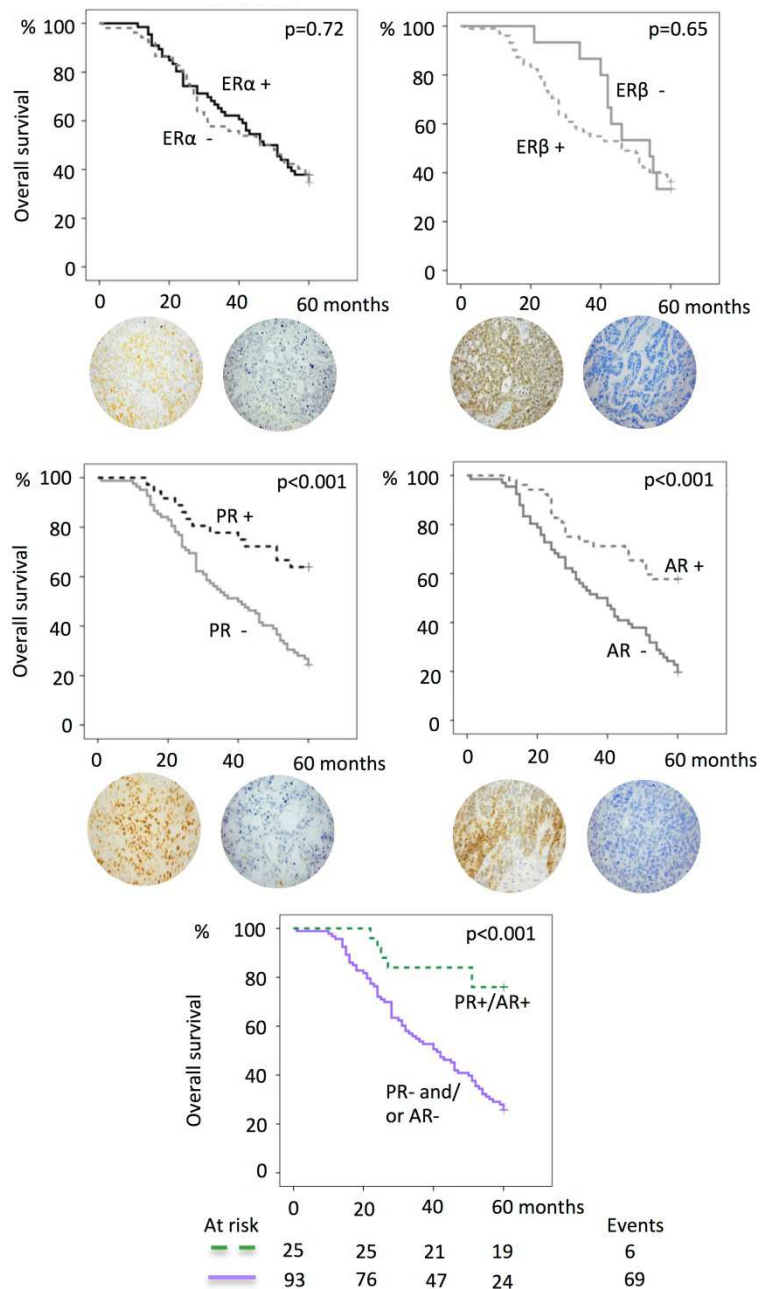| Endpoint	Variable	n (events)	HR	95% CI		p
5-year PFS	PR+/AR+ vs. PR- and/or AR-	101 (69)	0.32	0.13	0.79	**0.014**
5-year OS	PR+/AR+ vs. PR- and/or AR-	101 (64)	0.24	0.080	0.70	**0.009**

Figure 15. Overall survival curves outlined using the Kaplan-Meier method for positive vs. negative staining for the respective receptors analyzed in study IV. IHC stainings are displayed under each plot, with positive IHC receptor staining to the left and negative receptor staining to the right. p-values are calculated using the Log Rank test.

Since treatment data were missing for 21% of the patients, the impact of chemotherapy on the results was difficult to evaluate. Platinum containing chemotherapy vs. no chemotherapy was therefore included in a separate multivariable analysis (stability analysis) and was found to only slightly dilute the independent effect of co-expression of PR and AR (and, as expected from the decreased sample size, weakened the evidence of an effect). We also assessed the effect of co-expression of PR and AR in the serous and endometrioid tumor subsets separately, due to the great imbalance of histologies in the study. These analyses too showed that the favorable prognosis associated with PR+/AR+ status remained within each subset, and thus we felt assured that we could report the analyses in this study using the whole cohort (*i.e.* adjusted for histopathological subtype instead of separated into serous and endometrioid tumors) and withut including the variable chemotherapy.

In addition to the positive prognostic effect seen in women whose tumors co-expressed PR and AR, we also assessed the additional effect of co-expression, *i.e.* whether the effect of co-expression was greater than the combined positive effects expected from the independent effects of the tumor being PR+ and AR+. This so called interaction analysis revealed an evident additional effect of PR/AR co-expression which remained even in a multivariable analysis adjusted for the previously mentioned clinical factors (PFS, p for interaction=0.004 and OS, p for interaction=0.016) and is outlined in Figure 16.
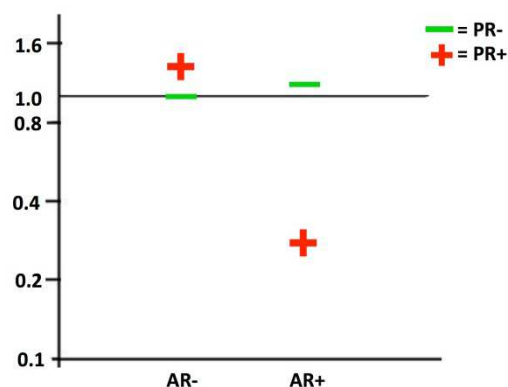
Figure 16. A graphical view of the independent effects on overall survival of PR and AR in relation to absent receptor expression (PR-/AR-). The *interaction effect* of co-expression of PR and AR (PR+/AR+) is visible as PR has an effect only within the AR+ tumors (and vice-versa for AR). The graph is based on a multivariable analysis adjusted for stage, grade, age at diagnosis, *BRCA1* and *BRCA*2 mutation status, and histology. PR-/AR- is used as reference (HR 1.0).

To further explore the prognostic relevance of hormone receptor expression, we analyzed the mRNA levels of the genes *ESR1*, *ESR2*, *PGR,* and *AR*, encoding ERα, ERβ, PR, and AR respectively, using the dataset originally used to describe the molecular subtypes of ovarian cancer and outlined in study III [58]. The mRNA levels of the respective genes were assessed in relation to the different subtypes, *i.e.* C1/mesenchymal, C2/immunoreactive, C3/borderline and LGSOC, C4/differentiated, C5/proliferative, and C6/low-grade endometrioid. This revealed higher median mRNA levels of *PGR* in the C3/borderline and C6/endometrioid subtypes, in line with the high frequency of PR protein expression reported in borderline tumors compared with malignant tumors and in endometrioid tumors compared with serous tumors [132, 240]. Likewise, the higher median mRNA levels of *ESR1* observed in the C2/immunoreactive, C4/differentiated, and C6/endometrioid subtypes seem logical. The C2 and C4 subtypes consist of serous malignant tumors associated with the best prognosis of the aggressive C1, C2, C4, and C5 signatures, in line with the notion of higher ERα protein expression in tumors with less malignant potential and a previous report showing that ERα expression was prognostically favorable in serous and endometrioid malignant tumors [172, 240]. Slightly higher median mRNA levels of *ESR2* were also observed in the C3/borderline and C6/endometrioid subtypes, which can probably be explained in the same way as the association between these subtypes and *PGR*. In a review article from 2007 it was also noted that ERβ protein expression might be protective against malignant transformation, which would be in line with our finding [299]. The *AR* mRNA levels were similar across the different subtypes.

We assessed the potential association between the mRNA levels and survival. The median mRNA levels for the respective genes were used as cut-offs, allowing for comparisons of low vs. high expression (*i.e.* below compared with equal or higher levels) and thus resembling the negative vs. positive protein expression comparisons previously described. Using the whole dataset, no associations were seen between the respective genes and prognosis, or for dual high *PGR* and high *AR* levels compared with the other possible outcomes. When assessed in the different subtypes, a weak tendency towards an association between high *PGR* levels and 3-year OS in the C2/immunoreactive subtype and between low *PGR* levels and 3-year OS in the C5/proliferative subtype were observed. The most apparent association, though, was revealed for *ESR1*, where evidence of a weak association between high *ESR1* levels and 3-year PFS and 3-year OS were observed in the C5/proliferative subtype (PFS, HR 0.45 [95% CI 0.19-1.0], p=0.058; OS, HR 0.19 [0.041-0.89], p=0.035). This contrasts to the lack of impact of ERα protein expression on survival in our study cohort, and may reflect the fact that two different cohorts were used in this study, and/or that regulation of hormone receptor expression is more complex and our study approach too simple to truly capture this. Both these potential explanations are further supported by the fact that weak evidence of an effect on survival (3-year PFS) of dual high *PGR*

and *AR* levels was only observed in the C2/immunoreactive subtype (PFS, HR 0.43 [0.17-1.0], p=0.063), *i.e.* the very strong finding on the protein level was not readily captured on the mRNA level. On the other hand, differences in mRNA levels between the molecular subtypes were exactly what we hypothesized and indeed variability was seen. As previously noted, the C2/immunoreactive subtype is the serous subtype associated with the best survival, supporting the observation of a weak association of high *PGR/AR* levels in this particular subtype [54, 58].

Ovarian cancers harboring *BRCA1* mutations most commonly develop as HGSOC. Such tumors are expected to have a lower expression of hormone receptors, in line with *BRCA1* mutated breast cancers often being hormone receptor negative [304]. We could, however, not reveal any association between (lack of) hormone receptor expression and *BRCA1* or *BRCA2* mutations. This observation supports similar findings in a previous study that also assessed the expression of ERα, ERβ, PR, and AR in a matched cohort of 22 ovarian cancers with *BRCA1* mutations and in 22 sporadic controls, revealing no differences in hormone receptor expression between the two groups [305]. A possible explanation for hormone receptor expression being prognostically favorable lies in differences in proliferation rate, as proposed in study III (associations between borderline ovarian tumors and the luminal A breast cancer subtype) and supported by the differences in receptor expression frequency in benign, borderline, and malignant ovarian tumors previously discussed here [171, 297]. This hypothesis is contradicted by the lack of differences in hormone receptor expression in *BRCA1* mutated *vs*. *BRCA1* and *BRCA2* wildtype ovarian tumors in *e.g.* our study. The latter observation may in fact imply that the hormone receptor status is prognostically far less important than the HRD in *BRCA1* mutated tumors, with HRD also sensitizing these tumors to cytotoxic agents, [306]. Besides, a comparison of hormone receptor expression in *BRCA2* mutated and wildtype tumors may potentially be more clarifying, supported by *BRCA2* mutated breast cancers showing a more variable and often positive hormone receptor expression than *BRCA1* mutated breast cancers [304].

Our finding of dual PR/AR positivity as a prognostically favorable factor requires validation, but is supported by the evidence of an effect in the interaction analysis. The biological background for this result also needs clarification, *e.g.* in relation to the cellular benefit from receptor co-expression and the limited effect of endocrine treatment in ovarian cancer [175, 176, 178, 184, 186, 188, 307]. Explanations for the lack of response to endocrine treatment may include treatment of different histopathological subtypes, limited numbers and heavily pre-treated patients, and lack of stratification for hormone receptor expression status. Likewise, AR expression has been reported to decrease after chemotherapy treatment, suggesting that adjuvant treatment may be more efficient [308]. Another explanation is that the regulation of hormone signaling is somewhat different in ovarian compared with breast cancer, supported by the very weak relationship between hormone receptor expression on the protein and mRNA levels seen in this study, and

contrasting to the strong predictive value of hormone receptor expression in relation to endocrine treatment observed in breast cancer [309].

Functional interactions between PR and AR are insufficiently described, although ERα may constitute an important link. Together with its ligands ERα regulates the expression of PR, and androstenedione constitutes a link between progesterone and the estrogen precursor testosterone, as outlined in Figure 17. Considering that estrogens also have a direct genotoxic effect, *i.e.* a non-receptor mediated effect, this may suggest a plausible explanation for the general lack of anti-estrogen response, but the slightly better effect of aromatase inhibitors, which prevent the conversion of androgen to estrogens rather than blocking the ERs, that has been reported in ovarian cancer [182, 184, 310].
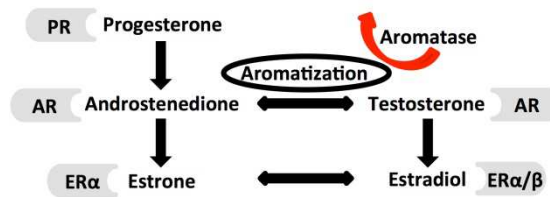

Figure 17. Overview of the relationship between progesterone, androgens, and estrogens as well as their receptors.

Recently, work by Jason Carroll's group was published in Nature, reporting novel functional interactions between PR and ERα, where progesterone was revealed to modulate the chromatin binding and transcriptional effects of ERα in breast cancer cells [241]. This is in line with the low-proliferative status of breast cancers co-expressing ERα and PR. Loss of PR could potentially cause, rather than be an effect of, altered ERα activity. This takes us back to the potential interactions between PR, AR, and, possibly, ERα. Several studies, including ours and as discussed previously in this text, have reported prognostic effects of hormone receptor expression in ovarian cancer. Although the results are sometimes contradictory, what remains is that the receptor function probably is of greater importance than the receptor expression, and may hold the key to whether hormone receptors actually do affect ovarian cancer survival and, if so, in which subgroup of tumors endocrine treatment may have a role. Our study did not identify associations between the mRNA and protein levels of hormone receptors, but the receptor expression in the different molecular subtypes varied, and there seems to be an additional effect of co-expression of PR and AR. Until the functional questions are resolved, future studies should preferably take histologic as well as molecular subtypes into consideration when assessing the role of hormone receptors in ovarian cancer in order to reveal true relationships.

80

Conclusions and Future Perspectives

Studies I-II

These studies demonstrate:

- Differences in gene expression profiles between Lynch syndrome-associated and sporadic ovarian cancers with deregulation of genes related to cell growth, proliferation, and apoptosis.

- No differences in expression levels of p-mTOR, PTEN, or EGFR in Lynch syndrome-associated and sporadic ovarian cancer.

- Separate clustering of Lynch syndrome-associated and sporadic serous and endometrioid ovarian cancers.

- Involvement of several chromatin modifiers, which may represent potential therapeutic targets, in clear cell ovarian cancers identified through targeted deep sequencing.

Studies I and II are explorative and identify areas for further investigation rather than provide firm conclusions. As for similarities and differences between Lynch syndrome-associated and sporadic ovarian cancer investigated in study I, we revealed involvement of proliferation and cell growth genes, though other markers for targeted therapies would also be relevant to investigate. Furthermore, the distinct clustering of serous and endometrioid ovarian cancers in hereditary and sporadic clusters was striking compared with the clear cell cancers that were seemingly unaffected by the hereditary influence. Since both endometrioid and clear cell cancers are overrepresented in ovarian cancers linked to Lynch syndrome, the differences in gene expression profiles between these histopathologic subtypes warrant further investigations.

An area of great interest is the similarities (or differences) between Lynch syndrome-associated cancers of different histologies and of different organs of onset. Comparison of, *e.g.*, the gene expression profiles as well as the mutation spectra in colorectal, endometrial, and ovarian cancers would potentially reveal pertinent information regarding the tumor dependence upon the origin of detection and/or the hereditary influence. Such an approach would provide a more unbiased estimate of potential targets relevant for future clinical trials. Furthermore, detailed studies of metachronous tumors in *MMR* mutation carriers may be useful to

predict why some individuals develop colorectal and endometrial cancer, and some colorectal and ovarian cancer (apart from the relevance of the specific *MMR* gene mutations that is) and how they are best managed. Definition of key carcinogenic steps may contribute to refined risk estimates and surveillance strategies. Furthermore, the ability to stratify mutation carriers according to risk of cancer development would potentially be of interest for preventive interventions.

Study II indicates that the seemingly homogenous OCCCs indeed show extensive heterogeneity, although they share features such as altered chromatin remodeling. The finding of frequent mutations in *KMT2C/MLL3* warrants validation, as does the involvement of other genes involved in remodeling of chromatin. Small molecules targeting chromatin modifiers are currently under evaluation in OCCCs, but a larger clear cell cohort is needed to truly assess the utility thereof. Exome sequencing would also be of interest to assess key targets outside of the preconceived genes. Gene expression profiling using a cancer directed platform instead of whole-genome expression profiling may also be clarifying, although less comprehensive. Ongoing preclinical studies of therapeutics directed against ARID1A and related proteins are promising. It would however also be interesting to assess the utility of TOP2A inhibitors, since such therapeutic agents already are available, as well as evaluating PI3K inhibitors in *ARID1A* mutated vs. wildtype OCCCs. The heterogeneity revealed in OCCCs warrants thorough stratification based on *e.g.* mutation spectrum, but other inter-tumor discriminators also need to be investigated.

Endometriosis is associated with endometrioid ovarian cancer and OCCC, and is thus relevant for studies of sporadic as well as Lynch syndrome-associated cancers. To date, we are probably not aware of the true endometriosis prevalence in relation to ovarian cancer development, since a cancer diagnosis, logically, is more important for clinical decision-making. Thus the first step would be to thoroughly investigate ovarian cancer tissue for signs of endometriosis. Thereafter, differences between endometriosis-related and endometriosis-unrelated cancers would be assessable. If such differences are present, they may potentially be of clinical relevance if they indicate different key driver changes. Such a study would also preferably assess the relation between anatomical endometriosis location and ovarian cancer development, including the histopathologic differentiation, as well as involvement of inflammation and immune response genes.

82

Studies III-IV

These studies demonstrate:

- Potential heterogeneity within the borderline ovarian tumors.

- Common alterations in the molecular subtypes of serous ovarian cancer and breast cancer beyond those previously described for high-grade serous ovarian cancer and basal-like breast cancer.

- Biological similarities between the serous borderline ovarian tumors and the normal-like and luminal A breast cancer subtypes, such as *e.g.* hormone dependency.

- A prognostic effect of co-expression of PR and AR was revealed for serous and endometrioid ovarian cancers.

- The prognostic benefit of PR/AR co-expression was greater than the combined effect caused by solitary expression of the individual receptors.

- No clear relationship between protein receptor expression and corresponding mRNA levels in an independent cohort, though the mRNA levels of the receptors varied between the molecular subtypes of ovarian cancer.

Studies III and IV were explorative and limited in size. As for similarities and differences between malignant, borderline, and benign ovarian tumors investigated in study III, we identified involvement of cell cycle genes, which is in line with a high proliferation rate in malignant tumors. Larger cohorts would, however, be needed for further investigations and for detection of small changes. A study with high power would require thousands of patients. This is not feasible due to the relatively low incidence of ovarian cancer and ovarian borderline tumors. Proportional numbers of the different tumor types, though, would potentially make the calculations more reliable. Based on the results by Curry *et al.*, who investigated 50 ovarian borderline tumors, approximately a hundred HGSOCs, LGSOCs, borderline, and benign tumors respectively would probably be needed in such a study. Exome sequencing of these tumors would be relevant to reveal information regarding carcinogenic transformation steps. Apart from requiring substantial resources such a project would also require widened collaborations to gather tumor material. It would, though, establish a solid platform for comparisons with the breast cancer subtypes. Genes of interest in both ovarian and breast cancer could then be sequenced and functional studies of *e.g.* genes involved in hormone signaling and in the MAPK/ERK pathway could be performed to investigate potential therapeutic targets. This would be relevant not least due to the need for refined therapeutic regimens for advanced and/or recurrent low-grade ovarian cancer.

An adjacent area of interest is liquid biopsies, which could be retrieved from *e.g.* ascites fluid. Investigation of ascites fluid at different time points during the course of disease may reveal information regarding acquired mutations and could potentially be relevant for prediction of disease progression and chemotherapy resistance. It could also be used for further stratification of patients, which may potentially be useful in future clinical trials. A liquid biopsy study should preferably aim at investigating one ovarian cancer subtype at a time to consider the tumor heterogeneity and to achieve sufficient power, but since this area is relatively new, a comprehensive investigation even of a limited number of well-characterized patients would be relevant.

The information in study IV on prognostic impact from PR/AR co-expression is promising, but needs further validation. Functional studies on the hormonal actions in ovarian cancer would be very interesting since they may reveal the true interactions between the receptors and the ligands and thus help predict whether and in which subgroup endocrine treatment would be feasible. Furthermore, insight into the non-receptor mediated, gene modulating effect of sex steroid hormones may be useful. Investigation of the hormonal interactions and functions between *e.g.* the fallopian tube and the ovary would be relevant to assess the potential hormonal dependence, and differences therein, in HGSOC and LGSOC.

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"We all need somebody to lean on"

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85

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86

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88

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106

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110

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