

DOUTORAMENTO
PATOLOGIA E GENÉTICA MOLECULAR

Identification of somatic and germline variants in tumor and circulating cell-free DNA samples of ovarian cancer patients

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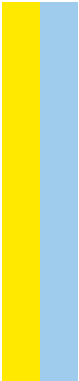


Ana Barbosa. Identification of somatic and germline variants in tumor and circulating cell-free DNA of ovarian cancer patients



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Aos meus Pais

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SUMMARY

Ovarian cancer (OC) is relatively rare but has poor prognosis. The high mortality rate is attributed to the lack of specific symptoms in the initial stages of the disease and of effective screening approach to detect the disease early. The 5-year survival of OC patients is strongly associated with the stage of the disease at diagnosis. The efforts to improve the outcomes of OC are focused on the development of a reliable biomarker to detect the disease in earlier stages, which is the key to reduction OC deaths, as well as to allow noninvasive predictive and prognostic genetic testing to improve therapy.

Circulating cell-free DNA (cfDNA) consists of small fragments of DNA released by the cells and that circulates freely in the bloodstream. In cancer patients, a fraction of cfDNA is derived from tumor cells, therefore containing the same genetic alterations, and it is called circulating tumor DNA (ctDNA). The use of ctDNA analysis, the so-called “liquid biopsy”, has recently received a lot of attention due to several features that make it a promising strategy. First, ctDNA provides a better representation of tumor genome than formalin-fixed paraffin-embedded (FFPE) tumor samples, as it comprises DNA released by tumor cells from primary tumor and metastases, minimizing the problem of inter-tumor and intra-tumor heterogeneity. Second, collection of blood samples is a minimally invasive procedure, allowing repeated sampling and “real-time” monitoring during the entire course of the disease. Liquid biopsies in OC have several potential applications, such as diagnosis, prognosis, evaluation of therapy response, monitoring the emergence of resistance to therapy and detection of disease relapse (reviewed in PAPER I).

In this thesis, we aimed to explore the use of next generation sequencing (NGS) on FFPE and cfDNA samples as alternatives for genetic testing in OC patients to detect both germline and somatic variants, which could be used as biomarkers for management of OC patients. Specifically, the aims of this thesis were: a) to design a customized NGS gene panel that includes all known OC cancer predisposing genes for the detection of pathogenic germline or somatic variants in a single test using FFPE tumor samples; b) to evaluate if the above strategy would increase the yield of clinically actionable pathogenic germline variants; c) to characterize the pattern of somatic variants of OC by NGS of FFPE samples using a custom gene panel that includes the most frequently mutated genes in the various histological subtypes of the disease; d) to evaluate the sensitivity of NGS of cfDNA for the detection of somatic variants as compared with testing FFPE tumor samples.

In PAPER II we used NGS of a customized panel of 10 genes associated with OC inherited predisposition for the analysis of FFPE tumor samples from 96 OC patients. This strategy allowed the detection of both somatic and germline variants in a single test and the identification of 7.4% additional patients with pathogenic germline variants in genes predisposing for OC other than *BRCA1* and *BRCA2*, namely *RAD51C*, *RAD51D* and *MSH6*, representing 35% of all pathogenic germline variants.

In PAPER III we used NGS of a custom panel of 27 genes on tumor and matched plasma samples from 96 OC patients, which were combined in two groups (treatment naive and post-treatment). Overall, at least one somatic variant present in the FFPE tumor sample was also detected in the matched plasma sample in 35.6% of the patients, a percentage that increased to 69.6% of the treatment-naive patients and 83.3% of those with stage IV disease, clearly showing the potential of ctDNA NGS analysis as a noninvasive alternative to identify somatic variants in these patients, namely those that have predictive value for targeted therapy. In fact, of the two treatment-naive patients with somatic *BRCA1* variants identified in FFPE tumor samples, we could detect in ctDNA a *BRCA1* frameshift somatic variant that was present in the tumor sample with a VAF of 53%, but not the one that had a VAF of only 5.4%. We also showed that ctDNA analysis can overcome the problem of inter- and intratumor heterogeneity, as exemplified by one patient diagnosed with bilateral ovarian tumors in which different somatic variants from both tumors were detected in ctDNA. Interestingly, as these bilateral tumors shared a rare combination of two of the three variants identified in the ctDNA, we could conclude that the two morphologically different tumors were clonally related and not synchronous independent neoplasias. Moreover, in the post-treatment group of patients, those with somatic variants detected in the plasma sample collected after surgery had poor prognosis when compared with patients with no detectable somatic variants after surgery, highlighting the potential of ctDNA analysis for the identification of patients at higher risk of recurrence.

The main conclusions of this thesis are: a) the use of a 10 gene panel for routine OC NGS genetic testing on FFPE allowed us to identify 7.4% patients with pathogenic germline variants in genes other than *BRCA1* and *BRCA2*; b) the strategy of gene panel tumor testing enables the identification of clinically actionable germline variants in a significant proportion of OC patients, while at the same time testing for somatic variants in the same genes, which may be a valuable information in patients with advanced stage disease that have run out approved therapeutic options, for the allocation into clinical trials or for the use of off-label therapy; c) the use of gene panel increases the chance to make available genetic counseling, presymptomatic genetic testing, and gynecological cancer prophylaxis to female who turn out to be healthy carriers of pathogenic germline variants; d) somatic variants can be detected in ctDNA from OC patients, potentially being an alternative for OC genetic testing when there is no tumor sample available and biopsy is not viable; e) the ctDNA analysis is also promising to be used to early detect OC, which could be very useful in healthy female relatives carriers of pathogenic germline variants in genes associated with inherited predisposition to OC; f) the ctDNA represents the mutational profiles from different tumor locations, overcoming the problem of inter-tumor and intra-tumor heterogeneity when performing genetic testing on FFPE tumor samples; g) the ctDNA analysis of plasma

samples collected after surgery can help to detect minimal residual disease after surgery, which could potentially be a prognostic biomarker identifying patients at higher risk of recurrence.

RESUMO

O cancro do ovário (ovarian cancer – OC) apesar de ser relativamente raro tem associada uma elevada taxa de mortalidade, que é atribuída à ausência de sintomas específicos nos estadios iniciais da doença e ao facto de não existirem métodos de rastreios eficazes que permitam diagnosticar precocemente a doença. A sobrevivência aos cinco anos para os doentes com OC está associada ao estadio da doença ao diagnóstico, sendo alta nos casos com doença localizada, mas diminui significativamente nos casos diagnosticados com doença avançada. Os esforços para melhorar o mau prognóstico associado ao OC estão focados no desenvolvimento de um biomarcador eficaz que permita detetar precocemente o cancro do ovário, que é a estratégia chave para reduzir a taxa de mortalidade, bem como permitir o teste genético não invasivo preditivo e de prognóstico para melhorar o tratamento.

O DNA circulante (circulating cell-free DNA – cfDNA) consiste em pequenos fragmentos de DNA libertados pelas células e que circulam livremente na corrente sanguínea. Nos doentes com cancro, uma fração deste DNA circulante é derivado das células tumorais, desta forma contem as mesmas alterações genéticas, e é designado de DNA tumoral circulante (circulating tumor DNA – ctDNA). A análise do ctDNA, que se chama de “biópsia líquida”, tem recentemente recebido muita atenção devido a várias características que tornam esta estratégia promissora. Primeiro, o ctDNA permite uma melhor representação do genoma tumoral do que as amostras de tumor fixadas em parafina, uma vez que engloba DNA libertado por células tumorais do tumor primário e das metástases, minimizando o problema da heterogeneidade tumoral. Segundo, a colheita de amostras de sangue periférico é um procedimento minimamente invasivo o que permite efetuar várias colheitas ao longo do tempo para uma monitorização da doença em “tempo real”. O potencial das biópsias líquidas no OC tem recebido muita atenção e tem várias possíveis aplicações, tais como o diagnóstico, prognóstico, avaliação da resposta à terapia, monitorizar a emergência de mecanismos de resistência à terapia e detetar a recorrência da doença (revisto no Artigo I).

O objetivo desta tese foi explorar o uso da sequenciação de nova geração (next generation sequencing – NGS) para a análise de amostras de tumor incluído em parafina e de cfDNA como alternativa ao teste genético em doentes com OC para detetar variantes germinativas e somáticas, o que pode ser usado como biomarcador para a monitorização destes doentes. Os objetivos específicos desta tese são: a) desenvolver um painel de NGS customizado que inclua todos os genes conhecidos como associados a predisposição para OC para a deteção de variantes somáticas e germinativas patogénicas num único teste usando amostras de tumor incluído em parafina; b) avaliar se esta estratégia aumenta a taxa de deteção de variantes patogénicas germinativas clinicamente acionáveis; c) caracterizar o padrão de variantes somáticas de OC através de análise por NGS de

amostras de tumor incluído em parafina usando um painel de genes que incluía os genes mais frequentemente mutados nos vários subtipos histológicos; d) avaliar a sensibilidade da técnica de NGS para análise de cfDNA para a detecção de variantes somáticas comparando com as análises das amostras de tumor.

No artigo II, usando um painel de NGS customizado contendo 10 genes associados a predisposição hereditária para o OC para analisar amostras de tumor incluído em parafina, foi possível identificar mais 7.4% de doentes com variantes patogénicas germinativas em outros genes além dos *BRCA1* e *BRCA2*, nomeadamente *RAD51C*, *RAD51D* e *MSH6*, representando 35% de todas as variantes patogénicas germinativas identificadas.

No artigo III, foi usado um painel customizado contendo 27 genes para analisar amostras de tumor e plasma de 96 doentes com OC, os quais foram divididos em dois grupos (ao diagnóstico e após tratamento). No global, pelo menos uma variante somática identificada na amostra de tumor foi também detetada na amostra de plasma correspondente em 35.6% dos doentes. Esta percentagem aumentou para 69.6% nos doentes ao diagnóstico e 83.3% destes doentes diagnosticados com estadió IV, demonstrando o potencial de análise de NGS em ctDNA como uma alternativa não invasiva para identificar variantes somáticas, nomeadamente as que têm valor preditivo para terapia dirigida. De facto, em dois doentes do grupo ao diagnóstico nos quais foram identificadas no tumor variantes somáticas no gene *BRCA1*, detetamos no ctDNA a variante somática frameshift que estava presente na amostra tumoral com uma frequência alélica de 53%, no entanto, não detetamos a variante com uma frequência alélica de 5.4%. Neste trabalho, também demonstramos que a análise do ctDNA pode ultrapassar o problema da heterogeneidade inter-tumoral e intra-tumoral, como exemplificado numa doente que tinha sido diagnosticada com tumores de ovário bilaterais foram detetadas três variantes no ctDNA, duas das quais partilhadas pelos dois tumores e uma exclusiva do tumor do ovário direito. Ao observarmos que estes tumores partilhavam duas variantes permitiu-nos concluir que estes tumores não serão síncronos independentes, mas sim tumores clonalmente relacionados. Adicionalmente, no grupo de doentes após tratamento, aquelas com variantes somáticas detetadas na amostra de plasma em que a colheita foi realizada após cirurgia têm pior prognóstico quando comparado com aquelas doentes nas quais não foram detetadas variantes somáticas após a cirurgia, realçando o potencial de análise do ctDNA para a identificação de doentes com maior risco de recorrência.

As principais conclusões desta tese são: a) o uso de um painel de 10 genes de NGS para o teste genético de amostras de tumor permitiu identificar 7.4% de doentes com variantes patogénicas germinativas em outros genes além dos *BRCA1* e *BRCA2*; b) a estratégia do teste genético no tumor usando um painel de genes permite a identificação de variantes germinativas clinicamente acionáveis numa proporção significativa de doentes

com OC, enquanto se testa simultaneamente variantes somáticas nos mesmos genes, o que pode ser uma informação importante no caso das doentes com estadio avançado que tenham esgotada todas as opções terapêuticas aprovadas, podendo ser usada para alocar as doentes em ensaios clínicos ou para uso de terapia off-label; c) o uso de um painel de genes aumenta a possibilidade de oferecer aconselhamento genético, teste genético pré-sintomático, e medidas de profilaxia às mulheres que venham a ser identificadas como sendo portadoras de variantes germinativas patogénicas; d) variantes somáticas podem ser detetadas no ctDNA de doentes com OC, podendo ser uma potencial alternativa para o teste genético em doentes com OC quando não existe amostra de tumor e quando a biópsia não é viável; e) a análise do ctDNA é também promissora para a deteção precoce de OC, que pode ser muito útil em mulheres familiares saudáveis portadoras de variantes germinativas patogénicas em genes associados a predisposição hereditária para OC; f) o ctDNA representa o perfil mutacional de diferentes locais do tumor, ultrapassando o problema da heterogeneidade inter-tumoral e intra-tumoral do teste genético realizado em amostras de tumor em parafina; g) a análise do ctDNA de amostras de plasma obtidas após a cirurgia pode ajudar na deteção de doença residual mínima após a cirurgia, o que pode potencialmente ser um biomarcador de prognóstico, identificando as doentes com um maior risco de recorrência.

PUBLICATIONS

Ao abrigo do nº2, alínea a, do artigo 31º do Decreto-Lei n.º 230/2009 de 14 de setembro, fazem parte integrante desta tese de doutoramento os seguintes manuscritos aceites para publicação:

PAPER I

Potential clinical applications of circulating cell-free DNA in ovarian cancer patients

Ana Barbosa, Ana Peixoto, Pedro Pinto, Manuela Pinheiro and Manuel R. Teixeira

Published in Expert Reviews in Molecular Medicine

PAPER II

Gene panel tumor testing in ovarian cancer patients significantly increases the yield of clinically actionable germline variants beyond *BRCA1/BRCA2*

Ana Barbosa, Pedro Pinto, Ana Peixoto, Joana Guerra, Carla Pinto, Catarina Santos, Manuela Pinheiro, Carla Escudeiro, Carla Bartosch, João Silva and Manuel R. Teixeira

Published in Cancers

PAPER III

Next generation sequencing of tumor and matched plasma samples: identification of somatic variants in ctDNA from ovarian cancer patients

Ana Barbosa, Pedro Pinto, Ana Peixoto, Joana Guerra, Manuela Pinheiro, Catarina Santos, Carla Pinto, Carla Escudeiro, Carla Bartosch, Rui Santos, Andreia Brandão, João Silva, Manuel R. Teixeira

Submitted to Frontiers in Oncology

LIST OF ABBREVIATIONS

BOT	Borderline ovarian tumor
BSO	Bilateral salpingo-oophorectomy
CA125	Cancer antigen 125
CCC	Clear cell carcinoma
CNA	Copy number alterations
CT	Computed tomography
ddPCR	Droplet digital PCR
DSB	Double strand breaks
EC	Endometrioid carcinoma
EOC	Epithelial ovarian carcinoma
EMA	European Medicines Agency
FA	Fanconi anemia
FIGO	International Federation of Gynecology and Obstetrics
HBOC	Hereditary breast ovarian cancer syndrome
HE4	Human Epididymis Protein 4
HGSOC	High-grade serous ovarian carcinoma
HR	Homologous recombination
LOC	Lifetime ovulatory cycles
LS	Lynch syndrome
LGSOC	Low-grade serous ovarian carcinoma
MBT	Mucinous borderline tumor
MC	Mucinous carcinoma
MMR	Mismatch repair
MRI	Magnetic resonance imaging
MSI	Microsatellite instability
NGS	Next-generation sequencing
NCCN	National Comprehensive Cancer Network
NHEJ	Non-homologous end-joining
OC	Ovarian Cancer
PARP	Poly-(ADP-ribose) polymerase
PARPi	Poly-(ADP-ribose) polymerase inhibitor
PDS	Primary debulking surgery
PET	Positron emission tomography
PFI	Platinum-free interval
PFS	Progression free survival
ROMA	Risk of Ovarian Malignancy Algorithm
RRSO	Risk-reducing salpingo-oophorectomy

LIST OF ABBREVIATIONS

SBT	Serous borderline tumor
UMI	Unique molecular identifier
WHO	World Health Organization

INTRODUCTION

1. Cancer Epidemiology

Cancer comprehends more than 100 distinct diseases with a diversity of risk factors and arises as a consequence of changes in the genome of cancer cells, which leads to its uncontrolled proliferation and invasion across normal tissue boundaries, as well as to the capacity to metastasize to distant organs. Worldwide, there were 18.1 million cases and 9.6 million cancer deaths estimated in 2018 [1]. According to the World Health Organization (WHO), cancer is the second leading cause of death globally, right after ischemic heart diseases, being responsible for about one in six deaths worldwide [2]. In 2016, in terms of premature mortality, cancer was the leading cause of death in 55 countries and the second in 79 countries [3]. Cancer incidence is rapidly growing worldwide mainly due to the growth and aging of global population, as well as to socioeconomic development that is associated with many risk factors [4]. The global cancer burden is predicted to increase by about 60% from 18.1 million in 2018 to 29.4 million cases in 2040, based on population aging and growth [3]. Access to early detection strategies and more effective therapies can substantially improve the survival of cancer patients. In fact, the number of cancer survivors is rising worldwide. In 2018, there were about 43.8 million cancer survivors diagnosed within the preceding five years [3]. Technological advances have allowed to increase the knowledge about cancer biology. Consequently, cancers can be classified according to their molecular features and this stratification may reveal risk factors. The identification of somatic mutations in tumor genomes can provide new clues to cancer etiology. The development of new biomarkers can also have impact in reducing cancer burden since they can improve early detection and the identification of individuals at higher risk to develop cancer for screening or surveillance [5].

1.1 Ovarian cancer epidemiology

Ovarian cancer (OC) is the eight most common cancer and the seventh cause of death from cancer among women worldwide, with an estimated 295,414 new cases and 184,799 cancer deaths in 2018 (Figure 1a). In Portugal, OC accounts for 3.4% of all cancer among women and 5.2% of cancer deaths [1]. OC incidence and mortality rates vary geographically according to the level of development, being higher in more economically developed regions, with highest estimated rates being observed in Australia, New Zealand, Central and Eastern Europe and North America and lowest in less economically developed regions such as Africa and South Central Asia (Figure 1b) [1]. This heterogeneous pattern of worldwide incidence distribution may be related with differential exposure to risk and protective factors that are highly related with cultural habits and lifestyle practices, as well as with the inconsistency of cancer registration among countries.

INTRODUCTION

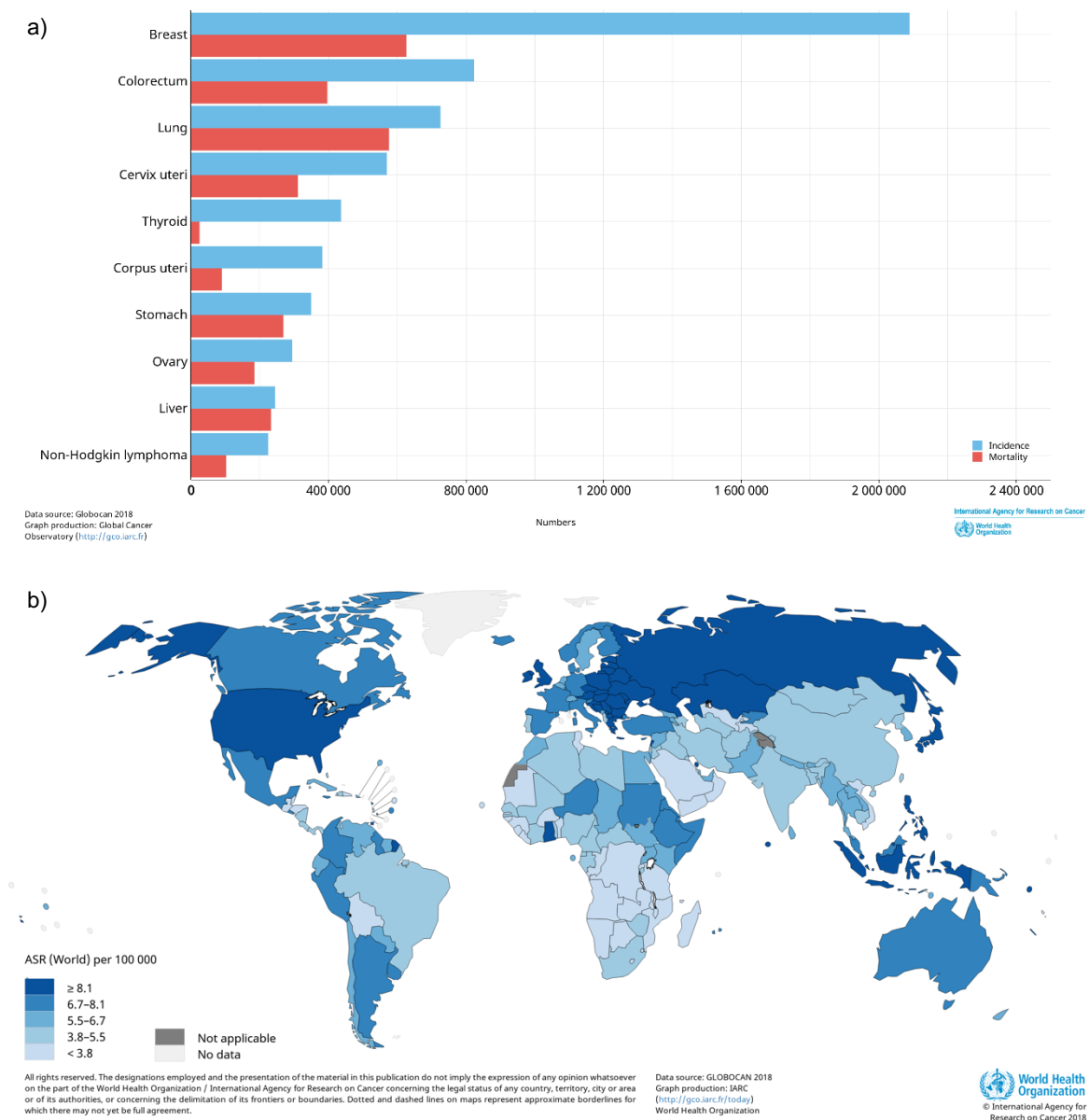


Figure 1: Estimated number of incident cases and death worldwide, females, all ages (a) and estimated age-standardized incidence rates (world) in 2018, ovary, all ages (b).

Although OC is a relatively rare cancer, it has the highest mortality rate among gynecological malignancies. The main factor that contributes to the high mortality of OC is the advanced stage of disease at diagnosis, largely due to the lack of specific symptoms and effective early detection strategies. The overall survival for OC patients is about 49.1%, but varies with the disease stage at diagnosis. The 5-year survival in patients diagnosed with localized disease is 92.6%, but decreases to 74.8% and 30.3% in patients diagnosed with regional and distant disease, respectively [6].

1.2 Risk factors for ovarian cancer

Several factors have been associated with an increased risk of developing OC, such as obesity, use of postmenopausal hormonal therapy and talcum powder use, but none of these has a strong correlation like genetic factors and reproductive history. Epidemiological studies showed that OC risk is associated with age progression, genetic predisposition, late age of menopause, nulliparity and postmenopausal hormone replacement therapy. On the other hand, the use of oral contraceptives, parity, breastfeeding, prophylactic oophorectomy and bilateral tubal ligation are reported as decreasing the risk of developing OC (Table 1) [7].

Table 1: Risk factors for ovarian cancer

Increased Risk
<i>Factors inherent to patient</i>
- Increasing age - Personal history of breast cancer
<i>Genetic factors</i>
- Family history of ovarian cancer - Hereditary breast and ovarian cancer syndrome - Lynch syndrome - Pathogenic germline variants in other genes predisposing to OC
<i>Reproductive factors</i>
- Nulliparity - Early menarche - Late menopause
Decreased Risk
<i>Reproductive factors</i>
- Use of oral contraceptives - Pregnancy - Breastfeeding
<i>Surgical interventions</i>
- Salpingo-oophorectomy - Tubal ligation

1.2.1 Genetic risk factors

Among all the risk factors that are associated with increased risk of developing OC, the most important are the genetic factors and several genes predisposing to OC have been

identified (Table 2). About 23% of OC have been related with hereditary conditions and approximately 65-85% of hereditary OC are associated with pathogenic germline variants in the *BRCA1* or *BRCA2* genes [8]. Germline variants in the mismatch repair (MMR) genes account for 10-15% of all hereditary OC. Besides the *BRCA1*, *BRCA2* and MMR genes, other genes of moderate penetrance have been identified as increasing the risk of developing OC, such as *RAD51C*, *RAD51D*, *BRIP1*, *ATM* and *PALB2* [9-11].

The most significant known genetic risk factor for OC is the inheritance of deleterious variants in *BRCA1* and *BRCA2*, which is the cause of the hereditary breast and ovarian cancer (HBOC) syndrome, an autosomal dominant inherited predisposition to breast and OC. *BRCA1* is a tumor suppressor gene that encodes a protein involved in the repair of double strand breaks (DSB) through the homologous recombination (HR) repair pathway, cell cycle checkpoint control and maintenance of genomic stability. *BRCA1* is part of a multi-subunit protein complex, called *BRCA1*-associated genome surveillance complex (BRASC) and acts with other tumor suppressors, DNA damage sensors and signal transducers. *BRCA2* encodes a protein that is also involved in repairing DNA damage through HR mechanism, mediating the recruitment of *RAD51* to the DSB through the formation of a *BRCA1*-*PALB2*-*BRCA2* complex [12, 13]. Pathogenic germline variants in *BRCA1* or *BRCA2* are found in 13% to 18% of OC patients and are associated with a higher risk of developing OC [8, 14-16]. When compared with women in the general population, for whom the lifetime risk to develop OC is 1.6% [17], the cumulative risk of developing OC at age 70 is 44% and 17% for *BRCA1* and *BRCA2* pathogenic variants carriers, respectively [18].

Lynch syndrome (LS), is an autosomal dominant cancer-susceptibility disorder characterized by predisposition to a spectrum of cancers, such as stomach, ovary, pancreas, upper urologic tract and brain tumors, in addition to the most prevalent colorectal and endometrial cancer [19]. This syndrome is characterized by inheritance of inactivating mutations in MMR genes, such as *MLH1*, *MSH2*, *MSH6* and *PMS2*, or large deletions of the *EPCAM* gene that is located upstream of the *MSH2* gene. These genetic alterations impair the ability of the MMR system to recognise and repair DNA mismatches, leading to microsatellite instability (MSI) and an increased risk of developing cancer [20-22]. Pathogenic germline variants in MMR genes are found in less than 1% of unselected OC cases [23]. *MSH2* and *MLH1* account for 80-90% of the cases [23], occurring at frequencies of 0.1-0.8% and 0.05-0.1%, respectively. *MSH6* germline variants are found in 0.2-0.3% of the cases, and *PMS2* in 0.2% [23-25]. The cumulative lifetime risk for OC associated with LS is dependent of the affected MMR gene. The cumulative risk for OC at age 80 is 4-20% for *MLH1*, 8-38% for *MLH2*, 1-13% for *MSH6* and 3.0% for *PMS2* [26, 27].

RAD51C and *RAD51D* are members of the *RAD51* family, which are proteins involved in the repair of DNA DSBs through HR, acting in the creation of HR complexes with other

members of the RAD51 family [28]. Studies on the prevalence of germline variants in these genes in OC vary widely, ranging between 0.57% and 2.5% for *RAD51C* and 0.16% and 0.57% for *RAD51D*, among patients unselected for age and family history [8, 24, 29-31]. The cumulative risk of OC by age 80 years was estimated to be 11% and 13% for *RAD51C* and *RAD51D* pathogenic variant carriers, respectively [32].

The *BRIP1* gene encodes a DNA helicase that binds directly to the BRCT repeats of BRCA1, having an important role in the repair mechanism of DNA DSBs [33]. Deleterious *BRIP1* germline variants occur in frequencies ranging between 0.2% and 1.4% among OC patients [8, 24, 30, 34] and women carriers of pathogenic germline variants have a cumulative OC risk of 5.8% by age 80 years [34].

The *PALB2* gene plays an important role in maintaining genome integrity through its role in the HR pathway. PALB2 binds to BRCA2 promoting its localization and stability in nuclear structures, being important for its recombination repair functions and recruitment of RAD51 to DSB sites [35]. *PALB2* pathogenic germline variants were found in 0.2-0.6% of OC patients [8, 24, 36] and the estimated risk for the development of OC was 4.8% by age of 80 years [37].

The *ATM* gene encodes a protein kinase that responds to damages in DNA by phosphorylating key proteins involved in DNA repair. ATM is involved in early recognition of DNA DSBs, and also in the coordination of cell-signaling pathways, response to oxidative stress, mitochondrial function and apoptosis [38]. *ATM* pathogenic germline variants were found in 0.6% of OC patients [39] and are associated with an increased risk for OC, with an estimated relative risk of 2.85 [40].

Table 2: Genes associated with increased risk for ovarian cancer.

Gene	Prevalence (%)	Evidence for increased risk	Absolute risk (%)	References
<i>ATM</i>	0.6	Strong	2.85	[39-41]
<i>BRCA1</i>	13	Very strong	44	[8, 14-16, 18, 41]
<i>BRCA2</i>	18	Very strong	17	[8, 14-16, 18, 41]
<i>BRIP1</i>	0.2-1.6	Strong	5.8	[8, 24, 30, 34, 41]
<i>EPCAM</i>	-----	Limited	-----	[41]
<i>MLH1</i>	0.05-0.1	Strong	4-20	[23, 25-27, 41]
<i>MSH2</i>	0.1-0.8	Strong	8-38	[23, 25-27, 41]
<i>MSH6</i>	0.2-0.3	Strong	1-13	[23, 25-27, 41]
<i>PALB2</i>	0.2-0.6	Strong	4.8	[8, 24, 36, 37, 41]
<i>PMS2</i>	0.2	Limited	3	[23, 25-27, 41]
<i>RAD51C</i>	0.57-2.5	Strong	11	[8, 24, 29-32, 41]
<i>RAD51D</i>	0.16-0.57	Strong	13	[8, 24, 29-32, 41]

1.2.2 Postmenopausal hormone replacement therapy

Hormone replacement therapy is widely used for the relief of postmenopausal symptoms. However, several studies showed that the use of postmenopausal hormone therapy is associated with an increased risk of developing OC in postmenopausal women [42, 43]. Compared with women who never used hormone therapy, the relative risk range was 1.19-1.69 for current users. Furthermore, the risk for OC increases with long-term use of hormone therapy with an estimated relative risk about 1.45-1.51 and 2.15 among women who had used hormone therapy for 5 years and 10 years, respectively, compared with never-users [44-46].

1.2.3 Reproductive factors

The ovulation process is crucial for the development of epithelial subsurface inclusion cysts from which a great proportion of OC can develop. Incessant ovulation has been associated with OC development and there is an association between the number of lifetime ovulatory cycles (LOC) and the risk of OC. Several hypotheses have been proposed regarding the contribution of ovulation in the OC carcinogenesis, including the acute pro-inflammatory environment (cytokines, reactive oxygen species created and steroids) that follows ovulation which creates a DNA damage-rich environment and the exposure to steroid hormones and direct damage caused by ovulation on the ovarian surface epithelium [47]. A large prospective study observed that the risk of OC increases with the increase in the number of LOC. Women in the 90th percentile of LOC were almost twice as likely to be diagnosed with OC when compared to women in the 10th percentile. Furthermore, a 14% increase in OC per five-year increase in LOC was observed [48]. Thus, epidemiologic factors that increase the number of ovulatory cycles, such as early age of menarche, late age of menopause and nulliparity, are associated with an increased risk of developing OC. On the other hand, factors that interrupt ovulation, such as pregnancy, breast feeding and use of oral contraceptives, are associated with a reduction of OC risk [49]. The use of oral contraceptives has been shown to reduce the risk of developing OC by 40% to 56% and the benefit of risk reduction is greater in long-term users [50].

2. Ovarian cancer histopathology

There is a wide variety of benign and malignant ovarian tumors. However, there are three fundamental groups: epithelial, stromal and germ cell. Malignant germ cell tumors are rare, accounting for about 3% of OC, and occur mainly in children and young women. Less common are the potentially malignant sex cord-stromal tumors, which account for about 1-2% of OC. Malignant stromal tumors are also rare and can occur at all ages. Malignant

epithelial ovarian carcinomas (EOC) are the most common OC, accounting for 90% of the cases [51]. EOC can be further subclassified in various histological subtypes that can be divided in two groups, Type I and Type II, according to the dualistic model of epithelial ovarian carcinogenesis. The dualistic model was proposed based on morphological and genetic studies in order to unravel the molecular pathways involved in the carcinogenesis of OC and its association with histopathological classification [52]. Type I OC constitutes a minority of epithelial lesions and includes low-grade serous carcinoma (LGSOC), endometrioid carcinoma (EC), clear cell carcinoma (CCC) and mucinous carcinoma (MC). The carcinogenic processes of type I OC are assumed to result from endometriosis, inflammation and incessant ovulation [53-55]. Typically, this type of OC tends to have a clinically indolent behavior, is associated with precursor lesions, is usually diagnosed at an early stage, and has a good prognosis. Type II tumors account for the majority of EOC and include the most common high-grade serous carcinomas (HGSOC), carcinosarcomas, and undifferentiated carcinomas. This type of tumors develop rapidly, are highly aggressive, and frequently present at advanced stages. Precursor lesions have not been clearly described and this type of tumors may develop *de novo* from the surface epithelium of ovary and/or tubal [52, 56, 57]. The main molecular feature that differentiates type I from type II tumours is the relative genetic stability of type I tumors in comparison with the high chromosomal instability observed in type II tumors. Furthermore, type II tumours are characterized by mutations in *TP53*, which are rare in type I tumors [57]. Although once considered as a single entity, OC is a highly heterogeneous disease that can be subdivided into distinct histological subtypes having different epidemiologic features, risk factors, precursor lesions, clinical presentation, molecular patterns, and outcomes (Table 3) [51]. According to the 2014 World Health Organization (WHO) diagnostic criteria, EOC are classified into five main histological subtypes: HGSOC (70%), EC (10%), CCC (10%), MC (3%), and LGSOC (<5%) [58, 59].

Table 3: Molecular characterization and precursor lesions of EOC histological subtypes.

Histological subtypes	Precursor lesions	Molecular features
High-grade serous	STIC	<i>TP53, BRCA1, BRCA2</i>
Low-grade serous	Serous borderline tumors	<i>BRAF, KRAS</i>
Mucinous	Cystadenoma	<i>KRAS, CDKN2A, PIK3CA, TP53</i>
Endometrioid	Endometriosis	<i>ARID1A, CTNNB1, PTEN</i>
Clear cell	Endometriosis	<i>ARID1A, PIK3CA, PTEN loss</i>

STIC, serous tubal intraepithelial carcinoma

2.1 High-grade serous carcinomas

HGSOC is the most common EOC histological subtype, accounting for over 70% of all EOCs [60]. Approximately 80% of HGSOC patients are diagnosed with advanced stage disease. This histological subtype is characterized by acquired or inherited mutations in genes involved in different DNA repair pathways. *TP53* is the most commonly mutated gene, occurring in approximately 96% of the HGSOC [61]. Most HGSOC are sporadic and approximately 18% are attributable to germline variants in *BRCA1* and *BRCA2*. Taking together germline and somatic variants, *BRCA1* and *BRCA2* variants occur in about 23% of HGSOC [14]. Additional genes were found to be mutated, but only in 2-6% of HGSOC. Genomic analysis have shown that about 50% of HGSOC have defects in HR repair, due to both germline and somatic mutations in *BRCA1* and *BRCA2*, epigenetic inactivation of *BRCA1*, as well loss-of-function mutations in other genes involved in HR pathway [61]. The combination of *TP53* mutations with *BRCA1/BRCA2* inactivation results in chromosomal instability and consequently in the accumulation of copy number alterations (CNA), a frequent feature of HGSOC [61].

The fallopian tube has been identified as the site of origin of HGSOC due to the identification of atypical tubal epithelial cells in a great proportion of OC patients. During the examination of tissues removed from risk-reduction salpingo-oophorectomy (RRSO), in carriers of *BRCA1/BRCA2* pathogenic germline variants, the presence of lesions in the fallopian tube was observed, named serous tubal intraepithelial carcinoma (STIC), characterized by DNA damage and *TP53* mutations [62]. The evolutionary analysis revealed that somatic *TP53* mutations detected in STICs were also observed in HGSOC located in the fimbrial ends of fallopian tube, thus concluding that STICs are precursor lesions of HGSOC [63].

2.2 Low-grade serous carcinomas

LGSOC is a relatively uncommon histological subtype of OC, representing less than 5% of all EOC [60, 64]. On average, women with LGSOC are diagnosed at a younger age when compared with women diagnosed with HGSOC [65]. LGSOC patients have better prognosis when compared with HGSOC, mainly due to the relative slow growth and the higher possibility of achieving no residual disease at primary surgery. However, these tumors are typically more unresponsive to chemotherapy compared with HGSOC and approximately 70% of the patients will die from the disease mainly due to the chemo-resistance nature of these carcinomas [66]. The response rate to chemotherapy is less than 5%, which is in contrast to the 80% response rate to primary chemotherapy in patients with HGSOC [67]. Approximately 6-7% of LGSOC have its origin on the progression of serous

borderline tumors (SBT), which are considered a precursor lesion of LGSOC and are histologically intermediate between cystadenoma and adenocarcinoma, by presenting cellular atypia but with absence of stromal invasion [68, 69]. Although little is known about the molecular drivers, LGSOC are characterized by the activation of the mitogen-activated protein kinase (MAPK) pathway, observed in up to 80% of the LGSOC, due to mutations in the *KRAS* or *BRAF* genes that encode proteins that are upstream regulators of this pathway [70]. LGSOC are characterized by mutually exclusive mutations in *KRAS* or *BRAF* at frequencies of 33% and 54%, respectively [71]. In contrast with HGSOC, *TP53* mutations and CNA are rare in LGSOC, which also seems to not be associated with *BRCA1/BRCA1* mutations.

2.3 Mucinous carcinomas

MC is the rarest of the five EOC histologic subtypes, representing 3-4% of all EOC [60]. Most of the patients are diagnosed with early-stage disease and have a good prognosis after primary surgery, with a 90% 5-year survival. However, cases with extraovarian spread have poor prognosis [72, 73]. It is believed that MC arises from benign cysts that develop to borderline tumors and then to invasive carcinomas [60]. The rarity of MC has limited its characterization at a molecular level. It is known that *KRAS* mutations are an early event in MC carcinogenesis, being the most common molecular alteration, found in about 44% of MC. *KRAS* mutations were also observed in mucinous borderline tumors (MBT), supporting the process of carcinogenesis from low-malignant mucinous lesions [74]. MC are also characterized by *ERBB2* amplification which occurs in a frequency of 19%. *KRAS* mutations and *ERBB2* amplification are nearly mutually exclusive events co-occurring only in 6% of all MC [74]. Mutations in *TP53*, *CDKN2A*, *PIK3CA* are also recurrent, occurring in 57%, 19% and 14% of MC, respectively [75].

2.4 Endometrioid carcinomas

EC account for approximately 10% of all EOC, are more frequent in perimenopausal or postmenopausal women and most of the tumors are diagnosed at an early stage and tend to have a better prognosis when compared to other histological subtypes [60]. EC is thought to arise from pelvic endometriosis, which is a well-established precursor lesion [76]. EC is commonly diagnosed with synchronous endometrial tumors [77] and is frequently observed in patients with LS [78]. About 11% of EC have loss of expression of MMR genes, characteristic of LS [79]. The molecular profiling of EC is characterized by prevalent mutations in *ARID1A*, *CTNNB1*, and *PTEN*, which are found mutated in 30%, 38-50%, and

20% of EC, respectively [80, 81]. EC are also characterized by microsatellite instability, occurring in about 12% to 19% of the cases [81].

2.5 Clear cell carcinomas

CCC represent approximately 10% of all OC, are usually diagnosed at early stages (stage I-II), and have a poor sensitivity to chemotherapy. Similarly to EC, there is a strong epidemiological association with pelvic endometriosis [76] that, together with atypical adenofibroma of the ovary, are considered precursor lesions of CCC [76, 82]. CCC are characterized by mutations in *ARID1A*, *PIK3CA*, and *PTEN*. *ARID1A* is the most frequently mutated gene, being detected in 40% to 57% of CCC [83-85]. There is a speculation that *ARID1A* mutations play a role in the neoplastic transformation of endometriosis into CCC, as identical *ARID1A* mutations were observed in CCC and in contiguous endometriotic lesions [84]. Several studies reported that a great proportion (58% to 71%) of CCC with *ARID1A* mutations also carried activating *PIK3CA* mutations [83, 86]. Activating mutations in *PIK3CA* and loss of *PTEN* occur in about 35% and 37% of CCC, respectively [87, 88].

3. Ovarian cancer screening and diagnosis

3.1 Clinical presentation

Most women with OC are diagnosed late in life, with a median age of diagnosis of 63 years [6]. OC may not cause early signs or symptoms leading to delays in diagnosis. Furthermore, initial OC symptoms include abdominal discomfort, abdominal bloating, fatigue, and gastrointestinal dysfunction, which are not specific and can overlap with symptoms of more common gastrointestinal and gynecological conditions [89]. Consequently, the use of symptoms to trigger medical evaluation for OC is likely to result in a diagnosis of the disease in one of 100 women in the general population [90]. Thus, OC diagnosis often occurs at advanced stages, when the symptoms are more severe and indicative, such as peritoneal carcinomatosis. This is the reason why OC has been named the “silent killer”, because it spreads without the manifestation of symptoms. Unlike cancers in many other sites, for the ovary there is no anatomical barrier preventing the spread of tumor cells into the peritoneal cavity. Thus, in advanced stages of disease, patients often manifest symptoms associated with ascites and abdominal distension, anorexia and shortness of breath due to pleural effusion [89]. Despite the efforts to develop a reliable screening approach for OC, only about 20% of OC are diagnosed in early stages, when the tumor is still limited to the ovaries and 90% of these patients can be cured with standard treatments [91].

3.2 Ovarian cancer screening

Despite the efforts that have been made to implement screening strategies, there is currently no validated population-based screening for OC. Given the prevalence of OC, approaches for early detection should have a sensitivity and specificity above 75% for early-stage disease, in order to achieve a positive predictive value of at least 10% [91]. Several studies have been evaluating the combination of cancer antigen 125 (CA125) and ultrasound for use as screening approach in the general population. The prostate, lung, colorectal and ovarian (PLOC) randomized clinical trial enrolled 78.216 women to evaluate the use of CA125 and transvaginal ultrasound as a screening tool for OC and no reduction in overall OC mortality was observed [92]. Results from the large UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS), which evaluated the effect of CA125 levels for OC screening followed by transvaginal ultrasound as secondary screening approach, showed no significant mortality reduction [93].

3.3 Ovarian cancer diagnosis

In symptomatic and/or high-risk women, physical examination, transvaginal ultrasound and biomarker CA125 blood test have been widely used as primary assessment for OC [94, 95]. However, none of these methods are sensible and specific enough for this type of cancer. The use of CA125 in early detection is questionable when used alone, because increased levels are found in only 50-60% of patients with early stage ovarian cancer and can also appear in other types of cancer, as well as in benign diseases [96], being therefore an unspecific marker for OC. Transvaginal ultrasound provides information regarding tumor size, complexity and location, which can help to determine the likelihood for cancer, but it also detects benign ovarian lesions [97]. The definitive diagnosis requires a tumor biopsy for histopathologic evaluation. However, ovarian biopsy is difficult to be performed due to anatomical limitations and has been discouraged due to the risk of spillage [98]. Thus, in the presence of elevated levels of CA125 and ovarian masses detected through ultrasound examination, surgery is usually necessary for diagnosis and disease staging, as well as for the initial treatment [99]. This screening strategy of ovarian cancer based on the combination of CA125 biomarker levels and imaging has therefore limited sensitivity and results in excessive and unnecessary surgeries [100].

3.4 Ovarian cancer staging

The staging system allows to assign patients into prognosis groups for which there are established treatments and helps in the exchange of information through standard terminology. OC staging requires surgery to assess the cancer extension at diagnosis and

uses the 2014 International Federation of Gynecology and Obstetrics (FIGO) staging classification (Table 4). Stage I defines tumors confined to the ovaries or fallopian tubes and peritoneal washings. Stage II OC is defined as extension to other pelvic organs, including tumors that have directly extended to adjacent organs but have not yet metastasized. Stage III includes tumors that had spread into peritoneal surfaces involving both pelvic and abdominal peritoneum. Finally, stage IV disease includes all OC with distant metastases, excluding peritoneal metastases [101].

Table 4: International Federation of Obstetrics and Gynecology Ovarian, Fallopian Tube and Peritoneal Cancer Staging System (2014).

Stage I. Tumor limited to ovaries or fallopian tube(s)
IA: Tumor limited to one ovary or fallopian tube, no tumor on ovarian or fallopian tube surface and no malignant cells in the ascites or peritoneal washings
IB: Tumor limited to both ovaries or fallopian tubes
IC: Tumor limited to one or both ovaries or fallopian tubes and with one of the following substages:
IC1: Surgical spill
IC2: Capsule ruptured before surgery or tumor on ovarian or fallopian tube surface
IC3: Malignant cells in the ascites or peritoneal washings
Stage II. Tumor involves one or both ovaries or fallopian tubes with pelvic extension or primary peritoneal cancer
IIA: Extension and/or implants on uterus and/or fallopian tubes and/or ovaries
IIB: Extension to other pelvic intraperitoneal tissues
Stage III. Tumor involves one or both ovaries or fallopian tubes, or primary peritoneal cancer with cytologically or histologically confirmed spread to the peritoneum outside the pelvis and/ or metastasis to the retroperitoneal lymph nodes
IIIA1: Positive retroperitoneal lymph nodes only
IIIA1(i): Metastasis up to 10 mm in greatest dimension
IIIA1 (ii): Metastasis more than 10 mm in greatest dimension
IIIA2: Microscopic extrapelvic peritoneal involvement with or without positive retroperitoneal lymph nodes
IIIB: Macroscopic peritoneal metastasis beyond the pelvis up to 2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes
IIIC: Macroscopic peritoneal metastases beyond the pelvis more than 2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes (includes extension of tumor to capsule of liver and spleen without parenchymal involvement of either organ)
Stage IV. Distant metastasis excluding peritoneal metastases
IV: Distant metastasis excluding peritoneal metastases
IVA: Pleural effusion with positive cytology
IVB: Parenchymal metastases to extra-abdominal organs (including inguinal lymph nodes and lymph nodes outside of the abdominal cavity)

4. Ovarian cancer treatment

The treatment of OC has evolved from a “one-size-fits-all” approach, becoming more precise regarding the use of surgery, systemic and targeted therapy. The management of OC patients will continue to evolve as new predictive biomarkers are discovered helping to select patients most likely to respond and benefit from specific therapies [102]. Currently, the first-line treatment of newly diagnosed OC relies on primary tumor debulking surgery to

no residual disease followed by treatment with chemotherapy, or chemotherapy administered before surgery followed by interval debulking surgery and additional cycles of chemotherapy [103].

The initial treatment recommendation for newly diagnosed OC is surgical cytoreduction. The main goal of surgery is to achieve the maximal resection of the disease. The results from the surgery are referred as suboptimal when at the end of the surgery there are disease focus above 1cm, optimal when there is residual disease (<1cm), or complete resection when there is no evidence of residual disease at the end of the surgery. “Optimal” cytoreduction has been associated with better outcomes, being the most important prognostic factor for survival in OC patients [104]. For patients who are diagnosed with disease confined to the pelvis, total hysterectomy (I-IIA) and bilateral salpingo-oophorectomy (BSO) is recommended. Adjuvant chemotherapy for these OC stages is controversial, however six cycles of single-agent carboplatin are recommended in patients with intermediate and high-risk disease (intermediate risk: IA and IB grade 2; high risk: IC all grades, IB and IC grade 2 and 3, clear cell histology). For advanced OC (stage IIb-IIIc) the standard initial treatment is cytoreductive surgery followed by platinum-based chemotherapy [105]. If maximal cytoreduction is not achieved an interval surgery is recommended in patients who respond to chemotherapy or with stable disease [104]. In patients who are not candidates for primary debulking surgery due to extensive disease burden that is unlikely to be optimally cytoreduced, or due to the clinical condition of the patient, neoadjuvant chemotherapy with interval debulking surgery should be considered [105].

After surgery, adjuvant chemotherapy is recommended. For early-stage OC patients the use of adjuvant chemotherapy depends on the disease stage and histology. Patients diagnosed with stage I are not treated with chemotherapy after surgery, but patients with stage II or above and patients with any stage of HGSOc and CCC undergo adjuvant systemic chemotherapy [103]. The most active therapeutic agents against OC are platinum analogues (cisplatin or carboplatin) combined with a taxane (paclitaxel or docetaxel) [106]. OC patients have good response to initial chemotherapy, with a response observed in about 80% of the patients, however, recurrence after first line platinum-taxane is very frequent.

After completing primary treatment with surgery and chemotherapy, for stage II-IV OC patients with stable disease or patients with complete response, maintenance therapy is recommended [103]. The main goal of maintenance therapy is to prolong the progression free survival (PFS), delaying subsequent disease progression and consequently improving the overall survival of OC patients. For stage II-IV disease patients with somatic or germline pathogenic variants in *BRCA1* or *BRCA2*, maintenance therapy with Poly-(ADP-ribose) polymerase inhibitors (PARPi) is an option [103], since its approval for first-line maintenance

therapy in BRCA mutated advanced EOC [107]. The SOLO-1 randomized clinical trial demonstrated a great improvement in PFS with Olaparib when compared with placebo as maintenance therapy for advanced OC patients with germline or somatic pathogenic variants in *BRCA1* or *BRCA2* who were in complete or partial response after first-line platinum-based chemotherapy. Furthermore, the risk of progression or death was 70% lower and the median PFS for Olaparib was not reached after a median follow-up of 41 months compared to 13.8 months for placebo [107]. PARP plays a crucial role in the repair of DNA single-strand breaks. The inhibition of this enzyme leads to the accumulation of single-strand breaks, as well as DSB which are normally repaired by HR repair. When there is a deficiency in the HR repair system, the accumulation of unrepaired DSB leads to cell death. This phenomenon of cell death in consequence to inactivated DNA repair pathways is called “synthetic lethality”, that is the mechanism underlying the efficacy of PARPi (Figure 2), occurring in cells with deficiency in HR repair, where *BRCA1/BRCA2* proteins play an essential role [108].

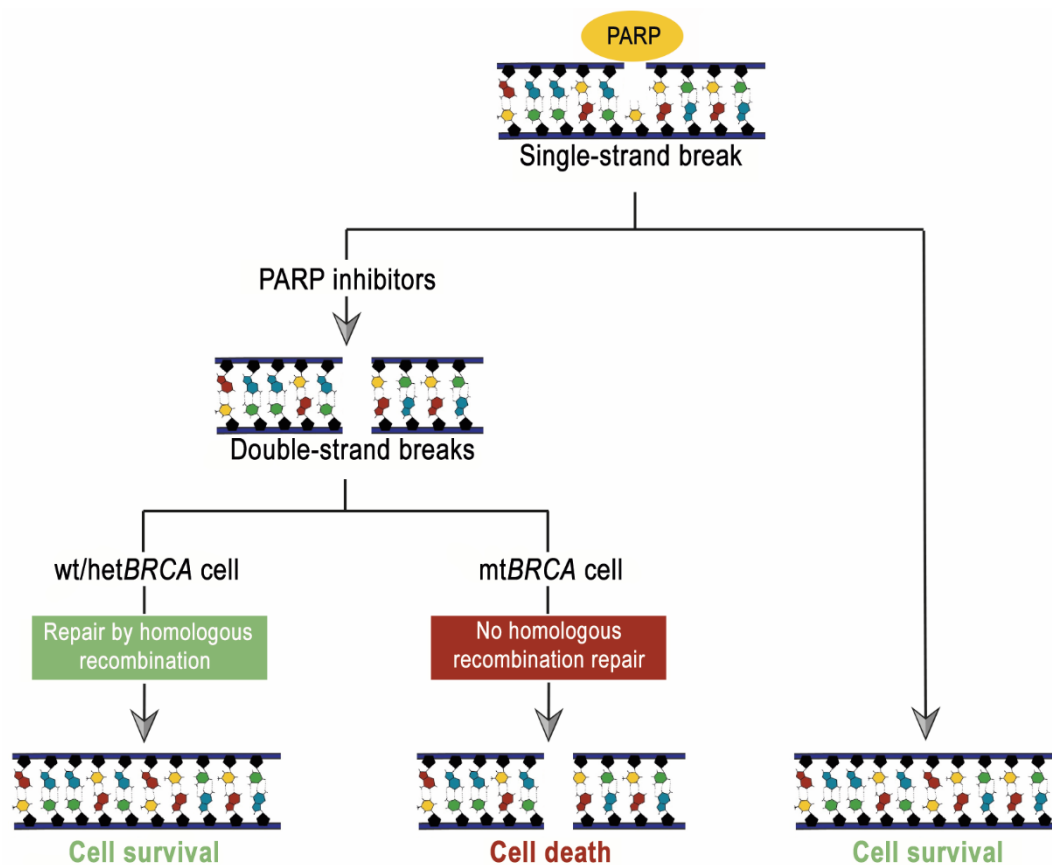


Figure 2: The principle of synthetic lethality therapy with PARP inhibitors (PARPi). PARP is involved in the repair of DNA single-strand breaks. When single-strand breaks are not repaired, they can result in double-stranded breaks during replication that can be repaired through homologous recombination mechanisms, in which *BRCA1* and *BRCA2* play key roles. *BRCA* mutated (mt*BRCA*) tumor cells are deficient in DNA repair by homologous recombination, which is the principle of targeted synthetic lethality therapy with PARPi. When PARP is inhibited, double-strand DNA breaks cannot be accurately repaired resulting in chromosomal instability and consequently cell death. In normal cells, with at least one functional copy of *BRCA1* and *BRCA2* (wt/het*BRCA*), the DNA repair process remains functional.

Despite the achievement of a complete clinical response, about 25% and 80% of patients with early-stage and advanced stage disease, respectively, will recur. About 25% of OC patients recur within six months after completing the platinum-based chemotherapy and 60% recur more than six months after completing chemotherapy. Recurrent OC is incurable in 75% of advanced OC, and the main goals of the treatment are to control the disease and the symptoms and to maintain quality of life [104]. Thus, after completing treatment, all patients who have had a complete response are recommended to undergo a surveillance to monitor disease recurrence [109]. The guidelines recommend follow-up visits every 2-4 months during the first two years, then 3-6 months for three years, and annually after five years. The surveillance should include physical examination and measurement of CA125 levels. Most of the patients have no symptoms at disease relapse and recurrence is detected by an increase in CA125 levels [103]. In spite of the improvements regarding primary treatment of OC, the outcomes after recurrence remain poor. As in diagnosis, measurement of CA125 levels to detect recurrence early lacks sensitivity and specificity and the early treatment of recurrence based on raised CA125 levels alone showed no improvement of the outcomes [103].

After a recurrence is diagnosed, several factors should be considered in the treatment decision, such as the performance status, the level of disease extension and the presence of adverse reactions to previous therapies. The standard treatment of recurrent OC includes chemotherapy, which is chosen based on platinum-free interval (PFI) [105]. PFI is defined as the time elapsed between the last cycle of platinum-based chemotherapy and disease relapse. Patients who develop disease recurrence within six months from the completion of first line platinum-based chemotherapy are considered to have “platinum-resistant” disease, those who develop disease recurrence between six and 12 months are considered to have “partially platinum-sensitive” disease and patients progressing with an interval of more than 12 months are considered to have “platinum-sensitive disease” [110]. Patients with platinum-resistant disease have a poor prognosis with a short overall survival, usually less than 12 months. Treatment of these patients is focused on quality of life and symptomatic control [105]. Treatment of patients with platinum-sensitive disease should consider previous tolerance to chemotherapy and performance status of the patient. These patients respond to re-challenge with platinum chemotherapy, which is the standard treatment [102, 111]. Since 2014, maintenance therapy with PARPi has become standard of care for relapsed HGSOC patients with platinum-sensitivity, with *BRCA1/BRCA2* somatic or germline variants and who have responded to second-line chemotherapy. Olaparib was the first PARPi approved as maintenance therapy for OC based on two randomized trials, SOLO-2 and Study-19, which demonstrated that Olaparib significantly increased the PFS

in platinum-sensitive recurrent OC patients compared to placebo (19.1 vs 5.5 months; 8.4 vs 4.8 months) [112, 113].

5. Genetic testing

Nowadays, genetic testing in OC patients is important to identify carriers of pathogenic germline variants in genes associated with increased risk of developing cancer, as well as to identify patients who could benefit from targeted therapies. Traditionally, *BRCA1/BRCA2* genetic testing has been performed in order to identify families with an increased risk of developing cancer and OC patients have been referred for testing based on family history of cancer, with *BRCA* testing offered only to those patients with a family history of breast and/or OC. However, this seems not to be the best approach, as some studies showed that more than 30% of women with pathogenic germline variants had no family history of breast or OC [8]. With the approval of PARPi, *BRCA1* and *BRCA2* have become predictive biomarkers of potential benefit from treatment with PARPi. Consequently, all patients diagnosed with OC have been referred for *BRCA1* and *BRCA2* genetic testing (germline and somatic) to guide treatment decisions in addition to familial risk assessment regardless of family history of breast or OC [114]. Recent studies showed that 18–24% of OC patients carry germline variants in genes associated with OC risk and, of these, 16–18% had germline variants in *BRCA1* or *BRCA2* genes and 4–6% had variants in other genes such as *BRIP1*, *RAD51C*, and *RAD51D* [8, 24]. Thus, the use of multigene panels in genetic testing, instead of single-gene analysis, seem to be the best strategy. Despite the advantages, this approach has some critical issues, such as the exponential identification of variants of uncertain significance (VUS).

6. Risk reduction and prevention of ovarian cancer

Since there are no effective screening strategies to detect OC early, the mortality from OC can be reduced with the implementation of prevention strategies. The strategies which contribute to OC prevention include the use of oral contraceptives, tubal ligation and RRSO [115]. Risk reduction and prevention strategies are based on genetic testing that enables the identification of women who are at high risk of developing OC, such as those with deleterious germline variants in genes predisposing to OC. For these women, taking oral contraceptives should be considered as its use has been associated with a 40% to 50% lifetime risk reduction for OC [116]. Tubal ligation has also been associated with a decreased risk of OC. In women at risk for OC, tubal ligation was associated with a 34% overall risk reduction [117]. However, the most proven method for OC risk reduction is RRSO and multiple studies have shown to have a great efficacy. Although women who are

at high risk according to family history or carriers of pathogenic germline variants are screened every 3-6 months, the prophylactic removal of both ovaries is generally recommended [41]. Several studies showed a significant risk reduction of approximately 80% among women carriers of *BRCA1* and *BRCA2* deleterious germline variants [118]. A meta-analysis showed that RRSO reduces the risk of OC, fallopian tube and peritoneal cancer in about 80%, as well as a 77% reduction in all-cause mortality [119]. Current guidelines recommended RRSO for carriers of *BRCA1* and *BRCA2* deleterious germline variants at age 35-40 years and 40-45 years, respectively. For *BRIP1*, *RAD51C* and *RAD51D* carriers RRSO should be considered at 45-50 years (Table 5). For women who do not choose RRSO, the National Comprehensive Cancer Network (NCCN) guidelines recommend screening through transvaginal ultrasound combined with measurement of CA125 starting at age 30-35 years, although there is no clear benefit [120].

Table 5: Genes associated with risk of EOC and cancer risk management recommendations according to NCCN guidelines [41].

Gene	Ovarian cancer risk	Ovarian cancer management
<i>ATM</i>	Potential increased risk of OC	RRSO: evidence insufficient; manage based on family history
<i>BRCA1</i>	Increased risk of OC	Consider RRSO at 35-40 years
<i>BRCA2</i>	Increased risk of OC	Consider RRSO at 40-45 years
<i>BRIP1</i>	Increased risk of OC	Consider RRSO at 45-50 years
MMR genes¹ and <i>EPCAM</i>	Increased risk of OC	RRSO: evidence insufficient; manage based on family history
<i>NBN</i>	Mixed evidence for increased risk of OC	RRSO: evidence insufficient; manage based on family history
<i>PALB2</i>	Increased risk of OC	RRSO: evidence insufficient; manage based on family history
<i>RAD51C</i>	Increased risk of OC	Consider RRSO at 45-50 years
<i>RAD51D</i>	Increased risk of OC	Consider RRSO at 45-50 years

¹ *MLH1, MHS2, MSH6, PMS2.*

7. Ovarian cancer biomarkers

About four decades have passed since the discovery of the expression of CA125 in OC. Despite the great advances that have been made over these 40 years, such as the improvement of understanding OC biology, classification of OC based on molecular features and the development of new therapies, CA125 continues to be the only biomarker used in OC for screening and diagnosis, as well as for monitoring treatment response. Serum biomarker CA125 is a glycoprotein expressed by a great proportion of OC and has been used for OC diagnosis with levels above 35 U/mL defined as the maximum cut-off value of the normal range. However, CA125 has a high false positive rate and poor

sensitivity and specificity as a biomarker for OC, being elevated in only 50% of stage I OC, as well as in benign gynecological diseases and in non-gynecological malignancies [121]. Several studies have been performed to identify new biomarkers in an attempt to improve the sensitivity and specificity for OC and among a wide spectrum of biomarkers, the Human Epididymis Protein 4 (HE4) has been reported as the most promising. HE4 is a glycoprotein that was found to be overexpressed in OC, mainly in serous and endometrioid histologic subtypes [122]. Although some studies have demonstrated that HE4 can be useful in the differential diagnosis of ovarian masses [123], this is not a perfect biomarker as it was found increased in some benign diseases, in lung cancer, as well as other gynecological cancer, such as endometrial and vulvar cancers [124]. Similar to CA125, HE4 is not sufficiently sensitive and specific to detect OC early. For this reason, these biomarkers were included in an algorithm in an attempt to improve the features of these biomarkers. The Risk of Ovarian Cancer Malignancy (ROMA) algorithm associates levels of CA125, HE4 and menopausal status to assess the risk of EOC in women diagnosed with a pelvic mass [125]. Since its development, several studies compared the usefulness of ROMA compared with CA125 alone, however the inconsistent results precluded its application in clinical practice [126].

Today, we still lack a highly sensitive and specific biomarker for OC. Thus, the development of reliable biomarkers for OC is still necessary for management of OC patients and might have a great impact in prognosis and survival. Circulating cell-free DNA (cfDNA) has been highlighted as a potential non-invasive alternative biomarker with several clinical applications, such as early detection, diagnosis and evaluation of treatment responses, which will be addressed next.

PAPER I

Potential clinical applications of circulating cell-free DNA in ovarian cancer patients

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Potential clinical applications of circulating cell-free DNA in ovarian cancer patients

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Abstract

Circulating cell-free DNA (cfDNA) consists of small fragments of DNA that circulate freely in the bloodstream. In cancer patients, a fraction of cfDNA is derived from tumour cells, therefore containing the same genetic and epigenetic alterations, and is termed circulating cell-free tumour DNA. The potential use of cfDNA, the so-called 'liquid biopsy', as a non-invasive cancer biomarker has recently received a lot of attention. The present review will focus on studies concerning the potential clinical applications of cfDNA in ovarian cancer patients.

Introduction

Mandel and Métais (Ref. 1) reported in 1948 the presence of nucleic acids in plasma of both healthy individuals and patients with various diseases. Following this discovery, the interest about cell-free DNA (cfDNA) reawakened in the 1960s when the detection of circulating cfDNA was reported in the serum of patients with systemic lupus erythematosus (Ref. 2). Interestingly, Leon et al. (Ref. 3) demonstrated in 1977 that cancer patients had higher levels of serum cfDNA than healthy individuals and that significantly higher cfDNA levels were present in patients with metastatic disease when compared with those without metastases. Furthermore, these investigators found that the levels of cfDNA decreased in up to 90% of the patients after radiation therapy (especially in those who responded well to treatment), whereas patients with persistently high or increased cfDNA levels had a poor prognosis. The observation that cancer patients had higher levels of cfDNA than healthy individuals was later extended by a study showing that patients with malignant disease of the gastrointestinal tract had significantly higher levels of cfDNA when compared with patients with benign disease (Ref. 4). A decade later, Stroun et al. (Ref. 5) extracted and purified DNA from plasma of cancer patients which was shown to consist of double-stranded DNA fragments of human origin ranging up to 21 kb in length. Soon after it was shown that the DNA extracted from plasma of cancer patients presented decreased strand stability and enhanced DNA synthesis, features that are typical of DNA from malignant cells and that were not observed in plasma DNA from healthy individuals (Ref. 6). These findings clearly showed that part of plasma DNA in cancer patients had its origin from tumour cells, being referred to as cell-free tumour DNA (ctDNA).

The issue of cfDNA attracted little attention during these early days, but its importance was recognised in 1994 with the detection of mutated *KRAS* gene in the plasma of patients with pancreatic carcinoma, which harboured the same mutation (Ref. 7). Soon after, several studies reported the presence of microsatellite alterations (Refs 8, 9), *TP53* mutations (Ref. 10), and aberrant promoter hypermethylation of tumour suppressor genes (Refs 11, 12) in cfDNA from cancer patients. Over the last two decades, many other tumour-related genetic and epigenetic alterations have been detected in plasma and serum from cancer patients, such as point mutations, rearranged genomic sequences, copy number variations (CNVs), microsatellite instability, loss of heterozygosity and DNA methylation (Ref. 13).

There are several features that make analysis of cfDNA a promising strategy in cancer patients: first, ctDNA provides a better representation of the tumour genome since it comprises DNA released by tumour cells from both primary tumour and metastases, minimising the problem of inter- and intra-tumour heterogeneity (Ref. 14); second, it is possible to quantify cfDNA and its concentration seems to reflect the tumour burden, making cfDNA measurements potentially useful to monitor tumour dynamics (Ref. 15) and third, blood samples can be drawn by minimally invasive methods, presenting lower risk than tissue biopsy, allowing repeated sampling and 'real-time' monitoring during the entire course of the disease.

The origin and biological characteristics of cfDNA

Recent studies have confirmed that, although cfDNA levels vary between individuals, higher levels are found in cancer patients. Indeed, small amounts are detected in the plasma from healthy individuals, with a plasma concentration range of 1.8–118 ng/ml (Refs 4, 16), while

cancer patients present a range of 59–819 ng/ml. Furthermore, it has been shown that the proportion of plasma ctDNA varies from 10 to 90% of the total cfDNA (Ref. 17) and can be derived from the primary tumour, metastases and circulating tumour cells (CTCs) in cancer patients, thereby representing the entire tumour burden (Ref. 18).

Some aspects concerning the biology of cfDNA remain poorly understood, such as the mechanism behind DNA release by cells, the process of clearance from the circulation and the biological impact of cfDNA. The assumption that cfDNA has its origin from dying cells that release their content into the bloodstream has become the most highlighted hypothesis. In healthy individuals, cfDNA is thought to derive from apoptotic cells, since normal plasma samples mainly contain DNA fragments of 180 bp and multiples of this (characteristic of apoptotic cells) and fragments larger than 2000 bp are not detected (Ref. 19). In cancer patients, apoptosis has also been proposed as the origin of cfDNA based on several observations showing that their plasma and serum DNA presents a ladder pattern after electrophoresis, which is identical to the pattern displayed by apoptotic cells (Ref. 17). However, this hypothesis is questionable because apoptosis is a mechanism supposedly lost by cancer cells and this characteristic ladder pattern was also observed for actively released DNA (Ref. 20). Necrosis has also been pointed out as a mechanism of tumour DNA release into the bloodstream, since the presence of plasma DNA fragments larger than 10 000 bp, typical of cells dying via necrosis, have been observed (Ref. 17). However, the decrease of plasma DNA levels reported following radiation therapy (Ref. 3) confronts this hypothesis, as an initial increase of cfDNA following radiation therapy would be expected if necrosis was the main mechanism of DNA release by tumour cells. Another hypothesis commonly advanced is the lysis of CTCs, although this is clearly not the predominant pathway for DNA release because CTCs are considered extremely rare (Ref. 21) and unlikely concentrations of CTCs would be necessary to explain the ctDNA concentrations found in plasma and serum of cancer patients (Refs 22, 23). A fourth possible and plausible possibility is that proliferating tumour cells actively release DNA into the bloodstream (Ref. 20) through the same mechanism as observed in *in vitro* cultivated lymphocytes (Ref. 24). Be that as it may, the main mechanism of DNA release has still not been determined and it is very likely that more than one mechanism is involved (Fig. 1).

The stability and clearance mechanism of cfDNA from plasma is also not completely understood and a few possibilities have been proposed. Although there are no studies in cancer patients, it is likely that ctDNA may be rapidly removed from bloodstream analogously to the rapid clearance of foetal DNA from maternal blood (Ref. 25). Several studies have shown that cfDNA has a relatively short half-life (Refs 25, 26) through a process that involves plasma nucleases (Ref. 25), but several studies in experimental animals indicate that liver, spleen, and kidneys are also involved in the removal of cfDNA from plasma (Refs 27, 28, 29).

Regardless of the mechanism involved in the origin of cfDNA, the fact is that elevated levels are present in the plasma of cancer patients and several questions have been raised about its potential biological impact. Experimental studies with tumour-bearing rats showed that their plasma DNA could be incorporated into the genome of cultured cells and transform them (Ref. 30). Based on these experiments and the reported presence of oncogenes in plasma of cancer patients, the 'hypothesis of genomestasis' was proposed which postulates the transformation of normal cells through the uptake of ctDNA (Ref. 30). This theory could eventually explain the development of metastases in distant target organs due to transfection of dominant oncogenes released from the primary tumour and present in the plasma (Ref. 30).

Clinical applications of 'liquid biopsies' in ovarian cancer

Ovarian cancer is the seventh most common cancer and the eighth most common cause of cancer-related death in women. In 2010, 239 000 new cases and 152 000 deaths were registered worldwide (Ref. 31). The lack of specific symptoms and effective screening methods explains the high mortality rate, as most ovarian carcinomas are diagnosed at an advanced stage in which surgery and chemotherapy are not curative. The 5-year survival of early detected ovarian cancers (stage I) is about 90%, but decreases to 30–50% and 13% for stage III and stage IV disease, respectively (Ref. 32), so a new strategy for early detection of this disease is an unmet need.

Epithelial ovarian cancer (EOC) is the most common type of ovarian cancer, accounting for over 95% of the cases, and is divided into five subtypes: high-grade serous (HGSC), endometrioid, clear cell, mucinous and low-grade serous (LGSC) carcinomas. These subtypes are considered as five distinct diseases because they present different morphologies, patterns of genetic alterations, precursor lesions, responses to therapy and prognosis (Refs 33, 34). The mutational spectrum of the various histological subtypes is the following: HGSC is genetically characterised by frequent *TP53* mutations and less frequent but recurrent mutations in *BRCA1*, *BRCA2*, *NF1*, *RBI* and *CDK12*; LGSC presents frequently *BRAF* and *KRAS* mutations; mucinous tumours have recurrent *KRAS* mutations; endometrioid carcinomas have high rates of *PTEN*, *ARID1A*, *CTNNB1* and *PIK3CA*; and clear cell carcinomas have prevalent mutations of *HNF1*, *ARID1A* and *PIK3CA* (Refs 33, 35, 36, 37). Ovarian tumours are also characterised by epigenetic changes, and among other genes, *BRCA1*, *RASSF1*, *MLH1*, *CDKN2A* and *ARH1* are found frequently silenced through promoter hypermethylation (Refs 38, 39). Moreover, DNA copy number aberrations and chromosomal abnormalities are frequently identified in ovarian carcinomas (Refs 37, 40). The well-established genetic characterisation of ovarian carcinomas makes possible the use of ctDNA as a blood based liquid biopsy.

The potential of 'liquid biopsy' in ovarian cancer management has gained increasing attention and has several possible applications, including diagnosis, prognosis, evaluation of therapy response, monitoring the emergence of resistance during the course of treatment and disease relapse prediction (Fig. 2). Several of these applications have also been evaluated and demonstrated to be promising in other types of cancer (Refs 41, 42, 43, 44, 45, 46, 47, 48, 49, 50). Herein, we will present an overview of the relevant discoveries using cfDNA and its potential applications in ovarian cancer. The literature review was performed using the PubMed database and the search terms included 'ovarian cancer', 'circulating cell-free DNA', 'circulating tumor DNA', 'circulating nucleic acid', 'plasma DNA' and 'liquid biopsy'. All the relevant articles published until the end of 2017 were included in the review (Table 1).

Screening and diagnosis of ovarian cancer

Nowadays, screening for ovarian cancer in symptomatic and high-risk women is done through ultrasound examination and measurement of the biomarker CA125 (Refs 51, 52) but none of these methods are sensitive and specific enough for this type of cancer. The use of CA125 in early detection is questionable, because increased levels are found in only 50–60% of patients with early stage ovarian cancer and can also appear in other types of cancer and benign diseases (Ref. 53), being therefore a non-specific marker for ovarian cancer. Like CA125, ultrasound also detects benign ovarian lesions (Ref. 54). In the presence of elevated levels of CA125 and ovarian masses detected through

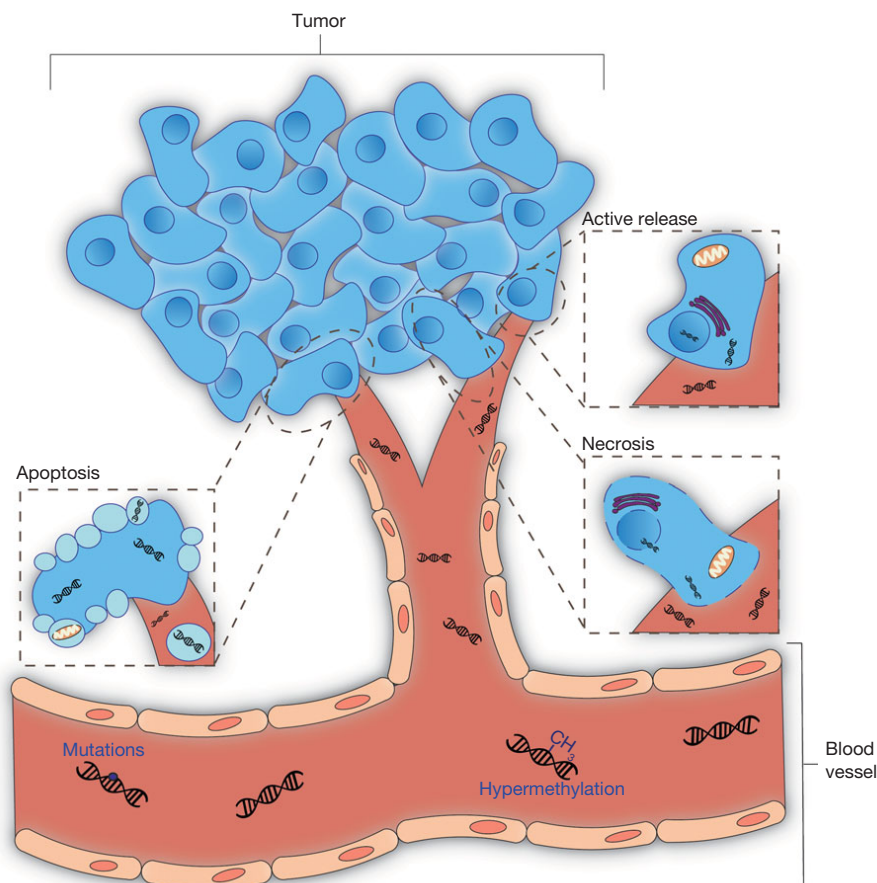


Fig. 1. The origins of cell-free tumour DNA. Tumour cells can release DNA into the blood stream by three different mechanisms, such as apoptosis, necrosis and spontaneous active release, making tumour-related genetic and epigenetic alterations detectable in blood.

ultrasound examination, surgery is usually necessary for diagnosis and disease staging, as well as for the initial treatment (Ref. 55). This screening strategy of ovarian cancer based on the combination of biomarker levels and imaging has therefore limited positive predictive value and leads to excessive and unnecessary surgeries (Ref. 56). For these reasons, there is a clear need to develop new biomarkers for screening and diagnosis of ovarian cancer.

There has been an increasing interest in the use of cfDNA and several studies have evaluated this approach for disease diagnosis. Some studies focused on the quantification of total levels of cfDNA and all of them found significantly increased levels in ovarian cancer patients when compared with healthy individuals and with patients with benign ovarian diseases (Refs 57, 58, 59, 60). When comparing healthy controls with women with benign ovarian diseases, none of the studies found statistically significant differences in total cfDNA levels. The sensitivity and specificity ranges of total cfDNA levels for ovarian cancer diagnosis were 87–91.5% and 85–89.5%, respectively (Refs 58, 59). Zhou et al. (Ref. 61) conducted a meta-analysis using data from nine studies, including 462 ovarian cancer patients and 407 controls, to

evaluate the potential of cfDNA as a biomarker for the diagnosis of ovarian cancer. The pooled sensitivity and specificity of cfDNA were 70 and 90%, respectively, suggesting that the diagnostic accuracy of cfDNA has unsatisfactory sensitivity but acceptable specificity. Shao et al. (Ref. 59) reported that cfDNA presents a sensitivity and specificity of 88.9 and 89.5%, which are higher than those of other ovarian cancer biomarkers such as CA125 (75 and 52.6%) and HE-4 (80.6 and 68.4%). When they combined cfDNA levels with CA125 and HE-4, they observed an improvement of the sensitivity up to 91.67%. The study developed by Zachariah et al. (Ref. 60) included an additional group of women with ovarian endometriosis and the cfDNA levels in ovarian cancer patients were not significantly increased when compared with this group. However, total cfDNA levels presented a sensitivity and specificity of 74 and 69%, respectively, to discriminate between ovarian cancer patients and healthy controls and 74 and 67%, respectively, to separate ovarian cancer patients from patients with benign ovarian diseases (Ref. 60). Interestingly, Kamat et al. (Ref. 58) found that preoperative cfDNA levels in patients with early stage disease were significantly higher when compared with controls and patients with benign ovarian

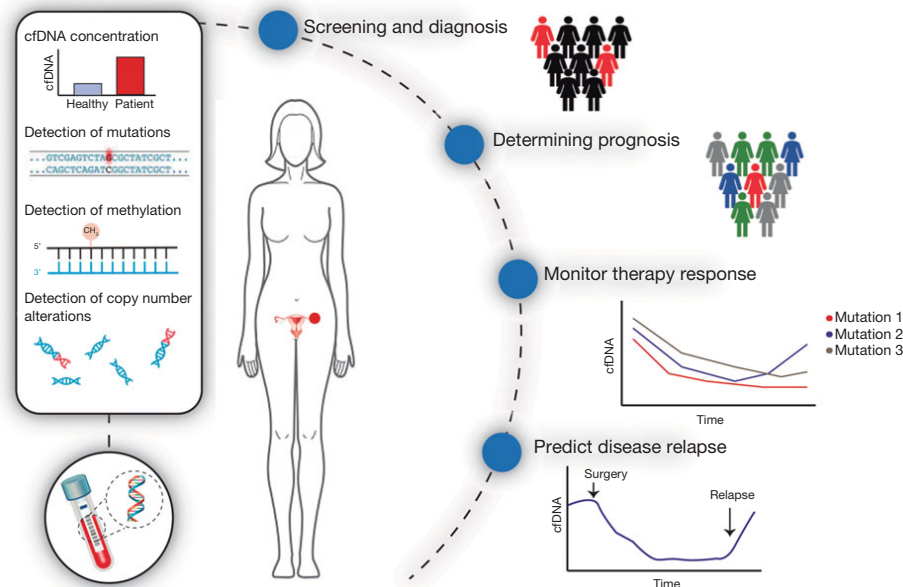


Fig. 2. Clinical applications of liquid biopsies in ovarian cancer patients. cell-free tumour DNA (cfDNA) can be obtained from peripheral blood of ovarian cancer patients. Analyses of cfDNA can involve quantification of total cfDNA levels or detection and measurement of genetic and epigenetic alterations such as point mutations, hypermethylation and copy number alterations. This strategy may allow screening and diagnosis of disease and can be used to estimate patient prognosis. The periodic monitoring of cfDNA might give information about therapy response, allowing detection of drug resistance mutations and prediction of disease relapse.

diseases. Regarding the correlation between cfDNA levels and clinicopathological characteristics, patients with stage I–II tumours presented lower cfDNA levels compared with patients with stage III–IV, indicating that cfDNA levels reflect the burden of disease (Ref. 59).

Other studies were more specific and attempted to quantify the presence of tumour-specific genetic alterations in cfDNA to evaluate its potential for screening and diagnosis of ovarian cancer. One of these studies, developed by Otsuka *et al.* (Ref. 62), was the first to demonstrate that tumour-specific somatic mutations can be detected in plasma DNA of ovarian cancer patients. They found that 44% of the patients involved in the study had detectable *TP53* mutations in the tumour tissue and 16.7% of those had the same mutations detectable in the plasma DNA. Regarding patients with advanced disease, the detection rate of *TP53* mutations was 28.6%. This study also showed that *TP53* mutations were undetectable in plasma after surgery, but in one patient the *TP53* mutation became again detectable 16 months after surgery and the patient died 2 months later. Regarding the detection rate, similar results were obtained in another study also based on tumour-specific *TP53* mutations in cfDNA, with a concordance rate between tumour and plasma DNA of 30% (Ref. 63). On the other hand, Pereira *et al.* (Ref. 64) quantified tumour-specific mutations in serum of 44 patients with a range of gynaecological cancers, including 22 with ovarian cancer, to explore the use of cfDNA to predict tumour presence, detected through computed tomography (CT) imaging and findings at the time of surgery. Interestingly, they found six cases with detectable cfDNA levels and negative CT imaging in which tumours were later detected in surgery. When compared with the presence of tumour at surgery, cfDNA presented a sensitivity and

specificity of 81 and 99%, respectively, to detect the presence of ovarian cancers and, on average, cfDNA predicted the disease recurrence approximately 7 months earlier than CT scans (Ref. 64). In a more recent study reporting the development of an ultrasensitive approach to detect alterations in frequently altered cancer genes in cfDNA, the authors analysed plasma samples from 42 ovarian cancer patients and detected alterations in 71% of them. Furthermore, 68% of these patients presented stage I–II disease suggesting the potential of cfDNA for early detection of disease (Ref. 65).

Chromosomal alterations might also contribute to increase sensitivity in cfDNA analysis. Aberrant chromosomal rearrangements previously identified in ovarian carcinomas were subsequently detected in pre-surgery plasma DNA samples of eight of 10 patients (Ref. 66). Undetectable chromosomal junctions in post-surgery plasma DNA samples were consistent with the lack of detectable disease, while the continued presence of genomic rearrangements in post-surgery DNA plasma samples was consistent with the observed presence of the disease (Ref. 66). Cohen *et al.* (Ref. 67) used a commercially non-invasive prenatal testing that identifies foetal aneuploidy through sequencing of DNA in the maternal plasma to evaluate its potential for ovarian cancer diagnosis. The algorithm chosen to identify genomic gains or losses presented a sensitivity of 40% for ovarian cancer detection, with approximately the same frequency for early and late stage disease, and a specificity of 94%. Another study was able to demonstrate a difference in copy number changes of a few genes between patients with ovarian cancer and those with benign ovarian tumours (Ref. 68). Furthermore, Vanderstichele *et al.* (Ref. 69) have recently reported a promising method for presurgical prediction of disease in patients with adnexal masses. This

Table 1. Studies evaluating the use of cfDNA in ovarian cancer patients

Study	Application	Cohort	Sample	Method	Target
Kamat et al. (Ref. 57)	Diagnostic	19 EOC patients and 12 HC	Plasma	Real-time PCR	Total cfDNA levels
Kamat et al. (Ref. 58)	Diagnostic and prognostic	164 EOC patients, 49 BOD patients and 75 HC	Plasma	Real-time PCR	Total cfDNA levels
Shao et al. (Ref. 59)	Diagnostic	36 EOC patients, 22 BOD patients and 19 HC	Serum	bDNA technique	Total cfDNA levels
Zachariah et al. (Ref. 60)	Diagnostic	21 EOC patients, 24 BOD patients, 23 patients with endometriosis and 36 HC	Serum and plasma	Real-time PCR	Total cfDNA levels
Otsuka et al. (Ref. 62)	Diagnostic	27 EOC patients	Plasma	F-SSCP	<i>TP53</i> mutations
Swisher et al. (Ref. 63)	Diagnostic and prognostic	137 EOC patients	Plasma or serum	Ligase detection reaction	<i>TP53</i> mutations
Pereira et al. (Ref. 64)	Diagnostic and prognostic	22 EOC patients in a group of 44 patients with gynaecologic cancer	Serum	Droplet digital PCR	Tumour-specific mutations
Phallen et al. (Ref. 65)	Diagnostic	42 EOC patients, 158 patients with other types of cancer and 44 HC	Plasma	NGS	58 cancer related genes
Harris et al. (Ref. 66)	Diagnostic	10 EOC patients	Plasma	Quantitative PCR	Tumour-specific chromosomal rearrangements
Cohen et al. (Ref. 67)	Diagnostic	32 EOC patients	Plasma	Percept™ NIPT Kit	Whole-genome
No et al. (Ref. 68)	Prognosis	36 EOC and 16 BOD patients	Serum	Real-time PCR	<i>B2M</i> , <i>RAB25</i> , <i>CLDN41</i> and <i>ABCF2</i> CNV
Vanderstichele et al. (Ref. 69)	Diagnostic	68 patients with adnexal mass and 44 HC	Plasma	NGS	Whole-genome
Wu et al. (Ref. 70)	Diagnostic	47 EOC patients, 14 BOD patients and 10 HC	Plasma	Methylation-specific PCR	<i>RASSF2A</i> methylation
Ibanez de Caceres et al. (Ref. 71)	Diagnostic	50 EOC patients, 10 BOD patients and 20 HC	Plasma or serum	Methylation-specific PCR	<i>BRCA1</i> and <i>RASSF1A</i> methylation
Melnikov et al. (Ref. 72)	Diagnostic	33 EOC patients and 33 HC	Plasma	MethDet56	Hypermethylation
Liggett et al. (Ref. 73)	Diagnostic	30 EOC patients, 30 BOD patients and 30 HC	Plasma	MethDet56	Hypermethylation
Capizzi et al. (Ref. 78)	Therapy response and prognostic	22 EOC patients and 50 HC	Plasma	Real-time PCR	Total cfDNA levels
Wimberger et al. (Ref. 79)	Therapy response and prognostic	62 EOC patients and 28 HC controls	Serum	Quant-iT PicoGreen dsDNA Kit	Total cfDNA levels
Steffensen et al. (Ref. 80)	Therapy response prognostic	144 EOC patients	Plasma	Real-time PCR	Total cfDNA levels
Dobrzycka et al. (Ref. 81)	Prognosis	126 EOC patients	Plasma	PCR-RFLP	<i>KRAS</i> mutations
Parkinson et al. (Ref. 82)	Prognosis	40 EOC patients	Plasma	Digital PCR	<i>TP53</i> mutations
Gifford et al. (Ref. 83)	Therapy response and prognostic	138 EOC patients	Plasma	Methylation-specific PCR	<i>MLH1</i> methylation
Kamat et al. (Ref. 88)	Therapy response	Orthotopic in vivo ovarian cancer model	Plasma	Real-time PCR	Total cfDNA levels
Choudhuri et al. (Ref. 89)	Therapy response	100 EOC patients	Plasma	Real-time PCR	Total cfDNA levels
Murtaza et al. (Ref. 90)	Therapy response	3 EOC patients	Plasma	Exome sequencing	Gene mutations
Christie et al. (Ref. 93)	Therapy response	30 EOC patients	Plasma	NGS	<i>BRCA1</i> and <i>BRCA2</i>
Weigelt et al. (Ref. 94)	Therapy response	19 EOC patients	Plasma	NGS	<i>BRCA1/2</i> and 141 additional genes

BOD, benign ovarian disease; bDNA, branched DNA; CNV, copy number variation; EOC, epithelial ovarian cancer; F-SSCP, fluorescence-based single-strand conformation polymorphism; HC, healthy controls; NGS, next-generation sequencing; NIPT, non-invasive prenatal testing; PCR-RFLP, PCR-restriction fragment length polymorphism.

method for ovarian cancer detection is based on chromosomal instability detected by whole-genome sequencing on cfDNA obtained from plasma of women with adnexal masses. They observed that chromosomal instability was significantly higher in ovarian cancer patients compared with healthy individuals and patients with benign disease. CNAs detected in tumour biopsies were also detectable in cfDNA, with a median Spearman correlation coefficient of 0.51. This method yielded a greater sensitivity and specificity (74 and 91%) to detect malignancy when compared with CA125 (86 and 64%).

Some investigators tried to use epigenetic alterations to evaluate the use of cfDNA in screening and diagnosis of ovarian cancer (Refs 70, 71, 72, 73). In most studies, the samples collected from patients with benign ovarian diseases and healthy controls showed no promoter hypermethylation, suggesting tumour-specificity. When looking at methylation status of the *RASSF2A* gene, Wu *et al.* (Ref. 70) found that the gene promoter was methylated in 51.1% of tumour samples and in 36.2% of the corresponding plasma samples. In a similar study, Ibanez de Caceres *et al.* (Ref. 71) tested a panel of six genes and observed a concordance rate of 82% in gene methylation pattern between tumour DNA and serum DNA, with a specificity to detect ovarian cancer of 100%. Two other studies used a panel of 56 genes and presented sensitivity and specificity ranges of 85–90% and 61–86.7%, respectively, for the detection of ovarian cancer (Refs 72, 73). For the discrimination between malignant and benign ovarian diseases the sensitivity and the specificity were 73.3 and 80%, respectively. Furthermore, for the first time, the possibility to differentiate benign ovarian diseases from healthy controls was demonstrated, with a sensitivity of 90% and a specificity of 76.7% (Ref. 73).

The screening of asymptomatic individuals is the most ambitious application of liquid biopsies. Efforts are being conducted by several companies to develop a liquid biopsy test for early detection of cancer in the general population (Ref. 74). In early 2018, Cohen *et al.* (Ref. 75) reported the results of a liquid biopsy test, called CancerSEEK, which can detect and localise eight types of cancer (ovarian, breast, liver, stomach, pancreatic, oesophageal, lung and colorectal) through the assessment of protein biomarkers levels and cfDNA mutations. The specificity of this test was greater than 99% and the median sensitivity among the eight types was 70%, which is low for the purpose of cancer screening. In addition, CancerSEEK was able to localise the cancer in a median of 83% of the patients. Despite the promising results, the authors recognise several limitations of the study and the need of prospective studies in a larger population.

Determining prognosis

Prognostic factors are used to estimate the likelihood of recurrence and survival. The prognostic factors established for ovarian cancer include: age of patient, tumour histology, tumour stage, tumour grade, residual disease after initial debulking surgery, volume of ascites and disease status at second-look and CA125 levels (Refs 76, 77). Several studies have been conducted to evaluate the potential of cfDNA as a prognostic factor for ovarian cancer.

Based on preoperative total levels of cfDNA, ovarian cancer patients with higher levels had decreased median survival compared with patients with low preoperative cfDNA levels, presenting a 2.38-fold increased risk of death from disease (Ref. 58). Concerning patients submitted to treatment, higher cfDNA levels before chemotherapy were associated with high risk of disease relapse and presence of distant metastasis, whereas after chemotherapy higher levels were associated with higher risk of disease progression (Ref. 78) and poor overall survival (Ref. 79). In multi-resistant ovarian cancer patients treated with Bevacizumab, higher

total levels of cfDNA were correlated with worse progression free and overall survival (Ref. 80).

Some other studies were based on the identification of genetic or epigenetic alterations in certain genes instead of relying on quantification of total cfDNA levels. No *et al.* (Ref. 68) found that lower copy number of the *RAB25* gene before surgery was associated with an increased risk of death from ovarian cancer, being suggested as an independent prognostic factor for poor disease-free and overall survival. The detection of tumour-specific *TP53* or *KRAS* mutations in plasma from ovarian cancer patients was associated with decreased overall survival, with a mean of 21–28 months for ovarian cancer patients with detectable mutations and a mean of 54–58 months for patients with no detected mutations in plasma (Refs 63, 81). Recently, Parkinson *et al.* reported that patients in whom a decrease of up to 60% of *TP53* mutant allele frequency was observed after one cycle of chemotherapy had a significantly longer time to progression than patients whose *TP53* mutant allele frequency after decreased by 60% or less (Ref. 82). On the other hand, Pereira *et al.* (Ref. 64) found that undetectable levels of tumour-specific mutations in serum samples of ovarian cancer patients obtained after chemotherapy were associated with improved progression free and overall survival. Finally, the detection of acquired *MLH1* promoter methylation in cfDNA of ovarian cancer patients at disease relapse after chemotherapy was found significantly associated with poor overall survival after disease progression (Ref. 83).

Monitoring the response to therapy

Ovarian cancer is treated with one or a combination of strategies. Surgery, apart from its role for diagnosis and disease staging, is usually the first step in the treatment of ovarian cancer and adjuvant chemotherapy is administrated after surgery to patients with a significant risk of recurrence (Ref. 84). Although ovarian cancer is sensitive to chemotherapeutic drugs and most patients respond to initial treatment, most patients will develop disease recurrence. Routinely, measurement of CA125 levels should be done during the course of treatment and increasing levels can be indicative of treatment failure (Ref. 85). The main factor of disease recurrence is the emergence of drug resistance by tumour cells and several molecular mechanisms have been shown to be involved (Refs 86, 87). Evaluation during the treatment course is important to anticipate the emergence of drug resistance, so that other strategies can be used promptly.

cfDNA may play a relevant role in disease monitoring and several studies have been performed for this purpose. Using an orthotopic *in vivo* ovarian cancer model, Kamat *et al.* (Ref. 88) evaluated the kinetics of cfDNA levels in response to therapy and observed a variation over time. More specifically, they observed an initial rise correlated with increased apoptosis rate of tumour cells (an increase of 63% at post-treatment day 1), followed by a rapid decline (20% lower at day 3 and 83% at post-treatment day 10). The key finding of this study is that cfDNA levels closely reflect tumour burden. Studies in which total levels of cfDNA were evaluated in ovarian cancer patients presented similar results, showing higher levels of cfDNA before chemotherapy and a significant decrease in total cfDNA levels after chemotherapy (Refs 78, 79, 89).

Murtaza *et al.* (Ref. 90) evaluated the genomic evolution in response to chemotherapy using exome sequencing of serial cfDNA samples from advanced cancer patients, three of which were affected with ovarian cancer. In one of the ovarian cancer cases, they observed an increasing allelic frequency of a truncating mutation in the *RBI* gene, previously described as being associated with therapy resistance (Ref. 91), thereby showing that exome sequencing analysis of cfDNA represents a novel

non-invasive strategy to evaluate the evolution of therapy response and to identify the emergence of drug resistance.

On the other hand, Martignetti et al. (Ref. 92) reported a case of a patient with ovarian cancer treated with surgery and chemotherapy who experienced multiple tumour recurrences for a period of four years. At the time of diagnosis, the patient presented a CA125 level of 40 U/ml and during the subsequent four years the CA125 levels were above the normal limit only three times, whereas a series of biopsies proved the presence of tumour. When looking for genomics-based biomarkers, they found a fibroblast growth factor receptor 2 fusion transcript in the tumour tissue that was also detectable in plasma or serum DNA from all 28 blood samples collected since the initial surgery, indicating the continued presence of tumour cells and explaining the multiple recurrences of the disease.

Finally, there are several studies regarding the detection of reversion germline mutations of *BRCA1/2*, which are associated with acquired resistance to chemotherapy. Christie et al. (Ref. 93) evaluated the possibility to detect reversion germline mutations of *BRCA1/2* in cfDNA from patients with HGSOC. Reversion mutations were only detected in tumours from patients with recurrence disease and in 60% of these patients the reversion mutations were detected in cfDNA. In another study conducted by Weigelt et al. (Ref. 94), *BRCA1* or *BRCA2* reversion mutations were identified in cfDNA from 21% of platinum resistant ovarian cancer patients, indicating that cfDNA sequencing may aid the selection of patients for PARP inhibition therapy (Refs 93, 94). In fact, with the emergence of targeted therapies in oncology, one of the main applications of liquid biopsies is the detection of gene mutations to steer therapy decisions. Furthermore, this approach can help to detect mutations which are known to be associated with acquired resistance. For example, nearly all *EGFR* mutated non-small cell lung cancer patients develop resistance under tyrosine kinase inhibitors (TKIs) therapy, being the T790M mutation the most common mechanism (Ref. 44). According to ESMO and NCCN guidelines, liquid biopsy is a valid alternative to tissue re-biopsy in the identification of resistance mechanism to *EGFR* TKI therapy (Ref. 95). Furthermore, according to Committee for Medicinal Products for Human Use (CHMP) of the European Medicines Agency, liquid biopsy can be used to predict response to *EGFR* TKI therapy when a tumour sample is not available (Ref. 96). It is well possible that similar strategies to detect and monitor the molecular mechanisms of targeted therapy may enter clinical practice for other cancer types, namely in serous ovarian cancer in which treatment with PARP inhibitors may be used in *BRCA1/BRCA2* mutation carriers (Ref. 97).

Conclusions

The lack of an effective screening method for early detection, combined with the acquired resistance to commonly used chemotherapeutics, makes this gynaecological malignancy a worldwide health burden. Thus, there is a growing need for better tools to improve the poor outcome underlying ovarian cancer. The findings summarised here emphasise the potential use of cfDNA as a liquid biopsy for early diagnosis and molecular characterisation of ovarian cancer, as well as for monitoring response to therapy and identifying the emergence of drug resistance mechanisms.

Among the studies reviewed, better results were obtained in those using genetic or epigenetic alterations in ctDNA, which present both higher sensitivity and specificity when compared with those which used total cfDNA levels. Genetic and epigenetic alterations found in ctDNA are representative of the tumour and are absent in normal cfDNA, whereas the total levels of

cfDNA can be increased as a result of benign conditions or other malignancies. Furthermore, regarding the correlation between findings in tumour and plasma, better results were observed when more sensitive techniques were used, such as next-generation sequencing (NGS) and droplet digital polymerase chain reaction (ddPCR). However, patient's recruitment was enriched for individuals diagnosed with ovarian cancer in the majority of the studies, so the reported sensitivities and specificities of cfDNA for ovarian cancer detection do not necessarily apply to the general population.

The molecular characterisation of tumours through cfDNA is particularly interesting with the emergence of targeted therapies for ovarian cancer, mainly in situations in which a tumour sample is not available and biopsy is not viable. Due to the distinct patterns of genetic alterations of each histological subtype, as well as the molecular heterogeneity in the pathogenesis and therapy resistance mechanisms in ovarian cancer, NGS of cfDNA with a customised gene panel, may be a good strategy to improve the diagnosis and clinical management of this disease. Using bioinformatic tools for the detection of CNVs using NGS data may improve the accuracy of cfDNA to detect the different histologic subtypes of ovarian carcinomas.

Although there are several advantages in the use of ctDNA as a screening tool, some limitations also exist. Major limitations are the low concentrations of ctDNA in plasma and the need of high sensitive techniques such as digital PCR (dPCR) and NGS for the detection of tumour specific alterations. NGS has the advantage of simultaneously analyse alterations in several genes and recent advances, such as the introduction of molecular barcodes, will enable the detection of low frequency variants.

Although the report of the detection of ctDNA in cancer patients preceded the detection of foetal DNA in blood of pregnant women, a reliable diagnostic test based on cfDNA was first established as a non-invasive pre-natal test (NIPT). NIPT rapidly evolved to a frequent test used for screening Down syndrome and other aneuploidies and is already implemented in public prenatal care in several countries worldwide (Ref. 98). In comparison, the development of a liquid biopsy approach to be applied in oncology has been more difficult and is taking longer due to several factors. Cancer is a pathologic and complex process, a dynamic and heterogeneous disease with multiple variables, while pregnancy is a physiological process with minimal variables. Furthermore, the percentage of foetal cfDNA in the mother's circulation is relatively high from 9 weeks of pregnancy, while the fraction of ctDNA in cancer patients is frequently low and variable even among patients with the same disease stage. Consequently, it is more difficult to standardise liquid biopsy assays based on ctDNA analysis for the management of cancer patients (Ref. 99). Liquid biopsies are being evaluated in large studies in order to validate their use in the management of cancer patients, with 322 clinical trials registered on the National Institute of Health database on early October 2018. Standardisation of the methodology from blood collection to cfDNA analysis is essential and there is an ongoing clinical trial for the development and comparison of different cfDNA detection techniques (NTC01350980). Furthermore, there are two ongoing clinical trials for the development of a blood test for ovarian cancer diagnosis based on cfDNA (NCT02441140 and NCT03485651). In addition, the International Ovarian Tumor Analysis Group (IOTA) is exploring the potential of cfDNA to be included in their disease prediction models (NCT01698632). It is expected that results from large-scale and multicentre clinical trials will support the use of liquid biopsies in clinical practice also for ovarian cancer in a not too distant future.

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AIMS

In this study, we aimed to explore the use of next generation sequencing (NGS) on FFPE and cfDNA samples as alternatives for genetic testing in OC patients to detect both germline and somatic variants, which could be used as biomarkers for management of OC patients. Specifically, the aims of this thesis were:

- a) to design a customized NGS gene panel that includes all known OC cancer predisposing genes for the detection of pathogenic germline or somatic variants in a single test using FFPE tumor samples;
- b) to evaluate if the above strategy would increase the yield of clinically actionable pathogenic germline variants;
- c) to characterize the pattern of somatic variants of OC by NGS of FFPE samples using a custom gene panel that includes the most frequently mutated genes in the various histological subtypes of the disease;
- d) to evaluate the sensitivity of NGS of cfDNA for the detection of somatic variants as compared with testing FFPE tumor samples.

PAPER II

**Gene panel tumor testing in ovarian cancer patients
significantly increases the yield of clinically actionable
germline variants beyond *BRCA1/BRCA2***

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Article

Gene Panel Tumor Testing in Ovarian Cancer Patients Significantly Increases the Yield of Clinically Actionable Germline Variants beyond *BRCA1/BRCA2*

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Simple Summary: Germline and somatic variant testing of the *BRCA1* and *BRCA2* genes are important to predict treatment response to PARP inhibitors in ovarian cancer patients. However, germline variants in other genes besides *BRCA1* and *BRCA2* are associated with ovarian cancer predisposition, which would be missed by a genetic testing aimed only at treatment decision. We aimed to evaluate the yield of clinically actionable germline variants using next-generation sequencing of a customized panel of 10 genes for the analysis of pathology samples of ovarian carcinomas. We identified clinically actionable germline variants in a significantly higher proportion of ovarian cancer patients when compared with genetic testing focused only on *BRCA1* and *BRCA2*. This strategy increases the chance to make available genetic counseling, presymptomatic genetic testing, and gynecological cancer prophylaxis to female relatives who turn out to be healthy carriers of deleterious germline variants.

Abstract: Since the approval of PARP inhibitors for the treatment of high-grade serous ovarian cancer, in addition to cancer risk assessment, *BRCA1* and *BRCA2* genetic testing also has therapeutic implications (germline and somatic variants) and should be offered to these patients at diagnosis, irrespective of family history. However, variants in other genes besides *BRCA1* and *BRCA2* are associated with ovarian cancer predisposition, which would be missed by a genetic testing aimed only at indication for PARP inhibitor treatment. In this study, we aimed to evaluate the yield of clinically actionable germline variants using next-generation sequencing of a customized panel of 10 genes for the analysis of formalin-fixed paraffin-embedded samples from 96 ovarian carcinomas, a strategy that allows the detection of both somatic and germline variants in a single test. In addition to 13.7% of deleterious germline *BRCA1/BRCA2* carriers, we identified 7.4% additional patients with pathogenic germline variants in other genes predisposing for ovarian cancer, namely *RAD51C*, *RAD51D*, and *MSH6*, representing 35% of all pathogenic germline variants. We conclude that the strategy of reflex gene-panel tumor testing enables the identification of clinically actionable germline variants in a significantly higher proportion of ovarian cancer patients, which may be valuable information in patients with advanced disease that have run out of approved therapeutic

options. Furthermore, this approach increases the chance to make available genetic counseling, presymptomatic genetic testing, and gynecological cancer prophylaxis to female relatives who turn out to be healthy carriers of deleterious germline variants.

Keywords: ovarian cancer; tumor testing; next generation sequencing; multi-gene panel; genetic predisposition; clinically actionable alterations

1. Introduction

Ovarian cancer (OC) is the third most common malignant disease among patients with gynecological neoplasia, being the eighth most common cancer and the eighth most common cause of cancer-related death in women [1]. The lack of specific symptoms and effective early detection strategies contributes to the high mortality rate, as most OC are diagnosed at an advanced stage [2]. Epithelial ovarian cancer (EOC) is recognized as a heterogeneous disease mainly represented by five histological subtypes (high-grade serous, low-grade serous, clear cell, endometrioid, and mucinous) that are characterized by distinct clinical and molecular features. Despite this knowledge, the initial standard treatment has typically been maximal effort cytoreductive surgery, with platinum-paclitaxel chemotherapy being the standard of care treatment [3,4]. Despite the usually good initial response, about 70–80% of patients will relapse within the subsequent three years and have limited treatment options thereafter [5,6].

The first poly(ADP-ribose) polymerase (PARP)-inhibitor (PARPi) received the approval from the European Medicines Agency (EMA) in 2014 for use as a maintenance therapy in relapsed high-grade serous ovarian cancer (HGSOC) patients with platinum-sensitivity and with *BRCA1/BRCA2* somatic or germline variants [7,8]. The same treatment was approved later for first-line maintenance therapy in *BRCA* mutated advanced EOC [9]. The mechanism underlying the efficacy of PARPi is synthetic lethality, which occurs in cells with deficiency in homologous recombination (HR) repair, where *BRCA1/BRCA2* proteins play an essential role. When base excision repair (BER) is blocked by PARPi and thereby inhibits single strand break repair, the combined effect with HR results in accumulation of DNA aberrations and cell death [10]. Since PARPi approval, *BRCA* status became a relevant criterion to select patients who may benefit from this therapy or to identify the main responders to this class of drugs. Consequently, OC patients have been referred for *BRCA1* and *BRCA2* genetic testing (germline and somatic) to guide treatment decisions in addition to familial risk assessment regardless of family history of breast or OC [11]. This seems to be the best approach as some studies showed that more than 30% of women with germline variants had no family history of breast or OC [12].

Although most are sporadic, it has been reported that about 23% of OC are associated with hereditary predisposition [12], and a large proportion of these cases are due to inherited variants in *BRCA1* and *BRCA2* genes. Women with germline variants in these genes are at higher risk of developing OC, when compared with women in the general population, with the lifetime risk being 44% and 17% for *BRCA1* and *BRCA2*, respectively [13]. The use of next-generation sequencing (NGS) with multi-gene panels may allow the identification of other genes associated with inherited predisposition to OC [12]. Recent studies with blood samples showed that 18–24% of OC patients carry germline variants in genes associated with OC risk and, of these, 16–18% had germline variants in *BRCA1* or *BRCA2* genes and 4–6% had variants in other genes such as *BRIP1*, *RAD51C*, and *RAD51D* [12,14]. The identification of carriers of deleterious germline variants in OC predisposition genes allows the use of prophylactic surgery for a disease that currently has no effective screening allowing early diagnosis.

Many laboratories are now performing *BRCA1/BRCA2* testing in formalin-fixed paraffin-embedded (FFPE) tumor samples to detect germline and somatic variants to identify, in a single test, those patients with somatic and germline variants who are most likely to benefit from PARPi therapy. Using this strategy, there is a risk that patients without *BRCA1/BRCA2* variants are not evaluated for other genes

predisposing to OC, thereby missing the opportunity for presymptomatic testing and prophylactic measures. Eventually, they might subsequently be referred for germline NGS testing of other genes, which increases the sequencing costs [11]. In this study, we designed a customized NGS gene panel that includes all known OC cancer predisposing genes for the detection of pathogenic germline variants in a single test using FFPE tumor samples. We show that this strategy significantly increases the yield of clinically actionable pathogenic germline variants in a single NGS test, which can subsequently be evaluated in a peripheral blood sample by Sanger sequencing for its eventual constitutional origin after genetic counseling.

2. Results

2.1. Variant Analysis

Of 96 FFPE OC samples, one (OC65) was excluded from further analysis because it did not pass quality filters. Among the ten genes included in the NGS panel (*BRCA1*, *BRCA2*, *BRIP1*, *MLH1*, *MSH2*, *MSH6*, *PMS2*, *RAD51C*, *RAD51D*, and *TP53*), a total of 3457 variants was annotated and classified among the remaining 95 tumor samples. After variant filtering 97 variants remained, of which 57 were classified as pathogenic/likely pathogenic in ClinVar. Among the variants not classified in ClinVar, two variants were nonsense, seven were frameshift variants, two were canonical +1 and −2 splice site variants, and one was an inframe variant, all of which were considered pathogenic since there is strong evidence of pathogenicity [15]. Additionally, there were 28 missense variants not classified, with unknown significance or conflicting interpretations of pathogenicity. The potential pathogenicity of these missense variants was evaluated through in silico prediction tools, and 19 of these variants were predicted to have a deleterious impact on the protein. We, therefore, retained 88 variants across seven genes, of which 69 (77.3%) were pathogenic and 19 (22.7%) were missense variants of uncertain significance (VUS). Of 95 tumor samples analyzed, variants in the genes under study were identified in 66 (69.5%).

2.2. Germline Variants

After variant filtering, all variants of the genes of interest identified by NGS in the tumor of the 66 patients were tested in peripheral blood samples, and only those which were confirmed to be germline are included in this report. Of the 88 variants detected, 26 variants were confirmed to be germline across seven of the 10 genes included in the panel. Out of the 26 germline variants identified, 20 (76.9%) were pathogenic variants and six (23.1%) were VUS. In 69 of the 95 (73.7%) patients included in the study, no germline variants among the 10 genes tested were identified. Pathogenic variants were found in 20 patients (21.1%): seven in *BRCA1*, six in *BRCA2*, four in *RAD51D*, two in *RAD51C*, and one in *MSH6*. Germline VUS were identified in six patients (6.3%) in *BRCA2*, *MSH6*, *TP53*, and *BRIP1* (Figure 1 and Table 1).

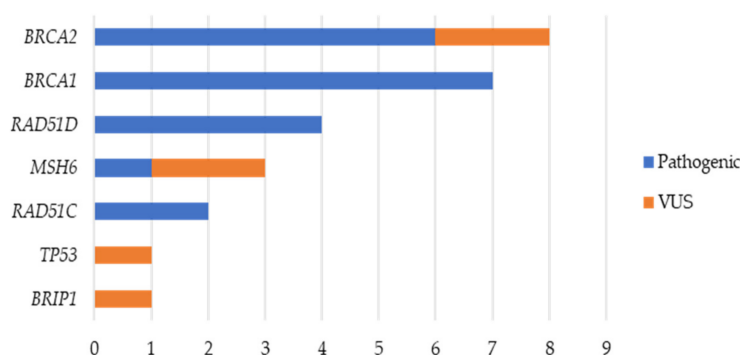


Figure 1. Spectrum of germline variants (pathogenic and variants of uncertain significance (VUS)).

Table 1. Germline variants identified among 95 ovarian cancer patients with next generation sequencing of a 10-gene panel.

Patient	Gene	HGVS Coding	HGVS Protein	VAF	ClinVar	MetaLR ¹	MetaSVM ¹	C-Score ¹	Varsome	Additional Tests	Personal History (Age ²)	Family History
OC09	BRCA1	c.2037delinsCC	p.(Lys679AsnfsTer4)	60%	Pathogenic	N/A	N/A	N/A	N/A	N/A	OC (46)	4 × OC, 2 × PaC, 1 × GC
OC11	BRIP1	c.2477A>G	p.(Asn826Ser)	74%	VUS	D	D	26.3	VUS	N/A	OC (39)	1 × CRC, 1 × L
OC12	BRCA1	c.211A>G	p.(Arg71Gly)	81%	Pathogenic	N/A	N/A	N/A	N/A	N/A	OC (58)	2 × BC, 1 × PC
OC16	RAD51C	c.709C>T	p.(Arg237Ter)	81%	Pathogenic	N/A	N/A	N/A	N/A	N/A	OC (57)	1 × OC
OC17*	RAD51D	c.748del	p.(His250ThrfsTer2)	68%	Pathogenic	N/A	N/A	N/A	N/A	N/A	OC (57)	2 × OC, 2 × BC, 1 × CRC
OC19	BRCA1	c.3331_3334del	p.(Gln1111AsnfsTer5)	81%	Pathogenic	N/A	N/A	N/A	N/A	N/A	OC (61)	4 × BC, 1 × OC
OC23	BRCA1	c.3817C>T	p.(Gln1273Ter)	92%	Pathogenic	N/A	N/A	N/A	N/A	N/A	OC (46)	6 × BC, 1 × CRC
OC24	MSH6	c.3848_3862del	p.(Ile1283_Tyr1287del)	45%	N/A	N/A	N/A	N/A	N/A	IHC: loss of expression	OC (51), UC (51)	2 × BC, 1 × CRC, 1 × LC
OC29	BRCA2	c.5073dup	p.(Trp1692MetfsTer3)	81%	Pathogenic	N/A	N/A	N/A	N/A	N/A	OC (48), BC (48)	3 × BC, 3 × PC, 3 × CRC
OC30*	RAD51D	c.748del	p.(His250ThrfsTer2)	81%	Pathogenic	N/A	N/A	N/A	N/A	N/A	OC (73)	2 × OC, 2 × BC, 1 × CRC
OC31	RAD51C	c.709C>T	p.(Arg237Ter)	77%	Pathogenic	N/A	N/A	N/A	N/A	N/A	OC (62)	1 × BC
OC36	BRCA2	c.8036A>G	p.(Asp2679Gly)	36%	CIP	D	D	31	LP	N/A	OC (59)	1 × CRC, 1 ×
OC37	MSH6	c.1729C>T	p.(Arg577Cys)	43%	VUS	D	D	34	VUS	IHC: normal; MSI: normal	OC (68)	1 × LC
OC39	BRCA1	c.3331_3334del	p.(Gln1111AsnfsTer5)	69%	Pathogenic	N/A	N/A	N/A	N/A	N/A	OC (63)	2 × BC, 1 × CRC
OC40	BRCA1	c.3331_3334del	p.(Gln1111AsnfsTer5)	58%	Pathogenic	N/A	N/A	N/A	N/A	N/A	OC (49), OC (50)	—
OC50	BRCA2	c.9364G>C	p.(Ala3122Pro)	88%	VUS	D	D	31	LP	N/A	OC (62)	1 × CRC, 1 × BC, 1 × PC
OC52	BRCA1	c.2037_2038insC	p.(Lys680GlnfsTer3)	54%	Pathogenic	N/A	N/A	N/A	N/A	N/A	OC (52)	2 × BC, 1 × PC
OC54	RAD51D	c.748del	p.(His250ThrfsTer2)	93%	Pathogenic	N/A	N/A	N/A	N/A	N/A	OC (48)	2 × BC, 1 × PC, 3 × CRC
OC55*	RAD51D	c.748del	p.(His250ThrfsTer2)	56%	Pathogenic	N/A	N/A	N/A	N/A	N/A	OC (54)	2 × OC, 2 × BC, 1 × CRC
OC60	BRCA2	c.7975A>G	p.(Arg2659Gly)	62%	Pathogenic	N/A	N/A	N/A	N/A	N/A	OC (56)	3 × BC, 1 × PaC, 1 × CRC
OC72	BRCA2	c.5073dup	p.(Trp1692MetfsTer3)	56%	Pathogenic	N/A	N/A	N/A	N/A	N/A	OC (60)	1 × PaC, 1 × PC
OC73	BRCA2	c.5073dup	p.(Trp1692MetfsTer3)	86%	Pathogenic	N/A	N/A	N/A	N/A	N/A	OC (44)	2 × BC, 1 × CRC
OC79	MSH6	c.3182T>C	p.(Leu1061Pro)	48%	VUS	D	D	24.8	VUS	IHC: loss of expression	OC (49)	1 × CRC
OC88	BRCA2	c.27>G	p.Met1?	93%	Pathogenic	N/A	N/A	N/A	N/A	N/A	BC (59), OC (69)	1 × BC, 1 × PC
OC91	TP53	c.845G>A	p.(Arg282Gln)	47%	VUS	D	D	35	LP	N/A	OC (58), TC (60), PaC (60)	1 × GC, 1 × CRC, BC
OC93	BRCA2	c.8488-1G>A	—	57%	Pathogenic	N/A	N/A	N/A	N/A	N/A	OC (65)	1 × BC, 1 × PaC

BC: Breast cancer; CIP: conflicting interpretations of pathogenicity; CRC: Colorectal cancer; GC: gastric cancer; IHC: Immunohistochemistry; L: Lymphoma; LC: Lung cancer; LP: Likely pathogenic; MSI: Microsatellite instability; N/A: not available; OC: ovarian cancer; PaC: Pancreatic cancer; PC: Prostate cancer; UC: uterine cancer; VAF: variant allele frequency; VUS: variant of unknown significance. * OC17, OC30, and OC55 belong to the same family. ¹ Missense variants were retained if they were predicted to be damaging (D) by MetaLR, MetaSVM, and CADD-score (>15). ² Age at diagnosis.

2.2.1. *BRCA1* and *BRCA2* Germline Variants

Of all patients included in this study, 91 (95.8%) were previously tested for *BRCA1* and *BRCA2* variants during clinical testing with BRCA Tumor MASTR™ Plus Dx, and 50 (52.1%) were part of the cohort of a previously published study [16]. Of these 91 patients, 15 had *BRCA1/BRCA2* germline variants, and all these variants were detected in this study using the custom panel (Table S1). Among the 95 OC patients included in this study, we identified 15 germline variants in *BRCA1/BRCA2* (seven in *BRCA1* and eight in *BRCA2*). All seven variants found in *BRCA1* were pathogenic, five frameshift, one nonsense, and one missense variant. Of the eight variants found in *BRCA2*, three were frameshift, one was a splice site variant at the canonical site -1, and four were missense variants. Of the four missense variants found in *BRCA2*, two were classified as pathogenic, and two were VUS (c.8036A>G and c.9364G>C). The *BRCA2* germline variant c.8036A>G, p.(Asp2679Gly), was identified in patient OC36, diagnosed with HGSOC at 56 years. This variant is a missense VUS predicted to have a deleterious impact on protein by in silico tools and is considered as likely pathogenic in Varsome. However, there is no family history of either breast or OC. Furthermore, this variant was included in a study aimed to assess the clinical relevance of *BRCA2* VUS missense variants and was classified as benign [17]. The variant *BRCA2* c.9364G>C, p.(Ala3122Pro), was identified in patient OC50, diagnosed with HGSOC at 62 years. This variant is predicted to have a deleterious effect on protein by in silico tools and is classified as likely pathogenic in Varsome. In the patient's family there are four relatives who have been affected with cancer, namely breast, prostate, and colorectal cancer. However, this variant was classified as likely benign in a recent study aimed to classify missense VUS variants in *BRCA1* and *BRCA2* using a multifactorial likelihood model [18].

2.2.2. *RAD51C* and *RAD51D* Germline Variants

The nonsense variant c.709C>T, p.(Arg327Ter), was detected in *RAD51C* in a proband diagnosed with HGSOC at age of 57 (OC16), who had a first cousin diagnosed with OC (Figure 2a). This variant was also identified in patient OC31, whose family has a history of other cancer types, such as breast, gastric, throat, and leukemia (Figure 2b).

The *RAD51D* frameshift variant c. 748del, p.(His250ThrfsTer2), was found in patient OC54 with a family history of colorectal, breast, prostate, and gastric cancers (Figure 3a). This variant was also detected in three patients (OC17, OC30, and OC55) belonging to the same family (seemingly unrelated to the previous), in which there is a history of breast and colorectal cancer (Figure 3b).

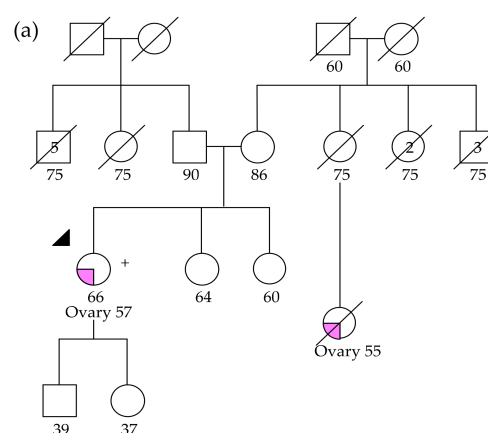


Figure 2. Cont.

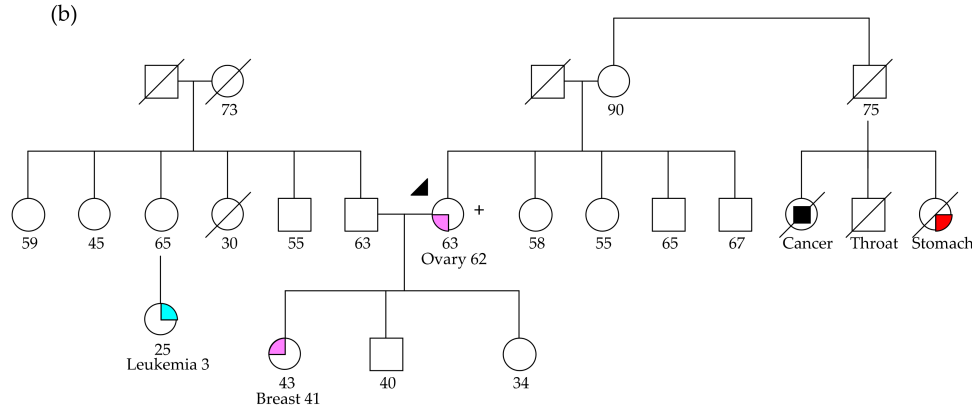


Figure 2. Pedigrees of patients with the *RAD51C* c.709C>T germline variant: (a) OC16 and (b) OC31. The index patients are indicated by an arrow.

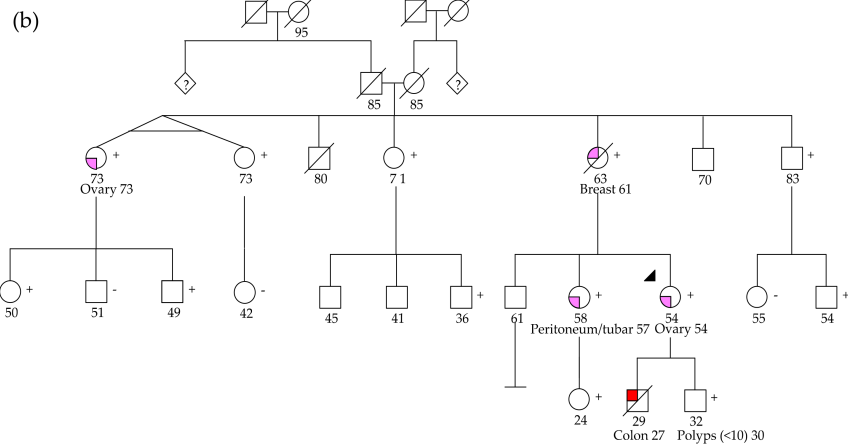
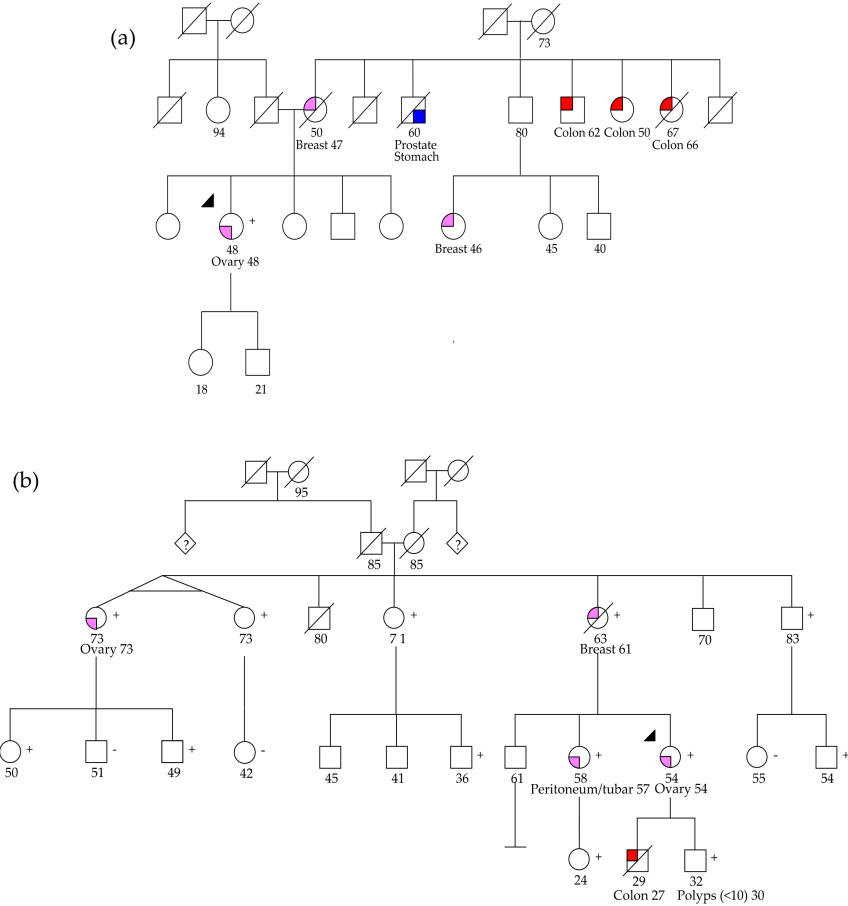


Figure 3. Pedigrees of patients with *RAD51D* c.748del germline variant. Family of patient OC54 (a) and OC17, OC30, and OC55 (b). The index patient is indicated by an arrow. Plus (+) and minus (−) signals indicate a positive and a negative result for germline genetic testing, respectively.

2.2.3. MSH6 Germline Variants

The *MSH6* inframe variant c.3848_3862del, p.(Ile1283_Tyr1287del), was identified in patient OC24, a woman diagnosed with endometrioid OC and uterine cancer at 51 years old, whose family has a history of colorectal, breast, and liver cancer (Figure 4). There is no classification for this variant in ClinVar, but it is described as a causal variant in the UMD database (www.umd.be). Immunohistochemistry analysis was performed in the ovarian tumor of the patient and showed loss of MSH6 expression. The somatic variant *MSH6* c.4001+1G>T was also identified in this patient, presumably representing the second-hit leading to biallelic inactivation typical of most cancer predisposition tumor suppressor genes.

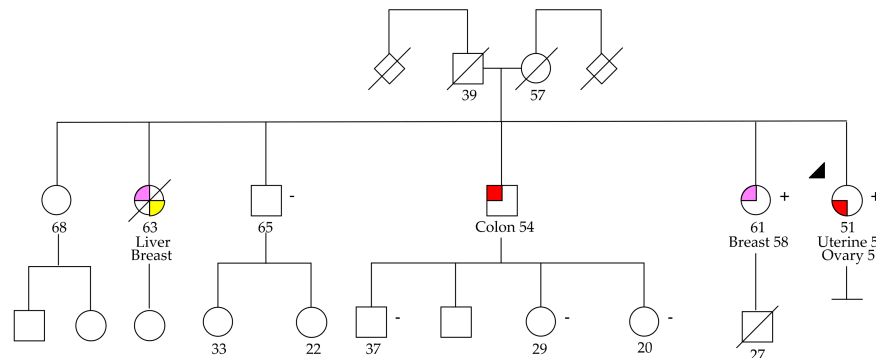


Figure 4. Pedigree of patient OC24 with the likely pathogenic *MSH6* c.3848_3862del germline variant. The index patient is indicated by an arrow.

Two *MSH6* missense VUS were detected in two patients. The *MSH6* c.1729C>T, p.(Arg577Cys), was identified in patient OC37 diagnosed with an ovarian carcinosarcoma at age 68. This variant was reported in ClinVar and in Varsome as uncertain significance, but it is predicted to be damaging by in silico tools. Immunohistochemistry and microsatellite instability analysis were performed in the tumor of the patient, and normal MSH6 expression and no microsatellite instability were observed, thus reducing the likelihood of the variant being associated with an increased risk for OC. The *MSH6* c.3182T>C, p.(Leu1061Pro) variant, was found in patient OC79, who was diagnosed with endometrioid OC at 49 years old and has two relatives affected by colorectal and skin cancer. In ClinVar, there is no classification for this variant, while in Varsome, it is classified as uncertain significance. However, immunohistochemistry analysis performed in the tumor of the proband showed loss of expression for MSH6, suggesting that this variant has a deleterious effect on the protein. Furthermore, we also identified in the OC the somatic variant *MSH6* c.1100A>G, p.(His367Arg), which is classified as pathogenic in ClinVar (reviewed by an expert panel) and could represent the second-hit leading to biallelic inactivation, but the scarce available data currently do not warrant the classification of the germline variant *MSH6* c.3182T>C other than a VUS.

2.2.4. BRIP1 Germline Variant

The *BRIP1* missense variant c.2477A>G, p.(Asn826Ser), was identified in patient OC11, diagnosed with HGSOE at the age of 39 years and family history of colon cancer and lymphoma. This variant is classified as VUS in ClinVar and in Varsome. However, it is predicted to be damaging by in silico tools

2.2.5. TP53 Germline Variant

The *TP53* missense variant c.845G>A, p.(Arg282Gln), was identified in patient OC91, diagnosed with pancreatic and thyroid cancer in addition to OC at age of 48. There is history of other cancers in the patient's family, such as stomach, colorectal, and bone cancer. This variant has conflicting interpretations of pathogenicity in ClinVar (five uncertain significance and two likely pathogenic

classifications). However, it was predicted to be damaging by in silico tools and is classified as likely pathogenic in VarSome.

3. Discussion

In this study, we aimed to evaluate the yield of germline variants using an NGS customized multi-gene panel for the analysis of ovarian carcinomas. Among the 95 OC patients analyzed, we found that 26 (27.4%) were carriers of rare germline variants in seven genes associated with genetic predisposition to OC (*BRCA1*, *BRCA2*, *BRIP1*, *MSH6*, *RAD51C*, *RAD51D*, and *TP53*). Of these 26 patients, 20 (77%) had pathogenic variants and six (23%) had missense VUS predicted to have a deleterious impact on the protein by in silico tools. Almost all patients included in this study were also tested for *BRCA1* and *BRCA2* somatic and germline variants using a clinically validated method [16], and this study was able to identify all 15 patients known to be carriers of deleterious *BRCA1/BRCA2* germline variants, which allowed validation of the custom panel we designed for those genes. The frequency of pathogenic *BRCA1* and *BRCA2* germline variants in this unselected series of OC patients was 13.7%, which is in concordance with previous studies that reported frequencies between 13% and 18% [12,16,19–21]. Furthermore, *BRCA1/BRCA2* tumor sequencing allows the detection of both germline and somatic variants in a single test, the latter having a frequency ranging from 4% to 7% [16,22,23]. Thus, about 20% of OC patients are likely to harbor germline or somatic *BRCA1/BRCA2* deleterious variants, which are predictive of response to PARPi. To initiate genetic testing of OC patients using a FFPE tumor sample is a strategy that has the potential to significantly decrease time and sequencing cost [11], as any clinically actionable variant detected in the initial NGS test can subsequently be evaluated by Sanger sequencing in a peripheral blood sample for its eventual constitutional origin after genetic counseling.

RAD51C and *RAD51D* are implicated in HR repair of DNA double-strand breaks, playing a key role in replication fork maintenance [24]. Germline variants in *RAD51C* and *RAD51D* are known to confer susceptibility to OC [25,26]. Studies on the prevalence of germline variants in these genes in OC vary widely, ranging between 0.57% and 2.5% for *RAD51C* and 0.16% and 0.57% for *RAD51D*, among patients unselected for age and family history [12,14,27–29]. For OC patients selected for family history, the prevalence of germline variants was found to be 1.27% and 0.88% for *RAD51C* and *RAD51D*, respectively [25,26]. The estimated relative risk for OC in carriers of germline variants in the *RAD51C* range from 5.88 to 7.55 [30,31] and from 6.30 to 7.60 for *RAD51D* germline variant carriers [26,31]. The National Comprehensive Cancer Network (NCCN) guidelines for breast and OC recommend that risk-reducing salpingo-oophorectomy (RRSO) should be considered beginning at age 40 to 50 years in women carriers of germline variants in these genes [32]. In this study, we identified two patients (2.1%) with a pathogenic variant in *RAD51C* and four patients (4.2%) with a pathogenic germline variant in *RAD51D*, together representing 30% of all pathogenic germline variants identified. The prevalence of germline variants in *RAD51C* in our cohort (2.1%) is in concordance with previous studies [12,28], while the observed frequency for *RAD51D* (4.2%) is higher than previously reported [26,29]. However, three of the patients with the germline variant in *RAD51D* turned out to be relatives, which may have spuriously increased the contribution of this gene in our study. Genetic counseling has already been offered to healthy relatives of this family, which so far allowed us to identify four female carriers of the germline variant, one of them already submitted to RRSO.

Germline variants in MMR genes (*MLH1*, *MSH2*, *MSH6*, and *PMS2*) are associated with Lynch Syndrome (LS) [33], which is characterized by predisposition to a spectrum of cancers such as stomach, ovary, pancreas, upper urologic tract, and brain tumors, in addition to the most prevalent colorectal and endometrial cancers [34]. A cumulative incidence of 13% at 75 years was observed for OC in carriers of *MSH6* germline variants [35], and the lifetime time risk was estimated to be up to 13.5% by age 70 [36,37]. We identified in one patient with both endometrial and ovarian cancer a *MSH6* germline inframe deletion, which is not described in the literature at the time of writing. Although this variant is predicted to lead to an inframe deletion of five aminoacids at the protein level,

we observed loss of MSH6 expression in the tumor by immunohistochemistry and detected a deleterious somatic *MSH6* mutation, presumably representing the second-hit leading to biallelic inactivation. Furthermore, this germline variant has been previously identified in our laboratory in another six index patients presenting clinical criteria for Lynch Syndrome, who also showed consistent MSH6 loss of expression in their tumors, allowing the classification of this variant as likely pathogenic. Because of this genetic diagnosis, the index patient was offered screening for additional tumors associated with Lynch syndrome. Furthermore, genetic counseling and presymptomatic genetic testing were offered to relatives, and carriers will be offered cancer surveillance recommended for Lynch syndrome, with consideration of bilateral salpingo-oophorectomy and hysterectomy after completed childbearing.

One of the side-effects of using larger NGS gene panels is the increased likelihood of finding VUS. On the other hand, it is important to report all additional information to improve variant classification, as some of the VUS may in fact be pathogenic and others may be benign, but with insufficient data to allow their classification either way. As shown in the Results section, we combined in silico, additional somatic mutation, protein expression, and segregation data to classify several germline missense variants, but six of them must remain classified as VUS with the information currently available. Of the two *BRCA2* VUS and two *MSH6* VUS we report here, the evidence favors the pathogenicity of one *MSH6* variant and the benign nature of the remaining three variants. The remaining two VUS were found in other cancer predisposing genes, namely *BRIP1* and *TP53*. *BRIP1* binds directly to the BRCT repeats of *BRCA1*, having an important role in the repair mechanism of double-strand DNA breaks [38]. Deleterious *BRIP1* germline variants have been described as conferring a moderately increased risk for OC [39], with an estimated relative risk of 3.41 [40], showing frequencies ranging between 0.9% and 1.36% [12,14,40]. Finally, *TP53* germline variants cause Li-Fraumeni syndrome, a relatively heterogeneous cancer predisposition syndrome characterized by increased risk of four core cancers (sarcoma, breast carcinoma, brain tumor, and adrenocortical carcinoma) [41], but known to increase the risk for other tumors, including OC [42]. The data we present here on these six VUS may contribute to improving their classification in the future and to clarify their eventual relevance for OC inherited predisposition.

The strategy we propose here using a 10 gene panel for routine OC NGS genetic testing on FFPE samples allowed us to identify 7.4% patients with pathogenic germline variants in genes other than *BRCA1* and *BRCA2*, namely *RAD51C*, *RAD51D*, and *MSH6*, significantly improving the yield of deleterious variants in OC predisposition genes to 21% of the patients tested, without increasing the sequencing costs. Besides the identification of women with inherited cancer predisposition, the use of the NGS multi-gene panel is also useful for the detection of pathogenic variants that are predictive biomarkers for targeted therapies. HR deficient tumors due to deleterious variants in genes other than *BRCA1* and *BRCA2* may be sensitive to PARPi [43–45]. In fact, one study showed that *RAD51C*-deficient cancer cells were highly sensitivity to Olaparib, significantly inhibiting tumor growth in a xenograft model [43], whereas Loveday and colleagues [26] showed that cells deficient in *RAD51D* were sensitive to Olaparib in a magnitude similar to the observed by silencing *BRCA2*. Furthermore, the Food and Drug Administration (FDA) recently approved Olaparib for the treatment of metastatic castration-resistant prostate cancer (mCRPC) patients with pathogenic germline or somatic variants in HR repair genes, including *RAD51C* and *RAD51D* [46]. However, the evidence that *RAD51C* and *RAD51D* variants are good predictive biomarkers of response to PARPi is still scarce. On the other hand, tumors with MMR deficiency display a phenotype of microsatellite instability, which is a predictive biomarker for immunotherapy [47], allowing the FDA approval of the use of pembrolizumab, an anti-PD1 therapy, for the treatment of solid tumors with high microsatellite instability (MSI-H) or MMR deficiency [48]. The use of multi-gene panel testing that allows the simultaneous detection of both somatic and germline variants in FFPE tumor samples may also be beneficial for the selection of patients to be allocated to clinical trials or for the use of off-label therapy, especially in those who experienced several disease relapses and have limited therapeutic options. Furthermore, since this

panel has a customizable design, new genes can easily be incorporated when new clinically actionable information becomes available.

4. Materials and Methods

4.1. Patients and Samples

The study cohort consisted of 96 women diagnosed with OC at the Portuguese Oncology Institute of Porto (IPO-Porto) from June 2016 to July 2019. The study was approved by the institutional review board (CI-IPOP-35-2016), and written informed consent was obtained from all patients. A FFPE tumor sample was obtained from each patient, after evaluation by a pathologist, who delimited areas with >50% cancer cells. Of all patients included in this study, 91 (95.8%) were previously tested in the clinical routine for *BRCA1* and *BRCA2* variants in tumor and blood using CE-IVD marked methodologies specific for those genes, and 50 patients (52.1%) were part of a study from our group that aimed to test the frequency of germline and somatic variants in a consecutive series of non-mucinous OC patients [16]. The clinicopathological features of OC patients included in the study are presented in Table 2. Patients were diagnosed with OC between 27 and 80 years (median 57 years of age), mostly at advanced stage (78.1% stage III-IV). All patients included in the study were recruited regardless of family history. However, three patients (OC17, OC30, and OC55) recruited at different times were known to belong to the same family when analyzing the results.

Table 2. Clinical features of the 96 patients included in the study.

Clinical Features		N (%)
Age, median (range years)		57 (27–80)
Figo Stage	Figo stage I	14 (14.6)
	Figo stage II	7 (7.3)
	Figo stage III	55 (57.3)
	Figo stage IV	20 (20.8)
Histological subtype	High-grade serous	72 (71.9)
	Low-grade serous	8 (8.3)
	Clear cell	6 (6.3)
	Endometrioid	7 (7.3)
	Mucinous	1 (1.0)
	Mixed	3 (3.1)
	Carcinosarcoma	2 (2.1)

4.2. DNA Extraction from FFPE Tumor Samples

DNA extraction from FFPE samples was performed using the Cobas[®] DNA Sample Preparation Kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol. Quality of DNA samples was measured with a Qubit[®] Fluorometer with the Qubit dsDNA HS assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

4.3. Next-Generation Sequencing and Bioinformatic Analyses

NGS was performed in 96 tumor samples using a customized QIASeq Targeted DNA Panel containing ten genes (*BRCA1*, *BRCA2*, *BRIP1*, *MLH1*, *MSH2*, *MSH6*, *PMS2*, *RAD51C*, *RAD51D*, and *TP53*) (QIAGEN, Antwerp, Belgium). These genes were selected according to international guidelines for OC screening and/or previously description in the literature as conferring a risk for the development of OC. Library preparation was performed according to the manufacturer's instructions, and final libraries were quantified on a 4200 TapeStation System (Agilent Technologies Inc, Santa Clara, CA, USA). Sequencing was carried out using a high-output kit in the NextSeq 550 System (Illumina, Inc., San Diego, CA, USA) in a 2 × 151 bp paired-end run. Sequencing alignment and variant calling were

performed using smCounter2 (QIAGEN, Antwerp, Belgium) [49], and the resulting .vcf files were imported to GeneticistAssistant™ software (SoftGenetics, LLC, State College, PA, USA) for variant annotation. All variants with minor allele frequency (MAF) greater than 1% and synonymous variants were excluded. For MAF filtering, data were obtained from the 1000 Genomes Project (1000G), Genome Aggregation Database (gnomAD), and Exome Aggregation Consortium (ExAC) databases. To screen germline variants, we used the variant allele frequency (VAF) as recommend by ASCO/AMP/CAP guidelines [15] using VAF above 30% as the cutoff, which was shown to be reliable in a previous study of our group [16]. In fact, since the present study is focused on testing tumor suppressor genes, which very often show loss of heterozygosity in the tumor, the VAFs of deleterious germline variants are actually expected to be higher than 50%, which is supported by our earlier finding of mean VAFs of 80% and 69% for *BRCA1* and *BRCA2* pathogenic germline variants, respectively, with the lowest VAF being 49% [16].

4.4. Variant Classification

All variants remaining after variant filtering that had been described as pathogenic/likely pathogenic in ClinVar or having literature evidence supporting their pathogenicity were retained as deleterious. Nonsense, frameshift, inframe deletion and/or insertion, as well as canonical splice site variants, were also retained since they are considered to have very strong evidence of pathogenicity [15]. The potential pathogenicity of missense VUS was evaluated using MetaLR and MetaSVM, which combine 10 in silico prediction tools (SIFT, PolyPhen-2 HDIV, PolyPhen-2 HVAR, GERP++, MutationTaster, Mutation Assessor, FATHMM, LRT, SiPhy, and PhyloP) and the maximum minor allele frequency (MMAF) from the 1000G project [50]. We also used Combined Annotation-Dependent Depletion (CADD), which is an integrative annotation built from more than 60 genomic features for scoring the deleteriousness of single nucleotide variants (SNVs) and insertion/deletion variants [51]. Missense VUS were retained only if they were predicted to be damaging by MetaLR, MetaSVM, and CADD. The potential clinical significance of germline VUS was also evaluated using the VarSome approach [52], which is a bioinformatic tool for clinical interpretation of variants according to the American College of Medical Genetics and Genomic (ACMG) and the Association for Molecular Pathology (AMP) guidelines. Variants described in ClinVar as benign/likely benign were classified as benign and discarded.

4.5. Germline Variants Validation

Variants suspected to be germline were confirmed by Sanger sequencing in peripheral blood samples. DNA was extracted from blood samples using a standard protocol. Sanger sequencing was performed in a 3500 Genetic Analyzer (Applied Biosystems Foster City, CA, USA) using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), following the manufacturer's instructions.

4.6. Microsatellite Instability and Immunohistochemical Analysis

Microsatellite instability analysis was performed using the Bethesda panel of markers (BAT25, BAT26, D2S123, D5S346, and D17S250) and according to the 1997 National Cancer Institute Guidelines, as previously described [53,54]. Assessment of MSH6 protein immunoexpression was performed as previously described [55].

5. Conclusions

We conclude that the strategy of reflex gene-panel tumor testing enables the identification of clinically actionable germline variants in a significantly higher proportion of OC patients, while at the same time testing for somatic variants in the same genes, which may be valuable information in patients with advanced disease that have run out of approved therapeutic options. Furthermore, this approach increases the chance to make available genetic counseling, presymptomatic genetic

testing, and gynecological cancer prophylaxis to female relatives who turn out to be healthy carriers of deleterious germline variants.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2072-6694/12/10/2834/s1>, Table S1: comparison of results obtained by the custom panel and by a clinically validated test regarding the BRCA1/BRCA2 deleterious variants.

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PAPER III

Next generation sequencing of tumor and matched plasma samples: identification of somatic variants in ctDNA from ovarian cancer patients

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Abstract

Genetic testing for the detection of somatic alterations is usually performed on formalin-fixed paraffin-embedded (FFPE) tumor samples. However, tumor molecular profiling through noninvasive ctDNA analysis may be particularly interesting with the emergence of targeted therapies for ovarian cancer (OC), mainly in situations in which tumor is not available and biopsy is not viable, also allowing representation of multiple neoplastic subclones in cases of intratumor or intertumor heterogeneity. Using a custom panel of 27 genes, next-generation sequencing (NGS) was performed on tumor and matched plasma samples from 96 OC patients, which were combined in two groups (treatment naïve and post-treatment). Overall, at least one somatic variant present in the FFPE tumor sample was also detected in the matched plasma sample in 35.6% of the patients, a % that increased to 69.6% of the treatment naïve patients and 83.3% of those with stage IV disease, clearly showing the potential of ctDNA NGS analysis as a noninvasive alternative to identify somatic variants in these patients, namely those that have predictive value for targeted therapy. In fact, of the two treatment-naïve patients with somatic *BRCA1* variants identified in FFPE tumor samples, we could detect in ctDNA a *BRCA1* frameshift somatic variant that was present in the tumor sample with a VAF of 53%, but not the one that had a VAF of only 5.4%. We also showed that ctDNA analysis can overcome the problem of inter- and intratumor heterogeneity, as exemplified by one patient diagnosed with bilateral ovarian tumors in which different somatic variants from both tumors were detected in ctDNA. Interestingly, as these bilateral tumors shared a rare combination of two of the three variants identified in the ctDNA, we could conclude that the two morphologically different tumors were clonally related and not synchronous independent neoplasias. Moreover, in the post-treatment group of patients, those with somatic variants detected in the plasma sample collected after surgery had poor prognosis when compared with patients with no detectable somatic variants after surgery, highlighting the potential of ctDNA analysis for the identification of patients at higher risk of recurrence. In conclusion, this study demonstrated that somatic variants in genes relevant to OC can be detected in plasma samples of a significant proportion of OC patients, supporting the use of NGS-based ctDNA testing for noninvasive tumor molecular profiling for targeted therapy and to stratify patients according to prognosis.

Introduction

Ovarian cancer (OC) is the third most common malignant disease among patients with gynecological neoplasia, being the eighth most common cancer and the eighth most common cause of cancer-related death in women [1]. OC is characterized by few and unspecific symptoms, late diagnosis, and poor survival. During the last decades, several efforts have been done to improve the outcomes for OC patients, through the development of new therapies. Since 2014, the treatment of OC has been improved with the emergence of the poly ADP-ribose polymerase inhibitors (PARPi) [2-5]. With the PARPi approval, BRCA status became an important predictor of response and susceptibility to this class of drugs in addition to family risk assessment. Therefore, tumor genetic testing in OC patients can provide predictive information to guide treatment with PARPi. Consequently, genetic testing (somatic and germline) for all women diagnosed with OC is recommended [6]. Comprehensive genetic testing is usually performed in formalin fixed paraffin embedded (FFPE) tumor samples and blood samples are used to verify whether the variants found in tumor are germline or somatic and to look for germline exon rearrangements [7]. FFPE tumor samples are often used for molecular profiling, as it is widely available and easy to use and to store. Furthermore, using these samples it is possible to select only tumor area for sequencing and to avoid contamination with normal tissue, which improves the sensitivity to detect somatic variants. However, the DNA extracted from FFPE samples is sometimes of poor quality and not suitable for molecular analysis [8], and may not constitute an ideal source for an accurate genetic characterization when inter- and intra-tumoral heterogeneity is present [9]. Furthermore, due to its invasive nature and associated risks, it is difficult to obtain additional tumor biopsies over the course of the disease. In the context of OC, there are other challenges, such as the difficulty of performing tumor biopsy due to anatomical limitations or risk of spillage [10], so the majority of tumor samples from OC patients are obtained at the time of the surgery. However, these samples may not be the most suitable for tumor molecular profiling, as several OC patients are treated with chemotherapy before debulking surgery, which can modify the tumor genetic profile as the result of mutational shifts induced by chemotherapy [7, 11].

Circulating-cell free DNA (cfDNA) consists of small fragments of DNA that are released by the cells believed to be through apoptotic and necrotic processes [12]. In cancer patients, a fraction of cfDNA is derived from tumor cells, therefore containing the associated genetic alterations, being named circulating cell-free tumor DNA (ctDNA) [13]. In the last decades, several studies showed that ctDNA has a great potential as a biomarker in several types of cancer [14]. The use of ctDNA, called liquid biopsy, in the management of cancer patients has several advantages as an alternative for tissue biopsies. First, liquid biopsy provides a

better representation of the overall tumor genome since it comprises DNA released by tumor cells from both primary tumor and metastases, minimizing the problem of inter- and intratumor heterogeneity [15]. Second, it is possible to quantify cfDNA and its concentration seems to reflect the tumor burden, making cfDNA measurements potentially useful to monitor tumor dynamics [16]. Third, blood samples are minimally invasive, allowing repeated sampling and “real-time” monitoring during the entire course of the disease. On the other hand, ctDNA is highly fragmented and its fraction in circulation can be as low as 0.01%, making its detection difficult, particularly in early-stage tumors [17]. Therefore, ctDNA detection requires high-sensitive approaches.

The potential of liquid biopsy in OC management has gained increasing attention and has several possible applications, including diagnosis, prognosis, evaluation of therapy response, monitoring the emergence of resistance during the course of treatment and disease relapse prediction. The vast majority of published studies in OC focused on the quantification of total levels of cfDNA in plasma or serum of OC patients [18]. However, the detection of mutations in ctDNA is expected to be more specific for those applications. Here, we aimed to evaluate the sensitivity of detection of tumor somatic variants in plasma samples from OC patients by comparing next generation sequencing (NGS) of FFPE tumor samples and paired cfDNA from 96 OC patients.

Material and methods

Patients and samples

This study included 96 tumor samples and 96 matched plasma samples from 96 women diagnosed with OC, collected between 2016 and 2019 at the Portuguese Oncology Institute of Porto (IPO-Porto). The study was approved by the institutional review board (CI-IPOP-35-2016) and written informed consent was obtained from all patients. FFPE tumor samples were obtained from all patients, after evaluation by a pathologist, who delimited areas with >50% cancer cells. From each patient, peripheral blood samples were obtained for plasma collection. Twenty-three plasma samples were collected from newly diagnosed patients, before any treatment. Six samples were collected after surgery and before treatment with chemotherapy. Twelve samples were collected at recurrence. The remaining 53 plasma samples were collected when patients had already received at least one cycle of chemotherapy, and 6 of these were collected at progression during treatment. For subsequent analysis, the samples were combined in two groups: treatment naive group and post-treatment group. The first includes patients from whom tumor and plasma samples were collected at diagnosis, before any treatment (n=23). In the second group includes those patients from whom plasma samples were obtained after treatment (n=73). All

patients included in this study were previously tested for germline variants in 10 genes in a study from our group that aimed to test the yield of germline variants using FFPE samples for NGS [19]. The clinicopathological features of OC patients included in the study are presented in Table 1.

DNA extraction

DNA extraction from FFPE samples was performed using the Cobas® DNA Sample Preparation Kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol. Quality of DNA samples was measured using the Qubit® Fluorometer with the Qubit dsDNA HS assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Peripheral blood samples collected in BD Vacutainer K₂EDTA (Becton Dickinson, Franklin Lakes, NJ, USA) tubes and plasma samples were obtained by centrifugation within 2 hours of the blood collection. We performed a two-step centrifugation, the first one 10 minutes at 1600g at room temperature. After centrifugation, the supernatant was collected and centrifuged 10 minutes at 6000g at room temperature, to remove remaining cells. Plasma supernatant was transferred to 1.5mL tubes and stored at -80°C until use. DNA was extracted from 3mL of plasma samples using the QIAamp circulating nucleic acid kit following the manufacturer's protocol (QIAGEN, Antwerp, Belgium).

Next generation sequencing and bioinformatic analysis

Next-generation sequencing (NGS) was performed in 96 tumor samples and matched plasma samples using a customized QIASeq Targeted DNA Panel containing 27 genes (QIAGEN, Antwerp, Belgium) previously described as frequently mutated in OC in the literature and COSMIC database (Table S1 – Supplementary data) [20-23]. Library preparation was performed according to the manufacturer's instructions and final libraries were quantified on a 4200 TapeStation System (Agilent Technologies Inc, Santa Clara, CA). Sequencing was carried out using a high-output kit in the NextSeq 550 System (Illumina, Inc., San Diego, CA, USA) in a 2x151 bp paired-end run. Sequencing alignment and variant calling were performed using Qiagen GeneGlobe Data Analysis Center (QIAGEN, Antwerp, Belgium). For plasma samples, sequencing alignment and variant calling was also performed using CLC Genomics Workbench 21 (CLC Bio, version 21.0.3). The resulting .vcf files were imported to GeneticistAssistant™ software (SoftGenetics, LLC, State College, PA, USA) for variant annotation. All variants with a minor allele frequency (MAF) greater than 1% and synonymous variants were excluded. For MAF filtering, data were obtained from the 1000 Genomes Project (1000G), Genome Aggregation Database

(gnomAD) and Exome Aggregation Consortium (ExAC) databases. Additionally, for tumor samples we excluded variants with allele frequency (VAF) lower than 5%.

Variant analysis

After variant filtering, all the variants that had been described as pathogenic/likely pathogenic in ClinVar or having literature evidence supporting their pathogenicity were retained as deleterious. Nonsense, frameshift, as well as canonical splice site variants, were also retained since they are considered to have very strong evidence of pathogenicity [24]. Given that the aim of this study is the analysis of detection of somatic variants in ctDNA, inframe deletion and/or insertion variants not classified were also retained. The potential pathogenicity of missense VUS was evaluated using MetaLR and MetaSVM, which combine 10 in silico prediction tools (SIFT, PolyPhen-2 HDIV, PolyPhen-2 HVAR, GERP++, MutationTaster, Mutation Assessor, FATHMM, LRT, SiPhy, and PhyloP) and the maximum minor allele frequency (MMAF) from the 1000G project [25]. We also used Combined Annotation-Dependent Depletion (CADD), which is an integrative annotation built from more than 60 genomic features for scoring the deleteriousness of single nucleotide variants (SNVs) and insertion/deletion variants [26]. Missense VUS were retained only if they were predicted to be damaging by MetaLR, MetaSVM, and CADD. Variants described in ClinVar as benign/likely benign were classified as benign and discarded.

Validation of germline and somatic variants

Variants present with a VAF at nearly 50% in plasma samples, suspected to be germline, were confirmed by Sanger sequencing in peripheral blood samples. Somatic variants were confirmed by Sanger sequencing in DNA extracted from FFPE samples. Sanger sequencing was performed in a 3500 Genetic Analyzer (Applied Biosystems Foster City, CA, USA), using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), following the manufacturer's instructions.

Results

Identification of somatic variants in tumor samples

Ninety-six FFPE samples were analyzed by NGS (mean UMI depth =1276). A total of 7255 variants were annotated and classified among all the samples with exception of cases OC65 and OC85 that did not pass the quality filters. After variant filtering, 335 variants remained, of which 99 were classified as pathogenic/likely pathogenic in ClinVar. Among variants not classified in ClinVar, 41 variants were nonsense, frameshift and variants located at canonical ± 1 or 2 splice sites and were therefore considered pathogenic [24].

Eight variants were inframe deletions and/or insertions that were also retained. The remaining 187 variants were missense alterations whose potential pathogenicity was evaluated through in silico prediction tools and 61 variants predicted to have deleterious impact on the protein were retained. After variant analysis, 209 variants were retained. At least one variant per tumor sample was identified, except in three cases (OC13, OC48 and OC63), in which no variants were identified. In addition to the 26 variants identified as germline in our previous study [19], 11 variants with a VAF at nearly 50% in plasma samples were tested in peripheral blood samples and were confirmed to be germline. Excluding the 37 germline variants, we retained 172 somatic variants, among 90 patients and across 23 of the 27 genes included in the panel (Figure 1). *TP53* variants were identified in 72 (78.0%) of the 94 samples, being the most prevalent mutated gene, followed by *BRCA1*, *ARID1A*, and *KRAS* in which variants were found in 9 (9.6%) samples each. Variants in *PIK3CA* were detected in eight (8.5%) samples, followed by *PTEN* and *RB1* that were found mutated in six (6.4%) samples each. Variants in *BRCA2*, *ATM*, *ATR* and *MSH6* were identified in five (5.3%) samples each. For the remaining genes, a frequency of mutations equal or less than two percent was observed.

Mutational concordance of tumor and plasma samples

We next analyzed 96 plasma samples by NGS (mean UMI depth = 513). Using Qiagen GeneGlobe Data Analysis Center, a total of 3414 variants were annotated and classified among the 96 plasma samples. After variant filtering, 239 variants remained, of which 43 were classified as pathogenic/likely pathogenic in ClinVar. Among variants not classified in ClinVar, 39 were nonsense, frameshift and variants located at canonical ± 1 or 2 splice sites, and were therefore considered pathogenic [24]. Five variants were inframe deletions and/or insertions that were also retained. The remaining 150 variants were missense alterations, of which 43 were retained because they were predicted to have deleterious impact on the protein by in silico tools. After variant analysis, we retained 130 variants, of which 37 were germline variants previously identified, which were excluded. Thus, 93 somatic variants were identified in plasma samples among 51 patients, with an average of two variants in each plasma sample. The overall mutational concordance between tumor and matched plasma samples was 26.7%. Considering only the group of untreated patients, at least one somatic variant was detected in matched plasma sample in 13 patients (56.5%). The detection rate of somatic variants in plasma samples was higher in patients with stage IV disease (83.3%). In patients diagnosed with stage III and stage I disease, somatic variants were detected in plasma samples in 46.2% and 50% of the cases, respectively. Comparing with tumor testing of the post-treatment patient group, at least one somatic

variant was detected in matched plasma sample in 17.9% of them. In six patients with plasma samples collected after surgery and before treatment with chemotherapy, none of the variants identified in tumor samples were detected in the matched plasma samples. In a subgroup of 12 patients from whom plasma samples were collected during follow-up at the time of disease recurrence, somatic variants were detected in 25% of the cases.

In an attempt to improve the sensitivity to detect somatic variants in plasma, we performed the bioinformatic analysis using the CLC Genomic Workbench, setting 0.2% as the minimum VAF. A total of 21232 variants were annotated and classified among the 96 plasma samples. After variant filtering 14280 variants remained, of which 1328 were classified as pathogenic/ likely pathogenic in ClinVar. Among the 12952 variants not classified, 2794 were nonsense, frameshift and variants located at canonical ± 1 or 2 splice sites which were considered pathogenic variants [24]. We also retained 1544 inframe deletion and/or insertion variants. The remaining 8614 were missense alterations, of which 2104 were retained as they were predicted to be deleterious by in silico tools. After variant analysis, 7770 variants were identified, of which 37 germline variants were excluded. Thus, 7733 somatic variants were identified among 96 plasma samples. On average, 80 variants were identified in each plasma sample. The overall mutational concordance between variants detected in tumor and matched plasma samples increased to 35.6%. Considering the group of untreated patients, the concordance between tumor and plasma samples for at least one somatic variant was 69.6%. Regarding patients diagnosed with advanced stage disease, at least one somatic variant initially identified in the tumor testing was detected in plasma samples of 83.3% and 61.5% of stage IV and stage III patients, respectively. Among four patients with early-stage disease (stage I), detectable tumor somatic variants were found plasma samples in three of them. In some patients with more than one shared variant between tumor and plasma sample, the hierarchy of VAF was concordant (Figure 2). Of the 14 patients with *BRCA1/BRCA2* somatic variants identified in tumor samples, two were from the naive treatment group (OC04 and OC28). These patients had a *BRCA1* frameshift somatic variant detected in tumor samples with a VAF of 5.4% and 53%, respectively. In patient OC28 the *BRCA1* somatic variant was also identified in the matched plasma sample at a VAF of 0.7%. Regarding the 67 patients of the post-treatment group, at least one somatic variant present in the tumor was detected in the plasma samples in 22.4% of the patients. In seven of these patients, in whom plasma samples were collected after surgery and before treatment with chemotherapy, somatic variants were detected in 42.9% of plasma samples. In 12 patients in whom plasma samples were collected at the time of disease recurrence, somatic variants were detected in 33.3% of the plasma samples.

Accessing inter- and intra-tumor heterogeneity in cfDNA

Performing the reverse analysis, in five patients belonging to the treatment naive group we detected pathogenic variants in cfDNA that were not previously identified in the tumor sample. For these patients, FFPE samples from metastasis and/or tumor from a different anatomic location were obtained. In patient OC05, a *CTNNB1* pathogenic variant (c.110C>G) was identified in the plasma sample with a VAF of 0.8%, which was not detected previously in the tumor sample (right ovary). This variant was further detected in the tumor sample from a different anatomic site (left ovary) in addition to *PIK3CA* and *ARID1A* variants previously identified in the tumor from the right ovary (Figure 3). Regarding the other four patients, the variants detected only in the plasma samples were not detected in the analyzed tumor samples from other anatomic sites.

Discussion

The analysis of ctDNA for tumor molecular profiling represents a potential and attractive alternative to that of FFPE tumor samples for the detection of actionable variants. However, the fraction of ctDNA can be extremely low in a high background of normal cfDNA, so that somatic variants may be present at very low VAF that require extremely sensitive techniques for their detection. Although PCR based methods, such as droplet digital PCR (ddPCR), are very sensitive, they have a low throughput on variant analysis and can only be used to screen for known variants. On the other hand, broader approaches, such as NGS, allow a more complete tumor molecular profiling, enable assessment of inter-tumor and intra-tumor heterogeneity and clonal evolution of the tumor, as well as identification of resistance mechanism to targeted therapy [9, 27-29]. Furthermore, recent advances, such as the introduction of unique molecular identifiers (UMIs) and adequate bioinformatic tools, can also help to increase the sensitivity and specificity [30].

The aim of this study was to evaluate the potential of cfDNA to allow detection of somatic tumor driver variants in OC patients, using a NGS approach with a custom gene panel to analyze 96 tumors and matched plasma samples from 96 patients. Initially, the bioinformatic analysis of tumor and plasma samples was performed using GeneGlobe, a web resource for the analysis of Qiagen's target enrichment panels. However, this is a closed platform that does not allow to define the settings for sequencing alignment and variant calling, including the minimum VAF, which is set at 0.5%. This is suitable for the analysis of FFPE tumor samples as we are looking for variants with a VAF above 5%. However, as somatic variants in ctDNA are present at allele frequencies below the cut-off defined in GeneGlobe, we tested a software that allows to adapt the settings for variant calling in ctDNA analysis. Thus, we reanalyzed plasma samples using CLC Genomics

Workbench, which allowed us to lower the minimum VAF to 0.2%, and as expected the overall detection rate of somatic variants in ctDNA improved from 26.7% to 35.6%. Using the most suitable software for ctDNA analysis, at least one somatic variant was detected in ctDNA from 69.6% of the patients from the naïve treatment group and in 83.3%, 61.5% and 75% of patients diagnosed with stage IV, stage III and stage I disease, respectively. Furthermore, of the two treatment-naïve patients with somatic *BRCA1* variants identified in FFPE tumor samples, we could detect in ctDNA a *BRCA1* frameshift somatic variant that was present in the tumor sample with a VAF of 53%, but not the one that had a VAF of only 5.4%. These findings support the potential of ctDNA NGS analysis for OC somatic genetic testing, especially when there is no tumor sample available for predictive genetic testing for targeted therapy. Our results are in agreement with those of a previous study by Phallen and colleagues [31], in which ctDNA alterations were detected in 71% of OC patients, including 75% and 83% of stage III and IV patients. Furthermore, as somatic variants were detected in a great proportion of patients diagnosed with stage I disease, ctDNA analysis also holds the promise to detect early-stage disease, namely as a screening test in healthy women carrying pathogenic germline variants in OC inherited predisposition genes. Recently, Cohen and colleagues [27] reported the development of a blood test based on liquid biopsy that can detect multiple types of cancer at earlier stages, including OC, and which combines the detection of variants in driver genes with the levels of circulating biomarker proteins to detect the presence of a cancer and to determine its origin. The median sensitivity of the test was 73% and 43% for stage II and stage I cancers, respectively.

In addition to noninvasive diagnosis and targeted therapy biomarker identification, ctDNA analysis also holds the promise to be used as a biomarker for prognosis, evaluation of therapy response, monitoring the emergence of resistance during treatment, and disease relapse prediction. This is well exemplified by our finding of somatic variants in ctDNA in the six patients belonging to the post-treatment group, from whom plasma samples were collected after surgery and before chemotherapy. All three patients with detectable somatic variants in plasma sample collected after surgery experienced disease relapse (and two of them have already died), while those patients with no detectable somatic variants in plasma after surgery are still disease-free. This suggests that ctDNA analysis can help detect microscopic residual disease after surgery with the potential to be used as prognostic biomarker for OC, allowing the identification of patients at higher risk of recurrence.

One of the advantages of cfDNA is the ability to integrate the detection of somatic alterations from both primary tumor and metastatic lesions, overcoming the problem of intra-tumor and inter-tumor heterogeneity. Our results demonstrated that tumor driver variants detected in ctDNA were not always identified in the FFPE sample initially tested. For instance, in a

plasma sample from a patient of the naive treatment group (OC05), in addition to the two variants previously detected in the tumor sample, we detected a *CTNNB1* pathogenic variant with a VAF of 0.8%. This patient was diagnosed with clear cell carcinoma (CCC) of the right ovary (the tumor initially tested with NGS) and poorly differentiated squamous cell carcinoma of the left ovary. To assess the possibility that this discrepancy may be caused by inter-tumor heterogeneity, we analyzed a tumor sample from the left ovary by Sanger sequencing. We confirmed that the *CTNNB1* variant detected in the plasma sample was derived from the tumor of the left ovary, in which we also detected the two variants identified in the tumor from the right ovary. Since these bilateral tumors shared a rare combination of two of the three variants identified in the ctDNA, we could conclude that the two morphologically different tumors were clonally related and not synchronous independent neoplasias, possibly originated from an endometriosis focus that was observed in the periphery of both tumors, which is a well-established precursor lesion of CCC [32]. The additional variant found in the tumor of the left ovary probably represents clonal divergence that may at least in part explain the different phenotype of the two tumors (CCC vs squamous cell). This observation highlights the ability to detect somatic variants in ctDNA that may be missed by analysis of FFPE samples, confirming that both sample types have complementary roles in managing cancer patients.

The somatic variants not detected in plasma samples may be present with a VAF below 0.2% and would likely be detected using a more sensitive approach. For the detection of variants at VAF as low as <0.2% by NGS, deep sequencing through sequencing the target regions with high coverage (>10,000x) can lower the percentage of false positives [33]. Furthermore, most of the cases with no somatic variants detected in ctDNA were those in which only one somatic variant was identified in the tumor sample. Including more genes in the NGS panel could increase the sensitivity, however it would also increase the sequencing costs. In the custom gene panel used in this study, we included genes described in the literature and the COSMIC database as frequently mutated (>10%) in OC. As OC is subclassified in five histological subtypes, which are characterized by different patterns of genetic alterations, it was necessary to include a relatively large number of genes to represent each subtype, resulting in a 27 gene panel. *TP53* variants were highly prevalent in this study (78.0%), as expected given that the majority of FFPE tumor samples were from HGSOC and this tumor type is characterized by *TP53* variants [20]. The remaining genes were found mutated in a frequency below 10% because they are associated with the other OC histological subtypes, which are less common and underrepresented in our cohort. Smaller panels specific for each histological subtype would reduce the sequencing costs and would help to improve the sequencing coverage and the sensitivity to detect low frequency variants. On the other hand, since several OC tumor types are relatively rare, it

may not be practical to have sequencing runs with only rare histological subtypes. One advantage of using a custom gene panel is the flexibility to manage the genes to include according to the purpose of the study.

Technical and biological factors can impact the concordance between NGS findings in tumor and plasma samples, eventually associated with false-negative and false-positive results for each sample type. False negatives might be explained by the limited amount of ctDNA extracted from plasma samples, which limits the detection of variants with low allele frequency. Weber and colleagues [17], reported that lower DNA input was associated with a decrease of variant calling precision. In our study, the limited amount of DNA extracted from plasma samples (average of 1.3 ng/ μ L), and consequently a DNA input lower than recommended (average of 17.7 ng), might have been a limitation for the detection of low frequency variants. The DNA input amount influences the number of unique molecular identifiers (UMIs) captured from the original DNA sample, and therefore the sequencing depth, which directly impacts the variant detection sensitivity. False positive results are another challenge when multiple variants are detected by NGS platforms, which has led to the implementation of strategies such as molecular barcodes in order to reduce errors introduced during library preparation [34].

In conclusion, this study demonstrated that somatic variants in genes relevant to OC can be detected in plasma samples of a significant proportion of OC patients, supporting the use of NGS-based ctDNA testing for noninvasive tumor molecular profiling for targeted therapy and to stratify patients according to prognosis.

Author Contributions

AB, PP, AP, JG, MP performed experiments. AB and PP analyzed data. AP, CB and RS provided samples. AB wrote the manuscript. AB and ABr created the plots. JS performed genetic counseling. MT designed and supervised the study. All the authors critically reviewed and approved the manuscript.

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Ethics Statement

Ethical reviewed and approved. The patients/participants provided their written informed consent to participate in this study.

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Tables

Table 1. Clinical features of the 96 patients included in the study.

Clinical features		N (%)
Age, median (range years)		57 (27-80)
Figo Stage	Figo stage I	14 (14.6)
	Figo stage II	7 (7.3)
	Figo stage III	55 (57.3)
	Figo stage IV	20 (20.8)
Histological subtype	High-grade serous	72 (71.9)
	Low-grade serous	8 (8.3)
	Clear cell	6 (6.3)
	Endometrioid	7 (7.3)
	Mucinous	1 (1.0)
	Mixed	3 (3.1)
	Carcinosarcoma	2 (2.1)

Figures

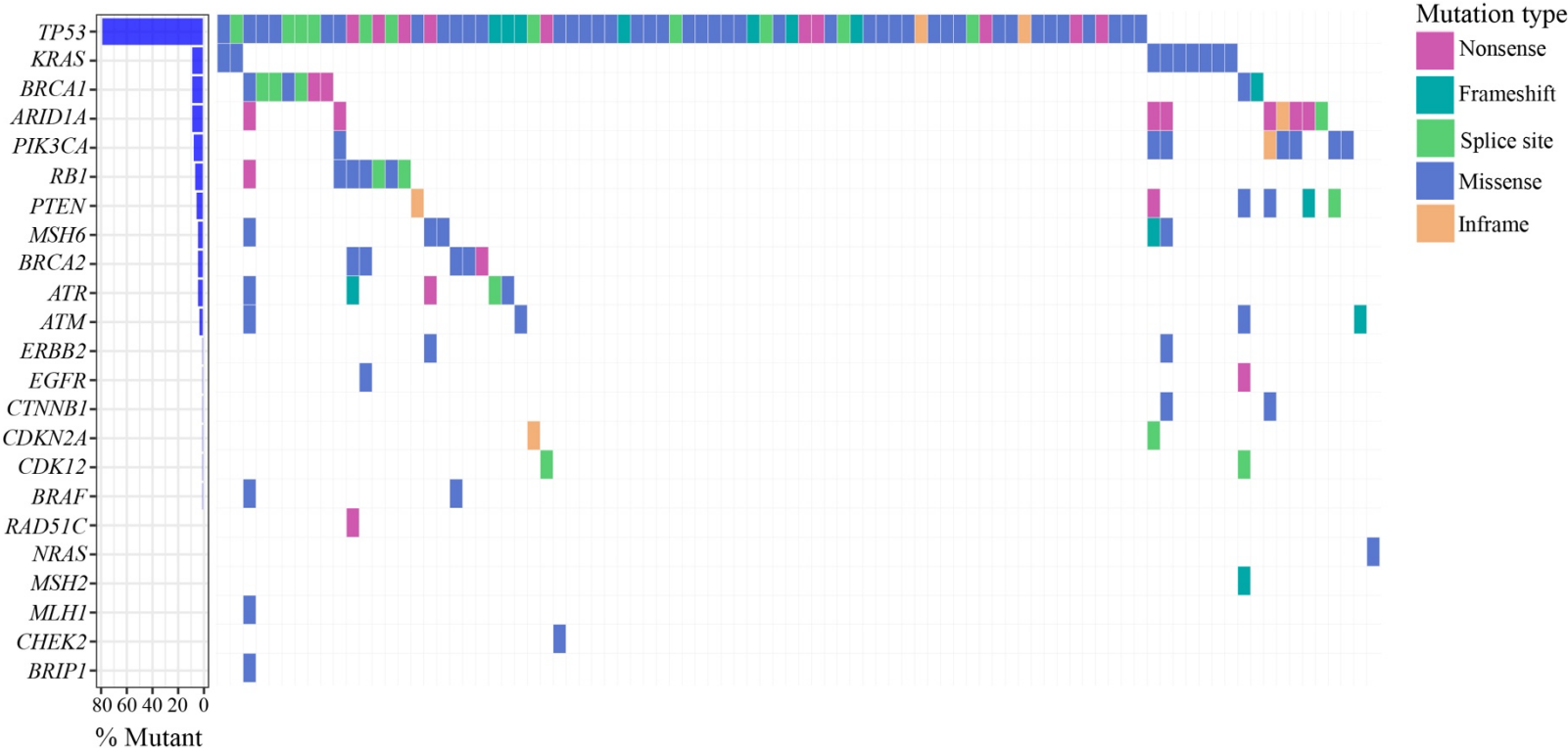


Figure 1: Spectrum of somatic variants identified in ovarian tumor samples.

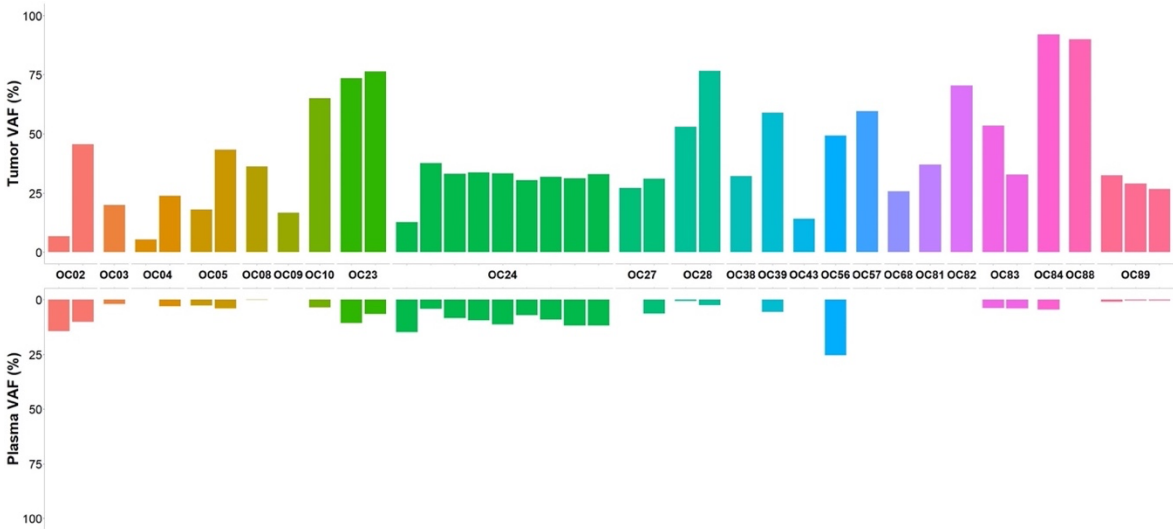


Figure 2: Concordance of variants calls between tumor and plasma samples from patients from treatment naive group. In patients with more than one somatic variant identified, the VAF for variants shared between matched tumor and plasma samples shows a concordance in hierarchy of VAF.

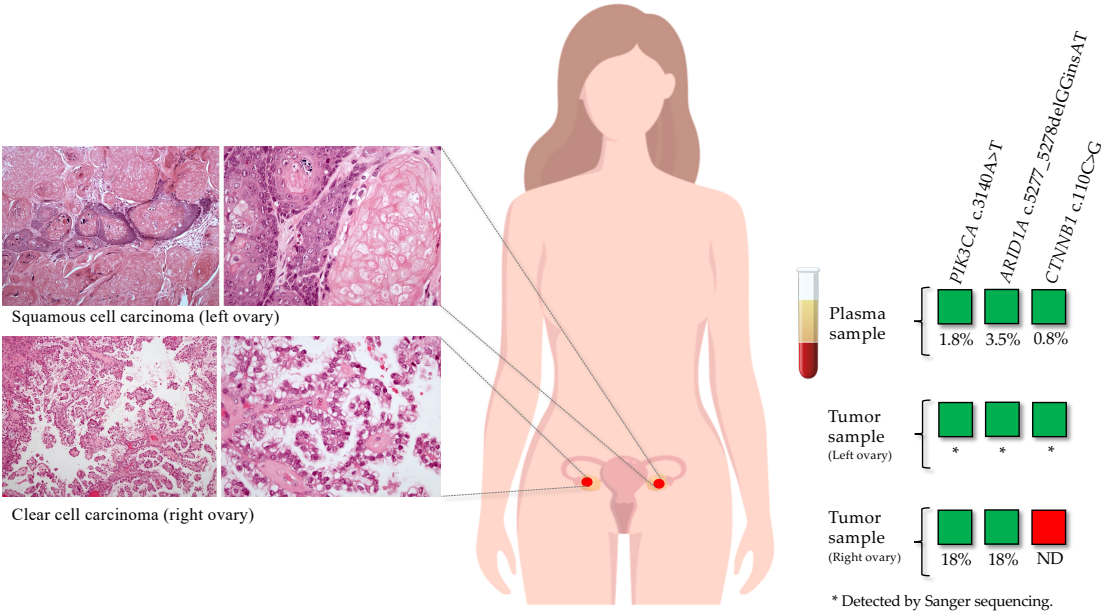


Figure 3: Inter-tumor heterogeneity detected by ctDNA analysis in a patient diagnosed with synchronous tumors on both ovaries. In tumor sample from the right ovary, selected for this study, a *PIK3CA* and a *ARID1A* pathogenic variants were identified. This tumor consisted of a clear cell carcinoma with a papillary growth pattern. In matched plasma sample, in addition to these two variants, a *CTNNB1* pathogenic variant was detected. We tested a tumor sample from the left ovary by Sanger sequencing, in which the three variants were identified. The left ovarian tumor consisted of a squamous cell carcinoma with abundant keratinization.

GENERAL DISCUSSION

Despite the continuous efforts and improvements over the past few decades, OC remains the most lethal cancer in women. The poor outcome of OC patients is closely related to the lack of effective screening approaches to detect the disease early, as OC diagnosed at advanced stage is associated with high morbidity and low survival rates. There is an unmet medical need for reliable biomarkers that could be used to detect asymptomatic disease, to stratify patients for target therapies, to identify patients at higher risk of recurrence, and to monitor treatment response and diagnosis of eventual disease relapse. Despite good response to initial treatment, most OC patients relapse and subsequent treatments with increased the toxicity will be needed, making it harder to treat and manage the patients. CA125, the currently used biomarker to diagnose and monitor OC patients, has poor sensitivity and specificity. Since there is no effective screening approach to detect OC early, the identification of women at higher risk to develop the disease, and to whom risk reduction strategies can be offered, is of extreme importance.

The majority of hereditary OC is attributed to pathogenic germline variants in the *BRCA1* or *BRCA2* genes and for several years the recommendations for genetic testing in OC patients were based on family history and were focused on those genes. However, the use of this criteria to select OC patients for genetic testing does not seems to be the most suitable, as a great proportion of women with pathogenic germline variants had no family history of breast or OC. In 2014 there was a paradigm shift in genetic testing of OC patients, with the approval of PARPi for the maintenance treatment of OC patients harboring *BRCA1* or *BRCA2* somatic or germline pathogenic variants and who respond to platin-based chemotherapy. Nowadays, all non-mucinous OC patients are recommended to be tested for *BRCA1* and *BRCA2* somatic and germline variants, namely because of the possible therapeutic implications. An earlier study from our group recommended to start the analysis on a tumor sample and that eventual deleterious variants predictive of better response to PARPi detected in the tumor should subsequently be evaluated in peripheral blood sample to determine its germline or somatic origin [14]. However, to test only for *BRCA1* and *BRCA2* variants due to their therapeutic implications lacks sensitivity to identify inherited predisposition to OC, as pathogenic germline variants in other genes are known to confer increased risk for OC. In Paper II, we report a tumor genetic testing strategy using a customized gene panel, containing 10 genes associated with risk for the development of OC, to identify somatic and germline variants in a single test. Using this strategy, we analyzed a series of 96 tumor samples from OC patients and we identified germline variants in 27.4% of the patients, in seven of the 10 genes included in the NGS gene panel (*BRCA1*, *BRCA2*, *BRIP1*, *MSH6*, *RAD51C*, *RAD51D* and *TP53*). Seventy-seven percent of these variants were classified as pathogenic in ClinVar and the remaining were VUS. *BRCA1* and *BRCA2* germline variants were found in 13.7% of the patients, reflecting the proportion of

patients with inherited predisposition to OC that one would identify if testing only for *BRCA1* and *BRCA2*. Using a 10 gene panel for OC genetic testing on FFPE tumor samples allowed us to identify 7.4% of patients with pathogenic germline variants in other genes beyond *BRCA1* and *BRCA2*, namely *RAD51C*, *RAD51D* e *MSH6*. The strategy we here propose improves the yield of diagnosis of inherited OC predisposition from 13% to 21% of the patients tested. This is extremely important, especially in the context of OC for which, as mentioned above, there are no effective screening approaches to detect disease at an early stage. This allows that more women, namely the healthy female relatives of the index patient that may subsequently be shown to be carriers of the familial pathogenic germline variant, have access to genetic counseling, presymptomatic genetic testing, and RRSO. This can be exemplified by a family in which we identified a pathogenic germline variant in the *RAD51D* gene. Genetic counseling was offered to healthy relatives of this family, which so far allowed us to identify four female carriers of this germline variant, and one of them was already submitted to RRSO.

The use of NGS of a gene panel has advantages but also brings some concerns. The major concern regarding the use of gene panels is that often results in the identification of VUS, mainly missense variants. However, the advent of NGS and the exponential identification of VUS led to the establishment of guidelines to define variant pathogenicity, thereby improving variant classification [127]. In our study, we detected six germline VUS whose pathogenicity was evaluated through the combination of in silico tools, additional somatic variants, protein expression studies, and segregation data. Five of these variants remained classified as VUS, while one in *MSH6* was classified as likely pathogenic, since this variant is predicted to have a deleterious impact at protein level, loss of *MSH6* expression was observed in the tumor, an additional pathogenic variant was detected in the tumor (presumably the second-hit), and it has been identified in six index patients with clinical criteria for LS.

Although tumor testing appears attractive, enabling the detection of somatic and germline variants in a single test, there are several factors that can influence its accuracy. FFPE samples, the most used material for tumor testing, present several challenges when used in molecular studies, as DNA extracted from this type of sample are fragmented and chemically modified. Furthermore, when using FFPE samples for genetic testing only a small section of the tumor is analyzed, which may not be the ideal for an accurate genetic testing given the possibility of inter-tumor and intra-tumoral heterogeneity. The use of ctDNA for genetic testing can help to resolve the problem of tumor heterogeneity, something that was demonstrated in our study that aimed to evaluate the detection of somatic variants in ctDNA (Paper III). In one patient, who was diagnosed with a CCC of the right ovary and a poorly differentiated squamous cell carcinoma of the left ovary, two somatic variants were

detected in the FFPE tumor sample from the right ovary. In the matched plasma sample, an additional pathogenic variant was detected, which was subsequently detected in the FFPE sample from the left ovary tumor. Thus, genetic testing on FFPE tumor samples may overlook intra-tumor and inter-tumor heterogeneity, which can be overcome by the analysis on ctDNA. Furthermore, as we found that these tumors shared a rare combination of two somatic variants, we concluded that these tumors were not independent but rather clonally related. The clonal progression of these tumors, accompanied by genotypic evolution, may explain the different phenotypes (CCC vs squamous cell). In addition to this advantage, the non-invasive nature of ctDNA analysis makes possible repeated sampling and 'real-time' monitoring during the entire course of the disease. There are several potential clinical applications of ctDNA analysis in OC management, such as diagnosis, prognosis, evaluation of therapy response, and the detection of disease relapse. Overall, we were able to detect somatic variants in ctDNA in 35.6% of the patients with detectable somatic variants in the tumor sample. In patients of the naive treatment group, the detection rate of somatic variants in ctDNA was 69.6%. In those patients, the detection rate was higher in the group diagnosed with stage IV disease (83%), followed by patients diagnosed with stage I OC (75%). These results demonstrated the potential of tumor molecular profiling through ctDNA analysis, which can be particularly interesting in the context of target therapy with PARPi, mainly in situations in which a tumor sample is not available and biopsy is not viable. Only two patients of the naive treatment group had somatic variants predictive to PARPi response in the tumor sample and in one of them the *BRCA1* somatic variant could be detected in ctDNA. OC genetic testing through ctDNA analysis can also detect both somatic and germline variants in the same test, as all the germline variants previously detected (Paper II) were easily identified in ctDNA presenting VAF around 50%, in concordance with a recently published study [128]. The ability to detect OC driver variants in ctDNA, even in patients with early-stage disease, suggests that ctDNA can be used to early detect OC, which would be very useful in healthy female relatives carriers of pathogenic germline variants in genes associated to inherited predisposition to OC. Recently, a test that combines the detection of variants in ctDNA with the levels of protein biomarkers to detect multiple cancers at early stages was announced [129]. The median sensitivity of this test to detect stage I and stage II cancer was 43% and 73%, respectively.

Our study also emphasizes the potential use of ctDNA as prognostic biomarker. Six patients of the post-treatment group plasma samples were collected after surgery and before chemotherapy and in three of them we detected somatic variants in ctDNA. These three patients experienced disease relapse, while patients with no detectable variants in plasma sample collected after surgery are still free from disease, highlighting the potential of ctDNA analysis for the identification of patients with higher risk of disease relapse.

Although ctDNA analysis holds promising as a biomarker with several potential applications in the management of OC patients, some limitations also exist (Paper I). The major limitation is the low fraction of ctDNA in a high background of normal cfDNA. Consequently, somatic variants are present at very low frequencies, with highly sensitive techniques being necessary for its detection, such as droplet-digital PCR (ddPCR). However, these techniques require a pre-knowledge of the variant to test, being more suitable in the evaluation of response to therapy or detection of disease relapse through the monitoring of variants previously identified in the tumor sample. NGS has the advantage of simultaneously detect variants in several genes and recent advances, such as the introduction of unique molecular barcodes (UMIs), can improve the detection of low frequency variants [130]. However, in our study we observed that the low concentrations of ctDNA that can be extracted from plasma samples have impact in the variant calling precision, mainly when using UMIs. Despite the high mean coverage (mean read depth = 27337) the mean UMI depth observed among all the samples (mean UMI depth = 513) was lower than the expected. The number of UMIs captured from the original DNA sample can be influenced by the DNA input amount and the number of sequenced UMIs have a direct impact on the variant detection sensitivity. Thus, the low coverage observed can be explained by the lower DNA input than the recommended for the library preparation due to the low concentration of ctDNA extracted from plasma samples. Thus, the collection of larger blood samples for plasma collection can help improving the sensitivity by increasing the amount of extracted ctDNA.

CONCLUSIONS

The main conclusions of this thesis are:

1. Using a 10 gene panel for routine OC NGS genetic testing on FFPE samples allowed us to identify 7.4% patients with pathogenic germline variants in genes other than *BRCA1* and *BRCA2*.
2. The strategy of gene panel tumor testing enables the identification of clinically actionable germline variants in a significant proportion of OC patients, while at the same time testing for somatic variants in the same genes, which may be valuable information in patients with advanced stage disease that have run out of approved therapeutic options for the allocation into clinical trials or for the use of off-label therapy.
3. The use of gene panel increases the chance to make available genetic counseling, presymptomatic genetic testing, and gynecological cancer prophylaxis to female who turn out to be healthy carriers of pathogenic germline variants.
4. Somatic variants can be detected in ctDNA from OC patients, potentially being an alternative for OC genetic testing when there is no tumor sample available and biopsy is not viable.
5. The ctDNA analysis is also promising to be used to early detect OC, which could be very useful in healthy female relatives carriers of pathogenic germline variants in genes associated with inherited predisposition to OC.
6. The ctDNA represents the mutational profiles from different tumor locations, overcoming the problem of inter-tumor and intra-tumor heterogeneity when performing genetic testing on FFPE tumor samples.
7. The ctDNA analysis of plasma samples collected after surgery can help to detect minimal residual disease after surgery, which could potentially be a prognostic biomarker identifying patients at higher risk of recurrence.

FUTURE PERSPECTIVES

The following point will be addressed in future studies:

1. In patients with detectable variants in ctDNA and from whom plasma samples were collected during the course of the disease, serial ctDNA samples will be analyzed, with the most appropriate method, to test its utility to evaluate therapy response and to anticipate the emergence of therapy resistance and disease relapse.
2. The use of ctDNA analysis to detect somatic variants in healthy relatives carriers of germline *BRCA1/2* variants undergoing cancer screening will be evaluated, using a combination of high-coverage amplicon-based NGS for the *BRCA1/2* genes (to test for second-hit mutations in germline mutation carriers) and identification of CNA and copy number neutral LOH at the respective *BRCA1/2* locus (to detect chromosome-level second-hit mutations). This strategy may allow early diagnosis of OC, as well as other neoplasia for which no prophylactic measures exist, namely peritoneal, prostatic, and pancreatic cancer.

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