Identifying blood-specific biomarkers for therapy-related clonal hematopoiesis in patients with hereditary or non-hereditary ovarian cancer

Inaugural Dissertation

zur

Erlangung des Doktorgrades

philosophiae doctor

der Medizinischen Fakultät

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»Alles hät sing Zick«

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Abbreviations

AAD ..... *age at first diagnosis*
ACMG ..... *American College of Medical Genetics and Genomics*
AML ..... *acute myeloid leukemia*
AMP ..... *Association for Molecular Pathology*
APL ..... *acute promyelocytic leukemia*
BC ..... *breast cancer*
CH ..... *clonal hematopoiesis*
CHIP ..... *clonal hematopoiesis of indeterminate potential*
CTx ..... *chemotherapy*
DDR ..... *DNA damage response*
DSB ..... *double strand breaks*
ExAC ..... *Exome Aggregation Consortium*
FAB ..... *French-American-British*
FFPE ..... *formalin-fixed paraffin-embedded*
FLOSSIES ..... *Fabulous Ladies Over Seventy*
g*BRCA1/2* ..... *BRCA1/2 germline*
GC-HBOC ..... *German Consortium for Hereditary Breast and Ovarian Cancer*
GMC ..... *geographically-matched controls*
HRR ..... *homologous recombination repair*
HSCs ..... *hematopoietic stem cells*
HSPCs ..... *hematopoietic stem and progenitor cells*
IARC ..... *International Agency for Research on Cancer*
LEAD ..... *Li-Fraumeni Syndrome Education and Early Detection*
LFS1 ..... *Li-Fraumeni cancer predisposition syndrome 1*
LOH ..... *loss-of-heterozygosity*
MDS ..... *myelodysplastic syndromes*
MMR ..... *DNA mismatch repair*
MRI ..... *magnetic resonance imaging*
NCCN ..... *National Comprehensive Cancer Network*
NGS ..... *next-generation sequencing*
OC ..... *ovarian cancer*
PARP ..... *poly ADP ribose polymerase*
PTVs ..... *protein-truncating variants*
PVs ..... *pathogenic variants*
RRSO ..... *risk-reducing salpingo-oophorectomy*
s-AML ..... *secondary acute myeloid leukemia*
SOP ..... *standard operating procedure*
t-AML ..... *therapy-related acute myeloid leukemia*
t-MDS ..... *therapy-related myelodysplatic syndrome*
t-MN ..... *therapy-related myeloid neoplasms*
VF ..... *variant fraction*
WHO ..... *World Health Organization*

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1.) Main publications (peer-reviewed)

Weber-Lassalle N*, Borde J*, **Weber-Lassalle K***, Horváth J, Niederacher D, Arnold N, Kaulfuß S, Ernst C, Paul VG, Honisch E, Klaschik K, Volk AE, Kubisch C, Rapp S, Lichey N, Altmüller J, Lepkes L, Pohl-Rescigno E, Thiele H, Nürnberg P, Larsen M, Richters L, Rhiem K, Wappenschmidt B, Engel C, Meindl A, Schmutzler RK, Hahnen E, Hauke J. (2019) **Germline loss-of-function variants in the *BARD1* gene are associated with early-onset familial breast cancer but not ovarian cancer.** *Breast Cancer Research* 2019 Apr 29;21(1):55. doi: 10.1186/s13058-019-1137-9. (Impact factor: 6.466; 2020)

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Summary

Every year, about 7,000 women in Germany are newly diagnosed with ovarian cancer (OC). In the general population, 1.2% of women will develop OC during their lifetime [1]. Approximately 18 to 26% of all OC cases exhibit a hereditary background [2-4]. Inherited germline pathogenic variants (PVs) in OC predisposition genes contribute to the development of familial OC. Therefore, germline testing for these variants from the patient's blood may provide important information about individual cancer risk of unaffected family members [5].

Multi-gene panel analysis in a routine diagnostic setting allows parallel screening for multiple OC predisposition genes. Gene panels include known and established OC genes as well as often candidate or research genes for which the association between PVs and cancer development has not yet been adequately elucidated. Women who carry germline PVs in *BRCA1* and *BRCA2* have the highest risk of developing breast cancer (BC) and OC during their lifetime [6-13]. Approximately, 39 to 44% of women with a germline PV in the *BRCA1* gene and 11 to 17% of women with a germline PV in the *BRCA2* gene will develop OC by the age of 70-80 years [11-13].

Most of the non-*BRCA1/2* OC susceptibility genes have been discovered due to direct interaction of the encoded proteins with BRCA1 or BRCA2 and their role in homologous recombination repair. The role of the gene BRCA1-associated RING domain 1 (*BARD1*) in BC/OC predisposition remained unclear due to controversial results of case-control investigations [14-16].

In the first part of the PhD thesis, I aimed to assess the role of germline PVs in *BARD1* in BC/OC predisposition in a sample of 4,469 *BRCA1/2*-negative female BC and 451 index patients with OC of the German Consortium for Hereditary Breast and Ovarian Cancer, and 2,767 geographically matched female control individuals. All samples were screened for protein truncating variants (PTVs) and potentially damaging rare missense variants in *BARD1*. Additional control databases (Exome Aggregation Consortium (ExAC); Fabulous Ladies Over Seventy (FLOSSIES)) were included for the calculation of odds ratios (ORs).

PTVs were detected in 23 of 4,469 BC index patients (carrier frequency=0.51%) and in 36 of 37,265 control individuals (carrier frequency=0.10%, OR=5.35; 95% confidence interval (CI)=3.17 to 9.04; $P<0.00001$), and no PTVs were identified neither in the 451 OC index patients nor in our previously published analysis of 523 OC patients enrolled in the observational AGO-TR1 trial [3, 17]. For BC, *BARD1*-mutated index patients showed a statistically significantly younger mean age at first diagnosis of 42.3 years (range: 24 to 60 years) compared with the overall study sample (mean: 48.6 years, range: 17 to 92 years;

P=0.00347). In the subgroup of BC, index patients with an AAD <50 years showed a statistically significant association with germline PTVs in *BARD1* (OR=7.43, 95% CI=4.26 to 12.98; *P*<0.00001) [17]. In contrast, germline PTVs in *BARD1* were not statistically significantly associated with BC in the subgroup of index patients with an AAD ≥50 years (OR=2.29; 95% CI=0.82 to 6.45; *P*=0.11217). Overall, rare and predicted damaging *BARD1* missense variants were statistically significantly more prevalent in BC index patients compared with control individuals (OR=2.15; 95% CI=1.26 to 3.67; *P*=0.00723).

Firstly, due to the significant association of germline PTVs in *BARD1* with early-onset BC, *BARD1* could be directly incorporated as "core gene" into routine diagnostic for germline testing. Screening for PVs in the *BARD1* gene will be now offered for all patients meeting the inclusion criteria of all 23 centers of the German Consortium for Hereditary Breast and Ovarian Cancer nationwide (Table 1). Secondly, intensified BC surveillance programs could be offered to women carrying PVs in the *BARD1* gene from now on [17].

Usually, DNA isolated from the blood of the patient or a family member seeking advice is used for genetic germline testing. Screening for PVs in the blood and the corresponding tumor tissue allows distinguishing between germline and acquired somatic PVs. Germline PVs in the tumor suppressor gene *TP53* are causative for the Li-Fraumeni cancer predisposition syndrome 1 (LFS1) [18] accompanied with the development of multiple (early-onset) tumors and a variety of tumor types [19-24]. Persons with a LFS1 diagnosis have to endure an intensified screening program that can harm the body (e.g. radiation) or may lead to psychological side effects and negative, emotionally exhausted impact concerning the whole family, caused either by the strict surveillance program or the permanent worry to detect cancer, especially when more than one relative is affected by LFS1 [25-27].

After paired analysis of the TruRisk® gene panel of blood and tumor DNA from 523 patients with OC (AGO-TR1 study) and 1,053 age-matched healthy female controls, three OC patients showed deleterious missense *TP53* variants with a low variant fraction (VF) that were present only in the blood samples but not or barely in the corresponding tumors [28]. The occurrence of blood-specific variants with a low VF may be caused by chemotherapy-induced and/or age-related clonal hematopoiesis (CH) [96, 104, 167]. Notably, these mutations were not causal for patients' OC. CH is defined as the premalignant stage, in which somatic mutations are restricted to the hematopoietic compartment and no other compartment of the body, and is a decisive step in the initiation and development of hematologic neoplasia e.g. myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) [29, 30].

Since the three OC patients with CH had completed first line taxane/platinum-based chemotherapy (CTx) at the time of blood draw, CTx was assumed to be the trigger for CH rather than age [28].

As direct translation from research to the clinic, these findings were integrated into the evaluation of *TP53* PVs found by germline genetic testing in routine diagnostics in the form of a standard operating procedure (SOP) to avoid false-positive genetic diagnoses of LFS1. Here, testing of a second tissue via next-generation sequencing (NGS) that is not derived from the hematopoietic system is required to exclude misdiagnosis of this fatal disease. Formalin-fixed paraffin-embedded (FFPE) tumor tissue should be the first choice for this purpose, because if the *TP53* PV is not present or present with a significantly lower VF than in blood, this indicates a blood-specific variant. This is particularly true if a different, tumor-specific somatic *TP53* PV is detected in the tumor [28]. Beside FFPE tumor material, normal tissue (e.g. hair follicles or fingernails) can be used for DNA isolation [31].

In addition, blood-specific PTVs were identified affecting the *PPM1D* gene that were originally thought to represent mosaic events leading to increased OC risks [32-34] in 24 out of 523 OC patients. All patients with these alterations had completed first line platinum-based CTx prior to blood draw or were currently treated with platinum, indicating CTx-induced CH [28]. These findings are reinforced by the fact that only one female individual from our control dataset carried a PTV in the *PPM1D* gene. Thus, a second biomarker, besides *TP53*, for chemotherapy-induced CH could be identified. Previous studies showed that CH is associated with elevated risks of hematologic cancer [30, 35]. In line with these results, the only patient with PVs in *TP53* and *PPM1D* did indeed develop AML.

Among patients who had CH-associated variants in the *TP53* and *PPM1D* genes, the proportion of patients carrying germline PVs in validated OC predisposition genes (*ATM, BRCA1/2, BRIP1, MSH2, MSH6,* and *RAD51C/D*) was 1.7-fold increased compared with the overall patient sample [28]. Based on these findings, a further study was conducted to investigate whether and to what extent germline cancer predisposition may be a risk factor for therapy-associated secondary hematologic events. In addition, the question arose whether there were other biomarkers for CTx-induced CH besides *TP53* and *PPM1D*.

To address these questions, a retrospective analysis was performed using a customized 10 gene panel, including the most prevalently altered CH-associated genes *ASXL1*, *DNMT3A, GNAS, JAK2, PPM1D, SF3B1, SH2B3, SRSF2, TET2,* and *TP53*, followed by amplicon-based NGS in 448 patients with OC enrolled in the AGO-TR1 trial [36]. CH-associated gene mutations were present in all 10 CH-related genes investigated with a high prevalence of 17%

(75/448) in the patients sample and VFs ranging from 0.03 to 0.37. Focusing on stratified subgroups, associations were shown with an advanced age at blood draw for CH-associated gene mutations in *PPM1D*, *SF3B1*, *SH2B3* and *TET2*, and prior platinum-based CTx exposure for CH-associated gene mutations in *PPM1D* and *TP53*, respectively [36].

Regarding the question of whether germline status in OC predisposition genes is related to increased incidence of CH-associated gene mutations, I demonstrated that positive germline *BRCA1/2* mutation status is not a risk factor for acquiring CH-associated gene mutations for all genes investigated. At the patient level, patients with heterozygous germline PV in the *BRCA1/2* risk genes are not more susceptible to CH than patients without these PVs. For patients investigated, alone, the number of prior lines of CTx seems to be the main risk factor for the development of therapy-related myeloid neoplasms (t-MN) [36].

In the future, our specific CH biomarker assay and monitoring of patients after chemotherapy exposure with blood sampling at defined time intervals and subsequent analysis for CH-associated gene mutations can be used to enable early detection of t-MN and optimized clinical management of patients at increased risk for these hematologic disorders. Moreover, further studies are required to assess whether the choice of treatment regimen needs to be adjusted to individual t-MN risk.

Zusammenfassung

Eierstockkrebs (OC) wird jedes Jahr bei etwa 7.000 Frauen in Deutschland neu diagnostiziert. Im Laufe ihres Lebens entwickeln 1,2% der Frauen in der Allgemeinbevölkerung ein OC [1], von diesen weisen etwa 18 bis 26% einen erblichen Hintergrund auf [2-4]. Da vererbte pathogene Keimbahnvarianten (PVs) in OC-Prädispositionsgenen zur Entwicklung eines familiären OC beitragen, kann eine Keimbahntestung auf diese Varianten im Blut der Patientinnen wichtige Informationen über das individuelle Krebsrisiko der nicht betroffenen Familienmitglieder liefern [5].

Multi-Gen-Panel-Analysen in der Routinediagnostik ermöglichen ein paralleles Screening auf mehrere OC-Prädispositionsgene. Diese Gen-Panels setzen sich aus bekannten, etablierten OC-Genen und häufig auch aus Kandidaten- oder sogenannten „Forschungsgenen“, für die der Zusammenhang zwischen PVs und Krebsentwicklung noch nicht hinreichend geklärt ist, zusammen. Das höchste Risiko im Laufe ihres Lebens an Brustkrebs (BC) und/oder OC zu erkranken, haben Frauen mit Keimbahn-PVs in den Genen *BRCA1* und *BRCA2* [6-13]. So entwickeln ungefähr 39 bis 44 % der Frauen mit einer Keimbahn-PV im *BRCA1*-Gen und 11 bis 17% der Frauen mit einer Keimbahn-PV im *BRCA2*-Gen bis zum Alter von 70-80 Jahren ein OC [11-13].

Während die meisten Nicht-*BRCA1/2*-OC-Suszeptibilitätsgene aufgrund der direkten Interaktion der kodierten Proteine mit BRCA1 oder BRCA2 und ihrer Rolle bei der Reparatur homologer Rekombination entdeckt wurden, blieb die Rolle des Gens BRCA1-assoziierte RING-Domäne 1 (*BARD1*) bei der BC/OC-Prädisposition aufgrund kontroverser Ergebnisse von Fall-Kontroll-Studien bisher unklar [14-16].

Im ersten Teil meiner Doktorarbeit habe ich daher das Auftreten von Keimbahn-PVs in *BARD1* bei der BC/OC-Prädisposition in einer Stichprobe von 4.469 *BRCA1/2*-negativen weiblichen BC- und 451 Index-Patientinnen mit OC des Deutschen Konsortiums für erblichen Brust- und Eierstockkrebs sowie 2.767 geographisch passenden weiblichen Kontrollpersonen untersucht [17]. Dabei wurden alle Proben auf Protein trunkierende Varianten (PTVs) und potenziell schädigende seltene Missense-Varianten in *BARD1* gescreent und zusätzliche Kontrolldatenbanken (Exome Aggregation Consortium (ExAC); Fabulous Ladies Over Seventy (FLOSSIES)) für die Berechnung der Odds Ratios (ORs) herangezogen.

Zwar konnten PTVs bei 23 von 4.469 BC-Index-Patienten (Trägerhäufigkeit=0,51%) und bei 36 von 37.265 Kontrollpersonen (Trägerhäufigkeit=0,10%, OR=5,35; 95% Konfidenzintervall (CI) =3,17 bis 9,04; P<0,00001) identifiziert werden [17]. Jedoch konnten weder bei der Gruppe der 451 OC-Index-Patienten noch in unserer zuvor veröffentlichten

Analyse von 523 OC-Patienten, die an der Beobachtungsstudie AGO-TR1 teilgenommen haben, PTVs detektiert werden [3, 17]. Im Falle von BC wiesen die *BARD1*-mutierten Indexpatienten ein statistisch signifikant jüngeres Durchschnittsalter von 42,3 Jahren (Spanne: 24 bis 60 Jahre) bei der Erstdiagnose (AAD) im Vergleich zur Gesamtstichprobe der Studie (Durchschnittsalter: 48,6 Jahre, Spanne: 17 bis 92 Jahre; *P*=0,00347) auf [17]. In der Gruppe der BC-Index-Patienten mit einem AAD <50 Jahre zeigte sich ein statistisch signifikanter Zusammenhang mit Keimbahn-PTVs in *BARD1* (OR=7,43, 95% CI=4,26 bis 12,98; *P*<0,00001). Im Gegensatz dazu waren Keimbahn-PTVs in *BARD1* in der Gruppe der Indexpatienten mit einem AAD ≥50 Jahren nicht statistisch signifikant mit BC assoziiert (OR=2,29; 95% CI=0,82 bis 6,45; *P*=0,11217). Insgesamt traten seltene und vorhergesagte schädliche *BARD1* missense Varianten bei BC-Indexpatienten im Vergleich zu den Kontrollpersonen statistisch signifikant häufiger auf (OR=2,15; 95% CI=1,26 bis 3,67; *P*=0,00723) [17].

Aufgrund der signifikanten Assoziation von Keimbahn-PVs in *BARD1* mit früh einsetzendem BC konnte das *BARD1*-Gen direkt als "Kerngen" in die Routinediagnostik für genetische Keimbahntests inkludiert werden. Somit wird das Screening auf PVs im BARD1-Gen nun bundesweit für alle Patientinnen angeboten, die die Einschlusskriterien aller 23 Zentren des Deutschen Konsortiums für erblichen Brust- und Eierstockkrebs erfüllen (Tabelle 1). Darüber hinaus kann Frauen, die PVs im *BARD1*-Gen tragen, von nun an eine intensivierte BC-Früherkennung angeboten werden [17].

Üblicherweise wird für genetische Keimbahntests DNA aus dem Blut der Patientin oder eines ratsuchenden Familienmitglieds isoliert. Dabei ermöglicht das Screening auf PVs im Blut und dem entsprechenden Tumorgewebe die Unterscheidung zwischen Keimbahn- und erworbenen somatischen PVs. Keimbahn-PVs im Tumorsuppressor-Gen *TP53* bilden die Ursache für das Li-Fraumeni-Krebsprädisposition-Syndrom 1 (LFS1) [18], welches mit der Entwicklung multipler (früh einsetzender) Tumoren und einer Vielzahl von Tumorarten einhergeht [19-24]. Personen mit einer solchen LFS1-Diagnose müssen ein intensives Screening-Programm über sich ergehen lassen, welches den Körper schädigen (z.B. durch Bestrahlung) oder zu psychologischen Nebenwirkungen führen und negative, emotional erschöpfende Auswirkungen auf die gesamte Familie haben kann, die entweder durch das strenge Überwachungsprogramm oder die ständige Sorge, Krebs zu entdecken, verursacht werden, insbesondere wenn mehr als ein Verwandter von LFS1 betroffen ist [25-27].

Nach der paarweisen Analyse mithilfe des TruRisk® Genpanels von Blut- und Tumor-DNA von 523 OC-Patientinnen (AGO-TR1-Studie) und 1.053 altersgleichen, gesunden, weiblichen

Kontrollen wiesen drei OC-Patientinnen schädliche Missense-*TP53*-Varianten mit einem reduzierten Variantenanteil (VF) auf, die nur in den Blutproben, aber nicht oder nur kaum in den entsprechenden Tumoren vorhanden waren [28]. Das Auftreten blutspezifischer Varianten mit reduzierter VF kann durch Chemotherapie-induzierte und/oder altersbedingte klonale Hämatopoese (CH) verursacht werden [96, 104, 167]. Bemerkenswert ist, dass diese Mutationen nicht ursächlich für die OC der Patientinnen waren. CH ist definiert als das prämaligne Stadium, in dem somatische Mutationen auf das hämatopoetische Kompartiment beschränkt sind und bildet einen entscheidenden Schritt bei der Initiierung und Entwicklung hämatologischer Neoplasien, z. B. des myelodysplastisches Syndrom (MDS) oder der akuten myeloischen Leukämie (AML) [28, 29].

Da die drei OC-Patientinnen mit CH zum Zeitpunkt der Blutentnahme eine Erstlinien-Chemotherapie (CTx) auf Taxan-/Platinbasis abgeschlossen hatten, wurde angenommen, dass die CTx der Auslöser für CH ist und nicht das Alter [28].

Als direkte Übertragung von der Forschung in die Klinik wurden diese Ergebnisse in die Bewertung der *TP53*-PVs, die durch Keimbahn-Gentests gefunden wurden, in der Routinediagnostik in Form einer Standardarbeitsanweisung integriert, um falsch-positive genetische Diagnosen von LFS1 zu vermeiden. Hierzu ist die Untersuchung eines zweiten Gewebes mittels Next-Generation-Sequencing (NGS) erforderlich, das nicht aus dem hämatopoetischen System stammt, um eine Fehldiagnose dieser tödlichen Krankheit auszuschließen. Daher sollte Formalin-fixiertes Paraffin-eingebettetes (FFPE-)Tumorgewebe die erste Wahl für diesen Zweck sein, denn wenn die *TP53*-PV nicht oder mit einer deutlich niedrigeren VF als im Blut vorhanden ist, deutet dies auf eine blutspezifische Variante hin. Dies gilt insbesondere dann, wenn im Tumor eine andere, tumorspezifische somatische *TP53*-PV nachgewiesen wird [30]. Neben FFPE-Tumormaterial kann auch normales Gewebe (z.B. Haarfollikel oder Fingernägel) für die DNA-Isolierung verwendet werden [31].

Darüber hinaus konnten bei 24 von 523 OC-Patienten blutspezifische PTVs identifiziert werden, die das *PPM1D*-Gen betreffen [28] und von denen ursprünglich angenommen wurde, dass sie Mosaikereignisse darstellen, die zu einem erhöhten OC-Risiko führen [32-34]. Alle Patienten mit diesen Veränderungen hatten vor der Blutentnahme eine platinbasierte Erstlinien-CTx-Therapie abgeschlossen oder wurden derzeit mit Platin behandelt, was auf eine CTx-induzierte CH hinweist [28]. Diese Ergebnisse werden durch die Tatsache bestärkt, dass nur eine weibliche Person aus unserem Kontrolldatensatz eine PTV im *PPM1D*-Gen aufwies. Somit konnte neben *TP53* ein zweiter Biomarker für Chemotherapie-induzierte CH identifiziert werden. Frühere Studien zeigten, dass CH mit einem erhöhten Risiko für

hämatologische Krebserkrankungen verbunden ist [29, 35]. In Übereinstimmung mit diesen Ergebnissen entwickelte die einzige Patientin mit PVs in *TP53* und *PPM1D* tatsächlich eine AML. Unter den Patientinnen, die CH-assoziierte Varianten in den Genen *TP53* und *PPM1D* aufwiesen, war der Anteil der Patientinnen mit Keimbahn-PVs in validierten OC-Prädispositionsgenen (*ATM*, *BRCA1/2*, *BRIP1*, *MSH2*, *MSH6* und *RAD51C/D*) im Vergleich zur gesamten Patientenstichprobe um das 1,7-fache erhöht [28].

Auf der Grundlage dieser Ergebnisse führte ich ein Follow-up durch, um zu untersuchen, ob und inwieweit eine Krebsveranlagung in der Keimbahn ein Risikofaktor für therapieassoziierte sekundäre hämatologische Ereignisse sein könnte. Darüber hinaus stellte sich die Frage, ob es neben den Genen *TP53* und *PPM1D* weitere Biomarker für eine CTx-induzierte CH gibt.

Zur Beantwortung dieser Fragenstellung führte ich eine retrospektive Analyse mit einem eigens designten 10-Gen-Panel durch, welches die am häufigsten veränderten CH-assoziierten Gene *ASXL1*, *DNMT3A*, *GNAS*, *JAK2*, *PPM1D*, *SF3B1*, *SH2B3*, *SRSF2*, *TET2* und *TP53* umfasste, gefolgt von einem Amplikon-basierten NGS bei 448 OC-Patienten, die an der AGO-TR1-Studie teilnahmen [36]. CH-assoziierte Genmutationen waren in allen 10 untersuchten CH-bezogenen Genen mit einer hohen Prävalenz von 17% (75/448) in der Patientenstichprobe und VFs zwischen 0,03 und 0,37 vorhanden. Bei der Betrachtung stratifizierter Gruppen wurden Assoziationen mit einem fortgeschrittenen Alter bei der Blutentnahme für CH-assoziierte Genmutationen in *PPM1D*, *SF3B1*, *SH2B3* und *TET2* sowie mit einer früheren platinbasierten CTx-Exposition für CH-assoziierte Genmutationen in *PPM1D* und *TP53* nachgewiesen [36].

In Bezug auf die Frage, ob der Keimbahnstatus in den Genen für die OC-Prädisposition mit einer erhöhten Inzidenz von CH-assoziierten Genmutationen zusammenhängt, konnte ich zeigen, dass ein positiver Keimbahnstatus für *BRCA1/2*-Mutationen kein Risikofaktor für den Erwerb von CH-assoziierten Genmutationen für alle untersuchten Gene ist [36]. Auf Patientenebene sind Patientinnen mit heterozygoter Keimbahn-PV in den *BRCA1/2*-Risikogenen nicht anfälliger für eine CH als Patientinnen ohne diese PVs. Bei den untersuchten Patientinnen scheint allein die Anzahl der vorangegangenen CTx-Linien der Hauptrisikofaktor für die Entwicklung einer therapiebedingten myeloischen Neoplasie (t-MN) zu sein [36].

In Zukunft kann unser spezifischer CH-Biomarker-Assay als Überwachung von Patientinnen nach einer Chemotherapie mit Blutentnahme in bestimmten Zeitabständen und anschließender Analyse auf CH-assoziierte Genmutationen eingesetzt werden, um eine frühzeitige Erkennung

von t-MN und ein optimiertes klinisches Management von Patientinnen mit erhöhtem Risiko für diese hämatologischen Erkrankungen zu ermöglichen. Darüber hinaus sind weitere Studien erforderlich, um zu bewerten, ob die Wahl des Behandlungsschemas an das individuelle t-MN-Risiko angepasst werden muss.

1 Introduction

1.1 Ovarian cancer

Every year, about 7,000 women in Germany are newly diagnosed with ovarian cancer (OC) (status 2016) [1]. The incidence rate is 11.1 in 100,000 women. This corresponds to a life-time risk for OC of 1 in 75 [1]. OC is the most lethal gynecologic cancer and the fourth most common cause of cancer-related death in women with an overall 5-year relative survival rate of 43% [37, 38]. The risk for developing OC increases with age and the median age at first diagnosis (AAD) is 68 years [1]. OC is a heterogeneous disease that in more than 90% originates from epithelial cells, but can also arise from cord-stromal or germ cells [39]. In the classification of tumor staging, the classification of the International Federation of Gynecology and Obstetrics (FIGO) is consistent with the TNM-(T=tumor size, N=spreading to the lymph nodes, M=metastases) classification [40]. Approximately 75% of cases are diagnosed in the advanced stages FIGO IIB–IV, when tumor cells have affected the lymph nodes and have spread to the pelvis or the entire abdomen or damage adjacent organs [41]. In these advanced stages, the 5-year survival rate is less than 40%. In contrast, only few cases are diagnosed at the early tumor stages FIGO I–IIA with a localized tumor and the 5-year survival rate is considered much more favorable at > 80% [42]. To date, there is no effective early detection for OC [37]. This represents the particular importance of diagnosing this disease as early as possible.

1.1.1 Hereditary ovarian cancer

Germline pathogenic variants (PVs) in OC predisposition genes can cause hereditary OC. Approximately 18 to 26% of all OC cases exhibit a hereditary background [2-4]. Hereditary OC often occurs at an earlier age than OC in the general population. Therefore, genetic testing for germline PVs in OC predisposition genes can provide important information about individual cancer risk [5]. In patients affected with OC and persons seeking advice, who met the inclusion criteria of the German Consortium for Hereditary Breast and Ovarian Cancer (GC-HBOC) for germline testing based on familial breast cancer (BC) and OC history (Table 1), genetic germline testing in a clinical setting can estimate patients' risk.

Due to the high mutation prevalence in some risk genes, such as breast cancer gene 1 (*BRCA1*, MIM*113705) and breast cancer gene 2 (*BRCA2*, MIM*600185), in OC patients who were not selected for family history, all OC patients under the age of 80 years can be

tested for germline PVs in OC risk genes from now on [3]. Subsequently, opportunities for cancer prevention for *BRCA1/2* mutation carriers or potential treatment options may be offered such as the treatment with poly ADP ribose polymerase (PARP) inhibitors [5, 41, 43]. In routine diagnostics, multi-gene panels are predominantly used to identify germline alterations in disease-associated genes [15]. The majority of the centers of the GC-HBOC use the TruRisk® multi-gene panel that was established by the GC-HBOC in 2015. The GC-GBOC comprises a network of 23 university centers across Germany, coordinated by the Center for Familial Breast and Ovarian Cancer Cologne[a]. Due to the continuous research of further risk factors, which modify the BC/OC risk, the gene panel is consecutively modified. The latest version of the TruRisk® gene panel consists of 34 BC and/or OC associated genes, either "core" genes with confirmed BC/OC risks and genes that are syndrome-associated (Lynch, Cowden, Peutz-Jeghers syndrome) with associations with BC/OC or so called candidate genes in which the BC/OC risks is still uncertain.

Table 1: Inclusion criteria for germline testing by the German Consortium for Hereditary Breast and Ovarian cancer (GC-HBOC)At least one criterion must be fulfilled for germline testing by the GC-HBOC [3, 41, 43-45]; yrs=years. *Genetic testing of women fulfilling these inclusion criteria under special contracts with the consortium centers.

Inclusion criteria for genetic germline testing in the German Consortium for Hereditary Breast and Ovarian cancer (GC-HBOC)
a. At least three women with breast cancer (independent of age of onset)
b. At least two women with breast cancer, one with diagnosis before 51 yrs
c. At least two women with ovarian cancer
d. At least one woman with breast and one woman with ovarian cancer
e. At least one woman with breast and ovarian cancer
f. At least one woman with breast cancer before the age of 36 yrs
g. At least one woman with bilateral breast cancer before the age of 51 yrs
h. At least one man with breast cancer and one woman with breast or ovarian cancer
*i. At least one women with triple-negative breast cancer before the age of 50 yrs**
*j. At least one woman with ovarian cancer before the age of 80 yrs**

[a] www.konsortium-familiaerer-brustkrebs.de

1.1.1.1 Ovarian cancer-associated and putative risk genes

BRCA1 and BRCA2

The two most prominent genes associated with hereditary OC are *BRCA1* and *BRCA2*. Both tumor suppressor genes are associated with high risks developing BC and OC by 70 to 80 years of age [6-13]. A collaborative study of the GC-HBOC demonstrated that germline PVs in the *BRCA1/2* genes were detected in 41.9% (95% confidence interval [CI] 36.1 to 48.0%) of families affected by OC [45]. Considering these genes individually, 29.6 % (95% CI 24.4 to 35.4%) of OC families showed germline PVs in *BRCA1* and 13.1 % (95% CI 9.5 to 17.7%) in *BRCA2*, respectively. The estimated cumulative lifetime risks of OC until the age 80 years for *BRCA1* mutation carriers is 44% (95% CI, 36 to 53%) and 17% (95% CI 11 to 25%) for *BRCA2* mutation carriers (Figure 1) [13]. Considering OC patients independently of their family history, 10% of all OC cases can be referred to germline PVs in the *BRCA1/2* cancer predisposition genes [46].

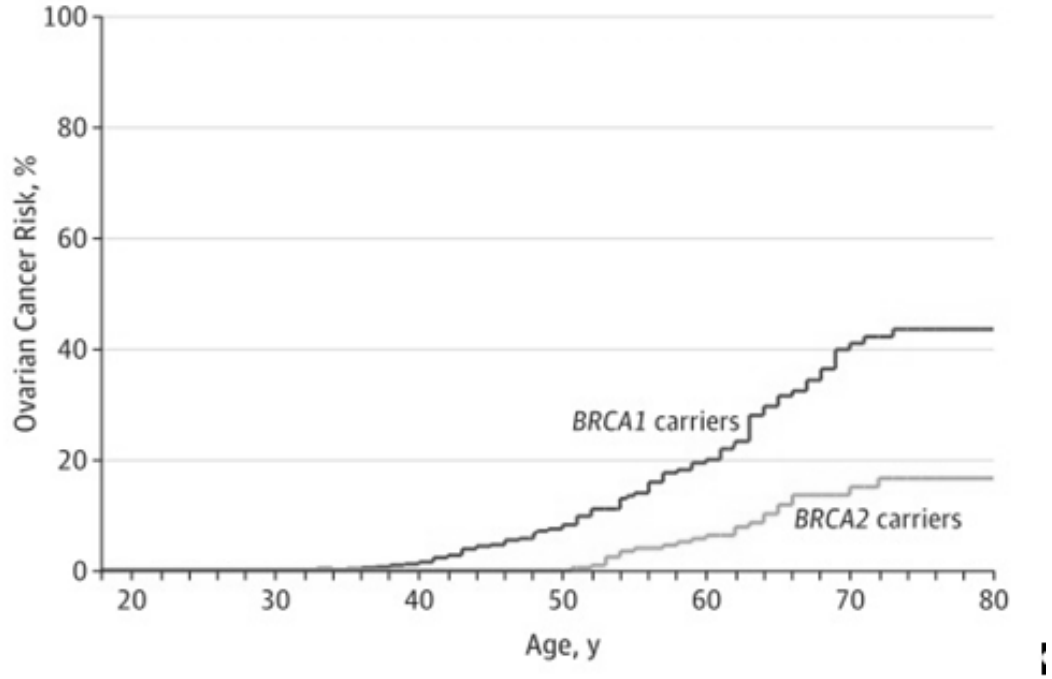

Figure 1: Kaplan-Meier estimates of cumulative risk of ovarian cancer among *BRCA1* and *BRCA2* mutation carriers. y=years. Adapted from Kuchenbaecker et al. [13]

The predisposition to cancer in *BRCA1* and *BRCA2* mutation carriers is caused by the fact that BRCA1 and BRCA2 play essential roles in DNA repair and are involved in the homologous recombination repair (HRR) pathway to fix DNA-double strand breaks (DSB). In the presence of DNA damage, healthy cells protect their genome integrity by cell cycle arrest and by activation of specialized DNA repair pathways [47]. Increased genomic instability results in a predisposition to cancer [48]. Heterozygous germline PVs in the *BRCA1* or *BRCA2* gene

cause an impaired HRR pathway. In tumors, total loss of the wild-type allele at the *BRCA1* or *BRCA2* locus, termed loss of heterozygosity (LOH), can be observed resulting in complete deficiency of HRR [48]. As a consequence, the repair process is shifted to more error-prone repair mechanisms (e.g. non-homologous end joining) which in turn can contribute to the development of cancer [49].

Additional OC predisposition genes

Besides *BRCA1/2* there are several OC predisposition genes that are associated with a moderate risk to develop hereditary OC, e.g. BRCA1 interacting Protein C-Terminal Helicase 1 (*BRIP1*, MIM*605882), RAD51 Paralog C (*RAD51C*, MIM*602774), RAD51 Paralog D (*RAD51D*, MIM*602954) [2, 3, 15, 50]. In case-control studies, carriers of a PV in the genes ATM Serine/Threonine Kinase (*ATM*, MIM*607585), the Partner and Localizer of BRCA2 (*PALB2*, MIM*610355) and tumor protein p53 (*TP53*, MIM*191170) were more prone to develop OC than non-carriers, though statistical significance was not achieved [2, 23]. The germline PV prevalence for *TP53* in individuals affected by OC is 0.30–0.81% [51, 52]. In a study comprising 7,768 OC patients unselected for family history, the germline mutation prevalence of these genes were 0.87% for *ATM*, 0.99% for *BRIP1*, 0.36% for *PALB2*, 0.79% for *RAD51C*, and 0.31% for *RAD51D* so that an increased lifetime risk for OC of affected individuals can be inferred [15]. Most of the non-*BRCA1/2* OC susceptibility genes encode for proteins that are involved in DNA repair mechanisms and interact directly with BRCA1 and BRCA2 (Figure 2) [2].

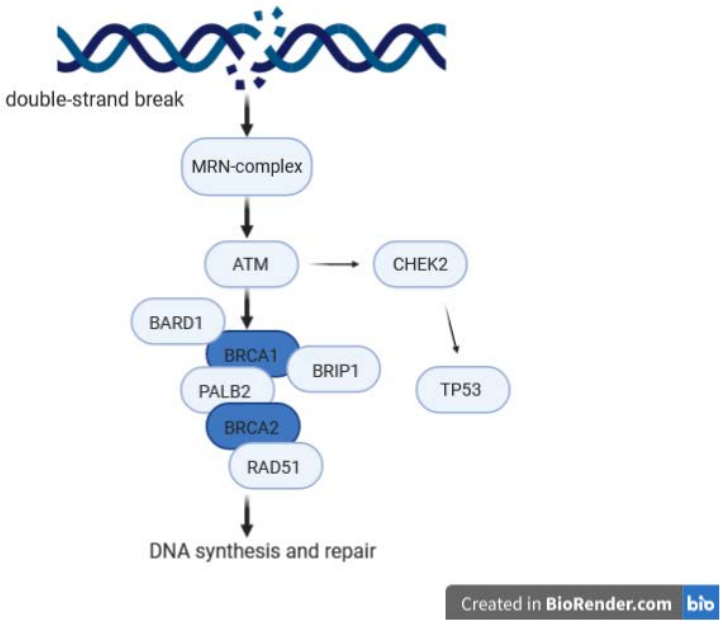

Figure 2: Homologous recombination repair pathway. The encoded proteins of *MRE11*, *RAD50* and *NBS1* (MRN complex) detect the DNA double-strand break and activate ATM, that recruits the BRCA complex with BARD1, BRIP1, PALB2 and RAD51 resulting in DNA synthesis and repair. Modified from Piombino et al. [53]

BARD1

The encoded proteins of *BARD1* (BRCA1-associated RING domain protein-1 gene, MIM*601593) and *BRCA1* share N-terminal RING finger and BRCA1 C-terminal (BRCT) domains, suggesting a high structural homology of both proteins [54]. These can either form homodimers via their N-terminal RING finger domains [55, 56] or more stable heterodimers involving amino acid residues 1-109 of the BRCA1 protein and amino acid residues 26-119 of the BARD1 protein [57] leading to an enhancement of tumor suppressor functions by acting on DSB repair and apoptosis initiation [58-61].

Due to the direct interaction of BRCA1 and BARD1 proteins, *BARD1* is considered as a BC/OC risk gene, but studies concerning the associated OC risk showed controversial results. Norquist et al. identified PTVs in 4 of 1,915 OC patients unselected for age or family history compared to 18 PTVs in 36,276 control individuals (OR=4.2, 95% CI=1.4 to 12.5, *P*=0.02) [2]. In contrast, Ramus et al. showed no significant association with OC in their study of 3,261 unselected patients with epithelial OC and 3,449 control individuals (4/3,261 patients, carrier frequency=0.12%, 2/3,449 patients, carrier frequency=0.06%, *P*=0.39) [16]. This non-significant result for OC is in line with the data provided by Lilyquist et al., which showed that germline PVs could be detected in 8 of 6,294 OC patients (carrier frequency=0.13%, risk ratio=1.28, 95% CI=0.55 to 2.51, *P*=0.59) [15]. Therefore, the role of PVs in the *BARD1* gene in OC predisposition remains unclear.

PPM1D

In the HRR pathway, ATM recruits the BRCA complex, phosphorylates CHEK2 and activates tumor suppressor protein p53 triggering expression of its target genes that control duration of the cell cycle arrest, senescence, or programmed cell death (Figure 2) [62]. The DNA damage response (DDR) pathway and its core component tumor suppressor p53 block cell cycle progression after genotoxic stress and represent an intrinsic barrier preventing cancer development [63]. The protein phosphatase $Mn^{2+/}Mg^{2+}$-dependent 1 delta (*PPM1D*, also called *Wip1*; MIM*605100) is a negative regulator of p53 that allows timely termination of the G2 checkpoint in DDR [64-66]. *PPM1D* has been suggested to act as an oncogene in a subset of tumors that retain wild-type p53[63]. PTVs cluster in the terminal exon (exon 6) of *PPM1D* leading to loss of a C-terminal degradation domain [67]. Previous studies have shown that mosaic *PPM1D* mutations are associated with increased BC/OC risks [32-34].

However, since these mutations are somatic, rather than germline, their mechanism of cancer association remains currently unclear [68].

Lynch-syndrome genes

In addition, germline PVs affecting the DNA mismatch repair (MMR) genes (*MLH1*, *MSH2*, *MSH6*, and *PMS2*) can cause the Lynch-syndrome [69, 70]. The Lynch-syndrome (MIM#120435), is an inherited cancer-susceptibility disorder with an increased risk to develop several cancer entities, affecting e.g. colon, endometrial and ovaries [70, 71]. In 7,768 OC cases, the germline PV prevalence was 0.1% for *MLH1*, 0.4% *MSH2*, 0.7% *MSH6*, and 0.4% for *PMS2* [15]. A report from the Prospective Lynch Syndrome Database showed estimated cumulative risks for developing OC until the age 75 years for carriers of germline PVs in *MLH1*of 11% (95% CI=7.4 to 19.7%), in *MSH2* of 17% (95% CI=11.8 to 31.2%), and in *MSH6* of 12% (95% CI=3.7 to 38.6%), respectively [72, 73]. In this study, germline PV carriers in the *PMS2* gene had lower risk of about 3 % (95% CI=0.5 to 43.3%) for OC compared to the other Lynch-syndrome genes, but regarding the width of the 95% CI the role of *PMS2* in OC development remains uncertain [73].

1.1.2 Clinical guidelines and recommendations for ovarian cancer patients

To date, there is no intensified surveillance provided for PV carriers in OC-associated risk genes, since there are currently no useful screening procedures that have been shown to lead to a benefit for the patient [37, 74].

Due to the poor prognosis of advanced OC combined with no effective early detection for women at an increased risk for OC, the National Comprehensive Cancer Network (NCCN) of the United States recommends and supports risk-reducing salpingo-oophorectomy (RRSO) as prevention method in clinical management. The recommendation is aimed in particular for patients carrying germline PVs in *BRCA1/2*, *BRIP1*, *RAD51C*, and *RAD51D* after completion of childbearing [74, 75]. In Germany, the same recommendations apply to carriers of germline PVs in the aforementioned genes [41]. In addition, recommendations on interventions for PV carriers of all established OC cancer predisposition genes are offered in the most recent 2020 GC-HBOC consensus [76].

RRSO is the method with the highest efficacy for reducing the risk of disease and mortality in hereditary OC. In *BRCA1/2* PV carriers a RRSO reduces the OC risk by about 80 to 90% [74, 77-80]. A general statement on the optimal timing of the intervention cannot be made precisely, because it makes a difference which gene is affected. Furthermore, family history, including youngest AAD, and desire to have children should be considered [41]. The average age for an RRSO in *BRCA1/2* mutation carriers is 40 years [77-79, 81].

1.2 Tumor protein p53-related cancers

1.2.1 Pathogenic variants in *TP53*

TP53 encodes p53, which is referred to the "guardian of the genome" and is involved in crucial cell functions including DNA replication and repair, epigenetic modifications, cell cycle arrest, apoptosis, autophagy, senescence, differentiation, antioxidant stress responses, and cellular energy metabolism [82-84]. Furthermore, when deleterious mutations are present, the functional impact of *TP53* mutations plays a critical role in carcinogenesis. The cumulative lifetime risk to develop cancer is 75% for male and almost 100% for female germline *TP53* mutation carriers [22].

Considering somatic PVs, *TP53* is the most frequently mutated gene in human tumors [85]. Somatic PVs are acquired during lifetime in somatic cells and are the most common cause of tumors. In contrast to germline variants, which originate in germ cells, they cannot be inherited directly from parents to children.

Distinction between germline and acquired/somatic PVs in BC/OC-associated genes requires in addition to blood sequencing, the analysis of a further tissue, which is not part of the hematopoietic compartment e.g. the corresponding tumor [84].

1.2.2 The Li-Fraumeni-syndrome 1

Germline PVs in the tumor suppressor gene *TP53* are causative for the Li-Fraumeni cancer predisposition syndrome 1 (LFS1) [18], which was first described in 1969 by Frederick Li and Joseph Fraumeni [86]. LFS1 is a very rare disease with an estimated prevalence of 1:5000 to 1:20.000 and follows an autosomal dominant inheritance [19]. The LFS1 is associated with an extensive cancer type spectrum, reaching from highly frequent tumor types such as breast cancer (27-31%), osteosarcoma (18-27%), adrenocortical carcinoma (6-18%), soft-tissue sarcoma (13-16%), to central nervous system tumors (9-11%) [19-24]. Individuals with LFS1 are also at an increased risk of developing hematologic tumors (leukemia and lymphomas; 2-4%), gastrointestinal cancers, and melanoma [21, 23]. LFS1-associated tumors can occur at any age but develop mainly in the childhood or beginning of adulthood [22, 87, 88].

LFS1 is characterized by early-onset of multiple specific cancers. Furthermore, the proportion of LFS1 patients developing more than one tumor during their lifetime is high: 15% develop second tumors, 4% third and 2% fourth tumors. Overall, 71% of multiple tumors are associated with LFS1 [89]. Clinical diagnosis of LFS1 can be characterized either by the classic criteria (Table 2) or the Chompret criteria (Table 3) [89].

Table 2: The classic criteria for the Li-Fraumeni syndrome.

The classic criteria for the Li-Fraumeni syndrome
• *Index patient with sarcoma before the age of 45 AND*
• *1st degree relative with carcinoma before the age of 45 AND*
• *Additional 1st or 2nd degree relatives with carcinoma before the age of 45 or sarcoma regardless of the age manifestation*

Besides the clinical criteria for LFS1 (Table 2), genetic testing for germline PVs in *TP53* is typically considered to confirm the diagnosis [19, 89]. Since *TP53* variants were also identified in families that do not meet the LFS1 criteria due to a different tumor spectrum, age of manifestation or sporadic occurrence of tumors, the Chompret criteria as recommendation for *TP53* germline screening were defined in 2001 and have been sequentially updated (Table 3) [23, 88, 89]. All patients who meet the modified "Chompret Criteria" should be tested for germline *TP53* variants.

Table 3: The Chompret criteria for the Li-Fraumeni Syndrome are an expanded version of the classic criteria aimed to identify affected families beyond the classic criteria listed above.

The Chompret criteria for the Li-Fraumeni Syndrome
• *Index patient with LFS1-associated tumor (breast cancer, soft tissue sarcoma, osteosarcoma, brain tumor, adrenal sarcoma leukemia, bronchoalveolar lung carcinoma, adrenocortical carcinoma before the age of 46 AND*
• *At least one 1st and 2nd degree relative with LFS1-associated tumor type (except BC, when the index patient is affected by BC) before the age of 56 OR*
• *Index patient with multiple tumors (except BC), of which two are LFS1-associated and the manifestation of the first tumor occurred before the age of 46 OR*
• *Index patient with adrenocortical sarcoma or choroid plexus carcinoma, regardless of family history OR*
• *Index patient with BC before the age of 31*

LFS1-causing p53 mutations mainly affect the DNA binding domain. In contrast to other tumor suppressor genes, such as *BRCA1* and *BRCA2*, in which PTVs play the major role in cancer development, the majority (74%) of mutations in the *TP53* gene are missense mutations [23]. Nonsense mutations account for 9%, splice variant for 8%, and frameshift mutations for 6% of PVs. There are only few deletions, duplications and other chromosomal rearrangements associated with LFS1 [88, 90].

Due to the high proportion of *TP53* missense mutations and the severe clinical consequences of PVs in *TP53*, it is of great importance to reliable determine whether missense variants are likely to be disease causing. For classification of a variant's pathogenicity, databases and tools

such as the International Agency for Research on Cancer (IARC) database (https://p53.iarc.fr/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) and for general population frequency estimates from the Genome Aggregation Database (gnomAD; https://gnomad.broadinstitute.org/) can be obtained [91, 92]. The IARC database uses data based on functional analyses of variants using different *in vitro* assays performed either in yeast or cultured cells to provide exact information for classification [83, 93, 94].

Moreover, missense variants should be interpreted and classified according to criteria based on international established classification systems e.g. the American College of Medical Genetics and Genomics (ACMG)/Association for Molecular Pathology (AMP) guidelines that have developed specifications for germline *TP53* variants [95]. The GC-HBOC uses criteria based on the IARC 5-class system for high-risk genes which is based on the guidelines issued by the Evidence-based Network for the Interpretation of Germline Mutant Alleles: (ENIGMA, http://enigmaconsortium.org/) Consortium (ENIGMA *BRCA1/2* Classification Criteria, Version 2.5.1, June 2017), as well as the above mentioned ACMG/ACGS guidelines [96]. The pathogenicity classes of germline variants are distinguished according to the relevance for a loss of function of the encoded protein: 1=neutral, 2=likely neutral, 3=uncertain evidence/no reliable evaluation, 4=likely relevant loss of function, and 5=relevant loss of function [97].

Besides the screening for PVs in *TP53*, clinical surveillance programs are recommended for patients with LFS1 [98]. Since there is no effective cancer prevention for patients carrying a germline mutation in *TP53*, clinical surveillance for early tumor detection is recommended [99]. In a long term study (from 2004 to 2015), Villani et al. demonstrated that the 5-year overall survival rate of germline *TP53* PV carriers that underwent a clinical surveillance protocol using physical examination and frequent biochemical and imaging studies (consisting of whole-body magnetic resonance imaging (MRI), brain MRI, breast MRI, mammography, abdominal and pelvic ultrasound, and colonoscopy) was significantly improved compared with the non-surveillance group (88.8% (95% CI=78.7 to 100) vs. 59.6% (95%CI=47.2 to 75.2); *P*=0.0132) [100]. However, the cross-over design and selection between the two groups may have biased the statistics of the study.

Despite of the clinical benefits, recommended surveillance programs such as the “Toronto protocol” or LFS1 Education and Early Detection (LEAD) are characterized by a tight frequency of foreseen examinations each year and can cause harm to the patient (Table 2) [25, 89, 101] e.g. radiation exposure can cause severe adverse side effects up to second tumors, such as myelodysplastic syndromes (MDS) or acute myeloid leukemia (AML) [35, 102].

To date, there is no reliable diagnostic tool available to detect early precursor states of therapy-related tumors.

Table 4: Overview of the routine examinations of the Li-Fraumeni Syndrome Education and Early Detection (LEAD) program of the University of Texas MD Anderson Cancer Center for children older than 16 years and adults. Modified from Villani et al. [103]

Cancer entitiy	Exams and tests	How often
General	A complete physical exam and check of these body systems: • Brain • Thyroid	Every 6 months
Adrenocortical tumor (ACT)	• Whole body MRI[1] • Blood tests	Annually
Breast (begin at age 20-25 years old)	Clinical breast exam by physician	Every 6 months
	Mammogram and MRI[1]	Annually
	Consider surgical removal of both breasts to prevent cancer (bilateral prophylactic mastectomy). For women treated for breast cancer, screening of remaining breast tissue should continue	Age and patient appropriate
Brain	Brain MRI[1]	Annually
Colon (begin at age 25 or 5 years before the earliest known colon cancer in the family)	• Colonoscopy • Esophagogastroduodenoscopy (EGD)	Every 2-5 years
Leukemia/Lymphoma	Blood Tests: CBC[2] with Differential	Annually
Melanoma	Skin Exam	Annually
Sarcoma	Whole Body MRI	Annually

[1]The whole body and brain MRI (Magnetic Resonance Imaging) are both performed on annual basis, staggered with a six months interval in between; the breast MRI should be performed at the same time (but on different days) as the brain MRI
[2]CBC= Complete Blood Count

Additionally, LFS1 accompanied by the screening program can lead to psychological and psychosocial side effects and negative, emotionally exhausted impact concerning the whole family, caused either by the strict surveillance program or the permanent worry to detect cancer, especially when more than one relative is affected by LFS1 [25-27].

1.3 Hematopoietic myeloid malignancies

1.3.1 Clonal hematopoiesis

Hematopoiesis is the process, in which cellular blood components are formed. The hematopoietic system, especially the bone marrow, is one of the most highly proliferating tissues in the human body, producing trillions of cells every day [104]. All blood cells are derived from only few hematopoietic stem cells (HSCs) located in the bone marrow with the unique ability to give rise to all of the different mature blood cell types and tissues.

The typical process of hematopoiesis involves the differentiation of the multipotent HSC into either the common myeloid or lymphoid progenitor: the lymphoid progenitor cells can differentiate into natural killer cells and T- and B-lymphocytes, whereas myeloid lineage cells can differentiate into red blood cells, platelets and white blood cells such as monocytes, neutrophils, basophils and eosinophils (Figure 3) [105-107].

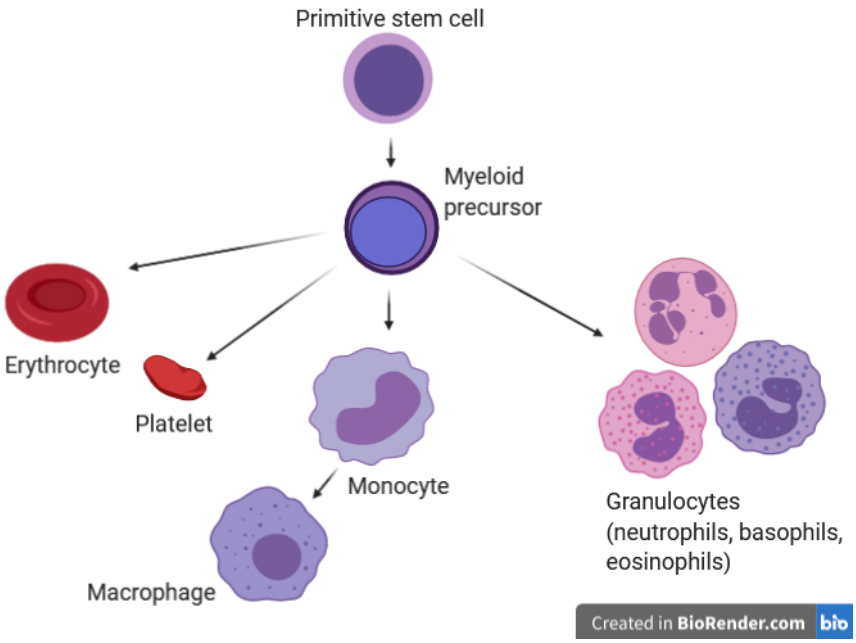

Figure 3: Blood cell development and differentiation in the bone marrow for myeloid lineage. Modified from Hallek et al. [108]

HSCs are self-renewing cells: when a HSC divides, it can either do it symmetrically, producing two daughter HSCs, or asymmetrically, producing both a HSC and a daughter cell (common progenitor cell) primed for differentiation and subsequent production of mature cells [109]. In addition, symmetric division of an HSC into two progenitors will effectively finish the clonal lineage, as the progenitor cells possess limited self-renewal potential. This hierarchy allows for rapid production of cells upon infection or blood loss (asymmetric) while safeguarding the hematopoietic system from mutation acquisition (symmetric), as most cells will terminally differentiate and die [110].

Thus, in a healthy body, the hematopoietic stem and progenitor cells (HSPCs) in the bone marrow multiply very slowly. Somatic mutations in e.g. proliferative or growth-promoting genes can stimulate an enhanced replication of HPSCs contributing to an imbalanced production of these cells that results in an increased numbers of clones [110]. If driver mutations in specific genes promote growth and fitness advantage in the clones compared to normal HSPC cells, mutated immature clones can spread into other tissues and may harm the body (clonal expansion) [111]. The premalignant stage, in which the somatic mutations only

affect the HPSCs and no other compartment of the body, is defined as clonal hematopoiesis (CH) and is a decisive step in the initiation and development of hematologic neoplasia e.g. MDS or AML [29, 30]. Due to the involvement of a wide spectrum of molecular functions in this complex and distinct pathogenesis, aberrant gene expression of the mutated clones cause the prevention of growth, development and function of the blood cells [112, 113]. These molecular functions comprise gene-specific differentiation, proliferation, cell survival, DNA-repair, cell distribution, chromatin instability, self-renewal, and cell-cycle checkpoint control as well as apoptosis [114].

The state, in which only a few HSPCs are affected by DNA alterations and do not cause any symptoms, is known as clonal hematopoiesis of indeterminate potential (CHIP) (Figure 4) [115]. Malignant transformation requires the gradual acquisition of various genetic alterations over the course of many cell divisions [116].

Thus, CH can develop into hematologic disorders such as MDS or AML in several stages. In addition, CH can also drive the risk to cardiovascular diseases and heart failure [117, 118].

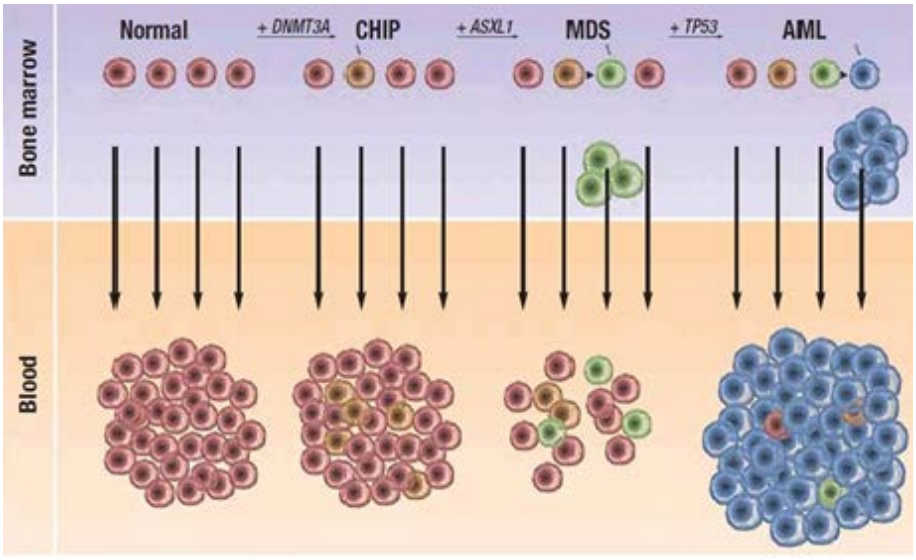

Figure 4: The stepwise development from polyclonal hematopoiesis to clonal hematopoiesis of indeterminate potential (CHIP), myelodysplastic syndrome (MDS), and acute myeloid leukemia (AML). Adapted from Heuser et al. [119]

1.3.1.1 Risk factors for clonal hematopoiesis

Advanced age is the major risk factor for developing CH [120]. For years, it is known that chronological aging is strongly associated with an accumulation of pre-leukemic mutations in HSPCs [30, 121-123].

In the human body, each cell derived from single HSCs achieves approximately 170 mutations in the whole genome per decade of life, whereas, in the exons (nearly 1% of the genome), only one mutation arises per decade of life [124, 125]. Nearly 95% of all adults,

each carrying approximately 10,000 to 200,000 HSCs in the body, harbor AML-related CH mutations [124, 126]. However, these somatic mutations usually affect only a very low percentage of cells (≤0.01%), and hence, show rarely the potential to cause hematologic malignancies. If next-generation sequencing (NGS) is performed, the fraction of affected cells can be assumed to be reflected in the variant fraction (VF), i.e., the proportion of sequencing reads showing the corresponding base alteration [110, 127].

Typically, CH-associated variants identified in the patient's blood show distinctly low VFs [30, 128, 129]. Since clones with very low VFs ≤10% are commonly observed in patients with CHIP, techniques with high sensitivity are needed to detect these mutations. Sanger sequencing is limited to VFs of approximately 10% and above, but targeted high-throughput sequencing employing multi-gene panels are capable of identifying mutations with VFs down to ≤0.001% [114]. Thus, NGS is able to identify CH events that would not be detected by Sanger sequencing due to their low VF [130, 131]. If VFs and the number of driver mutations increases in the clones, the risk for MDS/AML increases, too [132, 133]. Thus, increased VFs are predictors for the stepwise development from CH to AML [134].

In contrast to the usually low VFs of CH that are predominantly observed in the hematopoietic compartment, VFs close to 50 or 100% point towards either heterozygotic or homozygotic inherited germline variants, affecting one or both allele(s) of the individual are therefore occurring in principle in all tissues of the body.

Several studies revealed that recurrent somatic mutations in multiple genes, previously identified as drivers in MDS and AML [129, 135], are associated with age-dependent CH [30, 121, 122]. The first two genes identified to be associated with CH were the transcriptional regulators Tet Methylcytosine Dioxygenase 2 (*TET2*, MIM*612839) and DNA methyltransferase 3A (*DNMT3A*, MIM*602769) [136, 137]. In 2012, Busque et al. reported that 5% (10/182 individuals) of healthy elderly women with non-random-X-chromosome inactivation are carriers of a somatic PV in the *TET2* gene [136]. This phenomenon was the first example of CH and demonstrated that the inactivation of one X chromosome is favored over the other, resulting in an imbalanced amount of cells with each inactivated chromosome [138]. Mutations in the *DNMT3A* gene have been reported in about 22% of AML patients. These *DNMT3A* mutations occur often, namely in about 60% of patients, conjointly with mutations in other genes e.g. *FLT3* and *NPM1* gene [139, 140].

Further studies discovered the association between enriched somatic mutations in several genes with age-dependent CH, indicating a selective advantage of this clones [30, 122].

Among the most frequently mutated genes were several encoding for transcriptional regulators: Additional sex combs-like 1 (*ASXL1*, MIM*612990), spliceosome genes e.g. Splicing Factor 3B, Subunit 1 (*SF3B1*, MIM*605590) and Splicing factor, Serine/Arginine rich, 2 (*SRSF2*, MIM*600813), the Guanine Nucleotide binding protein complex locus (*GNAS*, MIM*139320), Janus Kinase 2 (*JAK2*, MIM*147796), *PPM1D*, SH2B Adaptor Protein 3 (*SH2B3*, MIM*605093), as well as the highly-penetrant cancer gene *TP53* [30, 121, 122, 127, 137, 141-143]. Jaiswal et al. showed an incidence of mutations in genes *ASXL1*, *CBL*, *DNMT3A*, *GNAS*, *GNB1*, *JAK2*, *SF3B1*, *SRSF2*, *TET2*, and *TP53* of <1% in the subgroup of individuals younger than the age of 40 years, in contrast to >20% in the subgroup of individuals aged 70 years and older. Moreover, they demonstrated a relationship of the occurrence of CH events with an increased risk of myeloid disorders, cardiovascular events, type-2 diabetes and even increased overall mortality [121]. A follow-up study confirmed these findings and demonstrated that in particular *DNMT3A*, *TET2*, *ASXL1* and *JAK2* mutations were associated with acute myocardial events which were linked to an increased coronary artery calcification process [117].

1.3.2 Myelodysplastic syndrome (MDS)

According to the WHO classification for AML, MDS as precursor condition can evolve to AML with myelodysplasia-related changes [144]. MDS is a biologically and clinically heterogeneous disease. It is characterized by dysplasia and ineffective hematopoiesis caused by impaired function of HSPCs, but in a milder form than AML [120, 145].

The annual incidence to develop MDS in Western countries is 5.3 to 13.1 cases per 100,000 individuals [146]. Approximately 30% of patients with MDS develop a progression to AML [147]. Diagnosis of MDS in suspected individuals, presenting cytopenia, which means a reduction in the number of mature blood cells, is performed via blood and bone marrow examination. The procedure consists of morphologic tests, cell counts, blast percentage, and the evaluation of cytogenetic data to exclude other diseases associated with bone marrow disorders [120, 148, 149].

1.3.3 Acute myeloid leukemia (AML)

AML is a life-threatening hematologic malignancy affecting the HSPCs of the human body [150]. This type of cancer is characterized by early precursors, known as blasts of myeloid cells, located in the bone marrow, that are not able to differentiate and proliferate uncontrolled. The immature blasts can spread rapidly in the bone marrow and may be

distributed throughout the body using the bloodstream. From there, they can infiltrate other types of tissues and damage organs [111].

As described above, CH can progress stepwise to AML due to a lot of risk factors. The rate of development of neoplasia in patients with CHIP is 0.5 to 1% per year and the progression to e.g. AML or MDS can last years or even decades [130]. In addition to aging, inherited genetic risk factors can cause *de novo* AML, although to a much lesser extent. Genetic disorders such as Down syndrome [151], Fanconi anemia and 5q-syndrome (loss of the long arm of chromosome 5, del(5q), MIM#153550) can also be the origin for AML. The deletion of the 5q-arm is the most frequent genomic loss in myeloid diseases, especially in MDS [152].

Hematopoietic stem cell transplantation, in which the recipient receives HSCs affected with CHIP from the donor, is also discussed as a risk factor, but corresponding studies yielded controversial results [153, 154].

Furthermore, external physical and chemical exposures can result in disorders of the hematopoiesis. Indeed, the observational data derived from atomic bomb survivors without a diagnosis of hematological malignancy reported that CH may be accelerated by radiation exposure resulting in peripheral blood monocytosis [155]. Additionally, exposure of chemotherapeutic agents especially to those that cause DNA damage (e.g. alkylating agents, taxanes, anthracyclines, and topoisomerase inhibitors), benzene, and nicotine as well as alcohol abuse are known risk factors for MDS/AML [156-160].

1.3.3.1 Incidence of AML

AML is a rare disease with an incidence that increases with age. The incidence to develop AML is slightly increased in early childhood (0-4 years), decreases in adolescence, but increases rapidly in elderly persons (Figure 5) [161]. Approximately 14,000 people (6,010 females, 7,900 males) are newly diagnosed with leukemia each year in Germany (status 2016). Of those, 25% of women and 22% of men are diagnosed with AML (Table 5) [162]. This corresponds to overall incidence rates in Europe, which observed rates of 3.49 males and 2.76 females in 100,000 European citizens suffering from AML [163]. In adults, men are more frequently affected by AML than women (Table 5) [164].

Table 5: Overall leukemia incidences for females and males, deaths and 5-year survival rates in Germany in 2016. Modified from Robert-Koch-Institute [162]

	Female	Male
Incidence (new cases per year)	6,010	7,900
Standardized disease rate	8.6*	13.5*
Deaths	3,710	4,542
Standardized mortality rate	4.0*	6.6*
5-year relative survival rate	57%	58%
10-year relative survival rate	49%	48%

*per 100,000 inhabitants

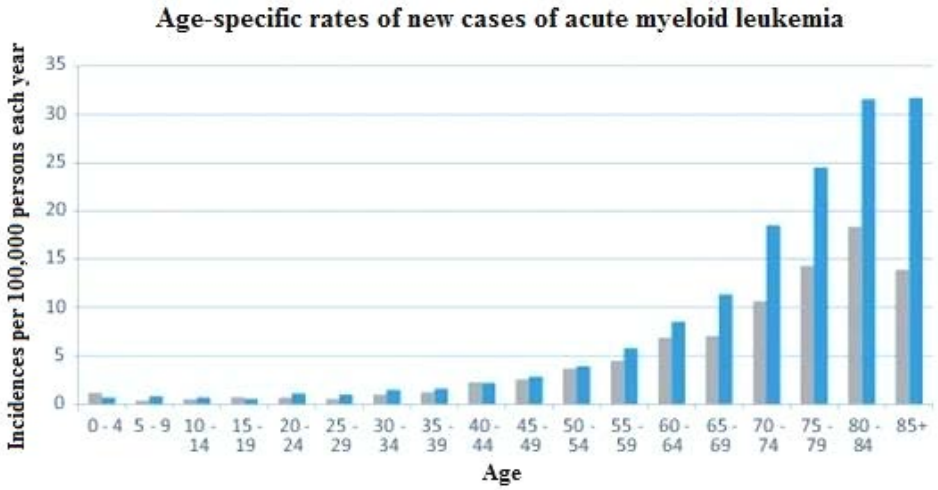

Figure 5: Age-specific rates of new cases of acute myeloid leukemia (AML) in Germany by gender. Selected registers, 2011 to 2013; incidences per 100,000 persons per year. blue=male; grey=female. Adapted from Kraywinkel et al. [161]

1.3.3.2 Symptoms and diagnosis of AML

The infiltration of the immature myeloid blasts can cause severe health problems such as anemia, infections and bleeding propensity in the whole body. According to the type of affected blood cells, the symptoms of AML patients are very heterogenic.

General initial symptoms are fatigue, exhaustion and pain due to the lack of mature erythrocytes that are not able to supply the cells with the essential sufficient oxygen [165].

Since leukocytes are responsible for the body's immune response, their deficiency leads to increased susceptibility to infections. Another frequent symptom can be bleeding, often under the skin, caused by impaired blood coagulation and low platelet count [166]. Due to the possible spread of the malignant cells into intestinal organs, the symptoms can be very extensive, ranging from vomiting, nausea and severe pain to enlargement of organs that prevent normal function [140, 166].

If AML is suspected, peripheral blood and bone marrow smear are morphologically examined [106]. For this purpose, at least 200 leukocytes on blood smears and 500 nucleated red blood cells (immature blasts) on bone marrow smears should be counted. In a healthy hematopoietic system, the nucleus is ejected during cellular differentiation before the cell is released into the bloodstream. A bone marrow or blood blast count ≥20% is considered a sufficient criterion for reliable diagnosis for most types of AML. Exceptions are the evidence of acquired recurrent chromosomal aberration defining AML such as translocations i.e. t(15;17), t(8;21), t(16;16), inversions i.e. inv(16) and some cases of erythroleukemia (Table 5) [144, 167].

1.3.3.3 Classification of AML

As mentioned before (see Chapter 1.1 Ovarian cancer), cancer staging provides helpful information about prognosis and treatment options. In contrast to solid tumors, leukemia such as AML can spread throughout the bone marrow or in some cases to other organs. Therefore, the classification of leukemia is different to other cancer types and involves more factors than staging of the main tumor and expansion/infiltration of tumor cells into other organs and tissues. There are two main ways of classifying AML: Firstly, AML can be classified by the French-American-British (FAB) classification system (Table 6), implemented in 1976 [168], which is based on cytomorphological aspects such as the cell type and the degree of differentiation [169]. The FAB classification according to the subtypes of AML can be very important, as its allows optimized treatment options [148]. However, the FAB system takes not all factors into account that are now known to affect prognosis [144]. Here, the nine subclasses play the major role in evaluating the prognosis of the patient.

Table 6: The French-American-British (FAB) classification system. Subtypes M0 through M5 all start in immature forms of white blood cells. M6 starts in very immature forms of red blood cells, while M7 starts in immature forms of platelets. Adapted from Bennet et al. [168, 169]

FAB class	Name of subtype	Frequency of cases
M0	Undifferentiated acute myeloblastic leukemia	<5%
M1	Acute myeloblastic leukemia with minimal maturation	15-20%
M2	Acute myeloblastic leukemia with maturation	25-30%
M3	Acute promyelocytic leukemia (APL)	5-10%
M4	Acute myelomonocytic leukemia	20-30%
M4 eos	Acute myelomonocytic leukemia with eosinophilia	Rare
M5	Acute monocytic leukemia	5-10%
M6	Acute erythroid leukemia	5%
M7	Acute megakaryoblastic leukemia	5-10%

Secondly, AML can be classified according to the World Health Organization (WHO) Classification of tumours of haematopoietic and lymphoid tissues that include more factors to modify the old FAB system. Because cytomorphologic assessments alone are often not informative for subgrouping, the WHO system is expanding the criteria to include cytogenetic and molecular genetic factors in addition to morphologic characteristics [144].

There are three major categories of the current classification (updated in 2016), firstly, *de novo* AML with recurrent genetic abnormalities, secondly, secondary AML with myelodysplasia-related changes (s-AML) and lastly therapy-related AML (t-AML). These three AML groups are divided into several subgroups (Table 7) [144].

Table 7: World Health Organization classification (WHO) for acute myeloid leukemia (AML) and related neoplasms. Modified from Arber et al. [144]; AML= acute myeloid leukemia; APL= acute promyelocytic leukemia; WHO=World Health Organization; *Provisional entry

World Health Organization Classification (WHO) for acute myeloid leukemia (AML) and related neoplasms	
AML with recurrent genetic abnormalities	
AML with t(8;21)(q22;q22.1); *RUNX1-RUNX1T1*	AML (megakaryoblastic) with t(1;22)(p13.3;q13.3);*RBM15-MKL1*
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*	*AML with *BCR-ABL1* gene fusion
ALP with t(15;17)(q24.1;q21.2); PML-*RARA*	AML with mutated *NPM1*
AML with t(9,11)(p21.3;q23.3); *MLLT3-KMT2A*	AML with biallelic mutations of *CEBPA*
AML with t(6;9)(p23;q34.1);*DEK-NUP214*	*AML with mutated *RUNX1*
AML with inv(3)(q21.3;q26.2) or t(3;3)(q21.3;q26.2); *GATA2, MECOM*	
AML with Myelodysplasia-Related Changes	
Therapy-related Myeloid Neoplasms	
AML, not otherwise specified (NOS)	
Myeloid Sarcoma	
Myeloid Proliferation Related to Down Syndrome	

1.3.3.4 The genetic landscape of MDS/AML

AML is characterized by a high degree of heterogeneity in terms of e.g. chromosomal abnormalities and gene mutations [170]. Somatic chromosomal abnormalities are found in approximately 50% of all newly detected AML cases [112, 170-172]. Their proportion is increased in patients with secondary leukemia or advanced age [112]. Chromosomal rearrangements as duplications, translocations, inversions and deletions are defined as aberrations that may change the whole structure of a chromosome that can result in *de novo* AML [125, 173]. According to the WHO criteria (Table 7), frequent chromosomal aberrations

were subdivided into different classes including well-characterized translocations such as t(15;17)(q24.1;q21.2), t(8;21)(q22;q22.1), t(9;11)(p21.3;q23.3), and the inversion inv(16)(p13.1q22) [170, 174-176]. Molecular studies have shown that these structural chromosome rearrangements can create fusion genes that alter their expression leading to functional activation of these genes that may contribute to the initiation or progression of leukemogenesis [177]. According to the WHO classification, the listed recurrent genetic abnormalities have important roles in predicting outcome, choice of treatment and overall survival [140, 144]. Patients with comparable better prognosis are those with functional inactivation of the core binding factors AML1 and CBFb. These cases include patients with AML and the balanced t(8;21)(q22;q22) or inv(16)(p13;q22), and acute promyelocytic leukemia (APL) with the translocation t(15;17). This translocation is always associated with APL and leads to the expression of PML-RARA fusion gene in hematopoietic myeloid cells [178]. Potential targeting of fusion genes has become a major focus for the development of novel therapeutics [179]. For example, all-trans retinoic acid ATRA that is able to alter the co-repressor interaction with the APL fusion protein is used in remission induction and has become a fundamental treatment of AML [180, 181].

In contrast to anomalies with “good” outcome, patients with large deletions e.g. loss of all or part of chromosome 5 or 7, or with translocations involving the long arm of chromosome 11q (e.g.11q23), or anomalies of chromosome 3 have a comparatively poor prognosis [112].

Besides leukemia-specific cytogenetic abnormalities, about half of patients suffering from AML present a karyotype without pathological findings [182]. Genomic studies have identified recurrent somatic PVs in patients with myeloproliferative neoplasms, including MDS and AML [183]. In some cases these PVs occur in genes with known roles in hematopoietic progenitors e.g. transcription (Nucleophosmin (*NPM1),* CCAAT Enhancer Binding Protein Alpha *(CEBPA)* Runt-related transcription factor 1 (*RUNX1),* and Wilms tumor 1 (*WT1*)-splicing (*SF3B1, SRSF2*) or signaling (*JAK2,* Neuroblastoma RAS Viral Oncogene Homolog *(NRAS),* Fms Related Receptor Tyrosine Kinase 3 *(FLT3),* and KIT Proto-Oncogene *(KIT*)) [112, 125, 129, 135, 166].

In other cases, genetic and functional studies have elucidated the role of specific mutations in altering epigenetic patterning in myeloid malignancies. Recent genetic and functional studies have identified PVs in epigenetic modifiers, including *ASXL1*, *DNMT3A*, enhancer of zeste homologue 2 (*EZH2*), isocitrate dehydrogenase 1 and 2 *(IDH1/2)* and *TET2* [114, 141, 184, 185]. Of note, *TET2* mutations are present in up to 25% of patients with a diagnosis of

myeloid malignancy, only few individuals had been found with mutations in established leukemia driver genes [136, 186].

ASXL1 and *DNMT3A* PVs are both highly recurrent in patients with *de novo* AML with an intermediate-risk cytogenetic profile and are independently associated with a poor outcome [187, 188]. Furthermore, germline PVs in the *TP53* gene occur in a subset of patients with AML and confer an exceedingly adverse prognosis, especially in therapy-related subtypes that may develop after ionizing irradiation [189-192]. Of note, PVs in *TP53* often occur concomitantly with chromosomal abnormalities known to be associated with *de novo* AML [140]. In *de novo* AML/MDS patients, PVs in the *PPM1D* gene occur only in <5% and are therefore likely to be of minor importance for disease development [131].

1.3.3.5 Prognosis of AML

AML is the most common form of leukemia in adults, yet continues to have the lowest survival rate of all leukemias [166]. Several decades ago there was only a little chance of recovery, but nowadays AML can be cured with intense treatment [193]. Intense intravenous chemotherapy is the main treatment for most patients affected by AML because it reaches all areas of the body, and therefore can reach and kill leukemic cells that spread throughout the body. The two most commonly used chemotherapeutic agents are a combination of cytarabine plus daunorubicin or cytarabine plus idarubicin [179]. As mentioned, the discovery of new molecular targets is giving rise to the development of novel selective agents for AML therapy. Two independent studies showed 5-year relative survival rate of 22.8 to 24% considering all AML patients in Germany [193, 194]. Looking at 5,277 patients with AML, Nennecke et al. showed a 5-year relative survival rate of 57.7% for both, females and males who are 50 years of age or younger. With increasing age the survival rate lowers down and drops rapidly to only 8% for patients who are older than 70 years of age (Figure 6). The reason for this rapid decrease is often the poor health condition in this subgroup [193]. As previously described, the classification of AML subtypes with recurrent genetic abnormalities also plays a crucial role with regard to prognosis. Additionally, t-AML induced by prior cytotoxic chemotherapy (CTx) or radiation is associated with a poorer outcome than *de novo* AML [102, 159, 195].

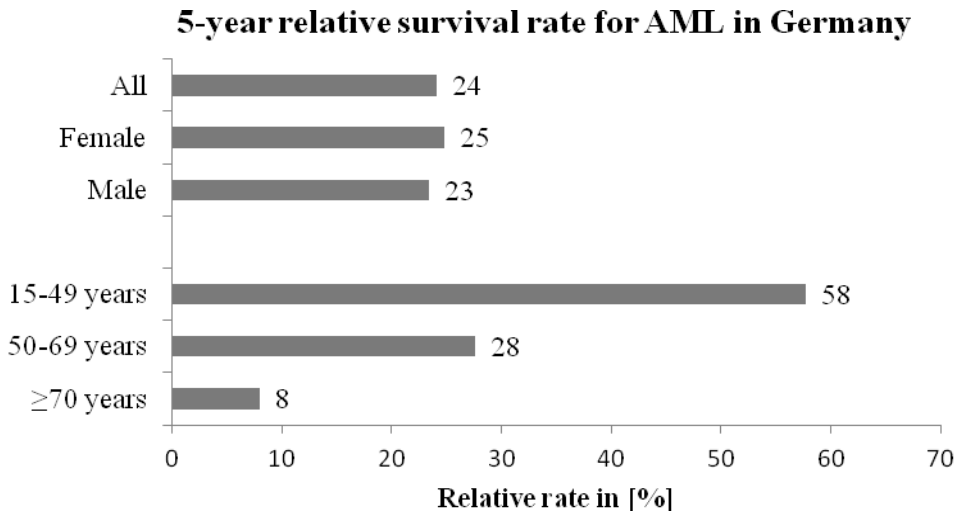

Figure 6: 5-year relative survival rates for acute myeloid leukemia (AML) in Germany 2008-2010. Relative rates are displayed in % for different age groups. Modified from Nennecke et al. [193]

1.3.4 Therapy-related myeloid neoplasms

Chemotherapeutic side effects are common in cancer treatment, especially those that harm the bone marrow [196]. During treatment, chemotherapeutic drugs spread through the whole body, destroying not only the targeted fast-growing and multiplying cancer cells, but can also affect healthy fast-growing cells e.g. hair follicles, cells in the digestive tract or blood forming cells in the hematopoietic compartment. This is why CTx can cause side effects such as hair loss, diarrhea, and anemia. In rare cases, exposing normal cells to cancer treatments such as CTx and radiation therapy can cause a new, different type of cancer developing many years after treatment [197]. This effect induced inadvertently by a physician, by medical treatment or diagnostic procedures is called iatrogenic [198].

Based on current investigations, several drugs are able to induce somatic mutations during CTx, driven by genotoxic stress, in the HSPCs, which can lead to CHIP and furthermore to the development of therapy-related myeloid neoplasms (t-MN) such as MDS and AML [29, 199]. These chemotherapeutic agents are used in neoadjuvant (before surgery) or adjuvant (after surgery) therapy of OC and are suspected of promoting the development of hematologic diseases [41]. The extent of the effects on disease risk varies by drug class.

Approved drugs for cancer treatment are DNA alkylating agents (e.g. cyclophosphamide, busulfan), antimetabolites, anthracyclines (e.g. doxorubicin, epirubicin), topoisomerase inhibitors and taxanes as well as PARP-inhibitors e.g. olaparib (approved in 2015) [200, 201]. Platinum derivatives (e.g. cisplatin, carboplatin) likewise appear to increase the risk to develop t-AML, but in a lower extent than DNA alkylating agents [201, 202]. Furthermore, the potential of PARP inhibitors, e.g. olaparib, to increase the risk of AML is unknown.

Hence, the majority of patients with OC receive platinum-based chemotherapeutic treatment; these patients have a significantly increased risk to develop t-AML [202, 203]. Previous studies demonstrate that pre-existing mutant clones with an acquired fitness advantage compared to normal clones can proliferate uncontrolled under selective pressure of CTx [204-207]. Wong et al. showed that *TP53* mutations associated with CH are resistant to CTx and compete after treatment with non-mutated cells [199].

Beside CH-associated mutations in *TP53*, PVs in the *PPM1D* gene were associated with prior exposure to CTx [199, 205, 208, 209]. CRISPR-Cas generated *PPM1D* mutant cells showed under cytotoxic conditions selective advantage towards growth and proliferation [63, 67, 206]. PVs in *PPM1D* typically cluster in the terminal exon (exon 6), leading to the loss of a C-terminal degradation domain [67]. A significant increase of *PPM1D* mutations in the terminal exon could be observed in patients with therapy-related MDS (t-MDS) and t-AML [206]. Overall, *PPM1D* is the second most frequently mutated gene, accounting for 20% of t-MN cases [206].

In summary, patients have an increased risk to develop hematologic secondary malignancies after treatment of solid tumor entities e.g. during or after the treatment of OC [210]. Swisher et al. identified deleterious somatic mutations in *TP53* and *PPM1D* in patients with OC, concluding a strong association with prior CTx and age at blood draw [208]. Furthermore, dose-dependent platinum-based treatment of patients with OC increases the frequency of somatic mosaic *PPM1D* mutations and the risk of therapy-related leukemia [202, 205].

In addition, t-AML patients have a poorer outcome compared with *de novo* AML patients [159]. Therefore, therapy-related CH plays a major role in t-MDS/t-AML pathogenesis [34, 35, 208].

2 Aims of the PhD thesis

Most of the non-*BRCA1/2* OC susceptibility genes have been discovered due to direct interaction of their encoded proteins with BRCA1 or BRCA2 and their role in HRR: since BARD1 can directly interact with the encoded protein of the BC/OC high-risk gene *BRCA1*, PVs in *BARD1* may lead to an enhancement of tumor suppressor functions (mitigation) by acting on double-strand break repair and apoptosis initiation. Due to controversial results of case-control investigations, the role of PVs in *BARD1* in cancer predisposition remains unclear.

In the first part of my PhD project, I investigated the role of the gene *BARD1* in OC predisposition by screening 451 familial index patients with OC for PVs in *BARD1*.

In the second part, the focus is on CH in patients with hereditary and non-hereditary OC. Here, the focus was on identifying novel biomarkers for therapy-related CH in patients with OC after CTx exposure. It is known that aging is strongly associated with an increased CH risk, resulting from the accumulation of pre-leukemic mutations in HSPCs. Besides the risk factor age, cytotoxic agents play a crucial role in the development of CH. The mosaic mutations in risk genes, more often detectable after CTx, seem to represent a selection advantage for the affected HSPCs, which ultimately results in therapy-related CH.

In our study cohort (observational AGO TR1 study), I screened for blood-specific PVs in ten genes associated with age-dependent CH (*ASXL1, DNMT3A, JAK2, GNAS, PPM1D, SF3B1, SH2B3, SRSF2,* and *TP53*) by performing a paired analysis in which the DNA isolated from the blood was compared with the FFPE tumor tissue.

Furthermore, the aim was to assess whether carriership of PVs in OC-associated genes, namely *ATM, BRCA1, BRCA2, BRIP1, MSH2, MSH6, PALB2, RAD51C*, *RAD51D,* and *TP53,* involved in DNA damage repair, predispose to the development of therapy-induced CH after CTx exposure.

3 Main publications

3.1 Germline loss-of-function variants in the *BARD1* gene are associated with early-onset familial breast cancer but not ovarian cancer.

Weber-Lassalle N*, Borde J*, **Weber-Lassalle K***, Horváth J, Niederacher D, Arnold N, Kaulfuß S, Ernst C, Paul VG, Honisch E, Klaschik K, Volk AE, Kubisch C, Rapp S, Lichey N, Altmüller J, Lepkes L, Pohl-Rescigno E, Thiele H, Nürnberg P, Larsen M, Richters L, Rhiem K, Wappenschmidt B, Engel C, Meindl A, Schmutzler RK, Hahnen E, and Hauke J.

Breast Cancer Res. 2019 Apr 29;21(1):55. doi: 10.1186/s13058-019-1137-9.

*authors contributed equally to this work

My own contributions

I supported the project conception and the definition of the study cohorts. DNA sample recruitment of 4,469 female index patients with BC, 451 index patients with OC, and 2,767 geographically-matched controls, as well as the preparation of blood-derived DNA samples was performed jointly with my colleague Julika Borde. Samples centrally stored in the national biobank of the Center for Hereditary Breast and Ovarian Cancer Cologne, were recruited by 16 centers of the GC-HBOC. I performed the coordination and the DNA quantification (standardization). Samples from the center Cologne were prepared by employing standardized workflow. I curated clinical and genetic data e.g. AAD, family history and receptor status of tumors to get more information about the role of the *BARD1* gene in pathogenesis.
Adjustment of the study sample was performed jointly with my colleague Nana Weber-Lassalle. In close cooperation with the writing team, consisting of Nana Weber-Lassalle, Julika Borde, PD Dr. Eric Hahnen, Prof. Dr. Rita K. Schmutzler, and Dr. Jan Hauke, I wrote the manuscript and designed the tables for the publication, and performed the proofreading of manuscript drafts.

Contributions of co-authors

Nana Weber-Lassalle (Center for Familial Breast and Ovarian Cancer, Center for Integrated Oncology (CIO), University of Cologne, Faculty of Medicine and University Hospital Cologne, Cologne, Germany) was involved in the project conception, definition of the study cohorts and the collection of available phenotype data including the stratification of the study

sample. She generated the data of the patients and controls, using NGS, and conducted variant classification. In accordance to internationally proposed guidelines, she calculated odds ratios as well as *P*-values with the Fisher´s exact test by using SPSS-statistics. She designed all primers for all class 4/5 variants in *BARD1* (identified by NGS) for Sanger sequencing.

Julika Borde (Center for Hereditary Breast and Ovarian Cancer, University of Cologne, Faculty of Medicine and University Hospital Cologne, Cologne) was involved in analyzing the clinical and genetic data, performed Sanger sequencing for confirmation of all pathogenic variants in the *BARD1* gene and checked data analysis as well as calculations of carrier frequencies and ORs. Corinna Ernst (Center for Hereditary Breast and Ovarian Cancer, University of Cologne, Faculty of Medicine and University Hospital Cologne, Cologne) performed the bioinformatic analyses of the NGS data (cases and controls). Dr. Holger Thiele, Prof. Dr. Nürnberg and Dr. Janine Altmüller coordinated the NGS runs using the HiSeq4000 sequencer at the Cologne Center for Genomics, University of Cologne, in Cologne. PD Dr. Eric Hahnen and Dr. Jan Hauke (Center for Hereditary Breast and Ovarian Cancer, University of Cologne, Faculty of Medicine and University Hospital Cologne, Cologne) designed the study, conducted and supervised variant classification. Both had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. All other authors provided DNA samples and/or basic clinical and genetic data. All authors read and approved the final manuscript.

Weber-Lassalle et al. Breast Cancer Research (2019) 21:55
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Breast Cancer Research

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Germline loss-of-function variants in the *BARD1* gene are associated with early-onset familial breast cancer but not ovarian cancer

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Abstract

Background: The role of the *BARD1* gene in breast cancer (BC) and ovarian cancer (OC) predisposition remains elusive, as published case-control investigations have revealed controversial results. We aimed to assess the role of deleterious *BARD1* germline variants in BC/OC predisposition in a sample of 4920 *BRCA1/2*-negative female BC/OC index patients of the German Consortium for Hereditary Breast and Ovarian Cancer (GC-HBOC).

Methods: A total of 4469 female index patients with BC, 451 index patients with OC, and 2767 geographically matched female control individuals were screened for loss-of-function (LoF) mutations and potentially damaging rare missense variants in *BARD1*. All patients met the inclusion criteria of the GC-HBOC for germline testing and reported at least one relative with BC or OC. Additional control datasets (Exome Aggregation Consortium, ExAC; Fabulous Ladies Over Seventy, FLOSSIES) were included for the calculation of odds ratios (ORs).

Results: We identified LoF variants in 23 of 4469 BC index patients (0.51%) and in 36 of 37,265 control individuals (0.10%), resulting in an OR of 5.35 (95% confidence interval [CI] = 3.17–9.04; $P < 0.00001$). *BARD1*-mutated BC index patients showed a significantly younger mean age at first diagnosis (AAD; 42.3 years, range 24–60 years) compared with the overall study sample (48.6 years, range 17–92 years; $P = 0.00347$). In the subgroup of BC index patients with an AAD < 40 years, an OR of 12.04 (95% CI = 5.78–25.08; $P < 0.00001$) was observed. An OR of 7.43 (95% CI = 4.26–12.98; $P < 0.00001$) was observed when stratified for an AAD < 50 years. LoF variants in *BARD1* were not significantly associated with BC in the subgroup of index patients with an AAD ≥ 50 years (OR = 2.29; 95% CI = 0.82–6.45; $P = 0.11217$). Overall, rare and predicted damaging *BARD1* missense variants were significantly more prevalent in BC index patients compared with control individuals (OR = 2.15; 95% CI = 1.26–3.67; $P = 0.00723$). Neither LoF variants nor predicted damaging rare missense variants in *BARD1* were identified in 451 familial index patients with OC.

Conclusions: Due to the significant association of germline LoF variants in *BARD1* with early-onset BC, we suggest that intensified BC surveillance programs should be offered to women carrying pathogenic *BARD1* gene variants.

Keywords: Early onset breast cancer, Ovarian cancer, *BARD1*, Germline mutations

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Background

The BRCA1-associated RING domain protein-1 (BARD1) was initially reported as a BRCA1-interacting protein by Wu et al. in 1996 [1]. The BRCA1 and BARD1 proteins show high structural homology, as they share *N*-terminal RING finger domains and BRCA1 C-terminal (BRCT) domains. Both proteins can form homodimers via their *N*-terminal RING finger domains [2, 3] but preferentially form more stable heterodimers involving amino acid residues 1–109 of the BRCA1 protein and amino acid residues 26–119 of the BARD1 protein [4]. The interaction between BARD1 and BRCA1 promotes tumor suppressor functions by acting in double-strand break repair and apoptosis initiation.

While the role of the *BRCA1* gene (MIM *113705) in breast cancer (BC) and ovarian cancer (OC) predisposition is well established [5], the role of *BARD1* (MIM *601593) in BC/OC predisposition remains elusive. Several case-control studies have investigated the association between deleterious germline variants in *BARD1* and the risk of developing female BC. Slavin et al. identified deleterious *BARD1* variants in 7 of 2134 *BRCA1/2*-negative familial BC patients (carrier frequency = 0.33%) and reported *BARD1* as a moderate-risk BC predisposition gene with an odds ratio (OR) of 3.18 (95% confidence interval [CI] = 1.34–7.36; P = 0.012) [6]. The considerably larger investigation of 28,536 BC patients of European ancestry by Couch et al. [7] revealed a carrier frequency of 0.18% (52/28,536) in BC patients and an OR of 2.16 (95% CI = 1.31–3.63; P = 0.00226). In contrast, however, recent studies by Lu et al. and Castéra et al. encompassing 9639 and 3667 patients with BC, respectively, did not show a significant association of deleterious *BARD1* variants with overall BC risk [8, 9]. Studies investigating the association of deleterious *BARD1* germline variants with OC risk, likewise, showed contradictory results. Norquist et al. identified protein-truncating germline variants in 4 of 1915 OC patients unselected for age or family history and in 18 of 36,276 control individuals, resulting in an OR of 4.2 (95% CI = 1.4–12.5; P = 0.02) [10]. In contrast, Ramus et al. were unable to demonstrate a significant association with OC in their study of 3261 unselected patients with epithelial OC and 3449 control individuals (4/3261, carrier frequency = 0.12%; 2/3449, carrier frequency = 0.06%; P = 0.39) [11]. Lilyquist et al. found deleterious *BARD1* germline variants in 8 of 6294 OC patients (carrier frequency = 0.13%) and calculated a nonsignificant risk ratio of 1.28 (95% CI = 0.55–2.51; P = 0.59) for OC [12]. Taken together, the role of deleterious *BARD1* germline variants in BC/OC predisposition remains unclear. In this study, we investigated the prevalence of deleterious *BARD1* germline variants in a sample of 4469 familial BC and 451 familial OC index patients of the German Consortium for Hereditary Breast and Ovarian Cancer (GC-HBOC) and 2767 geographically matched female controls (GMCs).

Methods

Study sample

A total of 4469 index patients with BC (mean age at first diagnosis [AAD] 48.6 years; range 17–92 years), 451 index patients with OC (mean AAD 53.4 years; range 18–85 years), and 2767 GMCs were screened for germline variants in *BARD1* (transcript NM_000465.3). All patients met the inclusion criteria of the GC-HBOC for germline testing [13] (Additional file 1: Table S1) and had at least one relative with BC or OC. Index patients with a personal history of both BC and OC were not included in this study. All patients were screened for pathogenic germline variants in BC/OC predisposition genes in a routine diagnostic setting using the TruRisk® gene panel of the GC-HBOC and tested negative for pathogenic *BRCA1/2* germline variants. Of the 4469 familial BC index patients, 3651 had a BC family history and no OC family history. Of the remaining 818 BC index patients, at least one family member with OC was reported. GMCs were aged 40 years and above and were cancer-free at the time of blood draw (mean age at blood draw 64.2 years; range 40–92 years). Written informed consent was obtained from all patients and controls; ethical approval was granted by the Ethics Committee of the University of Cologne (07-048). Two publicly accessible control datasets were used in this study (Table 1). From the Exome Aggregation Consortium (ExAC) [14], we requested a dataset of individuals of European, non-Finnish ancestry, excluding samples from The Cancer Genome Atlas (TCGA). This dataset comprises a total of 27,173 samples that were analyzed by whole-exome sequencing. The Fabulous Ladies Over Seventy (FLOSSIES) project provides a dataset of 7325 women of European American ancestry (https://whi.color.com). All participating women have remained cancer-free until at least 70 years of age. Blood-derived DNA samples of all participants were screened for variants in 27 established or suggested BC predisposition genes, including *BARD1*.

Next-generation sequencing (NGS)

Genomic DNA was isolated from venous blood samples. NGS and data analyses were carried out at each participating GC-HBOC center using Illumina sequencing platforms (MiSeq or NextSeq; Illumina, San Diego, CA, USA), employing the customized hybridization capture-based TruRisk® gene panel for target enrichment (manufactured by Agilent, Santa Clara, CA, USA; or Illumina). The diagnostic pipelines of the labs involved have been successfully tested in European Molecular Genetics

Table 1 Prevalence of heterozygous germline loss-of-function (LoF) variants identified in the *BARD1* gene (transcript NM_000465.3) in controls and index patients with breast cancer (BC) or ovarian cancer (OC) according to family history and age at first diagnosis (AAD). A total of 26 heterozygous germline LoF variants were listed in the ExAC database (Exome Aggregation Consortium, non-Finnish Europeans (NFE); excluding The Cancer Genome Atlas data (TCGA); as of June 2016); 8 heterozygous germline LoF variants were listed in the FLOSSIES database ("Fabulous Ladies Over Seventy"; American-European ancestry); 2 heterozygous germline LoF variants were identified in geographically matched female controls (GMCs); 23 germline LoF variants were found in 4469 familial index patients with BC; no heterozygous germline LoF variant was found in 451 familial index patients with OC. Univariate logistic regression was performed to estimate odds ratios (ORs) and 95% confidence intervals (CIs). When considering ExAC NFE nonTCGA controls only, ORs were similar to those given in Table 1 which consider all controls ([A]OR = 5.40, 95% CI = 3.08–9.47, $P < 0.00001$; [B]OR = 5.46, 95% CI = 3.02–9.88, P < 0.00001; [C]OR = 5.13, 95% CI = 1.79–14.74, $P = 0.01084$; [D]OR = 12.16, 95% CI = 5.68–26.03, $P < 0.00001$; [E]OR = 7.51, 95% CI = 4.15–13.58, $P < 0.00001$; [F]OR = 5.58, 95% CI = 2.69–11.60, $P = 0.00007$; [G]OR = 2.74, 95% CI = 0.83–9.08, $P = 0.11082$; [H]OR = 2.32, 95% CI = 0.81–6.64, $P = 0.11396$, [I]OR = 1.58, 95% CI = 0.21–11.66, $P = 0.47806$)

Study sample	Heterozygous carriers/number of tested individuals	Carrier frequency (%)	OR	95% CI	*P* value (Fisher's exact test)
ExAC NFE nonTCGA	26/27,173	0.10	/	/	/
FLOSSIES	8/7325	0.11	/	/	/
GMCs	2/2767	0.07	/	/	/
All controls	36/37,265	0.10	/	/	/
Familial BC index patients	23/4469	0.51	5.35[A]	3.17–9.04	< 0.00001
Relative(s) with BC only	19/3651	0.52	5.41[B]	3.10–9.44	< 0.00001
Relative(s) with OC	4/818	0.49	5.08[C]	1.81–14.31	0.01046
AAD < 40	9/782	1.15	12.04[D]	5.78–25.08	< 0.00001
AAD < 50	19/2662	0.71	7.43[E]	4.26–12.98	< 0.00001
AAD 40–49	10/1880	0.53	5.53[F]	2.74–11.16	0.00005
AAD 50–59	3/1145	0.26	2.72[G]	0.84–8.83	0.10969
AAD ≥ 50	4/1807	0.22	2.29[H]	0.82–6.45	0.11217
AAD ≥ 60	1/662	0.15	1.57[I]	0.21–11.43	0.47891
Familial OC index patients	0/451	/	/	/	/
Relative(s) with BC only	0/379	/	/	/	/
Relative(s) with OC	0/72	/	/	/	/

Quality Network (EMQN) schemes. Predictions of large genomic rearrangements (LGRs) on the basis of NGS data are prone to give false-positive results and thus require validation. To date, no multiplex ligation-dependent probe amplification (MLPA) assay for the *BARD1* gene is commercially available. Thus, we did not include LGRs in our investigation [15].

Variant classification

Variant classification was performed as previously described [16]. Briefly, all genetic variants were classified using a five-tier variant classification system as proposed by the International Agency for Research on Cancer (IARC) Unclassified Genetic Variants Working Group, namely, deleterious = class 5, likely deleterious = class 4, variant of uncertain significance (VUS) = class 3, likely benign = class 2, and benign = class 1. Variants reported to occur in large outbred control reference groups at an allele frequency of > 1% were generally considered benign. Loss-of-function (LoF) variants were defined as nonsense, frameshift, or essential splice site mutations affecting invariant splice sites or the last nucleotide of an exon. Missense variants were defined as potentially damaging when predicted deleterious by the in silico prediction tools SIFT and MutationTaster (Alamut version 2.10 as of November 9, 2017). Missense variants with a minor allele frequency (MAF) of < 0.1% in ExAC (non-Finnish Europeans; excluding TCGA data; as of June 2016) were defined as rare. All pathogenic (class 4/5) germline variants identified in patients and GMCs were confirmed by Sanger sequencing.

Results

In our study sample of 4469 familial BC index patients, 23 patients carried heterozygous germline LoF variants in *BARD1*, resulting in a carrier frequency of 0.51% (Table 1). One *BARD1*-mutated BC index patient additionally carried a heterozygous germline LoF variant in the *CHEK2* gene (patient 5; c.902del, p.Glu301Glyfs*; Additional file 1: Table S2). The remaining 22 *BARD1*-mutated index patients tested negative for pathogenic variants in further BC/OC predisposition genes (*ATM*, *BRCA1*, *BRCA2*, *CDH1*, *CHEK2*, *PALB2*, *RAD51C*, *RAD51D*, and

TP53). Information regarding the hormone receptor (estrogen receptor [ER]/progesterone receptor [PR]) and human epidermal growth factor receptor 2 (HER2) status of the tumor was available for 20/23 *BARD1*-mutated index patients with BC (Additional file 1: Table S2). Most *BARD1*-mutated index patients with BC developed hormone receptor-positive (ER-positive: 15/20; PR-positive: 11/20) and HER2-negative tumors (20/20). A triple-negative tumor phenotype was reported for 4 of 20 *BARD1*-mutated index patients with BC (Additional file 1: Table S2).

The carrier frequency observed in 4469 familial index patients with BC was elevated compared with the carrier frequencies observed in control datasets, which ranged from 0.07% (GMCs) to 0.11% (FLOSSIES) (Table 1). The comparison of carrier frequencies in the study sample of 4469 familial index patients with BC (23/4469, carrier frequency = 0.51%) and all control individuals (36/37,265, carrier frequency = 0.10%) revealed an OR of 5.35 (95% CI = 3.17–9.04; $P < 0.00001$) (Table 1). The subgroup of index patients with BC and heterozygous germline LoF variants in *BARD1* showed a younger mean AAD of BC (42.3 years; range 24–60 years) compared with the overall sample of index patients with BC (48.6 years; range 17–92 years), with differences reaching levels of significance ($P = 0.00347$; Student's t test). When comparing LoF variant prevalence in the subgroup of index patients with BC and an AAD < 40 years and all control individuals, an OR of 12.04 (95% CI = 5.78–25.08; $P < 0.00001$) was observed (Table 1). An OR of 7.43 (95% CI = 4.26–12.98; $P < 0.00001$) was observed when stratified for an AAD < 50 years. Heterozygous germline LoF variants in *BARD1* were not significantly associated with BC in the subgroup of 1807 BC index patients with an AAD ≥ 50 years, although the ORs were marginally elevated (Table 1). All heterozygous germline LoF variants in *BARD1* identified in patients with BC and in control individuals are listed in the supplements (Additional file 1: Table S3).

Data on proven pathogenic *BARD1* missense variants are currently lacking [17–22]. To examine the potential association of missense variants in *BARD1* with BC risk, we focused on potentially damaging rare missense variants (MAF < 0.1%), which were predicted to be damaging by the SIFT and MutationTaster algorithms. The carrier frequency of potentially damaging rare *BARD1* missense variants was 0.18% for all control individuals (66/37,265; Additional file 1: Table S4). Rare *BARD1* missense variants predicted to be damaging by both tools were significantly more prevalent in index patients with BC compared with control individuals (17/4469, carrier frequency = 0.38%; OR = 2.15; 95% CI = 1.26–3.67; $P = 0.00723$; Additional file 1: Table S4). A slightly elevated association was observed for potentially damaging rare *BARD1* missense variants affecting the two BRCT domains spanning the amino acid residues 560–653 and 667–777 (9/4469, carrier frequency = 0.20%; OR = 2.42; 95% CI = 1.15–5.09; $P = 0.03398$; Additional file 1: Table S4).

In summary, *BARD1* appears to be a risk gene for early-onset familial BC. To avoid a recruitment bias by OC, we next stratified the study sample according to family history. In the subgroup of 3651 index patients with BC and without an OC family history (mean AAD 48.3 years; range 19–91 years), 19 patients carried heterozygous germline LoF variants in *BARD1*, resulting in a carrier frequency of 0.52% and an OR of 5.41 (95% CI = 3.10–9.44; $P < 0.00001$) compared with all control individuals (Table 1). In the subgroup of 818 index patients with BC and at least one relative with OC (mean AAD 50.1 years; range 17–92 years), 4 index patients carried heterozygous germline LoF variants in *BARD1* (carrier frequency = 0.49%) and an OR of 5.08 (95% CI = 1.81–14.31; $P = 0.01046$) compared with all control individuals. Thus, an OC family history did not affect the prevalence of *BARD1* LoF variants. The analysis of 451 familial index patients with OC (mean AAD 53.4 years; range 18–85 years) did not reveal heterozygous germline LoF variants in *BARD1* (Table 1), and none of the patients with OC carried potentially damaging rare *BARD1* missense variants.

Discussion

We did not observe evidence that deleterious *BARD1* gene variants predispose for OC. LoF germline variants in *BARD1* could neither be detected in 451 familial OC index patients investigated in this study nor in our previously published analysis of 523 consecutive OC patients enrolled in the observational AGO-TR1 study [23]. Our data are in line with the data provided by Ramus et al. [11] and the largest investigation to date of 6294 OC cases by Lilyquist et al. [12], which showed a similar *BARD1* mutation prevalence in OC patients and controls. The weak association previously described by Norquist et al. ($P = 0.02$) [10] was based on the identification of 4 *BARD1*-mutated individuals in a study sample of 1915 unselected OC patients. Of note, Norquist et al. indicated that these results should be interpreted with some caution as 2 of the 4 *BARD1* mutation carriers also had mutations in *BRCA1* [10]. Overall, it appears likely that deleterious germline *BARD1* variants do not predispose for OC.

In study samples selected for (positive) cancer family history, the prevalence of deleterious variants in established risk genes is generally higher than in unselected cases. In our study focusing on 4469 index patients with familial BC, we demonstrate a significant association of heterozygous germline LoF variants in *BARD1* and overall BC (OR = 5.35; 95% CI = 3.17–9.04; $P < 0.00001$). This

association is comparable with that described by Slavin et al. (OR = 3.18; 95% CI = 1.34–7.36; $P = 0.012$), a study that also focused on index cases with familial BC. In study samples unselected for family history, the observed ORs were lower (e.g., Couch et al.: OR = 2.16; 95% CI = 1.31–3.63; $P = 0.00226$) [7] and even nonsignificant (e.g., Castéra et al.: OR = 2.00; 95% CI = 0.74–4.10) [8, 9]. Thus, it appears worthwhile to stratify study results by family history and possibly AAD, as shown in the current study. We demonstrate a significant association of heterozygous germline LoF variants in *BARD1* and the risk of early-onset BC (Table 1), a finding which may have important implications for the clinical management of women carrying pathogenic *BARD1* variants. Due to the pronounced association with early-onset BC (AAD < 40 years: OR = 12.04; AAD < 50 years: OR = 7.43), we suggest that *BARD1* should be included in multigene panels for BC risk assessment and, due to the comparatively young AAD of BC observed, intensified BC surveillance programs should be offered to women carrying pathogenic variants in *BARD1*.

Conclusions

No significant association between *BARD1* germline LoF variants and familial OC was observed. For BC, the significant association of heterozygous germline LoF variants in *BARD1* with early-onset BC (AAD < 50 years) suggests that intensified BC surveillance programs should be offered to women carrying pathogenic variants in *BARD1*.

Additional file

Additional file 1: Table S1. Inclusion criteria of the German Consortium for Hereditary Breast and Ovarian Cancer (GC-HBOC) for *BRCA1* and *BRCA2* germline testing. **Table S2.** Genotype, phenotype and cancer family history of familial BC index patients carrying heterozygous germline loss-of-function (LoF) variants in the *BARD1* gene (transcript NM_000465.3). **Table S3.** Prevalence of heterozygous germline LoF variants identified in the *BARD1* gene (transcript NM_000465.3). **Table S4.** Potentially damaging rare missense variants identified in the *BARD1* gene (transcript NM_000465.3). (DOCX 76 kb)

Abbreviations

AAD: Age at first diagnosis; BC: Breast cancer; CI: Confidence interval; EMQN: European Molecular Quality Network; ER: Estrogen receptor; ExAC: Exome Aggregation Consortium; FLOSSIES: Fabulous Ladies Over Seventy; GC-HBOC: German Consortium for Hereditary Breast and Ovarian Cancer; GMC: Geographically matched female control; HER2: Human epidermal growth receptor 2; IARC: International Agency for Research on Cancer; LGR: Large genomic rearrangement; LoF: Loss-of-function; MAF: Minor allele frequency; NGS: Next-generation sequencing; OC: Ovarian cancer; OR: Odds ratio; PR: Progesterone receptor; TCGA: The Cancer Genome Atlas; VUS: Variant of uncertain significance

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Availability of data and materials

The FLOSSIES and ExAC control datasets are available at https://whi.color.com and http://exac.broadinstitute.org.

Authors' contributions

NWL, JB, KWL, RKS, JHa, and EHa wrote the manuscript. NWL, JB, KWL, JHa, and EHa analyzed the clinical and genetic data. CoE performed bioinformatic analyses of NGS data. NWL, JB, KWL, JH, DN, NA, SK, CoE, VGP, EH, KK, AEV, CK, SR, NL, JA, LL, EPR, HT, PN, ML, LR, KR, BW, CE, AM, RKS, EHa, and JHa provided DNA samples and/or clinical and genetic data. All authors read and approved the final manuscript. EHa and JHa had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Ethics approval and consent to participate

Written informed consent was obtained from all patients and control individuals, and ethical approval was granted by the Ethics Committee of the University of Cologne (07-048).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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3.2 Diagnosis of Li-Fraumeni Syndrome: Differentiating *TP53* germline mutations from clonal hematopoiesis: Results of the observational AGO-TR1 trial.

Weber-Lassalle K*, Harter P*, Hauke J, Ernst C, Kommoss S, Marmé F, Weber-Lassalle N, Prieske K, Dietrich D, Borde J, Pohl-Rescigno E, Reuss A, Ataseven B, Engel C, Stingl JC, Schmutzler RK, Hahnen E.

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My own contributions

After screening of 523 unselected patients (observational AGO-TR1 study, NCT02222883) with primary diagnosis of OC (n=281) or platinum-sensitive recurrent OC (n=242) for deleterious variants in the *TP53* (MIM# 191170) and *PPM1D* (MIM# 605100) genes by hybridization capture-based NGS using Agilent SureSelect XT protocol (Agilent, Santa Clara, CA, USA), I evaluated the data regarding both genes. I compared the results from blood-derived DNA and the DNA from corresponding FFPE tumor tissue. I screened for blood-specific deleterious variants using the varbank (https://varbank.ccg.uni-koeln.de/ (assessed June 2018)) and data generated by Freebayes v1.1.0 (https://arxiv.org/abs/1207.3907; https://github.com/ekg/freebayes) provided by Corinna Ernst.

For classification of the *TP53* variants, I employed the International Agency for Research on Cancer (IARC) *TP53* database (http://p53.iarc.fr/ R18 (April 2016) (assessed January 2018)), ClinVar/UMD *TP53* database (https://www.ncbi.nlm.nih.gov/clinvar/ (last updated July 2016) (assessed July 2018), https://p53.fr/tp53-database (assessed July 30, 2018)), and used the Seshat tool (http://vps338341.ovh.net/ (assessed July 2018)) for predictions regarding damaging or deleterious potential of *TP53* variants.

I included the blood-derived DNA samples of 1,053 cancer-free female individuals as controls from a study on civilization diseases (LIFE, https://life.uni-leipzig.de/) in the biobank of the GC-HBOC and standardized all samples via DNA concentration measurement.

I prepared the multiplexed custom primer pairs for the ten gene panel and screened all samples for *TP53* and *PPM1D* variants in the LIFE control data set using a customized target enrichment 48.48 amplicon-based panel Access Array system (Fluidigm, San Francisco, CA, USA). Afterwards, I sequenced via NGS, the LIFE controls using the Mid Output Kit v2 and

the NextSeq500 (Illumina, San Diego, CA, USA). For verification of the *TP53* variants, I evaluated the re-analysis of the AGO-TR1 study cohort generated by the target enrichment amplicon-based Access Array system (Fluidigm, San Francisco, CA, USA).

I curated the AGO-TR1 patient's data including tumor histology, age, chemotherapy before blood draw, type of chemotherapy, OC predisposing pathogenic germline mutations (*BRCA1/2, RAD51C*) to stratify the results according to the before mentioned characteristics. For evaluation and interpretation of the results, I performed the Student's t-test and the calculation of ORs.

I wrote the first manuscript draft including literature search, preparation of all tables and figures in close cooperation with my supervisors Prof. Dr. Rita K. Schmutzler and PD Dr. Eric Hahnen. I updated the manuscript after critical revision of the co-authors and performed its submission for publication to the scientific journal.

Contributions of co-authors

PD Dr. Philipp Harter (Department of Gynecology & Gynecologic Oncology, Kliniken Essen-Mitte, Essen) supervised and coordinated the clinical part of the AGO-TR1 study. Dr. Jan Hauke (Center for Hereditary Breast and Ovarian Cancer, Center for Integrated Oncology (CIO), Medical Faculty, University Hospital Cologne, Cologne) evaluated the results of the AGO-TR1 study and classified the pathogenic OC predisposition variants. Corinna Ernst (Center for Hereditary Breast and Ovarian Cancer, Center for Integrated Oncology (CIO), Medical Faculty, University Hospital Cologne, Cologne) performed the bioinformatics processing for the amplicon-based Fluidigm Access Array™ data including de-multiplexing, mapping and variant calling, as well as applying variant fraction and annotation filters for the hybridization capture-based NGS-derived variants. Furthermore, Corinna Ernst supported the conceptualization of statistical data evaluation. Nana Weber-Lassalle (Center for Hereditary Breast and Ovarian Cancer, Center for Integrated Oncology (CIO), Medical Faculty, University Hospital Cologne, Cologne) validated the designed primers for *TP53* and *PPM1D*, provided, enriched and sequenced 523 blood-derived DNA samples; provided clinical and genetic data; read and approved the final manuscript. PD Dr. Dimo Dietrich (Department of Otolaryngology, Head and Neck Surgery, University Hospital Bonn, Bonn) extracted the DNA from FFPE tumor blocks. For DNA isolation from FFPE tumor samples, hematoxylin and eosin-stained tissue sections were analyzed by an experienced pathologist at the Institute of Pathology at the University Hospital Bonn, Germany. Julika Borde (Center for Hereditary Breast and Ovarian Cancer, Center for Integrated Oncology (CIO), Medical Faculty,

University Hospital Cologne, Cologne) was involved in the recruitment of the LIFE study controls and supported the NGS process by performing DNA sample preparation. Furthermore, Julika is responsible for the biobank of the GC-HBOC. Dr. Esther Pohl-Rescigno (Center for Hereditary Breast and Ovarian Cancer, Center for Integrated Oncology (CIO), Medical Faculty, University Hospital Cologne, Cologne) designed the primers for *TP53* and *PPM1D* for the target enrichment amplicon-based Access Array system. Alexander Reuss (Coordinating Center for Clinical Trials, Philipps-University of Marburg, Marburg) supervised and coordinated the clinical data of the AGO-TR1 study and performed the biometrics. Prof. Dr. Julia C. Stingl (Research Division, Federal Institute for Drugs & Medical Devices, Bonn) was involved in the study design. PD Dr. Eric Hahnen (Center for Hereditary Breast and Ovarian Cancer, Center for Integrated Oncology (CIO), Medical Faculty, University Hospital Cologne, Cologne) as head of the research group and Prof. Dr. Rita K. Schmutzler (Center for Hereditary Breast and Ovarian Cancer, Center for Integrated Oncology (CIO), Medical Faculty, University Hospital Cologne, Cologne) as director of the Center for Familial Breast and Ovarian Cancer supervised my work and coordinated the trial at the Center for Familial Breast and Ovarian Cancer Cologne and were part of the writing team. All other authors provided DNA samples and/or basic clinical and genetic data. All authors read and approved the final manuscript.

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RESEARCH ARTICLE

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Diagnosis of Li-Fraumeni Syndrome: Differentiating *TP53* germline mutations from clonal hematopoiesis

Results of the observational AGO-TR1 trial

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Abstract

The Li-Fraumeni cancer predisposition syndrome (LFS1) presents with a variety of tumor types and the *TP53* gene is covered by most diagnostic cancer gene panels. We demonstrate that deleterious TP53 variants identified in blood-derived DNA of 523 patients with ovarian cancer (AGO-TR1 trial) were not causal for the patients' ovarian cancer in three out of six TP53-positive cases. In three out of six patients, deleterious TP53 mutations were identified with low variant fractions in blood-derived DNA but not in the tumor of the patient seeking advice. The analysis of the TP53 and PPM1D genes, both intimately involved in chemotherapy-induced and/or age-related clonal hematopoiesis (CH), in 523 patients and 1,053 age-matched female control individuals revealed that CH represents a frequent event following chemotherapy, affecting 26 of the 523 patients enrolled (5.0%). Considering that TP53 mutations may arise from chemotherapy-induced CH, our findings help to avoid false-positive genetic diagnoses of LFS1.

KEYWORDS
chemotherapy, clonal hematopoiesis, Li-Fraumeni syndrome, PPM1D, TP53

1 | INTRODUCTION

Deleterious germline mutations in the *TP53* gene (MIM# 191170) cause the Li-Fraumeni cancer predisposition syndrome (LFS1, MIM# 151623). LFS1 presents with a variety of tumor types and the *TP53* gene is therefore covered by most diagnostic cancer gene panels. Due to the central role of the TP53 protein in tumor initiation, the classification of deleterious *TP53* mutations identified in blood-derived DNA as

disease-causing appears to be self-evident. The interpretation of *TP53* variants identified in a germline diagnostic setting, however, remains challenging. Firstly, most pathogenic *TP53* mutations are missense mutations (Bouaoun et al., 2016) and cannot be easily classified based on mutation type. Secondly, *TP53* mutations may arise *de novo*, leading to somatic mosaicism (Forsberg, Gisselsson, & Dumanski, 2017). Next-generation sequencing (NGS) allows detecting genetic variants with a high read depth. While inherited, heterozygous germline variants usually show a variant fraction (VF) of approximately 50%, *TP53* variants with a VF below 50% were described, suggesting *de novo* somatic mosaic variants (Weitzel et al., 2017). Using blood-derived DNA, Swisher et al. (2016) identified deleterious somatic mosaic *TP53* variants in 10 out of 686 patients with ovarian cancer (OC). Paired neoplastic tissue was available for four women with *TP53* mutations—in no case, the *TP53* mutation was identified in the tumor-derived DNA. The evidence for deleterious *TP53* mutations identified purely in blood-derived DNA but not in the tumor of the patient seeking advice requires further validation since it may have severe implications for genetic counseling.

2 | MATERIALS AND METHODS

Genomic DNA was isolated from venous EDTA blood samples using standard methods. Using blood-derived DNA, we screened 523 unselected patients with primary diagnosis of OC ($n = 281$) or platinum-sensitive recurrent OC ($n = 242$) and 1,053 cancer-free female control individuals for deleterious variants in the *TP53* (MIM# 191170) and *PPM1D* (MIM# 605100) genes by hybridization capture-based NGS (Agilent SureSelect XT protocol). The patient cohort was previously screened for pathogenic germline mutations in established cancer predisposition genes (observational AGO-TR1 study). Healthy controls were recruited by a study on civilization diseases (LIFE study, https://life.uni-leipzig.de/). The studies were approved by the local ethic committees. All participants gave their written informed consent. The study sample and the methodologies were previously described in detail (Harter et al., 2017). The AGO-TR1 study protocol was approved by the ethical committee of the Landesaerztekammer Nordrhein (Nr. 2014340) and registered (NCT02222883); all patients gave written informed consent prior to any study related procedure. The hybridization capture-based NGS method was suitable for the analysis of DNA derived from either blood- or formalin-fixed paraffin-embedded (FFPE) tumor samples (Agilent SureSelect XT protocol). For DNA isolation from FFPE tumor samples, hematoxylin and eosin-stained 3 μm tissue sections were analyzed by an experienced pathologist at the Institute of Pathology at the University Hospital Bonn, Germany. Tumor areas containing >80% tumor nuclei were chosen for DNA isolation using standard techniques. DNA quantification was performed using a Nanodrop® ND-1000 spectral photometer (NanoDrop Technologies, Wilmington, DE).

For the verification of *TP53* variants identified in blood/ tumor-derived DNA by hybridization capture-based NGS, *TP53*-positive DNA samples were re-analyzed using an amplicon-based Fluidigm Access Array 48.48 system (Fluidigm, San Francisco, CA) for target enrichment, covering all coding *TP53* exons and exon-flanking intronic sequences (*TP53* reference transcript NM_000546.5). DNA libraries were sequenced in 150 base paired end mode with the Mid Output Kit v2. on a Nextseq 500 device (Illumina, San Diego, CA). For evaluation of the variants, BCL files were demultiplexed, converted into FASTQ format using bcl2fastq2 Conversion Software v2.19.1.403. Sequence reads were mapped to the human reference genome assembly GRCh37 including decoy sequences (hs37d5) using Burrows-Wheeler Aligner (BWA) v0.7.15 (Li & Durbin, 2009), and target-specific primer sequences were removed using BAMClipper v1.1.1 (Au, Ho, Kwong, Chan, & Ma, 2017). The Genome Analysis Toolkit (GATK) v3.8 (DePristo et al., 2011; McKenna et al., 2010) was used for realignment of insertions and deletions and quality recalibration. Variant calling was performed using Freebayes v1.1.0 (https://arxiv.org/abs/1207.3907; https://github.com/ekg/freebayes), claiming a minimum alternate allele fraction of 0.05 (via argument –min-alternate-fraction), and base and mapping qualities of at least 20 (via arguments –min-base-quality and –min-mapping-quality). All positions found by FreeBayes (Garrison E, Marth G. Haplotype-based variant detection from short-read sequencing. *arXiv preprint arXiv:1207.3907 [q-bio.GN]* 2012) with a minimum alternative allele fraction of 0.05 and a minimum read depth of 60 were considered as putative variants, irrespective of the predicted genotype. Read depths and base counts of positions with alternative allele fractions below 0.05 were obtained via the mpileup utility of samtools v1.9, with minimum mapping and base qualities set to 20 (via arguments -min-MQ and –min-BQ).

3 | RESULTS

In our patient sample, potentially deleterious missense variants in the *TP53* gene were identified in blood-derived DNA of six out of 523 patients with OC using hybridization capture-based NGS (Table 1). The VFs of three *TP53* variants detected in three patients (#1–#3) were 55%, 50% and 49%, respectively, compatible with VFs usually observed for germline variants. In the remaining three patients (#4–#6), four *TP53* variants with lower VFs of 34%, 26%, 17%, and 7%, respectively, were observed (Table 1). DNA samples derived from paired neoplastic tissue from all six patients were analyzed by NGS. *TP53* variants with a VF of approximately 50% in blood-derived DNA (patients #1–#3) were also present in the corresponding tumor samples (Table 1). The VFs were elevated in the tumor samples of two patients (#1, #2), suggesting loss of the wild-type *TP53* alleles. In contrast, the *TP53* variants with lower VFs (patients #4–#6) were not or only barely detectable in the corresponding tumor. Of note, different somatic *de novo TP53* variants were observed only in the tumors of the latter three patients (Table 1), which were classified nonfunctional in the IARC *TP53* database (Bouaoun et al., 2016) and pathogenic according to the UMD *TP53* database and Seshat *TP53* variant classification tool (Soussi, Leroy, & Taschner, 2014). Two variants listed in the ClinVar database were classified likely pathogenic/pathogenic. In summary, these data suggest that deleterious *TP53* variants identified in blood-derived DNA were not causal for the patients' cancer in three out of six cases.

TABLE 1 Potentially deleterious *TP53* variants identified by NGS in 523 unselected patients with OC

Patient (age at first diagnosis, y)	Histologic subtype	1st line chemotherapy	Blood draw (after completing 1st line chemotherapy, m)	*TP53* variant cDNA[a]	*TP53* variant protein (exon number)	Variant fraction blood (total reads)	Variant fraction tumor (total reads)	Classification		
								IARC *TP53* database[b]	Seshat[c]	ClinVar[d] / UMD *TP53* database[e]
#1 (49)	High grade serous	Carboplatin, Paclitaxel	63	c.643A>G	p.(Ser215Gly) (6)	**55%** (205/373)	**77%** (477/620)	Non-functional	Deleterious	LP/LP
#2 (51)	Mucinous	None	n.a.	c.374C>T	p.(Thr125Met) (4)	**50%** (338/676)	**62%** (57/92)	Non-functional	Deleterious	LP/LP
#3 (45)	High grade serous	Bevacizumab, Carboplatin, Paclitaxel	2	c.523C>T	p.(Arg175Cys) (5)	**49%** (184/377)	**46%** (139/302)	Partially functional	Uncertain	Uncertain/PP
#4 (27)	Serous/ papillary	Carboplatin, Paclitaxel	15	c.482C>A	p.(Ala161Asp) (5)	**34%** (216/636)	**<1%** (2/203)	Non-functional	Deleterious	Uncertain/LP
				c.823T>A	p.(Cys275Ser) (8)	**26%** (121/462)	**0%** (0/166)	Non-functional	Uncertain	Not listed/VUS
				c.818G>A	p.(Arg273His) (8)	**0%** (0/462)	**63%** (103/163)	Non-functional	Pathogenic	P/P
#5 (58)	High grade serous	Carboplatin, Paclitaxel	9	c.1177G>C	p.(Asp393His) (11)	**17%** (61/361)	**0%** (0/144)	Functional	Not listed	Not listed/VUS
				c.646G>A	p.(Val216Met) (6)	**0%** (0/381)	**39%** (114/292)	Non-functional	Pathogenic	LP/P
#6 (66)	High grade serous	Bevacizumab, Carboplatin, Paclitaxel	27	c.711G>A	p.(Met237Ile) (7)	**7%** (24/338)	**0%** (0/196)	Non-functional	Pathogenic	LP/P
				c.775G>T	p.(Asp259Tyr) (7)	**0%** (0/362)	**51%** (93/182)	Non-functional	Pathogenic	Not listed/P

Alternative allele fractions ≥5% were considered true positive variant calls. For each patient, age at first diagnosis in years (y), histologic subtype, type of chemotherapy, time of blood draw after completing 1st line chemotherapy in months (m) and the *TP53* variants are given (including their VFs in blood- and tumor-derived DNA). Patient #6 carried a heterozygous pathogenic germline variant in the *BRCA2* gene (c.5496dupT, p.(Asn1833*fs)). The variant was present in blood (VF 48%, 208/433 reads) and in the corresponding tumor (VF 72%, 305/423 reads). The remaining five patients tested negative for deleterious germline variants in cancer predisposition genes (Harter et al., 2017). PLD, pegylated liposomal doxorubicin; n.a., not applicable; LP, likely pathogenic; PP, possibly pathogenic; P, pathogenic; VUS, variant of uncertain significance.

[a] *TP53* transcript NM_000546.5.

[b] http://p53.iarc.fr/ R18 (April 2016) (assessed January 2018).

[c] http://vps338341.ovh.net/ (assessed July 2018).

[d] https://www.ncbi.nlm.nih.gov/clinvar/ (last updated July 2016) (assessed July 2018).

[e] https://p53.fr/tp53-database (assessed July 30, 2018).

TABLE 2 Technical verification of potentially deleterious *TP53* variants (NM_000546.5) identified by NGS in blood-derived DNA of 523 unselected patients with OC

Patient	*TP53* variant cDNA	*TP53* variant protein	Variant fraction blood hybrid capture (total reads)	Variant fraction blood amplicon-based (total reads)
#1	c.643A>G	p.(Ser215Gly)	**55%** (205/373)	**47%** (1,686/3,588)
#2	c.374C>T	p.(Thr125Met)	**50%** (338/676)	**38%** (1,479/3,870)
#3	c.523C>T	p.(Arg175Cys)	**49%** (184/377)	**41%** (1,709/4,169)
#4	c.482C>A c.823T>A	p.(Ala161Asp) p.(Cys275Ser)	**34%** (216/636) **26%** (121/462)	**30%** (2,084/6,945) **20%** (1,253/6,266)
#5	c.1177G>C	p.(Asp393His)	**17%** (61/361)	**18%** (372/2,068)
#6	c.711G>A	p.(Met237Ile)	**7%** (24/338)	**6%** (295/4,910)

Using a hybrid capture technique for target enrichment (Agilent SureSelect), seven potentially deleterious *TP53* variants were identified in six patients. Alternative allele fractions ≥5% were considered true positive variant calls. All seven potentially deleterious *TP53* variants were independently verified by NGS using an amplicon-based technique (Fluidigm) for target enrichment, with a considerably higher read depth. For each patient, the *TP53* variants including their VFs in both independent assays are shown.

All *TP53* variants identified in blood-derived DNA using hybridization capture-based NGS were independently verified using an amplicon-based assay for target enrichment prior to NGS, with similar VFs observed (Table 2). Thus, *TP53* variants listed in Table 1 represent true positive NGS variant calls. No additional *TP53* variants were observed in the verification analysis.

The occurrence of deleterious mutations with a low VF may be caused by chemotherapy-induced and/or age-related clonal hematopoiesis (CH), in which the deleterious mutations only affect the hematopoietic stem and progenitor cells in the bone marrow and no other compartments of the body (Genovese et al., 2014; Jaiswal et al., 2014; Swisher et al., 2016). To differentiate whether the occurrence of *TP53* variants with low VFs may be chemotherapy-induced and/or age-related, we analyzed 1,053 cancer-free female control individuals for deleterious variants in the *TP53* gene by hybridization capture-based NGS. In this large control sample with a mean age at blood draw of 59.3 years (range 19–80) similar to the mean age at blood draw in all 523 patients enrolled in the AGO-TR1 trial (59.9 years, range 18–93), no pathogenic *TP53* variant and no other *TP53* variant with a low VF was observed, suggesting that age-related CH affecting the *TP53* gene represents a rare event.

At the time of the blood draw, patients with deleterious *TP53* variants with a low VF in blood had completed first line taxane/platinum-based chemotherapy (Table 1). Consequently, we suggested that the low VF-variants observed in the *TP53* gene were chemotherapy-induced rather than age-related. Mutations affecting the *PPM1D* gene were originally thought to represent mosaic events leading to predisposition to OC (Ruark et al., 2013). Subsequent studies have elucidated that such events are enriched in the peripheral blood of patients with prior chemotherapy (Coombs et al., 2017). In our study sample, 24 out of 523 patients (4.6%) carried truncating variants affecting the *PPM1D* gene (Table 3), with generally low VFs (≤40%) in blood-derived DNA which were not compatible with heterozygous germline alterations. In corresponding tumor-derived DNA samples, *PPM1D* variants were not or only barely detectable (Table 3). Traces of mutant alleles in the tumor may be explained by infiltration of the tumor tissue with blood cells. Of note, 18 out of 24 *PPM1D*-positive patients had completed first line platinum-based chemotherapy prior to blood draw. In five cases, blood was drawn during 1st line chemotherapy (Table 3). In the age-matched control sample ($n = 1{,}053$), *PPM1D* variants were extremely rare with only one 77-year-old woman carrying a nonsense variant with a low VF affecting the *PPM1D* gene (c.1654C>T, p.(Arg552*), VF 15%, 30 out of 198 reads).

In summary, 26 out of 523 (5.0%) patients enrolled in the AGO-TR1 trial carried *TP53* and/or *PPM1D* variants with low VFs in blood-derived DNA (23× *PPM1D* only, 2× *TP53* only, 1× *PPM1D* and *TP53* [patient #6 in Tables 1 and 3]) versus one out of 1,053 (0.1%) in age-matched control individuals (1× *PPM1D*). Thus, the event of a CH is substantially enriched following standard chemo therapy and most likely account for the *TP53* variants with low VFs observed in this study. Notably, the overall prevalence of pathogenic germline mutations in validated OC predisposition genes *ATM* (MIM# 607585), *BRCA1* (MIM# 113705), *BRCA2* (MIM# 600185), *BRIP1* (MIM# 605882), *MSH2* (MIM# 609309), *MSH6* (MIM# 600678), *RAD51C* (MIM# 602774), *RAD51D* (MIM# 602954) according to Lilyquist et al. (2017) in the AGO-TR1 study was 25.2% (132 out of 523) (Harter et al., 2017). In the subgroup of patients showing variants with low VFs in the CH-associated genes *TP53* and *PPM1D*, the proportion of germline mutation carriers was elevated (41.7%, 10/24; Table 3) compared with the overall patient sample, though not reaching levels of significance.

4 | DISCUSSION

We demonstrate that deleterious *TP53* variants identified in blood-derived DNA of patients with OC (AGO-TR1 trial, NCT02222883) were not causal for the patients' cancer in three out of six *TP53*-positive cases. The paired analysis of blood/tumor-derived DNA of OC patients along with the analysis of 1,053 age-matched healthy female control individuals revealed that, in three out of six patients, *TP53* mutations with low VFs arise from chemotherapy-induced CH. To avoid false-positive molecular genetic diagnoses of LFS1, we suggest that conspicuous *TP53* test results in patients who received chemotherapy prior to blood draw should be complemented with additional tissue testing, excluding the hematopoietic compartment (e.g., tumor tissue). Following these analyses, we now consider the

TABLE 3 Loss-of-function (LoF) variants in the *PPM1D* gene were identified by NGS in 24 out of 523 unselected patients with OC

Patients (age at first diagnosis, y)	Histologic subtype	1st line chemotherapy	Blood draw (after completing 1st line chemotherapy, m)	*PPM1D* variant cDNA (NM_003620.3), protein	Variant fraction blood (total reads)	Variant fraction tumor (total reads)	Germline mutation status gene, cDNA, protein, VF, total reads in blood (B) versus tumor (T)
Patients with pathogenic/likely pathogenic germline variants in established predisposition cancer genes							
#7 (72)	High grade serous	BIBF 1120 or placebo,Carboplatin, Paclitaxel	47	c.1538del, p.(Leu513*)	43% (248/576)	0% (0/301)	*RAD51C*, c.224dup, p.(Tyr75*), B:49%(292/596), T:80%(214/268)
#8 (58)	High grade serous	Bevacizumab, Carboplatin, Paclitaxel	29	c.1731dup, p.(Leu578Thrfs*8)	40% (215/635)	1% (8/827)	*BRCA2*, c.3264dup, p.(Gln1089Serfs*10), B:51%(240/471), T:49%(196/401)
#9 (50)	High grade serous	Carboplatin, Paclitaxel	80	c.1714C>T, p.(Arg572*)	40% (97/242)	<1% (3/1,063)	*BRCA1*, c.3108dup, p.(Lys1037Phefs*13), B:47%(117/248), T:57%(402/706)
#10 (61)	High grade serous	Carboplatin, Paclitaxel	39	c.1654C>T, p.(Arg552*)	23% (117/507)	2% (16/1,038)	*BRCA1*, c.3481_3491del, p.(Glu1161Phefs*3), B:39% (196/503), T:38% (350/921)
#6 (66)	High grade serous	Bevacizumab, Carboplatin, Paclitaxel	27	c.1535dup, p.(Asn512Lysfs*16) c.1440del, p.(Ala481Profs*2)	12% (50/417) 7% (35/509)	0% (0/213) 0% (0/717)	*BRCA2*, c.5496dup, p.(Asn1833*), B:48%(208/433), T:72%(305/423)
#11 (62)	High grade serous	Bevacizumab, Carboplatin, Paclitaxel	21	c.1521_1522insT, p.(Met508Tyrfs*20)	11% (31/275)	<1% (3/664)	*BRCA1*, c.2475del, p.(Asp825Glufs*21),B:51%(148/291), T:74%(474/640)
#12 (62)	Missing	Missing	Missing	c.1534_1535del, p.(Asn512Phefs*15)	10% (34/344)	Tumor n.a.	*BRCA1*, c.4689C>G, p.(Tyr1563*),B:45%(167/371), T:n.a.
#13 (50)	High grade serous	Carboplatin, Paclitaxel	45	c.1535dup, p.(Asn512Lysfs*16) c.1451del, p.(Leu484*)	10% (46/461) 6% (33/542)	0% (0/741) 0% (0/714)	*BRCA2*, c.4965C>G, p.(Tyr1655*), B:51%(275/528), T:65%(291/448)
#14 (46)	High grade serous	Carboplatin, Paclitaxel	135	c.1535del, p.(Asn512fs)	6% (27/446)	<1% (1/994)	*BRCA2*, c.7976G>A, p.(Arg2659Lys), B:48%(175/364), T:80%(483/604)
#15 (64)	High grade serous	Carboplatin, Paclitaxel	69	c.1280G>A, p.(Trp427*)	5% (17/349)	0% (0/401)	*RAD51C*, c.502A>T, p.(Arg168*), B:48%(200/416), T:57%(212/372)
Patients without pathogenic/likely pathogenic germline variants in established predisposition cancer genes							
#16 (76)	High grade serous	Bevacizumab, Carboplatin, Paclitaxel	During 1st line	c.1280G>A, p.(Trp427*)	36% (313/869)	<1% (2/264)	Negative
#17 (63)	High grade serous	Carboplatin, Paclitaxel	39	c.1432del, p.(Cys478Alafs*5)	33% (193/586)	<1% (8/999)	Negative
#18 (70)	Serous/papillary (grade unknown)	Bevacizumab, Carboplatin, Paclitaxel	During 1st line	c.1456_1460del, p.(Ile486*)	33% (237/718)	<1% (2/791)	Negative

(Continues)

TABLE 3 (Continued)

Patients (age at first diagnosis, y)	Histologic subtype	1st line chemotherapy	Blood draw (after completing 1st line chemotherapy, m)	*PPM1D* variant cDNA (NM_003620.3), protein	Variant fraction blood (total reads)	Variant fraction tumor (total reads)	Germline mutation status gene, cDNA, protein, VF, total reads in blood (B) versus tumor (T)
#19 (61)	High grade serous	Carboplatin, Paclitaxel	37	c. 1430del, p.(Asn477Ilefs*6)	31% (131/423)	<1% (7/1,027)	Negative
#20 (76)	High grade serous	Carboplatin, Paclitaxel	30	c.1618G>T, p.(Glu540*)	29% (194/669)	0% (0/1,094)	Negative
#21 (68)	High grade serous	Bevacizumab, Carboplatin, Paclitaxel	20	c.1535dup, p.(Asn512Lysfs*16)	27% (134/496)	<1% (4/561)	Negative
#22 (73)	Serous/papillary (grade unknown)	Carboplatin, Paclitaxel	67	c.1407del, p.(Asp470Ilefs*13)	26% (100/386)	0% (0/793)	Negative
#23 (63)	High grade serous	Carboplatin, Paclitaxel	112	c.1422del, p.(Glu475Lysfs*8)	17% (85/498)	tumor n.a.	Negative
#24 (62)	High grade serous	Carboplatin, Paclitaxel	48	c.1636delC, p.(Leu546*)	11% (55/503)	0% (0/964)	Negative
#25 (65)	High grade serous	Bevacizumab, Carboplatin, Paclitaxel	During 1st line	c.1432del, p.(Cys478Alafs*5*)	10% (76/755)	0% (0/987)	Negative
#26 (71)	Missing	Carboplatin, Paclitaxel	18	c.1403C>G, p.(Ser468*) c.1654C>T, p.(Arg552*)	8% (57/709) 6% (44/741)	1% (9/755) <1% (8/962)	Negative
#27 (76)	Clear cell	Carboplatin	During 1st line	c.1281G>A, p.(Trp427*)	6% (28/462)	0% (0/969)	Negative
#28 (72)	High grade serous	Bevacizumab, Carboplatin, Paclitaxel	During 1st line	c.1417_1430del, p.(Pro473Leufs*3)	6% (43/719)	0% (0/811)	Negative
#29 (63)	High grade serous	Carboplatin, Paclitaxel	22	c.1602delT, p.(Phe534Leufs*5)	5% (25/500)	<1% (2/461)	Negative

All LoF variants affect the last exon (exon 6) of the *PPM1D* gene. Of the 24 *PPM1D*-positive individuals, 10 (42%) were shown to carry likely pathogenic or pathogenic germline variants in established OC predisposition genes. The remaining 14 patients were tested negative for likely pathogenic or pathogenic germline variants in established OC predisposition genes. For each patient, age at first of diagnosis (years), histologic subtype, type of chemotherapy, time of blood draw after completing 1st line chemotherapy (months) and the *PPM1D* variants are given (including their VFs in blood- and tumor-derived DNA). Germline mutation status is shown in the right column, including the variants and their VFs in blood- and tumor-derived DNA. PLD, pegylated liposomal doxorubicin; n.a., not applicable.

TP53 germline variants observed in the patients #1–#3 (Table 1) likely pathogenic.

Genovese et al. (2014) proposed that CH is associated with increased risks of hematologic cancer. In line with this suggestion patient #6, the only patient who was shown to carry both, *TP53* and *PPM1D* variants, developed an acute myeloid leukemia. Whether chemotherapy-induced CH may be a risk factor for therapy-associated secondary hematologic malignancies needs to be clarified in larger prospective studies. In the subgroup of patients showing CH-associated alterations in the *TP53* and *PPM1D* genes, however, the proportion of patients carrying germline mutations in validated OC predisposition genes was 1.7-fold higher than in the overall patient sample. Thus, we suggest that patients with a heterozygous inactivation of OC predispositions genes are prone to chemotherapy-induced CH. Whether germline cancer predisposition may be a risk factor for therapy-associated secondary hematologic events needs to be clarified.

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CONFLICT OF INTEREST

Philipp Harter: Consulting or Advisory Role-AstraZeneca, Roche/Genentech, Tesaro, Clovis, Pharmamar Lilly, Sotio; Research Funding-AstraZeneca (Inst); Travel, Accommodations, Expenses-Medac; Stefan Kommoss: Honoraria-Astra Zeneca, Roche; Consulting or Advisory role-Roche, Tesaro, Astra-Zeneca; Travel, Accommodations, Expenses-Tesaro, PhermaMar, AstraZeneca; Katharina Prieske: Travel, Research funding-Medac Oncology; Honoraria-Astra Zeneca, Roche. Beyhan Ataseven: Advisory Role-Tesaro and Roche/Genetech; Honoraria-Roche/Genentech, AstraZeneca, Amgen; Travel Support-Roche/Genentech; Rita K. Schmutzler: Honoraria-AstraZeneca; Consulting or Advisory Role-AstraZeneca; Research Funding-AstraZeneca (Inst); Eric Hahnen: Honoraria-AstraZeneca, Consulting or Advisory Role-AstraZeneca, Research Funding-AstraZeneca (Inst); Konstantin Weber-Lassalle, Jan Hauke, Corinna Ernst, Frederik Marmé, Nana Weber-Lassalle, Dimo Dietrich, Julika Borde, Esther Pohl-Rescigno, Alexander Reuss, Christoph Engel, Julia Stingl, no relationships to disclose. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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3.3 Clonal hematopoiesis-associated gene mutations in a clinical cohort of 448 patients with ovarian cancer

Weber-Lassalle K, Ernst C; Möllenhoff K, Reuss A, Baumann K; Jackisch C; Hauke J; Dietrich D; Borde J, Park-Simon TW; Hanker L; Prieske K, Schmidt S; Weber-Lassalle N, Pohl-Rescigno E, Kommoss S, Marmé F; Heitz F, Stingl JC, Schmutzler RK; Harter P; Hahnen E.

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My own contributions

For the paired analysis of blood-derived DNA and DNA extracted from FFPE tumor samples, I performed the screening of ten leukemia-associated genes in 551 corresponding tumor samples in 496 patients with OC. In advance, I measured amplifiable DNA-concentration via qPCR (TaqMan copy number assay using primers for the *FTH1* gene (Thermo Fisher Scientific, Waltham, MA, USA)); and applied a customized target enrichment 48.48 amplicon-based panel Access Array system (Fluidigm, San Francisco, CA, USA) for screening of blood-specific variants using the ten-gene panel. The sequencing of the 551 tumor samples was performed using the Mid Output kit v2 and the NextSeq500 (Illumina, San Diego, CA, USA). For classification of the *TP53* variants, I checked the International Agency for Research on Cancer (IARC) *TP53* database (http://p53.iarc.fr/ R18 (April 2016) (assessed January 2018)), ClinVar/UMD *TP53* database (https://www.ncbi.nlm.nih.gov/clinvar/ (last updated July 2016) (assessed July 2018), https://p53.fr/tp53-database (assessed July 30, 2018)), and used the Seshat tool (http://vps338341.ovh.net/ (assessed July 2018)) for predictions regarding damaging or deleterious potential. I analyzed the AGO-TR1 patient data including tumor histology, AAD and age at blood draw, chemotherapy before blood draw, type of chemotherapy, as well as genetic data such as OC predisposition gene mutations (*BRCA1/2, RAD51C*). For evaluation and interpretation of the results from the customized gene panel, I performed the Fisher's exact test for calculation of significance levels and ORs. I drafted the manuscript including literature search, preparation of all tables and most figures in close cooperation with my colleague Corinna Ernst (Center for Familial Breast and Ovarian Cancer, Center for Integrated Oncology (CIO), University of Cologne, Faculty of Medicine and University Hospital Cologne, Cologne, Germany) and my supervisors Prof. Dr. Rita K. Schmutzler (Center for Familial Breast and Ovarian Cancer, Center for Integrated Oncology (CIO), University of Cologne, Faculty of Medicine and University Hospital Cologne,

Cologne, Germany) and PD Dr. Eric Hahnen (Center for Familial Breast and Ovarian Cancer, Center for Integrated Oncology (CIO), University of Cologne, Faculty of Medicine and University Hospital Cologne, Cologne, Germany). I contextualized the results with recent studies and discussed them in detail. I updated the manuscript after critical revision of the co-authors and submitted the final approval of the manuscript for publication to *JNCI*.

Contributions of co-authors

Corinna Ernst (Center for Familial Breast and Ovarian Cancer, Center for Integrated Oncology (CIO), University of Cologne, Faculty of Medicine and University Hospital Cologne, Cologne, Germany) performed the entire bioinformatic data processing, starting with de-multiplexing and mapping up to variant calling as well as developed filtering. Furthermore, Corinna Ernst developed the binary logistic regression approach for investigation of association of CH with age, germline mutation status and drug treatment including statistical analyses, and conceptualization and data visualization. Kathrin Möllenhoff (Institute of Medical Statistics and Computational Biology, Faculty of Medicine, University of Cologne, Cologne) supported the statistical analysis by introducing Haldane-Anscombe correction and age-dependent prediction of CH probabilities. Alexander Reuss (Coordinating Center for Clinical Trials, Philipps-University of Marburg, Marburg) supervised and coordinated the clinical data acquisition of the AGO-TR1 study and performed the biometrics analysis. Dr. Jan Hauke (Center for Hereditary Breast and Ovarian Cancer, Center for Integrated Oncology (CIO), Medical Faculty, University Hospital Cologne, Cologne) evaluated the results of the AGO-TR1 study and classified the pathogenic OC predisposition variants. PD Dr. Dimo Dietrich (Department of Otolaryngology, Head and Neck Surgery, University Hospital Bonn, Bonn) extracted the DNA from FFPE tumor blocks. For DNA isolation from FFPE tumor samples, hematoxylin and eosin-stained tissue sections were analyzed at the Institute of Pathology at the University Hospital Bonn, Germany. Julika Borde (Center for Familial Breast and Ovarian Cancer, Center for Integrated Oncology (CIO), University of Cologne, Faculty of Medicine and University Hospital Cologne, Cologne, Germany) was involved in the recruitment of the Leipziger Forschungszentrum für Zivilisationserkrankungen (LIFE) study controls and supported the NGS. Furthermore, Julika Borde maintains the biobank of the German Consortium for Hereditary Breast and Ovarian Cancer (GC-HBOC). Sandra Schmidt (Center for Familial Breast and Ovarian Cancer, Center for Integrated Oncology (CIO), University of Cologne, Faculty of Medicine and University Hospital Cologne, Cologne, Germany) extracted the genomic DNA from EDTA venous blood

samples using standard methods and performed capture-based target enrichment NGS. Nana Weber-Lassalle (Center for Familial Breast and Ovarian Cancer, Center for Integrated Oncology (CIO), University of Cologne, Faculty of Medicine and University Hospital Cologne, Cologne, Germany) validated the designed primers for *TP53* and *PPM1D*, provided, enriched and sequenced 523 blood-derived DNA samples via amplicon-based NGS; provided clinical and genetic data; read and approved the final manuscript. Dr. Esther Pohl-Rescigno (Center for Familial Breast and Ovarian Cancer, Center for Integrated Oncology (CIO), University of Cologne, Faculty of Medicine and University Hospital Cologne, Cologne, Germany) designed the primers for *TP53* and *PPM1D* for the target enrichment amplicon-based Access Array system. Prof. Dr. Julia C. Stingl (Institute of Clinical Pharmacology, University Hospital of RWTH Aachen, Aachen, Germany) was involved in the study design. PD Dr. Philipp Harter (Department of Gynecology & Gynecologic Oncology, Kliniken Essen-Mitte (KEM) Evang. Huyssens-Stiftung/Knappschaft GmbH, Essen, Germany) supervised and coordinated the clinical part of the AGO-TR1 study. PD Dr. Eric Hahnen (Center for Familial Breast and Ovarian Cancer, Center for Integrated Oncology (CIO), University of Cologne, Faculty of Medicine and University Hospital Cologne, Cologne, Germany) as leader of the research group and Prof. Dr. Rita K. Schmutzler (Center for Familial Breast and Ovarian Cancer, Center for Integrated Oncology (CIO), University of Cologne, Faculty of Medicine and University Hospital Cologne, Cologne, Germany) as director of the Center for Familial Breast and Ovarian Cancer supervised and coordinated the study were part of the writing team. All other authors provided DNA samples and/or basic clinical and genetic data. All authors read and approved the final manuscript.

Clonal Hematopoiesis-Associated Gene Mutations in a Clinical Cohort of 448 Patients With Ovarian Cancer

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Abstract

Background: Cancer patients are at risk of secondary therapy-related myeloid neoplasms (t-MNs). Acquired blood-specific mutations in clonal hematopoiesis (CH)-associated genes are t-MN risk factors and their occurrence associated with cancer therapy and age. Patients with ovarian cancer (OC) showed a particularly high prevalence of CH-associated gene mutations, which may additionally be explained by the high proportion of a hereditary disease cause in this cancer entity.

Methods: We performed a retrospective analysis of 448 OC patients enrolled in the AGO-TR1 study; 249 were enrolled at primary diagnosis and 199 at platinum-sensitive recurrence. Analyses included the most frequently altered CH-associated genes (*ASXL1*, *DNMT3A*, *GNAS*, *JAK2*, *PPM1D*, *SF3B1*, *SH2B3*, *SRSF2*, *TET2*, *TP53*). Results were analyzed according to the *BRCA1/2* germline (g*BRCA1/2*) mutation status. All statistical tests were 2-sided.

Results: Advanced age at blood draw and a high number of prior platinum-based chemotherapy lines were risk factors to acquire CH-associated gene mutations, with gene-specific effects observed. Binomial logistic regression suggested increased probabilities for g*BRCA1/2* mutation carriers to acquire CH-associated *PPM1D* and *TP53* gene mutations (*PPM1D:* odds ratio=4.30, 95% confidence interval=1.48 to 12.46, *P*=0.007; *TP53*: odds ratio=6.20, 95% confidence interval=0.98 to 53.9, *P*=0.06). This observation was due to a statistically significantly increased number of platinum-based chemotherapy lines in g*BRCA1/2* mutation carriers vs noncarriers (*PPM1D*: mean [SD] = 2.04 [1.27] vs 1.04 [0.99], *P*<0.001; *TP53*: mean [SD] =2.83 [1.33] vs 1.07 [1.01], *P*<0.001). No interaction between platinum-based chemotherapy and g*BRCA1/2* mutation status with the occurrence of CH-associated gene mutations was observed.

Conclusion: A positive g*BRCA1/2* mutation status is not a risk factor to acquire CH-associated gene mutations. OC patients may benefit from monitoring CH-associated gene

mutations, especially following carboplatin exposure. Future clinical studies are required to assess whether treatment regimen should be adapted according to individual t-MN risks.

Patients with cancer are at elevated risk of subsequent therapy-related myeloid neoplasms (t-MNs) such as acute myeloid leukemia (tAML) and myelodysplastic syndrome (tMDS). The recent MSK-IMPACT study analyzed blood-derived DNA from 24,146 patients with 56 different primary tumor types. In a subgroup of 10,138 patients with curated and detailed clinical data, older age at blood draw and cancer therapy prior to blood draw correlated with the presence of clonal hematopoiesis (CH), as shown by acquired mutations in CH-associated genes [1]. In the MSK-IMPACT study, patients with ovarian cancer (OC) showed a particularly high prevalence of mutations in CH-associated genes and it was hypothesized that cancer-specific differences may be due to interactions between mutations in specific genes and specific regimen of cancer therapy [1]. Most OC patients received cytotoxic treatment regimen, which was also most common in the overall MSK-IMPACT study sample. A unique feature of OC is a high prevalence of pathogenic germline mutations in the *BRCA1/2* (g*BRCA1/2*) cancer predisposition genes, which explain more than 10% of all OC cases irrespective of the patients´ cancer family history [2]. *BRCA1/2* mutation carriers with OC show a more favorable therapy response and survival than noncarriers with OC [3, 4], which simultaneously may be associated with an increased risk to acquire CH-associated gene mutations. To assess whether the g*BRCA1/2* mutation status modifies (either directly or indirectly) the association of drug treatment with the occurrence of CH-associated gene mutations, we performed a retrospective analysis of 448 OC patients enrolled in the observational AGO-TR1 study (NCT02222883). Our analyses included the most prevalently altered CH-associated genes *ASXL1*, *DNMT3A, PPM1D,* and *TET2*, along with *GNAS, JAK2, SF3B1*, *SH2B3, SRSF2,* and *TP53* [5, 6]. The prevalence of CH-associated gene mutations was assessed according to age at blood draw, number of prior platinum-based chemotherapy lines, and g*BRCA1/2* mutation status.

Methods

Study sample

A total of 523 consecutive OC patients were enrolled in the AGO-TR1 study. The AGO-TR1 study protocol was approved by the Ethics Committee of the Landesaerztekammer Nordrhein (No. 2014340) and registered (NCT02222883, ClinicalTrials.gov). All patients were at least 18 years of age and gave their written informed consent prior to enrollment. Demographic data, disease characteristics, and family history of the overall study sample were described previously [7].

Targeted next generation sequencing (NGS)

Blood-derived DNA was available from all 523 patients enrolled in the AGO-TR1 study. Genomic DNA was isolated from venous EDTA blood samples collected between March and November 2015 by employing a chemagic™ MSM instrument and using the chemagic Prime DNA Blood 4k Kit H24 (PerkinElmer chemagen Technology GmbH, Baesweiler, Germany). For DNA isolation from formalin-fixed and paraffin-embedded (FFPE) tumor samples, hematoxylin and eosin-stained 3µm tissue sections were analyzed and tumor areas containing >80% tumor nuclei were chosen for DNA isolation which was performed as described previously [8]. Tumor-derived DNA was available from 478 of the 523 patients enrolled in the AGO-TR1 study. A customized 48.48 amplicon-based gene panel (Access Array®, Fluidigm, San Francisco, CA, USA) was used for target enrichment, which was suitable for the amplification of DNA isolated from both blood samples and FFPE tumor samples. Panel design was performed using the web-based D3 Assay Design tool (Fluidigm). The gene panel covered the entire coding regions and exon-flanking sequences (±2 nt) of 10 CH-associated genes, namely *ASXL1* (MIM*612990, NM_015338), *DNMT3A* (MIM*602769, NM_175629), *GNAS* (MIM*139320, NM_000516.5), *JAK2* (MIM*147796, NM_004972), *PPM1D* (MIM*605100, NM_003620), *SF3B1* (MIM*605590, NM_012433), *SH2B3* (MIM*605093,

NM_005475), *SRSF2* (MIM*600813, NM_003016), *TET2* (MIM*612839, NM_001127208), and *TP53* (MIM*191170, NM_000546). Overall, the gene panel covered 130 sequencing target regions (Supplementary Table S1). NGS of the barcoded amplicons was performed by using a NextSeq 500 sequencing device and Mid-Output v2 kits (Illumina, San Diego, CA, USA). All DNA samples were centrally analyzed at the Center for Familial Breast and Ovarian Cancer, University Hospital Cologne, Germany. Raw BCL files were de-multiplexed using bcl2fastq2 Conversion Software v2.19 (available at https://support.illumina.com). Sequence reads were mapped to the human reference genome assembly GRCh37, including decoy sequences (hs37d5), using BWA-MEM of Burrows-Wheeler Aligner (BWA) v0.7.15 [9]. Target-specific primer sequences were removed using BAMClipper v1.1. [10]. Reads were filtered for reads mapped in proper pairs using samtools [11]. Variant calling was performed using FreeBayes v1.0.0 [12] on a merged BAM file including RG-tagged reads from blood and tumor samples per sample ID. FreeBayes was run under specification of --min-mapping-quality 20, --min-base-quality 20, --min-coverage 1000, and --min-alternate-fraction 0.03, as well as --use-duplicate-reads to account for the characteristics of amplicon sequencing.

Quality control

All NGS analyses of blood- and tumor-derived DNA samples with an overall mean sequencing coverage >1,000x were included in this investigation; data of 448 patients met this quality criterion and were processed further. All sequencing targets with a mean sequencing coverage >1,000x in both blood- and tumor-derived DNA in the study sample of 448 patients were included; 11 sequencing targets were excluded. This allowed the comparative analysis of 119 sequencing targets in 448 patients (**Supplementary Table 1**).

Variant filtering

We excluded variants located within interspersed repeats and low complexity sequence regions as defined by RepeatMasker [13]. Erroneous variant calls caused by technical artifacts were assumed to occur recurrently and to accumulate within the same sequencing run, with normally distributed variant fractions (VFs). Therefore, only variant calls with VFs reaching a modified Z-score ≥3.5 [14] considering all VFs at the corresponding locus within the sequencing run were considered. All variants with a VF ≥0.03 in the blood sample were analyzed further.

Variants identified in blood-derived DNA were considered blood-specific *i)* if the variant position was covered at least 500x in the corresponding tumor-derived DNA, *ii)* if the tumor VF did not exceed 0.10, and *iii)* if the log2 ratio of the blood VF vs the tumor VF was >1. Blood-specific variants were annotated with respect to the specified transcripts using SnpEff [15]. Frameshift variants, nonsense variants, and variants located at the canonical splice sites +/-2bp were defined as protein-truncating variants (PTVs). Missense variants and in frame indels were defined as non-PTVs. Intronic variants outside the canonical splice sites and synonymous variants were excluded from this investigation. These analyses identified a total of 655 blood-specific variants in the overall study sample. All blood-specific variants were filtered for putative pathogenic effects in cancer development using OncoKB [16]. Variants were annotated using the MafAnnotator utility of OncoKB Annotator v3.0.0 (https://github.com/oncokb/oncokb-annotator) without specification of a particular tumor type. Of the 655 blood-specific variants, 101 were classified as (likely) oncogenic according to oncoKB Annotator, subsequently referred to as ´CH-associated gene mutations´.

Statistical analysis

Analyses were run under R v3.6. Welch's t-test and Fisher's exact test were used to assess the association between the patients' age at blood draw, exposure to drug treatment, and g*BRCA1/2* mutation status with the occurrence of CH-associated gene mutations. Benjamini-Hochberg adjustment was applied for multiple testing correction. All statistical tests were two-sided with *P* values ≤0.05 considered statistically significant. Association of counts of CH-associated gene mutations per individual with age and number of treatment lines were assessed via Spearman‘s correlation using R‘s cor.test()function. Binomial logistic regression was employed to investigate the association of age at blood draw, exposure to drug treatment, and g*BRCA1/2* mutation status with the occurrence of CH-associated gene mutations using R’s glm() utility. Models were fitted using iteratively reweighted least squares and served as input for age-dependent predictions of probabilities for the occurrence of CH-associated gene mutations in dependence to number of prior platinum-based chemotherapy lines and g*BRCA1/2* mutation status employing R’s predict() utility. *P* values were obtained applying two-sided Wald tests. An interaction term between g*BRCA1/2* mutation status and number of prior platinum-based chemotherapy lines was included in the binomial logistic regression model to assess whether g*BRCA1/2* mutation status modifies the association of drug treatment with CH-associated gene mutations.

Results

CH-associated gene mutations were present in all 10 CH-related genes investigated and most prevalent in *DNMT3A* (n=33) and *PPM1D* (n=30), followed by *TET2* (n=12), *ASXL1* (n=8), *TP53* (n=7), *JAK2* (n=4), *SRSF2* (n=3), *GNAS* (n=2), *SF3B1* (n=1), and *SH2B3* (n=1). All 101 CH-associated gene mutations are listed in the **Supplementary Table 2**. VFs ranged from 0.03 (minimum cut-off) to 0.37, with a mean VF of 0.10 (**Supplementary Table 2**).

CH-associated mutations in the *PPM1D* gene, all PTVs, clustered in the terminal exon 6, in accordance with previous findings [17].

Demographic data of the 448 patients included in this investigation are presented in the **Supplementary Table 3**. Of the 448 patients, 249 were enrolled in the AGO-TR1 study at primary OC diagnosis and the remaining 199 patients at platinum-sensitive OC recurrence. On the patient level, 79 (17.6%) of the 448 OC patients carried at least one CH-associated gene mutation. Among these 79 patients, 64 patients carried one, 11 carried two, 3 carried three and 1 patient carried six CH-associated gene mutations (**Figure 1A**). The occurrence of CH-associated gene mutations was statistically significantly associated with the age at blood draw (Spearman's rho=0.17, $P<0.001$, **Figure 1A**). In the overall study sample of 448 patients, the mean age at blood draw was 59.8 years (range = 18 to 93 years, standard deviation (SD) = 12.3 years). Patients with at least one CH-associated gene mutation in any of the investigated genes were statistically significantly older at the time of blood draw than patients without (79 vs 369 patients, mean age=64.2 (SD=10.4) vs58.8 (SD=12.5) years, Benjamini-Hochberg-adjusted Welch's t-test $P=0.001$, **Figure 1B**). Regarding the individual genes, the difference reached statistical significance for *PPM1D* (24 vs 424 patients, mean age = 67.2 (SD=8.7) vs 59.3 (SD=12.4) years, $P=0.006$), as well as for *TET2* (11 vs 437 patients, mean age = 69.7 (SD=10.3) vs 59.5 (SD=12.3) years, $P=0.01$), *SF3B1* and *SH2B3*. In the latter two genes, however, only a single CH-associated gene mutation was identified (**Figure 1B**).

Of the 448 patients, 303 patients received drug treatment at least 30 days before blood draw (mean age at blood draw = 60.3 years, range = 20 to 85 years, SD=11.6 years); the remaining 145 patients did not receive drug treatment at least 30 days before blood draw (mean age at blood draw = 58.6 years, range = 18 to 93 years, SD=13.7 years). All 303 patients received a carboplatin-based chemotherapy (**Supplementary Table 3**); only 4 of the

303 patients received carboplatin and cisplatin. The standard dose in primary therapy was carboplatin AUC5 for 6 cycles, which was usually combined with taxane (**Supplementary Table 3**). For the treatment of relapsed disease, multiple mostly platinum-based therapies are possible treatment options (**Supplementary Table 3**).

The number of platinum-based chemotherapy lines at least 30 days before blood draw ranged from 1 to 6 (**Figure 1C**; 169 patients received one platinum-based chemotherapy line, 97 received two, 27 three, and 10 patients received four to six platinum-based chemotherapy lines). Overall, the 79 patients carrying at least one CH-associated gene mutation in any of the investigated genes received statistically significantly more platinum-based chemotherapy lines starting at least 30 days prior to blood draw than the 369 patients without (mean = 1.43 (SD=1.23) vs 1.02 (SD=0.97), Benjamini-Hochberg-adjusted Welch's t-test P=0.004, **Figure 1D**). This difference was mainly driven by CH-associated gene mutations in the *PPM1D* gene (mean = 2.04 (SD=1.27) vs 1.04 (SD=0.99), P<0.001, **Figure 1D**) and the *TP53* gene (mean = 2.83 (SD=1.33) vs 1.07 (SD=1.01), P<0.001, **Figure 1D**), which were exclusively identified in patients who received at least one platinum-based chemotherapy line starting at least 30 days prior to blood draw. In addition, CH-associated gene mutations in *GNAS*, *JAK2*, *SF3B1*, and *SH2B3* were exclusively identified in patients who received prior platinum-based chemotherapy, though the differences in mean numbers of platinum-based chemotherapy lines received did not reach levels of statistical significance (**Figure 1D**).

In the overall study sample of 448 OC patients, 92 patients (20.7%) carried pathogenic g*BRCA1/2* mutations (**Supplementary Table 3**). The occurrence of at least one CH-associated gene mutation was not statistically significantly associated with a positive g*BRCA1/2* mutation status, neither overall (19 occurrences in 92 g*BRCA1/2*-positive vs 60 in 356 g*BRCA1/2*-negative patients, Fisher's exact test P=0.44), nor in the subgroup of 303

patients who received platinum-based chemotherapy at least 30 days before blood draw (17 in 70 vs 45 in 233, *P*=0.40).

To visualize potential gene-specific effects of the age at blood draw, the number of prior platinum-based chemotherapy lines, and the g*BRCA1/2* mutation status on the age-dependent occurrence of CH-associated gene mutations, we employed a binomial logistic regression model for the most commonly affected genes *ASXL1*, *DNMT3A*, *PPM1D*, *TET2*, and *TP53* (**Figure 2A-E**). CH-associated gene mutations in the *ASXL1* gene did not associate with any of the potential risk factors investigated here (**Figure 2A**). CH-associated gene mutations in the *ASXL1* gene have recently been shown to be enriched in current or former smokers which may explain the lack of a statistically significant association in our study which did not stratify for smoking behavior [1]. CH-associated gene mutations in the *DNMT3A* and *TET2* genes associated with the age at blood draw, but not with the number of prior platinum-based chemotherapy lines or g*BRCA1/2* mutation status (**Figure 2B** and **C**). CH-associated gene mutations in the *PPM1D* gene associated with all three factors, i.e. age at blood draw, number of prior platinum-based chemotherapy lines, and *BRCA1/2* mutations status (age odds ratio (OR)=1.10, 95%CI=1.05 to 1.16, *P*<0.001; chemotherapy lines OR=1.97, 95%CI=1.39 to 2.83, P<0.001; g*BRCA1/2* OR=4.30, 95%CI=1.48 to 12.46, *P*=0.007; **Figure 2D**). CH-associated gene mutations in the *TP53* gene associated with the number of prior platinum-based chemotherapy lines, but not statistically significantly with *BRCA1/2* mutation status and the age at blood draw (age OR=1.01, 95%CI=0.92 to 1.11, *P*=0.84; chemotherapy lines OR=2.55, 95%CI=1.42 to 4.73, *P*=0.002; g*BRCA1/2* OR=6.20, 95%CI=0.98 to 53.9, *P*=0.06; **Figure 2E**).

Testing for interaction between the number of platinum-based chemotherapy lines and *BRCA1/2* mutation status with the occurrence of CH-associated gene mutations as an outcome, revealed no statistically significant association, neither for *PPM1D* (interaction

OR=1.02, 95%CI=0.49 to 2.20, *P*=0.95) nor for *TP53* (interaction OR=1.69, 95%CI=0.52 to 7.19, *P*=0.42) (**Supplementary Table 4**). However, a positive g*BRCA1/2* mutation status was statistically significantly associated with a younger age at onset (mean age at diagnosis = 52.5 (SD=8.9) vs 59.8 (SD=12.8) years, Welch's t-test *P*<0.001, **Supplementary Table 3**) along with a higher number of platinum-based chemotherapy lines at least 30 days prior to blood draw (mean = 1.39 (SD=1.15) vs 1.01 (SD=0.98), Welch's t-test *P*=0.004), which may explain the increased probability to acquire CH-associated gene mutations at younger ages (**Figure 2**).

Discussion

In this clinical cohort of 448 OC patients, we demonstrated a high prevalence of CH-associated gene mutations, affecting approximately one in six patients. Of note, integration of OncoKB annotation for CH classification may represent a conservative CH calling approach, which may lead to missing some less common CH-associated gene mutations.

In patients with t-MN, CH-associated gene mutations were most prevalent in the *ASXL1*, *DNMT3A*, *PPM1D*, *TET2* and *TP53* genes, respectively, whereas CH-associated gene mutations in the *PPM1D* and *TP53* genes were statistically significantly enriched in patients with t-MN compared with *de novo* MN [18]. The MSK-IMPACT study demonstrated that cancer therapy with radiation, platinum (especially carboplatin), and topoisomerase II inhibitors preferentially selects for CH-related mutations in DNA damage response genes, including *TP53* and *PPM1D* [1]. Concordant with these findings, we identified CH-associated gene mutations in the *TP53* and *PPM1D* genes exclusively in patients who received at least one line of platinum-based chemotherapy prior to blood draw. CH-associated gene mutations in the *PPM1D* and the *TP53* genes were identified in 28 of the 303 patients (9.2%) who received carboplatin-based regimen, of which two patients carried CH-associated gene

mutations in both genes. These 28 patients received first chemotherapy line 92 days to 11.5 years before blood draw (mean=3.4 years, SD=2.8 years; data not shown), with a mean number of 2.14 prior chemotherapy lines (range=1 to 6, SD=1.30). For the remaining 275 of the 303 patients who received carboplatin-based regimen prior to blood draw but did not show CH-associated gene mutations in the *TP53* and *PPM1D* genes, the time between first chemotherapy and blood draw ranges from 31 days to 16.1 years (mean=2.5 years, SD=2.5 years), with a mean number of 1.56 chemotherapy lines (range=1 to 5, SD=0.77).

Our results point towards a rather indirect association between g*BRCA1/2* mutations and a higher probability to accumulate CH-associated gene mutations in the *PPM1D*, *TP53* and probably other genes that accumulate CH-associated gene mutations in response to chemotherapy, which may have implications for the clinical management of patients with g*BRCA1/2*-associated hereditary cancers such as high grade serous OC and triple-negative breast cancer [7, 19]. In the multivariate analysis, we did not observe a statistically significant interaction between *gBRCA1/2* mutation status and the number of platinum-based chemotherapy lines with the occurrence of CH-associated gene mutations in *PPM1D* and *TP53* genes, respectively, though minor effects can not be excluded. In our study sample, g*BRCA1/2* mutation carriers received a statistically significantly higher number of platinum-based chemotherapy lines prior to blood draw than noncarriers, which is most likely due to a more favorable therapy response and survival benefit observed for g*BRCA1/2* mutation carriers [3, 4].

There had been a concern that g*BRCA1/2* mutation carriers may be more prone to tNM after administration of PARP inhibitors such as niraparib or olaparib: The ENGOT-OV16/NOVA and SOLO2/ENGOT-Ov21 trials revealed increased t-MN rates in the niraparib and the olaparib arms, respectively, vs the placebo arms [20, 21]. A follow-up investigation of the SOLO2/ENGOT-Ov21 trial, however, revealed that PARP inhibition did not increase the

t-MN risk vs placebo [22]. Rather, a trend was seen for a higher t-MN incidence with an increasing number of prior platinum-based chemotherapy lines. These results support our findings of an indirect association with g*BRCA1/2* gene mutations due to higher numbers of prior platinum-based chemotherapy lines.

Monitoring of patients after chemotherapy exposure with blood draws at defined time intervals and subsequent analysis for CH-associated gene mutations may allow optimized clinical management that considers the patients´ individual t-MN risk. Future clinical studies are required to assess the potential necessity to adapt the choice of treatment regimen according to the individual t-MN risk. For example, the MSK-IMPACT study suggested a lower CH risk following cisplatin or oxaliplatin than following carboplatin.

In the AGO-TR1 study sample, pathogenic germline mutations in non-*BRCA1/2* OC predisposition genes were observed [7]. Their prevalence, however, was too low to perform meaningful calculations. A further limitation of our study is the focus on 10 CH-associated genes only, i.e. additional genes that accumulate CH-associated gene mutations following chemotherapy such as *CHEK2* were not considered. A stringent minimum VF of 0.03 was chosen due to the NGS target enrichment used, which is more error-prone than hybrid capture techniques. The AGO-TR1 trial did not assess t-MN as an endpoint.

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Data Availability

All relevant data are shown in the main manuscript and the Supplementary Materials.

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Figure Legends

Figure 1. Associations between age at blood draw and number of prior platinum-based chemotherapy lines with the occurrence of clonal hematopoiesis (CH)-associated gene mutations.

(A) Number of CH-associated gene mutations per patient according to the age at blood draw. **(B)** Boxplots for age at blood draw in years stratified by noncarriers (no CH) and carriers (CH) of CH-associated gene mutations and Benjamini-Hochberg-adjusted Welch's t-test P values. Mean lines in boxes correspond to mean values. **(C)** Number of CH-associated gene mutations according to number of prior platinum-based chemotherapy lines. Marker sizes correspond to the number of observed samples. **(D)** Boxplots for numbers of platinum-based chemotherapy lines received stratified by noncarriers (no CH) and carriers (CH) of CH-associated gene mutations and Benjamini-Hochberg-adjusted Welch's t-test *P* values. Mean lines in boxes correspond to mean values.

Figure 2. Risk prediction for CH-associated gene mutations based on binomial logistic regression dependent on age at blood draw (Age), number of prior platinum-based chemotherapy lines (PtLines) and *BRCA1/2* germline mutation status (g*BRCA1/2*). Coefficients (β), standard errors (SE), odds ratios (OR), 95% confidence intervals (CI), and P values (*P*, two-sided Wald test) as obtained from fitting a binomial logistic regression model for the observation of CH-associated gene mutations in *ASXL1*, *DNMT3A*, *PPM1D*, *TET2*, and *TP53* **(A-E)**.

Figure 1

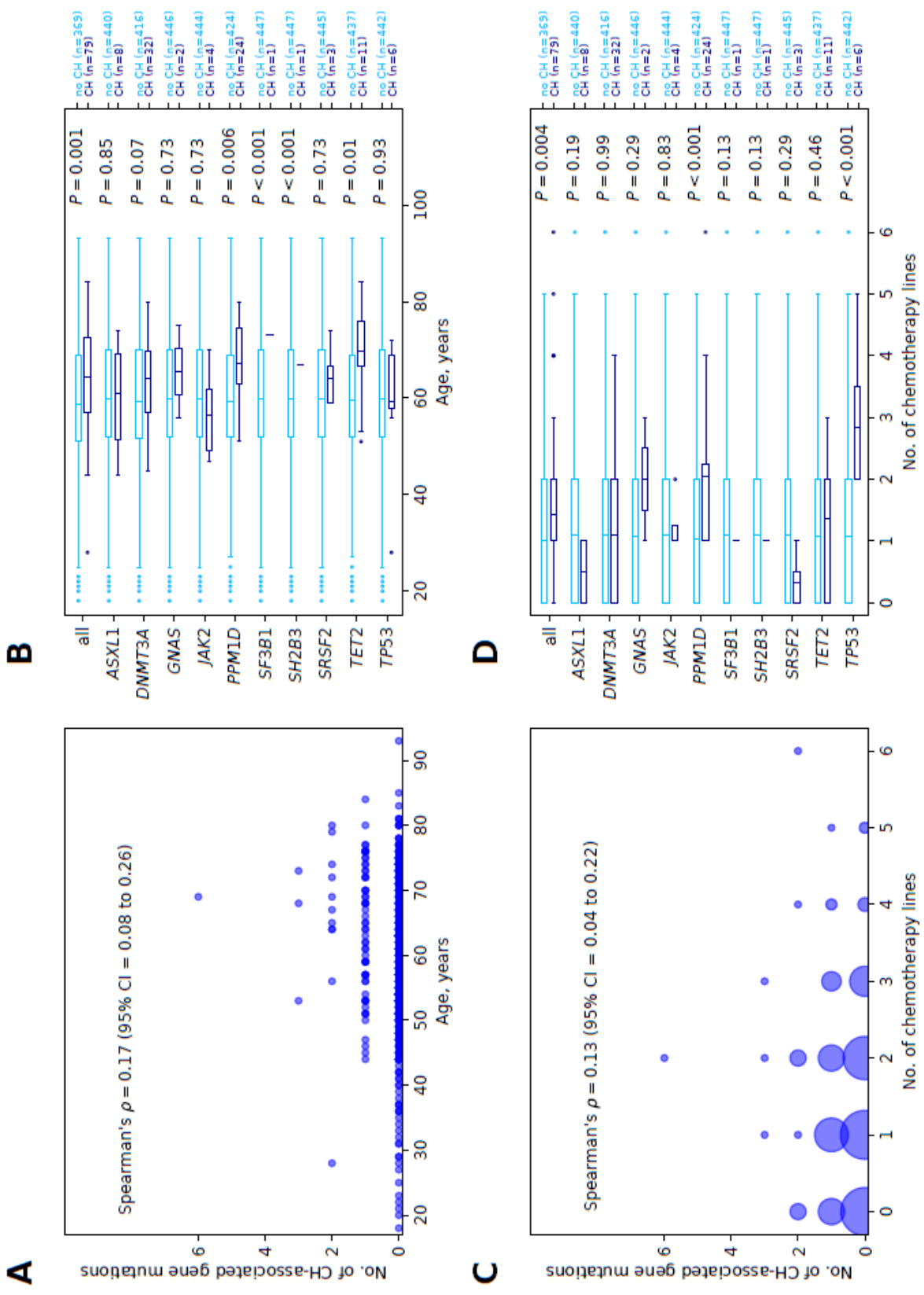
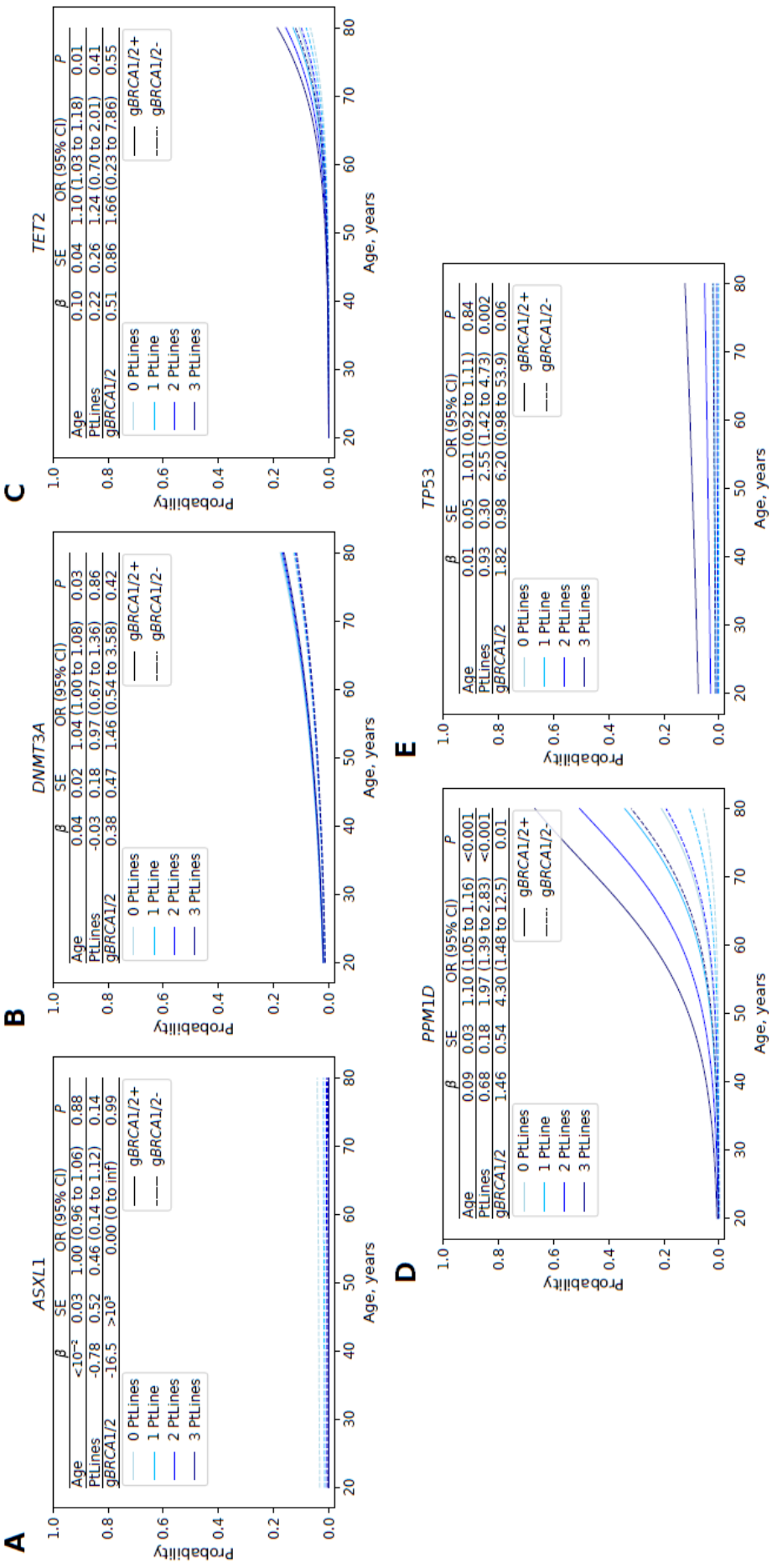

A
B
C
D
No. of CH-associated gene mutations
Age, years
Spearman's ρ = 0.17 (95% CI = 0.08 to 0.26)
Spearman's ρ = 0.13 (95% CI = 0.04 to 0.22)
No. of chemotherapy lines
all
ASXL1
DNMT3A
GNAS
JAK2
PPM1D
SF3B1
SH2B3
SRSF2
TET2
TP53
P = 0.001
P = 0.85
P = 0.07
P = 0.73
P = 0.73
P = 0.006
P < 0.001
P < 0.001
P = 0.73
P = 0.01
P = 0.93
P = 0.004
P = 0.19
P = 0.99
P = 0.29
P = 0.83
P < 0.001
P = 0.13
P = 0.13
P = 0.29
P = 0.46
P < 0.001

Figure 2


A
ASXL1
β SE OR (95% CI) P
Age <10⁻³ 0.03 1.00 (0.96 to 1.06) 0.88
PtLines -0.78 0.52 0.46 (0.14 to 1.12) 0.14
gBRCA1/2 -16.5 >10³ 0.00 (0 to inf) 0.99
B
DNMT3A
β SE OR (95% CI) P
Age 0.04 0.02 1.04 (1.00 to 1.08) 0.03
PtLines -0.03 0.18 0.97 (0.67 to 1.36) 0.86
gBRCA1/2 0.38 0.47 1.46 (0.54 to 3.58) 0.42
C
TET2
β SE OR (95% CI) P
Age 0.10 0.04 1.10 (1.03 to 1.18) 0.01
PtLines 0.22 0.26 1.24 (0.70 to 2.01) 0.41
gBRCA1/2 0.51 0.86 1.66 (0.23 to 7.86) 0.55
D
PPM1D
β SE OR (95% CI) P
Age 0.09 0.03 1.10 (1.05 to 1.16) <0.001
PtLines 0.68 0.18 1.97 (1.39 to 2.83) <0.001
gBRCA1/2 1.46 0.54 4.30 (1.48 to 12.5) 0.01
E
TP53
β SE OR (95% CI) P
Age 0.01 0.05 1.01 (0.92 to 1.11) 0.84
PtLines 0.93 0.30 2.55 (1.42 to 4.73) 0.002
gBRCA1/2 1.82 0.98 6.20 (0.98 to 53.9) 0.06
0 PtLines
1 PtLine
2 PtLines
3 PtLines
gBRCA1/2+
gBRCA1/2-
Probability
Age, years

4 Additional co-authored publications

4.1 Performance of breast cancer polygenic risk scores in 760 female *CHEK2* germline mutation carriers

Borde et al. (2020) Journal of the National Cancer Institute, doi: 10.1093/jnci/djaa203

Abstract

"**Background:** Genome-wide association studies (GWAS) suggest that the combined effects of breast cancer (BC)-associated single nucleotide polymorphisms (SNPs) can improve BC risk stratification using polygenic risk scores (PRSs). The performance of PRSs in GWAS-independent clinical cohorts is poorly studied in individuals carrying mutations in moderately penetrant BC predisposition genes such as *CHEK2*.
Methods: 760 female *CHEK2* mutation carriers were included; 561 women were affected with BC, of whom 74 developed metachronous contralateral BC (mCBC). For PRS calculations, two SNP sets covering 77 (SNP set 1, developed for BC risk stratification in women unselected for their *BRCA1/2* germline mutation status) and 88 (SNP set 2, developed for BC risk stratification in female BRCA1/2 mutation carriers) BC-associated SNPs were used. All statistical tests were two-sided.
Results: Both SNP sets provided concordant PRS results at the individual level (r = 0.91, $P<2.20\times10^{-16}$). Weighted cohort Cox regression analyses revealed statistically significant associations of PRSs with the risk for first BC. For SNP set 1, a hazard ratio (HR) of 1.71 per standard deviation of the PRS was observed (95% confidence interval [CI] = 1.36 to 2.15, $P=3.87\text{x}10^{-6}$). PRSs identify a subgroup of *CHEK2* mutation carriers with a predicted lifetime risk for first BC that exceeds the surveillance thresholds defined by international guidelines. Association of PRS with mCBC was examined via Cox regression analysis (SNP set 1 HR=1.23, 95%CI = 0.86 to 1.78, $P=0.26$).
Conclusion: PRSs may be used to personalize risk-adapted preventive measures for women with *CHEK2* mutations. Larger studies are required to assess the role of PRSs in mCBC predisposition." [211]

My own contributions

I prepared the DNA samples for the amplicon-based target enrichment access array, followed by NGS. I curated DNA samples and clinical data. Furthermore, I read and revised the final manuscript.

4.2 The *GPRC5A* frameshift variant c.183del is not associated with increased breast cancer risk in *BRCA1* mutation carriers.

Klaschik et al. (2019) International Journal of Cancer, 144(7):1761-1763, doi:10.1002/ijc.32016

Summary

In this published letter, the authors answer with respect to a publication of Sokolenko and colleagues (2014) [212]. The authors demonstrate that the heterozygous c.183del (p.R61Sfs*59) frameshift variant in *GPRC5A* (orphan G-protein coupled receptor, family C, group 5, member A) gene; OMIM *604138) does not contribute to BC risk as a germline modifier in *BRCA1* germline mutation carriers. No significant accumulation of this variant was observed in 1,707 BC index cases with PVs in *BRCA1* (15/1707; carrier frequency 0.88%) compared with 3,451 BC index patients negative for PVs in the *BRCA1* or *BRCA2* genes (21/3,451; carrier frequency 0.61%; OR=1.45, 95%CI=0.75–2.82, *P*=0.273), and 3,308 geographically matched control individuals (26/3,308; carrier frequency 0.79%; OR=1.12, 95% CI=0.59–2.12, *P*=0.730). Furthermore, the *GPRC5A* c.183del (p.R61Sfs*59) variant in the BC index patients with *BRCA1* haploinsufficiency was also not significantly enriched compared with 27,133 control from the ExAC data base (165/27,133; carrier frequency 0.61%; OR = 1.45, 95% CI = 0.85–2.46, *P*=0.169).

Recent data published by the Consortium of Investigators of Modifiers of *BRCA1/2* (CIMBA) emphasized that large cohorts are required to identify gene-disease associations; thus, the germline data presented by Sokolenko et al. might be biased by the limited sample size.
Although GPRC5A protein expression was also dysregulated in patients with breast cancer, its expression level obtained by CRISPR/Cas9-induced GPRC5A knockout (KO) using the MDA-MB-231 cell line was not associated with tumor stage, lymph node status, histological grading or histological tumor type, or with overall and recurrence-free survival. Investigations of proliferation (PCNA) and apoptosis (caspase-3) markers did not reveal any differences between the GPRC5A KO and GPRC5A wild type (WT) cell clones. Additionally, the authors did not observe any difference in the sensitivity to the DNA-damaging agent carboplatin of the GPRC5A KO and GPRC5A WT cell clone, suggesting no effect in DNA repair capacity. Thus, the role of *GPRC5A* in breast cancer initiation and progression seems to be of minor importance [213].

My own contributions

I prepared DNA samples for genetic analysis and recruited clinical data from patients, as well as curated genetic data. Furthermore, I also revised and approved the final manuscript.

4.3 Gene panel testing of 5,589 *BRCA1/2*-negative index patients with breast cancer in a routine diagnostic setting: results of the German Consortium for Hereditary Breast and Ovarian Cancer.

Hauke et al., (2018) Cancer Med, Apr;7(4):1349-1358, https://doi.org/10.1002/cam4.1376

Abstract

"The prevalence of germ line mutations in non- *BRCA1/2* genes associated with hereditary breast cancer (BC) is low, and the role of some of these genes in BC predisposition and pathogenesis is conflicting. In this study, 5,589 consecutive BC index patients negative for pathogenic *BRCA1/2* mutations and 2,189 female controls were screened for germ line mutations in eight cancer predisposition genes (*ATM*, *CDH1*, *CHEK2*, *NBN*, *PALB2*, *RAD51C*, *RAD51D*, and *TP53*). All patients met the inclusion criteria of the German Consortium for Hereditary Breast and Ovarian Cancer for germ line testing. The highest mutation prevalence was observed in the *CHEK2* gene (2.5%), followed by *ATM* (1.5%) and *PALB2* (1.2%). The mutation prevalence in each of the remaining genes was 0.3% or lower. Using Exome Aggregation Consortium control data, we confirm significant associations of heterozygous germ line mutations with BC for *ATM* (OR: 3.63, 95%CI: 2.67–4.94), *CDH1* (OR: 17.04, 95%CI: 3.54–82), *CHEK2* (OR: 2.93, 95%CI: 2.29–3.75), *PALB2* (OR: 9.53, 95%CI: 6.25–14.51), and *TP53* (OR: 7.30, 95%CI: 1.22–43.68). *NBN* germ line mutations were not significantly associated with BC risk (OR: 1.39, 95%CI: 0.73–2.64). Due to their low mutation prevalence, the *RAD51C* and *RAD51D* genes require further investigation. Compared with control datasets, predicted damaging rare missense variants were significantly more prevalent in *CHEK2* and *TP53* in BC index patients. Compared with the overall sample, only *TP53* mutation carriers show a significantly younger age at first BC diagnosis. We demonstrate a significant association of deleterious variants in the *CHEK2*, *PALB2*, and *TP53* genes with bilateral BC. Both, *ATM* and *CHEK2*, were negatively associated with triple-negative breast cancer (TNBC) and estrogen receptor (ER)- negative tumor phenotypes. A particularly high *CHEK2* mutation prevalence (5.2%) was observed in patients with human epidermal growth factor receptor 2 (HER2-) positive tumors."[214]

My own contributions

I prepared DNA samples and curated clinical and genetic data. I curated clinical data from patients. Furthermore, I read and revised the final manuscript.

4.4 *BRIP1* loss-of-function mutations confer high risk for familial ovarian cancer, but not familial breast cancer.

Weber-Lassalle et al., (2018) Breast Cancer Research, Jan 24;20(1):7, https://doi.org/10.1186/s13058-018-0935-9

Abstract

"**Background:** Germline mutations in the *BRIP1* gene have been described as conferring a moderate risk for ovarian cancer (OC), while the role of *BRIP1* in breast cancer (BC) pathogenesis remains controversial.
Methods: To assess the role of deleterious *BRIP1* germline mutations in BC/OC predisposition, 6341 well characterized index patients with BC, 706 index patients with OC, and 2189 geographically matched female controls were screened for loss-of-function (LoF) mutations and potentially damaging missense variants. All index patients met the inclusion criteria of the German Consortium for Hereditary Breast and Ovarian Cancer for germline testing and tested negative for pathogenic *BRCA1/2* variants.
Results: *BRIP1* LoF mutations confer a high OC risk in familial index patients (odds ratio (OR) = 20.97, 95% confidence interval (CI) = 12.02–36.57, $P<0.0001$) and in the subgroup of index patients with late-onset OC (OR = 29.91, 95% CI = 14.99–59.66, $P<0.0001$). No significant association of *BRIP1* LoF mutations with familial BC was observed (OR = 1.81 95% CI = 1.00–3.30, $P=0.0623$). In the subgroup of familial BC index patients without a family history of OC there was also no apparent association (OR = 1.42, 95% CI = 0.70–2.90, $P=0.3030$). In 1027 familial BC index patients with a family history of OC, the *BRIP1* mutation prevalence was significantly higher than that observed in controls (OR = 3.59, 95% CI = 1.43–9.01; $P=0.0168$). Based on the negative association between *BRIP1* LoF mutations and familial BC in the absence of an OC family history, we conclude that the elevated mutation prevalence in the latter cohort was driven by the occurrence of OC in these families. Compared with controls, predicted damaging rare missense variants were significantly more prevalent in OC ($P=0.0014$) but not in BC ($P=0.0693$) patients.
Conclusions: To avoid ambiguous results, studies aimed at assessing the impact of candidate predisposition gene mutations on BC risk might differentiate between BC index patients with an OC family history and those without. In familial cases, we suggest that *BRIP1* is a high-risk gene for late-onset OC but not a BC predisposition gene, though minor effects cannot be excluded." [215]

My own contributions

I prepared DNA samples and curated clinical/genetic data. I read and revised the final manuscript.

5 Discussion

5.1 Germline loss-of-function variants in the *BARD1* gene are associated with early-onset familial breast cancer but not ovarian cancer.

Germline PVs in the *BRCA1/2* genes are identified in approximately 24% of BC and/or OC index patients who met the inclusion criteria of the GC-HBOC for genetic germline testing (Table 1) [45]. Besides *BRCA1/2,* there are several OC predisposition genes that are associated with a moderate risk to develop hereditary OC e.g. BRCA1 interacting Protein C-Terminal Helicase 1 (*BRIP1,* MIM*605882), RAD51 Paralog C (*RAD51C,* MIM*602774), and RAD51 Paralog D (*RAD51D*, MIM*602954) [2, 3, 15, 50].

Most of the non-*BRCA1/2* OC susceptibility genes have been discovered due to direct interaction of the encoded proteins with BRCA1 or BRCA2 and their role in HRR: the role of the gene BRCA1-associated RING domain 1 (*BARD1*) in OC predisposition remained unclear due to controversial results of case-control investigations [14-16]. The PVs were classified according to criteria based on international established classification systems (IARC, ENIGMA, ACMG, ACGS) [96]. In our study, no evidence was found that PVs in the *BARD1* gene predispose for OC [17]. No PVs in *BARD1* could be observed in 451 familial OC index patients or in our previously published analysis of 523 OC patients (AGO-TR1 study) [3, 17]. Our data are in line with the data provided by Ramus et al. [16] and by Lilyquist et al. [15], which showed a similar *BARD1* mutation prevalence in OC patients and controls. However, a significant association as previously described by Norquist could not be confirmed [2]. This result may be biased, because two of the four identified *BARD1* mutation carriers also showed germline mutations in the *BRCA1* gene [2]. Therefore, it is very likely that germline PVs in *BARD1* do not predispose to OC.

Testing for an enriched PV rate in *BARD1* in BC patients, a statistically significant association of heterozygous germline PVs in *BARD1* and BC (OR=5.35; 95% CI=3.17 to 9.04; $P<0.00001$) in 4,469 index patients with familial BC could be confirmed [17]. For calculation of the ORs, NGS analysis of 2,767 geographically-matched female controls (GMC) was peformed and additional control databases (Exome Aggregation Consortium (ExAC); Fabulous Ladies Over Seventy (FLOSSIES) were included. This result is similar to the data of Slavin et al. (OR=3.18; 95% CI=1.34 to 7.36; $P=0.012$), who also studied index cases with familial BC [216].

Of note, in cohorts previously selected for familial cancer history, the prevalence of deleterious variants in established risk genes appears usually higher than in unselected cases.

Thus, the observed ORs in unselected BC patients were lower (e.g., Couch et al.: OR=2.16; 95% CI=1.31 to 3.63; *P*=0.00226) [217] and partly also not statistically significant (e.g., Castéra et al.: OR=2.00; 95% CI=0.74 to 4.10 [218] and Lu et al. [219]).

After stratification for age, BC index patients with heterozygous germline PVs in *BARD*1 showed a younger mean AAD of BC (mean=42.3 years; range: 24 to 60 years) compared with the overall sample of BC index patients (mean=48.6 years; range; 17 to 92 years; *P*= 0.00347; Student's t test). Heterozygous germline PVs in *BARD1* were associated with BC index patients with an AAD <40 years (OR=12.04, 95% CI=5.78 to 25.08; *P*< 0.00001), but not with the subgroup of BC index patients with AAD ≥ 50 years (OR=7.43, 95% CI=4.26 to 12.98; *P* < 0.00001) [17].

Rare *BARD1* missense variants, predicted (potentially) damaging according to the prediction tools i) SIFT [220] and ii) MutationTaster2 [221] (Alamut version 2.10 as of November 9, 2017) were significantly more prevalent in BC index patients compared with control individuals (OR=2.15; 95% CI=1.26 to 3.67; *P*=0.00723) [17].

Taken together, a statistically significant association of *BARD1* germline PVs with early-onset BC, but not OC was confirmed [17]. This indicates that despite many similarities in BC and OC carcinogenesis, there are also PVs in genes specific for either BC or OC risk. As translation of the identification of novel risk genes into BC risk assessment, the findings of my PhD thesis could be directly implemented into the routine diagnostic for genetic germline testing. Based on the consensus recommendations of the GC-HBOC working group "Klinische Konsequenzen" which develops standards for the clinical management for carriers of PVs predisposing for BC, the *BARD1* gene was incorporated as "core" gene into the TruRisk® panel analysis. Screening for PVs in the *BARD1* gene will be now offered for all patients meeting the inclusion criteria of the GC-HBOC for genetic germline testing nationwide. Due to the significant association of heterozygous germline PVs in *BARD1* with early-onset BC (AAD <50 years) intensified BC surveillance programs should be offered to women carrying PVs in *BARD1*. In families with PVs in *BARD1*, predictive tests for family members seeking advice can be offered as well to decide on risk-adjusted preventive measures.

5.2 Diagnosis of Li-Fraumeni Syndrome: Differentiating *TP53* germline mutations from clonal hematopoiesis: Results of the observational AGO-TR1 trial.

Multi-gene panel analyses in routine diagnostics provide important information about the patients‘risk to develop hereditary BC/OC. Usually, DNA isolated from the blood of the patient or a family member seeking advice is used for genetic germline testing. In case of the *TP53* gene, the majority (74%) of PVs are missense mutations that are causal for cancer development [88].

The paired analysis of blood and tumor-derived DNA of 523 patients with OC and unselected for family history, revealed that in three patients (#1-3) deleterious missense *TP53* variants with VFs around 50% (49%, 50%, and 55%) were present in both tissues, clearly suggesting heterozygous germline variants [28]. Corresponding VFs in tumor samples were increased in two patients (#1 and #2), indicating loss of wild-type TP53 alleles.

In three patients (#4-6), four *TP53* missense PVs with low VF (34%, 26%, 17%, and 7%), were identified in the blood, but not or only barely in the corresponding tumor [28]. However, further somatic *de novo TP53* variants were discovered in the tumor, which were classified non-functional according to the IARC TP53 database [222] and pathogenic according to the UMD TP53 database and Seshat TP53 variant classification tool [223], suggesting that the *TP53* missense PVs, which are only found in the patients' blood, were blood-specific and not causal for the patients' cancer [28].

The occurrence of blood-specific variants with a low VF may be caused by chemotherapy-induced and/or age-related CH [96, 104, 167].

Furthermore, neither *TP53* PVs nor other *TP53* variants with low VFs were observed in 1,053 cancer-free female age-matched controls. The mean age at blood draw of the control group was 59.3 years (range 19 to 80), and 59.9 years (range 18 to 93) for the 523 patients enrolled in the AGO-TR1 study [28]. This indicates that age-related CH affecting the *TP53* gene is very rare.

Since the three patients (#4-6) with CH had completed first line taxane/platinum-based chemotherapy at the time of blood draw, CTx is thought to be the trigger for CH, not age.

To clarify whether a PV is causal for the BC/OC or blood-specific and therefore should attributed to CH or somatic mosaicism, in which the PV is restricted to particular organs or tissues, testing of a second tissue via NGS that is not derived from the hematopoietic system is required. The authors propose FFPE tumor tissue as the first choice for this purpose, because if the *TP53* PV is not present or present with a significantly lower VF than in blood,

this indicates a blood-specific variant. As mentioned before, this is particularly true if a different, tumor-specific somatic *TP53* PV is detected in the tumor [28]. Beside tumor material, FFPE normal tissue, e.g. hair follicles or fingernails, can be used for DNA isolation [31].

A low VF identified in the patient's blood is a first indicator of a CH-associated mutation. However, CH can be present even with a VF around 50%, usually observed for heterozygous PVs affecting the germline, to over 80% [224]. If only VFs alone are considered, misdiagnosis may occur, followed by fatal clinical decisions e.g. in LFS1. This is precisely the error that Mitchell et al. uncovered by showing a misdiagnosis of LFS1 with a VF of 50% in *TP53* that was not causal for the disease [225]. It should further be noted that a very low VF of the PV, usually <10%, may also be explained by leukocyte infiltration into the tumor tissue [226]. Additionally, it must be pointed out that saliva is not suitable for the clarification of a putative CH, as it is infiltrated by a notable amount of leukocytes.

As direct translation from research to the clinic, these findings were integrated into the evaluation of *TP53* PVs found by germline genetic testing in routine diagnostics. Here, the modified standard operating procedure (SOP) ‘Further investigation after detection of a class 4/5 *TP53* variant in DNA isolated from blood‘ was established at the Center for familial Breast and Ovarian Cancer in Cologne and subsequently standardized for the 23 centers of the GC-HBOC (Figure 7), whereby class 4/5 refer to the pathogenicity classes of the IARC system (section 1.2.2). The classes 4 and 5 indicate that the variants are likely pathogenic and pathogenic, respectively. The SOP is similar to the recently introduced surveillance guidelines/diagnostic workup for LFS1 and hereditary *TP53* related cancers presented by Batalini et al. and Evans et al. [92, 227]. The most important goal of this new approach is to avoid false-positive molecular-genetic diagnoses of LFS1. This applies particular to LFS1 patients, who go through clinical surveillance programs for early tumor detection with a high frequency of examinations that may burden the patient’s body combined with psychological and psychosocial effects and problems [25-27].

Mutations affecting the *PPM1D* gene were originally thought to represent mosaic events leading to increased OC risks [32-34]. However, since these mutations are somatic, rather than germline, their mechanism of cancer association is currently unclear [68]. Subsequent studies have elucidated that such events are enriched in the peripheral blood of patients with prior chemotherapy [35]. In our study sample, 24 out of 523 patients (4.6%) carried PTVs affecting the *PPM1D* gene with generally low VFs (≤40%) in blood-derived DNA [28]. Notably, the PTVs clustered in the terminal exon (exon #6) of *PPM1D*, leading to the loss of

a C-terminal degradation domain [28]. In corresponding tumor-derived DNA samples, *PPM1D* variants were not or only barely detectable suggesting CTx-induced CH events. Indeed, all patients with PTVs in *PPM1D* had completed first line platinum-based chemotherapy prior to blood draw or were currently treated with platinum. The occurrence of a CTx-induced CH is reinforced by the fact that only one female individual from our control dataset carried a PTV in the *PPM1D* gene. Thus, a second biomarker, besides *TP53*, for chemotherapy-induced CH could be identified.

Previous studies showed that CH is associated with elevated risks of hematologic cancer [30, 35]. Consistent with these results, patient #6, the only patient with PVs in *TP53* and *PPM1D*, did indeed develop AML.

Among patients who had CH-associated alterations in the *TP53* and *PPM1D* genes, the proportion of patients carrying germline PVs in validated OC predisposition genes (*BRCA1/2, BRIP1, MSH2, MSH6, RAD51C* and *D*) was 1.7-fold increased compared with the overall patient sample [28]. Based on these findings, a further study was conducted to investigate whether and to what extent germline cancer predisposition may be a risk factor for therapy-associated secondary hematologic events.

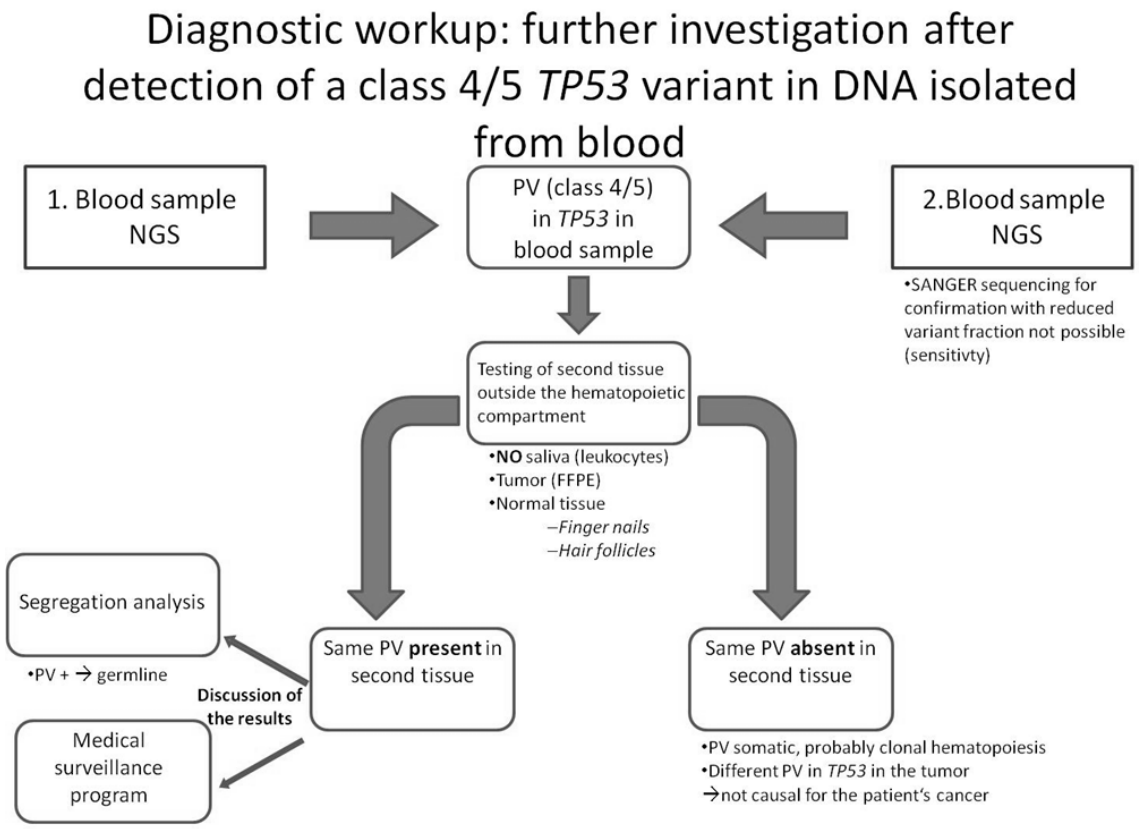


Figure 7: Recommended workup for a (likely) pathogenic variant (PV, class 4/5) in *TP53*. This standard operating procedure is integrated into the evaluation of PVs in *TP53* in routine diagnostic at the Center for Familial Breast and Ovarian Cancer Cologne and centers of the GC-HBOC. LOH=loss-of-heterozygosity; LFS1=Li-Fraumeni-syndrome 1, NGS=next-generation sequencing

5.3 Clonal hematopoiesis-associated gene mutations in a clinical cohort of 448 patients with ovarian cancer

Due to severe CTx side effects, patients with cancer are at elevated risk of secondary therapy-related myeloid neoplasms (t-MNs) [228]. Acquired blood-specific mutations in CH-associated genes were identified as t-MN risk factors and several studies have demonstrated a correlation between the age and/or CTx exposure of study participants with the occurrence of these mutations [30, 122, 202]. Of note, the overall prevalence of germline PVs in validated OC predisposition genes in the AGO-TR1 study was 25.2% (132 out of 523 patients) [3].

In a previous study examining the AGO-TR1 cohort, a particularly high prevalence of CH-associated gene mutations in patients with OC was revealed, suggesting that germline PVs in established OC risk genes may play a role in CH pathogenesis in this cancer entity [28]. Additionally, *BRCA1/2* germline (g*BRCA1/2*) mutation carriers with OC show a significantly more favorable response to treatment and better overall survival than OC patients without germline PVs in *BRCA1/2* [229, 230], which may be attributed to an increased risk to develop CH-associated gene mutations.

To address this hypothesis, I performed a paired blood and tumor analysis using a customized 10 gene panel followed by amplicon-based NGS in 448 patients with OC (249 with primary diagnosis of OC and 199 with platinum-sensitive recurrence) enrolled in the observational AGO-TR1 study [36]. I could demonstrate a high prevalence of CH-associated gene mutations, namely in 17.6% (79/448 patients) in OC patients, carrying at least one CH-associated gene mutation. Among these 79 patients, 64 patients carried one, 11 carried two, 3 carried three and 1 patient carried six CH-associated gene mutation [36]s.

CH-associated gene mutations were present in all 10 CH-related genes investigated and most prevalent in *DNMT3A* (n=33) and *PPM1D* (n=30), followed by *TET2* (n=12), *ASXL1* (n=8), *TP53* (n=7), *JAK2* (n=4), *SRSF2* (n=3), *GNAS* (n=2), *SF3B1* (n=1) and *SH2B3* (n=1) with a mean VF of 0.10 and VFs ranging from 0.03 (minimum cut-off) to 0.37.

Focusing on stratified subgroups, gene-specific associations with the accumulation of CH-associated gene mutations were shown with i) prior CTx exposure (number of CTx lines starting at least 30 days prior to blood draw), ii) an advanced age at blood draw and iii) a positive g*BRCA1/2* mutation status [36].

Previous studies revealed that CH-associated gene mutations were enriched in the *TP53, PPM1D, ASXL1, DNMT3A* and *TET2* genes after CTx [28, 199, 206, 209, 231]. In addition, Hsu et al. showed that mutations in these genes were most frequent in patients with t-MN [206]. Of note, these CH-associated gene mutations were significantly enriched in the

PPM1D and *TP53* genes in patients with t-MN compared with *de novo* MN and may provide a survival advantage due to improved growth and proliferation to clonal HSCs by retaining self-renewal capabilities and blocking differentiation and/or by potentiating the DNA damage response pathway without activation of apoptosis [63, 67, 110, 206].

Furthermore, the MSK-IMPACT trial has shown that cancer treatment with external beam radiation, CTx with platinum (especially carboplatin) and topoisomerase II inhibitors preferentially selects for CH-associated mutations in DDR genes, including the *TP53* and *PPM1D* genes [224].

According to the NCCN guidelines, platinum-based therapy is the first choice of treatment in all stages of OC [75]. Concordant with these results, CH-associated gene mutations were identified in the *PPM1D* and *TP53* genes exclusively in patients who had received at least one carboplatin-based CTx line starting at least 30 days prior to blood draw (28/303 patients, 9.2%) [36]. Of note, the 79 patients who had at least one CH-associated gene mutation in one of the genes studied received a statistically significantly higher number of platinum-based CTx lines than the 369 patients without this mutation (P=0.004). However, this effect was mainly caused by the above-mentioned CH-associated gene mutations in the *PPM1D* (P<0.001) and *TP53* gene (P<0.001), indicating that prolonged platinum exposure in the form of a high number of prior platinum-based CTx lines is associated with the accumulation of CH-associated gene mutations in these genes.

Furthermore, all identified *PPM1D* CH associated gene mutations accumulate in the terminal exon (exon #6) of *PPM1D* [36]. This clustering of truncating mutations in the terminal exon of *PPM1D* was previously described and demonstrated in *in vitro* data [206, 208, 232]. The underlying mechanism, here, is that gain-of-function mutations in the terminal exon of *PPM1D* after CTx exposure impair the p53-dependent G1 checkpoint leading to inhibition of DNA damage activation of p53 [63, 67, 206]. Hsu and colleagues demonstrated that the *PPM1D*-mutated clones exhibited a selective advantage towards growth in the case of cisplatin treatment. This effect was eliminated upon administration of a PPM1D inhibitor (GSK2830371), confirming the mutation-specific selective advantage [206].

Among the remaining genes, CH-associated gene mutations in *GNAS*, *JAK2*, *SF3B1*, and *SH2*B3 were identified exclusively in patients with prior platinum-based CTx. However, differences in the mean number of platinum-based CTx lines in the patients in concern received did not reach a statistically significant level [36].

Checking for increased occurrence of CH-associated gene mutations with advanced age, I could confirm an association of age-dependent CH in the *PPM1D*, *TET2*, *SF3B1* and *SH2B3* genes, but only a single CH-associated gene mutation was detected in the latter two [36].

The occurrence of CH-associated gene mutations in *ASXL1* shows no statistically significant association with any of the three potential risk factors considered. *ASXL1* has recently been associated with smoking [224], but there was no information on smoking behavior in our study sample.

In the overall study sample, 20.5% of the patients (92/448 patients) carried germline PVs in *BRCA1/2* [36]. Here, no direct association between g*BRCA1/2* mutations and a higher likelihood of accumulation of CH-associated gene mutations in the *PPM1D*, *TP53* genes and probably other genes could be identified, suggesting an increased occurrence of CH-associated gene mutations in response to platinum-based CTx. Furthermore, in multivariate analysis did not detect a statistically significant interaction between g*BRCA1/2* mutation status and the number of platinum-based CTx lines with the occurrence of CH-associated gene mutations in the *PPM1D* or *TP53* genes, although minor effects cannot be excluded [36]. In our study sample, OC patients with a positive g*BRCA1/2* mutation status received a statistically significantly higher number of platinum-based CTx lines before blood draw than OC patients without a g*BRCA1/2* PV, most likely due to a more favorable response to therapy and survival benefit observed in g*BRCA1/2* mutation carriers [229, 230]. These findings may have clinical implications for the management of patients with *BRCA1/2*-associated hereditary cancers, especially high grade serous OC and triple-negative BC (TNBC), in terms of the choice of treatment regimens [233, 234]. For patients affected by TNBC with positive g*BRCA1/2* mutation status were recently shown to have an increased pathological response following CTx exposure compared with patients without germline PVs in *BRCA1/2* [235].

In addition to the effects of platinum-based CTx, there have been concerns that g*BRCA1/2* mutation carriers may be more vulnerable to developing t-NM after treatment with PARP inhibitors such as niraparib or olaparib. Two trials, the ENGOT-OV16/NOVA and SOLO2/ENGOT-Ov21 trial, showed increased t-MN rates in the niraparib and the olaparib arms, respectively, compared with the placebo arms [236, 237]. However, a follow-up of the SOLO2/ENGOT-Ov21 trial, found that PARP inhibition did not increase the t-MN risk compared with placebo [238]. Rather, a trend emerged: as the number of prior platinum-based CTx lines increased, the t-MN risk also increased. These results support our hypothesis of an indirect association with *BRCA1/2* germline mutations due to higher numbers of prior platinum-based CTx lines.

6 Conclusion

In the first part of my PhD project, I could show that germline PVs in the *BARD1* gene, which is a direct interaction partner of the encoded protein of *BRCA1*, are associated with early-onset of familial BC, but not with OC. Thus, *BARD1* can be excluded as OC predisposition gene. Based on the consensus of the GC-HBOC working group "Klinische Konsequenzen" the *BARD1* gene was incorporated as "core" gene into the TruRisk® panel analysis as BC risk gene. Thus, intensified BC surveillance programs specifically for younger women carrying germline PVs in *BARD1* can be offered from now on.

In the following part of the project, focusing on CH, I was able to identify two robust biomarkers (*PPM1D* and *TP53*) for chemotherapy-related CH. Since, there is no diagnostic tool available to detect early precursor states of therapy-related tumors, the new findings were directly integrated in the evaluation of *TP53* mutations in routine diagnostics by an updated SOP for the Center for Familial Breast and Ovarian Cancer Cologne, and subsequently for all centers of the GC-HBOC.

Due to the new SOP, the detection of PVs in the tumor suppressor gene *TP53* in the blood sample of an index patient in the context of genetic counseling for familial BC/OC alone does not allow a statement regarding its clinical relevance. It must be investigated whether the PV may be causal for the BC or OC.

Therefore, to clarify the association of PV with tumorigenesis, it is necessary to apply the SOP and examine a second tissue that is not derived from the hematopoietic system. This procedure also allows preventing misdiagnosis of LFS1.

In our secondary analysis of the AGO-TR1 study, I could demonstrate that a positive g*BRCA1/2* mutation status is not a risk factor to acquire CH-associated gene mutations. Therefore, patients with heterozygous germline PVs in the risk genes *BRCA1/2* are not more susceptible to CH than patients without these PVs**.** For patients investigated, alone, the number of prior lines of CTx appears to be the main risk factor for developing t-MN.

Our specific CH biomarker assay and monitoring of patients after chemotherapeutic exposure with defined time-interval blood draws followed by subsequent analysis for CH-associated gene mutations may allow early detection of t-MN and an optimized clinical management of patients at increased risk for these hematological diseases in the future.

7 Outlook

In the future, new interdisciplinary networks must be established to provide the best possible advice and care for cancer patients with CH-associated gene mutations. This could be created with the help of direct care pathways starting with the identification of such variants in routine diagnostics and ending with referral to hematologists/oncologists or other physicians e.g. cardiologist. A first approach is the recently established National Center for Familial Tumors (NCFT), which is integrated into the Center for Integrated Oncology (CIO) of the University Hospital of Cologne, with the main goal to perform direct translation from genetics to the clinic for different tumor entities, e.g. through multimodal early detection programs.

To exclude PV as the cause of the underlying cancer in the case of *TP53*, genetic analysis requires the examination of a second tissue by NGS, in addition to the blood sample, that does not originate from the hematopoietic system. Furthermore, multi-gene panel analyses for the evaluation of BC and OC risks should no longer be limited to solid tumor entities, but should additionally include biomarkers in the form of genes or regions known to harbor CH-associated gene mutations to enable preventive measures against possible t-MDS or t-AML. This should be considered especially if the patient has received prior chemotherapy.

After molecular genetic analysis, all results should be discussed in an interdisciplinary genetic diagnostics board and subsequently evaluated taking further patient data such as age at onset of disease, tumor subtype, family history and information on the administration of chemotherapeutic agents prior to blood draw into account.

Future clinical trials are needed to assess whether the choice of treatment regimen needs to be adjusted to individual t-MN risk. For example, the MSK-IMPACT study suggests that the risk of developing CH-associated gene mutations is lower with cisplatin or oxaliplatin than with carboplatin.

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10 Appendices

10.1 Kurzfassung der Dissertationsschrift in deutscher Sprache

Identifizierung blutspezifischer Biomarker für therapiebedingte klonale Hämatopoese bei Patientinnen mit erblichem oder nicht erblichem Eierstockkrebs

Im Rahmen meines Dissertationsprojektes konnte ich im ersten Teil zeigen, dass pathogene Varianten (PVs) in der Keimbahn im *BARD1*-Gen, einem direkter Interaktionspartner des kodierten Proteins von BRCA1, mit dem frühen Auftreten von familiärer Brustkrebs (BC), nicht aber mit dem Ovarialkarzinom (OC) in Verbindung gebracht werden. Somit kann *BARD1* als OC-Prädispositionsgen ausgeschlossen werden. Auf der Grundlage dieser Ergebnisse wurde das *BARD1*-Gen von der Arbeitsgruppe "Klinische Konsequenzen" des Deutschen Konsortiums für erblichen Brust- und Eierstockkrebs neu bewertet und in einem Konsens als "Kerngen" in die TruRisk®-Panel-Analyse als BC-Risikogen aufgenommen. Damit können von nun an intensivierte BC-Überwachungsprogramme speziell für jüngere Frauen angeboten werden, die Keimbahn-PVs in *BARD1* tragen.
Im zweiten Teil des Projektes konnte ich zwei robuste Biomarker (die Gene *PPM1D* und *TP53*) für eine Chemotherapie-bedingte klonale Hämatopoese (CH) identifizieren. Da es kein diagnostisches Werkzeug gibt, um frühe Vorstufen, wie eine CH, von therapiebedingten Tumoren zu erkennen, wurden die neuen Erkenntnisse direkt in die Bewertung von *TP53*-Mutationen in der Routinediagnostik durch eine aktualisierte SOP für das Zentrum Familiärer Brust- und Eierstockkrebs in Köln integriert und anschließend auf alle Zentren des Deutschen Konsortiums für erblichen Brust- und Eierstockkrebs ausgeweitet.
Da der Nachweis von PVs im Tumorsuppressorgen *TP53* in der Blutprobe einer Indexpatientin im Rahmen der genetischen Beratung für familiäres BC/OC alleine keine Aussage über die klinische Relevanz zulässt, muss im Folgenden abgeklärt werden, ob die PV kausal für die Tumorentstehung ist. Hierfür ist es zwingend notwendig ein zweites Gewebe zu untersuchen, welches nicht aus dem hämatopoetischen System stammt, um Fehldiagnosen (z.B. hinsichtlich des *TP53*-assoziierten Li-Fraumeni-Syndroms) zu vermeiden.
In unserer Sekundäranalyse der AGO-TR1-Studie konnten demonstriert werden, dass ein positiver *BRCA1/2*-Mutationsstatus in der Keimbahn kein Risikofaktor für den Erwerb CH-assoziierter Genmutationen ist. In Folge dessen sind Patientinnen mit heterozygoter Keimbahn-PV in den Risikogenen *BRCA1/2* nicht anfälliger für den Erwerb einer CH als Patientinnen ohne diese PVs. Allein die Anzahl der vorangegangenen Chemotherapie-Linien scheint bei den untersuchten Patientinnen der Hauptrisikofaktor für die Entwicklung einer therapiebedingten myeloischen Neoplasie (t-MN) zu sein.
Blutentnahmen in definierten Zeitintervallen bei Patienten nach einer Exposition mit Chemotherapeutika, gefolgt von einem spezifischen Biomarker-Assay auf CH-assoziierte Genmutationen könnten in Zukunft sowohl eine frühzeitige Erkennung von t-MN als auch eine optimierte klinische Behandlung von Patienten mit erhöhtem Risiko für die zuvor angesprochenen hämatologischen Erkrankungen ermöglichen.

von René Konstantin Weber-Lassalle

aus dem Zentrum Familiärer Brust- und Eierstockkrebs der Universität zu Köln

10.2 Erklärung

Erklärung II

Hiermit versichere ich, dass ich die von mir vorgelegte Dissertation selbstständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von PD Dr. Eric Hahnen aus dem Zentrum Familiärer Brust- und Eierstockkrebs der Uniklinik Köln betreut worden.

Übersicht der Publikationen:

Germline loss-of-function variants in the *BARD1* gene are associated with early-onset familial breast cancer but not ovarian cancer. Weber-Lassalle N, Borde J, Weber-Lassalle K, Horváth J, Niederacher D, Arnold N, Kaulfuß S, Ernst C, Paul VG, Honisch E, Klaschik K, Volk AE, Kubisch C, Rapp S, Lichey N, Altmüller J, Lepkes L, Pohl-Rescigno E, Thiele H, Nürnberg P, Larsen M, Richters L, Rhiem K, Wappenschmidt B, Engel C, Meindl A, Schmutzler RK, Hahnen E, Hauke J. *Breast Cancer Res.* 2019 Apr 29;21(1):55. doi: 10.1186/s13058-019-1137-9.

Diagnosis of Li-Fraumeni Syndrome: Differentiating *TP53* germline mutations from clonal hematopoiesis: Results of the observational AGO-TR1 trial. Weber-Lassalle K, Harter P, Hauke J, Ernst C, Kommoss S, Marmé F, Weber-Lassalle N, Prieske K, Dietrich D, Borde J, Pohl-Rescigno E, Reuss A, Ataseven B, Engel C, Stingl JC, Schmutzler RK, Hahnen E. *Hum Mut.* 2018 Dec; 39(12):2040-2046. doi: 10.1002/humu.23653.

Clonal hematopoiesis-associated gene mutations in a clinical cohort of 448 patients with ovarian cancer. Weber-Lassalle K, Ernst C; Möllenhoff K, Reuss A, Baumann K; Jackisch C; Hauke J; Dietrich D; Borde J, Park-Simon TW; Hanker L; Prieske K, Schmidt S; Weber-Lassalle N, Pohl-Rescigno E, Kommoss S, Marmé F; Heitz F, Stingl JC, Schmutzler RK; Harter P; Hahnen E. *J Natl Cancer Inst.* 2021 Dec 28;djab231. doi: 10.1093/jnci/djab231.

Ich versichere, dass ich alle Angaben wahrheitsgemäß nach bestem Wissen und Gewissen gemacht habe und verpflichte mich, die obigen Angaben betreffenden Veränderungen, dem Promotionsausschuss unverzüglich mitzuteilen.

08.11.2022 .....

Datum

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René Konstantin Weber-Lassalle