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Edited by
Priya Ranjit Bhosale and Elena Ioana Braicu

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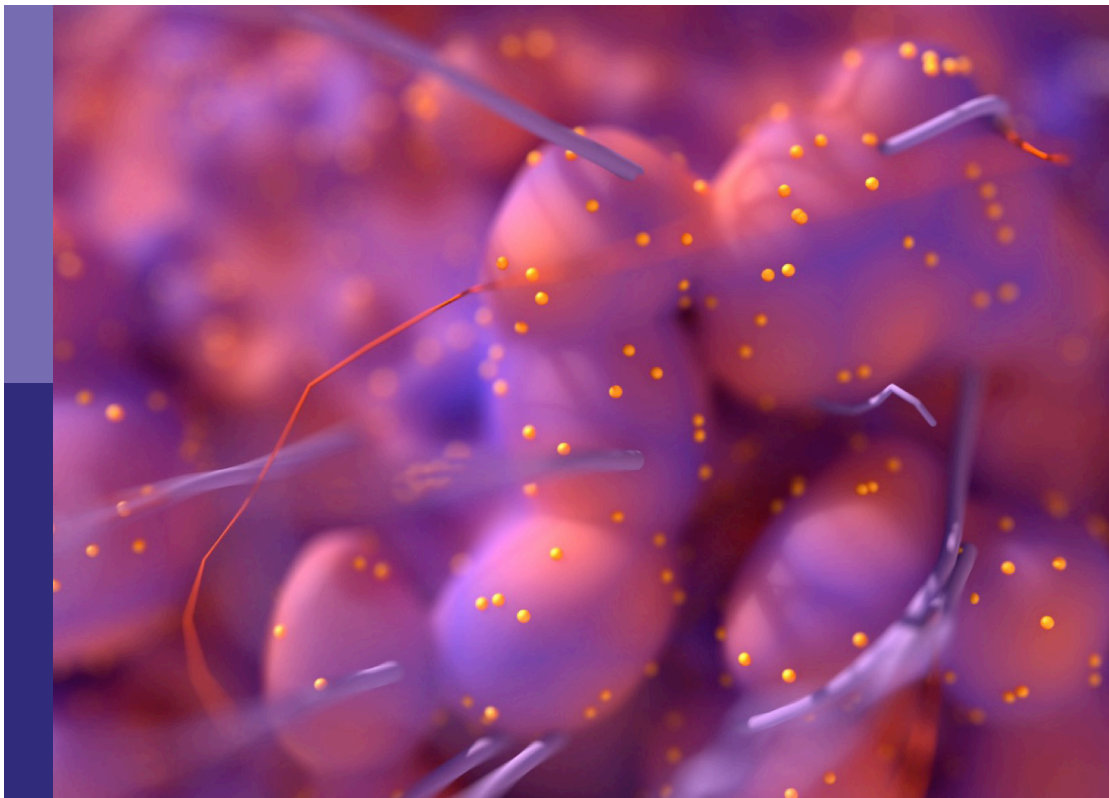

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Women in gynecological oncology vol II: 2022

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hnRNP E1 Regulates HPV16 Oncogene Expression and Inhibits Cervical Cancerization

*Li Song[1], Rui Mao[2], Ling Ding[1], Zhiqiang Tian[3,4], Mingxuan Zhang[1], Jiahao Wang[1], Ming Wang[1], Yuanjing Lyu[1], Chunliang Liu[1], Meijuan Feng[1], Haixia Jia[1] and Jintao Wang[1]**

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hnRNP E1 (heterogeneous nuclear ribonucleoprotein E1) is an important RNA-binding protein (RBPs) that plays a vital role in tumor development. Human papillomavirus 16 (HPV16) contains numerous sites that can bind to RNA/DNA and may be modified by multiple RBPs, which contribute to HPV gene expression and HPV-associated cancer development. However, the effects of hnRNP E1 on HPV16 oncogenes in the development of cervical lesions remain unclear. A total of 816 participants with different grades of cervical lesions were enrolled in a community-based cohort established in Shanxi Province, China. The Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) databases were used to analyze the association between hnRNP E1 mRNA expression and cervical lesions. Cells with up_ and down_regulated hnRNP E1 were established. hnRNP E1 functions were evaluated using cell counting kit-8, flow cytometry analyses, and chromatin immunoprecipitation sequencing. Our results showed that hnRNP E1 expression was linearly dependent on the severity of the cervical lesions. Low expression of HPV16 E2, high expression of E6, and a low ratio of E2 to E6 could increase the risk of cervical lesions. hnRNP E1 expression was correlated with HPV16 oncogene expression. hnRNP E1-relevant genes were involved in the dopaminergic synapses, Wnt signaling pathway, gnRH secretion, and mTOR signaling pathway. hnRNP E1 significantly inhibited cell proliferation, induced apoptosis, arrested the cell cycle at the G0/G1 stage, and decreased HPV16 E6 expression. Our results indicate that hnRNP E1 could downregulate HPV16 E6 oncogene expression and inhibit cervical cancerization, which sheds new light on preventing the carcinogenicity of HPV across a range of diseases by regulating RNA-binding proteins.

Keywords: hnRNP E1, cervical cancerization, HPV16 E6, HPV16 E2/E6 ratio, regulation

INTRODUCTION

Cervical cancer is the fourth most frequently diagnosed cancer in women globally (1). In contrast to the downward trends in developed countries, the incidence of cervical cancer in China has increased significantly. It is estimated that by 2020, there will be 0.11 million new cases and 0.06 million deaths in China (2). Shanxi Province, China has a notably high incidence of cervical cancer cases, with 5.42 associated deaths per 100,000 women in 2014 (3), which was about two times more than the national average (4).

High-risk human papillomavirus (HPV) persistent infection, especially HPV16 infection, accounts for more than 50% of HPV cases (5) and plays an important role in cervical carcinogenesis (6). The HPV16 genome comprises approximately 8000 bp of double-stranded circular DNA that can be divided into early genes (E1, E2, E4, E5, E6, and E7), late genes (L1 and L2), and long control regions. HPV16 E6, which induces and maintains cellular transformation, is an important oncogenic protein. Specifically, E6 and E7 expressions are necessary to drive proliferation in infected cells and for progression to high-grade lesions and cancer development. HPV16 E2 is considered to be the main inhibitor of E6 and E7 oncogene expression (7, 8). Integration of the HPV16 genome into host chromosomes is a vital event in cervical carcinogenesis, which usually causes disruption of E2, loss of regulation of E6, and subsequently E6 overexpression (9). Therefore, E2 and E6 play an important role in HPV integration and carcinogenesis and have attracted extensive attention as key genes for integration. However, the regulation of HPV gene expression depends on intracellular RNA processing and is usually modified by RNA binding proteins (RBPs) (10).

As RBPs, heterogeneous nuclear ribonucleoproteins (hnRNPs), contribute multiple functions to nucleic acid metabolism through post-transcriptional regulation (11). hnRNP E1 is a member of the hnRNP family and contains three K homology (KH) domains. It is required to achieve greater RNA/DNA binding affinity and specificity (12). HPV16 has two characteristic promoters, early promoter p97, and late promoter p670. In addition, the HPV16 genome covers 5′splice sites, 3′splice sites, and two polyadenylation sites. These sites provide the structural basis for specific binding to a variety of RBPs and their protein complexes (13). Studies have shown that hnRNP E1 is involved in multiple pathological processes (14, 15). hnRNP E1 expression is associated with numerous tumor types, such as liver cancer (16), pancreatic cancer (17), gastrointestinal adenocarcinomas (18), prostate cancer (14), and thyroid carcinoma (19). However, to the best of our knowledge, the association between hnRNP E1 expression and cervical cancerization remains unclear. Pillai et al. (20) used an immunohistochemical method to detect hnRNP E1 in cervical tissue with a small sample size. The results showed that the expression level of hnRNP E1 decreased gradually with the increase in the severity of the disease. Our previous study showed that high expression of hnRNP K, which is similar to hnRNP E1 in structure and function, could increase the risk of cervical lesions (21). Collier et al. (22) found that hnRNP E1 inhibited the translation of L2 mRNA of the HPV16 late gene. However, the association between hnRNP E1 and HPV16 and the progression of cervical carcinogenesis has not been reported.

Based on the specificity with which the unique KH structural domains of hnRNP E1 bind with RNA/DNA, considering that HPV16 provides RNA/DNA binding sites, we hypothesized that hnRNP E1 may be crucial to HPV16 oncogenes expression and cervical carcinogenesis. Our previous population-based results showed that hnRNP E1, HPV16 E2, and E6 are closely linked to cervical cancer development (23). In the present study, we analyzed the expression changes of hnRNP E1 in different cervical pathological stages. We further explored the potential function and mechanism of hnRNP E1 in cervical lesions *in vitro*. Our findings may serve as a foundation for elucidating novel molecular targets against HPV16 and new prognostic and predictive biomarkers for cervical cancer.

MATERIALS AND METHODS

Study Population

There were 11984 participants, including 11938 women from the community cohort established in Jiexiu City and Yangqv County, Shanxi Province, China from June to September 2014, and 46 women suspected of cervical cancer from the Shanxi Cancer Hospital. All participants followed inclusion criteria: a) married, b)18-65 years, c) had resided in Shanxi for at least 1 year, d) volunteered to participate in the study, and the exclusion criteria were: a) pregnancy, b) history of hysterectomy or cervical conization, current or prior malignancy, c) had other tumors, d) received chemotherapy or radiotherapy. After completing the ThinPrep cytologic test (TCT) of 11,938 women, 858 women were diagnosed with atypical squamous cells of undetermined significance and above (ASC-US+). Then, after 77 women were excluded, 781 women with ASC-US+ underwent HPV genotyping and histopathological examination. Of the 781 women, 469 were diagnosed by pathology as normal cervical (NC), 236 as low-grade cervical intraepithelial neoplasm (CIN I), and 71 as high-grade cervical intraepithelial neoplasm (CIN II/III) and 5 as squamous cell carcinoma of the cervix (SCC). Meantime, we collected 35 SCC patients diagnosed by pathology from the hospital. Ultimately, 816 participants were involved in the study (**Figure 1**). All participants signed informed consent, and the study was approved by the institutional review committee of Shanxi Medical University (2013–003). No potentially identifiable human images or data are presented in this study.

Data and Sample Collection

All participants were interviewed face-to-face to collect information about sociodemographic characteristics, personal hygiene behavior, lifestyle, menstrual state, sexual life, and history of the personal disease through a structured questionnaire. Cervical swabs were collected with a cervical brush and stored at 4°C and completed HPV typing within 24 hours. Cervical biopsy specimens were collected and stored in a -80°C refrigerator immediately.

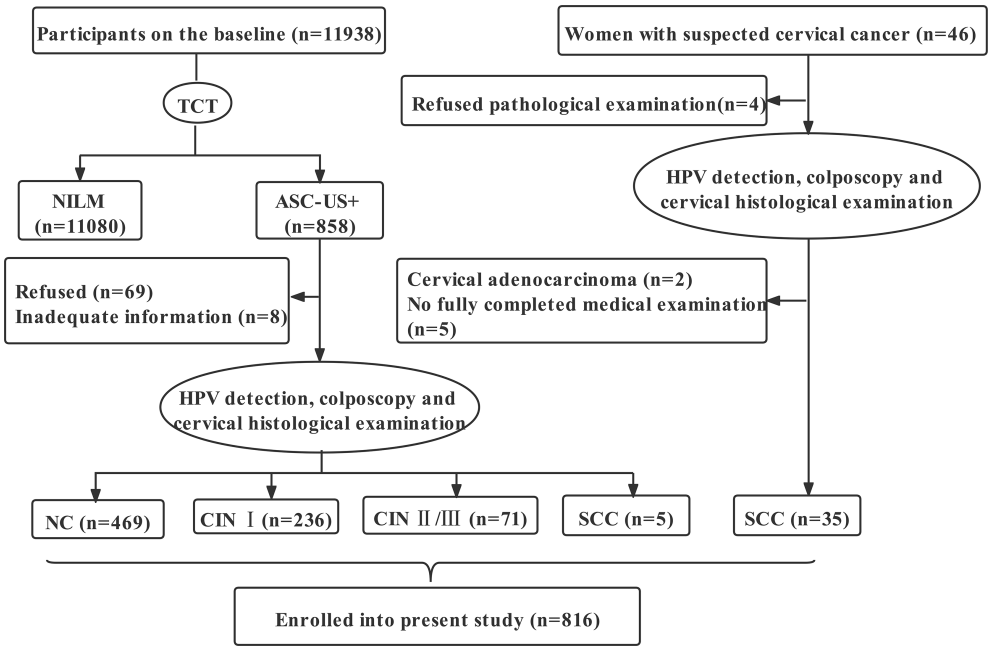

FIGURE 1 | Flowchart of participants in the study. TCT, ThinPrep cytologic test; NILM, negative for intraepithelial lesion or malignancy; ASC-US+, atypical squamous cells of undetermined significance and above; NC, normal cervical; CIN I, low-grade cervical intraepithelial neoplasm; CIN II/III, high-grade cervical intraepithelial neoplasm; SCC, squamous cell carcinoma; HPV, human papillomavirus.

HPV Detection

HPV16 infection assay by flow-through hybridization technology, DNA extraction, and HPV genotyping have previously been described in detail (24, 25). Briefly, HPV-DNA in cervical swabs was extracted by HPV-DNA extraction kit and amplified by PCR. HPV genotyping was performed using an HPV Geno Array Test Kit (HybriBio Ltd, Chaozhou, China) according to the manufacturer's instructions. Twenty-one HPV genotypes can be identified. In the study, HPV16 positive was defined as the participants with HPV 16 single infection or multiple infections with other HPV genotypes.

Cell Culture

Cervical cancer cell lines (SiHa and C33A) were obtained from the Chinese Academy of Sciences (Beijing, China). SiHa cells were cultured in high glucose Dulbecco's modified eagle medium (Hyclone, USA), and the minimum essential medium (Boster Inc, China) was used to culture C33A cells. All the cell lines were supplemented with 10% fetal bovine serum of 5% CO_2 at 37°C.

Plasmid Transfection

hnRNP E1 cDNA plasmids (Genechem Co., Ltd., Shanghai, China) and hnRNP E1 shRNA (Sangon Biotech, Co., Ltd., Shanghai, China) were used to up_ and down_ regulate the expression of hnRNP E1, respectively. When the cell confluence reached 75%, the transfection complex composed of plasmid, transfection reagent (Roche Applied Science, Germany), and culture medium were added to the 6-well plates. DNA plasmids encoding Green fluorescent protein (GFP) were performed to establish the best transfection efficiencies and conditions.

Western Blotting

Proteins were extracted from cells or tissues and added to the RIPA buffer. Cracking on ice for 30_60 minutes. The supernatant was collected by centrifugation at 4°C and 12000 rpm for 15 minutes. BCA protein assay kit (Boster Inc, China) was used for protein quantification. SDS-PAGE gel electrophoresis was performed. Then, the protein was transferred to the nitrocellulose membrane and 5% skimmed milk powder blocked the nonspecific antigen. The primary antibody was added and incubated at 4°C overnight. After washing the membrane, horseradish peroxidase-labeled IgG (secondary antibody) was added at 37 °C for 1h. Densitometric analysis was performed by Image Lab. In the assay, antibodies included rabbit anti-hnRNP E1 (Abcam, UK. ab74793, 1:1000), mouse anti-HPV16 E2 (Abcam, UK. ab17185, 1:1000), mouse anti-HPV16 E6 (Abcam, UK. Ab70, 1:1000), mouse anti-β-actin (Boster Inc, China, 1:300).

Quantitative Real-Time RT-PCR

TRIzol Reagent (Invitrogen, CA, USA) was used for extracting total RNA from cultured cells. cDNA was prepared by reverse transcription using TransScript one-step gDNA Removal and cDNA Synthesis SuperMix kit (Transgen Biotech, China). Quantitative real-time RT-PCR (RT-qPCR) was carried out using the QuantiNova SYBR Green PCR kit (QIAGEN GmbH, Germany). PCR was carried out following the manufacturer's

protocol. The PCR primer information was shown in **Table S1**. β-actin was used as the internal control. The relative mRNA levels were defined by using the $2^{-\triangle\triangle Ct}$ method.

Cell Proliferation, Cycle, and Apoptosis Assays

10μl CCK8 stock solution (Dojindo Laboratories, Japan) were added to each cultured 96-well plate at the stages of post-transfection 12h, 24h, 36h, 48h, and 72h, further incubated for 2h at the 37°C. The absorbance value at 450 nm was measured by a microplate reader. To analyze the cell cycle, cultured cells were collected, counted, and added 500μl 70% pre-cooling ethanol to centrifugated cells, overnight at 4°C. The next day, centrifugated cells were treated with 500μl RNaseA/Propidium Iodide and kept away from light at 25°C for 30 min. Subsequently, the cell cycle was detected by flow cytometry (FCM). Cell apoptosis was detected using an Annexin V-APC/Propidium Iodide apoptosis detection kit (KGA1030, KeyGEN Biotech, China), then, cell proliferation indexes (PI) were evaluated by a formula of (S+G2/M)÷(G0/1+S+G2/M)×100%.

Chromatin Immunoprecipitation Sequencing and Bioinformatics Analysis

Chromatin immunoprecipitation (ChIP) experiments were performed on SiHa cells that had not been treated with any plasmid. The SimpleChIP Enzymatic Chromatin IP Kit (no. 9003; Cell Signaling Technology, Danvers, MA, USA) was used to prepare cross-linked chromatin for ChIP. Briefly, the cells were fixed with 1% formaldehyde for 10 min, followed by cell and nuclear lysis. Cross-linked DNA was sonicated and ranged in size from 200 to 900 bp. DNA/protein complexes were immunoprecipitated overnight using anti-hnRNP E1 (#8534, Cell Signaling Technology, Danvers, MA, USA) antibodies. After reverse cross-linking and DNA purification, enriched DNA was detected by PCR. The primers used were listed in **Table S1**. The quality control analysis of the ChIP experiment was presented in **Figure S1**. Sequencing was performed at BGI-Shenzhen (Shenzhen, China) using an BGISeq-500 system. The authors acknowledge that the data presented in this study must be deposited and made publicly available in an acceptable repository, prior to publication. Frontiers cannot accept a manuscript that does not adhere to our open data policies.

The raw data were aligned to hg19 using SOAPaligner/SOAP2 (26). Peak calling was conducted by MACS (Model-based Analysis of ChIP-Seq) (27). The Gene Ontology (GO) terms (28) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses (29) were performed in R software using the "clusterProfiler" to show enrichment closely related to hnRNP E1_relevant genes. A two-sided Fisher's exact test and the "enrichplot" and "ggplot2" packages were used to visualize the top 10 enriched terms and KEGG pathways.

Statistical Analysis

Analyses were performed using SPSS 22.0, R 4.0 and graphics were generated using GraphPad Prism 7.0. Significance testing between groups was performed by analysis of variance (ANOVA), Bonferroni's multiple comparison tests, The *post hoc* Least Significant Difference (LSD) test, the Kruskal-Wallis H test, and the Chi-square test. Possible associations were analyzed using Spearman's rank-order correlation. Kaplan-Meier survival analyses were performed in R 4.0 software using the "survival" and "survminer" to assess the relevance of hnRNP E1 expression and prognosis of the patient with cervical cancer. All analyses were two-sided and α=0.05.

The raw gene expression profiles of GSE9750 and GSE75132 were downloaded from the Gene Expression Omnibus (GEO) and used to analyze the expression of hnRNP E1 in cervical cancer and its relationship with HPV. GSE9750, containing 24 normal cervical samples and 33 cervical cancer samples. GSE75132 included data from six participants with persistent HPV16 infection and participants without HPV. The mRNA expression and clinical information of 304 patients with cervical cancer were downloaded from The Cancer Genome Atlas (TCGA) portal (https://portal.gdc.cancer.gov/).

RESULTS

1. Demographic Characteristics of Participants and Factors Related to Cervical Lesions

We analyzed the demographic characteristics and factors related to cervical lesions of 816 participants. The average age of 816 women was 47 ± 12 years (range 19_65 years). Of all participants, 79.5% had received education in junior high school and above. The distribution of marital status, age, education level, and occupation among different cervical lesions groups was not significant (P>0.05); however, early age of first sexual intercourse, multiple gravidities, low bathing frequency, and low vaginal cleaning frequency were found to be associated with increased risk of CIN and cervical cancer(P<0.05).

2. HPV16 Gene Expression in Multistage Cervical Cancerization

The prevalence of HPV16 in NC, CIN I, CIN II/III, and SCC were 8.53%, 14.41%, 40.85%, and 67.50%, respectively, with significant differences, and showed an upward trend with the aggravation of cervical lesions (χ^2_{trend}=113.560, P<0.001). The results were displayed in **Table 1**. Subsequent results showed that HPV16 E2 protein levels in the NC and CIN I groups were significantly higher than those in the CIN II/III and SCC groups. The HPV16 E6 protein levels in the NC and CIN I groups was significantly lower than those in the CIN II/III and SCC groups (**Table 1**). There were significant differences in the ratio of E2 to E6 in different groups (H=71.392, P<0.001), showing a decreasing trend from NC to CIN I, CIN II/III, and SCC (**Table 1**).

3. hnRNP E1 Expression in Multistage Cervical Cancerization and Associations With HPV16 E2 and E6

To explore hnRNP E1 expression patterns in cervical lesions, hnRNP E1 protein expression was detected using western

TABLE 1 | Associations between HPV16 genes expression and cervical lesions.

Group	N	HPV16 infectionn (%)	HPV16 E2 M(Q)*	HPV16 E6 M(Q)*	Ratio of E2/E6 M(Q)*
NC	469	40(8.53)	1.72(0.28)^{a}	0.18(0.08)^{a}	9.89(4.75)^{a}
CIN I	236	34(14.41)	1.71(0.34)^{a}	0.17(0.06)^{ab}	9.39(3.20)^{a}
CIN II/III	71	29(40.85)	1.08(0.86)^{bc}	0.47(0.78)^{c}	2.13(3.72)^{bc}
SCC	40	27(67.50)	0.86(0.48)^{c}	1.25(0.67)^{d}	0.69(0.85)^{b}
		χ^2_{trend}=113.560, P<0.001	$H^{\#}$=46.207, $P < 0.001$	$H^{\#}$=66.848, $P < 0.001$	$H^{\#}$=71.392, $P < 0.001$

**median (quartile range); a/b/c/d, different letters indicate significant differences at least $P<\alpha'$ (α'=0.05/6 = 0.0083); $^{\#}$overall comparison among different groups.*

blotting. With the progress of cervical lesions, hnRNP E1 protein expression levels gradually decreased (**Figure 2A**). The average hnRNP E1 expression level in NC (2.081 ± 1.708, n=469) was 2.30 times higher than that in SCC (0.906 ± 0.844, n=40). Moreover, data from the GEO dataset (GSE9750) showed that hnRNP E1 mRNA was more highly expressed in NC samples than in SCC samples (**Figure 2B**). We further investigated the associations between hnRNP E1 and HPV in cervical lesions and found that the expression levels of hnRNP E1 protein or mRNA in the HPV-negative group were significantly higher than those in the HPV-positive group (P<0.05), as shown in **Figures 2C**, **D**. Spearman's rank correlation analysis showed positive correlations between hnRNP E1 expression and HPV16 E2 (r_s=0.397, P<0.001) and the ratio of HPV16 E2 to E6 (r_s=0.584, P<0.001) and a negative correlation between hnRNP E1 and HPV16 E6 (r_s =-0.584, P<0.001), as shown in **Figures 2E, F**.

4. Correlations Between hnRNP E1 and Prognosis of the Patient With Cervical Cancer Based on TCGA Database

We further analyzed the relationship between hnRNP E1 expression and clinicopathological characteristics in cervical

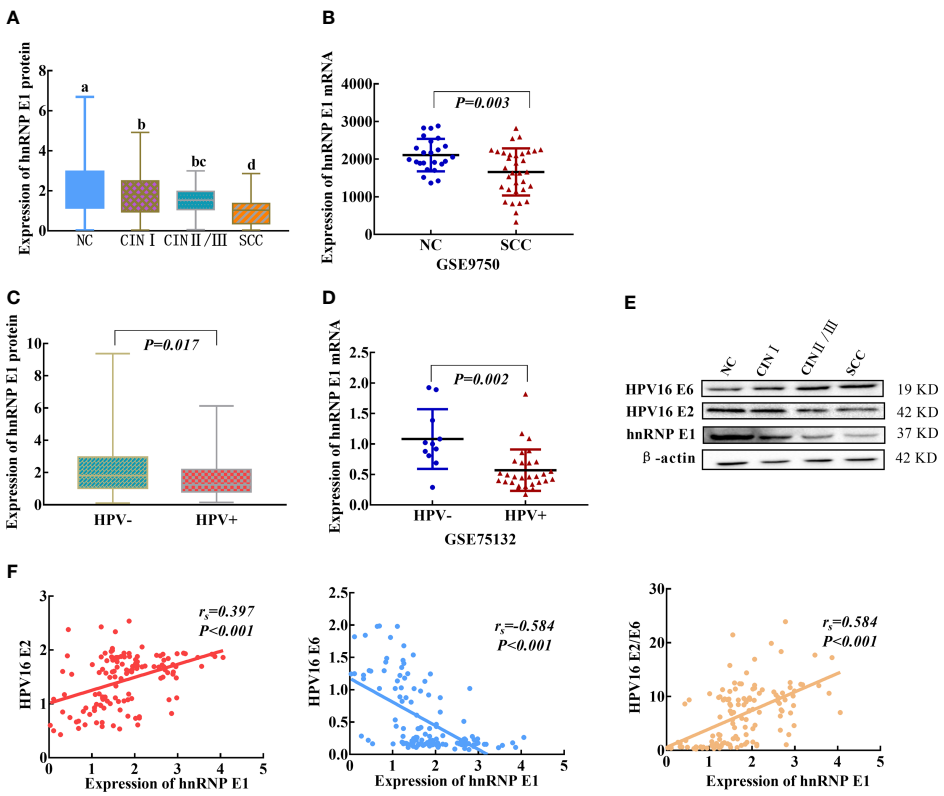

FIGURE 2 | hnRNP E1 expression and correlations with HPV16 E2 and E6 in cervical lesions. **(A)** hnRNP E1 protein expression levels in the clinical samples. **(B)** The hnRNP E1 mRNA expression levels in NC and SCC samples, data were obtained from GEO dataset. **(C)** The hnRNP E1 protein expression levels in HPV+ and HPV- samples. **(D)** The hnRNP E1 mRNA expression levels in HPV+ and HPV- samples, data were available from GEO dataset. **(E)** hnRNP E1, HPV16 E2 and E6 expression levels was detected using western blotting in cervix tissues. β-actin was used as loading control. **(F)** Correlations between hnRNP E1, HPV16 E2 and E6 in cervical lesions. a/b/c/d, different letters indicate significant differences at least $P<\alpha'$(α'=0.05/6 = 0.0083). HPV+ represents HPV-positive samples, and HPV- represents HPV-negative samples.

cancer based on the TCGA database. The Kruskal-Wallis rank-sum test showed that there was no significant correlation between hnRNP E1 expression and T stage (*P=0.609*), N stage (*P=0.078*), M stage (*P=0.203*), International Federation of Gynecology and Obstetrics (*FIGO*) stage (*P=0.178*), pathological type (*P=0.602*) and therapy (*P=0.448*) of cervical cancer (**Figures 3A–F**). To better understand the relevance of hnRNP E1 expression and the prognosis of the patient with cervical cancer, we performed Kaplan-Meier survival analysis. Patients were divided into high-hnRNP E1 and low-hnRNP E1 groups based on the median value. Results showed that the overall survival rate was not significant between the high-hnRNP E1 and low-hnRNP E1 groups *(P>0.05*, **Figure 3G**). Although our results have revealed the subtle prognostic value of hnRNP E1, compared with the low expression of hnRNP E1, high expression of hnRNP E1 has a better prognosis.

5. Functional Enrichment Analysis of Binding Sites Relative to hnRNP E1

Based on the main purpose of this study was to explore the role of hnRNP E1 on the regulation of HPV16 oncogene expression in cervical cancerization, we conducted ChIP-seq in the untreated SiHa cell line. A total of 121 potential targets for hnRNP E1 across the human genome in SiHa cells were identified, including 357 binding sites (peaks). The average peak length was 161 bp, and the length was mainly distributed between 100_500 bp. To further analyze the potential biological functions of annotated genes related to hnRNP E1, GO and KEGG enrichment analyses were performed, and the screening criteria was *P-value*<0.3. According to the functional annotation in the GO database, the most significant biological process (BP) terms were peptidyl-threonine phosphorylation, peptidyl-serine phosphorylation, peptidyl-threonine modification, and peptidyl-serine modification, and

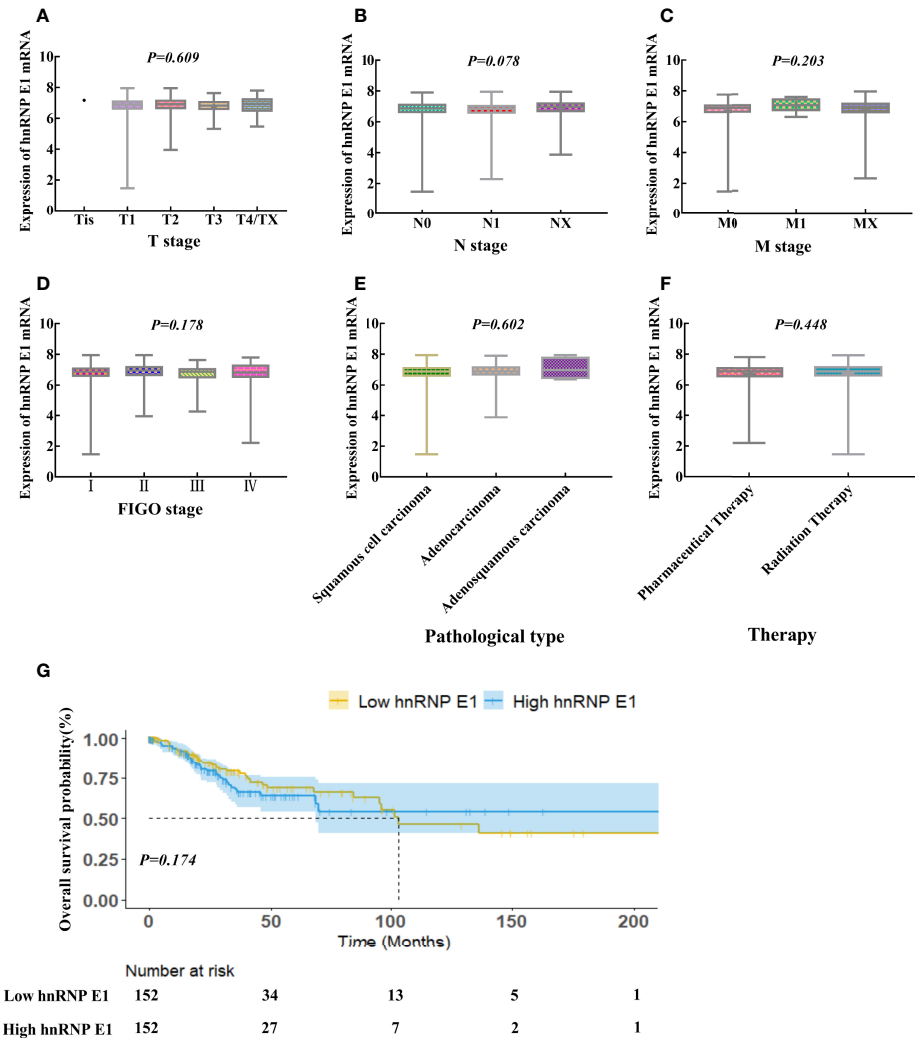

FIGURE 3 | Correlations between hnRNP E1 and prognosis of the patient with cervical cancer based on TCGA database. hnRNP E1 mRNA expression levels in the different T stage **(A)**, N stage **(B)**, M stage **(C)**, International Federation of Gynecology and obstetrics (FIGO) stage **(D)**, Pathological type **(E)** and Therapy **(F)**. **(G)** Kaplan-Meier survival curves were performed for the overall survival probability.

Hippo signaling. When focusing on cellular components (CC), the most highly represented categories were the cell cortex, protein phosphatase type 2A complex, histone deacetylase complex, presynaptic active zone, and magnesium-dependent protein serine/threonine phosphatase complex. The main functional categories of molecular function (MF) were related to protein serine kinase activity, on-membrane spanning protein tyrosine phosphatase activity, heme transmembrane transporter activity, flap endonuclease activity, and inositol 1,4,5 trisphosphate binding. GO enrichment terms of BP, CC, and MF for hnRNP E1 annotated genes are shown in **Figures 4A, C, E**. Based on the KEGG pathway enrichment analysis, hnRNP E1_relevant genes were involved in the dopaminergic synapse, Wnt signaling pathway, gnRH secretion, mTOR signaling pathway, pathways of neurodegenerative diseases, sphingolipid signaling pathway, AMPK signaling pathway, proteoglycans in cancer, and long-term depression. (**Figure 4G**). The top 10 most enriched functions were obtained to construct a network (**Figures 4B, D, F, H**). It was worth noting that we found that hnRNP E1 relevant genes were enriched in HPV infection pathway. These results suggested that hnRNP E1 may play a key role in HPV-induced cervical lesions. Detailed information was listed in **Table S2**.

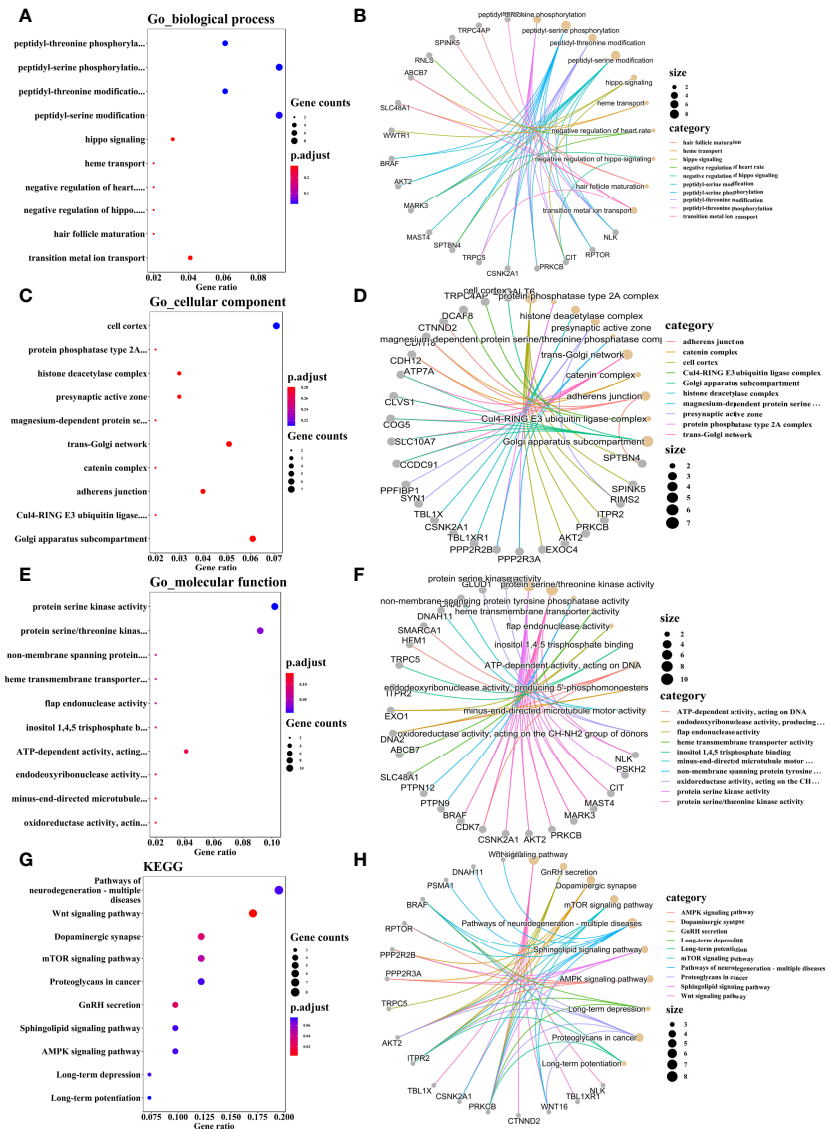

FIGURE 4 | GO and KEGG pathway enrichment analyses of hnRNP E1 related genes. Note: BP, biological process. CC, cellular components. MF, molecular function. GO, Gene Ontology. KEGG, Kyoto Encyclopedia of Genes and Genomes.The top 10 significant BP **(A)**, CC **(C)**, MF **(E)**, and KEGG pathways **(G)**. Construction of the enrichment BP_genes network **(B)**, CC_genes network **(D)**, MF_genes network **(F)**, and pathways_genes network **(H)**. **(A, C, E, G)** The x axis reflects gene ratio, and the y axis represents the KEGG and GO terms. The node size represents the gene count. The node colors represent adjusted *P* values. **(B, D, F, H)** The size of the circle indicates the number of enriched genes. Lines represent linkages of genes and enriched terms.

6. hnRNP E1 Inhibits Cervical Cancer Cell Proliferation *in Vitro*

To further explore the biological role of hnRNP E1 in cervical cancer, cultured SiHa, and C33A cell lines were transduced with hnRNP E1 overexpression plasmid or shRNA. OE indicates the hnRNP E1 overexpression group and NC-OE indicates the relative control group. KD indicates the hnRNP E1 knockdown group and NC-KD indicates the relative control group. Overexpression and knockdown efficiencies of hnRNP E1 were confirmed by RT-qPCR and western blotting (**Figure 5A**). As shown in **Figure 5B**, compared with the control group, overexpression of hnRNP E1 (OE) in SiHa and C33A cells resulted in decreased cell viability (NC-OE, $P<0.05$), and it was found that the inhibition rate of viability in hnRNP E1 overexpression was significantly higher than that of the control cells after transfection (0.36% ± 0.02% vs. 0.05% ± 0.04%, $P<0.05$; 0.28% ± 0.03% vs. 0.10% ± 0.03%, $P<0.05$). Conversely, knockdown of hnRNP E1 in SiHa and C33A cells significantly promoted cell growth with an increase in the cell proliferation rate at 48 h after transfection (0.23% ± 0.01% vs. 0.03% ± 0.03%, $P<0.05$; 0.22% ± 0.02% vs.0.03% ± 0.02%, $P<0.05$).

Next, the cell cycles were analyzed by FCM. As shown in **Figure 5C**, overexpression of hnRNP E1 increased the percentage of G0/G1 phase cells in SiHa and C33A cells (77.33% ± 1.00% vs. 69.38% ± 1.09%, $P<0.05$; 69.31% ± 0.90% vs.63.82% ± 0.41%, $P<0.05$) and reduced the proportion of S/G2/M cells and PI (22.68% ± 0.83% vs.30.62% ± 1.09%, $P<0.05$; 30.73% ± 0.87% vs.36.18% ± 0.41%, $P<0.05$). Conversely, hnRNP E1 knockdown in SiHa and C33A cells markedly attenuated the percentage of G0/G1 cells (62.71% ± 0.47% vs. 71.87% ± 0.67%, $P<0.05$; 60.52% ± 0.28%

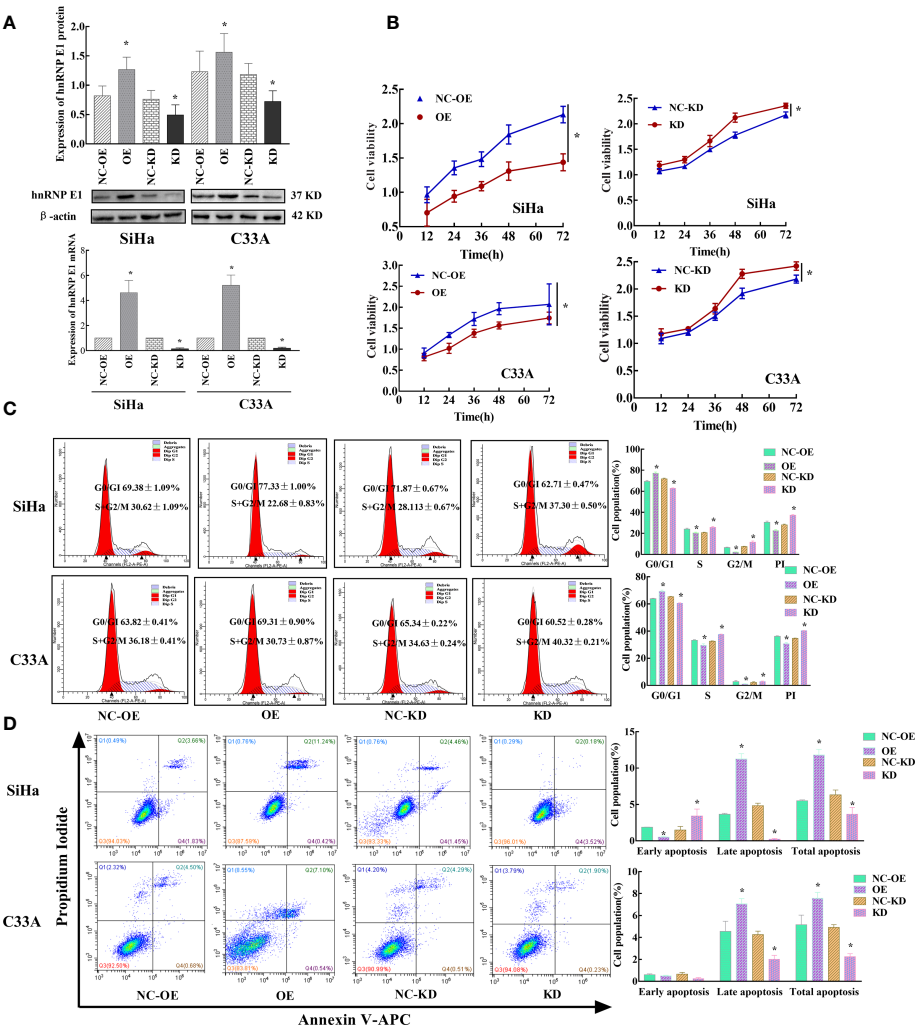

FIGURE 5 | Effects of hnRNP E1 on cell biological function in cervical cancer cell lines. Note: OE indicates hnRNP E1 overexpression group and NC-OE indicates the relative control group. KD indicates hnRNP E1 knockdown group and NC-KD indicates the relative control group. *$P < 0.05$. **(A)** Stably transfected hnRNP E1-modified cervical cancer cells were identified by RT-qPCR and western blotting. **(B)** hnRNP E1 overexpression strongly suppressed cell viabilities in SiHa and C33A cells. hnRNP E1 knockdown markedly enhanced cell viabilities in SiHa and C33A cells. **(C)** hnRNP E1 arrests the cell cycle from the G0/G1 to the S phase. **(D)** hnRNP E1 promotes cell apoptosis.

vs. 65.34% ± 0.22%, $P<0.05$), but increased the proportion of S/G2/M phase and PI (37.20% ± 0.50% vs. 28.13% ± 0.67%, $P<0.05$; 40.32% ± 0.21% vs. 34.63% ± 0.24%, $P<0.05$). These results suggest that hnRNP E1 affects cell cycle progression and G0/G1 phase arrest. Additionally, we compared the changes in cell cycles and proliferation index of SiHa and C33A cells modified by hnRNP E1 and found that the changes in PI in SiHa cells were higher than those in C33A cells (**Figure S2A**). To understand hnRNP E1-induced cell apoptosis, we performed FCM on hnRNP E1-intervened SiHa and C33A cell lines. These results indicated that the total apoptotic rate was significantly increased after the upregulation of hnRNP E1 in SiHa and C33A cell lines (11.81% ± 0.77% vs. 5.52% ± 0.11%, $P<0.05$; 7.57% ± 0.53%, vs. 5.16% ± 0.88%, $P<0.05$), as shown in **Figure 5D**. Conversely, knockdown of hnRNP E1 in SiHa and C33A cells considerably reduced the total apoptotic rate (3.64% ± 0.95% vs. 6.33% ± 0.67%, $P<0.05$; 2.25% ± 0.27% vs.4.92% ± 0.25%, $P<0.05$). These results were consistent with our work in this population and indicated that hnRNP E1 played a tumor suppressor role in cervical cancer. Furthermore, we analyzed changes in the apoptotic rate of SiHa and C33A cells modified by hnRNP E1 and found that the changes in the early and late apoptotic rate of SiHa cells were superior to those of C33A (**Figure S2B**).

7. hnRNP E1 Effects on HPV16 Oncogene and Subsequent Changes in Cell Biological Function

Our population-based study identified a negative correlation between hnRNP E1 and HPV16 E6 and a positive correlation between hnRNP E1 and HPV16 E2 protein expression. We confirmed that hnRNP E1 induced different biological changes in SiHa and C33A cells *in vitro*. Next, to test whether hnRNP E1 regulated HPV16 oncogene expression, HPV16 E2 and HPV16 E6 were assessed in SiHa cells. As shown in **Figure 6A**, hnRNP E1 overexpression downregulated HPV16 E6 expression at both the mRNA and protein levels but increased the ratio of HPV16 E2 to E6 at the mRNA level. Conversely, hnRNP E1 knockdown markedly enhanced HPV16 E6 expression at both the mRNA and protein levels but decreased the HPV16 E2 to E6 ratio at the mRNA level (**Figure 6A**).

We further analyzed the relationship between HPV16 E6 expression, cell proliferation, and apoptosis. The expression of HPV16 E2 and E6 were detected in SiHa cells by RT-qPCR and western blotting. The PI and total apoptosis rate were evaluated using FCM. Our results indicated that as HPV16 E6 expression decreased, the proliferation indices of SiHa cells were reduced ($P<0.05$), while the total apoptosis rate increased ($P<0.05$). Conversely, as HPV16 E6 expression increased, the PI of SiHa cells increased ($P<0.05$), while the total apoptosis rate was attenuated ($P<0.05$), especially in hnRNP E1-modified cells (**Figure 6B**). Taken together, these findings indicated that hnRNP E1 may act as a tumor suppressor by downregulating HPV16 E6 expression.

DISCUSSION

In this study, we reviewed and compared hnRNP E1 expression in participants with and without HPV and explored the relationship between hnRNP E1 expression levels and cervical lesion development. Mechanistically, hnRNP E1 expression may be a significant factor affecting HPV carcinogenicity by downregulating HPV16 oncogene expression and mitigating cancerization.

In the past decade, the contribution of HPV16 persistent infection to invasive cervical cancer has been well-documented (30).

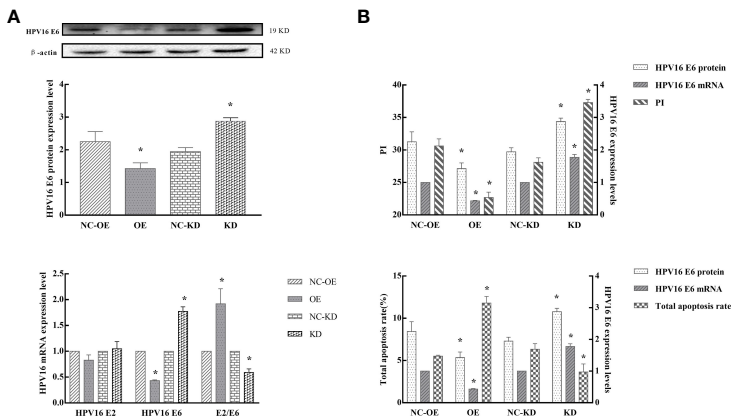

FIGURE 6 | hnRNP E1 effects on HPV16 oncogene and subsequent changes in cell biological function. Note: OE indicates hnRNP E1 overexpression group and NC-OE indicates the relative control group. KD indicates hnRNP E1 knockdown group and NC-KD indicates the relative control group. *$P < 0.05$. **(A)** The expression of HPV16 E2, and E6 were detected in SiHa cells by RT-qPCR and western blotting. **(B)** The relationship between HPV16 E6 expression, cell proliferation, and apoptosis in hnRNP E1-modified cells. The expression of HPV16 E2, and E6 were detected in SiHa cells by RT-qPCR and western blotting. The PI and total apoptosis rate were evaluated using flow cytometry analyses. As HPV16 E6 mRNA and protein expression levels decreased, the proliferation indices of SiHa cells decreased, while the total apoptosis rate increased. As HPV16 E6 expression increased, the proliferation indices of SiHa cells increased, while the total apoptosis rate decreased.

The HPV16 prevalence was 3.7% in women residing in Shanxi Province, which exceeded the national average (0.78%) in 2014 (31). We observed that HPV16 infection rates were 8.53%, 14.41%, 40.85%, and 67.50% in the NC, CIN I, CIN II/III, and SCC groups, respectively. This further corroborated that HPV16 infection is a crucial etiological factor for cervical lesions, especially CIN II/III and SCC. Therefore, it is essential to carry out HPV screening to prevent the occurrence and progression of cervical cancer. HPV16 E2 is crucial for transcriptional regulation, DNA replication, viral genome tethering, and viral DNA packaging (32). Xue et al. (33) found that E2 was highly expressed in CINI compared with that in CIN II/III through immunohistochemistry. The present study revealed that HPV16 E2 low expression was closely related to the development of cervical lesions, especially CIN II/III and SCC. HPV16 E6 is a multifunctional oncoprotein that mediates various biological events, such as promoting the degradation of p53, regulating the transcription of cell cycle-associated genes, activating telomerase, and contributing to immune response and cell communication (34, 35). Wang et al. (36) suggested that HPV16 E6 could promote the migration and invasion of cervical cancer cells. Our previous study combined with this study indicated that HPV16 E6 increases the risk of cervical cancerization (37). BPV E2 expression results in specific inhibition of HPV E6 gene expression in cells and considerable growth inhibition[8]. HPV16 DNA integration and virus E2 gene damage often occur in cervical carcinogenesis, suggesting that inactivation of the HPV E2 gene allows high expression of the E6 gene, which can promote the growth of cervical epithelial cells (38). Our study showed that the ratios of HPV16 E2 to E6 in CIN II/III and SCC groups were significantly lower than those in the NC and CIN I groups. This observation was supported by Choi et al. (39) who discovered that the mean HPV16 E2/E6 ratio decreased significantly following a linear trend from CIN II/III to SCC. The ratio of E2 to E6 may be a sign of cancer progression.

hnRNP E1 was downregulated in numerous tumors (40). Zhang et al. (14) reported that hnRNP E1 functions as a tumor suppressor in gastric cancer. The expression of hnRNP E1 and miRNA-3978 in peritoneal metastasis of gastric cancer was inhibited (15). Pillai et al. (20) discovered that hnRNP E1 expression decreased from 86% in CIN I to 68% in CIN II/III and 40% in cervical cancer. Pathak et al. (41) also found that hnRNP E1 expression decreased progressively from the normal cervix (100%) to squamous intraepithelial lesions (75%) and cervical cancer (52.6%). The present study showed that with the development of cervical lesions, hnRNP E1 expression decreased from NC to CIN and SCC. Although the survival analysis based on the TCGA database revealed a subtle prognosis value of hnRNP E1. These results indicated that low hnRNP E1 expression contributed to the risk of cervical lesions and promoted the progression of cervical cancerization. hnRNP E1 may be considered a biomarker for the early detection of cervical carcinogenesis.

To understand the binding capacity of hnRNP E1 at the genome-wide level, we conducted ChIP-seq in the untreated SiHa cell line. Our data further supported that hnRNP E1 relevant genes were associated with a series of GO terms related to cell proliferation, metabolism, and apoptosis. KEGG pathway enrichment analysis revealed that hnRNP E1 relevant genes were mainly involved in the Wnt signaling pathway, GnRH secretion, dopaminergic synapse, mTOR signaling pathway, pathways of neurodegeneration-multiple diseases, sphingolipid signaling pathway, and AMPK signaling pathway. The aberrant Wnt/β-catenin, mTOR, and MAPK signaling pathways facilitate cancer cell proliferation and differentiation (42–44). GnRH is known primarily as a neuroendocrine decapeptide that is essential for maintaining the reproductive state (45). Most notably, we found that hnRNP E1 relevant genes were enriched in HPV infection pathway, although the effect was not significant. These results suggested that hnRNP E1 may play a key role in HPV-induced cervical lesions.

To better understand the biological mechanism of hnRNP E1 in cervical lesions, we performed *in vitro* plasmid transfection. Our results demonstrated that hnRNP E1 inhibited cervical cancer cell proliferation, promoted apoptosis, and arrested the cell cycle in the G0/G1 phase. *In vitro* research has shown that the deletion of hnRNP E1 reduced the expression of p27, a key regulator of the cell cycle, and promoted carcinogenesis (46). Overexpression of hnRNP E1 reduced the expression of p53 (47) and played a vital role in the cell's biological function and DNA damage response (48). In addition, we confirmed that hnRNP E1 induced different biological changes in SiHa and C33A cells *in vitro*. The changes in biological function in SiHa cells were greater than those in C33A cells. Given the key tumor-suppressive role of hnRNP E1 in cervical cancerization, it may have great therapeutic potential for cervical cancer. hnRNP E1 can inhibit the translation of HPV16 L2 mRNA *in vitro* (22). However, it remains unclear whether hnRNP E1 can regulate the HPV16 early gene. Our results demonstrated that hnRNP E1 was positively correlated with HPV16 E2 or E2/E6 ratio and negatively correlated with HPV16 E6. In addition, *in vitro* experiments verified that hnRNP E1 overexpression diminished the expression of HPV16 E6 but improved the ratio of HPV16 E2 to E6. In contrast, hnRNP E1 knockdown significantly increased HPV16 E6 expression but attenuated the ratio of HPV16 E2 to E6. The relationship between hnRNP E1 and HPV16 E2 protein in SiHa cells was not found in this study, which may be related to the fact that during HPV integration, E2 is destroyed in SiHa and may not produce functional E2 (49). E6 is one of the earliest-expressed genes following HPV infection, containing the selective binding site for hnRNP E1 (50). The KH domain in hnRNP E1 may reduce the expression of HPV16 E6 by binding with its regulatory element. Interestingly, our results showed that with a decrease in HPV16 E6 expression, the proliferation index of SiHa cells decreased, and the total apoptosis rate increased. Conversely, with the increase in HPV16 E6 expression, the proliferation indices of SiHa cells increased while the total apoptosis rate decreased, and this effect was more significant when hnRNP E1 was modified. Our findings suggest that HPV16 E6 low expression inhibits the proliferation and promotes cervical cancer cell apoptosis, and this effect may be regulated by hnRNP E1.

Our study does, however, have several limitations. First, our study was based on a cross-sectional study, prospective cohort studies can be conducted in the future to further explore the effect of hnRNP E1 on the prognosis of cervical lesions and HPV16 gene expression. Second, we only selected two cervical cancer cell lines for *in vitro* experiments, the conclusions will be further demonstrated in a variety of cervical cancer cell lines. Lastly, the *in vivo* effect of hnRNPE1 overexpression in the xenograft of cervical cancer cell lines was unclear, future relevant animal experiments will need to be done.

In conclusion, our study indicated that abnormally low expression of hnRNP E1 could promote cervical lesion development and was closely linked with HPV16 E2, E6 expression, and the E2 to E6 ratio. hnRNP E1 suppresses tumor growth *in vitro* by inhibiting proliferation and promoting apoptosis. hnRNP E1 relevant genes were significantly enriched in cervical cancer-related pathways. Moreover, hnRNP E1 significantly downregulated the expression of HPV16 E6. Upregulating hnRNP E1, particularly incorporating the control patterns of HPV16 infection, may offer advances in the control of cervical cancer. Owing to the complexity of HPV gene expression and post-transcriptional regulation, in-depth studies are needed to understand the underlying mechanisms of hnRNP E1.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: GEO with accession GSE203023: (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE203023).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institutional review committee of Shanxi Medical University (2013–003). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JTW conceived the project. RM, LD, and ZT designed the experiments. MZ and JHW performed the experiments. MW and YL contributed reagents, materials, and analysis tools. CL, MF, and HJ integrated, analyzed, and interpreted all data. JTW contributed to the supervision of the work. LS wrote the manuscript with the assistance and final approval of all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.905900/full#supplementary-material

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Importance of the endometrial immune environment in endometrial cancer and associated therapies

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Endometrial cancer is rising in prevalence. The standard treatment modality of hysterectomy is becoming increasingly inadequate due primarily to the direct link between endometrial cancer and high BMI which increases surgical risks. This is an immunogenic cancer, with unique molecular subtypes associated with differential immune infiltration. Despite the immunogenicity of endometrial cancer, there is limited pre-clinical and clinical evidence of the function of immune cells in both the normal and cancerous endometrium. Immune checkpoint inhibitors for endometrial cancer are the most well studied type of immune therapy but these are not currently used as standard-of-care and importantly, they represent only one method of immune manipulation. There is limited evidence regarding the use of other immunotherapies as surgical adjuvants or alternatives. Levonorgestrel-loaded intra-uterine systems can also be effective for early-stage disease, but with varying success. There is currently no known reason as to what predisposes some patients to respond while others do not. As hormones can directly influence immune cell function, it is worth investigating the immune compartment in this context. This review assesses the immunological components of the endometrium and describes how the immune microenvironment changes with hormones, obesity, and in progression to malignancy. It also describes the importance of investigating novel pathways for immunotherapy.

KEYWORDS

endometrial cancer, immunotherapy, microenvironment, adiposity, levonorgestrel

Introduction

Endometrial cancer (EC), or cancer originating in the uterine epithelium, is the most prevalent gynecological cancer in the developed world (1, 2). Rates of EC are rising, and prevalence is increasing in younger people. This is due, in part, to the rise in obesity; increased adiposity raises estrogen production and adipokine release which results in an oncogenic signal, stimulating endometrial cell proliferation (3). The predominant treatment for EC is hysterectomy which sometimes includes bilateral salpingo oophorectomy and pelvic lymph node dissection. Late-stage disease can also receive adjuvant chemotherapy or radiation. While this treatment pathway is effective, hysterectomy is an invasive procedure for early-stage disease which removes the fertility of the patient and is high-risk for those with a high body mass index (BMI). The use of the levonorgestrel-loaded intra-uterine system (LNG-IUS) circumvents these issues in patients who respond, however, response rates to LNG-IUS treatment for EC are as low as 40% (4). In EC, as with many cancers, the tumor microenvironment plays a significant role in cancer progression and response to therapy. This includes both the interaction between the tumor and stroma and the interaction between the tumor and infiltrating immune cells. Immune cells in the normal endometrium play important roles in protection from external pathogens, aiding fertilization, and tolerance and maintenance of pregnancy. Significant infiltration of immune cells characterize certain subtypes of EC, suggesting that immunotherapies may be effective as therapeutic alternatives or adjuvants to surgery in a subgroup of patients. As such, the literature examining the immunological tumor microenvironment (iTME) of EC has focused on the potential for specific EC subtypes to respond to immunotherapy. Within this literature, emphasis has been placed on examining immune checkpoint inhibitors (ICI). However, ICI success is variable and most ICIs for treatment of EC are still in clinical trial stage. A summary of this literature has been recently reviewed by Cao et al. (5). Investigating alternative methods of immunomodulation could increase the scope of therapies available, making personalized treatment for EC more feasible. A more holistic understanding of the composition of EC-infiltrating immune cells and their function within the iTME is warranted and could provide evidential support for the use of other types of immunotherapies in this context. Here, we aim to review the evidence describing the iTME of EC and how this may be influenced by increasing adiposity, discuss the successes and pitfalls of immunotherapies for EC treatment, and provide recommendations to fill the knowledge gaps that exist within this body of literature.

Endometrial cancer classification

The pathogenesis of EC is the over-proliferation of endometrial glands resulting in an abnormal gland-to-stroma ratio (6). Currently, diagnosis and classification of EC is based largely on the histological phenotype of the tumor cells biopsied by pipelle or curettage and, following surgical intervention, on the primary tumor. Histological subtypes include endometrioid, clear cell, serous, and mucinous. Endometrioid endometrial carcinoma (EEC) is the most common subtype, accounting for between 75 – 90% of cases (7, 8). EEC is strongly associated with prolonged estrogen exposure and has the best prognosis due to its often early presentation with abnormal bleeding (9). Serous carcinomas contribute to approximately 10% of EC cases (10), whereas clear cell and mucinous endometrial carcinomas are rare, collectively contributing to less than 5% (9).

In addition to histological grading, The Cancer Genome Atlas (TCGA) categorized EC into four prognostically distinct molecular subtypes using whole genome sequencing, irrespective of histology (11). These are: microsatellite instability high (MSI-H), DNA polymerase ϵ (*POLE*) mutated, copy number low, and copy number high (11). *POLE* mutated tumors have the highest progression-free survival rates, while copy number low and MSI-H tumors are intermediate risk and copy number high tumors have the poorest prognosis (11). Diagnostic testing must be resource, time and cost-effective, and whole genome sequencing does not fall into these parameters. To combat this, Talhouk et al. extrapolated TCGA classifications into groups that are defined using a combination of immunohistochemistry (IHC) markers and targeted DNA sequencing, creating a 'proactive molecular risk classifier for endometrial cancer', or ProMisE (12). The four molecular subtypes thus became: mismatch repair deficient (MMRd), *POLE* exonuclease domain mutant (*POLE*mut), p53 wild type/nonspecific molecular profile (NSMP), and p53 abnormal (p53abn). Since their conception, validation and confirmation of the ProMisE molecular subtypes has been conducted to identify whether these subtypes have different therapeutic outcomes. This topic has been recently comprehensively reviewed by Mitric and Bernardini (13). For example, the PORTEC3 clinical trial investigated the benefit of adjuvant combined chemotherapy and radiotherapy compared to chemotherapy alone in patients with high grade and/or stage endometrial cancer (14). Subsequent analysis separated trial participants into molecular subtype based on the ProMisE guidelines (12) and found a significant benefit for patients with p53abn tumors receiving combined chemoradiotherapy compared to chemotherapy alone ($P = 0.019$) (15). Furthermore, those with *POLE*mut EC had an excellent recurrence free survival regardless of treatment (15).

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Immunity in the normal endometrium

To better understand the immunological characteristics of EC, it is important to contextualize the role of immune cells within the homeostatic immune interactions of the normal endometrium. The healthy endometrial immune environment is characterized by cyclic shifts in immune cell proportions and functionality due to hormonal changes throughout the menstrual cycle and its direct exposure to environmental pathogens and the uterine microbiota. Local immune cells protect against pathogens and support endometrial remodeling during menstruation, conception, and pregnancy (16). Reproductive hormones are likely to be the dominant factor driving immunoregulation in the endometrium, as these hormones generally induce immunological suppression to facilitate conception. This idea has been reviewed extensively elsewhere (17). In non-immune cells, the estrogen receptor (ER) and progesterone receptor (PR) are intracellularly located and function as transcription factors. While expression of ER by immune subsets is widely accepted (18, 19), the exact mechanism of progesterone immunomodulation is not fully understood. Early studies of endometrial-derived immune cells show no overlap of CD45 and PR expression (20–22), and RNA sequencing has found no detectable nuclear PR expression in T cells (23). Notwithstanding, progesterone demonstrably influences T cell function, observed by a decrease in T cell expression of the inflammatory cytokines interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α), and an increase in expression of the regulatory cytokine IL-4 (23). Progesterone also hinders T cell proliferation and activation (24). T cell-intrinsic expression of PR therefore remains the prevailing paradigm (25–28). Alternative mechanisms of progesterone signaling include membrane progesterone receptors, indirect signaling *via* stromal cells, and signaling *via* the glucocorticoid receptor (23, 29–31).

Progesterone, which begins to increase following ovulation (Figure 1), exerts immunosuppressive activity through intrinsic and extrinsic mechanisms to regulate immune cell function and trafficking, respectively (24, 32). For example, the release of the cytolytic molecule, perforin, from $CD56^+$ cells is inhibited by progesterone (32). Additionally, the declining levels of progesterone towards the late-secretory phase triggers a pro-inflammatory signaling cascade which leads to macrophage and neutrophil recruitment and the release of degradative enzymes required for menstruation. The resulting sterile inflammation and tissue remodeling processes, triggered during the decline of progesterone, are tightly regulated to promote scarless healing (33, 34). The environment then switches from pro- to anti-inflammatory as the cycle continues (35–37). There is also evidence suggesting that the uterine microbiota may play a role in immune regulation (38).

Pre-menopause, the proportion of immune cells within the endometrium fluctuates with the menstrual cycle, which has led to variable reports of immune cell distribution. The proportion of T cells is higher in the proliferative, compared to the secretory, phase (39). This is likely due to a large increase in NK cell numbers (40) rather than a decrease in T cell numbers, as absolute T cell counts are stable throughout the menstrual cycle (16, 41). Post-menopausal uteri have similar T cell proportions to those in the proliferative phase but contain a higher proportion of granulocytes (39), potentially as a result of endometrial atrophy characteristic of menopausal uteri. T cells are the dominant immune cell subset throughout the female reproductive tract, including the endometrium, where they represent 1-5% of all endometrial tissue cells (39, 42) and 40-80% of $CD45^+$ immune cells, depending on menstrual cycle stage (39, 43) (Figure 2). In addition to residing within the epithelium and being scattered amongst stromal cells, endometrial immune cells form lymphoid aggregates (LAs).

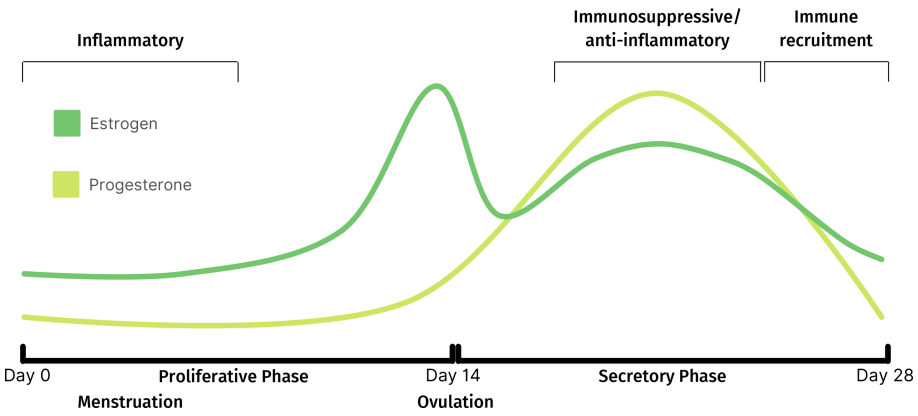

FIGURE 1
Fluctuations of estrogen and progesterone throughout normal menstrual cycle and corresponding immune activity.

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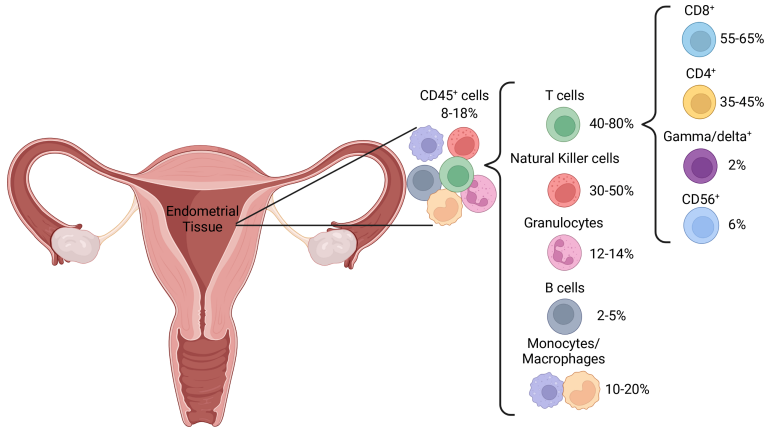

FIGURE 2
Distribution of immune cell populations in normal endometrial tissue.

LAs are comprised of a B cell core surrounded by $CD8^+$ T cells, which are themselves surrounded by macrophages and some NK cells (44). LAs begin to develop during the end of the proliferative phase, becoming larger during the secretory phase, and are absent post-menopause (45). While the existence of LAs is well established in early research (44–47), recent research confirming their existence is lacking, and their function remains unclear. The majority of endometrial T cells express the co-receptor CD8, which is likely due to the prominence of $CD8^+$ T cells within LAs (44, 48, 49). However, the effector functions of endometrial $CD8^+$ T cells may extend beyond their traditional function of cytotoxicity, as reflected by the functional plasticity displayed by decidual $CD8^+$ T cells during pregnancy (50). Moreover, $CD3^+$ T cells obtained from the endometrium during the secretory phase exhibit limited cytotoxic capacity compared to those obtained during the proliferative phase (51). This suggests that the LAs, which develop during the secretory phase, play a regulatory role that coincides with potential blastocyst implantation. It has also been postulated that the presence of LAs in the basalis stroma, an inner portion of the endometrium that is not shed during menstruation, is a means of maintaining immune presence during menstruation (52). This would allow the immune system to quickly re-infiltrate the regenerating endometrium post-menstruation.

Endometrial T cells also include a significant proportion of $CD4^+$ T cells, at approximately 40% of T cells (40). $CD4^+$ T cells, also known as helper T cells, play an integral role in the immune response by activating other effector cells such as macrophages, $CD8^+$ T cells and B cells (53). The function of endometrial $CD4^+$ T cells is less well established than their $CD8^+$ counterparts, however the balance of the $CD4^+$ T cell subsets Th1/Th2/Th17/Treg appears to be important in maintaining pregnancy (54), and this balance must be present prior to conception (55). For example, the pre-pregnant and pregnant endometrium is more inclined towards a Th1 phenotype than a Th2 phenotype (55), and a decrease in the proportion of Th1 $CD4^+$ T cells in the endometrium could lead to recurrent miscarriage (56). Also present in the endometrium are $\gamma\delta^+$ and $CD3^+CD56^+$ T cells (40), referred to as innate-like T cells due to their non-specific nature and functional similarity to components of the innate immune system (57). These cells may provide additional regulatory tone. Additionally, decidual $CD3^-CD56^+$ NK cells express high levels of CD56 (58). There is a significant increase in $CD56^{bright}$ cells during the periovulatory period, which is thought to occur through NK cell trafficking from peripheral lymphoid tissues, as well as proliferation in the uterine mucosa (59, 60). As $CD56^{bright}$ NK cells are believed to represent the immature and regulatory precursors of their cytotoxic $CD56^{dim}$ counterparts (61), it is likely that the endometrial NK cell compartment favors cytokine production and regulatory functions over cytotoxicity. Increasing levels of estrogen and luteinizing hormone prior to ovulation increases adhesion of natural killer (NK) cells in the uterus (59). This is also thought to be a necessary precursor to successful pregnancy, as these decidual NK cells play important roles in tissue remodeling, embryonic development, trophoblast invasion and placentation, and are seldom cytotoxic unless primed by pathogens (62).

The phagocytic cells of the innate immune system – granulocytes, monocytes and macrophages, collectively make up 25% of the $CD45^+$ endometrial immune milieu, whereas B cells are comparably rare regardless of hormonal cycle stage, at no more than 5% (17, 39, 43, 63). Endometrial B cells exist primarily within LAs, but have also been detected in the stroma (45, 64). The function of B cells within the endometrium is not well established, as was concluded in a systematic review by Shen et al. (63). However, it is possible that the B cells within LAs function as well-placed antigen presenting cells to the

surrounding T cells, as they express activation markers such as CD69, HLA-DR and CD83 to a higher degree than their peripherally derived counterparts (65). Over 70% of $CD68^+$ endometrial macrophages are alternatively activated, identified by co-expression of CD163 (66). Alternatively activated macrophages, otherwise known as M2 macrophages, traditionally express anti-inflammatory cytokines such as interleukin (IL)-10 and participate in wound healing and tissue re-modeling (67). Major histocompatibility complex (MHC)-II, CD80 and CD86 expression on endometrial macrophages is low (66). As these proteins are involved in antigen presentation to $CD4^+$ T cells, it is possible that endometrial macrophages have limited capacity to stimulate a $CD4^+$ T cell response.

Collectively, our current understanding suggests that the healthy endometrium is biased towards a regulatory immune environment, while retaining the capacity to rapidly switch to traditional immune defense mechanisms due to the presence of classical type 1 effector cells. However, the majority of research on the immunological state of the normal, non-pregnant endometrium was conducted over 20 years ago, leaving room for confirmation of these findings with present-day technologies.

Immunity in the cancerous endometrium

The relevance of the iTME for EC diagnosis and treatment is highlighted by the fact that EC molecular subtypes differ both in their tumor-infiltrating immune cell density and prognosis. $CD8^+$ T cell infiltrates generally indicate a good prognosis (68–70), and advanced stage ECs have lower T cell density (71). The working hypothesis to support this finding is that tumors with fewer somatic mutations produce lower levels of immunogenic antigens. As such, these tumors can avoid detection by cytotoxic T cells and are more likely to advance to a late stage (72). Indeed, copy number low EC tumors have the second highest mortality rate (73). The correlations between molecular subtype and tumor-infiltrating immune cell abundance and phenotype have been investigated using IHC. A high proportion of $CD8^+$ T cells in *POLE*mut and MMRd tumors was discovered, with most expressing the immunological checkpoint molecule, programmed cell death protein 1 (PD-1) (74). The binding of the PD-1 ligand, PD-L1, to PD-1 restricts T cell function, and is a key mechanism of immune tolerance in cancer (75). As such, high PD-1 expression by tumor-infiltrating T cells is a target for PD-1 inhibition by immune checkpoint inhibitors. Effective inhibition of PD-1 prevents the binding of PD-L1, ameliorating immune tolerance by the PD-1/PD-L1 pathway and elevating T cell efficacy (76). The PD-1/PD-L1 pathway is discussed in more detail in the section on targeting the immune microenvironment further on in this review.

The abundance of tumor-infiltrating immune cells in *POLE*mut and MMRd subtypes has been validated by multiple studies (74, 77, 78) and is likely to contribute to the comparatively high survival rates of these subtypes as well as their clinical responsiveness to immunotherapy. Indeed, MMRd tumors had significantly higher $CD3^+$ cells expressing both PD-1 and PD-L1 compared to both NSMP and p53abn subtypes (79). While the advantage of PD-1 inhibition in high PD-1 expressing T cells is clear, the advantage of PD-L1 expression on T cells is more enigmatic. Recent evidence has elucidated a bi-directional role of PD-L1 expressed by tumor-associated T cells whereby it can act as both ligand and receptor. This gives T cells the ability to induce immunosuppressive phenotypes in other immune infiltrates such as macrophages, while also repressing its own differentiation into an effective anti-tumor cell (80). Furthermore, the tumor itself can induce T cell PD-L1 expression to enhance immune tolerance (80).

Tumor-infiltrating immune cells have been implicated as prognostic indicators in a range of cancers including melanoma, endometrial, ovarian, and colorectal cancer (81–83), and are positively associated with EC disease severity (77). The prognostic ability of an increase in particular immune subsets is less clear. For example, the number of regulatory T-cells (Treg) is both positively and negatively associated with overall survival in NSMP (84) and p53abn EC (77), respectively, suggesting a potential molecular subtype-specific role for these T cells. In parallel to significant T cell infiltration, EC is also characterized by an influx of $CD3^-CD56^+$ NK cells in tumor tissue compared to non-tumor tissue (85). Tumor-resident NK cells, as identified by the expression of CD103, have attenuated functionality as they express the inhibitory molecules TIGIT (T cell immunoreceptor with Ig and ITIM domains) and TIM-3 (T cell immunoglobulin and mucin-domain contaoining-3) (85). It is not known whether inhibitory signals from the tumor are causing this effect, or whether the cells are exhausted by other means, but it is likely that the result is immune tolerance of the cancer.

Tumor associated macrophages (TAMs) are a diverse subgroup of tumor-infiltrating immune cells derived from monocytes with traditionally cytotoxic and phagocytic attributes (86), but have also been implicated in cancer tolerance (87). It is therefore important to distinguish cancer tolerant TAMs from cancer intolerant TAMs to determine their prognostic significance. However, doing so is not simple. TAMs are traditionally defined as either M1 or M2, which represent the extremes of macrophages functional state – pro-inflammatory and regulatory, respectively. Importantly, these states are not mutually exclusive and as such this dichotomous nomenclature paradigm is beginning to shift to include additional (sub) categories of macrophage activation states (88). In EC, increased total TAM count, identified by positive IHC staining for CD68, was observed in both tumor and stromal tissues collected from EC patients compared to controls of benign

pathology (89). However, there was no significant correlation between TAM density and cancer progression, so it remains unclear whether measuring TAM density in EC has any clinical or prognostic implications. Further investigation using IHC identified an increasing density of macrophages in the stromal compartment of EC as the severity of the disease increased, with fewer macrophages in the EC precursor endometrial hyperplasia, and the highest density observed in non-endometrioid EC, which tend to be more aggressive than EEC (90). However, the density of TAMs expressing CD163, a marker of M2 macrophages, was similar across EC histologies (90), suggesting that TAM infiltration in more aggressive EC tumors is predominantly comprised of the pro-inflammatory M1-like subtype. Conversely, co-culture of EC cell line-derived exosomes with a monocyte cell line can induce an M2-like macrophage phenotype (91), suggesting that tumor cells can polarize macrophages towards immune tolerance. Taken together, these data provide some evidence of the state and function of TAMs in EC but further investigation is warranted.

EC does not seem to be heavily infiltrated by B cells (74), but their role in EC disease progression warrants further investigation in light of recently described antigen-independent mechanisms (92). Specifically, the binding of dimeric IgA, but not monomeric IgG, to the polymeric immunoglobulin receptor (pIgR) initiates cell-intrinsic inflammatory, endoplasmic reticulum stress and pro-apoptotic pathways, thereby leading to improved patient survival. Albeit antigen-independent, this mechanism is of broad relevance to EC, and pIgR is quasi-universally expressed in EC cells. Additionally, tertiary lymphoid structures (TLS) are a major source of B cells in EC (93). TLS are similar to LAs in that they are comprised of a B cell core surrounded by T cells. They may play an important role in EC protection, as their absence is related to more progressive disease (93).

In summary, the iTME of EC is characterized by the infiltration of innate and adaptive immune cell subsets with anti-tumoral activity, and the molecular subtype of the cancer affects the immune infiltration. However, the literature is skewed towards describing the role of T cells in this context. Since the cytotoxic capacity of tumor-resident $CD8^+$ cells is actively restricted by the iTME (94), the quantification of T cell subset or NK cell density is only meaningful if paired with established functional markers such as PD-1, TIM-3 or CD163. Furthermore, alongside the quantification of effector cells, it is important to consider the influence of regulatory immune cell subsets such as Tregs (95) and myeloid derived suppressor cells (96) on effector cell function. Pairing functional markers and regulatory immune subsets with immune cell density would give a more accurate picture of the interactions within the iTME, and thus would allow researchers to develop tools that work specifically to reduce the capacity of those that are advantageous to cancer progression.

Hormone therapy in endometrial cancer

Endometrial cancer is commonly described as a hormone-driven cancer. This refers primarily to the most common subtype, EEC. As mentioned previously, prolonged exposure to high levels of bioavailable estrogen is the main driver of this histological subtype. The natural antagonist of estrogen is progesterone. This has prompted the use of levonorgestrel (LNG), a synthetic progestogen, as a novel treatment for early-stage EEC. While LNG administration is hormonal therapy, it is important to consider the immunological side-effects of such treatment, whether beneficial or detrimental. LNG activates the PR, binding with three times more affinity than natural progesterone (97). As discussed previously, the presence of PR in immune subsets is yet to be unequivocally determined. It is therefore important to note that LNG has an over 40-fold higher affinity to PR as compared to the glucocorticoid receptor (97) and would thus work more effectively on PR-expressing cells. The antagonistic effect of progesterone is also observed in immune cells, with progesterone-treated cells exhibiting an attenuated phenotype (23, 24). For example, progesterone causes the differentiation of $CD4^+$ T cells towards a Th2 profile (23), which is generally regarded as anti-inflammatory. This mechanism is thought to be a major driver of pregnancy tolerance, as progesterone increases substantially during early pregnancy. To support this notion, progesterone can also dose-dependently reduce the activation status of human peripheral $CD4^+$ T cells (24).

It is plausible that, alongside antagonizing estrogen, the use of LNG on early-stage EEC is dampening the immune response to EC. The downstream effects of this should be investigated as LNG treatment becomes more widely established. There is little information on the effect of LNG on endometrial immune cell populations. It appears that LNG has an immunoregulatory effect, with increased IL-10 expression by $CD4^+$ and $CD8^+$ T cells post-stimulation and significantly more endometrium-resident regulatory T cells in healthy LNG-IUS users compared to controls on no contraception (98). LNG also appears to reduce immunological surveillance, with fewer $CD4^+$ and $CD8^+$ endometrial T cells in LNG-IUS users compared to controls (98). Conversely, $CD4^+$ and $CD8^+$ T cells were more likely to express the activation markers CD38 and HLA-DR (98) indicating that, despite reduced numbers, the T cell compartment is in an increased state of activation with LNG-IUS use. While the argument has been made that the presence of a foreign body in the uterus results in a local inflammatory response (99), whether the observed increase of these activation markers is caused by a foreign body reaction or the LNG itself is not yet known. One study demonstrated that the endometrial transcriptome from LNG-IUS users exhibited more inflammatory markers and immune activation than those using the copper intra-uterine device, which was indistinguishable from non-

IUS users (100). This would support the hypothesis that LNG regulates the endometrial immune compartment beyond a foreign body reaction. It must also be stated that, collectively, the studies discussed in this section up to this point investigate the effects of progesterone and LNG in the normal endometrial immune microenvironment. There is scope to explore whether these findings hold true of progesterone and LNG treatment within the iTME of EC.

Obesity, immunology and endometrial cancer

The link between obesity and EC is well established. Obesity, defined by the World Health Organization (WHO) as a BMI > 30 (101), is the leading modifiable risk factor for EC. The rise in EC incidence has been directly related to the obesity epidemic (102). Between 40% to 60% of EC incidence in the United States and the United Kingdom has been ascribed to excess weight (103, 104). All measures of increased adiposity (waist-to-hip ratio, hip and waist circumference, weight gain and high BMI) increase the relative risk of EC development (105). Moreover, there is a positive association between increasing BMI and EC mortality, with a hazard's ratio of 1.43 (confidence interval 1.26-1.61) per 5 kg/m^2 increase in BMI (106). Bariatric surgery and the subsequent weight loss associated with it has been shown to reduce the relative risk of developing EC (107), which highlights the interconnectedness of increased body mass with this hormone-sensitive cancer. One of the molecular mechanisms explaining this relationship is the production of aromatase by adipocytes, which is an enzyme that cleaves androgens into estrogens (108). Increasing adiposity contributes more aromatase, and consequently more estrogen, to the endometrial environment, to directly promote endometrial cell proliferation. Obesity also reduces the amount of hormone-binding globulin, a carrier molecule which reduces the activity of estrogen molecules (108). These combined molecular processes result in elevated levels of bioavailable estrogen. Obesity also contributes to an increase in other EC risk factors including anovulation and polycystic ovarian syndrome, culminating in a higher risk profile for EC.

As obesity is associated with chronic inflammation (109), a recent study assessed the link between inflammation and weight loss on the endometrial iTME in participants classed as high-risk for EC due to having a BMI > 40 kg/m^2. Participants received either bariatric surgery or a low-calorie diet to support weight loss. Blood and endometrial biopsies were taken to assess a range of immune markers using IHC and tissue imaging including CD68 (a pan-macrophage marker), CD56 (an NK cell marker), CD3 (a pan-T cell marker), CD8 (a cytotoxic CD8$^+$ T cell marker), FOXP3 (a transcription factor of Tregs), and PD-1 (110). The authors found that weight and BMI were inversely correlated to CD8$^+$ T cell infiltration but found no significant difference in any other immune subsets examined. This relationship between BMI and reduced CD8$^+$ T cell infiltration in EC has been corroborated by another recent study (3). Thus, obesity specifically reduces endometrial immune surveillance by CD8$^+$ cytotoxic T cells. This contrasts with what has been observed in peripheral blood of obese but otherwise healthy women, where CD8$^+$ cell count was higher in obese compared to non-obese healthy women. Women with increasing BMI also had higher white blood cell counts in general than those in the normal index range (111). These findings suggest that increasing adiposity can reduce CD8$^+$ T cell trafficking and tumor infiltration. Furthermore, CD8$^+$ T cells from the tumor of obese MC38 mice produce less IFN-γ than non-obese mice (3). IFN-γ is a major anti-tumoral effector cytokine, thus demonstrating CD8$^+$ T cell reduced functionality. Importantly, immune suppression and reduced tumor infiltration can be reversed with weight loss. In one case study, a patient with EC receiving an LNG-IUS underwent vertical sleeve gastrectomy for weight loss. Endometrial biopsies were analysed after 6 months. The patient lost 26 kg which correlated with an increase in CD3$^+$ and CD8$^+$ T cell tumor infiltration (3).

NK cells are known to be impaired in obese individuals, with fewer circulating and resident NK cells present with reduced anti-tumor functionality (112–114). Weight loss appears to ameliorate the effect (115). This evidence is in keeping with our current understanding of T cell behavior and distribution within the tumor. It is therefore likely that NK cells are similarly affected by increasing adiposity and could be driven by a change in tumor metabolism to increase consumption of fatty acids, which can have immunoregulatory effects (116). A conflicting study found no significant difference in peripheral NK cell number or cytotoxic capacity in lean compared to overweight or obese participants of similar age (117). This study opposes the current knowledge on NK cells and obesity and, as such, warrants further investigation.

Interestingly, increased BMI was found to positively correlate with the effectiveness of immunotherapy using pembrolizumab, an anti-PD-1 monoclonal antibody, in 36 PD-L1$^+$ gynaecologic cancers including MSI high EC (118). PD-L1 positivity is an important control in this study as it means that BMI is the variable, not PD-L1 expression, making the data on increased BMI and efficacy of pembrolizumab more reliable. This data suggests that, although high BMI appears to suppress cytotoxic T cell capacity, immunotherapy may more effectively ameliorate the immunosuppressive iTME in individuals with higher BMI by overcoming adiposity-related immunosuppression. The mechanistic origin of the association between immunotherapeutic response and high BMI remains to be explored. However, it is intriguing to consider that obesity-associated intestinal hyperpermeability may prime immune effector functions by mediating the anticancer activity of ICIs through the circulation of bacterial metabolites, similarly to chemotherapy (119, 120). In

summary, the immunosuppressive effect of obesity in EC could be a driver of immune-resistance in this highly obesity-associated cancer. Weight loss can reverse these effects and so should be incorporated into awareness campaigns and clinical management of EC. Increased BMI may be related to effective immunotherapy in this context, so further investigation is warranted here.

Targeting the immune environment in endometrial cancer: Beyond checkpoint inhibition

Cancer immunotherapies involve targeting the immune system to drive a specific immunological function. This leads to greater outcomes in patients whose therapy can be tailored to their needs (121). Personalized medicine in the form of immunotherapies are gaining traction as adjuvant treatments for EC. These immunotherapies work best in *POLE*mut and MMRd tumor profiles due to their high mutation burden and associated increase in the production of immunogenic antigens (122). Despite this, the only immunotherapies currently FDA-approved are pembrolizumab and dostarlimab (both PD-1 inhibitors) for patients with MMRd and a pembrolizumab/lenvatinib (a multitargeted tyrosine kinase inhibitor) combination as a second-line therapy for MMR proficient patients (123). Immunotherapies for EC and the possibility for immune phenotypes to be used as prognostic indicators have been reviewed extensively elsewhere (79, 124–127). Here, we focus on the important role of the iTME for the therapeutic success of immunotherapies.

'Immunotherapies' technically include any therapy which targets the immune system to achieve a beneficial therapeutic outcome, but are mostly restricted to cancer vaccines, ICI therapies or passive infusion of cancer-specific T cells (124). Immune checkpoint inhibition, particularly the inhibition of the PD-1/PD-L1 (128) and cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) pathways (129), together with TIM-3 pathway (130), have received great attention in the last decade and have put a spotlight on the immune system for treatment of cancer. These pathways, when engaged by ligands expressed by the tumor, provide inhibitory signals to the associated T cells with the exception of the engagement of TIM-3, which induces T cell apoptosis (129). The therapeutic success of ICIs depends on neoantigen immunogenicity and the presence of neoantigen-specific T cells, which are highly variable across patient populations. This limits the therapeutic efficacy against EC and is reflected by an overall response rate of only 13% in confirmed PD-L1 positive, albeit mostly non-MSI-H status, EC tumors with pembrolizumab treatment (131). Based on this, non-MSI-H tumors may be intrinsically less responsive to ICI than MSI-H tumors regardless of their PD-L1 expression pattern. Mechanistically, although PD-L1 expression might be high, which would indicate that the tumor is expressing immunosuppressive signals, T cell PD-1 expression may be low which would reduce the efficacy of the therapy. This is in line with the theory that immunotherapy works best in tumors with high mutational burden such as MSI-H, a term which can be used synonymously with MMRd. Indeed, a recent clinical trial in confirmed MMRd stage two or three rectal cancer patients found a 100% response rate after 6 months of treatment with another PD-1 inhibitor, dostarlimab (132). Although this study had a small sample size of just 12 patients and additional follow-up is needed to determine response in the remaining 4 patients who have not completed treatment, and to assess recurrence and response duration in those that do respond, this result is nevertheless a powerful indicator of how molecular subtype can be used to inform personalized treatment. Interestingly, recent *in silico* analyses unexpectedly demonstrated that MSI-H and non-MSI-H EC tumors are similarly infiltrated by immune cells (133), suggesting important functionality differences in the iTME between these EC subtypes. Highlighted here is the importance of assessing multiple parameters when implementing personalized treatment pathways, such as PD-1/PD-L1 positivity, MSI status and infiltrating immune cell phenotype and function.

MHC class I loss has been proposed as a possible mechanism of resistance to PD-1 inhibition (134) since this loss prevents neoantigen recognition by neoantigen-specific $CD8^+$ T cells irrespective of PD-1 expression. MHC downregulation has indeed been documented in 42% of MMRd tumors (134). Therapeutic resistance to PD-1/PD-L1 ICI may also originate from the heterogenous expression of PD-1 in the iTME, and may be potentially overcome by targeting other inhibitory molecules such as TIM-3 as an addition or alternative to PD-1 targeting (135). Therefore, further categorisation of the specific deficiency of the MMRd tumor may be necessary to facilitate optimal treatment. TIM-3 expression is differentially expressed in immune infiltrates of different EC subtypes, with preferential expression in MMRd as compared to MMR intact tumors (136). However, more work is required to delineate the respective contributions of tumor versus immune cell specific TIM-3 expression and their relevance for TIM-3 targeted ICI. As TIM-3 expression on cancer cells predicts response to PD-1 targeted ICI in other solid cancers (137), the consistent expression of TIM-3 on both MMRd and MMR intact tumor cells (136) suggests that ICI approaches combining PD-1 and TIM-3 (138) may provide therapeutic benefit in EC irrespective of subtype or mutational burden. Additionally, the inhibition of CD47, a ligand for the macrophage-associated signal-regulatory protein α (SIRPα) has been shown to increase phagocytosis of EC tumor cells by TAMs (139), although CD47- SIRPα pathway inhibition has not yet been considered for clinical trial.

Importantly, an integrative approach to immunotherapy against EC requires the consideration of hormonal therapy and its strong influence on the iTME. As a number of conventional

cancer treatment modalities such as radiotherapy and chemotherapy have been described to indirectly harness the immune system (120, 140), it is conceivable that LNG treatment may also play a significant role in future approaches to EC immunotherapy. Comparably, androgen deprivation therapy has been used as an immunotherapy in prostate cancer, whereby blocking the androgen receptor ameliorates T cell function by increasing $CD8^+$ T cell expression of the pro-inflammatory cytokine, IFN-γ (141). We have described LNG's intrinsic immunosuppressive activity in a previous section, suggesting that treatment with LNG may reduce the capacity of the iTME to control cancer growth. However, such effect could be partly counteracted by LNG-mediated early EC cell death by starving EC of bioavailable estrogen, which enhances cancer-associated antigen presentation and immune responses, a mechanism previously described for radiotherapy (142).

Discussion

Due to the inconsistent classification system of EC, the work examined in this review largely uses either histological or molecular subtype, but not both. As it is impossible to infer a histological subtype based on a molecular subtype and vice versa, interpreting the role of infiltrating immune cell proportion and phenotype within EC subtypes across studies is difficult. Future research, both within the scope of understanding the iTME of EC and beyond, would benefit from incorporating both histological and molecular classifications. Although this is a more costly exercise, it may facilitate discoveries and is likely to be pivotal for personalizing treatment. It would be futile to investigate the iTME in EC and not comment on EC subtype, particularly molecular subtype, which can directly influence the iTME. Additionally, many studies are retrospective in design, utilizing publicly available TCGA data. Prospective studies would allow for more versatile experimental conditions, such as how therapeutics influence the immunologic phenotype and affect EC pathogenesis. Current literature is overwhelmingly skewed towards the immune profiles of MMRd and *POLE*mut tumors, however we understand very little about the iTME of the other molecular subtypes which may not be classed as immunogenic but could benefit from immunotherapy. Additionally, there is conflicting evidence regarding the clinical relevance of immune infiltration in patients with high versus low BMI, as well as the mechanism of the observed superior response to immunotherapy in high BMI patients. Trialing methods of immunotherapy other than the T cell PD-1/PD-L1 pathway, such as TIM-3 or the macrophage-associated CD47-SIRPα pathway, could expand the clinical arsenal of immunotherapy. Macrophages constitute up to 20% of $CD45^+$ cells in the benign endometrium and, although there is little information on their presence in EC, it is feasible that their phagocytic capabilities could be harnessed for antitumor response.

Additionally, there is a large body of work from the 20^{th} century regarding the immunogenic state of non-malignant endometrium that needs to be verified using present day technologies. We are yet to unequivocally determine the expression of PR on immune cells and are therefore unable to investigate the specific molecular pathways induced by progesterone, and its derivatives, in these cells. LNG is currently being used as a contraceptive and therapeutic for early-stage EEC, yet its effect on the endometrial immune landscape remains largely unexplored. The little evidence we have suggests that LNG, although effective in some cases at inhibiting EC growth by restricting responsiveness of tumor to estrogen, may contribute to an immunosuppressive state. It is crucial that the effect of LNG on endometrial-resident immune cell subsets be explored further to ensure that any detrimental effects on the immune landscape are included in the risk assessment of the treatment when used as either a contraceptive or cancer therapeutic.

Immune cell count and proportion have both been investigated as prognostic markers for EC, but evidence is needed to identify which proportions of immune cells confer prognostic advantages or disadvantages in each EC subtype before these measurements can be of clinical benefit. A comprehensive review of studies examining the tumor infiltrating immune cells of EC and clinical outcome would consolidate the research and provide a framework for new research opportunities. Furthermore, literature investigating infiltrating immune cells in EC is predominantly restricted to the role of T cells. Although some have identified a role for NK cells, tumor associated macrophages (TAMs) and B cells, further research into the participation of non-T-cell immune cells is warranted. NK cells are well known for their cytotoxic capacity and are a major part of the iTME. Their importance, as well as the role of other immune subsets, should not be overlooked. While evidence suggests a major role of $CD8^+$ cytotoxic T cells in adiposity-driven immune suppression in EC, there is controversy regarding the role of NK cells in this context. Additionally, although we understand the role of obesity in driving immune suppression in non-EC participants, the role of immunity in obesity-related EC pathogenesis is yet to be explored.

The immune landscape of endometrial cancer remains incompletely understood. In the non-cancerous endometrium, immune cells are under the influence of cyclic hormonal regulation. At this time, the dominating immune phenotype appears to be regulatory. When the endometrium becomes malignant, many immunological changes are induced which tend to vary based on the molecular classification of the cancer. As such, molecular classification of EC should be incorporated into standard-of-care practices, as well as future research. Furthermore, EC is increasingly associated with obesity, the combination of which is typified by fewer tumor infiltrating cytotoxic T cells with limited cytotoxic capacity. Importantly, this can be reversed with weight loss. Novel treatments for EC such as LNG-IUS and ICIs have (potentially opposing) direct

impacts on the iTME and gaining a deeper understanding of the immunological mechanisms of these treatments could lead to personalized, novel treatments and treatment regimens. Progesterone has immunosuppressive effects. Hormonal treatment of inoperable early-stage EEC using LNG-IUS can be effective in preventing EC proliferation, but its immunosuppressive effects could contribute to the low response rates observed for this therapy. Immunotherapies for adjuvant treatment of EC are still in their infancy and show promise for molecular subtypes characterized by high immune infiltration, but their use does not necessarily need to be restricted to such subtypes. A focus on characterizing the response to immunotherapies across the spectrum of subtypes is likely to yield important insight into their effectiveness in previously understudied subtypes. We recommend exploring novel mechanisms of immune activation and exhaustion, for example investigating immunotherapies targeting macrophages so as not to limit our capacity for immune manipulation to the PD-1/PD-L1 pathway.

Author contributions

HV wrote the first draft of the manuscript. KH, MC, OG, and CH edited sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Incidence of pelvic high-grade serous carcinoma after isolated STIC diagnosis: A systematic review of the literature

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Objective: Serous tubal intraepithelial carcinoma (STIC) is a precursor lesion of pelvic high-grade serous carcinoma (HGSC). Information on treatment and outcome of isolated STIC is rare. Therefore, we reviewed systematically the published literature to determine the incidence of subsequent HGSC in the high- and low-risk population and to summarize the current diagnostic and therapeutic options.

Methods: A systematic review of the literature was conducted in MEDLINE-Ovid, Cochrane Library and Web of Science of articles published from February 2006 to July 2021. Patients with an isolated STIC diagnosis and clinical follow-up were included. Study exclusion criteria for review were the presence of synchronous gynaecological cancer and/or concurrent non-gynaecological malignancies.

Results: 3031 abstracts were screened. 112 isolated STIC patients out of 21 publications were included in our analysis with a pooled median follow-up of 36 (interquartile range (IQR): 25.3-84) months. 71.4% of the patients had peritoneal washings (negative: 62.5%, positive: 8%, atypic cells: 0.9%). Surgical staging was performed in 28.6% of all STICs and did not show any malignancies. 14 out of 112 (12.5%) patients received adjuvant chemotherapy with Carboplatin and Paclitaxel. Eight (7.1%) patients developed a recurrence 42.5 (IQR: 33-72) months after isolated STIC diagnosis. Cumulative incidence of HGSC after five (ten) years was 10.5% (21.6%). Recurrence occurred only in *BRCA1* carriers (seven out of eight patients, one patient with unknown *BRCA* status).

Conclusion: The rate of HGSC after an isolated STIC diagnosis was 7.1% with a cumulative incidence of 10.5% (21.6%) after five (ten) years. HGSC was only observed in *BRCA1* carriers. The role of adjuvant therapy and routine surveillance remains unclear, however, intense surveillance up to ten years is necessary.

Systematic Review Registration: https://www.crd.york.ac.uk/prospero/, identifier CRD42021278340.

KEYWORDS

serous tubal intraepithelial carcinoma (STIC), high-grade serous carcinoma (HGSC), precursor, peritoneal carcinomatosis, incidence, treatment, outcome

Introduction

Serous tubal intraepithelial carcinoma (STIC) in the fimbriated end of the fallopian tube is regarded as the precursor lesion of pelvic (i.e. ovarian or peritoneal) high-grade serous cancer (HGSC) (1–3). Women with proven *BRCA* germline mutations have an increased risk of 10-60% for developing ovarian cancer. For these women, a risk-reducing salpingo-oophorectomy (RRSO) is therefore recommended and presents the most effective method of prevention so far (4, 5). Occult carcinoma and/or STIC is detected in approximately 10-15% of these cases (1), isolated STIC is detected in approximately 2% (6). Metachronous peritoneal carcinomatosis after RRSO in high-risk patients occurs in approximately 4.5% (7) and predominantly in *BRCA1* mutation carriers, usually within 5 years (8). Moreover, STIC diagnosis accompanies more than half of the cases with sporadic ovarian, tubal or primary peritoneal cancer (1). The incidence of STIC in patients with a normal risk of ovarian cancer is uncertain; however, a Canadian study reported STIC in eight out of 9392 women (<0.01%) with benign diagnoses (9). Accordingly, a recently published population-based, retrospective cohort study of all individuals in British Columbia, Canada, who underwent opportunistic salpingectomy or a control surgery (hysterectomy alone or tubal ligation), showed that the opportunistic salpingectomy group had significantly fewer serous and epithelial ovarian cancers than the control group (10). In the future, opportunistic salpingectomies will likely increase in routine surgery as a strategy for epithelial ovarian cancer prevention.

The SEE-FIM (Sectioning and Extensively Examining the FIMbria) protocol helps pathologists to detect these STIC lesions and is nowadays established for RRSOs after its first publication in February 2006 (11). Women with a proven isolated STIC lesion are at substantial risk to develop advanced HGSC and the metastatic pattern of a STIC remains unclear (6, 12, 13). Furthermore, consistent information on diagnostic necessities and therapeutical consequences for patients with STIC is lacking so far since most of the literature is focusing on pathological features (7).

The aim of this review was to determine the incidence of HGSC following a proven, isolated STIC diagnosis to discuss the management and follow-up of these women. Additional outcomes comprised the description of therapeutic and diagnostic options for STICs in the clinical routine.

Methods

Literature search and eligibility criteria

Our systematic review is based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (14). It is registered at PROSPERO (CRD42021278340).

Three electronic bibliographical databases including MEDLINE (*via* Ovid), the Cochrane Central Register of Controlled Trials (CENTRAL) and Web of Science were searched systematically from February 2006 to July 2021 (15). In February 2006, the SEE-FIM protocol was initially introduced to detect STICs in routine diagnostics regularly (11).

The search strategies for each database were conducted by a librarian from the Johannes Gutenberg- University Mainz according to the PICOS criteria (16). All search strategies included index terms as well as free text related to STIC. The search strategies are provided in the supplementary material (appendix A). The search was performed on 28th July 2021. Furthermore, studies included in related systematic reviews and meta-analyses were screened for eligibility. A de-duplication of database search results in EndNote was performed according to Bramer (17). Grey literature, such as conference abstracts, were not included.

Study inclusion criteria for review were the pathological diagnosis of isolated STIC and clinical follow-up. Patients with a STIC and a positive cytology were also included to maintain consistency with previous publications on this subject (7). Serous intraepithelial neoplasia is also known as STIC and was included (18). Study exclusion criteria for review were missing clinical data (follow-up) and publications restricted to pathological information only. In addition, the presence of synchronous gynaecological cancer and/or concurrent non-

gynaecological malignancies were exclusion criteria. Patients with a STIC diagnosis at RRSO and with an upstaging to a HGSC at the following surgical staging were not included, since the HGSC might have been overlooked at the initial surgery. Meta-analyses, systematic reviews, literature review and case reports were not included. Results should be interpreted accordingly. Only the latest published data were reported in case of articles that were an update of previously published patients.

Data extraction

Title and abstract screening, as well as full-text screening, were conducted by two review authors (V.C.L and A.L.) independently. A third independent reviewer (M.J.B) was contacted in case of disagreements between the first two reviewers. Data extraction was performed by V.C.L. and re-checked independently by A.L. using a predefined EXCEL spread sheet. The following information was collected: age, personal history of breast cancer, genetic predispositions, surgical indications, preoperative serum CA-125 levels, preoperative pelvic ultrasound, surgical procedure, peritoneal washings, adjuvant treatment (e.g. completion surgery, chemotherapy), and follow-up.

Risk of bias assessment

For each cohort study adequateness was assessed by the following criteria based on a systematic review of Van der Hoeven in 2018 (19): STICs should be diagnosed according to predefined pathological criteria and by an expert pathologist. The reporting bias included the description of the original cohort size, the genetic predisposition, median or mean age at surgery, information about clinical staging and adjuvant treatment for the patients with STIC. The indication was considered adequate if the surgery and the treatment of STIC took place according to a predefined protocol. The reported follow-up was seen as adequate if the follow-up was given in months or years describing the presence or absence of recurrence.

Data synthesis and analysis

A pooled incidence of subsequent HGSC with a corresponding confidence interval (CI) after an isolated STIC diagnosis was calculated for all patients with an isolated STIC and follow-up. The median is shown with interquartile ranges (IQR) if possible. The Kaplan-Meier estimation was used to calculate the cumulative incidence of HGSC.

Staging procedures, adjuvant treatment and their outcome were described. Due to the limited number of recurrences, a risk stratification as well as a statistical analysis of the associations between staging, chemotherapy and recurrence was not performed (19).

A statistical analysis was carried out using SPSS version 27 (SPSS, Chicago, IL, USA).

Results

In total, 3031 records were screened and 21 articles met our inclusion criteria as shown in the PRISMA flow chart in Figure 1.

We were able to include 112 patients out of these 21 articles (Table 1 and Table 2 for detailed information; Table 3 for overview). Median age was 52.3 (46.3-60) years. 71 (63.4%) patients were *BRCA1* carriers, 18 (16.1%) patients were *BRCA2* carriers. Eight (7.1%) patients were either *BRCA1* or *2* positive. Four patients (3.6%) had a high risk and four patients a low risk of ovarian cancer. The *BRCA* status was unknown for five (4.5%) patients. Two (1.8%) were *BRCA* negative. One patient had a *PALB2* mutation (31). RRSO was performed in 100 patients due to *BRCA* mutations or high-risk personal or family history. An opportunistic salping(o-oophor)ectomy was performed in the remaining twelve patients with an isolated STIC during surgery for benign reasons (ovarian cyst, cholecystectomy) (12, 34–36). In some cases, additional procedures were performed, mostly hysterectomies. All individual procedures are listed for each study in Table 1. Peritoneal washing during RRSO/surgery was reported in 80 (71.4%) cases of which nine (8%) were positive and one (0.9%) showed atypical cells. Six out of these nine patients had immediate reoperation for surgical staging. One patient declined the offer and opted for observation with CA-125 biannually and clinical review yearly for 3.5 years, and afterwards was discharged to the local medical officer (26). All of the surgical stagings showed no pathological findings and no subsequent HGSC was described in the follow-up.

The surgical staging procedures mostly included omentectomy and in some cases a pelvic and paraaortic lymph node dissection (see Table 1).

In the study of Wethington and colleagues, all patients with an isolated STIC were offered a surgical staging, including hysterectomy, omentectomy and in five cases pelvic and paraaortic lymph node dissections. All procedures were without pathological findings. Three patients declined a surgical staging (6). Postoperative imaging as staging was hardly reported. Four out of 12 patients in the cohort of Wethington had an additional postoperative imaging without pathological findings (6).

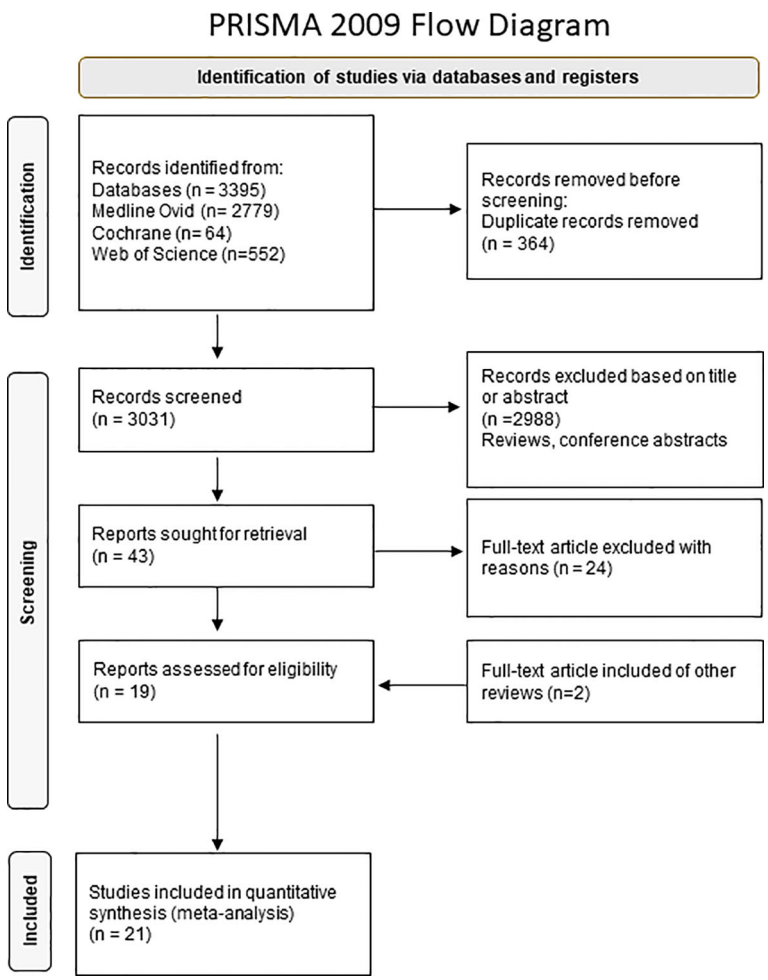

FIGURE 1
Flowchart of literature selection.

14 out of 112 (12.5%) patients received adjuvant chemotherapy consisting of a combination of Carboplatin and Paclitaxel. Five out of nine patients with a positive washing received chemotherapy as well as seven patients with a negative cytology and one patient with a non-reported cytology. Follow-up mostly included clinical observation with CA-125 yearly. Pooled median follow up was 36 months (IQR: 25.3-84).

Eight out of 112 patients developed a subsequent HGSC (7.1%, 95% CI 2.3-12%), listed in Table 4. Pooled median time to recurrence were 42.5 (IQR: 33-72) months. The five (ten)- year-HGSC rate was 10.5% (21.6%), determined by the Kaplan-Meier estimation (Figure 2). The latest HGSC recurred 118 months after the diagnosis of STIC at RRSO/surgery. Seven out of eight patients were *BRCA1* carriers and one patient had an unknown *BRCA* status since STIC was detected after revaluation of a salpingectomy during cholecystectomy (36). No *BRCA2* carrier presented a recurrence in the selected studies. A recurrence occurred in four patients with a negative peritoneal washing, in three patients in which no pelvic washing was done and in one patient without a reported peritoneal cytology at the time of the first surgery.

Risk of bias assessment

The risk of bias assessment is shown in appendix B. In 10/21 (48%) studies, STIC was diagnosed according to predefined pathological criteria. 17/21 (81%) studies reported the mutation status for the cohort. 11/21 (52%) studies operated according to a predefined protocol and only one study had a predefined treatment protocol for STIC. In general, adjuvant treatment was adequately described in 13/21 (61%) studies. Two studies had a predefined protocol for the follow-up of patients with STIC. Finally, 18/21 (86%) studies reported an adequate follow-up for patients with STIC.

TABLE 1 Detailed characteristics of all included patients with isolated STIC (white: high-risk cohort; grey: low-risk cohort).

Reference	Number of cases (STIC/ total RRSO or performed surgeries)	Median age (range) or mean age in years	Previous cancer	*BRCA* status/ mutation status	CA 125	Pelvic USG	Cytology outcome at RRSO/ surgery	Additional procedure at RRSO/ surgery and outcome
				High-risk cohort				
Blok 2019 (13)	4/527	54 (47.8-67)	Breast (1)	BRCA1 (3) BRCA2 (1)	Normal (4)	Normal (4)	Negative (2) ND (2)	
Carcangiu 2006 (20)	3/50	52.7 (+/-7.2)	Breast (3)	BRCA1 (3)	Normal (3)	Normal (3)	Negative (2) NR (1)	TAH with USO (1)
Conner 2014 (21)	11/349	49 (41-53)	Breast (2)	BRCA1 (5) BRCA2 (1) BRCA1 or 2 (5)	ND (11)	ND (11)	Negative (6) NR (5)	
Gornjec 2020 (22)	3/155	62 (+/-8.2)	NR (3)	BRCA1 (2) High-risk (1)	Normal (3)	Normal (3)	Negative (3)	
Lamb 2006 (23)	4/113	49.5 (46.3-61.8)	NR (4)	BRCA1 (3) BRCA2 (1)	NR (4)	NR (4)	Positive (1) Negative (3)	
Miller 2017 (24)	3/70	47.4 (+/-8.6)	NR (3)	BRCA1 (3)	NR (3)	NR (3)	Negative (3)	Peritoneal and omental biopsies (3): negative
Minig 2018 (25)	3/359	56.3 (+/-5.7)	Breast (1)	BRCA1 or 2 (3)	Normal (3)	Normal (3)	Negative (2) Positive (1)	
Poon 2016 (26)	3/138	52.3 (49-57)	Breast (1)	BRCA1 (2) BRCA2 (1)	NR (3)	NR (3)	Negative (1) Positive (atypical cells) (1) ND (1)	
Powell 2013 (27)	16/407	52.5 (47.5-61.8)	NR (16)	BRCA1 (13) BRCA2 (3)	Normal (14) ND (2)	Normal (11) ND (2) Ovarian cyst (2) Hydrosalpinx (1)	Negative (13) Positive (3)	
Reitsma 2013 (28)	3/360	54.3 (+/-3.8)	Breast (1)	BRCA2 (2) BRCA2 UV (1)	Normal (3)	Normal (3)	Negative (3)	
Ricciardi 2017 (29)	7/411	54 (43-67)	Breast (6)	BRCA1 (7)	Normal (7)	Normal (7)	Negative (7)	
Rudaitis 2020 (30)	7/71	45 (43-52)	Breast (1)	BRCA1 (7)	ND (7)	Normal (7)	NR/ND (7)	
Rush 2020 (31)	9/644	47 (42.5-57.5)	Breast (4)	BRCA1 (6) BRCA2 (2) PALB2 (1)	Normal (9)	NR (9)	Negative (7) Positive (2)	TLH (5) TAH (2)
Selmes 2015 (32)	1/93	40	Breast (1)	BRCA1 (1)	NR (1)	NR (1)	ND (1)	
Van der Hoeven 2018 (19)	2/235	56.5 (+/-26.2)	Breast (1)	BRCA1 (2)	Normal (2)	Normal (2)	ND (2)	
Wethington 2013 (6)	12/593	48.5 (44.3-66.5)	Breast (2)	BRCA1 (5) BRCA2 (4) BRCA2 rearrangement (1) Unknown, but high risk (2)	Normal (12)	Normal (10) ND (2)	Negative (11) Positive (1)	Serous adenofibroma (1) Endosalpingiosis (1)
Zakhour 2016 (33)	9/257	57.1 (49.5-66.5)	No (9)	BRCA1 (8) BRCA2 (1)	Normal (12)	Normal (12)	Negative (7) Negative: Atypic cells (1) ND (1)	HE (2)

(Continued)

TABLE 1 Continued

Reference	Number of cases (STIC/ total RRSO or performed surgeries)	Median age (range) or mean age in years	Previous cancer	*BRCA* status/ mutation status	CA 125	Pelvic USG	Cytology outcome at RRSO/ surgery	Additional procedure at RRSO/ surgery and outcome
				Low-risk cohort				
Chay 2016 (12)	5 (unknown)	52 (48.5-63)	Breast (1) NR (3)	BRCA1 (1) NR (4)	NR (4) Elevated (1)	Ovarian mass (1) Ovarian cyst (1)	NR (1) ND (4)	USO + USE (1): ovarian fibroma (1) TAH (2); Ovarian cyst (1) Endometriosis(2) Hydrosalpinx(1)
Morrison 2015 (34)	3 (unknown)	58 (+/- 7.2)	NR (3)	NR (3)	NR (3)	NR (3)	NR (3)	HE (3) Uterine leiomyomas (2)
Rabban 2014 (35)	3/522	64 (+/- 18.5)	No (3)	Negative (1) ND (2)	NR (3)	Adnexal cyst (3)	NR/ND (3)	USO (1)
Tomasch 2020 (36)	1/98	57	NR (1)	Unknown (1)	NR (1)	NR (1)	NR (1)	Cholecystectomy and bilateral prophylactic salpingectomy

HE, hysterectomy; ND, not done; NR, not reported; RRSO, risk reducing salpingo-oophorectomy; STIC, serous tubal intraepithelial carcinoma; TAH, total abdominal hysterectomy; TLH, total laparoscopic hysterectomy; USE, unilateral salpingectomy; USG, ultrasound scan test; USO, unilateral salpingo-oophorectomy; UV, unknown variant. If possible, median age with interquartile range was calculated.

Discussion

Summary of main results and results in the context of published literature

In our review, the rate for subsequent HGSC after an isolated STIC diagnosis was 7.1%. In literature, recurrence rates in patients with isolated STIC ranged from 0-22% (21, 26, 28, 33), mostly due to small numbers of patients per study. One systematic review reported a rate of 4.5% in 2015 (7) and a more recent one in 2018 a rate of 11% (19). Our rate of recurrence may be more accurate since all patients with isolated STIC and available follow-up of the current literature were included. It is important to note that our rate might be probably increased with a longer follow-up of patients after an isolated STIC diagnosis, because the pooled median follow-up was 36 months and the pooled recurrence was detected more than half a year later after 42.5 months. A long follow-up is necessary to be able to determine the real incidence of HGSC. Our study determined a high and clinically relevant cancer risk for HGSC after STIC diagnosis of 10.5% (21.6%) after five (ten) years according to the Kaplan-Meier estimation. This again underlines the importance of a long follow-up, especially if we consider that the latest recurrence occurred almost 10 years after initial surgery. During the preparation of the manuscript, Steenbeek and colleagues published a systematic review about the risk of peritoneal carcinomatosis after RRSO with similar results in February 2022. They report a five- and ten- year- risk of developing peritoneal carcinomatosis of 10.5% and 27.5% after RRSO, respectively (37). Due to the prior closure of our data collection, we could not include their newly published STIC cases.

Interestingly, only *BRCA1* carriers developed a subsequent HGSC. In general, *BRCA1* carriers have the highest risk of occult neoplasia at RRSO (31). For all *BRCA* mutation carriers, a 3.5% cumulative risk for peritoneal cancer after prophylactic oophorectomy was reported after 20 years of follow-up (38). One STIC patient had a *PALB2* gene mutation which is also involved in hereditary breast and ovarian cancer but insufficiently determines the ovarian cancer risk (39, 40).

Strengths and weaknesses

We present a comprehensive review on published clinical outcomes and treatment modalities of patients with isolated STIC. Our strength is that our study contains the largest patient collective with isolated STIC and follow-up in the high-risk and especially the low-risk population so far. An increase in the number of STIC patients in the low-risk population is expected because opportunistic salpingectomies are recommended during routine surgery to prevent epithelial ovarian cancer (10).

However, our study reanalysed published data. The quality of collected data was low and with significant risk of bias (see appendix B). The latter included incomplete clinical data, heterogeneous follow-up data, e.g. only the mean data was

TABLE 2 Follow-up of patients with STIC included in our systematic review in alphabetical order (white: high-risk cohort; grey: low-risk cohort).

Reference	STIC cases with follow-up	Surgical staging after positive washings at RRSO	Surgical staging after negative washings at RRSO	Surgical staging after unreported or nor performed washings at RRSO	Chemotherapy	Median follow-up (range) or mean (+/- SD) in months	Status at follow-up	Recurrence	Additional information on recurrences	Additional information
				High-risk cohort						
Blok 2019 (13)	4		ND (2)	ND (2)	ND (4)	62.1 (3.1-131.3)	Alive (2) NED (2)	PPSC (2)	Recurrence after 80 and 118 months: both with unknown cytology at RRSO, no staging or chemotherapy thereafter	
Carcangiu 2006 (20)	3		ND (2)	ND (1)	ND (3)	44 (+/- 40.3)	NED (3)			
Conner 2014 (21)	11		Surgical staging (3) (no details): negative ND (3) ND (5)		6 cycles C/P (2) 2 cycles C/P (1) ND (8)	60 (24-84)	Alive (11)	Yes (1)	Elevated serum CA125 and ascites (1) 48 months after RRSO, but no tissue diagnostic	
Gornjec 2020 (22)	3		Surgical staging (3): negative		ND (3)	29 (15-51)	NED (3)			Staging procedure not described
Lamb 2006 (23)	4	"second look operation" (1): negative	ND (3)		6 cycles C/P (1; positive washing) 3 cycles C/P (1)	28 (unkown)	NED (4)			
Miller 2017 (24)	3	–	NR (3)		NR (3)	32.5 (+/- 24.7)	NED (3)			Data for cohort, not exclusively for STIC patients
Minig 2018 (25)	3	OE, PPALND(1): negative	ND (2)		ND (3)	23 (+/- 10.8)	NED (3)			
Poon 2016 (26)	3	Offered (1): Surgical staging versus observation: Patient opted for observation with CA-125 and clinical review 6/12 for 3.5 years. Patient discharged to LMO with yearly CA-125.	ND (1), Observation	Offered (1): Surgical staging versus observation. Patient opted for observation with yearly CA-125.	ND (3)	79.3 (+/- 31.9)	NED (3)			
Powell 2013 (27)	16	Staging surgery without lymph node excision (2)/with lymph node excision (1)	Staging surgery without lymph node excision (4)/with lymph node excision (2)		ND (12) 6 cycles C/P (2, positive washing) 3 cycles C/P (2,	79 (59.5-100.5)	NED (15)	Yes (1)	43 months after RRSO: omental deposits	

(Continued)

TABLE 2 Continued

Reference	STIC cases with follow-up	Surgical staging after positive washings at RRSO	Surgical staging after negative washings at RRSO	Surgical staging after unreported or nor performed washings at RRSO	Chemotherapy	Median follow-up (range) or mean (+/- SD) in months	Status at follow-up	Recurrence	Additional information on recurrences	Additional information
					negative washing, no surgical staging)					
Reitsma 2013 (28)	3		ND (3)		NR (3)	12 (+/- 12.5)	NED (3)			
Ricciardi 2017 (29)	7		Surgical staging (4): laparoscopic HE, OE, random peritoneal biopsies and peritoneal washing: No malignancies		ND (7)	30 (9-84)	NED (7)			
Rudaitis 2020 (30)	7	NR/ND	NR/ND	NR/ND	ND (7)	54 (37.2-63.6)	NED (7)			
Rush 2020 (31)	9	NR	NR		6 cycles C/P (2; positive washings) 3 cycles C/P (2; negative washings)	144 (42-192)	NED (9)			
Selmes 2015 (32)	1			NR (1)	NR(1)	22	NED (1)			
Van der Hoeven 2018 (19)	2			ND (2)	ND (2)	78 (59-96)	Dead of disease (1)	Yes (1)	36 months after RRSO, died 59 months after RRSO at disease Deceased due to breast cancer (1)	
Wethington 2013 (6)	12	Surgical staging (1): TAH, omentectomy, biopsies: negative	TH(7), OE (7) Peritoneal biopsies (6) Diaphragm biopsies (3) PLND (1) PPALND (5) Surgical staging		ND (12)	28 (20-33.8)	NED (12)			4 patients had additional postoperative imaging: normal

(Continued)

TABLE 2 Continued

Reference	STIC cases with follow-up	Surgical staging after positive washings at RRSO	Surgical staging after negative washings at RRSO	Surgical staging after unreported or nor performed washings at RRSO	Chemotherapy	Median follow-up (range) or mean (+/- SD) in months	Status at follow-up	Recurrence	Additional information on recurrences	Additional information
			declined (3) or performed outside the hospital (1): negative							
Zakhour 2016 (33)	9		ND (8)	ND (1)	ND (9)	81.3 (38.5-109.5)	NED (9)	PPSC (2)	Recurrences after 32 and 42 months: both with negative cytology and negative HE at RRSO, no staging or chemotherapy thereafter	
Low-risk cohort										
Chay 2016 (12)	5			Surgical staging (1): vaginal HE, peritoneal washing, OE, PPALND sampling: no malignancies	ND (5)	25 (11.5-83)	NED (5)	No (5)		2° diagnosis of TNBC (BRCA1 mutation) 2° diagnosis of colon cancer (HGSC)
Morrison 2015 (34)	3			ND (3)	4 cycles C/P (1)	6.7 (+/- 4.6)	NED (3)	No (3)		Non-prophylactic setting
Rabban 2014 (35)	3			Surgical staging (1): HE, completion salpingo-oophorectomy, lymph node dissection, OE: No malignancies	ND (3)	14 (+/- 10.1)	NED (3)		–	
Tomasch 2020 (36)	1			ND (1)	ND (1)	28	Peritoneal carcinomatosis (HGSC)	Yes (1)		Prophylactic salpingectomy at the time of elective laparoscopic cholecystectomy. No clinical data available. STIC was overseen and later on detected with the SEE-FIM protocol.

C/P, Carboplatin/Paclitaxel; HE, hysterectomy; HGSC, high-grade serous cancer; LAVH, laparoscopic-assisted vaginal hysterectomy; LMO, local medical officer; NED, no evidence of disease; OE, omentectomy; PLND, pelvic lymph node dissection; PPALND, pelvic and paraaortic lymph node dissection; PPSC, primary peritoneal serous carcinoma; RRSO, risk-reducing salpingo-oophorectomy, SEE-FIM, Sectioning and Extensively Examining the FIMbria; STIC, serous tubal intraepithelial carcinoma; TNBC, triple negative breast cancer.

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TABLE 3 Overview of the 112 included STIC patients.

Characteristics	112 patients
Median age (years)	52.3 (46.3-60)
BRCA status (%)	71 (63.4)
-BRCA1	18 (16.1)
-BRCA2	8 (7.1)
-BRCA1 or 2	9 (8.0)
-Low risk/unknown	
Peritoneal washing at RRSO/surgery (%)	70 (62.5)
-Negative	9 (8.0)
-Positive	1 (0.9)
-Atypical cells	32 (28.6)
-Not done/not reported	
Surgical staging (%)	32 (28.6); no malignancies detected
-Performed	
-Not done/not reported	80 (71.4)
Adjuvant treatment (%)	14 (12.5)
-Chemotherapy	
Recurrence (%)	8 (7.1)
Median time to recurrence with interquartile range (months)	42.5 (33-72)
Time to latest recurrence (months)	118
Risk for HGSC (%)	10.5
-5 years	21.6
-10 years	
Median follow-up (months)	36 (25.3-84)

HGSC, high-grade serous carcinoma; RRSO, risk-reducing salpingo-oophorectomy; STIC, serous tubal intraepithelial carcinoma.

given, the lack of data regarding a standard diagnostic, staging, treatment and surveillance. An important confounder in many studies was the short follow-up period after the STIC diagnosis, which might disguise the real rate of subsequent HGSC after RRSO/salpingectomy.

The impact of STIC in a low risk population is difficult to assess due to the lack of information. A Canadian study reported STIC in eight out of 9392 women (<0.01%) with benign diagnoses who had a normal risk of ovarian cancer using the SEE-FIM protocol (9). However, the SEE-FIM protocol was not routinely applied in non-RRSO surgery in the past and therefore published data on the incidence of STIC low-risk populations should be interpreted with caution. In general, diagnosing STIC is challenging with only moderate reproducibility. A recently published systematic review suggests not only the use of the SEE-FIM protocol, but also evaluation by a subspecialized pathologist, rational use of immunohistochemical staining, and obtaining a second opinion from a colleague to secure the diagnosis (41). Furthermore, there can also be a HGSC unrelated to a STIC diagnosis. Another bias is that STIC patients with positive washings were included to maintain the comparability to previous studies (7). However, not every patient received a subsequent surgical staging to eliminate the risk of a HGSC. The impact of positive peritoneal washings remains unclear as well. The routine use of peritoneal biopsies during RRSO does not seem to improve the detection of occult malignancies (42). In our review, nine patients had a positive washing and six

TABLE 4 Characteristics of the eight STIC patients with subsequent HGSC.

Patient number	Age at RRSO/ surgery	Menopausal status at RRSO/surgery	*BRCA* gene involved	Peritoneal washing	Additional procedures at RRSO/surgery	Surgical staging	Adjuvant treatment	Recurrence	Time to recurrence (months)
1 (Blok 2019) (13)	46	premenopausal	BRCA1	ND	ND/NR	ND	ND	PPSC	118
2 (Blok 2019) (13)	53	premenopausal	BRCA1	ND	ND/NR	ND	ND	PPSC	80
3 (Conner 2014) (21)	46	NR	BRCA1	negative	ND	ND	ND	Elevated serum CA125 and ascites	48
4 (Powell 2013) (27)	49	NR	BRCA1	negative	HE	ND	ND	Omental deposits	43
5 (Tomasch 2020) (36)	57	NR	Low risk (unknown)	ND	Cholecystectomy	ND	ND	Peritoneal carcinomatosis (HGSC)	28
6 (Van der Hoeven, 2018) (19)	54	NR	BRCA1	NR	ND	ND	ND	Recurrence at peritoneum, omentum, uterus	36
7 (Zakhour 2016) (33)	50	NR	BRCA1	negative	HE	ND	ND	PPSC	97
8 (Zakhour 2016) (33)	60	NR	BRCA1	negative	ND	ND	ND	PPSC	104

HE, Hysterectomy; HGSC, high-grade serous carcinoma; PPSC, primary peritoneal serous carcinoma; ND, not done; NR, not reported; RRSO, risk-reducing salpingo-oophorectomy; STIC, serous tubal intraepithelial carcinoma.

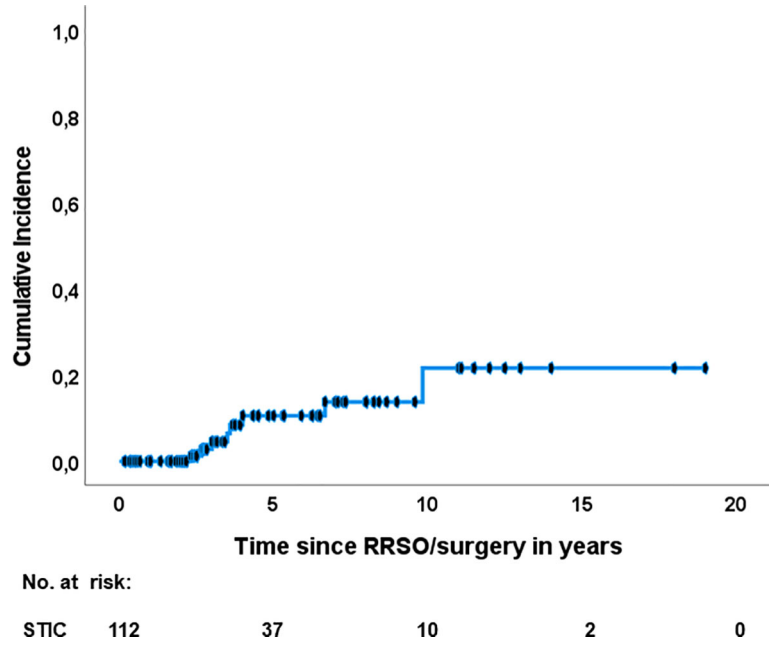

FIGURE 2
Recurrence of HGSC after isolated STIC diagnosis. RRSO risk reducing salpingo-oophorectomy; STIC, serous tubal intraepithelial carcinoma.

underwent surgical staging without pathological findings. No patient with a positive washing developed a recurrence. According to the study of Wethington 15% of the peritoneal washings were positive at the time of RRSO and therefore recommended as a component of RRSO (6).

Implications for practice and future research; conclusion

Clinical management of STIC is still a matter of debate. It is important that patients are informed about their potential risk of developing pelvic HGSC after a STIC diagnosis. A surgical staging should be considered (43), especially in cases of a positive peritoneal washing at initial RRSO/surgery to reduce the risk of synchronous HGSC. A surgical staging mostly included hysterectomy, omentectomy, pelvic and paraaortic lymph node dissection and peritoneal washing in the published studies. In case of a positive peritoneal washing at initial surgery, which implies circulating malignant cells in the peritoneal cavity, some institutions offered adjuvant chemotherapy. The latter usually comprised six cycles of Carboplatin and Paclitaxel. However, if the surgical staging is without evidence of disease, observation remains a reasonable option and avoids possible chemotherapy-induced adverse events (6). Adjuvant chemotherapy for intraepithelial neoplasia is not recommended any longer (31, 43). A radiological staging was rarely reported in our study.

Routine surveillance is recommended for the next years of follow-up, because the time from STIC to invasive cancer has been suggested to be approximately seven years and has guided the recommendation for RRSO in *BRCA1* patients at the age of 35–40 years (44). This is coherent with the findings of Stanciu and colleagues who published seven cases with isolated STICs. Two of these patients (28%) developed peritoneal HGSC within 53 and 75 months after RRSO. The publication was not included in our study, because the follow-up of the five other patients with isolated STIC was missing (45).

To date, no effective screening tool exists to monitor STIC patients (26). Most of the published studies included annual clinical check-ups with pelvic ultrasound and in some cases routine evaluation of serum CA-125. *BRCA* status should be checked in cases of isolated STIC as well. No routine screening for ovarian HGSC should be offered to women of the general population. Two prospective randomized trials could not reduce ovarian cancer mortality with simultaneous screening of CA-125 and transvaginal ultrasound compared with usual care in the normal population (46, 47).

To summarize, several questions concerning STIC remain unclear and the therapy may require an individualized treatment plan. We are urgently in need of registries for longer follow-up data of STIC patients to assess the real incidence of HGSC after a STIC diagnosis. Future multicentre and international efforts are needed to generate a large cohort of patients with STIC to allow further subgroup analyses, e.g. regarding histopathological characteristics. In the meantime, systematic reviews will help to gather information and to define and update guidelines for the management of STIC.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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A comparison of four technologies for detecting p53 aggregates in ovarian cancer

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The tumor suppressor protein p53 is mutated in half of all cancers and has been described to form amyloid-like structures, commonly known from key proteins in neurodegenerative diseases. Still, the clinical relevance of p53 aggregates remains largely unknown, which may be due to the lack of sensitive and specific detection methods. The aim of the present study was to compare the suitability of four different methodologies to specifically detect p53 aggregates: co-immunofluorescence (co-IF), proximity ligation assay (PLA), co-immunoprecipitation (co-IP), and the p53-Seprion-ELISA in cancer cell lines and epithelial ovarian cancer tissue samples. In 7 out of 10 (70%) cell lines, all applied techniques showed concordance. For the analysis of the tissue samples co-IF, co-IP, and p53-Seprion-ELISA were compared, resulting in 100% concordance in 23 out of 30 (76.7%) tissue samples. However, Co-IF lacked specificity as there were samples, which did not show p53 staining but abundant staining of amyloid proteins, highlighting that this method demonstrates that proteins share the same subcellular space, but does not specifically detect p53 aggregates. Overall, the PLA and the p53-Seprion-ELISA are the only two methods that allow the quantitative measurement of p53 aggregates. On the one hand, the PLA represents the ideal method for p53 aggregate detection in FFPE tissue, which is the gold-standard preservation method of clinical samples. On the other hand, when fresh-frozen tissue is available the p53-Seprion-ELISA should be preferred because of the shorter turnaround time and the possibility for high-throughput analysis. These methods may add to the understanding of amyloid-like p53 in cancer and could help stratify patients in future clinical trials targeting p53 aggregation.

KEYWORDS

p53, protein aggregation, ovarian cancer, immunofluorescence staining, immunoprecipitation, ELISA, proximity ligation assay

Introduction

Protein misfolding, aggregation, and amyloid formation have been associated with neurodegenerative diseases such as transmissible spongiform encephalopathies (TSEs), Alzheimer's disease, Parkinson's disease, and even diabetes type 2. The disease-causing agents of TSEs, such as Creutzfeldt-Jakob disease or bovine spongiform encephalopathy (BSE), are prions. They are a subclass of amyloid proteins with the unique characteristic of being infectious. Amyloids are aggregated proteins with enriched β-sheet structures running perpendicular to the fibril axis, resulting in a fibrillar structure (1). Misfolded forms of key proteins in neurodegenerative diseases, such as β-amyloid or tau in Alzheimer's disease and α-synuclein in Parkinson's disease, share some of the characteristics of prions and have therefore been named prionoids or prion-like proteins (2).

Intriguingly, the p53 protein has shown amyloid-like behavior, thereby, adding cancer to the class of protein aggregation diseases. A so-called aggregation-prone sequence has been identified in the hydrophobic core of the DNA-binding domain (3, 4). This region gets exposed upon conformational changes caused by mutation, leading to the formation of amyloid-like protein aggregates (4). The amyloid-like structures formed by mutant p53 protein have been detected in the cytoplasm and the nucleus in different types of cancer cell lines and tumors (3, 5–8). It has been proposed that misfolded mutant p53 protein exhibits an amyloid-like behavior by converting correctly folded wild-type p53 to a misfolded amyloid conformation (9). Moreover, mutant p53 can cause co-aggregation not only of wild-type p53 but also of other members of the p53 protein family, namely p63 and p73. Therefore, the amyloid-like behavior of p53 can provide a mechanistic explanation for the dominant-negative and gain-of-function (GOF) effects of p53 (3, 10). Recently, it has been shown that amyloid p53 exerts another characteristic feature of prions, which is cell-to-cell transmission leading to the induction of amyloid formation in neighboring cells (8).

TP53 mutations are found in more than 50% of cancer cases. The mutation rate varies across different cancer types, including ovarian cancer (OC) in which *TP53* mutations are the most frequent genetic alteration and the hallmark of precancerous lesions. The most dominant OC subtype, high-grade serous ovarian cancer (HGSOC), is characterized by an almost ubiquitously presence of *TP53* mutations (11). The ability of p53 to form amyloid-like structures has been observed in HGSOC cells exhibiting cancer stem cell properties, where it is associated with chemoresistance (12, 13). This finding attracts attention to amyloid-like p53 as a new potential therapeutic target. The first inhibitor targeting aggregated p53, ReACp53, was shown to diminish p53 amyloid formation and rescue the p53 function *in vitro* and in pre-clinical testing *in vivo* (4). Further, the combination of carboplatin and ReACp53 enhanced tumor cell targeting in OC cancer cell lines and patient-derived HGSOC organoids (14).

Robust, sensitive, and reproducible methods to detect and characterize amyloid p53 are a prerequisite for future studies unraveling their clinical relevance and possible therapeutic intervention.

Currently, the state-of-the-art method for the detection of p53 aggregates is the immunofluorescence co-localization assay (co-IF) based on the co-localization of p53 and amyloid structures (5–7, 10, 12, 15–18). For the detection of those amyloid structures, various antibodies or amyloid-specific dyes are available, including Thioflavin T, Congo Red, the anti-oligomer antibody A11, and the anti-amyloid fibrils antibody OC. Both amyloid-specific dyes bind amyloid fibrils with β-sheet-rich structures, but their specificity remains limited. The A11 antibody detects prefibrillar oligomers, which are immunologically distinct from the fibrillar oligomers that are recognized by the OC antibody. Neither one detects natively folded proteins and monomers (19). However, co-staining of two epitopes in the same subcellular compartment does not prove that both epitopes are present within one molecule and therefore, co-IF does not allow specific detection of p53 aggregates. Co-immunoprecipitation using A11 or OC antibodies for the pull-down of amyloid protein followed by immunoblotting to analyze the p53 levels in the amyloid fractions showed that p53 is present as amyloid aggregates (16, 20).

In an earlier study, we developed a highly-sensitive ELISA-based assay, the p53-Seprion-ELISA, for the detection of p53 aggregates in cancer cell lines and fresh-frozen tissue (21). This assay is based on a high-molecular-weight polymeric ligand, selectively binding aggregated proteins including amyloid oligomers, proto-fibrils, and fibrils (22). The Seprion ligand was previously used to isolate and quantify aggregated forms of prion protein (23). By combining the Seprion ligand with an anti-p53 antibody, the ELISA specifically detects high-molecular-weight p53, but neither monomers, naturally occurring tetramers, or octamers. The most recent method for detecting p53 aggregates is the proximity ligation assay (PLA). This technique is based on two primary antibodies, which are bound by oligonucleotide-labeled proximity probes that form a DNA circle when bound in close proximity. The DNA circle serves as a template for the rolling-circle amplification (RCA) and the amplified DNA is detected by fluorescently labeled detection probes (24). The resulting distinct fluorescent spots can be quantified *via* microscopy or flow cytometry. The PLA has been successfully applied to detect oligomeric p53 aggregates in nuclear inclusion bodies in ovarian cancer tissue biopsies (7).

In the present study, we aimed at comparing the state-of-the-art technique co-immunofluorescence (co-IF) with novel assays such as the proximity ligation assay (PLA), co-immunoprecipitation (co-IP), and the p53-Seprion-ELISA (Figure 1) in cancer cell lines and ovarian cancer tissues.

Materials and methods

Cell culture

Nine ovarian cancer cell lines (COV644, OAW42, COV318, COV362, ES2, OVCAR3, TYK-nu, 59M, and COV504) and one cervical cancer cell line (ME-180), either obtained from ATCC or kindly provided from Els Berns (Erasmus MC, Rotterdam, Netherlands) were grown in RPMI-1640 (Gibco/Life Technologies) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% CO_2. STR-DNA-profile analysis was used for cell-line verification. In addition, *TP53* mutations specified for each cell line in the IARC TP53 Database were confirmed by Sanger sequencing (Supplementary Table S1).

Patient cohort

Formalin-fixed paraffin-embedded (FFPE) tumor tissues from 78 patients diagnosed with ovarian cancer were collected at the Department of Obstetrics and Gynecology, Medical University of Innsbruck, Austria, and analyzed using co-IF. The study was approved by the local ethics committee (AN3507) and all patients gave their written informed consent. The *TP53* mutation status was identified by a functional yeast-based assay (FASAY) combined with Sanger sequencing as described previously (25, 26). Additionally, in a subgroup of 30 patients pulverized fresh-frozen tissue was available and therefore analyzed using co-IP and the p53-Seprion-ELISA.

Immunofluorescence co-localization assay

The cancer cell lines were washed twice with PBS, fixed with 3.7% formaldehyde, and permeabilized with 0.3% Triton X-100 and 0.1% sodium citrate. Nonspecific antigenic sites were blocked using 5% BSA in PBS for 1h. Further, cells were labeled with primary antibodies: mouse anti-p53 antibody DO-1 (1:200, sc-126, Santa Cruz Biotechnology) and rabbit anti-amyloid oligomer A11 antibody (1:200, AB9234, Merck Millipore) or the anti-amyloid fibrils OC antibody (1:200, AB2286, Merck Millipore) for 2h at room temperature (RT) in a humidified chamber. Next, the cells were incubated with secondary anti-rabbit Alexa Fluor 488-conjugated (1:500, Life Technologies) and anti-mouse Alexa Fluor 568-conjugated (1:500, Life Technologies) antibodies for 1h at RT in the dark. Nuclear counterstain was done by incubation with DAPI (4', 6-diamidino-2-phenylindole) solution. Between the blocking and staining steps, the cells were washed three times with PBS. The samples were analyzed using a laser scanning confocal microscope SP5 (Leica Microsystems).

FFPE tissues were deparaffinized in xylene and hydrated in decreasing concentrations of isopropanol. Briefly, antigen retrieval was performed by heat-induced epitope retrieval (HIER), while sections were immersed in 10 mM citrate buffer at pH 6.0 for two 5-minute intervals at 900 Watt using a microwave. To eliminate fixation-caused autofluorescence, sections were incubated in 1% sodium borohydride (Sigma Aldrich) three times. Nonspecific antigenic sites were blocked using 5% BSA/PBS. Next, sections were labeled with anti-p53 DO-1 (1:200) and anti-amyloid oligomer A11 (1:400) primary antibodies overnight in a humidified chamber at 4°C. The samples were incubated with secondary anti-rabbit Alexa Fluor 488-conjugated (1:750) and anti-mouse Alexa Fluor 568-conjugated (1:750) antibodies for 2h at RT in the dark. Nuclear counterstain was done by incubation with DAPI solution. Between the blocking and staining steps, the cells were washed three times with PBS.

All samples were analyzed using a laser scanning confocal microscope SP5 (Leica Microsystems).

Proximity ligation assay

Cell lines were harvested, washed with PBS, and cytospins were prepared. The proximity ligation assay (PLA) was performed according to the manufacturer's instructions using the Duolink PLA Kit (Sigma-Aldrich). To specifically detect p53 aggregates, the primary antibodies anti-p53 DO-1 (1:200) and

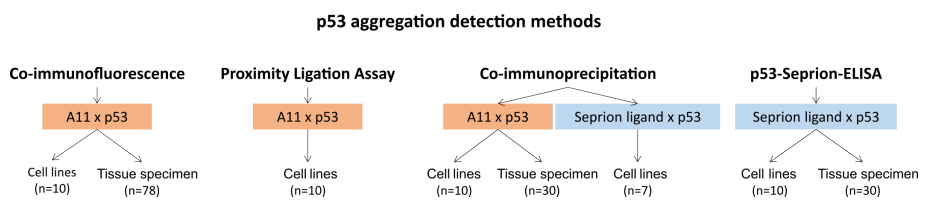

FIGURE 1
Overview of the p53 aggregation detection methods evaluated in this study.

the anti-amyloid oligomer A11 antibody (1:200) were used. Peripheral blood mononuclear cells (PBMCs) of a healthy individual were included as a negative control. The slides were analyzed using a LSM 780 confocal microscope (Zeiss).

Co-immunoprecipitation and immunoblot

1,000,000 cells were seeded in Petri dishes and harvested at 80% of confluency. The cells as well as the tissue samples (approx. 15 mg) were lysed with 0.5% Triton X-100/PBS and incubated on ice for 1h. After sonication and centrifugation, the protein concentration of the supernatant was adjusted to 1 mg/ml. To prevent non-specific binding to the IP antibody, a lysate pre-purification step was performed with 1 mg/ml of the lysates. Therefore, the samples were incubated with 20 µl of Dynabeads® Protein G (Life Technologies) for 1h at 4°C. For antibody binding, the pre-purified lysate was immunoprecipitated with the rabbit anti-amyloid oligomer A11 antibody (1:1000) overnight at 4°C. For the control reaction, the tissue lysates were incubated without the A11 antibody or only the lysis buffer with the A11 antibody. Then, the samples were incubated with 40 µl of Dynabeads® Protein G 1h at 4°C. The beads were washed with lysis buffer, resuspended in 2x Laemmli buffer, and incubated at 95°C for 5 minutes. Samples were resolved by SDS-PAGE. Separated proteins were transferred to a nitrocellulose membrane. The membranes were blocked in Odyssey Blocking Buffer (LI-COR) and incubated with the mouse anti-p53 DO-1 primary antibody (1:200). Next, the membranes were labeled with anti-mouse IRDye®800 CW secondary antibody (LI-COR) and imaged using an Odyssey Scanner.

The Seprion-based co-immunoprecipitation was performed for 7 of 10 cancer cell lines (COV644, COV318, COV362, ES2, OVCAR3, TYK-nu, and COV504) and for 30 fresh-frozen OC samples. Crude lysates were incubated with Seprion-coated magnetic beads (Protein Aggregation Detection (PAD)-beads, Microsens Biotechnologies) according to the manufacturer's instructions. Briefly, cell/tissue lysates were incubated with capture buffer and shaken by vibration at RT for 30 minutes. Beads were washed with wash buffers 1 and 2 and resuspended in 2x Laemmli buffer. Samples were resolved by SDS-PAGE as described above.

p53-Seprion-ELISA

The aforementioned ten cancer cell lines were harvested at 70-80% confluency using Accutase (Sigma-Aldrich) and lysed with 1% Triton X-100/PBS for 30 minutes on ice. 12,500 cells per well were used as standard concentration. A 2.5% (w/v) lysate was prepared by lysing the pulverized tissues in the appropriate amount of ice-cold RIPA buffer complemented with protease inhibitor (Sigma-Aldrich). The lysates were incubated on ice for 5 minutes, immediately frozen on dry ice, and stored at -80°C until further analysis. All tissue samples were analyzed within 4 days after preparation.

The p53-Seprion-ELISA was performed as described previously (21). The tissue specimens were diluted 1:20 with ultrapure water and the anti-p53 (DO-1, Santa Cruz Biotechnologies) was used for the detection of p53 aggregates. All lysates were measured in triplicates and the average blank was subtracted from all sample replicates. The absorbance values were normalized to total protein concentration according to the recently published formula:

$$\frac{absorbance}{total\ \ protein} * 1000$$

Samples with a p53 aggregation value below 1 were considered as negtive and samples with a value greater than 1 were considered as positive.

Statistical analysis

The association between the categorical variables p53 protein expression, p53 aggregation, and histological subtypes of OC was determined using Cramer's V and Fisher's exact test. The level of significance was set at $p < 0.05$. All statistical analyses were performed using R Studio (version 4.0.3).

Results

Detection of p53 aggregates in cancer cell lines using co-immunofluorescence, proximity ligation assay, co-immunoprecipitation, and the p53-Seprion-ELISA

The aim of this study was to compare state-of-the-art co-immunofluorescence staining with novel technologies to detect p53 aggregates and assess the method's applicability in cancer cell lines as well as tumor tissue specimens. Nine ovarian and one cervical cancer cell lines were evaluated by co-immunofluorescence (co-IF), proximity ligation assay (PLA), co-immunoprecipitation (co-IP), and the p53-Seprion-ELISA.

By using co-IF we were able to detect a strong co-localization of the p53 and the A11 antibody in the nucleus of all cell lines carrying a *TP53* missense mutation (Figure 2A). In the cell lines OAW42 (wild-type) and COV504 (frameshift (FS) deletion), only single cells showed co-localization of both antibodies. In the cell line COV644 (wild-type) neither p53 nor A11 expression was detected. In the remaining cell lines (ME-180, 59M) no p53 expression was detected, but they were found positive for the expression of amyloid proteins. In a subset of cell lines (ME-180, OAW42, COV318, COV362, ES2, OVCAR3, 59M, and COV504) the co-localization of p53 and amyloid fibrils, detected by the OC antibody, was evaluated. Again, in all

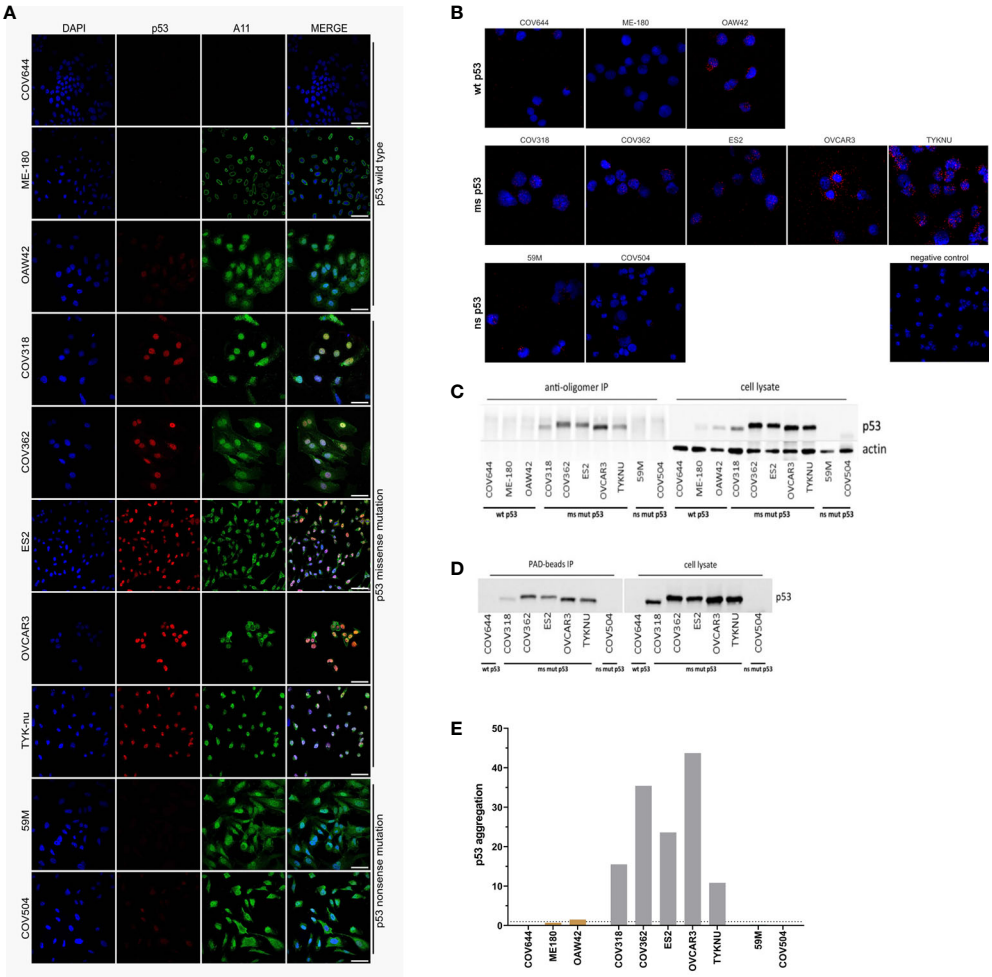

FIGURE 2
Detection of p53 aggregates in ovarian and cervical cancer cell lines. **(A)** Immunofluorescence co-localization assay (co-IF) using an anti-p53 (red) and an anti-oligomer (A11, green) antibody. Nuclear counterstaining was performed using DAPI. Scale bars: 50 µm. **(B)** Proximity ligation assay (PLA): red dots indicate p53 aggregates and nuclei in blue. **(C)** Co-immunoprecipitation (co-IP) was performed by using the anti-oligomer A11 antibody for the pull-down of amyloid proteins. Immunoblots for p53 were performed to show that p53 was present as oligomeric aggregates. **(D)** Amyloid proteins were immunoprecipitated with Seprion-coated beads (PAD-beads) and an immunoblot was performed to show that the aggregates consisted of p53. **(E)** The p53-Seprion-ELISA was performed to specifically detect p53 aggregates Dashed line, cut-off value for positive samples (p53 aggregation >1). Absorbance values were normalized to the total protein concentration. Grey, missense mutated cell lines; brown, wild-type p53 cell lines.

missense mutated cell lines (COV318, COV362, ES2, and OVCAR3) co-localization of both antibodies was detected. In the wild-type (ME-180 and OAW42) and nonsense mutated (59M and COV504) cell lines only OC staining but no p53 staining was detected (Supplementary Figure S1).

In contrast to co-IF, the proximity ligation assay (PLA) allows determining whether the p53 and A11 antibodies bind in close proximity, demonstrating that p53 is present as oligomeric aggregates. In concordance with the co-IF results, we could observe PLA signals in all missense mutated cell lines (Figure 2B). The number of PLA dots varied considerably between the different cell lines, with the most signals observed in the OVCAR-3 and the TYK-nu cell lines. Intermediate levels were observed in the ES-2 and COV362 cell lines, and a weak signal in the COV318. In contrast to the co-IF results, the COV504 cell line was PLA negative, whereas the nonsense mutated 59M cell line, which was co-IF negative, resulted in a low amount of PLA signals in single cells.

The third method, co-IP, also allows the specific detection of aggregated p53. The A11 antibody was used to isolate the amyloid fractions, followed by immunoblotting to verify if p53 is present as oligomeric amyloid. Again, in all missense mutated cancer cell lines p53 aggregates could be detected to various extents (Figure 2C). The highest amount of p53 aggregates was detected in OVCAR3 and COV362 cell lines. In contrast to co-IF and PLA, no p53 aggregates were detected in wild-type or

nonsense mutated cell lines. Furthermore, as an alternative to the A11 antibody, in seven cell lines, the Seprion ligand (PAD-beads) was applied to pull down the amyloid aggregates. The results of the Seprion-based co-IP were 100% concordant with the A11-based co-IP (Figure 2D).

Finally, the previously published p53-Seprion-ELISA was applied (Figure 2E). Consistently, p53 aggregates were detected in all missense mutated cell lines. The highest number of p53 aggregates was detected in the OVCAR3 and COV362 cell lines. None of the nonsense mutated cell lines and wild-type bearing cell lines, except the OAW42 cell line, formed p53 aggregates.

To sum up, all methods detected p53 aggregates in missense mutated cell lines (Table 1). The PLA was the only method detecting p53 aggregates in the 59M cell line, whereas only co-IF detected amyloid p53 in the COV504 cell line. In the OAW42 cell line all methods, except A11-based co-IP, showed the presence of p53 aggregates. In summary, in 7 out of 10 cell lines the applied methods showed 100% concordance.

Detection of p53 aggregates in ovarian cancer tumor tissue using three different methods

To validate our findings, co-IF, A11-based co-IP, and the p53-Seprion-ELISA were applied to detect p53 aggregates in ovarian cancer tissues. FFPE tissue specimens of 78 patients were analyzed using co-IF. Due to economic reasons (high costs, no tissue microarrays were available) the PLA was not applied on the FFPE samples. In a subset of 30 patients, fresh-frozen tissue was available; therefore, these patients were also analyzed using A11-based co-IP and the p53-Seprion-ELISA. The clinical pathological information of all patients is summarized in Supplementary Table S2. 51 out of 78 (65.4%) samples carried a *TP53* mutation. The most frequent mutations were missense mutations in 45 of 51 (88%) cases. In 5 of 51 (10%) cases FS deletions were present and in 1 of 51 (2%) cases a nonsense mutation was detected.

In 38 of 78 (48.7%) samples p53 aggregates could be detected by using co-IF (Figure 3A; Supplementary Figure S2, Supplementary Table S3). 33 out of 38 (87%) positive samples carried a *TP53* missense mutation, one sample a FS deletion, whereas the remaining four samples harbored wild-type p53. In 22 out of 38 (58%) positive samples strong co-localization in almost all cancer cells could be detected, whereas in the remaining 16 (42%) positive samples co-localization was detected in just a few cancer cells. Of note, there was a strong association with p53 protein expression (Cramer's V = 0.787, Fisher's $p<0.001$, Supplementary Table S4), however, 8 samples were p53 positive but A11 negative, suggesting that p53 protein expression did not necessarily lead to the formation of p53 aggregates. Moreover, we did not find a statistically significant association between histological subtypes and p53 protein expression or p53 aggregation (Supplementary Table S5).

The p53-Seprion-ELISA detected p53 aggregates in 15 of 30 (50%) fresh-frozen tissue samples (Figure 3B; Supplementary Table S3). By using co-IP, p53 aggregates were detected in 17 of 30 (57%) fresh-frozen tissue samples, all of them harboring a *TP53* missense mutation (Figure 3C; Supplementary Table S3). Tumors with *TP53* wild-type or FS deletions were negative. Again, all of the positive samples carried a *TP53* missense mutation. Interestingly, in both assays, of four samples carrying the R273H mutation, one showed a very high aggregation level, two samples showed moderate p53 aggregation, and one sample was negative.

TABLE 1 Comparison of the techniques applied in the detection of p53 aggregates *in vitro*.

Cell line	*TP53* status	Protein change	P53 aggregation detection method				
			co-IF	PLA	co-IP		p53-Seprion-ELISA
			(A11 x p53)	(A11 x p53)	(A11 x p53)	(Seprion ligand x p53)	(Seprion ligand x p53)
COV318	missense	I195F	+	+	+	+	15.5
COV362	missense	Y220C	+	+	++	++	35.4
ES2	missense	S241F	+	+	+	+	23.6
OVCAR-3	missense	R248Q	+	++	++	++	43.7
TYK-nu	missense	R175H	+	++	+	+	10.8
59M	FS deletion	H193KfsX49	–	+/-	–	n.e.	0.2
COV504	FS deletion	P322fsX13	+/-	–	–	–	0
COV644	WT	–	–	–	–	–	0.1
ME-180	WT	–	–	–	–	n.e.	0.7
OAW42	WT	–	+/-	+	–	n.e.	1.5
P53 aggregation positive			7/10	7/10	5/10	5/7	6/10

"-", negative; "+/-", only some of the cells show a (weak) signal; "+", positive; "++", strong signal; "n.e.", not evaluated; "FS deletion", frameshift deletion; "WT", wild-type.

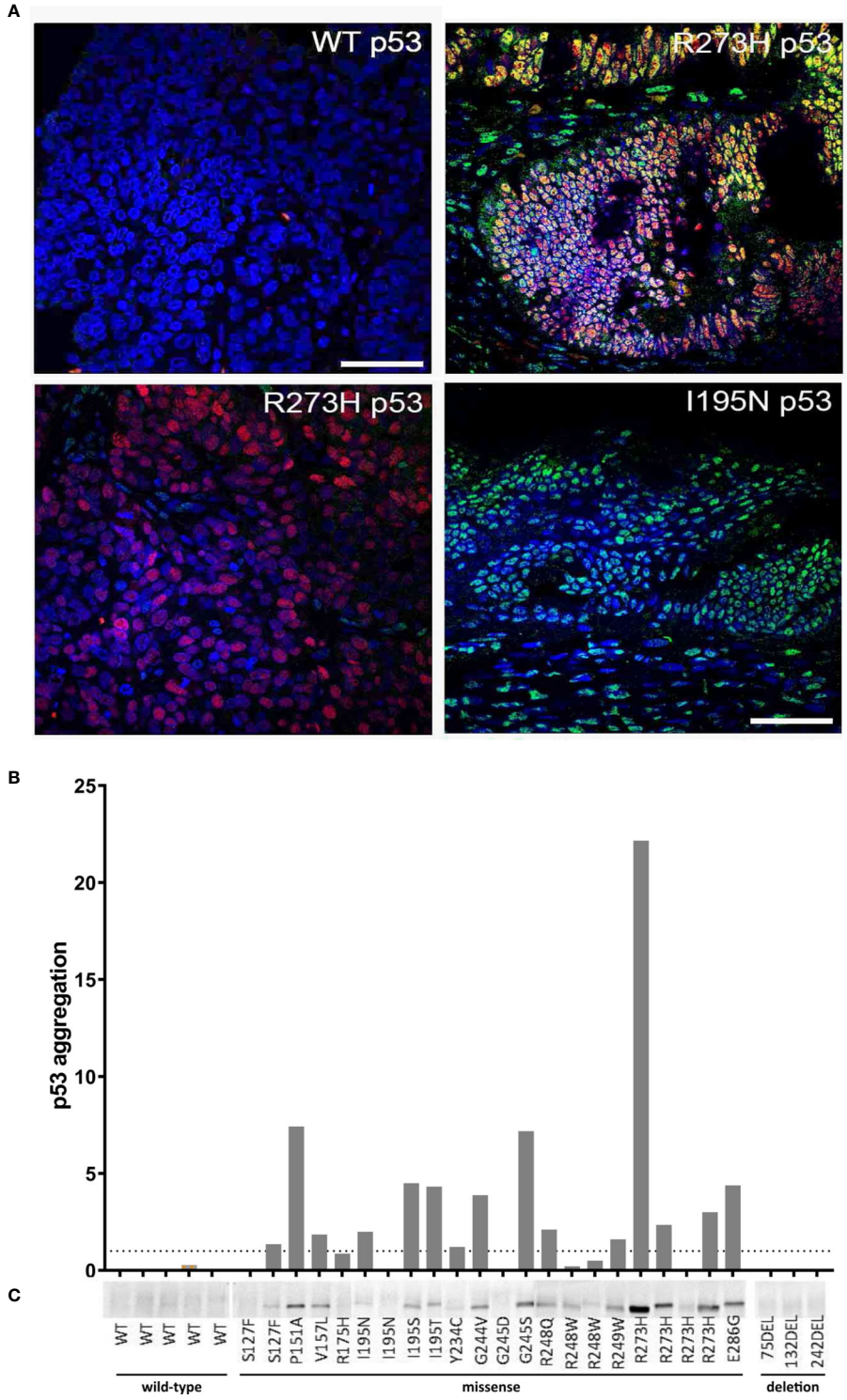

FIGURE 3
Detection of p53 aggregates in ovarian cancer (OC) tissue specimens. **(A)** Four representative OC FFPE tissue samples, which were analyzed by co-IF using an anti-p53 (red) and anti-amyloid (A11, green) antibody' are shown. Nuclear counterstaining was performed using DAPI. Scale bar = 50 µm. **(B)** Detection of p53 aggregates in 30 fresh-frozen OC tissue samples by using the p53-Seprion-ELISA. Absorbance values were normalized to the total protein concentration Dashed line, cut-off value for positive samples (p53 aggregation >1). **(C)** Co-IP was performed by using the anti-oligomer A11 antibody for the pull-down of amyloid proteins. Immunoblots for p53 were performed to show that the amyloid oligomers consisted of p53.

In summary, 100% concordance between all three methods could be achieved in 23 out of 30 (76.7%) samples. Co-IP and the p53-Seprion-ELISA, the methods that specifically detect p53 aggregates, showed concordance in 28 of 30 (93.3%) samples (Figure 4, Supplementary Table S3).

Discussion

Highly sensitive and specific methods are the pre-requisites for the evaluation of p53 aggregates in patients' biomaterials and interpretation of their clinical relevance. Our study provides a comprehensive comparison of four different technologies for the detection of p53 aggregates, involving state-of-the-art techniques as well as new innovative approaches (Figure 1). We were able to demonstrate 70% (cell lines) and 76.7% (tissue samples) concordance between optimized techniques.

Different conformational dyes and antibodies have been used to detect p53 aggregates by co-IF (5, 7, 27). Thioflavin and Congo Red are the traditional fluorescent dyes used for the detection of amyloid proteins. Nonetheless, both dyes have several limitations as they can cause false-positive results due to unspecific binding and Thioflavin does not detect amyloid oligomers and protofibrils (28). In our study, we focused on the A11 antibody that detects sequence-independent amyloid oligomers, but not monomers or fibrils, as with the OC antibody fewer p53 aggregates positive cell lines were identified (co-IF: OC: 4/8 positive cell lines vs A11: 7/10 positive cell lines; Supplementary Figure S1 and Figure 2A). In addition, the novel Seprion ligand, previously used for the detection of PrP^{Sc}, β-amyloid, α-synuclein, and huntingtin, was evaluated (22, 29–36). Co-IF, PLA, co-IP, and the p53-Seprion-ELISA showed 100% concordance in 7 of 10 (70%) cell lines (Table 1). Co-IF and PLA detected p53 aggregates in 7 of 10 cell lines, followed by the p53-Seprion ELISA (6/10), and A11-based co-IP (5/10). The Seprion-based co-IP resulted in 5/7 positive cell lines. When detecting p53 aggregates, it is critical to keep in mind that different types of amyloids are detected by the various conformation-dependent dyes, antibodies, and ligands. In the present study, the A11-based co-IF, PLA, and co-IP detect oligomer-like p53, while the Seprion-based approaches detect a wider range of amyloid proteins including also fibril-like p53 (Table 2). These differences might explain why we could not achieve 100% concordance between all applied techniques.

To evaluate the applicability of these methods in patient samples, primary ovarian cancer tissues were analyzed using co-IF, A11-based co-IP, and the p53-Seprion-ELISA. Although the PLA resulted in a high detection rate in the cell lines and would be the method of choice for the analysis of FFPE tissues, its application is rather expensive on large tissue sections and tissue microarrays should be the preferred sample type; however, these were not available. We show concordant results between the three techniques in 76.7% of samples and the p53 aggregates detection rate ranged from 48.7% to 56.7%. All positive samples harbored a *TP53* missense mutation. Additionally, co-IF detected four positive wild-type samples and one positive sample with a FS deletion. Moreover, in our study, the R273H missense mutation showed differences in the ability to form

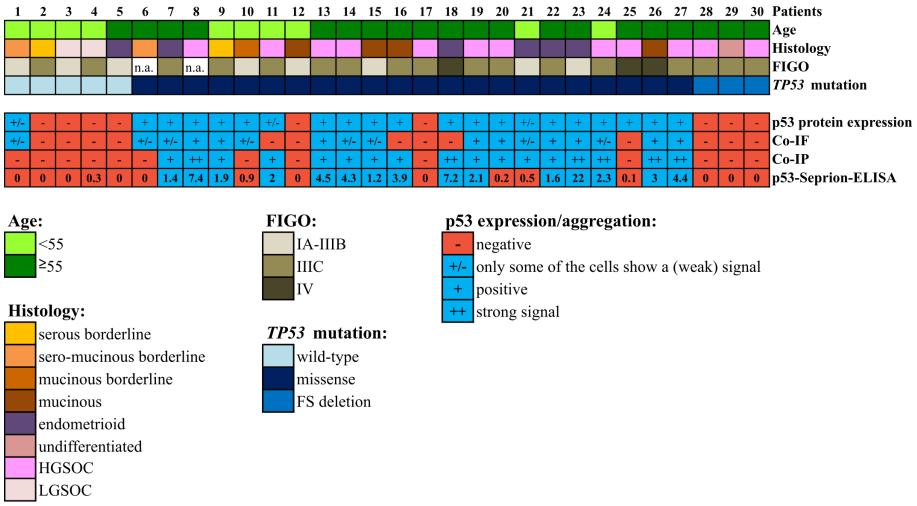

FIGURE 4
Comparison of the three techniques for the detection of p53 aggregates in 30 ovarian cancer tissue samples. Co-IF and co-IP were performed using the anti-p53 and the anti-oligomer A11 antibodies. "FIGO", International Federation of Gynecology and Obstetrics; "Co-IF", Immunofluorescence co-localization assay. "Co-IP", Co-immunoprecipitation; "HGSOC", high-grade serous ovarian cancer; "LGSOC", low-grade serous ovarian cancer. "n.a."; not available.

TABLE 2 Overview of methods for the detection of p53 aggregates in cell lines and tissue specimens.

	co-IF	PLA	co-IP	Seprion-based co-IP	p53-Seprion-ELISA
Binding agents	A11, p53 DO-1 antibody	A11, p53 DO-1 antibody	A11, p53 DO-1 antibody	Seprion ligand, p53 DO-1 antibody	Seprion ligand, p53 DO-1 antibody
Principle	Co-localization	Close proximity of two epitopes	Isolation of protein complexes	Isolation of protein complexes	Sandwich ELISA
What is detected	Amyloid p53 oligomers	Amyloid p53 oligomers	Amyloid p53 oligomers	Amyloid p53 oligomers, proto-fibrils, and fibrils	Amyloid p53 oligomers, proto-fibrils, and fibrils
Sample type	FFPE, cytopreparations	FFPE, cytopreparations	Fresh-frozen tissue and cell line lysates	Fresh-frozen tissue and cell line lysates	Fresh-frozen tissue and cell line lysates
Ease of use	Experience in fluorescence microscopy needed	Experience in fluorescence microscopy needed, Quantification of PLA dots requires either a scanning microscope with appropriate software or high-resolution images and subsequent analysis software (ImageJ, CellProfiler, …)	Easy	Easy	Very easy
Large scale	Only if applied on tissue microarrays	Only if applied on tissue microarrays	no	no	yes
Advantage	Use on FFPE, low costs, allows single-cell analysis	Use on FFPE, high sensitivity, high specificity, quantification, allows single-cell analysis	Semi-quantitative, high specificity	Semi-quantitative, high specificity	Quantification, high-throughput, high sensitivity, high reproducibility
Disadvantage	No quantification, limited specificity	Time-consuming, high costs	Fresh-frozen tissue needed, time-consuming, complex procedure, no single-cell analysis	Fresh-frozen tissue needed, time-consuming, complex procedure, no single-cell analysis	Fresh-frozen tissue needed, no single-cell analysis

amyloid-like structures in four ovarian cancer patients. For this mutation, a high propensity to form aggregates was reported previously in breast cancer (5). The positive wild-type samples as well as the varying p53 aggregation levels in patients with identical *TP53* mutation suggest that a missense mutation alone is not sufficient to increase the capability of p53 to form aggregates. It has been shown that inhibition of MDM2-mediated p53 degradation promoted the formation of wild-type p53 aggregates (12). Molecular interaction partners of p53 may also enhance the aggregation propensity of p53. For example, the transient interaction between mutant p53 (R175H) and the cellular chaperone heat shock protein 70 (HSP70) resulted in the increased half-life of mutant p53 and exposure of an aggregation-prone region. In the presence of MDM2, these two proteins can form amyloid-like aggregates (37). Furthermore, the heat shock protein 90 (HSP90) has been reported to interact with the p53 DNA-binding domain, leading to a structural change in the protein and the formation of a molten globule state, which is prone to aggregation (38). Additionally, the expression of specific p53 isoforms may have an impact on the capability of p53 to form amyloid-like structures as well. The wild-type Δ133p53β isoform has been shown to form aggregates in cancer cells and tumor biopsies (39). Moreover, the Δ40p53 isoform, which lacks the p53 transactivation domain, has been reported to have a high aggregation tendency in endometrial cancer cells (40).

The cell lines ME-180 and 59M as well as some of the tissue samples pointed out the limitations of the state-of-the-art co-IF and the need for more specific novel methods. These samples were p53 negative, but still showed abundant A11 staining (Figure 2A, Figure 3A), demonstrating that other amyloid proteins are present and co-localization of p53 and A11 antibodies in the same subcellular compartment does not necessarily mean that p53 aggregates are detected. The novel proximity ligation assay (PLA) is a powerful tool for the highly specific p53 aggregate detection as it only results in a fluorescent signal when the p53 and A11 antibodies are bound in close proximity indicating the presence of p53 aggregates. Another major advantage of the method is that the individual PLA dots can be quantified using freely available software tools such as ImageJ or CellProfiler (41–43). Moreover, the PLA can be performed on formalin-fixed paraffin-embedded (FFPE) tissue samples, which are often the only available source of clinical samples, whereas co-IP and ELISA are limited to fresh-frozen material. The disadvantages of the PLA are the extensive costs if tissue microarrays are not available and the relatively long duration of the procedure of 2 days. In contrast, the p53-Seprion-ELISA has a turnaround time of only 5 hours and

allows high-throughput analysis, but requires fresh-frozen tissue, which is often not archived in clinical routine.

In conclusion, we compared the state-of-the-art p53 aggregation detection method co-IF with co-IP and the novel PLA and Seprion technology. The PLA and p53-Seprion-ELISA are the only two methods allowing quantitative measurement of p53 aggregates. Taking into consideration that the most widely available source of tumor tissue is formalin-fixed and paraffin-embedded, the PLA outperforms co-IF in terms of sensitivity, specificity, and quantification of p53 aggregates. Wherever fresh-frozen material is available, the p53-Seprion-ELISA should be preferred as it allows rapid, high-throughput testing in contrast to co-IP. Our study provides the basis for the reliable detection of p53 aggregates in biological specimens to unravel the clinical significance of p53 aggregates, especially since potential p53-aggregation targeting drugs are currently under investigation and would open up new paths in cancer therapy. Moreover, mutated p53 is not the only tumor suppressor protein with enhanced aggregation tendency. In silico analyses demonstrated that protein aggregation is not a rare phenomenon, but far more common, and other tumor suppressor proteins, such as PTEN or Axin, have been identified to form amyloid-like structures (44–47). Our herein mentioned tools can be easily adapted to detect other types of amyloid-like proteins and help to evaluate their biological and clinical relevance in various cancer types.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of the Medical University of Innsbruck. The patients/participants provided their written informed consent to participate in this study.

Author contributions

RZ and NC designed the study. NH, KK, EM, AB, and EP performed the experiments and collected and interpreted the data. HF provided the clinical pathological information for all patients. NH, KK, and EM prepared the figures and tables, and wrote the manuscript. NH, KK, EM, AB, EP, HF, AZ, CM, RZ, and NC reviewed and revised the manuscript. All authors read and approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor EB declared a shared research group with the authors NC and RZ at the time of review.

Publisher’s note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.976725/full#supplementary-material

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Rationale for combination of paclitaxel and CDK4/6 inhibitor in ovarian cancer therapy — non-mitotic mechanisms of paclitaxel

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Taxanes and CDK4/6 inhibitors (CDK4/6i) are two families of successful anti-mitotic drugs used in the treatment of solid tumors. Paclitaxel, representing taxane compounds, has been used either alone or in combination with other agents (commonly carboplatin/cisplatin) in the treatment of many solid tumors including ovarian, breast, lung, prostate cancers, and Kaposi's sarcoma. Paclitaxel has been routinely prescribed in cancer treatment since the 1990s, and its prominent role is unlikely to be replaced in the foreseeable future. Paclitaxel and other taxanes work by binding to and stabilizing microtubules, causing mitotic arrest, aberrant mitosis, and cell death. CDK4/6i (palbociclib, ribociclib, abemaciclib) are relatively new cell cycle inhibitors that have been found to be effective in breast cancer treatment, and are currently being developed in other solid tumors. CDK4/6i blocks cell cycle progression at the G1 phase, resulting in cell death by mechanisms not yet fully elucidated. At first glance, paclitaxel and CDK4/6i are unlikely synergistic agents as both are cell cycle inhibitors that work at different phases of the cell cycle, and few clinical trials have yet considered adding CDK4/6i to existing paclitaxel chemotherapy. However, recent findings suggest the importance of a non-mitotic mechanism of paclitaxel in cancer cell death and pre-clinical data support rationale for a strategic paclitaxel and CDK4/6i combination. In mouse tumor model studies, drug sequencing resulted in differential efficacy, indicating complex biological interactions of the two drugs. This article reviews the rationales of combining paclitaxel with CDK4/6i as a potential therapeutic option in recurrent ovarian cancer.

KEYWORDS

chemotherapy, taxanes/taxol/paclitaxel, microtubules, mitosis, nuclear envelope, micronuclei, CDK4/6, drug resistance

Introduction

Taxane compounds are effective anti-mitotic cancer drugs which have successfully been used for more than 30 years, and are often cornerstones in the management of ovarian cancer today. These drugs work as microtubule stabilizing agents, interfering with mitosis of proliferating cancer cells. Another family of newly developed anti-cancer drugs, the CDK4/6 inhibitors (CDK4/6i), are effective in breast cancer treatment, and these inhibitors are actively being tested and expanded in other malignancies. CDK4/6i block cancer cell growth at the G1 phase of the cell cycle, while paclitaxel (and additional taxanes) targets cancer cells at the M phase (Figure 1). Additional mitotic inhibitors can act by blocking DNA replication (Figure 1), but there are no such agents with tolerable toxicity and sufficient efficacy available to be commonly used in clinics.

Although agents in either families are effective anti-cancer drugs, issues on efficacy, response rate, and development of drug resistance are limiting factors for both. An obvious interest is to combine these two useful classes of common anti-cancer drugs for more effective cancer treatment. New biological understanding of these agents may provide a rationale and

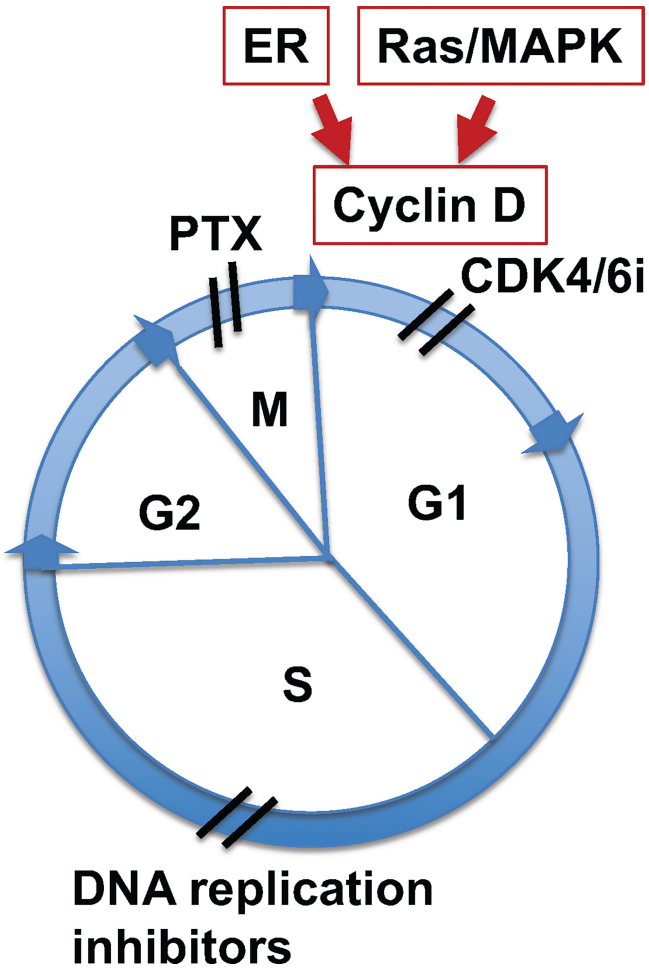

FIGURE 1
Paclitaxel and CDK4/6 inhibitors target different sites of the cell cycle. Illustration of sites of cell cycle targeted by paclitaxel and CDK4/6i. Mitogenic signaling by estrogen receptor (ER) or Ras/MAPK pathways induces cyclin D expression, which activates cyclin kinase 4 and 6 (CDK4/6) to initiate cell cycle through G1 phase. CDK4/6 inhibitors (CDK4/6i) block cycle kinase activities and arrest cells at early G1 phase. Paclitaxel (PTX) targets the function of spindle microtubules in cells at mitotic (M) phase, leading to aberrant mitosis and mitotic catastrophe. Additionally, mitotic inhibitors targeting DNA relication arrest cells at S phase.

strategy to develop an enhanced cancer treatment regimen using them in combination to capitalize on their potential synergistic mechanisms of action.

Taxanes as important common anti-cancer agents

Among many potential targets investigated for cancer therapy, stabilizing microtubules is one of the most effective strategies for cell kill *via* mitotic inhibition in many solid tumors (1–4). Paclitaxel is the first example of a microtubule stabilizing agent developed into a successful anti-cancer drug (4–7). Taxanes and non-taxane microtubule targeting agents remain common anti-cancer drugs, given their significant efficacy in multiple cancer types (4, 8–10). Taxol/paclitaxel, the first taxanes, was isolated from plant (Taxus brevifolia) as a cytotoxic anti-tumor agent (11–13). Currently, several taxane compounds, including paclitaxel, docetaxel, and cabazitaxel, are used as standard of care chemotherapeutic agents (14). Additional formulations of taxanes have been developed to improve delivery, including bound to albumin, and with additional nanoparticle carriers (15–18). Non-taxane microtubule stabilizing drugs, such as ixabepilone, are also tested and used in certain cancer types (9, 19, 20).

Paclitaxel is commonly used as a key component in front line therapy for epithelial ovarian cancer, and is given in combination with a platinum agent (cisplatin or carboplatin) (21–25). It also is utilized as a single agent in a dose dense (weekly) schedule to treat recurrent and drug (platinum agent)-resistant ovarian cancer (26–28). However, recurrent ovarian cancer progressively becomes refractory to continuous paclitaxel treatment, and the severity of side effects, such as peripheral neuropathy, correlates with accumulative drug dosage and often necessitates dose-reductions (29–33). Thus, strategies to enhance paclitaxel efficacy and to counter drug resistance are highly desirable and are actively sought (33–35). One strategy is to find potential synergistic combination with additional new agents, such as CDK4/6i.

Paclitaxel in microtubule stabilization, mitotic mechanisms, and mitotic catastrophe

Paclitaxel, and all other taxane and non-taxane microtubule stabilizing drugs, act by binding to alpha-tubulin subunits within microtubules, resulting in stabilization of the filaments (36–39). The discovery of this unique cytotoxic mechanism occurred in the 1970s-80s (4, 7, 40, 41), when paclitaxel was first extracted from the bark of the Pacific Yew tree (4, 6, 11–13). By interfering with microtubules in mitosis, paclitaxel causes cell growth arrest at M-phase by cytoskeleton paralysis (Horwitz, 1994; 42), and subsequent cell death by apoptosis (43, 44). However, the molecular details on the initiation of apoptosis by paclitaxel have been elusive. Some studies suggest that paclitaxel-mediated cancer cell death is independent of caspase activation and does not follow a classic mechanism of apoptosis (45, 46). In laboratory study and comparision of a panel of tumor lines treated with paclitaxel in xenograft tumor models, neither degree of mitotic arrest nor apoptosis appeared to correlate with the anti-tumor effect of paclitaxel (47). Furthermore, paclitaxel anti-tumor activity is also independent of p53 mutational status of the tumors (47).

Paclitaxel-treated cancer cells arrested at M-phase often then undergo aberrant mitosis (known as mitotic slippage), resulting in the formation of multiple micronuclei and consequential death (mitotic catastrophe) (41, 48–51). Moreover, both laboratory and clinical observations led to the thinking that in addition to acting as a mitotic inhibitor, paclitaxel has cytotoxic activity against cancer cells with non-mitotic mechanisms (29, 52–57). Proposed non-mitotic paclitaxel mechanisms include paclitaxel-induced phosphorylation of apoptotic protein bcl-2 (58), disruption of microtubule-mediated cellular transport (52), physical breaking of nuclear envelope by rigid microtubule bundles (59), stimulating of inflammatory activity by paclitaxel-induced nuclear fragmentation (60), and anti-angiogenic activity by damaging endothelial cells (61–64).

The concept of a non-mitotic mechanism for paclitaxel action is re-enforced by the lack of efficacy of mitotic inhibitory drugs developed more specifically to target mitotic machineries (65, 66). A better understanding of the non-mitotic mechanism and the complex processes underlying cancer cell kill is crucial to design drug combinations with taxanes to optimize rates of response and overcome taxane drug resistance.

Non-mitotic mechanisms of paclitaxel in inducing micronucleation and cell death by nuclear membrane rupture

Laboratory studies are fairly convincing that highly proliferative cells are sensitive targets for paclitaxel, as the drug preferentially kills proliferative cancer cells, which are more likely to be in M-phase (4, 7, 29, 41). Taxanes, however, also affect continuously growing non-cancer cell populations such as hair follicle matrix keratinocytes (67) and hemopoietic cells (32). Thus, the major side effects of paclitaxel include alopecia and neutropenia.

In contrast to cell culture models, only a small fraction of tumor cells *in vivo* are proliferative; despite this, most of the

cancer cells in patient tumors are sensitive to paclitaxel (53, 54). Moreover, cell killing efficacy does not correlate with mitotic index (47, 54). Experimenal and clinical observations suggest that paclitaxel also kills cancer cells at non-mitotic phases, and interfering with the function of microtubules in G1 or S phases of the cell cycle also contributes to cancer cell killing (53–57). A new study suggests that in paclitaxel-treated cancer cells, the stabilized and rigid microtubule bundles around the cancer cell nucleus pull the nuclear envelope membrane by physical force into multiple micronuclei (59, 68) (Figure 2). This finding provides a new addition to the well-accepted notion that paclitaxel acts as a mitotic inhibitor. Thus, in addition to proliferation, a malleable nuclear envelope caused by a defective nuclear envelope structural proteins (69a) provides another specificity of cancer cells for killing by paclitaxel, as non-neoplastic cells have a sturdier nuclear envelope and are more resistant to paclitaxel-induced breaking (59, 68).

The formation of numerous micronuclei following paclitaxel treatment (45, 70), referred to as "micronucleation" (60), may be the consequence of both aberrant mitosis (41, 49–51, 71) and nuclear breaking in non-mitotic cells with a weakened nuclear envelope (59, 68). In the presence of several types of pharmaceutical compounds (including CDK4/6i) to inhibit mitosis, paclitaxel was observed to induce micronucleation, suggesting a non-mitotic mechanism to break up the cancer nucleus (59, 68). These small micronuclei are observed to be unstable and often undergo sudden and irreversible rupture (72, 73). A likely reason is that the nuclear membrane is stretched in micronucleation, as the combined surface of multiple smaller spheres is much larger than a single sphere with the same volume. Either by mitotic or non-mitotic mechanisms, the formation of multiple micronuclei is likely important for the efficacy of paclitaxel in killing cancer cells (60, 68). One possible mechanism is that the genomic DNA released will trigger the cGAS-Sting cytoplasmic DNA sensing pathway to activate the inflammatory pathway (60, 74). Nevertheless, the rupture of the nuclear membrane, essentially compromising a key cellular organelle, may be sufficient to assume the demise of the paclitaxel-treated cancer cells (46, 59) (Figure 2).

Clinical efficacy of CDK4/6 inhibition

Small molecule compounds specifically targeting cell cycle kinases, including the CDK4/6 inhibitors, are new agents found to have activity in cancer treatment (75), and are commonly used in metastatic breast cancer. Additional indications in other solid tumors are currently under investigation (76–80).

The study of the mammalian cell cycle over several decades and the ultimate successful application of the knowledge to cancer therapy took a long road (80–82). Based on the identification and understanding of the cyclin-dependent kinase 4 (CDK4) and CDK6, the activator such as cyclin D1, and their multiple cyclin inhibitors, the concept of an inhibitor for cell cycle kinases to block cell cycle progression and tumor growth seems obvious (81). The first CDK4/6i to be developed and tested in clinical trial was palbociclib; however, the lack of efficacy of monotherapy in early studies limited the enthusiasm and delayed the clinical development. Fortunately, later trials showed a clear benefit of adding palbociclib to hormone antagonism therapy in metastatic breast cancer, leading to FDA approval of palbociclib in early 2015 (78–81). The details in the laboratory discoveries and clinical development of the CDK4/6i have been well reviewed in these (78–81) and many additional recent articles.

Following the initial success, many pharmaceutical companies independently developed additional CDK4/6i and are testing for their utility in combination therapy. Today,

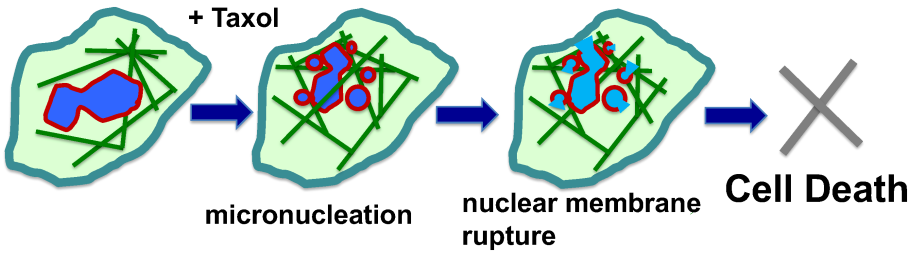

FIGURE 2
Mechanisms of paclitaxel-induced multiple micronucleation and nuclear membrane rupture in cancer killing. Paclitaxel (Taxol) induces mitotic catastrophe, resulting in micronucleation. In non-mitotic cells, the rigid microtubule filaments induced by paclitaxel can promote massive formation of micronuclei through nuclear budding of cells during interphase. The paclitaxel-bound rigid microtubule bundles pull and distort the nuclear envelope structure. As a result, the malleable cancer nuclear envelope breaks into multiple micronuclei (micronucleation). The proposal of physical force exerted by paclitaxel-induced rigid microtubule filaments in breaking malleable cancer nuclei provides a non-mitotic mechanism to generate multiple micronuclei. Paclitaxel also induces rigid microtubules and the breaking of nuclei of neoplastic cells and the formation of multiple micronuclei in both. The micronuclei derived from both mitotic and non-mitotic cells are defective in membrane structure and have high propensity for rupture and release of chromatin materials, resulting in cell death.

several CDK4/6 inhibitors, Ibrance (chemical name: palbociclib, developed by Pfizer.), Kisqali (chemical name: ribociclib, developed by Novartis), Verzenio (chemical name: abemaciclib, developed by Eli Lilly), have been developed and approved to treat metastatic breast cancer (78–80). Numerous clinical trials are ongoing to assess CDK4/6i in combination therapy to treat breast and additional cancer types. However, little information of CDK4/6i in ovarian cancer treatment has been reported yet, though substantial interests prompt ongoing efforts to evaluate a potential role in ovarian cancer treatment (83, 84).

De novo and acquired resistance to the combined treatments have been frequently observed, and alterations in both Rb and cell cycle regulation, and PI3K survival signaling pathway are potential mechanism of resistance (82, 83, 85). The CDK4/6i are exciting new drugs for cancer therapy, and ongoing studies and trials surely will add new mechanistic understanding to and improvement of clinical outcomes. Yet development of resistance to CDK4/6 inhibitors is already recognized as a limitation to this class. The rapidly accumulating information should allow the contemplation of strategy and design of rationale combinatorial therapies of CDK4/6i with other anti-cancer agents to overcome drug resistance and achieve superior treatment outcomes (80, 83, 86).

Combination therapy: Rationale for synergy between paclitaxel and CDK4/6 inhibitors

The utility of CDK4/6 inhibition as a component in a combined therapy regimen with additional agent(s) is an area of active investigation as CDK4/6i by itself lacks sufficient activity (81, 84, 86). One potential mechanism of synergy is that both inhibition of the mitogenic signaling pathway that regulates D-type cyclins, and blocking of CDK4/6 activities, are necessary for a synergized therapy to prevent tumor cell proliferation (81). Paclitaxel and CDK4/6i are expected to be antagonists, since arresting cells by CDK4/6i at the G1 phase of the cell cycle presumably limits cell kill by paclitaxel, which targets cells at M-phase. Consistently, in laboratory studies, CDK4/6 inhibitors were shown to reduce and prevent apoptosis of hair follicle matrix cells that normally results from paclitaxel treatment (67), and the inhibitors also rescued hematopoietic cell death from paclitaxel treatment (87). Thus, CDK4/6i, when used strategically, may reduce some side effects of paclitaxel treatment.

Although not yet met with general enthusiasm because of the theory of antagonism and some preliminary observations, clinical trials for a paclitaxel/CDK4/6i have been attempted and initiated for solid tumors (for example, NCT 04594005). So far, no outcome has been reported. Pre-clinical studies of a paclitaxel/CDK4/6i combination have been attempted and reported, some with positive results (84, 88–90). In breast cancer cells, although simultaneous exposure to palbociclib and paclitaxel produced an antagonistic effect, sequential treatment caused higher cell death than single agent alone (88). The authors suggested pretreatment with CDK4/6i may enhance the efficacy of paclitaxel for chemotherapy of triple negative breast cancer (88). CDK4/6 inhibition was found synergistic in combination with paclitaxel to suppress growth and induce apoptosis in K-Ras mutant lung adenocarcinoma cells (90). In the cases of lung cancer cells, addition of paclitaxel first followed by CDK4/6i had higher cancer cell killing than the reversed sequence (89).

Another pre-clinical study found that the sequences for the administration of the two drugs produced differential efficacy in mouse pancreatic tumor xenograft models (91, 92). In the study, treatment first with paclitaxel followed by CDK4/6i produced better tumor suppressing activity than when CDK4/6i was administrated first. The authors suggest that CDK4/6i impairs the ability of cancer cells to recover from chromosomal and DNA damage caused by prior treatment with paclitaxel (91).

With the realization of the non-mitotic mechanism of paclitaxel in killing cancer cells (68), a new rationale may motivate the study of adding CDK4/6i to paclitaxel regimen, especially to the dose dense treatment of metastatic breast and recurrent ovarian cancer (Figure 3). Furthermore, ongoing study will yield additional understanding of the potential mechanism(s) of CDK4/6 inhibition in damaging cancer cells, in addition to the cytostatic effects. An initial treatment of cancer cells with CDK4/6 inhibitors may prevent mitosis-targeting mechanism of paclitaxel cytotoxicity (Figure 3A). A possible better strategy may be first to allow full attainment of the robust cytotoxic activity of paclitaxel alone to the mitotic cancer cell population before exposing the remaining cells to CDK4/6 inhibitors when non-mitotic paclitaxel killing mechanism still can occur (Figure 3B). Additionally, inhibition of CDK4/6 may impair the recovery of damaged and micronucleated cancer cells from prior exposure to paclitaxel, further enhancing the efficacy (91).

Paclitaxel exhibits high activity against mitotic cells, but also can kill non-mitotic cancer cells (59, 68), such as that are expected to accumulate in the presence of CDK4/6 inhibition. Thus, the possibility of a paclitaxel and CDK4/6i combination as a chemotherapy regimen for ovarian cancer exists. With a well-considered drug scheduling to avoid antagonism and fostering the synergy of the two drugs, a treatment with higher efficacy and overcoming drug resistance to both paclitaxel and CDK4/6i may be developed.

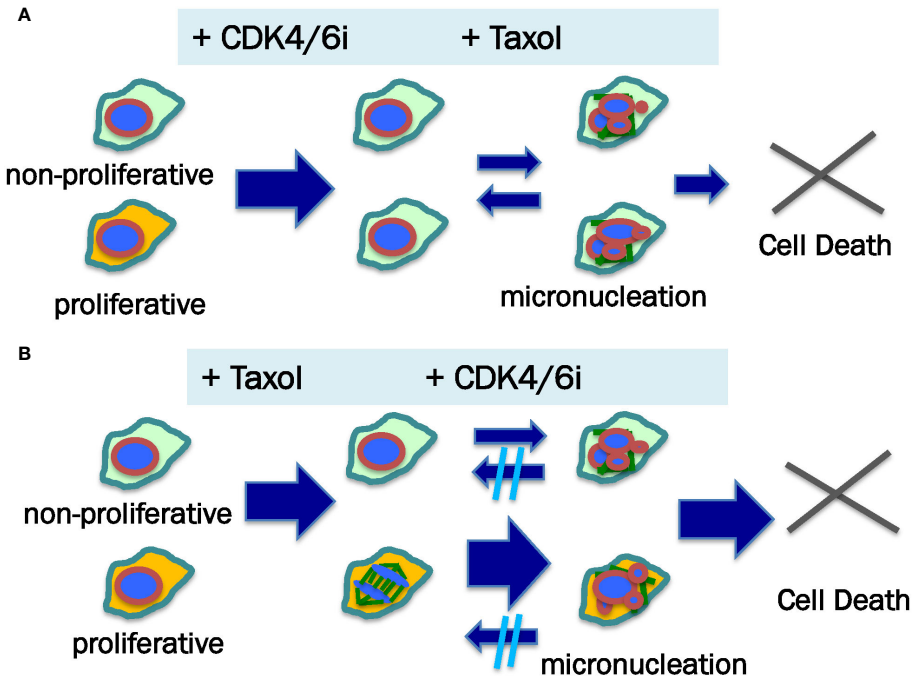

FIGURE 3
Proposed mechanism for sequence-dependent of paclitaxel and CDK4/6 inhibitor in killing cancer cells. Neoplastic cells within a tumor comprise proliferative (illustrated as yellow color cytoplasma) and non-mitotic (illustrated as green color cytoplasma) populations, which may respond to anti-cancer agents differently. **(A)** When CDK4/6i is added prior to paclitaxel (Taxol), the cancer cells are arrested at G1 phase, producing both cytotoxic and cytostatic effects. Subsequently, paclitaxel induces micronucleation and death of the non-mitotic cells. Some of the micronucleated cells may be able to recover. **(B)** In the case of paclitaxel addition first followed by CDK4/6i, both proliferative and non-mitotic cell populations undergo micronucleation, though mitotic cells more readily than non-mitotic cells form multiple micronuclei following paclitaxel stimulation (illustrated by small and bigger arrows). It is postulated that CDK4/6i treatment impairs the recovery of paclitaxel-induced damage to the nuclear structure (micronucleation). Thus, paclitaxel — CDK4/6i may have a higher cell killing outcome than CDK4/6i — paclitaxel sequences.

Prospective: Clinical trial design — drug scheduling and sequence

Currently, paclitaxel and additional taxane compounds are the key drugs in the management of several major solid tumors, as frontline therapy and salvage option. Eventual development of resistance and accumulative side effects limit the continuous application of the drugs. Thus, the possibility of adding the new anti-cancer drug, CDK4/6i, to the paclitaxel regimen is highly desirable to increase drug potency and overcome resistance (80, 86). With the findings of a non-mitotic mechanism of paclitaxel (59, 68), and the observation of differential activity of drug administrative sequences (91), a therapeutic trial may be designed with these rationales, as an example discussed above (Figure 3).

One approach may be the addition of CDK4/6i to dose dense paclitaxel treatment in patients with recurrent ovarian cancer and metastatic breast cancer (Figure 4). It may be suitable to use paclitaxel alone in the first two of a 7-cycle chemotherapy schedule, to eliminate most active proliferating cancer cells. In the subsequent 5 treatment cycles. CDK4/6i may be given in the last two days, based on the hypothesis that inhibition of CDK4/6 impairs the recovery of the damaged cancer cells following exposure for the previous 5 days with paclitaxel (91). Although paclitaxel is rapidly cleared from the circulation (1, 93), the drug is sequestered and persists within cells for several days (39, 94–96), where the drug stabilizes microtubules and produces additional cytotoxicity.

The proposed two drug combination and schedule has the benefit of both mitotic and non-mitotic mechanisms of paclitaxel action, plus growth inhibition and cytotoxicity bestowed by CDK4/6 inhibition, and thus are predicted to be a more effective therapy. It is not clear if there will be significant change in the side effect profile of either drugs when given in combination in the schedule designed. Both paclitaxel and CDK4/6i have tolerable side effects, and are both routinely used in clinics currently. The major side effects of paclitaxel are well documented: neutropenia/myelosuppression, alopecia, and peripheral neuropathy (32). No surprisingly, CDK4/6i suppresses cell proliferation and causes neutropenia/myelosuppression and alopecia (97). However, both agents inhibit cell cycle progression and may not be additive for cytotoxicity, and may be even antagonistic, as shown by

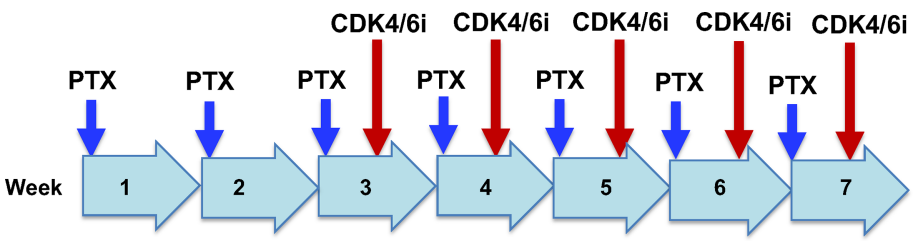

FIGURE 4
Potential clinical trial design for paclitaxel/CDK4/6i combination chemotherapy for recurrant ovarian cancer. Drug administration and schedule are illustrated for a hypothetical dose dense regimen of paclitaxel treatment of recurrant ovarian cancer. Paclitaxel (PTX) will be given alone in weeks 1 and 2. In weeks 3 to 7, paclitaxel will be given on day 1, and CDK4/6i will be administrated on day 6 of the week.It is postulated that paclitaxel alone in week 1 and 2 will eliminate the majority of proliferative cancer cells. In subsequent weeks, CDK4/6i given on day 6 will impair the recovery of damaged cancer cells from exposure to paclitaxel during the previous 5 days.

preclinical findings for CDK4/6 inhibitors in protecting paclitaxel-caused hair follicle damage (67), or paclitaxel in myelosuppression (87). Thus, side effects such as myelosuppression/neutropenia and alopecia may be lessened or more severe, as results of either protection of paclitaxel damage by CDK4/6i, or the combined damage to the stem cells, respectively. This rationale for a potential sequential drug administration based on the paclitaxel dose dense regimen (Figure 4), derived from pre-clinical studies and consideration, will only be verified or disproved by a clinical trial in cancer patients.

Author contributions

ES: developed concept, produced and analyzed research data, and edited manuscript; MH: developed concept and edited manuscript; MS: participated in concept development and edited manuscript; SG: participated in concept development and edited manuscript; X-XX: developed concept and wrote first draft of manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

MS is on the Stategic Council for GlascoSmithKlein (GSK).

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Impact of perioperative red blood cell transfusion, anemia of cancer and global health status on the prognosis of elderly patients with endometrial and ovarian cancer

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Introduction: Perioperative red blood cell (RBC) transfusions have been associated with increased morbidity and worse oncological outcome in some solid neoplasms. In order to elucidate whether RBC transfusions themselves, the preoperative anemia of cancer (AOC), or the impaired global health status might explain this impact on patients with endometrial cancer (EC) or ovarian cancer (OC), we performed a retrospective, single-institution cohort study.

Materials and methods: Women older than 60 years with EC or OC were included. The influence of RBC transfusions, AOC, and frailty status determined by the G8 geriatric screening tool (G8 score), as well as the clinical-pathological cancer characteristics on progression-free survival (PFS) and overall survival (OS), was determined by using the Kaplan-Meier method and the Cox regression analyses.

Results: In total, 263 patients with EC (n = 152) and OC (n = 111) were included in the study. Patients with EC receiving RBC transfusions were faced with a significantly shorter 5-year PFS (79.8% vs. 26.0%; $p < 0.001$) and 5-year OS (82.6% vs. 25.7%; $p < 0.001$). In multivariable analyses, besides established clinical-pathological cancer characteristics, the RBC transfusions remained the only significant prognostic parameter for PFS (HR: 1.76; 95%-CI [1.01–3.07]) and OS (HR: 2.38; 95%-CI [1.50–3.78]). In OC, the G8 score stratified the cohort

in terms of PFS rates (G8-non-frail 53.4% vs. G8-frail 16.7%; p = 0.010) and AOC stratified the cohort for 5-year OS estimates (non-anemic: 36.7% vs. anemic: 10.6%; p = 0.008). Multivariable Cox regression analyses determined the G8 score and FIGO stage as independent prognostic factors in terms of PFS (HR: 2.23; 95%-CI [1.16–4.32] and HR: 6.52; 95%-CI [1.51–28.07], respectively). For OS, only the TNM tumor stage retained independent significance (HR: 3.75; 95%-CI [1.87–7.53]).

Discussion: The results of this trial demonstrate the negative impact of RBC transfusions on the prognosis of patients with EC. Contrastingly, the prognosis of OC is altered by the preoperative global health status rather than AOC or RBC transfusions. In summary, we suggested a cumulatively restrictive transfusion management in G8-non-frail EC patients and postulated a more moderate transfusion management based on the treatment of symptomatic anemia without survival deficits in OC patients.

KEYWORDS

prognosis, ovarian cancer, endometrial cancer, transfusion, anemia of cancer, frailty

Introduction

It is still a matter of debate whether transfusions of red blood cells (RBC) alter the survival prognosis of patients with oncologic diseases or not (1). Furthermore, it remains unclear if RBC transfusions themselves, the underlying anemia of cancer (AOC), or the preoperative global health status influence the outcome in addition to the conventional tumor entity–specific risk factors (2–4).

Despite the effort of restrictive transfusion strategies, the transfusion indication of RBCs is still an essential treatment component especially in almost frail elderly cancer patients, requiring a major tumor reductive debulking surgery (5–7). Perioperative surgical transfusion rates in patients with gynecological malignancies range from 3% (8) to 77% (9–12). Transfusions of allogeneic RBCs can be life-saving in many circumstances and represent one of the main advances of modern medicine, particularly in oncology (13). Overall, RBC transfusions are safer than they have ever been, but there are still significant risks and impaired postoperative outcomes (14–16). Increased cancer recurrence rates and the risk of developing new malignancies are reported in transfused patients affected by solid cancers, mainly in colorectal and gastroesophageal cancer (17–19). The negative effects rely possibly on the transfusion-related immune modulations (20–23) because the activity of natural killer cells and T lymphocytes is reduced by allogeneic RBC transfusions (24). However, these endogenous defense cells are required to prevent quiescent cancer cell dissemination (25). With the exception of cervical carcinoma, the current literature on the effect of RBC transfusions among gynecological cancer patients is limited and partially controversial (14, 26–28).

AOC, one main reason for perioperative RBC transfusions, has been shown to be independently associated with an increased risk of adverse postoperative complications and an increased length of intensive care unit and hospital stay (29). Approximately every second cancer patient scheduled for major oncologic surgery while being anemic even prior to surgery at the time of diagnosis (30). Bleeding, nutritional deficiencies, hemolysis, reduced erythropoietin levels, and inflammation with increased hepcidin activity cause AOC (31). The state of functional iron deficiency as a well-established squeal of chronic anemia is often regarded as a consequence of chronic illness (32). Moreover, perioperative RBC transfusions due to severe AOC have been analyzed as an independent risk of poorer outcomes and adverse events in 941,496 operations from various disciplines (33). Finally, anemic patients undergoing cancer-related therapies suffer more often from advanced oncologic

Abbreviations: AOC, Anemia of cancer; BMI, Body mass index; CGA, Comprehensive Geriatric Assessment; 95%-CI, 95%-confidence interval; DSS, disease-specific survival; EC, endometrial cancer; ECOG, Eastern Cooperative Oncology Group; EQUATOR, Enhancing the QUAlity and Transparency Of health Research; FIGO, International Federation of Gynecology and Obstetrics; G8 score, G8 geriatric Screening tool; HR, hazard ratio; MNA, Mini Nutritional Assessment; OC, ovarian cancer; OS, overall survival; OR, odds ratio; PFS, progression free survival; RBC, red blood cell; SCS, Surgical Complexity Score; SIOG, International Society of Geriatric Oncology; STROBE, Strengthening the reporting of observational studies in epidemiology.

diseases and present more often with a limited global health status (34).

To which extent global health status besides AOC and entity-specific clinical-pathological cancer characteristics might influence the overall outcome depends on the following considerations. Gynecological malignancies mainly affect elderly patients (35, 36). Women with endometrial (EC) or ovarian cancer (OC) are at high risk of RBC transfusions due to a multitude of cancer and treatment-related factors (37, 38). In addition to the high median age of approximately 68 years at diagnosis (39) increased age is often associated with more aggressive and advanced diseases and requires extended, curatively intended surgical procedures (40, 41). However, the population older than 65 years of age is less likely to be treated in accordance with the recommendations of internal guidelines resulting in an overall worse outcome in elderly cancer patients (42, 43). Advanced diseases are often associated with an impaired individual global health status and malnourishment in the elderly population. This causes a not negligible impact on the decision to perform extensive surgical cytoreduction, possibly with multi-visceral resections to achieve complete macroscopic tumor resection (44–46). The "phenotype of frailty" can be defined as a multidimensional aging-related clinical syndrome of decreased homeostatic reserves and function due to various organ systems which could form a non-standardized definition of the global health status, especially in elderly cancer patients (47, 48). Frail patients are characterized by vulnerability to adverse health outcomes as well as the combination of dysregulation across various physiologic and molecular pathways (49). Various global health assessment tools exist in order to detect preoperative frailty (48, 50, 51). The G8 geriatric screening tool (G8 **s**core) is one of the most commonly used rapid geriatric screening questionnaires (52, 53). The G8 **s**core has been evaluated especially in oncological-surgical disciplines because of its main focus on nutrition, mobility, and comorbidities (54, 55).

We try to elucidate the impact of RBC transfusions, AOC, and the pre-surgical global health status on the outcome of elderly patients with EC and OC in a retrospective, single-institution cohort study.

Materials and methods

Study population

This retrospective cohort analysis reports data from women older than 60 years of age surgically treated at the University Medical Center Mainz – Johannes-Gutenberg University Mainz, Germany, between January 2008 and December 2019. Patients at all stages of EC and OC who were being operated on with curative intent were screened and included if they fulfilled the following criteria: 1) Patients with EC receiving a standardized primary staging operation including hysterectomy and bilateral salpingo-oophorectomy, with or without pelvic and para-aortic lymph node resection, depending on tumor stage, histological grade of differentiation, and histological subtype. 2) Patients with OC who underwent primary or interval tumor debulking surgery with maximal surgical effort. 3) Patients for whom the determination of the G8 **s**core was possible. 4) OC and EC patients for whom complete follow-up information was available.

Data collection

General patient information was gathered from our electronic hospital database SAP (Walldorf, Germany, 1972) and the archives, including clinical-pathological cancer characteristics such as tumor stage [TNM and FIGO (International Federation of Gynecology and Obstetrics) classification system (56) and histological grade] and surgical parameters (e.g., amount of blood transfusions, blood loss, or operating time). Perioperative RBC transfusions were defined as any transfusions within 24 h preoperatively, during surgery, or 24 h after surgery. Transfusions due to surgical bleedings within operative revisions were not included in the final evaluation. Preoperative hemoglobin was taken from the electronic patient record. The cutoff for preoperative anemia was chosen with a hemoglobin <12 g/dl, according to the definition of the World Health Organization for women (57). The patients' preoperative global health status was retrospectively assessed with the G8 **s**core based on the routine pre-surgical patient evaluation. This process has previously been described elsewhere (54, 58). Long-term follow-up including progression-free survival (PFS) and overall survival (OS) was performed by telephone calls, written inquiries to the patients or their physicians, and by checking the patient clinical records up to February 2021.

Clinical-pathological cancer characteristics and intraoperative treatment parameters

Clinical-pathological cancer characteristics were collected from patients' charts. Standardized operation reports were reviewed to extract the information on intraoperative blood loss, cut-seam time, and surgical radicality using the surgical complexity score (SCS). SCS was established by Aletti et al. in order to categorize the maximal surgical effort into a low, intermediate, and high level of complexity (44).

Frailty assessment – G8 geriatric screening tool

The G8 geriatric screening tool (G8 **s**core) established by Bellera et al. in 2012 was chosen as one of the most frequently used frailty evaluation tools recommended by the International Society of Geriatric Oncology (SIOG) to characterize the preoperative global health status (53). As a simple, time-saving, and reproducible questionnaire, the G8 **s**core consists of seven items from the Mini Nutritional Assessment (MNA) questionnaire with predefined answer options in combination with the chronological age (59–61). The several items assessed in the G8 **s**core are routinely recorded through a standardized health status self-assessment questionnaire in accordance with the MNA as a standard procedure during the pre-surgical consultation. Adding the missing item "biological-calendar age" allows us to calculate the G8 **s**core retrospectively for each patient. The main categories arise from physical performance status and mobility, nutrition, and comorbidities in combination with polypharmacy. The scoring system ranges from 17 points (not impaired at all: G8-non-frail) to a minimum of 0 points (heavily impaired: G8-frail) using the validated cutoff value of ≤14 points as an indicator of frailty (52). In various surgical disciplines, the G8 **s**core is validated to preoperatively identify frail patients, who could benefit from a full comprehensive geriatric assessment (CGA) after a two-step evaluation before major surgery (62).

Statistical analyses

The manuscript was written following the STrengthening the Reporting of Observational Studies in Epidemiology (STROBE)—a cohort checklist of the Enhancing the QUAlity and Transparency Of health Research (EQUATOR) network reporting guidelines (63). Statistical analyses were performed with the use of the SPSS statistical software program, version 27.0.1 (SPSS Inc, Chicago, IL, U.S.A.). Patients' characteristics are given in absolute and relative frequencies (categorical data). The frequency of distribution of categorical variables was compared with Fisher's exact test. For continuous data, normal distribution was explored using the Shapiro-Wilk test. Between-group differences (e.g., transfused vs. non-transfused or "G8-frail" vs. "G8-non-frail") were explored using either the Mann-Whitney U-test or a t-test to evaluate for significant differences. A *post-hoc* power calculation was used to underline the sufficient number of subjects using an alpha error rate of 0.05. The Cox proportional hazard regression model was used to determine the prognostic influence of preoperative hemoglobin results, perioperative RBC transfusions, and the preoperative frailty status assessed by the G8 **s**core. Furthermore, established entity-specific clinical-pathological cancer characteristics such as tumor stage at diagnosis (according to the FIGO stage), histological grade of differentiation, and histological subtype, as well as surgical parameters, were included in the Cox regression analyses. Firstly, a univariable Cox regression analysis for every single variable was performed. Secondly, variables with a p-value < 0.10 were included in the multivariable Cox regression analyses with a variable selection *via* backward elimination. In the Cox regression model, hazard ratios (HRs) with their 95%-confidence interval (95%-CI) and p-values were used. Kaplan-Meier estimates were used to describe PFS and OS after 5 years. Time points in months were the date of diagnosis which resulted in the operation date up to death (or recurrence) or last follow-up. Consequentially, PFS was defined as the length of time after the primary operation that a patient lives without a relapse. In the case of residual tumor burden, PFS was defined as the time after primary surgery until clinical or radiological progression of the disease was found. OS was measured from the date of operation to the date of death or last follow-up. The log-rank test was used to compare the survival curves. All tests were two-sided and a p-value of < 0.05 was considered exploratory, because no correction for multiple testing was performed.

Results

Endometrial cancer

Clinical-pathological cancer characteristics

A total of 338 patients were screened and, finally, 263 women entered the study. Out of them, 152 (57.8%) patients suffered from EC (Figure 1). The median follow-up time in this sub-cohort was 31.0 [8.0–68.5] months. The mean age of the study population did not differ between the two cancer entities (EC: 71.0 ± 7.4 years and OC: 70.9 ± 5.9 years) (Table 1).

A higher FIGO **s**tage of EC required significantly more RBC transfusions than lower FIGO **s**tages (transfused: FIGO III–IV: 42.1% vs. non-transfused FIGO III–IV: 9.6%; transfused: FIGO I–II: 57.9% vs. non-transfused FIGO I–II: 89.4%; p < 0.001). By histologic subtype, significantly, more women with endometrioid cancers were recorded in the EC-transfused cohort (63.2% vs. 36.8%; p = 0.005). The histological grade of differentiation was not associated with RBC transfusions (p = 0.774).

Surgical treatment parameters

The transfused EC patients (84.2%) received up to five RBC transfusions, mostly during surgical procedures (63.2%). AOC was associated with RBC transfusion indication (77.8% vs. 22.2%; p < 0.001). The mean cut-seam time was 142.4 min (± 82.2 min) with a mean intraoperative blood loss of 229.5 ml (± 422.8 ml). Forty-nine patients (32.0%) received laparoscopic surgery, 72 (49.7%) received open surgery, and the remaining 29 (18.3%) received vaginal surgery. In total, four operative

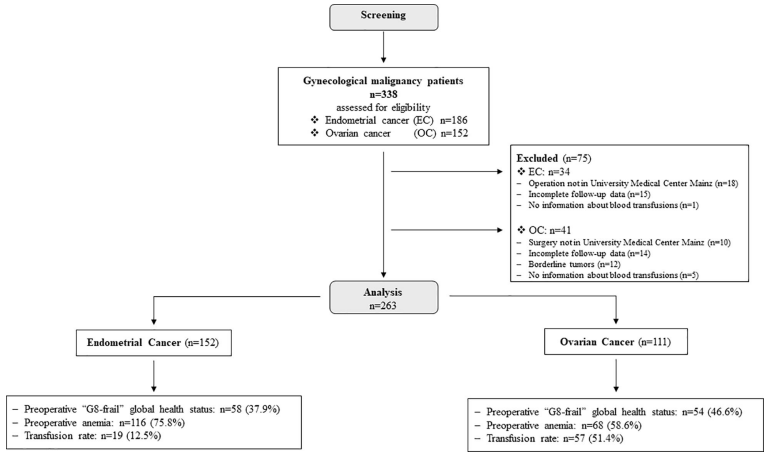

FIGURE 1
Consort-Statement.

revisions were necessary, two due to an incarcerated intestinal loop and two due to a subsequent postoperative hemorrhage. In both circumstances, one of two patients received RBC transfusions. A total of 144 EC patients (94.7%) were operated on without any residual tumor burden. Frail patients were operated on with the same surgical radicality as non-frail patients (data not shown). In total, 14 (9.2%) patients received adjuvant chemotherapy, which was completed in 12 (85.7%) patients. Moreover, 67 and (43.9%) women received adjuvant radiotherapy [61 (39.9%) brachytherapy, 5 (3.3%) percutaneous radiation, and 1 (0.7%) local radiation]. The indication was stage-appropriated in 137 (89.5%) cases.

Global health status

Preoperative global health status evaluation with the G8 score allocated 58 (38.9%) patients in the G8-frail and the remaining 91 (61.1%) patients in the G8-non-frail cohort. Frail patients received significantly more RBC transfusions than G8-non-frail patients (83.3% vs. 16.7%; $p < 0.001$). G8-frail patients receiving RBC transfusions were faced with the lowest survival rates compared to their non-frail and non-transfused counterparts (Table 3). The impact of intraoperative RBC transfusions on the survival rates was more pronounced than the influence of the preoperative frailty status (5-year OS: transfused: 25.7% vs. G8-frail: 49.7%).

Prognosis

Kaplan-Meier plots yielded 5-year statistically different OS rates for RBC transfusions (non-transfused: 82.6% vs. transfused: 25.7%; $p < 0.001$), AOC (non-anemic: 81.2% vs. anemic: 57.1%; $p < 0.001$), and global health status (G8-non-frail: 88.2% vs. G8-frail: 49.7%; $p < 0.001$) (Table 2). Overall, frail and transfused patients had the worst prognosis in the EC cohort (Figure 2). In the univariable Cox regression analysis, FIGO stage, histological grade of differentiation, postoperative residual tumor burden, and RBC transfusions, as well as preoperative frailty status, were associated with decreased survival rates for both, 5-year PFS and 5-year OS (all p-values < 0.05) (Table 3). In the multivariable analyses, besides selected clinical-pathological cancer characteristics (FIGO stage and histological grade of differentiation), only RBC transfusions retained their independent significance for both 5-year PFS and 5-year OS (all p-values < 0.05). However, AOC and G8-Status were not independently associated with PFS and OS (all p-values > 0.05).

Ovarian cancer

Clinical-pathological cancer characteristics

A total of 111 patients with OC (43.1%) met the inclusion criteria (Figure 1), and 51.4% of patients received RBC transfusions (Table 1). The conventional clinical-pathological cancer characteristics such as FIGO stage, histological grade of differentiation, and histological subtype did not differ between the transfused and not-transfused cohort. The median follow-up time was 26.0 [12.0–39.0] months.

Surgical treatment parameters

AOC was diagnosed in 37.4% of OC patients, and 47.3% of patients receiving RBC transfusions suffered from AOC. Otherwise, solely 26.9% of women with AOC did not receive RBC transfusions and 73.1% of non-anemic patients did not receive RBC transfusions ($p = 0.030$). The mean operation time was 260.0 min (± 122.7 min) with a mean blood loss of 1,015.32 ml (± 1468.82 ml). In total, 82 (78.1%) patients were treated with primary debulking surgery, and 23 (21.9%) patients received an

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TABLE 1 Patients'-characteristics gynaecological malignancies.

Parameter n (%) (+/- SD)	Endometrial Cancer (EC) total n=152	transfused n=19	non- transfused n=133	Ovarian Cancer (OC) total n=111	transfused n=57	non- transfused n=54
Mean age [years]	71.0 (+/- 7.4)			70.9 (+/- 5.9)		
			Clinical-pathological cancer characteristics			
Tumor Stage (FIGO-Stage)	**p<0.001**			p=0.483		
I	123 (80.9)	11 (57.9)	111 (84.1)	13 (11.2)	4 (7.0)	9 (16.7)
Ia	70 (46.1)			**Ia** 8 (6.9)		
Ib	53 (34.9)			**Ib** 1 (0.9)		
				Ic* 4 (3.4)		
II	7 (4.6)	0 (0.0)	7 (5.3)	6 (5.2)	3 (5.3)	3 (5.6)
				IIa 2 (1.7)		
				IIb 4 (3.4)		
III	12 (7.9)	2 (10.5)	10 (7.6)	74 (63.8)	36 (63.2)	35 (64.8)
IIIa	2 (1.3)			**IIIa1** 5 (4.3)		
IIIb	3 (2.0)			**IIIa2** 0 (0.0)		
IIIc1	5 (3.3)			**IIIb** 13 (11.2)		
IIIc2	2 (1.3)			**IIIc** 56 (48.3)		
IV	10 (6.6)	6 (31.6)	4 (3.0)	17 (14.7)	10 (17.5)	7 (13.0)
IVa	3 (2.0)			2 (1.7)		
IVb	7 (4.6)			15 (12.9)		
Histological Subtype	**p=0.005**			p=0.824		
Endo-metrioid	130 (85.0)	12 (63.2)	117 (88.0)	**Serous** 86 (74.1)	18 (31.6)	16 (29.6)
				low grade 5 (4.3)		
				high grade 81 (69.8)		
Others	23 (15.0)	7 (36.8)	16 (12.0)	**Others** 30 (25.9)	39 (68.4)	38 (70.4)
(serous, squamous, mucinous,)				(endometrioid, mucinous, clear cell)		
Histological grade of differentiation	p=0.774			p=0.560		
G1	75 (49.0)	7 (36.8)	67 (50.4)	6 (5.2)	2 (3.5)	4 (7.4)
G2	46 (30.1)	6 (31.6)	40 (30.1)	21 (18.1)	9 (15.8)	11 (20.4)
G3	30 (19.6)	4 (21.1)	26 (19.5)	87 (75.0)	44 (77.2)	39 (72.2)
			Red blood cell transfusion management			
Timing of transfusion						
preoperative		4 (21.1)		6 (10.5)		
intraoperative		12 (63.2)		42 (73.7)		
postoperative		3 (15.8)		9 (15.8)		
Number of transfusions						
≤ 5> 5		16 (84.2)3 (15.8)		38 (66.7)19 (33.3)		
			Preoperative anemia of cancer			
[g/dl]	**p<0.001**			**p=0.030**		
Haemoglobin < 12	35 (23.3)	14 (77.8)	21 (15.9)	40 (37.4)	26 (47.3)	14 (26.9)
Haemoglobin ≥ 12	115 (76.7)	4 (22.2)	111 (84.1)	67 (62.6)	29 (52.7)	38 (73.1)
			Global health status			
G8 Score	**p<0.001**			**p=0.031**		
G8-frail	58 (38.9)	15 (83.3)	43 (33.1)	51 (48.1)	32 (58.2)	19 (37.3)
G8-non-frail	91 (61.1)	3 (16.7)	87 (66.9)	55 (51.9)	23 (41.8)	32 (62.7)
			Surgical treatment parameters			
Postoperative Residual tumor burden	p=0.150			**p=0.017**		

(Continued)

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TABLE 1 Continued

Parameter n (%) (+/- SD)	Endometrial Cancer (EC) total n=152	transfused n=19	non- transfused n=133		Ovarian Cancer (OC) total n=111	transfused n=57	non- transfused n=54
None	144 (94.7)	16 (84.2)	127 (95.5)		67 (58.3)	27 (47.4)	37 (68.5)
Present	6 (3.9)	2 (10.5)	4 (3.0)		48 (41.7)	30 (52.6)	16 (29.6)
Unknown	2 (1.3)	1 (5.3)	2 (1.5)		0 (0.0)	0 (0.0)	0 (0.0)
SCS – Surgical Complexity Score	n.a.			*p=0.062*			
SCS 1					37 (33.3)	16 (28.1)	21 (39.6)
SCS 2					53 (47.7)	26 (45.6)	27 (50.9)
SCS 3					21 (18.0)	15 (26.3)	5 (9.4)
Completeness of systemic therapy	p=0.425			p=0.841			
No	2 (14.3)	0 (0.0)	2 (100.0)		13 (16.0)	6 (16.2)	7 (17.9)
Yes	12 (85.7)	3 (25.0)	9 (75.0)		68 (84.0)	31 (83.8)	32 (82.1)

EC, endometrial cancer; FIGO, International Federation of Gynecology and Obstetrics; G, histological grade of differentiation; G8 Score, G8 geriatric Screening tool; G8 frail, G8 geriatric Screening tool > 14 points; G8 non-frail, G8 geriatric Screening tool ≤ 14 points; OC, ovarian cancer; SD, standard deviation; SCS, Surgical Complexity Score.
n.a.: not applicable, n: number of patients.
*if the number of cases is small, the subdivision into IC1, IC2 and IC3 is waived.
bold written words: analyzed main categories.
bold written numbers: statistically significant results (p<0.05); italic written numbers: clinically relevant results (p<0.1).

interval debulking surgery. Nineteen (17.1%) operative revisions were performed due to deep and superficial wound dehiscence in eight (38.1%) cases, intestinal complications as anastomosis insufficiencies or peritonitis in seven (36.8%) cases, and surgical bleeding in four (21.1%) cases. Fourteen of these 19 (73.7%) patients required RBC transfusions. No residual tumor burden was achieved in 67 (58.3%) patients. RBC transfusions were associated with surgical radicality determined by the SCS (p = 0.062) and postoperative residual tumor burden (p = 0.017). Similar to the EC group, the OC patients mostly received five or fewer (66.7%) RBS intraoperatively (73.7%).

Global health status

The G8 **s**core allocated 51 (48.1%) patients to the G8-frail group and 55 (51.9%) patients to the G8-non-frail group. Significantly, more G8-frail OC patients were transfused than G8-non-frail (58.2% vs. 41.8%; p = 0.031). Frail patients were operated on with the same surgical intent as non-frail patients (data not shown). G8-frail patients were faced with an impaired PFS compared to their G8-non-frail counterparts (53.4% vs. 16.7%; p = 0.010) (Figure 2). No significant survival difference was recognized in terms of 5-year OS (G8-non-frail: 40.5% vs. G8-frail: 15.3%, p = 0.149) (Table 2).

Prognosis

RBC transfusions did not influence the prognosis in OC patients in terms of PFS (40.8% vs. 26.0%, p = 0.738) and OS (46.3% vs. 20.8%, p=0.073). The *post-hoc* power calculation for the dichotomous endpoint of the two independent sample studies was 82.1%. Anemic patients were faced with a worse outcome in terms of OS when compared with non-anemic patients (10.6% vs. 36.7%; p = 0.008) but not in terms of PFS (26.9% vs. 39.5%; p = 0.088). The univariable Cox regression analyses were shown in Table 3. In multivariable Cox regression analyses, solely the FIGO **s**tage and G8 **s**core retained independent significance as prognostic factors for PFS (HR: 6.52; 95%-CI [1.51–28.07] and HR: 2.23; 95%-CI [1.16–4.32], respectively). AOC missed the statistical level of significance (HR: 1.18; 95%-CI [0.59–2.38]). In terms of OS, the TNM tumor stage achieved statistical and AOC clinical significance (HR: 3.75; 95%-CI [1.87–7.53] and HR: 1.70; 95%-CI [0.92–3.15], respectively) in multivariable analyses.

Discussion

To the best of our knowledge, we here reported for the first time the relationship between RBC transfusions, AOC, and global health status with their possible impact on prognosis for cancer patients and try to elucidate the real source of the fundamental reason for performing a Cox regression analyses. In EC, RBC transfusions and selected clinical-pathological cancer characteristics impaired PFS and OS. In OC, the FIGO **s**tage and frailty status seemed to be the most important prognostic factors for PFS followed by the TNM tumor stage and AOC in terms of OS.

The correlation between perioperative RBC transfusions and postoperative outcome on survival seemed to be entity-specific and was discussed controversially in the current literature. Bogani and colleagues retrospectively examined 275 patients with locally advanced cervical cancer scheduled to undergo neoadjuvant chemotherapy plus radical surgery (15). They reported no association between RBC transfusions and worse disease-specific survival (DSS) (HR: 2.71; 95%-CI [0.91–8.03]). Contrastingly, in gastrointestinal tumor surgery, e.g., esophageal cancer resections, RBC transfusions have been correlated in 568 esophagectomies with a significantly poorer short- and long-term survival (PFS: HR:1.8:

TABLE 2 Estimated 5-year survival rates by Kaplan-Meier method.

	Endometrial Cancer			Ovarian Cancer		
	n (%)	PFS after 5 years [%], *p value*	OS after 5 years [%], *p value*	n (%)	PFS after 5 years [%], *p value*	OS after 5 years [%], *p value*
Red blood cell (RBC) transfusions	152	**<0.001**	**<0.001**	111	0.738	*0.073*
	133	79.8	82.6	54	40.8	46.3
non-transfused	(87.5)	26.0	25.7	(48.6)	26.0	20.8
transfused	19			57		
	(12.5)			(51.4)		
Preoperative anemia of cancer (AOC)	151	0.110	**<0.001**	110	*0.088*	**0.008**
	116	77.2	81.2	72	39.5	36.7
non-anemic	(76.8)	65.0	57.1	(65.5)	26.9	10.6
anemic	35			38		
	(23.2)			(34.5)		
G8 geriatric Screening tool (G8 Score)	150	*0.071*	**<0.001**	110	**0.010**	0.149
	92	82.1	88.2	56	53.4	40.5
G8-non-frail	(61.3)	65.4	49.7	(50.9)	16.7	15.3
G8-frail	58			54		
	(38.7)			(49.1)		
Frail – RBC transfusions	148	**0.003**	**<0.001**	99	**0.039**	0.170
G8-non-frail + non-transfused	87	86.0	90.2	38	47.2	45.5
	3	66.7	33.3	(38.4)	77.8	27.8
G8-non-frail + transfused	43	72.4	61.3	13	22.3	14.6
G8-frail + non-transfused	15	39.2	17.3	(13.1)	17.0	14.6
G8-frail + transfused				27		
				(27.3)		
				21		
				(21.2)		

AOC, anemia of cancer; OS, overall survival; PFS, progression free survival; RBC, red blood cell.
n = number of patients; G8 frail: G8 geriatric Screening tool > 14 points, G8 non-frail: G8 geriatric Screening tool ≤ 14 points
bold written words: analyzed main categories;
bold written numbers: statistically significant results (p<0.05); italic written numbers: clinically relevant results (p<0.1).

95%-CI: [1.2–2.5] and OS: HR: 2.2; 95%-CI [1.5–3.2], respectively) (17). Although we recorded a restrictive blood management with a transfusion rate of only 12.5% in the EC cohort, RBC transfusions retained its independent significance according to poorer 5-year PFS and 5-year OS. Uccella and colleagues similarly proved the association between RBC transfusions and a higher risk of recurrence in 331 women with EC (27). They hypothesized that RBC transfusions potentially promoted the intraabdominal spread of neoplastic cells due to the transitory perioperative immunodepression (64, 65). For elderly patients with OC, our data could not show an independent impact of the RBC transfusions but for AOC and clinical-pathological cancer characteristics on prognosis. Our results were in line with the findings of Warner and colleagues. They refuted an independent influence of RBC transfusions on survival in women with epithelial OC even if RBC transfusions were significantly associated with age, advanced stages of diseases, and higher surgical complexity (9). In contrast to these findings, Zhang and colleagues postulated a significant deleterious effect on cancer survival related to RBC transfusions in their retrospective study (66). Furthermore, De Oliveira and colleagues were able to demonstrate an association between advanced OC and RBC transfusions in their retrospective cohort investigation (67), although the radicality of tumor debulking surgery, as well as residual tumor burden as validated independent predictors of poor oncological outcomes, was not considered as a potential variable influencing the outcome in that study.

To clarify the indications of RBC transfusions in the perioperative setting, one might address the rule of AOC (2). Our results suggested a poorer 5-year OS for pre-surgical anemic EC and OC patients in Kaplan-Meier plots. In the multivariable Cox regression analyses, an independent prognostic influence was solely demonstrated in OC in terms of OS. Our results were comparable with the results of a recently published prospective trial with 192 patients by Chen and colleagues. "Specifically in obese, nondiabetic, elder, advanced stage but having relatively good performance status patients" a low preoperative hematocrit, lower than 35%, was a valuable predictor of OC women's poor prognoses (68). Contrastingly, Abu-Zaid and colleagues were not able to determine an independent prognostic association between AOC and OS in endometrioid-type EC in a retrospective cross-sectional study (69). Moreover, Abu-Zaid et al. demonstrated that poorer survival outcomes were predicted by preoperative AOC in patients with exclusively advanced FIGO stage EC in their subsequent systematic review and meta-analysis from 2021 (70). Possibly, our contrasting findings might be explained by the lower rate of 14.5% suffering from an advanced EC.

For the preoperative global health status in cancer patients, our study group could demonstrate the independent impact on

TABLE 3 Uni- and multivariable Cox-regression analyses for survival in patients with gynecological malignancies.

univariable	Endometrial Cancer						Ovarian Cancer					
	PFS			OS			PFS			OS		
	HR	95%- CI	p-value	HR	95%- CI	p-value	HR	95%- CI	p-value	HR	95%- CI	p-value
TNM-Tumor Stage	1.48	0.89-2.47	0.134	2.21	1.56-3.15	**<0.001**	2.56	1.48-4.42	**0.001**	3.41	1.86-6.22	**<0.001**
FIGO-Stage	1.87	1.34-2.61	**<0.001**	2.25	1.69-2.99	**<0.001**	6.21	1.91-20.18	**0.002**	1.96	1.32-2.89	**0.001**
Histological subtype	0.45	0.18-1.12	*0.087*	0.34	0.16-0.73	**0.006**	1.52	0.80-2.86	0.200	1.77	0.93-3.36	*0.083*
Histological grade of differentiation	1.93	1.19-3.12	**0.008**	2.26	1.44-3.55	**<0.001**	1.59	0.91-2.80	0.104	1.71	0.98-2.97	*0.057*
Postoperative residual tumor burden	2.22	1.06-4.65	**0.034**	2.95	1.71-5.08	**<0.001**	2.07	1.17-3.67	**0.012**	3.03	1.70-5.41	**<0.001**
SCS – Surgical Complexity Score				-			1.46	0.96-2.22	*0.078*	1.509	1.01-2.26	**0.045**
Completeness of systemic therapy				-			2.06	0.87-4.85	*0.071*	0.984	0.49-1.96	0.963
RBC transfusions	4.97	2.03-12.18	**<0.001**	7.48	3.48-16.08	**<0.001**	1.10	0.62-1.98	0.743	1.66	0.95-2.93	*0.078*
Preoperative anemia of cancer (AOC)	0.53	0.24-1.18	0.118	0.29	0.15-0.58	**0.001**	0.58	0.31-1.10	*0.097*	0.46	0.26-0.83	**0.010**
G8 geriatric Screening tool (G8 Score)	2.29	1.04-5.02	**0.040**	3.55	1.73-7.26	**0.001**	2.14	1.17-3.92	**0.014**	1.49	0.86-2.57	0.154
multivariable												
TNM-Tumor Stage		–		0.92	0.54-1.56	0.759	1.25	0.45-3.47	0.671	3.75	1.87-7.53	**<0.001**
FIGO-Stage	1.25	1.06-1.46	**0.007**	1.30	1.13-1.49	**<0.001**	6.52	1.51-28.07	**0.012**	1.10	0.12-9.89	0.932
Histological Subtype	3.17	0.82-12.33	*0.096*	3.83	1.15-12.74	**0.029**		-		1.38	0.70-2.73	0.351
Histological grade of differentiation	2.25	1.22-4.14	**0.009**	2.11	1.23-3.61	**0.007**		-		1.45	0.72-2.93	0.299
Postoperative residual tumor burden	1.29	0.51-3.25	0.586	1.29	0.57-2.91	0.543	1.35	0.70-2.63	0.375	0.83	0.42-1.67	0.605
SCS				–			1.08	0.65-1.79	0.778	1.03	0.64-1.65	0.899
Completeness of systemic therapy				–			1.81	0.68-4.80	0.231		-	
RBC transfusions	1.76	1.01-3.07	**0.046**	2.38	1.50-3.78	**<0.001**		-		0.85	0.43-1.68	0.643
Preoperative AOC		–		1.07	0.50-2.30	0.860	1.18	0.59-2.38	0.644	1.70	0.92-3.15	*0.090*
G8 Score	1.34	0.53-3.36	0.533	2.02	0.87-4.67	0.101	2.23	1.16-4.32	**0.017**		-	

95%-CI, confidence interval; FIGO, International Federation of Gynecology and Obstetrics; G8 Score, G8 geriatric Screening tool; HR, hazard ratio; OS, overall survival; PFS, progression free Survival; RBC, red blood cell; SCS, Surgical Complexity Score.
n = number of patients.
bold written words: analyzed main categories;.
bold written numbers: statistically significant results (p<0.05); italic written numbers: clinically relevant results (p<0.1).

postoperative prognosis (54, 55, 58). Consecutively, the frailty status was seen as a potential confounder in this study and was included in the multivariate analyses.

Unfortunately, the important question to be answered remains open: how to manage AOC in the elderly partially frail patients with EC or OC before major surgery? From the presented results, one might conclude to clarify AOC and determine global health status with validated geriatric screening tools, which was beneficial especially in OC patients. These mainly preoperatively detectable parameters seemed to give important insights into the patients' prognosis regardless of the administration of RBC transfusions. Especially in older women with OC, a multilayered and interdisciplinary diagnostic or rehabilitation program might be helpful to enable an individual therapy concept mainly in frail patients, as G8-frail patients were faced with a poor prognosis irrespectively of maximal surgical effort (58). A moderate transfusion management, based on the fact that RBC transfusions in symptomatic anemic OC patients, was not associated with an overall poorer outcome. However, a restrictive perioperative RBC transfusion management seemed to be much more important in patients with EC, most likely due to the lower surgical radicality and the overall better general condition. Boone and colleagues postulated a strongly restrictive transfusion policy in gynecologic oncology (71). In their retrospective chart review, they examined 582 women, 55.9 years of mean age, with various gynecological malignancies, receiving a total of 2,276 blood transfusions. Their hypothesis was based on the findings that solely women with symptomatic anemia with hemoglobin results <7 g/dl or an intraoperative blood loss of more than 1,500 ml should be transfused, without increased postoperative morbidity, concerning infections, thrombotic events, or mortality. Moreover, the American College of Surgeons National Surgical Quality Improvement Program (NSQIP) published data from 8,519 women, gynecologic surgically treated between 2010 and 2012, in a large-scale multi-institutional dataset. They reported an RBC transfusion rate of 13.8% with a significant higher transfusion-related composite morbidity (odds ratio (OR) = 1.85; 95%-CI [1.5–2.24]), including surgical site infections (OR=1.80, 95%-CI [1.39–2.35]) and length of hospital stay (non-transfused: 3.02 vs. transfused 7.17 days, p=<0.001) (72). However, Boureau and de Decker were able to examine that liberal transfusion strategies could show lower mortality rates, especially in a surgical ward in elderly cancer

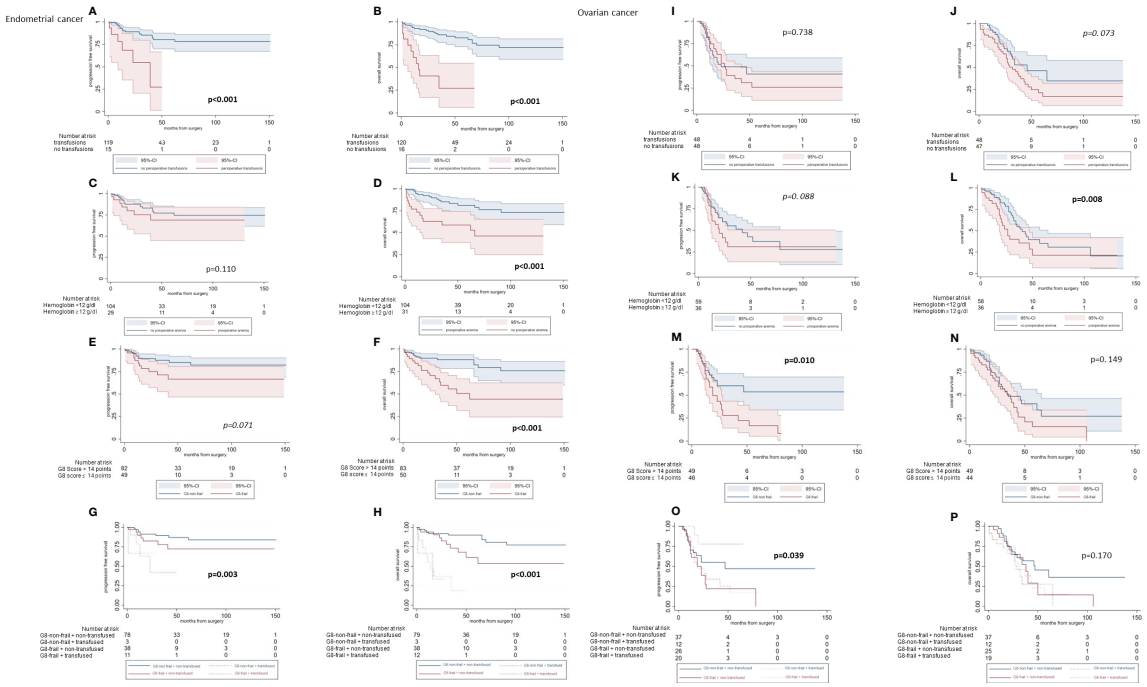

FIGURE 2
Statistical survival analyses according to the transfusion status of endometrial and ovarian cancer patients. **(A–H)** Endometrial cancer: Kaplan Meier curves. **(I–P)** Ovarian cancer: Kaplan Meier curves.

patients (73). They postulated that transfusion decisions should be based on "benefit/risk balance taking into account patients' symptoms". Further trials reviewed evidence-based indications for RBC transfusions, almost in conservative treated patient cohorts, and resumed the potential risks and complications of blood interventions (3). Considering the RBC transfusion management in gynecologic oncologic surgery, most of the current literature had been limited by small sample sizes of about 150 patients (38, 74). In addition, solely univariable analyses showed significant differences between the transfused and non-transfused study sub-cohort (16). If multivariable regression models were used to elucidate the possible effect of RBC transfusions on survival, key perioperative parameters such as surgical complexity or radicality as well as intra-operative blood loss and preoperative hemoglobin level were not included (27, 75, 76). We tried to overcome some of these limitations and demonstrated an independent impact of RBC transfusions and selected clinical-pathological cancer characteristics on the prognosis of patients with EC but not in OC.

By analyzing the impact of RBC transfusions, AOC, and the global health status in the context of known influential clinical-pathological cancer characteristics on the prognosis of EC and OC, this study tried to elucidate the underlying causative mechanism of these intertwined conditions causing a poorer prognosis and went beyond the pure description of an association between RBC transfusions in patients with EC and OC. Limitations arise from the retrospective nature of the data analyses limiting the generalizability of our findings. This might be relevant, particularly in terms of incomplete follow-up, which was successfully reduced to a minimum of 34 EC and 41 OC patients by reaching out to patients and physicians through different channels of communication and an extensive review of clinical records. Nevertheless, the large number of considered entity-specific clinical-pathological prognostic parameters as well as the multidimensional nature of the included patients regarded the frailty status and surgical aspects besides current clinical risk factors strengthen the validity of our results. Additionally, this work was carried out in a single institution. In contrast, the benefit of this single-center trial was the depth of data available, allowing for the analysis of possible confounding variables related to outcomes. Moreover, selection bias that could arise from the decision to transfuse was subjective and some practitioners might have been more liberal with transfusions than others, although the overall rate of perioperative RBC transfusions in this cohort was moderate at 28.3% and was in line with globally reported standards (67). Finally, multiple testing might regard as a weakness of retrospective data analyses.

In conclusion, in addition to the stage- and entity-dependent cancer prognosis, the prognostic impact of RBC transfusions was

detected only in patients with EC. In OC patients, the preoperative determination as "G8-frail" was associated with an independent worse oncological outcome. The different impact of RBC transfusions concerning the cancer entity could be firstly explained by the fact that EC patients in general were less likely to be as frail as OC patients. Secondly, the 5-year OS in the non-transfused OC cohort was fundamentally lower than in the EC cohort (46.3% vs. 82.6%). This survival disadvantage of the OC patient seemed to be explained by the fact that the diseases at diagnosis were more advanced, and the tumor biology, in general, seemed to be more aggressive. A standardized AOC clarification, as well as an evidence-based screening of frailty status, might be established in a preoperative diagnostic pathway to improve the individual cancer prognosis. However, due to the abovementioned limitations, a multi-centric or even prospective approach might be helpful to elucidate further information on our goal to improve the perioperative workup of cancer patients.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

Author contributions

KA, and MB conceived and designed the study. KA, MWS, AL, AH, VL, WW, CW, SK, RS, MS, RR, EH, WB and MB collected the data. KA, MWS and MB performed the statistical analysis. KA and MB wrote the manuscript. All authors critically revised the manuscript and contributed to the article and approved the submitted version.

Conflict of interest

KA reports personal fees from Eisai, Roche, MSD. MWS reports holding a patent WO 2021/176091 A1 not related to this study. SK received speaker Honoria, research funding and travek reimbursement from Vovartis Pharma GmbH Germany. MS reports personal fees from AstraZeneca, BioNTech, Daiichi Sankyo, Eisai, Lilly, MSD, Novartis, Pantarhei Bioscience, Pfizer, Roche, and SeaGen outside the submitted work. Institutional research funding from AstraZeneca, BioNTech, Eisai, Genentech, German Breast Group, Novartis, Palleos, Pantarhei Bioscience, Pierre Fabre, and SeaGen. In addition, MS has a patent for EP 2390370 B1 and a patent for EP 2951317 B1issued. RS reports honoraria and expenses from Roche Pharma AG and AstraZeneca GmbH. AH reports honoraria and expenses from AstraZeneca, FBA Frauenärzte BundesAkademie GmbH, KlarigoVerlag, MedConcept, Med public GmbH, Med update GmbH, Medicultus, Pfizer, Promedicis GmbH, Pierre Fabre Pharma GmbH, Softconsult, Roche Pharma AG, Streamedup! GmbH, Tesaro Bio Germany GmbH. I am consultant to PharmaMar, Promedicis GmbH, Pierre Fabre Pharma GmbH, Roche Pharma AG and Tesaro Bio Germany GmbH. I have received funded research from Celgene. MB reports honoraria and expenses from Pharma Mar, Astra Zeneca, Tesaro, GSK, Roche, Clovis Oncology.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Optimizing the use of lenvatinib in combination with pembrolizumab in patients with advanced endometrial carcinoma

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Introduction: The combination of lenvatinib plus pembrolizumab demonstrated a relevant clinical benefit in patients with endometrial carcinoma. The safety profile was consistent with the established profiles of each drug in monotherapy, with the most frequent adverse events being hypertension, an on-target effect, hypothyroidism, diarrhea, nausea, vomiting, loss of appetite, fatigue, and weight loss.

Areas covered: We first review the rationale based on the combination of a VEGFR inhibitor and an immune checkpoint inhibitor, highlighting the main pharmacokinetic and pharmacodynamic features of lenvatinib. Next, we focus on the common adverse events associated with lenvatinib and guide how to optimally prevent, detect, and manage them, while minimizing interruptions during lenvatinib treatment.

Discussion: The side effects profile of lenvatinib is very well known, being similar across different tumor types. Most toxicities can be preventable. An appropriate, proactive, and thorough management of lenvatinib toxicities during treatment is

required to maximize potential lenvatinib efficacy. Adverse events should be detected as early as possible, by both carefully monitoring the patient from lenvatinib initiation and preventing their occurrence. Patients should be followed also during treatment as some adverse events, e.g., cardiac dysfunction might appear later. Increased awareness on risk to benefit ratio among clinicians would be helpful to avoid dose interruptions or discontinuation of lenvatinib, with preferring other medical interventions and supportive care.

KEYWORDS

lenvatinib, pembrolizumab, endometrial cancer, tyrosine kinase inhibitor, immune response

Introduction

Endometrial cancer is the most common gynecologic malignancy, with an estimated 65,950 new cases and 12,550 deaths in 2022 in the United States (1). Although endometrial carcinoma is a disease usually associated with older age, it can present in women at any age. Most endometrial carcinomas result from a spontaneous mutation, but up to 30% of cases are associated with germline mutations in mismatch repair genes (deficient mismatch repair dMMR) or show microsatellite instability-high (MSI-H) (2). Front-line treatments for women with advanced endometrial carcinoma are established as platinum-based chemotherapy plus taxane and hormone therapy, especially in women with low-grade endometrioid tumors and smaller tumor volume (3). Second-line treatments for recurrent patients have been an unmet clinical need until the recent approval of pembrolizumab for patients with MSI-H (4). For patients without MSI-H/deficient MMR (dMMR), the combination of lenvatinib plus pembrolizumab has emerged as a potential therapeutic opportunity (5, 6).

In this review, we summarize the main molecular, pharmacokinetic, and pharmacodynamic features of the combination of lenvatinib and pembrolizumab and focus on the management of lenvatinib in endometrial carcinoma based on the results from clinical trials and the experience acquired in other tumors.

Role of VEGF in tumor immune editing and rationale for the combination of anti-VEGF and immunotherapy

Vascular Endothelial Growth Factor (VEGF) is a cytokine with dual action in tumor biology. On one hand, hypoxic cancer cells and vascular endothelial cells release VEGF that favors angiogenesis, tumor growth, invasion, and metastasis; on the other, VEGF induces the mobilization and proliferation of various cells, including regulatory T cells (Tregs), the release of immunosuppressive cytokines, thus leading to immune escape (7). The inhibition of VEGF receptors (VEGFR) with targeted drugs impacts immune response: dendritic cells show an increased antigen presentation, and T cells are activated in the priming phase and migrate from lymph nodes to tumor sites. In addition, anti-VEGFRs suppress the generation of Tregs, tumor-associated macrophages, and myeloid-derived suppressor cells at the tumor site and abrogate the expression of immunosuppressive cytokines such as TGF-β and IL-10. Therefore, these drugs reprogram the immunosuppressive tumor microenvironment into an immunostimulatory environment; under these conditions, immunotherapy with PD-1/PD-L1 antibodies further enhances the antitumor activity of T cells (7).

The combined antitumor activity of lenvatinib plus anti-PD-1 was investigated in animal models (CT-26 mice) (8). Treatment with lenvatinib or an anti-PD-1 alone significantly inhibited the *in vivo* tumor growth of CT26 isografts compared with the vehicle; however, the combination of lenvatinib plus anti-PD-1 drugs synergically suppressed tumor growth compared with either treatment alone. The activity was more evident in immune-competent animals than in their immunosuppressed counterparts (8). Therefore, the rationale to combine lenvatinib and pembrolizumab is based on the synergic effect that these drugs exert on the immune system.

Pharmacodynamic and pharmacokinetics of lenvatinib

Lenvatinib is a tyrosine kinase inhibitor that selectively blocks VEGFR, PDGFR, RET, and cKIT (9), with a higher potency, especially against VEGFR-2 and VEGFR-3, than other tyrosine kinase inhibitors (TKI) such as cabozantinib, pazopanib, and

sunitinib (10). Like other TKIs, lenvatinib binds the ATP binding pocket of kinases in its active conformation (9). The ATP binding site is a highly conserved domain between kinases, and this explains the relative lack of single kinase selectivity and the ability to act on multiple targets (9). This behavior may represent an advantage since lenvatinib can inhibit other receptors involved in angiogenesis.

Lenvatinib binds the ATP binding pocket in a peculiar mode. Indeed, kinetic studies revealed that lenvatinib had a rapid association rate constant and a relatively slow dissociation rate constant in complex with VEGFR2 and interacted with a region neighboring the kinase ATP-binding site of VEGFR2. This interaction may contribute to prolonging the binding time compared with that of other inhibitors, such as sorafenib (11).

In vivo data indicated that lenvatinib is extensively metabolized through non-P450-mediated pathways, including oxidation by aldehyde oxidase, glutathione conjugation with the elimination of the O-aryl group (chlorophenyl moiety), and combinations of these pathways followed by further biotransformation (e.g., glucuronidation, hydrolysis of the glutathione moiety, degradation of the cysteine moiety, and intramolecular rearrangement of the cysteinyl-glycine and cysteine conjugates with subsequent dimerization). In the liver, cytochrome P450 3A4 is the predominant isoform that metabolizes lenvatinib by methylation; however, hepatic metabolism is not relevant and, thus, *in vivo*, inducers and inhibitors of CYP 3A4 show a minimal effect on lenvatinib exposure. As expected from a low CYP 3A4 metabolism, no gender differences were observed in the PD and PK profile of lenvatinib, and no clinically relevant drug-drug interactions had been reported (12).

Lenvatinib half-life is of about 28 hours; therefore, it is enough to cover the entire period between administrations, but not so long as to ensure a reasonably short and fast clearance, in case of adverse reactions (10, 12).

Dosing

Lenvatinib showed a high binding to human plasma proteins, especially alpha1-glycoprotein and gamma-globulin, which increases the apparent volume of distribution at a steady-state (12). In addition, the distribution of drugs in tumor angiogenesis is always very difficult, due to the structural abnormalities of blood vessels. At treatment initiation, it is advisable to use the full lenvatinib dose to quickly saturate the distribution volume and achieve an effective concentration at a steady state that guarantees antitumoral activity. On the contrary, a progressive increase in concentration may need a considerable number of days or even weeks to reach the effective concentration and exposes the patient to subtherapeutic concentrations, which are not able to block tumor vascularization. In animal models, sunitinib showed transient antitumor effects as well as dynamic changes in the VEGF pathway: it initially blocked tumor growth, but after drug discontinuation, the tumor rapidly regrew (13). Therefore, dose titration and the use of lenvatinib at a sub-optimal concentration to prevent adverse reactions may be detrimental.

A randomized study specifically investigated the efficacy and safety of lenvatinib 18 mg versus 24 mg to understand whether a lower dose of lenvatinib would provide comparable efficacy but improved safety relative to the approved 24-mg/day starting dose in patients with thyroid carcinoma. The study did not demonstrate noninferiority of lenvatinib 18 mg compared to the approved dose and the 17% difference in the overall response rate at 24 weeks and overall response rate indicated that lenvatinib 24 mg provides a clinically relevant higher activity than the reduced dose; the safety analysis, on the contrary, did not reveal any advantage in terms of adverse reactions incidence and the overall safety profile of two dosings was similar (14). Therefore, starting at the recommended dose, with dose reductions if required, is important for optimizing lenvatinib treatment.

In endometrial cancer the approved dose is 20 mg once daily; due to both the strong molecular interaction with the target and the wide volume of distribution, bodyweight does not affect the antitumoral activity of lenvatinib and the approved dose can be used without adjusting for weight. For the sake of completeness, a retrospective study on 70 patients with endometrial carcinoma treated with the combination of lenvatinib and pembrolizumab showed that a lower starting dose of lenvatinib (14 mg daily) was as similarly effective and safe than the full dose, with a significantly lower prevalence of dose reductions (15). Further trials may better elucidate this point.

Data from the real world would be also helpful to further improve the management of endometrial cancer with lenvatinib. Up to date, few data are available: a Korean multicenter study described similar activity and discontinuation rates as clinical trials. Patients received the combination of lenvatinib and pembrolizumab for a median of 4.5 cycles, achieving the best objective response rate and disease control rate of 23.8% (95% CI, 11.9–38.1) and 76.2% (95% CI, 61.9–88.1), respectively. Overall, 56.2% of patients needed lenvatinib dose reduction once or more (16).

Lenvatinib plus pembrolizumab activity on endometrial cancer: Results from KEYNOTE 146 and KEYNOTE 775 trials

Recent advances in immunotherapy demonstrated the efficacy of pembrolizumab in solid tumors with MSI-H, dMMR, or with a high tumor mutational burden (4). Adding lenvatinib to an immune checkpoint inhibitor determined the

synergic effect, described above, which provided a rationale for clinical trials.

KEYNOTE 146, a phase Ib/II trial, investigated lenvatinib plus pembrolizumab beyond first-line treatment in selected advanced solid tumors, including endometrial cancer, and established the recommended phase II dose to be lenvatinib 20 mg orally daily with pembrolizumab 200 mg intravenously every 3 weeks. This trial enrolled patients with metastatic endometrial cancer, unselected for microsatellite instability or PD-L1, thus including those patients who were less or not responsive to immunotherapy (5, 6). Overall, 38.0% of patients achieved the objective response at 24 weeks and most patients showed a reduction in tumor size, although not sufficient to define a complete or partial response according to RECIST 1.1 criteria (6). These results led to the accelerated approval of lenvatinib plus pembrolizumab by the Food and Drug Administration for the treatment of advanced endometrial carcinoma that is not MSI-H or dMMR, after progression with prior systemic therapy.

In the phase III trial KEYNOTE 775, the combination was compared with chemotherapy of investigator's choice (doxorubicin or paclitaxel) beyond first-line treatment (17): in both patients with proficient-MMR (pMMR) and all-comers, lenvatinib plus pembrolizumab achieved a significantly longer progression-free survival (PFS), doubled overall response rate (ORR), and gained a longer duration of response. A *post hoc* analysis described the activity of lenvatinib plus pembrolizumab that was maintained in all histology subtypes, including serous and clear cell histology, which currently represent an unmet clinical need; prior therapy and platinum-free interval did not affect PFS (18).

In line with PFS data, the overall survival (OS) improved in all-comers and pMMR (17) and this result was consistent in all subgroups and histologic subtypes (18). The effect on OS was not influenced by one prior platinum-based treatment, but more than one previous platinum-based treatment reduced the response, thus suggesting that this treatment might be used early in the therapeutic strategy; the efficacy was maintained independently of platinum-free interval.

In November 2021, European Medicine Agency has approved lenvatinib plus pembrolizumab for the treatment of advanced or recurrent endometrial carcinoma in adults who have disease progression on or following prior treatment with a platinum-containing therapy in any setting and who are not candidates for curative surgery or radiation.

Safety data

Lesson from clinical trials

Concerning safety, in the setting of endometrial cancer, few institutes and clinicians have already gained experience with lenvatinib plus pembrolizumab; therefore, it is important to take into account the experience of pivotal trials and other diseases where the combination is already used in clinical practice. In addition, it should be considered that endometrial cancer prevalently affects elderly women with multiple comorbidities that can exacerbate adverse events potentially related to this treatment.

In KEYNOTE 775 trial, all patients experienced at least one adverse event related to therapy; 66.5% of patients required a dose reduction after adverse events, 33.0% discontinued the treatment, and 69.2% had a transient interruption to manage toxicities. The most frequently reported adverse events were: hypertension, an on-target effect, hypothyroidism, diarrhea, nausea, vomiting, loss of appetite, fatigue, and weight loss (17).

Toxicities are not related to subgroup populations, as the pMMR population showed a similar safety profile as all-comer. When adjusted for exposure, the most frequent adverse events were diarrhea, hypertension, and musculoskeletal disorders in both all-comer and pMMR populations. During the trial, these toxicities had been managed with dose reductions and interruptions of lenvatinib without observing a decreased activity of drugs. Indeed, despite dose reductions, median tumor size decreased over time (18). The median time to the first onset of most frequent adverse events occurred approximately 3 months after treatment initiation in both all-comer and pMMR populations: adverse events with the shortest median time to onset included hypertension and musculoskeletal disorders, while hypothyroidism, palmar-plantar erythrodysesthesia (PPES), and weight decrease, which can be considered as a cumulative effect of vomiting, nausea, and musculoskeletal disorders, had a long time to onset (Figure 1) (19). Therefore, some adverse events should be monitored and prevented from the beginning of the treatment. Furthermore, being proactive in the management of gastrointestinal adverse events may avoid weight loss. Patients' and clinicians' education and preventive strategies need to be implemented (18, 19).

Health-related quality of life (HRQoL) assessment in the KEYNOTE 775 revealed a similar profile between lenvatinib plus pembrolizumab and the chemotherapy of choice, thus supporting the favorable risk/benefit ratio of the combination (20).

Lesson from thyroid carcinoma and hepatocellular carcinoma

The lesson learned from thyroid carcinoma and hepatocellular carcinoma (HCC) might be useful to manage adverse events even in patients with endometrial carcinoma. In patients with radioiodine (RAI) refractory differentiated thyroid carcinoma, during the SELECT trial, which compared lenvatinib 24 mg to placebo, the most frequent adverse events included hypertension (68%), diarrhea (59%), fatigue (59%), stomatitis (36%), PPES (32%), and proteinuria (31%) (21). Similar adverse events were reported in clinical practice, with the most common

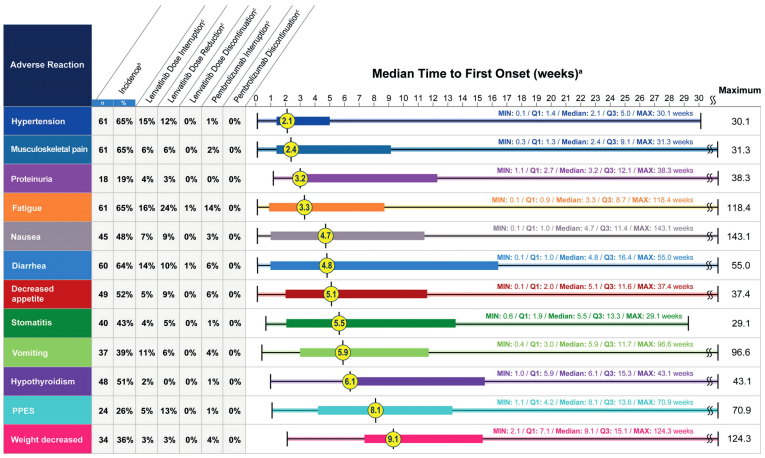

FIGURE 1
Post hoc analysis of time to first onset of selected adverse reactions. Reproduced with permission from (18).

being fatigue (13.6%) and hypertension (11.6%) (22). Hypertension, fatigue, and diarrhea appear relatively early during lenvatinib treatment. They can be managed with appropriate drugs, considering progressive dose reduction in case of ineffectiveness. Symptomatic biliary disorders (gallbladder and biliary duct disease) and cholecystitis (generally acalculous) were reported after 4 months of lenvatinib initiation or even later. The onset of symptoms and the peak of γ-glutaryl transferase levels corresponded to the highest weight loss during the first months of treatment. When these disorders required surgical intervention, presurgical lenvatinib interruption was shorter than one week (at least 48 hours before) and the treatment was resumed immediately after wound healing (23); in other cases, supportive care with ursodeoxycholic acid, when appropriate, and TKI dose reduction were used (24). Few cases of fistula and tumor-related bleeding were described after 10 weeks of treatment with lenvatinib; individual dose adjustments should be considered to manage this adverse reaction (25). Hemorrhage, acute coronary syndrome, and thrombosis/venous thromboembolism also occurred in clinical practice and should be kept into account in the management of lenvatinib (26). The ability to manage toxicities determined longer treatment duration and allowed to observation of late adverse events that could not be found in clinical trials. A retrospective study revealed new adverse events after 12 months of lenvatinib treatment: cardiovascular toxicity was the most common (57%) and no differences in the incidence of late adverse events were observed between younger (<65 years) and older patients (≥65 years), except for QTc prolongation that was more frequent in older people (27).

Patients with hepatocellular carcinoma frequently present a liver disease in addition to cancer. From a phase I trial, lenvatinib 12 mg once daily was determined to be the dose that achieved preliminary efficacy with manageable toxicity and was the recommended dose for patients with HCC with liver function as Child Pugh-A, while for patients with Child Pugh-B the recommended dose was 8 mg (28–30). Since weight loss determined an increase in the area under curve, dose adjustment according to bodyweight was suggested (31). In particular, in patients with HCC Child-Pugh class A starting doses of 12-mg and 8-mg for subjects over 60 kg and under 60 kg of body weight respectively, were used in the phase 3 REFLECT trial and are now the recommended doses for patients with HCC. The main toxicities that emerged from the phase III trial were hypertension (42.2%), diarrhea (38.7%), decreased appetite (34.0%), and weight loss (30.9%); PPES was reported by 26.9% of patients versus 52.4% in the group randomized to receive sorafenib (32). In clinical practice, similar adverse events were reported with a high incidence of hypertension, diarrhea, and anorexia/weight loss (33). Monitoring blood pressure and body weight from treatment initiation and educating the patient and his/her caregiver to promptly recognize any problem allow to manage these adverse events as well.

The combination of lenvatinib and pembrolizumab was tested also in other solid tumors - melanoma, renal cell carcinoma, squamous cell carcinoma of the head and neck, non-small cell lung cancer, or urothelial cancer- in a phase Ib/II open-label study in which all patients received the recommended phase II dose of lenvatinib 20 mg/day with pembrolizumab 200 mg every 3 weeks until disease progression or development of unacceptable toxicity. The combination confirmed a manageable safety profile in patients with these solid tumor types, with fatigue, hypertension, diarrhea, and hypothyroidism being the most common adverse reactions (34).

Even in patients with renal cell carcinoma enrolled in the CLEAR trial, lenvatinib plus pembrolizumab demonstrated a safety profile consistent with that previously described for each drug both as a single agent and combined. Adverse reactions that occurred or worsened during treatment led to a dose reduction of lenvatinib in 68.8% of patients treated with the combination. Again, interruptions and reductions were effectively utilized in the study, which allowed patients to continue to receive life-prolonging therapy for a longer period (35). The analysis of HRQoL data demonstrated that lenvatinib plus pembrolizumab had similar or favorable scores compared with those obtained by sunitinib, especially concerning the time to the definitive deterioration (36).

Lenvatinib-related adverse events: Incidence, time to the first onset, and suggestions for an appropriate management

Hypertension

Hypertension is one of the adverse events most frequently reported with lenvatinib and other anti-angiogenic; it is an on-target adverse event, directly related to the activity of these drugs on their target. Indeed, the inhibition of the VEGFR signaling pathway acts on nitric oxide-dependent processes and impairs endothelium-dependent vasodilation in the microvessels, as well as seems to enhance the vasomotor tone through the endothelin system with a mechanism that has not been elucidated yet (37). Regardless of specific angiogenic inhibitors, most patients experience an increase in blood pressure with a peak within the first weeks of treatment (37).

In KEYNOTE 146 trial, hypertension occurred in approximately 65% of patients treated with lenvatinib in the first two weeks of treatment (19). Prevention, early detection, and effective management of hypertension are important to minimize the need for dose interruptions and reductions. Before starting lenvatinib, blood pressure should be measured and eventually controlled with a stable antihypertensive therapy. Proactive management includes prompt, daily monitoring of blood pressure before the start of lenvatinib and from the first cycle both at the clinic visit and at home. Administration of angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, or beta-blockers is useful in the occurrence of a hypertensive peak; calcium channel blockers should be used with caution to avoid drug-drug interactions, which, however, are limited with lenvatinib (38–40). The choice of antihypertensive treatment should be individualized to the patient's clinical characteristics and follow standard medical practice: for previously normotensive subjects, monotherapy with one of the classes of antihypertensives should be started when elevated blood pressure is observed, while for patients already on hypertension treatment, increasing the dose of the current agent or adding a different class of antihypertensive should be appropriate (40). If antihypertensive agents are not effective or hypertension grade 3 occurs, lenvatinib dose reduction may be necessary (39).

Patients should be educated to reduce the risk factors that can enhance hypertension, such as smoking, alcohol consumption, stress, and lack of physical activity.

Musculoskeletal pain

Musculoskeletal pain was experienced in 65% of patients in the first 2.4 weeks of treatment (19). This adverse event should be managed with medications, including opioids (oxycodone, fentanyl, morphine), paracetamol, nonsteroidal anti-inflammatory drugs, and topical diclofenac (6).

Fatigue

In the clinical trial, fatigue was reported in 65% of patients, with a median time to the first onset of 3.3 weeks (19). Managing fatigue can be difficult; the initial step is to exclude any treatable cause that presents similar effects (e.g anemia) and assess the intensity level of fatigue if present. Then, periodic re-evaluations are recommended at routine and follow-up visits (41). Based on the experience in hepatocellular carcinoma, Grade 1 fatigue can generally be managed without interrupting lenvatinib. If fatigue of Grade≥2 occurs in the early phase of treatment, lenvatinib should be resumed at a reduced dose after fatigue is resolved, while it can be restored at a full dose if fatigue occurs after several weeks of treatment. Only if a patient cannot tolerate the symptoms of Grade 2 fatigue, lenvatinib should be discontinued (42). Education (i.e. coping strategies and good sleep hygiene), counseling, and both non-pharmacologic (physical activity, nutritious diet, and proper hydration) and pharmacologic interventions may be introduced to ameliorate fatigue, accounting that in many instances a combination of approaches must be used (39, 41). Lenvatinib dosing in the evening rather than in the morning may reduce daytime fatigue (39).

Stomatitis

Stomatitis or mucositis is frequently reported in patients receiving TKI and other targeted therapies. Stomatitis is a painful inflammation of the mucous lining of the mouth, whereas mucositis refers to inflammation or ulceration of the mucous membranes lining the digestive tract; both adverse events can make it difficult to speak, eat, or even open the mouth and generate discomfort for the patient. In KEYNOTE 146 trial, stomatitis occurred in 43% of

patients after a median of 5.5 weeks (19); only 5% of patients required a reduction of lenvatinib for this adverse event, while 25.5% of patients received symptomatic medications reported to manage this adverse event, such as dexamethasone, lidocaine, triamcinolone, nystatin, and mouth preparations (6). To minimize the risk of stomatitis and improve adherence to therapy, patient awareness and early intervention are important. Patients should be educated to avoid mint-flavored toothpaste, alcohol-containing mouthwash, and spicy or acidic foods and to maintain dental and oral care. Furthermore, before lenvatinib initiation and regularly during the treatment, accurate oral health is recommended. Topical lidocaine or steroid ointment may also be helpful for painful ulcerations, although for more severe stomatitis (grade ≥3) dose reductions or interruptions may be necessary (43).

Diarrhea

In KEYNOTE 146, 64% of patients experienced diarrhea, with a median time to the first onset of 4.8 weeks (19). Recommendations for lenvatinib-associated diarrhea management are consistent across tumor types. Diarrhea may be managed by making dietary changes, including avoiding caffeine, alcohol, spicy or fatty foods, dairy products, and foods high in insoluble fibers; writing a food diary may help identify particular items that exacerbate diarrhea. Patients with diarrhea should not become dehydrated; therefore, fluid intake should be increased, and electrolytes monitored and replaced when necessary. Prompt medical management of diarrhea should be established to prevent dehydration before any lenvatinib therapy dose interruption or reduction. When pharmacological intervention is necessary, loperamide is widely recommended; atropine-diphenoxylate, octreotide, codeine, or tincture of opium can be also prescribed (39). In case of Grade 3 diarrhea, lenvatinib should be interrupted until resolution, and resumed at a reduced dose; if diarrhea is persistent and becomes Grade 4 despite medical management, lenvatinib should be discontinued. Patients should be encouraged to complete a stool diary and report any concerns to their healthcare provider. Data from the clinical trials indicate that no patients discontinued pembrolizumab because of diarrhea and among patients who reported an adverse event 6% required pembrolizumab dose interruption, compared to 14% who needed lenvatinib dose interruption (19). Prescribing information of pembrolizumab should be considered to eventually interrupt or discontinue the drug in presence of diarrhea of grade 3 and 4 (or recurrent grade 3), respectively (44).

Nausea/vomiting

Nausea and vomiting were reported in 48% and 39% of patients, respectively (19). As for other TKIs, most cases of nausea and vomiting are of Grade 1 or 2. Optimal medical management to minimize gastrointestinal toxicity should be initiated before any lenvatinib interruption or dose reduction. Dietary modifications (i.e. avoiding chocolate, caffeine, alcohol, and nicotine) are suggested to prevent this adverse event, and antiemetics may alleviate symptoms. Caution should be paid in prescribing ondansetron as it may determine QTc prolongation (39).

Weight loss and anorexia

Decreased appetite was reported in 52% of patients, with a median time to onset of 5.1 weeks, while weight loss occurred in 36% of patients and had a late-onset after 9.1 weeks from starting the combination (19). Prophylactic recommendations for decreased appetite and weight loss include monitoring the patient's appetite and weight in each treatment cycle and encouraging a nutritious diet. In the case of Grade 1 and 2, anorexia dose interruption is effective to alleviate symptoms and lenvatinib treatment can be resumed at the same dose; in the case of Grade 3 anorexia occurring several days after lenvatinib initiation, dose interruption, and dose reduction should be considered (42). Antiemetic agents (prochlorperazine maleate or domperidone) can be prescribed, or oral nutrition support offered when underlying nausea is present (39).

Proteinuria

Proteinuria occurred in 19% of patients enrolled in the KEYNOTE 146 trial in the first 3 weeks (19). Before starting lenvatinib, it is mandatory to check renal function for the presence of proteinuria, and during the treatment monitoring patients with urine dipstick testing regularly is recommended (40). Special attention should be paid to patients with renal dysfunction caused by diabetes or hypertension during lenvatinib treatment. If Grade 1 in high-risk patients with edema, fluid collection, or elevated serum creatinine, lenvatinib treatment should be interrupted and spot urine or 24-hr urine should be checked to determine urinary protein and/or the urine protein-to-creatinine ratio. In the case of Grade 2 proteinuria, dose interruptions, adjustments, or discontinuation may be required (40).

Thyroid toxicity

Hypothyroidism was the most common thyroid toxicity described in the KEYNOTE 146 trial in 51% of patients; the median time to the first onset was 6.1 weeks (18). The American Thyroid Association 2015 guidelines for adult patients recommend monitoring thyroid function by testing thyroid-stimulating hormone (TSH) levels at baseline and regularly during treatment (39). Because patients with Grade 2 hypothyroidism tend to have no symptoms, patients requiring levothyroxine can be hard to identify; however, appropriate information on how to manage

hypothyroidism must be provided to clinicians (37). According to prescribing information for both lenvatinib and pembrolizumab, hypothyroidism either immuno-related or associated with lenvatinib should be treated per clinical practice with a substitutive therapy (44, 45).

PPES

PPES was reported in 26% of patients, with a median time to the first onset of 8.1 weeks (19); 11.1% of patients received medications, including emollients and protectants, and corticosteroids (6). Physicians and nurses should educate patients to care for the skin on their hands and feet before lenvatinib initiation, highlighting the importance of moisturizing hands and feet, the use of appropriate protective clothing, and the importance of sun protection. A change in the schedule of lenvatinib treatment should be considered according to the severity of PPES. In the case of Grade 1 PPES, lenvatinib treatment may be continued at the same dose with the use of moisturizing cream, and a hydrocolloid dressing for the feet may be considered; to manage Grade 2-3 PPES lenvatinib should be interrupted and steroid ointment should be used, after consulting a dermatologist. After recovery to a lower grade, lenvatinib treatment may be resumed at a reduced dose (40).

Alterations in cardiac function

In KEYNOTE 146 10% of patients reported QTc prolongation and cardiac dysfunction (6). The incidence was similar to that observed in the SELECT trial in radioiodine refractory patients where 9% of patients (2% had a cardiac adverse event of Grade 3) experienced cardiac dysfunction, including decreased left or right ventricular function, cardiac failure, or pulmonary edema (21). The increased risk of hypertension associated with lenvatinib may also determine an increased risk of cardiac disease. A baseline echocardiogram is recommended before starting lenvatinib and regularly during the treatment (at least once a year); administration of heart failure therapies is also recommended if indicated (43). Lenvatinib interruption should be considered for grade 3 cardiac dysfunction until resolution to grade 0 or 1. Upon resolution, lenvatinib can be resumed at a lower dose and blood pressure should be monitored daily and maintained within the normal range. Lenvatinib discontinuation should be considered for grade 4 cardiac dysfunction (43).

Osteonecrosis

Osteonecrosis is a rare adverse event associated with antiangiogenic agents, even if its incidence is unknown because it is not always monitored. Before starting lenvatinib, ortho-pan tomography and dental visit are suggested, and invasive dental procedures should be avoided during treatment.

Discussion

In clinical trials, the combination of lenvatinib plus pembrolizumab obtained a significant clinical benefit in terms of PFS and OS in patients with endometrial carcinoma. The safety profile was consistent with the established profiles of each drug in monotherapy. Immune-related adverse events reported in the study, including colitis, rash, hepatitis, and pneumonitis, are expected to occur with anti-PD-1 therapy, likely because of general immunologic enhancement. No new safety signals were identified, and the toxicity profile was manageable with supportive medications, dose interruptions, and/or lenvatinib dose reductions (19). Therefore, to translate advantages observed in trials to clinical practice and provide the maximal benefit from the treatment to patients, clinicians should learn how to manage lenvatinib and its potentially related adverse events.

An appropriate, proactive, and thorough management of lenvatinib toxicities is required to maximize lenvatinib efficacy. Adverse events should be detected as early as possible, by both carefully monitoring the patient from lenvatinib initiation and preventing their occurrence. Patients should be carefully followed also during treatment as some adverse events, e.g. cardiac dysfunction may appear later. Increased awareness on risk to benefit ratio among clinicians would also be helpful to avoid dose interruptions or discontinuation in case of adverse events, with preferring other medical interventions and supportive care, as the experience in thyroid carcinoma and hepatocellular carcinoma teaches (46).

Indeed, evidence in the setting of RAI-refractory differentiated thyroid carcinoma indicated that dose interruptions of more than 10% correlated to shorter PFS, thus limiting the clinical benefit of the drug (47). Furthermore, starting at a reduced dose did not show any advantage in the incidence of adverse events of grade 3, but provided a clinically relevant difference in terms of a lower overall response rate (14). Therefore, dose reductions are justified to manage adverse events, but not to prevent them.

Patients and their caregivers should be educated and made aware of the importance of adherence to treatment to optimize its effectiveness. On one hand, patients should be educated to reduce the risk factors associated with adverse events: smoking, alcohol consumption, stress, and lack of physical activity should be avoided to prevent hypertension, as well as nutrition counseling can be useful to reduce gastrointestinal disorders and weight loss. On the other, increasing patients' awareness of signs and symptoms of a potential adverse event can result in early detection and appropriate management before an excessive worsening. Effective communication between patients and their

physicians is a key factor in successful long-term treatment with lenvatinib.

To date, the experience of handling lenvatinib in endometrial carcinoma is very limited in clinical practice, and in this setting, patients are usually elderly with concomitant diseases that lenvatinib may exacerbate; a geriatric assessment is recommended upfront to effectively plan a monitoring activity and more tailored supports. A long-term follow-up may reveal further adverse events that clinical trials could not detect due to the short duration of treatment. Future data collection in the real world also in patients with endometrial carcinoma will allow to better address the management of adverse events and maximize the clinical benefit for all patients.

Conclusion

The toxicity profile associated with lenvatinib in endometrial cancer is similar to that reported in thyroid carcinoma and hepatocellular carcinoma, where lenvatinib is already used in clinical practice. Careful management of adverse events with prevention strategies, early detection, and proactive interventions allows patients to remain on full doses of lenvatinib as long as possible to gain maximal benefit from the treatment.

Author contributions

Each author gave a substantial contribution to designing the work and collecting data, revised critically for important intellectual content, approved the final version, and agreed to be accountable for all aspects of the work.

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Conflict of interest

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Can circulating PD-1, PD-L1, BTN3A1, pan-BTN3As, BTN2A1 and BTLA levels enhance prognostic power of CA125 in patients with advanced high-grade serous ovarian cancer?

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The most common subtype of ovarian cancer (OC) is the high-grade serous ovarian carcinoma (HGSOC), accounting for 70%–80% of all OC deaths. Although HGSOC is a potentially immunogenic tumor, clinical studies assessing the effectiveness of inhibitors of programmed death protein and its ligand (PD-1/PD-L1) in OC patients so far showed only response rates <15%. However, recent studies revealed an interesting prognostic role of plasma PD-1/PD-L1 and other circulating immunoregulatory molecules, such as the B- and T-lymphocyte attenuator (BTLA), butyrophilin sub-family 3A/CD277 receptors (BTN3A), and butyrophilin sub-family 2 member A1 (BTN2A1), in several solid tumors. Since evidence showed the prognostic relevance of pretreatment serum CA125 levels in OC, the aim of our study was to investigate if soluble forms of inhibitory immune checkpoints can enhance prognostic power of CA125 in advanced HGSOC women. Using specific ELISA tests, we examined the circulating PD-1, PD-L1, pan-BTN3As, BTN3A1, BTN2A1, and BTLA levels in 100 advanced HGSOC patients before treatment, correlating them with

baseline serum CA125, age at diagnosis, body mass index (BMI), and peritoneal carcinomatosis. A multivariate analysis revealed that plasma BTN3A1 ≤4.75 ng/ml (HR, 1.94; 95% CI, 1.23–3.07; p=0.004), age at diagnosis ≤60 years (HR, 1.65; 95% CI, 1.05–2.59; p=0.03) and absence of peritoneal carcinomatosis (HR, 2.65; 95% CI, 1.66–4.22; p<0.0001) were independent prognostic factors for a longer progression-free survival (PFS) (≥30 months) in advanced HGSOC women. However, further two-factor multivariate analyses highlighted that baseline serum CA125 levels >401 U/ml and each soluble protein above respective concentration cutoff were covariates associated with shorter PFS (<30 months) and unfavorable clinical outcome, suggesting that contemporary measurement of both biomarkers than CA125 only could strengthen prognostic power of serum CA125 in predicting PFS of advanced HGSOC women. Plasma PD-L1, PD-1, BTN3A1, pan-sBTN3As, BTN2A1, or BTLA levels could be helpful biomarkers to increase prognostic value of CA125.

KEYWORDS

BTLA, butyrophilins, serum CA125, circulating immune checkpoints, HGSOC, PD-1, PD-L1, prognostic factors

Introduction

Ovarian cancer (OC) is the seventh most frequently diagnosed tumor and the eighth leading cause of cancer death in women worldwide, with a 5-year relative survival rate of 49% (1, 2).

High-grade serous ovarian carcinoma (HGSOC) is the most recurrent subtype and represents 70%–80% of all OC deaths (3). Unfavorable prognosis of HGSOC is determined by tumor heterogeneity and therapy resistance (4). Standard treatment for OC includes surgery and platinum-based chemotherapy (5). Improvements in the progression-free survival (PFS) and overall survival (OS) were achieved by neoadjuvant chemotherapy followed by interval debulking surgery (3). Nevertheless, recurrence rate still remains elevated and about 70% of women with advanced OC relapses with a worse prognosis (6, 7). Immunotherapy, whose effectiveness was demonstrated in other tumors, including non-small cell lung cancer (8, 9), renal cell carcinoma (10, 11), and melanoma (12), has not yielded the expected results in OC (13), despite the presence of tumor-infiltrating lymphocytes (TILs) (14–16).

The most investigated immune checkpoint receptor is the programmed cell death protein 1 (PD-1), with its ligands, PD-L1, and PD-L2 (17). Although PD-L1 expression was detected in more than 50% of advanced OCs, early-phase trials on effectiveness of anti-PD-1/PD-L1 agents exhibited an overall response rate (ORR) between 8% and 60% and a median PFS of 2–10 months (4, 18).

Other immune checkpoints, including B- and T-lymphocyte attenuator (BTLA) (19), butyrophilin sub-family 3 member A1 (BTN3A1) receptor, pan-BTN3A, and butyrophilin sub-family 2 member A1 (BTN2A1) (20), showed an interesting immunomodulatory role in different tumors (11, 21, 22). The activation of these immunoregulatory molecules (including PD-1 and PD-L1) able to positively or negatively modulate anti-tumor immune response may allow, in some cases, cancer cells to overcome immune surveillance (23).

Recently, new evidence showed that investigating the soluble forms of inhibitory immune checkpoints may allow to obtain useful information about the evolution of cancer by predicting survival of patients affected by various tumors. Therefore, these studies suggested their potential use as prognostic biomarkers (11, 21, 22, 24–26). Since plasma is a biological sample that can be easily obtained with little invasiveness, evaluating the plasma concentrations of inhibitory immune checkpoints may provide us a more dynamic profile of the tumor microenvironment and a better overview of disease by overcoming the limitations arising from tissue biopsy (invasiveness, limited quantity of sample, and poor dynamism) (22).

Since evidence showed the potential prognostic relevance of pretreatment serum CA125 (Cancer antigen 125) levels in OC (27), the aim of our study was to investigate if soluble forms of inhibitory immune checkpoints, such as PD-1 (sPD-1), PD-L1 (sPD-L1), BTN3A1 (sBTN3A1), pan-BTN3As (pan-sBTN3As), BTN2A1 (sBTN2A1), and BTLA (sBTLA), can act as useful biomarkers to enhance prognostic power of serum CA125 in advanced HGSOC women.

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Patients and methods

Study cohort

We prospectively studied a cohort of 100 advanced HGSOC women enrolled at the two Sicilian hospital centers: "Sicilian Regional Center for the Prevention, Diagnosis and Treatment of Rare and Heredo-Familial Tumors" of the Section of Medical Oncology of University Hospital Policlinico "P. Giaccone" of Palermo (Italy) and Department of Gynecologic Oncology of the Hospital ARNAS Civico "Di Cristina Benfratelli" of Palermo (Italy).

The study (Protocol "TIC-OC v.1.1") was approved by the ethical committee (Comitato Etico Palermo 1) of the university-affiliated hospital A.O.U.P. "P. Giaccone" of Palermo (Italy).

The information regarding the age at diagnosis, personal history, histological subtype, grading, and International Federation of Gynecology and Obstetrics (FIGO) stages were anonymously collected for all recruited patients (Table 1) who had previously signed a written informed consent.

From May 2018 to July 2021, blood specimens were prospectively harvested from 100 patients with histological diagnosis of advanced HGSOC (stage IIIB–IV) at baseline, before surgery (surgical staging or cytoreductive surgery as clinically recommended), and starting first-line chemotherapy with Carboplatin AUC (area under the curve) 5 and Paclitaxel (175 mg/m^2) according to the current therapeutic strategies. Patients with Eastern Cooperative Oncology Group (ECOG) Performance Status (PS) ≥3 were excluded from the study.

An independent validation cohort of 24 advanced HGSOC women, enrolled at the Section of Medical Oncology of University Hospital Policlinico "P. Giaccone" of Palermo (Italy), was used to confirm the previously obtained data.

TABLE 1 Clinical and pathological characteristics of advanced HGSOC patients.

Characteristic	No. of Patients (%)
Total patients	*100*
Age at diagnosis (y):	
Median: 61	
Mean: 60	
Range: 27-79	
Age groups (y)	48 (48)
≤ 60	52 (52)
> 60	
FIGO stage [a]	
IIIB	23 (23)
IIIC	52 (52)
IV	25 (25)
Histological grade	
G1/2	0 (0)
G3	100 (100)
Histological subtype	
Serous	100 (100)
Other	0 (0)
OC	
Unilateral	64 (64)
Bilateral	36 (36)
Surgery	
Surgical staging	52 (52)
Cytoreductive surgery	48 (48)
Serum CA125 levels	
≤ 401	50 (50)
> 401	50 (50)
Peritoneal carcinomatosis	
Yes	43 (43)
No	57 (57)
BMI	
≤ 25	59 (59)
> 25	41 (41)
Smoker	
Yes	23 (23)
No	77 (77)

[a]AJCC Cancer Staging Manual 8th staging.
BMI, body mass index; CA125, cancer antigen 125; FIGO, International Federation of Gynecology and Obstetrics.

Analysis of plasma PD-1, PD-L1, BTN3A1, pan-BTN3As, BTN2A1, and BTLA dosages

Baseline peripheral blood specimens from untreated advanced HGSOC women were collected, processed for plasma isolation, and analyzed through specific enzyme-linked immunosorbent assays (ELISAs) as previously described (10, 11, 28) to determine plasma concentrations of sPD-1, sPD-L1, sBTN3A1, pan-sBTN3As, sBTN2A1, and sBTLA. In this analysis, the soluble forms of all six immune checkpoints were detected in the plasma rather than in the serum because serum concentrations have been shown to be 10 times lower than those detected in the plasma from the same blood sample. Probably, most of the tested biomarkers were apparently lost due to the clotting process. For this reason, only plasma samples were used in our investigation. Furthermore, a dilution of all samples in the ratio of 1–5 was performed before running the ELISAs in order to prevent interference processes due to the plasma matrix.

Since some discrepancies, concerning the performances, reproducibility, sensitivity and specificity, cross-reactivity, differences in quantification in the plasma and serum, and run temperature, were observed in other commercially available tests, specific ELISAs produced by the company DYNABIO S.A. (Parc de Luminy, Marseille, France) were used according to the previously described recommendations (21, 22). All specifications concerning the features of six ELISAs are reported in Supplementary Table S1.

In particular, these specific ELISAs were used because some assays were either not commercially available (tests for pan-BTN3A, BTN3A1, and BTN2A1) or were not satisfactory (lack of sensitivity, specificity, or reproducibility in our own preliminary studies). These ELISA tests not only showed very

good performances but also established conditions for optimal determination of concentrations of the six markers in blood (use of plasma instead of serum), solving the problem regarding the differences in quantification of proteins between the plasma and serum, which are detected when using other commercial kits. All six used ELISA tests showed good linearity and a high specificity. The linearity for sPD-1 measurement in the test ranges from 0.01 to 5.00 ng/ml, for sPD-L1 from 0.02 to 2.00 ng/ml, for pan-sBTN3As from 0.10 to 8.00 ng/ml, for sBTN3A1 from 0.05 to 8.00 ng/ml, for sBTN2A1 from 0.03 to 2.00 ng/ml, and for sBTLA from 0.25 to 8.00 ng/ml, as shown in Supplementary File 1. In addition, we tested the cross-reactivity between these six recombinant proteins, and, as expected, no signal was detected when the antibodies used did not correspond to the antigen.

Specific details on the experimental protocol regarding the used ELISA assays are reported in Supplementary File 2.

Data analysis

An analysis by receiver operating characteristic (ROC) curves (29) was carried out to identify the optimal concentration thresholds for each soluble form of immune checkpoints and other examined clinicopathological factors (CA125, age at diagnosis, and BMI) in order to divide HGSOC women based on long (≥30 months) versus short PFS (<30 months). The Kaplan–Meier method and log-rank test were applied to perform association analysis of biomarkers and other factors with PFS. We used univariate and multivariate Cox proportional hazard regression models to identify significant prognostic factors for PFS (22). MedCalc software v.18.2.1 for Windows (MedCalc Software, Ostend, Belgium) and GraphPad Prism software v. 9.0.0 (GraphPad Software, San Diego, CA) were used to generate and represent data (22). p-values <0.05 were considered statistically significant.

Results

Determination of the optimal thresholds to discriminate long versus short PFS advanced HGSOC patients

Using specific ELISAs, we performed the measurement of plasma levels of sPD-1, sPD-L1, sBTN3A1, pan-sBTN3As, sBTN2A1, and sBTLA in 100 advanced HGSOC women, before surgery and of starting first-line chemotherapy.

The optimal concentration cutoffs (Youden-index-associated criterion) to discriminate advanced HGSOC patients based on long (≥30 months) versus short PFS (<30 months) were determined for each circulating immune checkpoint through ROC analysis.

The best concentration cutoffs were 2.48 ng/ml for sPD-1 (AUC=0.60, p=0.04), 0.42 ng/ml for sPD-L1 (AUC=0.71, p=0.01), 4.75 ng/ml for sBTN3A1 (AUC=0.64, p=0.01), 13.06 ng/ml for pan-sBTN3As (AUC=0.65, p=0.008), 5.59 ng/ml for sBTN2A1 (AUC=0.64, p=0.02), and 2.78 ng/ml for sBTLA (AUC=0.62, p=0.02). The same analysis also allowed to establish the most suitable thresholds of three different considered factors: age at diagnosis, baseline CA125, and BMI. Therefore, the optimal thresholds for age at diagnosis, CA125, and BMI were, respectively, the following: 60 years (AUC=0.67, p=0.002), 401 U/ml (AUC=0.59, p=0.05), and 25 kg/m2 (AUC=0.62, p=0.01).

Using scatter plots by group, we graphically depicted the circulating levels of each immune checkpoint, ages at diagnosis, serum CA125 levels, and BMIs, dividing the advanced-stage HGSOC women into two groups at long versus short PFS based on each examined parameter (Figure 1).

As shown, most of advanced HGSOC women with PFS <30 months had higher plasma levels of biomarkers (above specific thresholds) and age at diagnosis >60 years, CA125 >401 U/ml, and BMI >25.

Low circulating levels of sPD-1, sPD-L1, sBTN3A1, pan-sBTN3As, sBTN2A1, and sBTLA correlate with a longer PFS in advanced HGSOC women

Since the clinical role of plasma immune checkpoints in predicting survival of advanced HGSOC women has yet to be elucidated, we carried out a Kaplan–Meier survival analysis in order to investigate the prognostic relevance of plasma sPD-1, sPD-L1, sBTN3A1, pan-sBTN3As, sBTN2A1, and sBTLA. The thresholds previously identified by ROC analysis allowed to distinguish advanced HGSOC patients on the basis of low and high plasma levels for each analyzed marker (below and above the specific cutoffs). Kaplan–Meier curves showed the relationship between plasma concentrations of immune checkpoints and PFS (Figures 2A–F).

Concentration cutoffs associated with favorable prognosis and longer PFS were determined for sPD-1 (≤2.48 ng/ml), sPD-L1 (≤0.42 ng/ml), sBTN3A1 (≤4.75 ng/ml), pan-sBTN3As (≤13.06 ng/ml), sBTN2A1 (≤5.59 ng/ml), and sBTLA (≤2.78 ng/ml) (Figures 2A–F). Instead, patients with plasma levels above established cutoffs exhibited a median PFS, which was from 6 to 16 months shorter compared to women with levels below the concentration cutoffs.

In particular, women with lower baseline concentrations showed the following median PFS values than those with higher levels: 30 versus 24 months for sPD-1 (95% CI, 24–36 *vs.* 17–30; log-rank p-value = 0.02); 40 versus 24 months for sPD-L1 (95% CI, 30–55 *vs.* 14–28; log-rank p-value <0.0001); 37 versus 21 months for sBTN3A1 (95% CI, 32–45 *vs.* 15–26; log-rank p-value <0.0001); 35 versus 21 months for pan-sBTN3As (95% CI, 30–45 *vs.* 15–26; log-

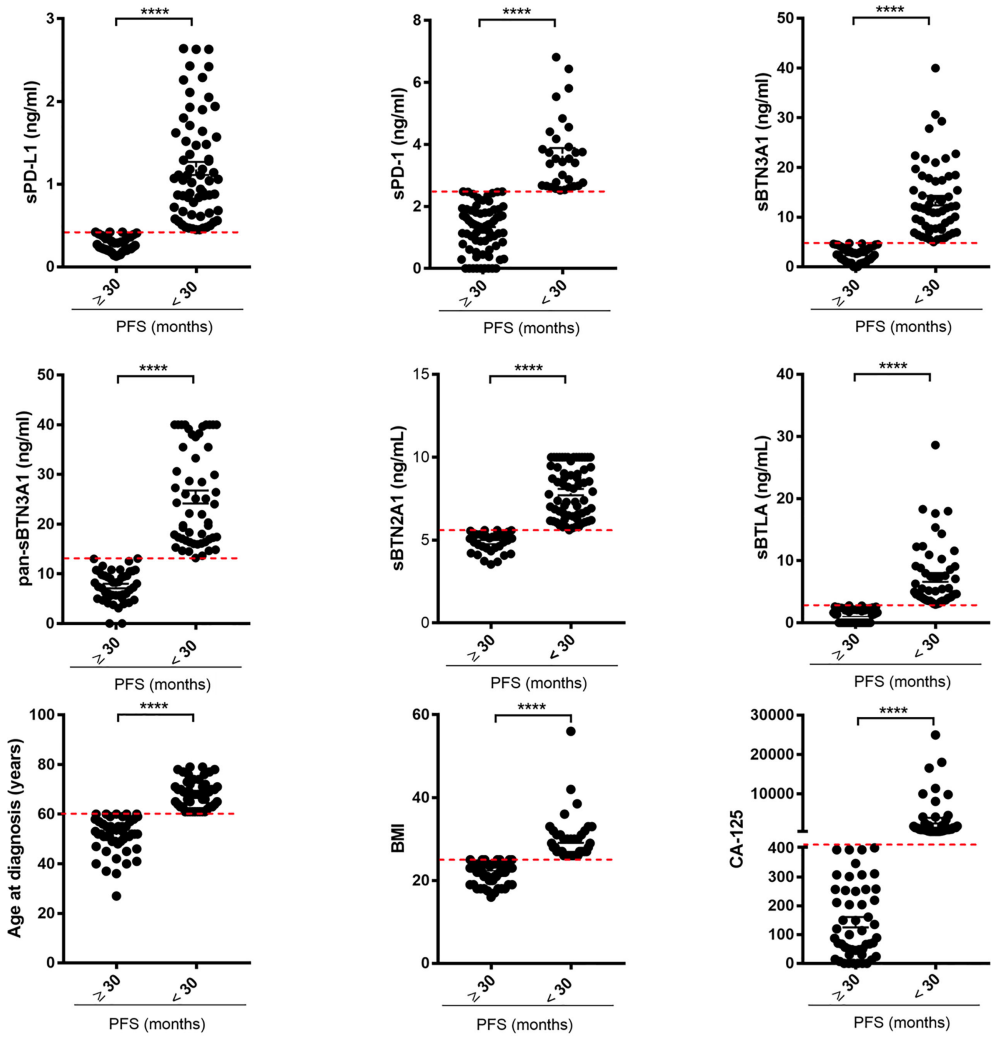

FIGURE 1
Scatter plots by group discriminating advanced HGSOC patients based on long versus short PFS for each examined factor. The plasma levels of each soluble protein, ages at diagnosis, BMIs, and baseline serous CA125 levels of advanced HGSOC patients were plotted for short (<30 months) versus long PFS (≥30 months). For each considered factor, the red dashed lines represent the optimal thresholds previously calculated by ROC analysis. The concentrations of each biomarker are reported in ng/ml. BMI, body mass index; CA125, cancer antigen 125; PFS, progression-free survival. ****p<0.0001.

rank p-value <0.0001); 32 versus 25 months for sBTN2A1 (95% CI, 24–41 *vs.* 20–29; log-rank p-value =0.004); and 32 versus 24 months for sBTLA (95% CI, 25–44 *vs.* 17–28; log-rank p-value =0.0002).

Interestingly, baseline levels of sPD-1, sBTN2A1, and sBTLA below their specific cutoffs showed a lower benefit in median PFS (6–8 months), while a greater advantage in median PFS (14–16 months) was associated with baseline plasma concentrations of sPD-L1, sBTN3A1, and pan-sBTN3As below their specific thresholds.

Furthermore, Kaplan–Meier analysis allowed to evaluate the association between PFS and serum CA125 levels, age at diagnosis, baseline BMI, or peritoneal carcinomatosis at onset (Figures 2G–J).

Advanced HGSOC women with age at diagnosis >60 years, serum CA125 >401 U/ml, BMI >25, or presence of peritoneal carcinomatosis showed shorter PFS and poor prognosis. Instead, a longer median PFS (from 10 to 21 months higher) was associated with age at diagnosis ≤60 years, serum CA125 ≤401 U/ml, BMI ≤25, or absence of peritoneal carcinomatosis (Figures 2G–J).

Specifically, median PFS values for patients with age at diagnosis ≤60 years, serum CA125 ≤ 401 U/ml, BMI ≤25, or absence of peritoneal carcinosis compared to values above the specific thresholds were the following: 32 versus 19 months for age at diagnosis (95% CI, 28–44 *vs.* 13–25; log-rank p-value <0.0001); 32 versus 22 months for serum CA125 (95% CI, 26–38 *vs.* 18–26; log-rank p-value =0.006) and for BMI (95% CI, 25–37 *vs.* 15–26; log-rank p-value = 0.007), respectively; and 38 versus

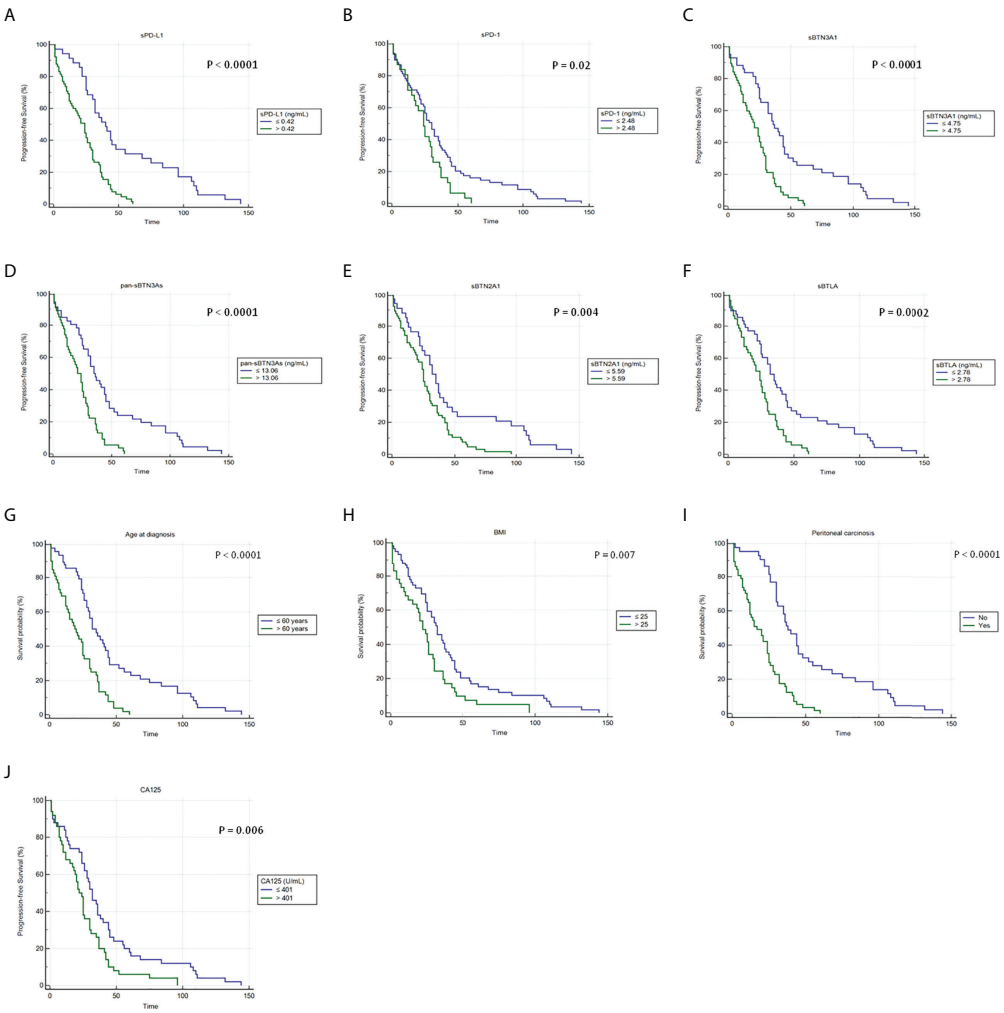

FIGURE 2
Kaplan–Meier analysis of progression-free survival in 100 advanced HGSOC patients with high and low plasma levels of **(A)** sPD-L1, **(B)** sPD-1, **(C)** sBTN3A1, **(D)** pan-sBTN3As, **(E)** sBTN2A1, and **(F)** sBTLA. In addition, Kaplan–Meier analyses showing the correlations between PFS and **(G)** age at diagnosis, **(H)** BMI, **(I)** presence of peritoneal carcinomatosis, and **(J)** baseline CA125 levels are shown. BMI, body mass index; CA125, cancer antigen 125.

17 months for peritoneal carcinomatosis (95% CI, 31–45 *vs.* 12–24; log-rank p-value <0.0001). No peritoneal carcinosis at diagnosis showed a greater gain in PFS.

Plasma BTN3A1 levels, age at diagnosis, and peritoneal carcinomatosis are independent prognostic factors for PFS in advanced HGSOC women

Following the previously obtained results, we carried out a multivariate analysis for PFS to correlate the circulating PD-1, PD-L1, pan-BTN3As, BTN3A1, BTN2A1, and BTLA levels with other clinicopathological factors, such as age at diagnosis, serum CA125 levels, baseline BMI, and peritoneal carcinomatosis. Cox proportional hazard regression models were used for univariable and multivariable analyses in order to evaluate the prognostic significance of all examined parameters (Table 2). The univariable analyses showed a significant association between PFS and age at diagnosis, pre-treatment serum CA125 levels, baseline BMI, peritoneal carcinomatosis at onset, and plasma concentrations of sPD-1, sPD-L1, sBTN3A1, pan-sBTN3As, sBTN2A1, and sBTLA. Conversely, the final multivariable Cox regression model highlighted that only the plasma concentration of sBTN3A1>4.75 ng/ml (HR, 1.94; 95% CI, 1.23–3.07; p =0.004), age at diagnosis >60 years (HR, 1.65; 95% CI, 1.05–2.59; p = 0.03), presence of peritoneal carcinomatosis (HR, 2.65; 95% CI, 1.66–4.22; p <0.0001) were statistically significant. The

TABLE 2 Univariate and multivariate analysis of biomarkers and other factors for PFS in advanced HGSOC patients.

Factor/biomarker	Univariate Cox regression		Multivariable Cox regression	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Age at diagnosis (>60 *vs.* ≤60 years)	2.57 (1.66–3.98)	<0.0001	1.65 (1.05–2.59)	0.03
Serum CA125 (>401 *vs.* ≤401 U/ml)	1.75 (1.16–2.65)	0.008	–	NS
BMI (>25 *vs.* ≤25 kg/m^2)	1.73 (1.14–2.61)	0.007	–	NS
Peritoneal carcinosis (yes *vs.* no)	2.28 (1.51–3.45)	0.0001	2.65 (1.66–4.22)	<0.0001
sPD-L1 (>0.42 *vs.* ≤0.42 ng/ml)	3.01 (1.85–4.89)	<0.0001	–	NS
sPD-1 (>2.48 *vs.* ≤2.48 ng/ml)	1.62 (1.04–2.50)	0.02	–	NS
sBTN3A1 (>4.75 *vs.* ≤4.75 ng/ml)	2.74 (1.75–4.30)	<0.0001	1.94 (1.23–3.07)	0.004
pan-sBTN3As (>13.06 *vs.* ≤13.06 ng/ml)	2.53 (1.63–3.94)	<0.0001	–	NS
sBTN2A1 (>5.59 *vs.* ≤5.59 ng/ml)	1.92 (1.22–3.03)	0.004	–	NS
sBTLA (>2.78 *vs.* ≤2.78 ng/ml)	2.18 (1.41–3.36)	0.0002	–	NS

BMI, Body Mass Index; CA125, Cancer antigen 125; HR, Hazard Ratio; NS, Not Significant.

other studied parameters did not show any statistically significant association. Thus, circulating sBTN3A1 ≤4.75 ng/ml (HR, 1.94; 95% CI, 1.23–3.07; p=0.004), age at diagnosis ≤60 years (HR, 1.65; 95% CI, 1.05–2.59; p=0.03), and absence of peritoneal carcinomatosis (HR, 2.65; 95% CI, 1.66–4.22; p<0.0001) have been shown to be independent prognostic factors for a longer PFS (≥30 months) in advanced HGSOC patients.

However, further two-factor multivariate analyses revealed that each circulating immune checkpoint (with levels above concentration cutoffs) individually correlated in a statistically significant way with baseline serum CA125 >401 U/ml levels, suggesting shorter PFS (<30 months) and poor prognosis (Supplementary Table S2).

Validation analysis

A further independent cohort of 24 peripheral blood samples from advanced HGSOC women was studied to validate the previously obtained results. A Kaplan–Meier survival analysis was carried out using the same concentration cutoffs adopted for leading cohort.

As previously observed, a significant inverse association between PFS and high plasma concentrations for each analyzed biomarker/factor was detected (Figure 3). This confirms and emphasizes our previous data obtained for the leading cohort (Figure 2).

In particular, advanced HGSOC women from validation cohort with lower baseline concentrations of each soluble protein exhibited the following median PFS values compared to those with higher concentrations: 48 versus 28 months for sPD-1 (95% CI, 45–57 *vs.* 19–31; log-rank p-value=0.03); 48 versus 24 months for sPD-L1 (95% CI, 45–57 *vs.* 15–31; log-rank p-value <0.0001) and sBTN3A1 (95% CI, 45–57 *vs.* 15–29; log-rank p-value <0.0001), respectively; 45 versus 28 months for pan-sBTN3As (95% CI, 31–53 *vs.* 17–29; log-rank p-value=0.004); 45 versus 19 months for sBTN2A1 (95% CI, 29–55 *vs.* 12–31; log-rank p-value = 0.0004); and 45 versus 24 months for sBTLA (95% CI, 31–53 *vs.* 17–28; log-rank p-value=0.0004).

Furthermore, an additional Kaplan–Meier analysis confirmed also for validation cohort that age at diagnosis ≤60 years, baseline serum CA125 ≤401 U/ml levels, BMI ≤25, or absence of peritoneal carcinomatosis were associated with a longer PFS (Figure 3). Particularly, advanced HGSOC patients with age at diagnosis ≤60 years or serum CA125 ≤401 U/ml showed a median PFS of 48 months versus 24 months of women with tumor diagnosed over 60 years of age (95% CI, 34–57 *vs.* 15–29; log-rank p-value=0.0002) or serum CA125 >401 U/ml (95% CI, 29–57 *vs.* 15–34; log-rank p-value=0.002). Women with BMI ≤25 or absence of peritoneal carcinosis showed higher median PFS values compared to those observed in women with opposite features: 45 versus 17 months for BMI (95% CI, 34–55 *vs.* 12–28; log-rank p-value <0.0001) or absence of peritoneal carcinosis (95% CI, 34–53 *vs.* 12–24; log-rank p-value <0.0001).

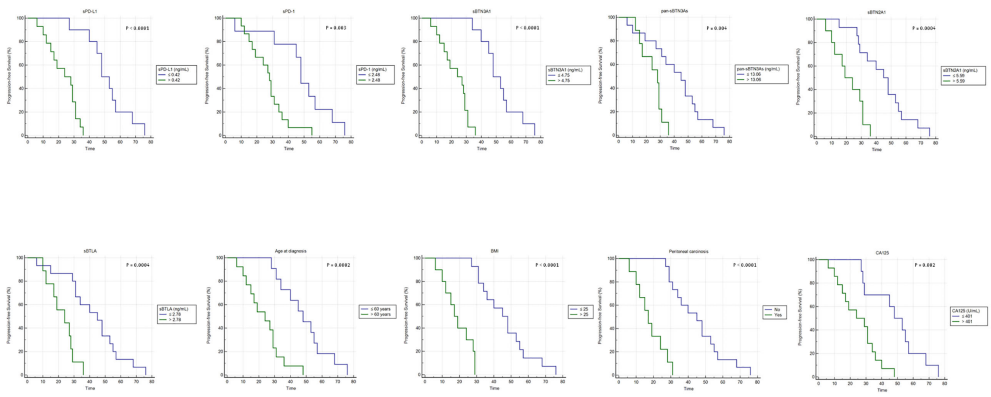

FIGURE 3
Kaplan–Meier analysis of progression-free survival in 24 advanced HGSOC patients from validation cohort. BMI, body mass index.

A further multivariate analysis conducted on validation cohort confirmed that low BTN3A1 concentrations (≤4.75 ng/ml) in plasma, age at diagnosis ≤60 years, and absence of peritoneal carcinosis are independent prognostic factors for a longer PFS in women with advanced HGSOC (Table 3). Lastly, also in this cohort, two-factor multivariate analyses suggested that baseline serum CA125 levels >401 U/ml and each soluble protein above respective concentration cutoff were covariates associated with shorter PFS and unfavorable clinical outcome (data not shown).

Discussion

Scientific research is continuously looking for new prognostic indicators able to predict patient survival, enhancing the therapy efficacy. Due to difficulty detected in early detection of OC, the identification of specific biomarkers could improve disease management and provide information helpful for predicting prognosis (30).

Among the numerous investigated biomarkers, CA125, also known as carbohydrate antigen 125, often considered the "gold

TABLE 3 Univariate and multivariate analysis of biomarkers and other factors for PFS in the validation cohort.

Factor/biomarker	Univariate Cox regression		Multivariable Cox regression	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Age at diagnosis (>60 *vs.* ≤60 years)	5.64 (2.03–15.6)	0.0002	8.12 (2.24–29.5)	0.001
Serum CA125 (>401 *vs.* ≤401 U/ml)	4.91 (1.66–14.5)	0.002	–	NS
BMI (>25 *vs.* ≤25 kg/m^2)	3.64 (1.47–9.03)	<0.0001	–	NS
Peritoneal carcinosis (yes *vs.* no)	5.86 (1.93–17.8)	<0.0001	12.7 (3.65–44.2)	0.0001
sPD-L1 (>0.42 *vs.* ≤0.42 ng/ml)	2.11 (1.01–4.42)	<0.0001	–	NS
sPD-1 (>2.48 *vs.* ≤2.48 ng/ml)	2.62 (1.13–6.07)	0.003	–	NS
sBTN3A1 (>4.75 *vs.* ≤4.75 ng/ml)	4.30 (1.55–11.9)	<0.0001	4.47 (1.30–15.3)	0.02
pan-sBTN3As (>13.06 *vs.* ≤13.06 ng/ml)	2.38 (0.63–9.07)	0.004	–	NS
sBTN2A1 (>5.59 *vs.* ≤5.59 ng/ml)	2.25 (1.01–4.98)	0.0004	–	NS
sBTLA (>2.78 *vs.* ≤2.78 ng/ml)	2.16 (0.71–6.55)	0.0004	–	NS

BMI, body mass index; CA125, cancer antigen 125; HR, hazard ratio; NS, not significant.

standard," has proven to be the most significant indicator involved in screening, detection, management, and survival of OC (31). Serum CA125 levels are measured before surgery in women diagnosed or with suspected diagnosis of OC. Approximately 80% of women affected by epithelial OC show high serum CA125 levels at diagnosis (normal range <35 U/ml) (32). High serum CA125 levels, related to tumor burden and FIGO stages (33), were detected in 50% of early stage disease and 92% of advanced tumors (34). However, several physiological and non-physiological factors affect normal serum CA125 levels, including premenopause, pregnancy (35), menstruation, smoking (34), old age, endometriosis (36), and several malignant conditions, such as breast cancer (37), mesothelioma (38), gastric cancer (39), non-Hodgkin's lymphoma (40), heart failure (41), and liver cirrhosis (42). In addition, BMI is also positively correlated with CA125 levels, and excess adipose tissue has been shown to lead to increased CA125 levels (34).

In the last years, the ability of PD-L1 and PD-1 to act as a marker for clinical outcome was evaluated by several studies (43), which demonstrated the association between their high expression and poor prognosis in patients harboring different tumors (44–46), including OC (47, 48). However, the prognostic value of tumor PD-L1/PD-1 is still controversial and has not been fully clarified yet in OC (49, 50). In addition, the evaluation of PD-L1/PD-1 expression in primary tumor does not always provide information about the evolution of metastatic disease, since these proteins are dynamic biomarkers (22).

Recently, several studies highlighted an association between poor prognosis and high plasma PD-1 and PD-L1 concentrations in different tumors (21, 25, 26, 28), although this correlation has been little studied, to date, in OC patients (51, 52).

Furthermore, in recent years, our research group analyzed the plasma levels of other immunomodulatory proteins, such as BTLA and butyrophilins, in individuals with different cancers (11, 21, 22).

Since several studies demonstrated the prognostic impact of pretreatment serum CA125 levels in predicting the optimal treatment strategy, clinical outcome, and survival in OC (27), our investigation focused on the search for potential correlations between serum CA125 and circulating levels of immunomodulatory molecules, such as sPD-L1, sPD-1, sBTN3A1, pan-sBTN3As, sBTN2A1 and sBTLA, in 100 advanced HGSOC women. In particular, the aim of our study was to investigate if soluble forms of these immune checkpoints may enhance prognostic power of CA125 in advanced HGSOC.

A survival analysis by Kaplan–Meier curves highlighted that plasma concentrations of each immunoregulatory protein were inversely correlated with PFS of advanced HGSOC patients, allowing to divide them into two subgroups on the basis of a longer (≥30 months) versus shorter PFS (<30 months). A benefit in median PFS ranging from 6 to 16 months was observed when circulating levels of soluble proteins were below the specific concentration thresholds. This suggests that, in the future, sPD-1, sPD-L1, sBTN3A1, pan-sBTN3As, sBTN2A1, and sBTLA could act as useful biomarkers for predicting survival of women with advanced HGSOC, enabling to improve patient clinical management and adopt personalized therapeutic strategies for some patients.

Additionally, our investigation also assessed the impact of age at diagnosis, serum CA125, baseline BMI, and peritoneal carcinomatosis at onset on survival of advanced HGSOC patients, suggesting the negative effect of age at diagnosis over 60 years, high serum CA125 levels (>401 U/ml), excess body weight (BMI > 25), or presence of peritoneal carcinomatosis on PFS.

Furthermore, a multivariate analysis performed to study the impact of different baseline covariates (circulating immunomodulatory proteins, age at diagnosis, serum CA125, BMI, and peritoneal carcinomatosis) on PFS revealed that only the plasma concentration of sBTN3A1>4.75 ng/ml, age at diagnosis >60 years, and presence of peritoneal carcinomatosis were independent prognostic factors for a shorter PFS (<30 months) of advanced HGSOC women. This suggests that circulating sBTN3A1 levels, age at diagnosis, and presence/absence of peritoneal carcinomatosis rather than serum CA125 levels should be considered before starting the therapeutic treatment in advanced HGSOC patients.

BTN3A1 showed a significant immunoregulatory function exerted through modulation of the anti-tumor immune response and activation of γδ T cells (53, 54). Since BTN3A1 is highly expressed in malignant tissues of HGSOC compared to benign ovarian tumors and normal tissues and is associated with poor clinical outcome (53, 55), our results about the correlation between high plasma levels of its soluble form and unfavorable prognosis in advanced HGSOC women are consistent with what was expected. In addition, targeting of BTN3A1 has been shown to transform BTN3A1 from an immunosuppressive to an immunostimulatory molecule, by inducing γδ T-cell-mediated anti-tumor cytotoxicity, resulting in the killing of specific tumor cells by γδ T cells. This may represent an interesting strategy for the treatment of tumors resistant to immunotherapy (55).

Finally, additional two-factor multivariate analyses highlighted that circulating levels of each immunomodulatory protein (sPD-1, sPD-L1, sBTN3A1, pan-sBTN3As, sBTN2A1, or sBTLA) were individually associated with serum CA125 levels, suggesting that contemporary measurement of both biomarkers than CA125 only could strengthen the prognostic power of serum CA125 in predicting PFS of advanced HGSOC women.

Although this investigation provides significant and useful information to current knowledge in the field, it presents some potential limitations, including the relatively limited number of analyzed patients in the leading cohort, the lack of a sufficiently large validation set able to test each new putative biomarker (despite data shows a good statistical power), and the potential interference of the plasma matrix during the dosage in plasma of immune checkpoints through ELISA assays (although a one-

fifth dilution of the samples seems to overcome this problem). In addition, a larger number of studies are needed to deeply investigate the releasing mechanisms (to date, unknown) of the soluble form of each immunoregulatory protein from tumors and/or stromal cells.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This study was reviewed and approved by Comitato Etico Palermo 1, University-affiliated Hospital A.O.U.P. "P. Giaccone" of Palermo (Italy)- (Protocol "TIC-OC v.1.1"). The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization: DF, JLI, AR, VB and VC. Sample collection and investigation: DF, CB, LRC, SC, MCDD, CF, MCL, LM, UR, SV, GP and VC. Experimental analysis: DF and JLI. Data curation and analysis: DF, CB and LRC, MCDD, SC, CF, MCL, LM and TDBR. Writing: DF, CB and LRC. Critical revision of the manuscript: DF, JLI, AR, VB, VC, SV and GP. Supervision: JLI, AR, VB and DF. The figures of the manuscript were conceived and designed by DF, CB, LRC, UR and TR. The tables were conceived and designed by DF, CB, LRC, UR, LM and TR. Literature data were acquired and analyzed by DF, CB, LRC, LM, UR and TR. All authors contributed to the article and approved the submitted version.

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Conflict of interest

JLI is cofounder of PanCa Therapeutics and PredicitngMed.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.946319/full#supplementary-material

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The immersive experience of virtual reality during chemotherapy in patients with early breast and ovarian cancers: The patient's dream study

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Background: A virtual reality experience (VRE) could represent a viable non-pharmacological intervention to reduce and better manage the main factors of psychophysical distress related to the diagnosis and treatment of cancer.

Aim: The *"Patient's Dream"* study was a two-arm randomized controlled trial conducted at the Regina Elena National Cancer Institute – IRCCS (Rome, Italy) from April 2019 to January 2020 to evaluate VRE impact in patients affected by breast or ovarian cancer. Before starting the first cycle of chemotherapy (CT), patients were randomized to receive the VRE (VRE arm) as "distraction therapy" or to entertain themselves with conventional means (control arm). The primary aims were the assessment of psychological distress, anxiety and quality of life between the two study arms. Secondary endpoints were the perceived time during the first course of CT and the acute and late toxicity.

Results: Fourty-four patients were enrolled, 22 patients were randomly assigned to the VRE arm and 22 to the control arm. Collected data underline the absence of prevalent disturbs of anxiety and depression in both groups. Nevertheless, even if the state anxiety values before and after CT decreased in both groups, this reduction was statistically significant over time only in the VRE arm. The duration of therapy perceived by patients undergoing distraction therapy was significantly shorter when compared to the control group. The use of VRE during the first CT cycle appeared to reduce asthenia outcomes.

Conclusion: Obtained data suggest that the VRE positively influenced the levels of state anxiety among cancer patients and support the continuous research on VRE as a distraction intervention, with the aim to meet the clinical need for effective nonpharmacologic adjunctive therapies.

Clinical trial registration: https://clinicaltrials.gov/ct2/show/NCT05234996, identifier NCT05234996.

KEYWORDS

virtual reality, breast cancer, ovarian cancer, anxiety, chemotherapy, perceived time

1 Introduction

The diagnosis of neoplastic disease is accompanied by an emotional complex process characterized by anxiety, depression, anger, and uncertainty about the present and future (1). The proposed treatments often cause anxiety and psychological distress further because of the toxicity profile and the frequent requirement for painful procedures (venipuncture, central venous access, invasive investigations) (2). Therefore, efforts to provide interventions to alleviate symptoms related to chemotherapy are an important area of research and improvement.

Evidence from the literature shows that an immersive virtual reality experience (VRE) can reduce procedural pain and anxiety in patients undergoing medical procedures, such as wound care or physical therapy for burn wounds, dressing changes for trauma injuries, procedures under local anesthesia, such as episiotomy repair and orthopedic surgery (3, 4). Indeed, virtual reality creates a sense of absorption in the virtual environment through special glasses and motion sensors, thus estranging the user from reality.

Based on these considerations and given the need for an integrated approach to managing cancer patients, VRE could represent a viable non-pharmacological intervention to reduce and better drive the main factors of psychological distress related to the diagnosis and treatment of cancer.

In the field of oncology, VRE can represent a "distraction therapy" tool that helps the cancer patient overcome physical limits and/or mental dictated by the disease condition (4, 5). This approach could also promote greater adherence to treatment with potential benefits on effectiveness and increase the host healthcare facility's confidence and approval rating.

The "Patient's Dream" was a prospective study designed to evaluate VRE impact in patients affected by breast or ovarian cancer. The present work assessed the improvement of psychological distress, anxiety and quality of life after a "distraction therapy" intervention by means of VRE, utilized during the first cycle of adjuvant chemotherapy (CT). The time perception by the patients during the treatment and acute and late toxicity were also assessed.

2 Patients and methods

2.1 Study design and setting

The "Patient's dream" study is a two-arm randomized controlled trial conducted at Regina Elena National Cancer Institute – IRCCS (Rome, Italy) from April 2019 to January 2020; no stratification factors were planned. Before starting the first cycle of chemotherapy, patients were randomized to receive the VRE (VRE arm) as "distraction therapy" or to entertain themselves with conventional means (control arm), such as listening to music, watching a TV program, reading newspapers, books, magazines or also doing nothing, according to the patient's preferences and for the entire duration of administration of the first CT cycle. A clinical team composed of three oncologists, three psychologists, one nurse and one expert VR operator supported the patients involved in the study.

The primary aims were the assessment of psychological distress, anxiety and quality of life between the two study arms. Secondary endpoints were the perceived time during the first course of CT and the acute and late toxicity.

The study was conducted in accordance with the ethical standards as laid down in the Declaration of Helsinki and its later amendments and within the protocol approved by the Central Ethics Committee (protocol registration number: RS 1105/18). Written informed consent was obtained from all participants included in the study. Clinical trial registration number: NCT05234996.

2.2 Patients

To be eligible for the study, all patients had to have a confirmed histological diagnosis of breast or ovarian cancer stage I–III, surgery as the first therapeutic approach, and be suitable to receive the first cycle of adjuvant CT, with or without a biological treatment according to specific cancer (regimens including anthracyclines/taxanes, anthracyclines/cyclophosphamide, carboplatinum/taxane, taxane alone combined or not with trastuzumab for breast cancer,

carboplatin/paclitaxel combined or not with bevacizumab for ovarian cancer). Patients must be aged ≥18 years, with a median Eastern Cooperative Oncology Group (ECOG) performance status of 0–2, life expectancy >12 months and ability to understand and sign the informed consent. Patients presenting a previous history of alcohol and/or drug addiction, disorder of vision and eyes, and a history of psychiatric pathologies were not eligible.

2.3 Study evaluations

In both study arms, patients were evaluated as follows: before the start of the first infusion of CT (T1) with the Hospital Anxiety and Depression scales (HADs), the State–Trait Anxiety Inventory for Adults (STAI) in Y1 forms for the State Anxiety and Y2 for the Trait Anxiety and with the European Organization for Research and Treatment of Cancer quality of life questionnaire, Core 30 (EORTC QLQ-C30); immediately at the end of the infusion (T2) with STAI Y1 and with the investigation of the perceived time; within 48 h from the first CT cycle (T3) with HADs, STAI Y1, EORTC QLQ-C30; 1 week after the first CT cycle (T4) with a patient-reported outcomes (PROs) questionnaire; within 48 h from the second cycle (T5) with STAI Y1 and PROs questionnaire (Table 1). The description of each evaluation tool is provided in the following sections.

2.3.1 Psychological evaluations

2.3.1.1 Psychological distress

The psychological distress was evaluated with the Hospital Anxiety and Depression scale (HADs) (6). The HADs is composed of 14 items, seven assess the anxiety status, and seven assess the depression status. The answers were given on a 4-point Likert scale (from 0 to 3) with a maximum of 21 points for anxiety and depression. A score ≥8 is the cut-off indicating the presence of anxiety or depression disorder. According to Carrol and collaborators, scores between 0 and 7 indicate a normal condition, scores between 8 and 10 indicate borderline cases, while scores ≥11 identify clinical cases (7).

2.3.1.2 Anxiety

Anxiety has been evaluated with the STAI (6) in Y1 forms for the State Anxiety and Y2 for the Trait Anxiety. Each form consists of 20 items answered on a 4-point Likert scale (from 0 to 3). The final score ranges from 20 to 60, higher score corresponds to major anxiety.

2.3.2 Quality of life assessment

Health-related quality of life (HRQoL) was assessed with EORTC QLQ-C30 Version 3.0 [eortc.org]. The questionnaire is made up of 30 items divided into 15 scales: Physical Functioning (PF), Role Functioning (RF), Social Functioning (SF), Emotional Functioning (EF), Cognitive Functioning (CF), Global QOL (QL), Fatigue (FA), Pain (PA), Nausea/Vomiting (NV), Appetite Loss (AP), Dyspnea (DY), Sleep Disturbances (SL), Diarrhea (DI), Constipation (CO), and Financial Impact of Disease (FI) (8). The score of each scale was obtained by a sum and a linear transformation and ranged from 0 to 100. In the functional scales, a higher score corresponded to better functioning of the area; in the symptomatic scales, a higher score corresponded to the worst of symptoms.

2.3.3 Effective time and perceived time

The effective time was defined as the time from the start to the end of chemotherapy infusion; the nurse of the study team checked this time. The perceived time was the time felt and reported by the patient from the start to the end of CT infusion.

2.3.4 Toxicity assessment

Toxicity was evaluated according to the Common Terminology Criteria for Adverse Events (CTCAE) v4.0 (9).

2.3.5 Patient-reported outcomes questionnaire

Before the CT infusion, the oncologist illustrated and delivered the PRO-CTCAE™ questionnaire to the patient to be reported on subsequent visits (10). It was composed of 124 items and covered 78 symptoms. Symptoms evaluated can be detected by one up to a maximum of three characteristics:

TABLE 1 Timeline of study measures.

	T1	T2	T3	T4	T5
HADs	X		X		
STAI Y1	X	X	X		X
STAI Y2	X				
Perceived Time		X			
EORTC QLQ-C30	X		X		
PROs				X	X

T1: before the start of the first infusion of CT; T2: immediately at the end of the infusion; T3: within 48 h from the first CT cycle; T4: one week after the first CT cycle; T5: within 48 h from the second CT cycle. HADs, Hospital Anxiety and Depression scales; STAI, State-Trait Anxiety Inventory for Adults; Y1 forms for the State Anxiety, Y2 for the Trait Anxiety; EORTC QLQ-C30, European Organization for Research and Treatment of Cancer quality of life questionnaire, Core 30; PROs, patient-reported outcomes.

presence (yes; no); frequency (never; rarely; occasionally; frequently; almost constantly); severity (none; mild; moderate; severe; very severe); and/or interference with usual or daily activities (not at all; some; a bit; a lot; very). Some PRO-CTCAE™ symptoms comprise only one, while others include two, and some include three characteristics.

2.4 Distraction therapy modalities

The VRE was administered using three VR headsets containing a selection of audiovisual productions made with 360° technology and selected based on content, plot and production dynamics. During the entire experience, an operator dedicated to patient care was present to allow the most comfortable experience possible.

Patients were trained on the functioning of the headset, their interface, and how to select the preferred contents. Once familiar with the controls, the operator equipped the patients with high-quality audio headphones to complete the immersive effect of the contents. At that point, the patients were free to use the contents in complete autonomy. In any case, the operator remained close to the patient for the entire duration of the experience, giving advice on the contents or on the commands to be used from time to time. The contents, some created for the occasion and others made available by audiovisual production companies specialized in 360° videos, were carefully selected based on pre-established criteria:

- High viewing comfort (no abrupt camera movement, low risk of nausea, clear images etc.);

- Relaxing and engaging content, such as concerts, walks in the European capitals, mountain nature trails, isolated and fascinating places, pristine, exotic beaches, and Yoga sessions;

- Duration and comfortable viewing time, never more than 10 minutes for single content (the patient chose 3 or 4 contents).

The administration of VRE began with the therapy and lasted a maximum of 60–90 minutes. The VR headset used to provide such experiences was Oculus Go, a standalone VR headset developed by Facebook Technologies in partnership with Qualcomm and Xiaomi. The Oculus Go was an all-in-one headset and did not need to be tethered to an external device to use. It was equipped with a Qualcomm Snapdragon 821 chipset and a single 5.5-inch LCD with a resolution of 1280 × 1440 pixels per eye and a refresh rate of 72 or 60 Hz, depending on the application. The headset used Fresnel lenses that were improved over those used in the company's previous headset, the Oculus Rift. It provided a field of view of about 101°, which gives the Go a display fidelity of 12.67 pixels per degree. Inputs were provided with a wireless controller that functions much like a laser pointer. The headset and controller utilized non-positional 3-degrees-of-freedom tracking, making it capable of seated or static-standing activities but unsuitable for room-scale applications.

2.5 Statistical analysis

The primary endpoint was the HADs scale. A sample size of 44 patients was needed to test an effect size (standardized mean difference among the 2 groups) of at least 0.70 considering a correlation of 0.50 and using repeated measurement analysis of variance (ANOVA) as a model. This sample size was determined to ensure a power of 80% at a significance level of 5%. Data were reported as mean and standard deviations, and the Student's t-test was used to compare mean values. The chi-square test assessed associations between categorical variables. All analyses were performed using the IBM-SPSS statistical software, version 22.0. No adjustments for multiple tests were made.

3 Results

A total of 44 patients were enrolled; 22 patients were randomly assigned to the VRE arm and 22 to the control arm. The characteristics of the patients are shown in Table 2.

Patients presented predominantly breast cancer submitted to surgery (17 patients [77%] in the experimental arm and 19 [86%] patients in the control arm) with HER2-negative phenotype (15 patients [68%] in the experimental arm and 12 [55%] patients in the control arm). Patients had an ECOG status predominantly equal to 0 (Table 2), and the most common CT regimen was anthracyclines + taxanes. Most of the patients were married (50% in the VRE arm and 77% in the control arm), had a master's degree (54% and 41% in VRE and control arm, respectively) and were employed (82% and 59% in VRE and control arm, respectively).

3.1 Psychological evaluations

3.1.1 Psychological distress

The HADs mean scores were below the cut-off at both considered time points T1 and T3, underlining the absence, in the whole sample, of anxiety and depression (Supplementary Table 1).

Stratifying patients by distress severity according to scores, in the VRE arm, stability in normal scores was reported between T1 and T3 (55% vs 52%, respectively), along with an increase in patients with borderline scores (14% at T1 vs 33% at T3) and a decrease in patients with pathological scores (32% at T1 vs 14% at T3). In the control arm, a decrease in patients with normal scores (64% at T1 vs 50% at T3) and an increase in both patients with borderline (14% at T1 vs 20% at T3) and pathological (23% at T1 vs 30% at T3) scores was reported from T1 to T3 (Supplementary Table 2).

Considering depression, results showed a similar trend in the two arms with a reduction in normal scores (77% at T1 vs 57% at T3 in the VRE arm; 86% at T1 vs 70% at T3 in the control arm)

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TABLE 2 Patient characteristics.

	VR arm (n = 22), n (%)		Control arm (n = 22), n (%)	
	Breast cancer, 17 (77.2%)	**Gynecological cancer, 5 (22.7%)**	**Breast cancer, 19 (86.4%)**	**Gynecological cancer, 3 (13.6%)**
Age (years), median (range)	51 (37–71)	50 (36–61)	50 (39–69)	52 (51–62)
Menopausal status:				
• Pre	8 (47.1)	0 (0)	12 (63.2)	0 (0)
• Post	9 (52.9)	5 (100)	7 (36.8)	3 (100)
BRCA status				
• Mutation	1 (5.9)	1 (20)	0 (0)	1 (33.3)
• Wild-type	10 (58.8)	3 (60)	5 (26.3)	1 (33.3)
• Not done	6 (35.3)	1 (20)	14 (73.7)	1 (33.3)
ECOG performance status:				
• 0	16 (94.1)	4 (80)	18 (94.7)	2 (66.6)
• 1	1 (5.9)	1 (20)	1 (5.2)	1 (33.3)
Hormonal receptors:				
• Negative	4 (23.5)	0 (0)	6 (31.6)	0 (0)
• Positive	13 (76.5)	0 (0)	13 (68.4)	0 (0)
HER2:				
• Negative	15 (88.2)		12 (63.2)	
• 2+/FISH+	0 (0)		2 (10.5)	
• 3+	2 (11.8)		5 (26.3)	
Surgery:				
• Radical	4 (23.5)	4 (80)	9 (47.4)	2 (66.6)
• Conservative	12 (70.5)	1(20)	10 (52.6)	1 (33.3)
• Unknown	1 (5.9)			
Chemotherapy regimens:				
• Antracyclines + taxanes	15 (88.2)	0 (0)	16 (84.2)	0 (0)
• Trastuzumab + taxane	2 (11.8)	0 (0)	3 (15.8)	0 (0)
• Carboplatin + taxane	0 (0)	5 (100)	0 (0)	3 (100)
State:				
• Maiden	1 (5.9)	0 (0)	1 (5.2)	0 (0)
• Cohabitant	1 (5.9)	1 (20)	2 (10.5)	0 (0)
• Married	10 (58.8)	4 (80)	15 (78.9)	2 (66.6)
• Separate	3 (17.6)	0 (0)	1 (5.2)	0 (0)
• Divorced	0 (0)	0 (0)	0 (0)	0 (0)
• Widow	2 (11.8)	–	0 (0)	–
Schooling:				
• Elementary school	1 (5.9)	–	1 (5.2)	–
• Middle school degree	1(5.9)	–	2	1 (5.2)
• High school degree	5 (29.4)	3 (60.0)	8	1 (5.2)
• Master's degree	10 (58.8)	2	8	1 (5.2)
Occupation:				
• Employee	14	3 (60)	6	1 (5.2)
• Trader craftsman	0 (0)	0 (0)	1 (5.2)	–
• Freelance	1 (5.9)	0 (0)	4	1 (5.2)
• Housewife	1 (5.9)	1 (20)	7 (36.8)	–
• Unemployed	0 (0)	0 (0)	0 (0)	–
• Retired	1 (5.9)	1 (20)	1 (5.2)	1 (5.2)

and an increase in borderline (14% at T1 vs 29% at T3, VRE arm; 9% at T1 vs 20% at T3, control arm) and pathological scores (9% at T1 vs 14% at T3, VRE arm; 5% at T1 vs 10% at T3, control arm; Supplementary Table 2).

3.1.2 Anxiety

In the VRE arm, a statistically significant reduction of the State Anxiety mean values was reported between T1 and T2 (45.9 ± 12.5 vs 33.4 ± 9.3, p<0.0001) and between T1 and T3 (45.9 ± 12.5 vs 40.9 ± 10.4, p=0.02). At T5, the observed mean value (41.9 ± 10.1) remains lower than the baseline value (Figure 1).

In addition, in the control arm, the State Anxiety mean values between T1 and T2 were statistically different (39.2 ± 9.5 vs 37.2 ± 9.0; p=0.04), albeit at a lower level. At T3 and T5, the observed mean values (41.6 ± 9.7 and 42.6 ± 10.8, respectively) tended to be higher than baseline (Figure 1).

Comparing the two groups, at T1 the State Anxiety was significantly higher in the VRE group than in the control group (45.9 ± 12.5 vs 39.2 ± 9.5; p=0.05) (Figure 1).

In the VRE group, at T1 there was a statistically significant difference between the Trait Anxiety (37.3 ± 8.6), and the State Anxiety (p=0.002) mean values. This difference was not statistically significant in the control group (Trait Anxiety: 40.6 ± 11.1; p=0.32). The Trait anxiety was not statistically different between the two groups (37.3 ± 8.6, VRE arm vs 40.6 ± 11.1, control arm; p=0.22).

3.2 Quality of life assessment

The mean scores related to the EORTC QLQ-C 30 questionnaire did not show statistically significant differences between the two groups considered time points, T1 and T3, except for constipation at T3 (p=0.02) (Supplementary Table 3).

3.3 Secondary endpoints

3.3.1 Effective and perceived time of treatment

In the VRE arm, 86% (n=19) of patients reported the perception of a shorter duration of CT compared to the effective treatment time. The median perceived time was of 104 minutes (range: 99–105) versus a median of 141 minutes (range: 135–145) of real duration (p<0.0001).

In the control arm, 76% (n=16) of patients perceived a longer than the effective duration of CT (median perceived time: 170 minutes, range: 165–174; median real duration: 155 minutes, range: 150–160; p<0.004).

The median reported perceived time was statistically different between the two groups (p=0.02).

3.3.2 Toxicity

All the enrolled patients were assessable for safety analysis. The most frequently reported treatment-related toxicities were mild to moderate (grades 1–2) (data not shown). The main toxicities reported after the first CT cycle were grade 2 alopecia and grade 3 and 4 neutropenia. They were similar in both arms (73% in the VRE arm vs 68% in the control arm for alopecia, 27% in both arms for neutropenia). Grade 3 emesis was less evident in the VRE arm than in the control arm, but not significantly (4% vs 14% respectively; p<0.24). In the VRE arm, asthenia was reported less than in the control arm (4% vs 36% respectively; p=0.008; Table 3).

3.3.3 PRO data analysis

The patients' perceptions investigated through the PROs at T4 (eight patients) and T5 (22 patients) showed a population

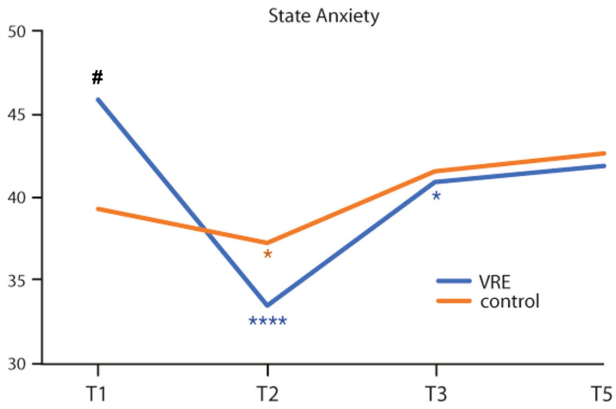

FIGURE 1
State anxiety mean values from the start to 48 hours from the end of the first chemotherapy cycle in the two study arms. *Intra-group variability. #Inter-group variability. Statistical significance: *,#p < 0.05; ****p < 0.0001.

with low toxicity. The statistical analysis between the time points considered was not studied for an imbalance of patients, so only T5 analysis was reported.

The analysis of the psychological variables included: insomnia (items 52), fatigue (items 53), anxiety (items 54), mood (items 55), sadness (items 56). At T5, all these were different between the VRE and control arm: severity and interference with usual or daily activities of insomnia [(9% vs 35% (p=0.04) and 14% vs 35% (p=not significant), respectively)]; a greater interference of the anxiety [(9% vs 35% (p=not significant), respectively)]; a perception of higher frequency and intensity in the mood [(14% vs 35% (p=not significant) and 0 vs 20% (p=0.03), respectively)]; a greater perception of interference in daily activities deriving from sadness (5% vs 14%, respectively); no difference was documented in fatigue item.

4 Discussion

In recent years, there has been an exponential development of VRE. Today, this entertainment intervention finds widespread application in various fields: from the fashion industry with digital dressing rooms to 360° VR photography, to the automotive sector and cinema, from the world of videogames to virtual museum visits. VR is not a new technology, but it is a tool that is going through radical evolution. There have been some advancements in industries with some interesting new possibilities. One of the most interesting and perspective-bending abilities of VR is the capability to immerse ourselves completely into an environment totally outside of our regular size, positioning us in a new relationship with the world. For instance, the growing field of immersive microscopy is putting doctors and scientists into microscopic worlds, giving them literal new perspectives of what is happening inside the human body, down to the scale of connected networks of neurons within the brain structure. Being able to visualize and manipulate the world at this scale holds incredible possibilities for solving medical problems, the potential of which has generated a lot of interest in the field from outside investors.

For the reason stated above, also in health, VRE is gaining increasing interest, finding potential areas for development and application in the field of diagnostics, therapy, training and prevention.

In breast and gynecological patients, psychological distress is present at diagnosis and after surgery (11). The first experiments to assess the impact of VRE in cancer patients undergoing CT dates back to 1999 (12). The results of the seven randomized clinical trials published from 1999 to 2011 supporting the use of the VRE reported a reduction of the symptoms related to distress, fatigue, anticipatory nausea, and perception of the duration time of CT administration (13).

Although encouraging, these results derive mainly from pilot studies with low or mixed samples and limited statistical power. They explored various relevant variables, including different settings (i.e., during chemotherapy, during painful procedures, during hospitalization, and during port access). Moreover, most were the result of experiments conducted by technology and contents are now considered obsolete. Specifically, at that time, most VREs were limited to graphic reconstruction of reality without further direct experience with VRE, highlighting the need for more modern and innovative technologies.

In the present study, 44 breast and ovarian cancer patients were randomly assigned to receive VRE or to entertain themselves with conventional means during the first cycle of adjuvant CT, with the aim to evaluate if patients in the VRE arm reported an early improvement of psychological, anxiety, and quality of life outcomes. In this study, thanks to applying the most modern technologies, it was possible to give a “dream” during adjuvant CT exposure, allowing patients to view and live an immersive global experience.

Results from HAD questionnaire underlined the absence of prevalent disturbs of anxiety and depression in both groups.

A prevalence of normal scores related to depression was reported in both groups, along with a similar trend over time. This result underlines the low impact of VRE on the depressive state.

Regarding anxiety, our findings showed a different distribution of scores over time. In the comparison between T1 and T3 in the VRE arm, there was an increase in patients with borderline scores and a decrease in patients with pathological scores. On the contrary, in the control group, an increase in both borderline and pathological scores were observed at T3.

TABLE 3 Grade 3 and 4 reported toxicities.

	VRE arm (n = 22), n (%)	Control arm (n = 22), n (%)
Alopecia (grade 2)	16 (72.7)	15 (68.1)
Neutropenia	6 (27.2)	6 (27.2)
Febrile neutropenia	2 (9.0)	4 (18.2)
Emesis	1 (4.5)	3 (13.6)
Hypertransaminasemia	1 (4.5)	1 (4.5)
Asthenia	1 (4.5)	8 (36.4)

This trend suggests the impact of VRE on the anxiety outcome, as supported by data relating to state anxiety. Indeed, even if the state anxiety values before and after the first CT cycle decreased in both groups, this reduction is statistically significant over time only in the VRE arm. This difference in the scoring trend could be related to the different scores in trait anxiety, which tend to be lower in the VRE group, indicating a population whose anxiety is predominantly situational, determined by a stressful event such as CT. VRE would therefore act more positively in a population that presents an increased state of anxiety related to the crisis event in the absence of an anxious basic structure.

QoL scores related to the EORTC QLQ-C 30 questionnaire overall compared the two study groups.

The duration of therapy perceived by patients undergoing distraction therapy was significantly shorter when compared to the control group, which reports a perception of time greater than the real duration of therapy.

Regarding toxicity data, the use of VRE during the first CT cycle appears to reduce asthenia outcomes. The PROs results analyzed at T5 were in line with the results of the STAI scale, confirming the presence of a better psychological state in the patients of the VRE group. Taken together, these data suggest that the VRE positively influenced the levels of state anxiety among patients. Moreover, even if a cost analysis goes beyond the scope of this study, we can speculate that a VRE intervention could reduce the costs related to drug therapy and support interventions for the anxiety management.

Our results were in line with other previous studies about the impact of VRE on the health system and during cancer treatments. These studies found that VRE improved patients' emotional well-being and diminished cancer-related psychological symptoms (4, 13–17) in different settings. Nevertheless, the time of the VRE exposition was very short in most of these experiences. VRE's impact on clinical variables involved in distress (pulse rate, blood pressure) has been investigated only partially. In this context, our study is the first to evaluate the use of VRE during the first cycle of CT in breast or ovarian cancer patients, thus analyzing a specific homogeneous subgroup of patients and with a methodologically improved study design. In addition, our study used a relatively high-tech VRE with highly interactive virtual worlds. Considering that immersion and interactivity impact VRE efficacy, stronger results might likely be obtained with more immersive and fully interactive experiences. In addition, few studies compared the efficacy of VRE with a concurrently randomized control group and, therefore, are at risk of bias.

Our study presents some limitations, mainly related to the sample's small size and the short-term analysis. In addition, from our data, it was not possible to identify the mechanism of action of VRE on anxiety nor the population that could benefit most from this strategy. Further studies are therefore needed to define the role of VRE in improving the psychological well-being of patients undergoing CT. This will allow virtual reality to be used more effectively in daily clinical practice.

Conclusion

In the era in which the quality of life of cancer patients is taking a fundamental role, it is a primary goal to improve the benefit of the cure and the life of long survival patients. Our study suggests that the use of VRE has some benefits on the state anxiety of the first cycle of CT. Since the first cycle of CT can not scan impact subsequent cycles, not only for toxicity related to treatment but also for emotional distress, this tool could also be useful for a more important acceptance of the treatment and compliance with the therapy.

Our results support the continuous research on VRE as a distraction intervention, with the aim to meet the clinical need for effective nonpharmacologic adjunctive therapies.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Central Ethics Committee (protocol registration number: RS 1105/18). The patients/participants provided their written informed consent to participate in this study.

Author contributions

Study design: AF, FG, DG. data collection and interpretation: All. manuscript writing: AF, VR, and CF. manuscript editing: All. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Authors FG, AG and GG are funders of Twiceout.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.960387/full#supplementary-material

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Clinical impact of soluble Neuropilin-1 in ovarian cancer patients and its association with its circulating ligands of the HGF/c-MET axis

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Background: Neuropilin (NRP) is a transmembrane protein, which has been shown to be a pro-angiogenic mediator and implicated as a potential driver of cancer progression. NRP-1 up-regulation in ovarian cancer tissue predicts poor prognosis. However, the clinical relevance of the soluble form of NRP-1 (sNRP-1) as a circulating biomarker in ovarian cancer patients is unknown.

Methods/patients cohort: sNRP-1 levels were quantified in a cohort of 88 clinically documented ovarian cancer patients by a commercially available sNRP-1 enzyme-linked immunosorbent assay (ELISA) kit (Biomedica, Vienna, Austria). Patients (81.8% with FIGOIII/IV) received primary cytoreductive surgery with the aim of macroscopic complete resection (achieved in 55.7% of patients) and the recommendation of adjuvant chemotherapy in line with national guidelines.

Results: Higher levels of sNRP-1 reflected more advanced disease (FIGO III/IV) and indicated a trend towards suboptimal surgical outcome, i.e. any residual tumor. sNRP-1 was neither related to the patients' age nor the *BRCA1/2* mutational status. Patients with higher sNRP-1 levels at primary diagnosis had a significantly reduced progression-free survival (PFS) (HR = 0.541, 95%CI: 0.304 - 0.963; p = 0.037) and overall survival (OS) (HR = 0.459, 95%CI: 0.225 - 0.936; p = 0.032). Principal component analysis showed that sNRP-1 levels were unrelated to the circulating hepatocyte growth factor (HGF) and the soluble ectodomain of its receptor the tyrosine kinase mesenchymal–epithelial transition (c-MET), suggesting that there is no proportional serological

concentration gradient of soluble components of the NRP-1/HGF/c-MET signaling axis.

Conclusions: In line with the previously shown tissue-based prognostic role, we demonstrated for the first time that sNRP-1 can also act as a readily accessible, prognostic biomarker in the circulation of patients with ovarian cancer at primary diagnosis. Given its known role in angiogenesis and conferring resistance to the poly ADP-ribose polymerase (PARP) inhibitor olaparib *in vitro*, our results encourage more detailed investigation into sNRP-1 as a potential predictive biomarker for bevacizumab and/or PARP-inhibitor treatment.

KEYWORDS

ovarian cancer, soluble neuropilin-1, prognosis, blood-based biomarker, retrospective analysis, HGF, c-MET

Introduction

Ovarian cancer is the leading cause of death among patients with gynecological malignancies and more than 70% of patients are diagnosed with advanced disease (1). The most important prognostic factor is the postoperative residual tumor burden (1, 2). The cornerstone of standard first-line treatment of advanced ovarian cancer involves surgical debulking, aimed at macroscopically complete tumor resection, followed by platinum/paclitaxel-based chemotherapy and maintenance treatment with the anti-angiogenic monoclonal antibody bevacizumab (3–6). More recently, in patients with ovarian cancer harboring homologous repair deficiency (HRD), defined by either the presence of a germline or somatic pathogenic breast cancer gene (*BRCA) 1/2* mutation and/or genomic instability, a combination of bevacizumab with the Poly ADP-ribose polymerase (PARP) inhibitor olaparib has been approved as maintenance therapy after response to first-line platinum-based chemotherapy (7). Likewise, the PARP inhibitor (PARPi) niraparib has been approved as sole maintenance therapy (without bevacizumab) after response to first-line treatment, independently of the HRD status (8). This very recent milestone of biomarker-guided, first-line PARPi treatment has been based on the knowledge that ovarian cancer with *BRCA1/2* mutations comprises a molecular Achilles' Heel that can be exploited by targeting HRD (9). Hence, treatment with PARPi led to a markedly improved progression-free survival in patients with HR-deficient ovarian cancer (7, 8).

Despite these therapeutic advances, many patients with ovarian cancer still face a poor overall prognosis (2, 10). Given this clinical challenge, the identification of novel blood-based predictive and/or prognostic biomarkers is of high clinical significance. This would drive personalized treatment of ovarian cancer patients and guide future drug target identification.

Neuropilin (NRP) is a 120-140 kDa type I transmembrane protein, which is actively involved in a variety of physiological processes, such as cardiovascular development, activity of regulatory T cells (Tregs) and neuronal guidance (11–13). Two neuropilin homologues have been identified in vertebrates, referred to as NRP-1 and NRP-2 (12). NRP-1 is strongly expressed in the tumor vasculature and is a high-affinity co-receptor for a number of vascular endothelial growth factor (VEGF) isoforms, particularly $VEGF_{165}$, resulting in an increased affinity of $VEGF_{165}$ for the extracellular domain of VEGFR2 (12, 14, 15). Therefore, NRP-1 has been shown to be a pro-angiogenic mediator and implicated as a potential driver of metastatic cancer progression. Besides its interaction with VEGFR2, NRP-1 acts as a co-receptor for a number of other extracellular ligands, such as semaphorins, hepatocyte growth factor (HGF) and transforming growth factor beta (TGF-β) (13).

Preclinical studies have suggested that NRP-1 expression is up-regulated in ovarian cancer tissue and correlates with advanced FIGO stage and lymph node metastasis (16, 17). Moreover, NRP-1 expression was associated with epithelial to mesenchymal transition (EMT) markers (18) and PARPi resistance (19). It was proposed that high NRP-1 expression in the primary tumor predicts poor prognosis in ovarian cancer patients (16). Since a tissue-based biomarker is restricted to the histological analysis of cancerous tissue, the identification of blood-based biomarkers is of high clinical interest in ovarian cancer diagnostics biomarkers because they offer relatively easy and safe sampling for follow-up analysis and disease monitoring. This is particularly true because tissue samples of ovarian cancer are typically only obtained at primary cytoreductive surgery. In

contrast, surgical treatment at first disease recurrence is clinically indicated and performed only in a specific subset of patients, i.e. in whome macroscopically complete tumor resection can be achieved (20). In addition to its transmembrane configuration, NRP-1 is also shed into circulation as soluble NRP-1 (sNRP-1), where it lacks the transmembrane and cytoplasmic domain. sNRP-1 is robustly detectable in human serum samples, as we have previously shown in patients with early breast cancer (21). We were able to demonstrate that breast cancer patients with low levels of sNRP-1 had a significantly better prognosis compared to patients with high levels of sNRP-1 (21). However, the clinical relevance of sNRP-1 and its potential prognostic value in patients with ovarian cancer is completely unknown.

The aim of this study was to profile sNRP-1 levels in serum samples of a comprehensive set of clinically documented ovarian cancer patients and to study its relation to patients' clinicopathological parameters and its prognostic relevance. Moreover, we compared sNRP-1 levels with levels of selected soluble components of NRP-1 interaction partners, i.e. soluble HGF (sHGF) and the soluble ectodomain of the tyrosine kinase mesenchymal–epithelial transition (c-MET), referred to as soluble/serum c-MET (sMET).

Patients and methods

Patient characteristics and healthy controls

Patients were recruited at the Department of Gynecology and Obstetrics at the Carl Gustav Carus University of Dresden, Technische Universität Dresden, Germany. Overall, 88 patients with histologically confirmed primary epithelial ovarian cancer (primary diagnosis from 2013-2019, 81.8% with FIGOIII/IV) were included. Inclusion criteria were: primary cytoreductive surgery at our hospital with the aim of macroscopic complete resection and the recommendation of adjuvant platinum-/paclitaxel-based chemotherapy in line with national guidelines. In the case of no contraindications, patients with a tumor stage of at least FIGO IIIb (50/72 patients, 69.4%) were additionally treated with the monoclonal antibody bevacizumab and enrollment in clinical trials was permitted. Exclusion criteria were: primary/neo-adjuvant chemotherapy, interval debulking surgery, treatment with hyperthermic intraperitoneal chemotherapy, benign disease or borderline tumors. Progression-free survival (PFS) and overall survival (OS) were calculated from the date of primary diagnosis (i.e. at the time of primary debulking surgery). 30 healthy women were also recruited. In order to be included in this study, these women must have had no past medical history of benign or malignant disease. The median age was 38 (range: 31 – 47 years). Written informed consent was obtained from all study participants and the study was approved by the Local Research Ethics Committee in Dresden (EK74032013). All study methodologies conformed to the standards set by the Declaration of Helsinki. The clinical data from the patients are summarized in Table 1. Tumor staging was documented according to the Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) (22), revised in 2014 (23). Hence, the revised version was used for all patients who underwent primary surgery from 2014 onwards. In agreement with national recommendations, genetic testing was offered and performed, if patients consented (24, 25). Given the significant oncological implementation, BRCA status was analyzed in all patients from whom genetic testing had been documented. Germline *BRCA*1/2 mutational status was available in 39/88 patients. It is important to note that HRD analyses were not routinely tested outside of clinical trials at the time of primary diagnosis (2013-2019) in this retrospectively analyzed patient cohort.

Serum preparation and detection of sNRP-1

Serum preparation from blood-samples obtained at primary diagnosis of ovarian cancer was performed, as described previously (26–28). Briefly, sample processing occurred within 1 h of blood drawing. After obtaining blood samples, they were incubated at room temperature (rt) for at least 30 min in order to allow complete blood coagulation. The cell-free serum fraction was obtained by centrifugation (8 min, 1800 g, rt) and was then immediately frozen at −80°C until further use. In order to compare pre-processing of control samples and patient samples were performed with the same protocol.

After complete thawing on ice, samples were immediately processed. The NRP-1 ELISA was performed as described previously (21). Briefly, 10 µl of the sample was used per well and the NRP-1 ELISA was conducted according to the manufacturer's protocol (Biomedica, Vienna, Austria). The absorbance was measured immediately at 450 nm with reference at 630 nm.

Statistical analysis

The statistical analysis was conducted with R, Version 3.6.2 and GraphPad Prism version 8.4.3 (GraphPad Software, La Jolla, CA, USA) as described previously (26–28), and listed in each figure legend. P-values < 0.05 were considered statistically significant. The Hodges-Lehman estimate was used to determine the estimated differences (ED) of medians. Uni- and multivariate Cox proportional hazards model regression analyses were performed and hazard ratios (HRs) are indicated

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TABLE 1 Patient characteristics.

Patients	N	88	
Age	median (range)	65 years	(23-82years) -
FIGO stage	1/11	16	18.2%
	III/IV	72	81.8%
Surgical debulking	residual disease	39	44.3%
	No residual tumor	49	55.7%
Histology	serous	78	88.6%
	non-serous	10	11.4%
Grading	high-grade (G3)	76	86.4%
	G1/G2	12	13.6%
BRCA1/2 mutational status	wtBRCA1/2	24	27.3%
	mBRCA1/2	15	17.0%
	unknown	49	55.7%
sNRP-1levels	median (range)	2.358 nmol/L(1.049-	- 5.126 nmol/L)
Progression-free survival	median (range)	30 months	(1 - 86 months)
	progression/death	49	55.7%
	no progression	39	44.3%
Overall survival	median (range)	42 months	(3 - 89 months)
	dead	33	37.5%
	alive	55	62.5%

with 95% confidence intervals (CI). The median (2.358 nmol/L) has been used to stratify patients into sNRP-1 high (n = 44) and sNRP-1 low (n = 44), unless specified otherwise. The optimized cut off analysis was performed using maximally selected rank statistics (maxstat package). Kaplan–Meier analyses were performed with significance levels indicated by log-rank (Mantel-Cox) analysis and HRs (Mantel-Haenszel) are shown with 95%CI. The correlation between sNRP-1 levels with age or cancer antigen 125 (CA125) was assessed by non-parametric Spearman correlation. Correlation-based principal component analysis was performed, using Pearson correlation.

Results

Soluble sNRP-1 levels at primary diagnosis of ovarian cancer

We analyzed the sNRP-1 level in a comprehensive cohort of 88 clinically documented ovarian cancer patients at primary diagnosis and compared it to the level of healthy controls (n = 30). There was no significant difference between median sNRP-1 in ovarian cancer patients *vs.* healthy controls (estimated difference (ED) = -0.15, 95% CI: -0.39 - 0.12; p = 0.24; Figure 1A). This was supported by the receiver operating characteristic (ROC) analysis, which failed to show any discrimination between patients and healthy controls by sNRP-1 levels (p = 0.24; Figure 1B), meaning that sNRP-1 cannot be considered as a *bona fide* diagnostic marker without additional parameters.

Correlation of sNRP-1 level with clinicopathological features of ovarian cancer

Higher levels of sNRP-1 reflected more advanced disease, indicated by a higher FIGO stage (ED = 0.42, 95%CI: 0.04 - 0.70; p = 0.04; Figure 2A). Moreover, higher sNRP-1 levels at primary diagnosis showed a non-significant but numerical trend to be associated with suboptimal surgical outcome (ED = 0.26, 95%CI: -0.01 - 0.53; p = 0.07; Figure 2B). There was also neither a correlation between sNRP-1 levels between high-grade *vs.* lower grading (low-grade and moderately-differentiated) ovarian cancer (ED = -0.29, 95%CI: -0.66 - 0.12; p = 0.16; Figure 2C) nor the patients' age (r = 0.20, 95%CI: -0.02 - 0.40; p = 0.06; Figure 2D).

The *BRCA*1/2 mutational status was available in 39 of 88 patients in our cohort (44.3%). Of those, 24/88 patients (27.3%) were *BRCA1/2* wild type (wt*BRCA1/2*), whereas in 15/88 patients (17.0%) a pathogenic *BRCA1/2* mutation (m*BRCA1/2*) had been detected. There was no significant difference in sNRP-1 levels between m*BRCA*1/2- *vs.* wt*BRCA1/2*-patients (ED = -0.01, 95%CI: -0.42 - 0.38; p=0.97; Supplementary Figure 1A). Information on CA125 at primary diagnosis was available in all patients (n = 88). We observed a correlation between sNRP-1 and CA125 (r = 0.22, 95%CI: 0.001 - 0.419; p = 0.04; Supplementary Figure 1B).

Taken together, sNRP-1 at primary diagnosis is unrelated to *BRCA1/2* mutational status, correlates with advanced disease and associates with surgical outcome by trend.

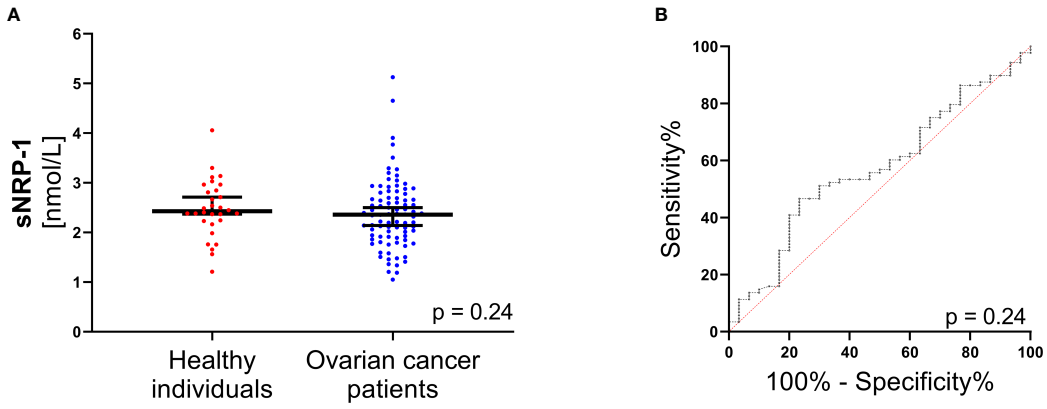

FIGURE 1
sNRP-1 levels in ovarian cancer at primary diagnosis. **(A)** Scatter plots comparing sNRP-1 levels in ovarian cancer patients (n = 88) and in healthy individuals (n = 30). The black horizontal lines indicate median sNRP-1 levels in each group, with error bars showing the 95%CI. P-value according to the non-parametric, two-sided Mann-Whitney test. **(B)** Receiver operating characteristic (ROC) analysis to determine the diagnostic ability of sNRP-1 levels to distinguish between ovarian cancer patients (n = 88) and healthy controls (n = 30). The respective area under the curve (AUC) values and the 95%CIs are indicated.

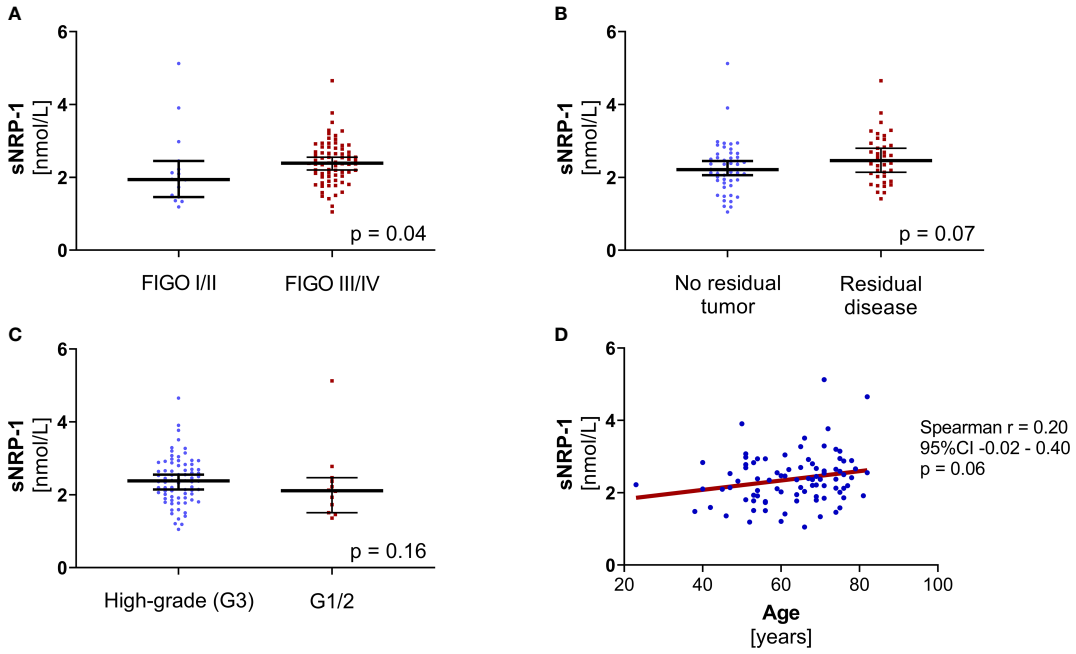

FIGURE 2
Association analyses of sNRP-1 with known clinical parameters. **(A)** The sNRP-1 levels of patients with advanced ovarian cancer (FIGO III/IV, n = 72) compared to patients with early-stage disease (FIGO I/II, n = 16), p = 0.04. **(B)** The sNRP-1 levels of patients (n = 39) with residual tumor compared to patients (n = 49) with no macroscopic tumor after cytoreductive surgery, p = 0.07. **(C)** The sNRP-1 levels of patients (n = 76) with high-grade ovarian cancer compared to patients (n = 12) with lower grading, p = 0.16. The black horizontal lines indicate the median sNRP-1 levels in each group with error bars, showing the 95%CI. P-values according to the non-parametric, two-sided Mann-Whitney test. **(D)** The correlation of sNRP-1 and age is shown, using non-parametric Spearman correlation (n = 88, p = 0.06) with simple linear regression (red line).

Prognostic relevance of sNRP-1

Using the median sNRP-1 level as a cut-off value, we stratified our study cohort into sNRP-1 high (>2.358 nmol/L) *vs.* sNRP-1 low (<2.358 nmol/L) patients and performed a Cox proportional hazards model regression and Kaplan-Meier analyses. We observed that higher sNRP-1 levels at primary diagnosis of ovarian cancer were associated with significantly reduced PFS (HR = 0.541, 95%CI: 0.304 - 0.963; p = 0.037) and OS (HR = 0.459, 95%CI: 0.225 - 0.936; p = 0.032) in the univariate but not multivariate analysis (Figure 3A). This was consistent with Kaplan-Meier analyses, indicating that higher sNRP-1 levels predict a significantly reduced PFS (HR = 0.54, 95%CI: 0.30 - 0.96; p = 0.03) and OS (HR = 0.46, 95%CI: 0.23 - 0.92; p = 0.03; Figures 3B, C). In the above analyses, we have used the median as cut off for grouping the patient into sNRP-1 high or sNRP-1 low. Another approach for dichotomizing a patient cohort with an optimized cut-off can be performed by maximally selected rank statistics. This resulted in the following cut offs: OS: >2.9805 nmol/L or PFS: > 2.3195 nmol/L. Using this optimized cut off as means to group our patient cohort into sNRP-1 high vs. sNRP-1 low, an even more pronounced prognostic relevance of sNRP-1 became evident in the Kaplan-Meier analysis (PFS: HR = 0.49, 95%CI: 0.28.-0.88; p = 0.02 and OS: HR = 0.12, 95%CI: 0.03 - 0.45; p = 0.002; Supplementary Figures 2A, B). It was also observed that higher sNRP-1 levels at primary diagnosis of ovarian cancer were associated with a significantly reduced PFS (HR = 0.491, 95%CI: 0.272 - 0.885; p = 0.018; Supplementary Figure 2C) in the univariate but not multivariate cox proportional hazards model regression analysis. Notably, Cox proportional hazards model regression analysis could not be performed for the OS analysis because the stratification using this optimized cut off did not meet the proportional hazards assumption.

This demonstrates that sNRP-1 can be considered as a blood-based prognostic biomarker in ovarian cancer patients. High levels of sNRP-1 indicate higher risk of disease recurrence and poor survival.

Association of sNRP-1 with serum levels of HGF and the soluble ectodomain of c-MET

In addition to its interaction with VEGFR2, NRP-1 is a co-receptor for a number of other extracellular ligands, including c-MET and HGF (13, 29, 30). We hypothesized that there could be an association between the level of sNRP-1 and associated ligands in the blood of ovarian cancer patients. We took advantage of our previous studies on ovarian cancer, which demonstrated the prognostic relevance of both sHGF levels and the soluble ectodomain of its receptor c-MET (sMET) (26, 27). Corresponding data on sHGF and sMET levels were available in 35/88 and 26/88 of our patients from two previous studies of our group, respectively (26, 27). This number of matching samples allowed us to investigate whether there was a proportional serological concentration gradient of sNRP-1 and its functionally related proteins sHGF and sMET. We performed

A

	Univariate analysis				Multivariate analysis			
	HR	Lower 95% CI	Upper 95% CI	p-value	HR	Lower 95% CI	Upper 95% CI	p-value
Progression free survival								
sNRP-1 low *vs.* high (median cut off)	0.541	0.304	0.963	**0.037**	0.643	0.357	1.158	0.141
Overall survival								
sNRP-1 low *vs.* high (median cut off)	0.459	0.225	0.936	**0.032**	0.546	0.264	1.129	0.103

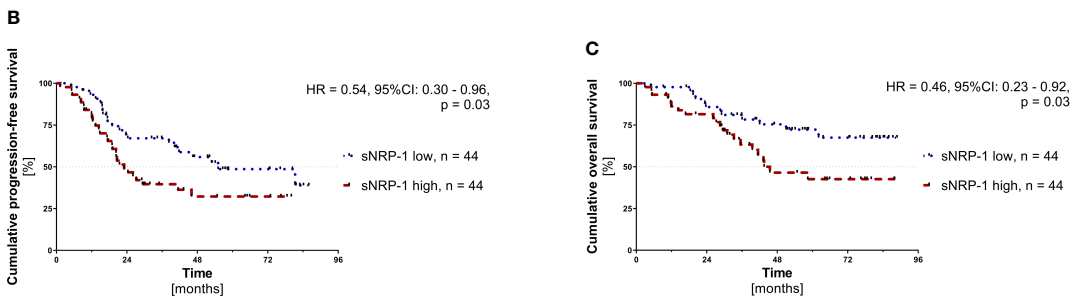

FIGURE 3
Prognostic relevance of sNRP-1. **(A)** Results from univariate and multivariate Cox proportional hazard regression model analyses of sNRP-1 low (n = 44) *vs.* sNRP-1 high (n = 44) are shown, including hazard ratio (HR) and 95%CIs and p-values. Kaplan-Meier analyses comparing **(B)** cumulative progression-free survival (PFS) and **(C)** cumulative overall survival (OS) of patients with ovarian cancer stratified as above. HR and 95%CI determined by Mantel Haenszel and p-value by log-rank (Mantel-Cox), as described in the methods section.

a principal component analysis, assessing all three serological biomarkers sNRP-1, sMET and sHGF. However, there was no significant correlation/clustering obtained by analyzing all three biomarkers (Figures 4A, B).

Discussion

This is the first study that investigated the clinical relevance of sNRP-1 in blood samples of patients with ovarian cancer at primary diagnosis, demonstrating that high sNRP-1 indicates advanced disease and poor prognosis.

This is supported by an earlier study, which demonstrated that NRP-1 upregulation in ovarian cancer indicated poor prognosis when analysing tissue, gene and protein expression levels (16). Our findings complement the pro-tumorigenic effects of NRP-1 in cancer cells, such as modulating EMT, evasion of contact inhibition or promoting angiogenesis (12, 14, 31, 32).

However, the origin and function of sNRP-1 is still unclear. Firstly, the pool of sNRP-1 could be derived, at least partially, from cancer cells or the tumour microenvironment. If true, one would assume that more aggressive tumors may release more sNRP-1. This is consistent with our observation that higher sNRP-1 levels correlate with a poor prognosis and advanced disease. However, median sNRP-1 levels did not significantly differ between healthy women and patients with ovarian cancer (Figure 1A). This finding is consistent with reports showing no significant difference of sNRP-1 in patients with non-advanced breast cancer or malignant vocal lesions compared to healthy

A

Correlation matrix (Pearson)			
	sNRP-1	sHGF	sMET
sNRP-1	**1.000**	0.189	-0.212
sHGF	0.189	**1.000**	0.306
sMET	-0.212	0.306	**1.000**

B

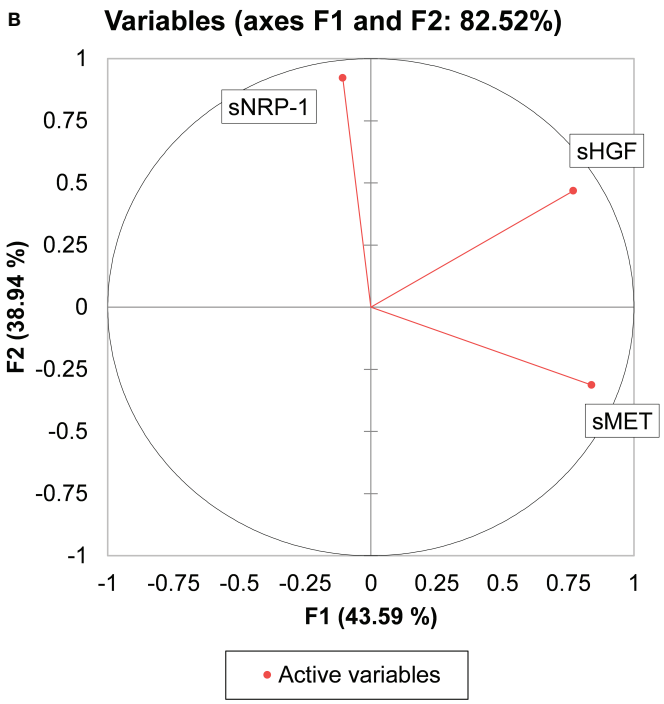

FIGURE 4
Principal component analysis using sNRP-1, sHGF and sMET levels. **(A)** A correlation matrix as a principal component analysis (Pearson) is shown with sNRP-1, soluble HGF (sHGF) and soluble ectodomain of c-MET (sMET). Values in bold are different from 0 with significance as $p < 0.05$. Biomarker levels were measured in blood samples of the same ovarian cancer patient at primary diagnosis. **(B)** Graphical representation of the principal component analysis of the three variables (sNRP-1, sHGF and sMET) contributing to 82.52% of the variability of the data set.

controls or patients with benign vocal cord lesions, respectively (22, 33).

The shedding rate may also influence sNRP-1 concentrations, which may differ by cancer type and could partially explain the above observations. Another circulating ovarian cancer biomarker (sMET) also offers prognostic relevance despite similar median levels in serum of patients and healthy controls (26). One can speculate whether the tumor microenvironment potentiates the effect of sNRP-1 once malignant transformation occurred. In a preliminary study, sNRP-1 levels in human serum ranged from a median of 4.62 nmol/L (range: 2.10 - 8.87 nmol/L) (34). Since the study did not disclose specific characteristics of study participants, one must speculate which factors contributed to sNRP-1 levels in these individuals.

Since both tissue and blood-based NRP-1 levels allow for prognostic stratification in ovarian cancer (16), further investigation should aim to investigate 1) the cellular processing of NRP-1, 2) its release from the tumor microenvironment in patients with ovarian cancer, and 3) determinants of its concentration in non-malignant physiological conditions.

Our exploratory study has certain limitations, i.e. the medium-sized patient cohort, a lack of comparison with tissue-based NRP-1 expression and the retrospective setting. Nonetheless, the strength of our study is that we can show prognostic relevance of our marker candidate in a well-documented patients cohort, considering all relevant clinicopathological parameters and including BRCA1/2 mutational status.

It is important to note that the present study refers to patients with a primary diagnosis of ovarian cancer from 2013-2019. At this time, the addition of the PARPi olaparib as maintenance treatment after response to first-line chemotherapy was restricted to patients with *BRCA1/2*-mutant advanced ovarian cancer. Only one patient with a germline *BRCA1* mutation received olaparib in our patient cohort as maintenance treatment following response to first-line chemotherapy. Given this is the first study describing a prognostic relevance of sNRP-1, it will be interesting to prospectively investigate the use of sNRP-1 in patients with HR-deficient ovarian cancer receiving maintenance therapy with bevacizumab and/or PARPi according to standard clinical practice (7, 8). Since NRP-1 promotes angiogenesis (35), previous studies have assessed whether it could predict response to bevacizumab at primary diagnosis. However, NRP-1 expression in ovarian cancer tissue failed to predict bevacizumab response in a retrospective analysis of the GOG-0218 clinical trial (36).

Interestingly, a previous study demonstrated a potential role of NRP-1 in conferring olaparib resistance *in vitro* (19). Both the pro-angiogenic activity of NRP-1 and its link to PARPi resistance would strongly suggest a potential use as a suitable auxiliary marker for predicting response to the combination of bevacizumab/olaparib in patients with ovarian cancer. This is of particular importance because PARPi treatment is expanding, resulting in an increasing number of patients with acquired (or primary) PARPi resistance in clinical practice. Furthermore, it would also be of clinical importance to determine the prognostic relevance of sNRP-1 in each subtype of ovarian cancer (37). Given the heterogeneous nature of ovarian cancer, this may also improve our understanding of sNRP-1 release and its correlation with tissue expression, if subtype-specific patterns are observed.

We have previously shown the use of sHGF and sMET as an independent prognostic biomarker in patients with ovarian cancer (26). HGF is a pleiotropic cytokine and a potent growth and pro-angiogenic factor that acts on its target cells by binding to the c-MET receptor. Moreover, HGF and c-MET also interacts with neuropilins (29, 38). However, we did not observe any correlation between sNRP-1, sHGF or sMET in a subset of corresponding patients' serum samples, indicating that there may not be a proportional serological concentration gradient of sNRP-1 and circulating HGF and/or c-MET. Considering the broad spectrum of NRP-1 interacting ligands (39), a combined analysis of sNRP-1 and other functionally related proteins may still yield a biomarker signature that would enable additional prognostic or predictive information.

Conclusion

We show for the first time, that NRP-1 is a blood-based prognostic biomarker, which could be easily implemented into routine clinical diagnostics of ovarian cancer. Our results encourage a prospective validation study to analyse whether sNRP-1 detection could be considered as an auxiliary predictive or prognostic tool in patients with ovarian cancer. This will be of future clinical relevance given its interaction with VEGF and conferring olaparib resistance *in vitro* (14, 19).

Data availability statement

Upon reasonable requests, the raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by ETHIKKOMMISSION AN DER TECHNISCHEN UNIVERSITÄT DRESDEN (EK74032013). The patients/participants provided their written informed consent to participate in this study.

Author contributions

DMK, JDK, TDR, LCH and PW made substantial contributions to the conception and design of the study. DMK, JDK, TL, AG, MG and PW contributed to the experimental work or to the acquisition of clinical samples/data or to the analysis/interpretation of the results. DMK, JDK, TL, TDR, and PW were involved in drafting the manuscript, creating figures and/or revising the manuscript. All authors read and approved the manuscript in its final version.

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Conflict of interest

DMK has a patent application pending regarding the use of HGF as a prognostic biomarker in ovarian cancer.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.974885/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Association of sNRP-1 with *BRCA1/2* mutational status and CA125. **(A)** Scatter plots with sNRP-1 levels of patients with known *BRCA1/2* mutational status are shown with *BRCA1/2* mutations (*mBRCA1/2*, n =15) and wild-type *BRCA1/2* status (*wtBRCA1/2*, n = 24), p = 0.97. **(B)** The correlation of sNRP-1 and log (CA125) is shown, using non-parametric Spearman correlation (n = 88, p = 0.04) with simple linear regression (red line).

SUPPLEMENTARY FIGURE 2
Prognostic information of sNRP-1 using an optimized cut-off. Kaplan-Meier analysis of patients with ovarian cancer comparing **(A)** cumulative progression-free survival according to sNRP-1 low (n = 42) and sNRP-1 high (n = 46), optimized cut-off PFS: 2.3195 nmol/L and **(B)** cumulative overall survival (OS) according to sNRP-1 low (n = 77) and sNRP-1 high (n = 11), optimized cut-off: 2,9805 nmol/L. HR and 95%CI determined by Mantel Haenszel and p-value by log-rank (Mantel-Cox), as described in the methods section. **(C)** Results from univariate and multivariate Cox proportional hazard regression model analyses for PFS of sNRP-1 low (n = 42) *vs*. sNRP-1 high (n = 46) are shown, including hazard ratio (HR) and 95%CIs and p-values. **(D)** Scatter plot is shown with sNRP-1 low (n = 42) and sNRP-1 high (n = 46) according to an optimized cut-off determined by maximally selected rank statistics for PFS, p < 0.0001. The black horizontal lines indicate the median sNRP-1 levels in each group with error bars, showing the 95%CI. P-value according to the non-parametric, two-sided Mann-Whitney test. **(E)** Graphical representation of cut-off determination by maximally selected rank statistics.

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Gender-related differences in career development among gynecologic oncology surgeons in Europe. European Network of Young Gynecologic Oncologists' Survey based data

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Introduction: Gender-related differences in career development are well known issues in various professions. An international survey on gender-related differences was performed among young gynecologic oncology surgeons in Europe to identify potential gender inequalities in career development.

Material and methods: A survey on demographics, clinical and academic working environment, family/parenting, career development, salary and leadership was sent to all members of the European Network of Young Gynecologic Oncologists (ENYGO), which is a network within the European

Society of Gynecologic Oncology (ESGO). Gynecologic oncology surgeons and obstetricians/gynecologists who actively work in this field in Europe were included in the study.

Results: Responses were analyzed from 192 gynecologic oncology surgeons of whom 65.1% (125/192) were female (median age 37, IQR: 34 - 42) and 34.9% (67/192) were male (median age 38, IQR: 36 - 41). Male reported to perform a median of 15 and female a median of 10 operations per month *(p* = .007). Among female, 24.8% had a leadership position vs. 44.8% among male, crude *OR* = 2.46, 95% *CI* 1.31-4.62, *p*<.01. When stratifying for age under 41 and having children, 36.7% of male and 5.6% of female had a leadership position, adjusted *OR* 10.8, 95% *CI* 3.28-35.64, *p*<.001. A significantly higher proportion of female compared to male believed they earned less than their gender counterparts at the same clinical position and with same qualifications (30.4% vs. 2.5%, *p*<.001). There was not a statistically significant gender difference in the academic qualification PhD degree or professorship (*p* = .92 and *p* = .64, respectively). In the previous year, male published more peer-reviewed articles than female (median 3 vs. median 2; *p* = .017).

Conclusion: This first comprehensive survey on gender-differences in gynecologic oncology in Europe revealed that there are gender gaps concerning several aspects during the critical time of career development in the young generation of gynecologic oncology surgeons. These gender gaps are particularly reflected by a lower rate of female leadership positions. ENYGO and ESGO are dedicated to work on solution to overcome the identified obstacles and to support closing gender gaps.

KEYWORDS

gender-related differences, gender inequalities, gynecologic oncology surgeons, career development, leadership, salary

Introduction

According to the report of the Organization for Economic Cooperation and Development countries (OECD), almost half of all medical doctors in 2019 were female (1). Female accounted for more than half of students entering PhD programs in the United States (2). Based on membership data of the European Society of Gynecological Oncology (ESGO), the proportion of female gynecologic oncologists has been constantly increasing in the last decade. While in 2009 more than two thirds of members were male, the gender distribution reached parity in 2019. An even faster growing trend is evident among the younger generation of gynecologic oncologists in Europe. According to the membership directory of the European Network of Young Gynecologic Oncologists (ENYGO), which is a network of ESGO comprising the young generation of the gynecologic oncology surgeons, 2/3 of members were female in 2019.

On the other side the published literature indicates that in the field of medicine female still face manyfold career barriers in comparison to their male colleagues. Female are reported to have less opportunities to research and to publish, are promoted more slowly and at a lower rate e.g. for professorship positions and are less paid than their male counterparts (3–9). Studies performed among medical professionals in different fields show that although female participation in medical schools and hospitals is increasing, male professionals still dominate at senior leading positions (10, 11).

To our knowledge there is not any published data on gender-related differences in career development among gynecologic oncology surgeons in Europe.

We performed a comprehensive survey consulting the young generation of gynecologic oncology surgeons and specialists in gynecology and obstetrics with a major focus on gynecologic oncology surgery, who are working in academic and

nonacademic surgical centers. The aim of this survey was to investigate potential gender differences and potential obstacles during the time of career development.

Methods

A survey consisting of 85 questions was designed and approved by the Institutional Review Board at the Medical School, University in Kielce, Poland (Number: 65/2021). The questionnaire consisted of questions related to demographics; clinical and academic working environment; family and parenting; career development; leadership including clinical director, head of department and head of division, as well as salary. The questions validity was thereafter assessed through a review of 12 ENYGO members who were selected to represent the different sociodemographic characteristics (gender, age, nationality, country of practicing, partner status, children, and sexual orientation). Each reviewer assessed the questions and response options to ensure their clearness and inclusivity with regards to diverse life and professional experiences.

ENYGO is a network within ESGO, which comprises of subspecialists in gynecologic oncology surgery who are ≤40 years of age, and of fellows in subspeciality training for gynecologic oncology surgery (all fellows in training independent of age), as well as of specialists in gynecology and obstetrics ≤40 who are actively working in the field of gynecologic oncology surgery. The survey was administrated anonymously to all ENYGO members in electronic format *via* the survey tool "SurveyMonkey" (SurveyMonkey Inc. Palo alto, CA, USA).

The first participating request was sent in October 2019. It was promoted during the ESGO conference in Athens in November of 2019 and after that, launch was repeated in January and February of 2020. The online software allowed respondents to complete the survey without answering all the questions, hence each question was not necessarily answered by all the respondents.

Inclusion criteria for distribution of this survey were ENYGO members who were practicing in Europe at the time of survey. Respondents who were not actively working in the field of gynecologic oncology surgery and were not practicing in Europe were excluded from the study.

Statistical analysis

Statistical data analysis was performed using statistical software SPSS version 26. Descriptive statistics was used to summarize the results of the questionnaire. As not all the questions were answered by all the respondents, the percentage was calculated using a number of respondents to the specific question as a denominator. Categorical and ordinal data associations were tested using Chi-squared or Fisher's exact test and Mann-Whitney U test. The Cochran-Mantel-Haenszel test of homogeneity was used in cases where controlling for gender or age with other significant factors had to be explored. All tests were two-tailed at the level of statistical significance $\alpha = 5\%$.

Results

Description of the study population

The survey link was sent to 745 ENYGO members. A total of 230 recipients replied after the survey was launched by the third time (response rate of 30.9%). Of them, 38 (16.5%) respondents were excluded, as they did not meet the inclusion criteria (31 were not working in Europe, 7 were not actively working in the field of gynecologic oncology surgery). Thus, answers from a total of 192 respondents were included in the final analysis with a median age 37 years (IQR: 35 - 42). Of them, 132 (68.6%) were gynecologic oncology surgeons, either subspecialists who have completed their training (n=84, median age 40 years, IQR: 36 - 44) or fellows in training (n=48, median age 36 years, IQR: 33 - 38), and 60 (31.4%) were specialists in gynecology and obstetrics who were actively working as surgeons in gynecologic oncology (median age 37 years, IQR: 34 - 42). Table 1 shows the general demographic characteristics of the respondents.

Clinical working environment and leadership

The obtained data about the clinical working environment are presented in Table 2.

There was a significant difference in the number of reported operative cases per month between male and female. Male reported a median of 15 and female a median of 10 cases per month (U (N_{male}= 29, N_{female}= 71) = 677, z = -2.71, p = .007, (η^2 = .072). The number of night shifts per month did not differ between male and female (median of 4 for both genders).

The data about the reported leadership positions in male versus female, as well as the subsequent stratifications are presented in Table 3.

There was a significant difference in reported leadership positions between male and female. 44.8% (30/67) of male compared to 24.8% (31/125) of female reported to have a leadership position, (crude OR 2.46, 95% CI 1.31-4.62, p = .005, χ^2 (1, N = 192) = 8.03, small effect size ϕ = .205).

When stratified for age under 41 and having children, the gender gap was the most pronounced with 36.7% (11/30) of male compared to 5.6% (2/36) of female having a leadership position (adjusted OR 10.8, 95% CI 3.28-35.64, p<.001, $\chi^2_{CMHtest}$ (1, N = 137) = 19.3, p<.001, χ^2_{BDtest} (3, N = 137) = 3.77, p = .29).

Among respondents with children, 96.4% (53/55) of female and 75.8% (25/33) of male used parental leave. 58.5% (31/53) of female

TABLE 1 Demographic characteristics.

	Male		Female		Total	
	n	%	*n*	%	*n*	%
Gender	67	34.9%	125	65.1%	192	
Age						
≤ 30	0	0.0%	17	13.6%	17	8.9%
31-40	49	73.1%	72	57.6%	121	63.0%
41-50	11	16.4%	30	24.0%	41	21.4%
51-60	5	7.5%	6	4.8%	11	5.7%
> 61	2	3.0%	0	0.0%	2	1.0%
Marital Status						
Single	4	8.3%	20	23.0%	24	17.8%
Married/partner	43	89.6%	64	73.6%	107	79.3%
Divorced	1	2.1%	3	3.4%	4	3.0%
Children						
Yes	38	79.2%	57	64.0%	95	69.3%
No	10	20.8%	32	36.0%	42	30.7%
Number of Children						
0	29	43.3%	68	54.4%	97	50.5%
1	13	19.4%	29	23.2%	42	21.9%
2	17	25.4%	22	17.6%	39	20.3%
≥ 3	8	11.9%	6	4.8%	14	7.3%
Parental leave						
Yes	25	75.8%	53	96.4%	78	88.6%
No	8	24.2%	2	3.6%	10	11.4%

TABLE 2 Clinical characteristics.

	Male		Female		Total		p - value
	n	%	*n*	%	*n*	%	
Working in academic oncology center							.76
Yes	42	85.7%	72	83.7%	114	84.4%	
No	7	14.3%	14	16.3%	21	15.6%	
Level of training							.59
Fellow in Gyn. Onc.	14	20.9%	34	27.2%	48	25.0%	
Gyn. Onc. Surgeon	32	47.8%	52	41.6%	84	43.8%	
Specialist in Ob/Gyn.	21	31.3%	39	31.2%	60	31.3%	
Number of operative cases per month							.006
< 5	1	2.2%	9	10.5%	10	7.5%	
5-10	9	19.1%	33	38.4%	42	31.6%	
> 10	37	78.7%	44	51.1%	81	60.9%	
Number of night shifts per month							.64
0	4	8.5%	9	10.7%	13	9.9%	
1-4	19	40.4%	42	50.0%	61	46.6%	
5-9	18	38.3%	25	29.8%	43	32.8%	
≥ 10	6	12.8%	8	9.5%	14	10.7%	

TABLE 3 Leadership position analysis.

Male	Female	Crude OR (95% *CI*)	p - value	χ^2	Effect size (ϕ)		
44.8% (30/67)	24.8% (31/125)	2.46 (1.31-4.62)	.005	(1, N=192) = 8.03	.205		
Stratified							
Male	Female	Adjusted OR (95% *CI*)	p - value	$\chi^2_{CMHtest}$	p - value	χ^2_{BDtest}	p - value
"Being married/living with a partner"							
48.8% (20/41)	17.5% (11/63)	3.89 (1.75-8.65)	.001	(1, *N* = 139) = 11.6	.001	(1, *N* = 139) = 0.64	.425
"Being married to a medical doctor/living with a partner who is a medical doctor"							
45.8% (11/24)	24.3% (9/37)	3.37 (1.46-7.75)	.004	(1, *N* = 115) = 8.58	.003	(1, *N* = 115) = 0.46	.496
Age under 41 years							
32.7% (16/49)	13.5% (12/89)	3.11 (1.53-6.36)	.002	(1, *N* = 192) = 10.3	.001	(1, *N* = 192) = 0.001	.993
Parental status (having children vs. not having)							
50% (19/38)	19.3% (11/57)	3.91 (1.75-8.73)	.001	(1, *N* = 137) = 11.6	.001	(1, *N* = 137) = 0.11	.74
Age under 41 and having children							
36.7% (11/30)	5.6% (2/36)	10.8 (3.28-35.64)	<.001	(1, *N* = 137) = 19.3	<.001	(3, *N* = 137) = 3.77	.29

used parental leave for a year or less (6 weeks to 12 months) and 41.5% (22/53) for more than a year (up to a max of 3 years). 66.7% (16/24) of male used parental leave for a maximum of six weeks (1 to 6 weeks) and 33.3% (8/24) for more than six weeks (up to a maximum of one year). Among both genders, there was no association between the leadership position and the length of parental leave (for female: χ^2 (1, N = 53) = 0.298, p = .56; for male: χ^2 (1, N = 24) = 2.1, p = .15).

The data on the question: "Is your achieved clinical position same, higher or lower in comparison to your opposite gender colleagues with the same experience and at approximately same age?" is presented in Figure 1. There was a significant medium to strong association between the perception of having achieved the "adequate" clinical position and gender. 45.6% (36/79) of female believed that their achieved clinical position was lower than the one of their male counterparts with approximately the same experience and age, 45.6% (36/79) believed it was the same, and 8.9% (7/79) of female believed that their position was higher. On the other side, 87.5% (35/40) of male believed that their achieved clinical position was the same as their female counterparts with approximately same experience and age, 12.5% (5/40) believe that it was higher, and none believed that their position was lower (χ^2 (2, N = 119) = 26.4, p<.001, medium to strong effect size V = .471).

On the question: "Are you happy with your current clinical achievements?", more than half of male respondents, i.e. 53.8% (21/39), declared that they were happy, and 46.2% (18/39) declared that they were not happy with their current clinical achievements, while 32.5% (26/80) of female respondents declared their happiness and more than half, i.e. 67.5% (54/80), their unhappiness with the current clinical achievements (*OR* 2.42, 95% *CI* 1.11-5.31, p = .025, χ^2 (1, N = 119) = 4.99, small effect size ϕ = .205).

30.4% (24/79) of female and 2.5% (1/40) of male believed that their salary was lower in comparison with their gender

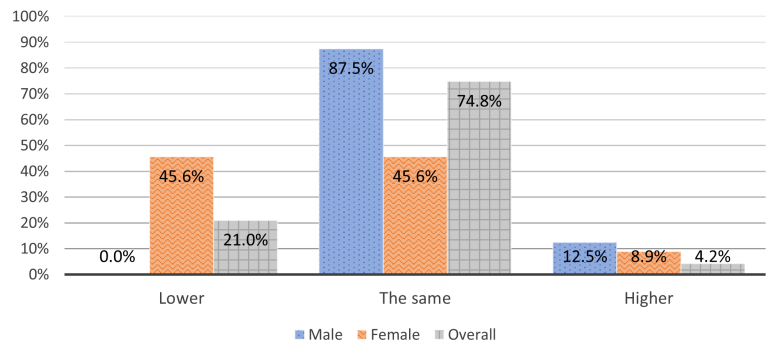

FIGURE 1
Is your achieved clinical position same, higher or lower in comparison to your opposite gender colleagues with the same experience and at approximately same age?

counterparts at the same position and clinical/academic qualifications, while 97.5% (39/40) of male and 69.6% (55/79) of female believed that their salary was the same or higher (*OR* 17.01, 95% *CI* 2.21-131.14, $p<.001$, χ^2 (1, N = 119) = 12.44, medium effect size ϕ = .323).

Academic working environment

Data about the academic working environment are presented in Table 4.

55.2% (37/67) of female respondents and 62.9% (22/35) of male respondents hold a PhD title (χ^2 (1, N = 102) = 0.55, p = .92, adjusted for Bonferroni correction). More male, 51.9% (14/27), than female, 40% (20/50), reported to have a professor position. However, this difference was not statistically significant (χ^2 (1, N = 77) = 0.99, p = .64, adjusted for Bonferroni correction).

Parental leave duration was no adverted factor for female to hold an academic qualification (PhD degree and/or professorship; χ^2 (2, N = 52) = 2.71, p = .258).

There was a significant small to intermediate effect between number of peer reviewed publications in the previous year and the gender. On average, male had more publications (median = 3) in the previous year than female (median = 1). The difference was significant U (N_{male}= 46, N_{female}= 86) = 1,488, z = -2.38, p = .017, η^2 = .042. When stratified for having children, the small to intermediate effect between number of publications in the previous year and gender remained. Male with children published more (median = 2) than female with children (median = 1) in the previous year. The difference was significant U (N_{male}= 36, N_{female}= 55) = 741.5, z = -2.06, p = .04, η^2 = .043.

A comparable number of males and females have received at least one medical grant/funding in the previous five years 45%

TABLE 4 Academic characteristics.

	Male		Female		Total		p - value
	n	%	*n*	%	*n*	%	
Academic qualifications:							.59
None	13	26.5%	30	34.5%	43	31.6%	
PhD	22	44.9%	37	42.5%	59	43.4%	
Professor	14	28.6%	20	23.0%	34	25.0%	
Professorship:							.11
Assistant Professor	3	21.4%	8	40.0%	11	32.4%	
Associate Professor	10	71.4%	7	35.0%	17	50.0%	
Full Professor	1	7.2%	5	25.0%	6	17.6%	
Did you have publications in the previous year?							.30
Yes	36	78.3%	60	69.8%	96	72.7%	
No	10	21.7%	26	30.2%	36	27.3%	
Number of full-text publications as first and last author in your medical career:							.024
0	17	37.0%	35	40.7%	52	39.4%	
1-3	18	39.1%	45	52.3%	63	47.7%	
4-6	10	21.7%	4	4.7%	14	10.6%	
> 6	1	2.2%	2	2.3%	3	2.3%	
Number of medical conferences (national and international) attended in the previous year:							.13
≤ 1	5	10.2%	21	24.1%	26	19.1%	
2-3	30	61.2%	47	54.0%	77	56.6%	
≥ 4	14	28.6%	19	21.8%	33	24.3%	
Number of conference presentations (oral and poster) in the previous year:							.012
0	6	13.0%	27	31.0%	33	24.8%	
1-4	29	63.0%	54	62.1%	83	62.4%	
5-9	8	17.4%	5	5.7%	13	9.8%	
	≥ 10	3	6.5%	1	1.1%	4	3.0%
Medical grants/funding in the previous 5 years:							.60
Yes	22	55.0%	48	60.0%	70	38.3%	
No	18	45.0%	32	40.0%	50	41.7%	

(18/40) of male vs. 40% (32/80) of female (χ^2 (1, N = 120) = 0.247, p = .60).

There was a significant intermediate effect between number of congress presentations in the previous year and gender. On average, male had more congress presentations (median = 3) than female (median = 2). The difference was significant U (N_{male} = 46, N_{female} = 87) = 2,651.5, z = 3.13, p = .002, η^2 = .071.

On the question: "Are you happy with your current academic achievements?", 27.3% (15/55) of female and 41.9% (13/31) of male confirmed their happiness with their current academic achievements, this association was not statistically significant (χ^2 (1, N = 86) = 1.94, p = .164).

Challenges for career development and barriers for gender parity

Among female, child planning was extremely important in 31.5% (17/54) of the respondents, considerably important in 37% (20/54) and not important at all in 13% (7/54), while among male it was extremely important in 7.5% (3/40) of the respondents, considerably important in 32.5% (13/40) and not important at all in 20% (8/40). Planning parenting was playing a major role in carrier development and there was a moderate positive correlation with gender (d = .35, p = .001).

On the question: "Do you think that the parental leave has affected your clinical career?", 48.1% (26/54) of female deemed that parental leave has affected their clinical carrier vs. 20% (4/20) of male (*OR* 3.71, 95% *CI* 1.11-12.57, p = .029, χ^2 (1, N = 74) = 4.79, small to moderate effect size ϕ = .26). When asked in which way has the parental leave affected their clinical career, 45.7% (21/46) of female stated that it was the lack of surgical activities while on leave, and 39.1% (18/46) assessed that due to the parental leave they have missed their career advancements.

On the question: "If the parental leave affected your academic career?", there was again a significant difference between male and female. 44.4% (24/54) female and 5.9% (1/17) man perceived that parental leave had adversely affected their academic career (*OR* 12.8, 95% *CI* 1.58-103.53, p = .004, χ^2 (1, N = 71) = 8.43, moderate effect size ϕ = .35). 42.5% (17/40) female stated that because of parental leave, they did not manage to actively participate in research projects and could not publish, 27.5% (11/40) stated that they did not get the desired academic position, 12.5% (5/40) stated that they did not have time to enroll in PhD studies and did not have time to work with students/fellows/residents.

A significant higher number of female 41.8% (23/55) than of male 14.3% (3/21) were feeling underestimated by their manager, because of using parental leave (*OR* 4.31, 95% *CI* 1.14-16.4, p = .024, χ^2 (1, N = 76) = 5.12, small to moderate effect size ϕ = .26).

The significant majority of female, 79.3% (65/82), see obstacles for career success for females in surgical gynecologic oncology compared to less than half of male, 46.2% (18/39), (*OR* 4.46, 95% *CI* 1.95-10.2, p<.001, χ^2 (1, N = 121) = 5.12, moderate effect size ϕ = .33). Figure 2 presents the perceived obstacles for career success among female in surgical gynecologic oncology.

Suggested ways for gender parity achievement

The suggested ways to increase the feasibility for a woman to be both a successful gynecologic oncology surgeon and a caring mother are presented in Figure 3.

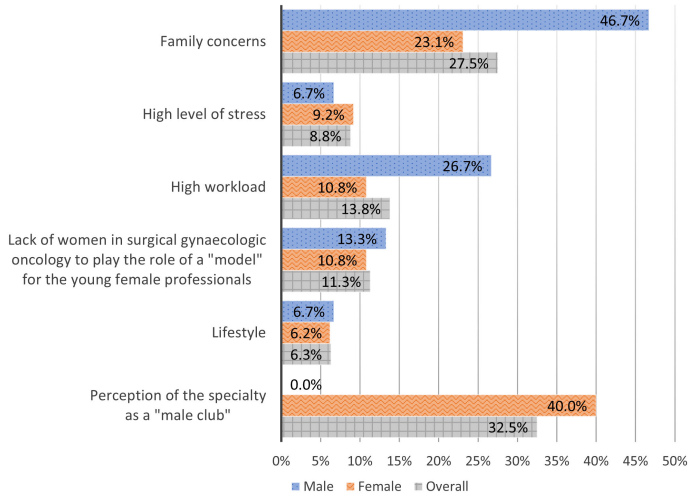

FIGURE 2
Perceived obstacles for career success among female in surgical gynecologic oncology.

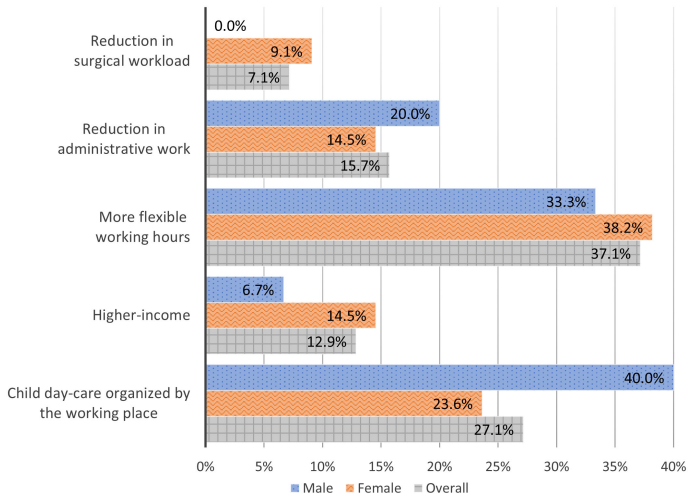

FIGURE 3
Suggested ways to increase the feasibility for a woman to be both a successful gynecologic oncology surgeon and a caring mother.

On the question: "According to you could ESGO and ENYGO help improving the gender disparity in the field of gynecologic oncology surgery?" 76.5% (62/81) of female and 55.6% (20/36) of male gave a positive answer. Table 5 presents selected individual suggestions on options how ESGO and ENYGO could help.

Figure 4 presents the answers to the question: "If you were now about to choose your career, what would you do?". 77.5% (31/40) of male and 70.9% (56/79) of female answered that they would stay with their career choice. 10% (4/40) of male and 19% (15/79) of female stated that they would choose a non-medical career. There was not a significant association between

TABLE 5 Selected individual suggestions on how ESGO and ENYGO could help improving the gender disparity in gynecologic oncology surgery.

- This survey is already helpful and is a good start in promoting female to reach parity and change the perspective of the old generation.
- Encourage and support female in their clinical and research fellowships.
- Organize hands on courses and courses in leadership especially designed for female.
- ESGO and ENYGO should promote female as role models at the conferences. At least 50% female at all of their committees. More female at leadership position in the both of the organizations. Also, at least 50% of conference speakers should be female.
- Provide a better network between colleagues from different centers.
- Successful female in this field should openly talk on conferences about managing family and work.
- Gender disparity issues should be analyzed within ESGO and ENYGO committees. The visuality of this problem should be increased at open forums. Discussions and public awareness initiatives are needed. Promotion of activities to spread awareness of this problem.
- Providing training scholarships and grants for female. Giving priority to applicants with children.
- Fellowships should be well paid and should be with a shorter duration. More regional meetings should be organized. Mentor-fellow system of training in the host hospitals for better acquisition of specific surgical skills.
- Female mentorship programs. Encouraging academic/clinical leads to facilitate and support female.
- Providing childcare during conference, financial support for female coming to conferences with children. Providing special time and places at congress for female surgeons with children.
- More online educational platforms.
- Promoting a culture of understanding of mothers.
- Promoting female surgical career and put in value the female's work in this field.
- Support pregnant female in being allowed to still do surgery. Setting up mentoring and fellowship programs, especially for female and encouraging hospitals to invest in the training of their female gynecologic oncology surgeons.
- To encourage young woman to do surgery.
- Flexible working models.
- Day-care organized by the clinical setting.

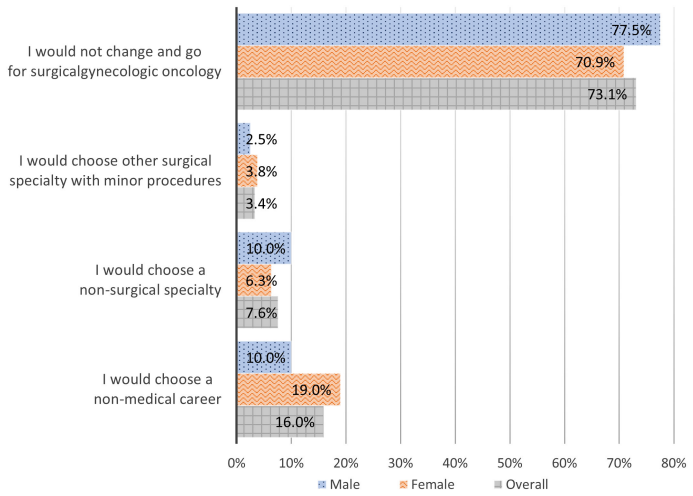

FIGURE 4
If you were now about to choose your career, what would you do?

gender and the current career choice χ^2 (3, N = 119) = 2.1, p = .55).

Discussion

In this study we report on gender disparities in the critical time of career development among the young generation of gynecologic oncology surgeons in Europe based on an international survey. To the best of our knowledge this is the first comprehensive evaluation of gender differences and experienced obstacles in carrier development among gynecologic oncology surgeons.

Self- reported data in our survey showed a higher exposure of male compared to female to operative procedures during their fellowships and as young specialists in gynecologic oncology surgery (Table 2). The predominance of male over female surgeons has been described before and is well known in different surgical disciplines, e.g. a Canadian population based retrospective study has included all surgical disciplines and reported that only 12.4% of identified surgeries were performed by females (12).

Although the majority of specialists in gynecology and obstetrics worldwide are nowadays female, the published literature reports on an underrepresentation of female in leadership positions (13, 14). The comparison of two observational studies on leadership positions in gynecology and obstetrics performed in the periods 2012/2013 and 2019/2020 in United States of America shows a slight increase in the percentage of female in leadership positions. In the period 2012/2013 20% of the chairs of departments were female; 36.1% of the vice chairs and 29.6% of the division directors (15). In the period 2019/2020 female were 29% of the chairs, 46% of the vice chairs, and 47% of the division directors (16).

Gender disparity in leadership position has been previously reported in the field of medical oncology as well. Banerjee et al. demonstrated a significant male dominance at leadership positions among respondents mainly practicing in Europe, 71% (11). Furthermore, a recent leadership study in the United States of America identified the absence of female gynecologic oncologists at cancer center director positions (17). Another study in the United States of America exposed that female constituted only a minority of all faculty in academic oncology institutions (medical oncology, radiation oncology and surgical oncology) and the low female representation was particularly pronounced at a leadership level (18).

In our study the male dominance at leadership positions was particularly displayed when focusing on leadership at younger age (under 41 years) and on younger age plus having children (Table 3). The adjusted odds ratio for male in a leadership position was more than 3 and notably more than 10 times higher, respectively, compared to female with these attributes in life. Parenting and domestic duties mainly carried out by female together with other factors probably contribute to hampered career advancement and gender disparity seen in leadership positions. Among high-achieving young physician-researchers it was reported that female with children spent a longer time on domestic duties and were more dedicated to childcare activities compared to male (19). Furthermore, the impact of gender and parenthood on physician's career success was investigated by Buddeberg-Fischer B. at al. who performed a prospective study on career development among young physicians in the first seven years after graduation in Switzerland. They found out a lower career success among female physicians, especially those

with children in comparison to their male colleagues. Moreover female physicians with children tended to work in smaller hospitals or private practices and aspired less often to senior hospital leading positions (20).

In our study a significantly higher proportion of female than male believed that they earn less than their gender counterparts at the same clinical position with comparable clinical and academic qualifications, with an impressive odds ratio of around 17. This estimate is leveled with data reported by Croft et al. who analyzed exact annual income sums among gynecologic oncologists in USA. They found that 75% of female gynecologic oncologists in academic settings make bellow the median salary calculated for the combined group of gynecologic oncologists of both genders (21). Further reports have robustly pointed out the gender reimbursement gap among medical oncologists in Europe and among health care providers in the USA (11, 22, 23).

With respect to PhD degrees and professorship positions, our finding in gynecologic oncology surgery are in contradiction to data reported in medical oncology by Elez et al. (24), who did report on a gender gap, while we did not reveal a gender disparity in our survey. However, our analysis showed that male published more than female. This is in line with previous gender related publication analyses in the field of gynecologic oncology (4, 25). Furthermore, our survey revealed that male with children published significantly more than female with children, which might again be related to female taking more responsibility in domestic duties and in childcare.

Planning parenting during training was of a higher importance for female than for male respondents in this survey, which is matching with published data among general gynecologists and gynecologic oncologists (26, 27). Such difference in perceived importance of family planning seems logical in light of gender specific influence on working opportunities and impact on career advancement by a decision for a family. Also, an initial career accomplishment and a subsequent child planning is feasible for male, but not for female. Fertility struggles were reported among gynecologic oncologists in United States of America with an impressive rate of 81% of females having sought infertility counseling (26). Indeed, parental leave was mainly utilized by female and covered a much longer timeframe. It was accompanied with a feeling of being underestimated by their supervisors and by the impression that parental leave has adversely affected their career, both academically and clinically due to lack of exposure to surgical procedures.

Both, male and female, recognize that there are obstacles in the career development for female in the field of gynecologic oncology surgery. The majority of male perceive the "family concerns" as the biggest obstacle for female, whereas female related their experienced carrier barriers mainly to gynecologic oncology being a closed "male club" besides the family concerns. Curiously, none of the male respondents perceived their subspecialty as a "male club". This finding underlines the importance of more females in leadership positions in gynecologic oncology to serve as role-models, to encourage female colleagues that they can succeed, and to support them during the process of their carrier development.

Among the offered options on how to increase the feasibility for a woman to be at a same time both a successful gynecologic oncology surgeon AND a caring mother (Figure 3) both genders agreed that more flexible working hours and child day-care organized by the working place could be helpful in this direction.

Valuable selected individual suggestions have been received on the open question on how ESGO and ENYGO could help to overcome gender disparity in gynecologic oncology surgery (Table 5): children friendly conferences with organized daycare and space for mothers and children, promotion of female as role models at ESGO and ENYGO conferences, reaching parity at ESGO and ENYGO committees and among invited conference speakers, open forums with discussion on current gender issues to increase awareness and prompt support for female in their clinical and research advancement, leadership academies for female, flexible working-models and day care organized by the working place.

Regardless of the numerous exposed challenges and barriers and the significant dissatisfaction in the field of clinical gynecologic oncology surgery, the majority of female would not change and would opt again for gynecologic oncology surgery.

Study strengths and limitations

To the best of our knowledge our study is first comprehensive evaluation of the gender-related differences among gynecologic oncology surgeons in Europe that provides an in-depth analysis of several specific problems. Also, the used qualitative methods allow a careful description of the broad spectrum of gender climate.

The biggest limitation of the study is that it is based on self-reported data. Also, there might be a selection bias with respect to those ENYGO members, who decided to fill in the survey. ENYGO is a diverse network of physicians interested in gynecologic oncology as it is the training and certification process in gynecologic oncology in various European countries. This leads to certain limitations in our study. Since this survey was sent only to ENYGO members, representing the younger generations of gynecologic oncology surgeons, our results give insights to gender issues mainly in the third and fourth decade of age only. Based on the presented data, a more focused survey on gender discrepancies is already planned to be distributed among all ESGO members, which could enable a further more profound analyses.

Conclusion

Although female present a rising proportion in the field of gynecologic oncology surgery in Europe, male prevail over

female as surgeons in operating theaters and dominate at leadership positions. Different factors related to family and childcare seem to adversely influence the clinical career advancement among female, while the effect of family planning and parenting in male seems comparably small.

It is not a lack of attractiveness or deficient wish of female to work in the field of gynecologic oncology surgery that explains the low number of females in leadership positions in this field. The majority of female would opt again for gynecologic oncology surgery. Obviously, there are obstacles in the critical time of career development that lead to a substantial attrition of female from training to leadership functions in gynecologic oncology.

ESGO and ENYGO aim to work on the implementation of measures and programs to overcome the identified obstacles, to close gender gaps, and support female to fully invest their skills, power and indispensable potential in the field of gynecological oncology surgery.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Medical School, University in Kielce, Poland (Number: 65/2021). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

TN performed the conception and design of the study. TN, MB, NN, KZ, JK-B, and ZR designed the survey. NN organized the database. TN and NN performed the statistical analysis. TN wrote the draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version. NC did the senior supervision and last revision of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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