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Cyclin E1 overexpression identifies a therapeutically relevant poor prognostic patient subgroup in high-grade serous ovarian cancer

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Cyclin-E1 protein overexpression, including through *CCNE1* gene amplification, is recognized as a poor prognostic factor in high-grade serous ovarian cancer (HGSOC) and is a promising predictive marker for investigational therapies targeting cell-cycle checkpoints. However, the demonstration of its clinical utility remains elusive due to inconsistent definitions of protein overexpression and gene amplification. This study characterizes Cyclin-E1 overexpression and *CCNE1* amplification prevalence and prognostic value in HGSOC using both original and public clinical cohorts. Fifty-nine percent of tumors overexpressed Cyclin-E1, more than half of which had no evidence of *CCNE1* gene amplification. The prevalence of *CCNE1* amplification varied across studies and was higher in interventional studies. Patients with Cyclin-E1 positive tumors had poorer outcomes after adjuvant therapy. Platinum-based chemotherapy increased Cyclin-E1 expression. These patients were less likely to benefit from PARP inhibitors (75% are BRCA-wildtype) or mirvetuximab-soravtansine (67% were not FRα-high), highlighting a distinct patient population in need of novel therapies.

High-grade serous ovarian cancer (HGSOC) is the most common and lethal subtype of epithelial ovarian cancer, accounting for approximately 70% of ovarian cancer cases and deaths, with a five-year survival rate of only 30–40% for advanced-stage disease. Despite initial platinum sensitivity in most patients, the majority develop platinum-resistant recurrence within 12–18 months, creating a critical unmet need. Major genetic drivers of HGSOC include loss of function mutations of *TP53*, *BRCA1/2*, and amplification of *CCNE1*, limiting the development of targeted therapies beyond PARP inhibitors. Cyclin E1, which is encoded by *CCNE1*, activates CDK2 to initiate the G1/S cell cycle transition, and high levels of Cyclin E1 lead to increased replication stress. Dysregulation of Cyclin E1 expression, including genomic amplification at *CCNE1*, is a pioneering event occurring early in the transformation of fallopian tube epithelium[1,2] and has been associated with poor prognosis and resistance to chemotherapy[3]. Interestingly, this effect may be indirect, reflecting the inherent chemosensitivity of tumors with BRCA mutations, which are mutually exclusive with *CCNE1*

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amplification[4,5]. Cyclin E1 protein expression is regulated at the DNA level by amplification, but other indirect mechanisms including mutations in *FBXW7* – a ubiquitin ligase that mediates Cyclin E1 degradation[6], or activation of transcription factors regulating G1/S transition, such as E2F1-3[7] can also lead to high levels of Cyclin E1 protein. Immunohistochemistry for Cyclin E1 protein status thus serves as a good general biomarker for Cyclin E1 activation as it captures all the underlying mechanisms of deregulation in one assay. *CCNE1* amplification status determined by next generation sequencing (NGS) may also have some relevance as a clinical biomarker given the ubiquity of this analyte on widely available NGS panels. *CCNE1* gene amplification typically results in high Cyclin E1 protein expression, but expression of similar levels or higher is also observed in many non-amplified tumors, suggesting that Cyclin E1 activity may be a more common oncogenic driver than previously thought[8–10]. *CCNE1* amplification or elevated Cyclin E1 protein expression have been proposed as biomarkers predicting HGSOC sensitivity to therapies targeting cell cycle and DNA damage repair regulators such as WEE1, CDK2, PLK1, ATR1 or PKMYT1[11–16], highlighting the need for better characterization of tumors expressing high level of Cyclin E1. Furthermore, antibody drug-conjugates (ADC), recently approved or in development, are expected to improve outcomes for a subset of patients with platinum-resistant ovarian cancer (PROC)[17–19]. However, the correlation between protein expression of the ADC target and Cyclin E1 is poorly understood but may prove critical in designing future clinical studies.

Here, we estimate the prevalence of Cyclin E1 activation across many published studies of tumors of HGSOC patients using genomic data to assess *CCNE1* amplification status as well as the relationship between *CCNE1* gene amplification and Cyclin E1 protein overexpression for a subset of patients. We supplement these studies with a retrospective analysis of Cyclin E1 protein and *CCNE1* gene amplification status in tumors of patients enrolled in clinical studies of the WEE1 inhibitor azenosertib. We estimate the fraction of tumors with gene amplification or positive protein expression and examine inter-cohort differences. We also investigate associations of Cyclin E1 protein expression with common molecular features - including the expression of ADC-targets– as well as treatment outcomes and discuss implications for using Cyclin E1 protein expression -including but not restricted to *CCNE1* amplification - as a clinically relevant biomarker that can inform patient management.

Results

The prevalence of *CCNE1* copy number amplification varies by assays and studies

We determined the prevalence of *CCNE1* amplification across 26 cohorts from 19 published studies, 5 real world datasets (RWD) and 2 Zentalis cohorts (Supplementary Table 1). Of those, 18 were observational, 6 interventional and 2 followed either a case-control design or investigated relapse tumors only (Fig. 1). The number of subjects in each cohort varied between 31 and 12857, with observational studies being larger than interventional studies (median 467 vs. 139). Of note, some studies sharing cohorts such as TCGA, AOCS or OTTA likely have overlapping subjects. While most studies were uniformly HGSOC, mixed ovarian histology was reported in at least 2. The assays used to identify the number of *CCNE1* gene copies were heterogeneous including in situ hybridization ($N = 5$), microarray ($N = 5$), panel or exome sequencing ($N = 11$), whole genome sequencing ($N = 2$) or quantitative PCR ($N = 3$). Similarly, the methods and thresholds used to determine the amplification status varied and ranged from as low as 3 copies (considered gains) to more than 6 or 8 copies, sometimes corrected for ploidy. Although not always specified, it is very likely that most studies analyzed archival tissue specimen collected at diagnosis or following neoadjuvant chemotherapy (NACT). However, the specimens from 2 studies were collected at relapse. Some of the cohorts were clearly selected (case-control design) or represented only patients with recurrent or drug-resistant disease (interventional studies). Additional selection bias may remain due to practical reasons such as tumor cellularity or requirement of pre-existing clinical or molecular data for inclusion.

In aggregate, the studies reported the amplification status of tumors on 33,841 patients (overlap not resolved, relapses and case-control studies excluded) for an overall prevalence of 12% and a median prevalence of 17%. Significant variability (8%-31%) was seen across cohorts and important differences were observed. The median prevalence in interventional studies, which typically only includes patients with more advanced disease (recurrent, PROC or PARPi resistant) was 22% (8%-29%), compared to 15% (8%-31%) in observational studies from patients not enrolled in clinical trials and with unknown clinical history. Variability in prevalence was also noticeable among the observational studies – including in large cohorts (15% in GENIE-MSK vs 11% in GENIE-DFCI). Compared to RWD from diagnostic laboratories (FMI, Tempus, Caris), large observational cohorts from academic institutions or consortiums (AOCS, TCGA, GENIE) reported higher prevalence (aggregated prevalence 20% vs 10%), perhaps reflecting some ascertainment bias between patients seen at tertiary care centers versus those profiled by large diagnostic laboratories. Variability in prevalence was also observed between the two Zentalis cohorts (A:29%, B:8% from FMI-xT and Caris-MI Profile assays respectively), likely reflecting methodological differences between the vendors since the same trend was observed in their respective RWD (FMI-Insight:16%, Caris/George: 9%). Finally, the prevalence in the Zentalis-A cohort (29%) likely overestimates the expected prevalence in PROC. Indeed, the prevalence was higher in the Zentalis-A1 cohort (31%) which preferentially enrolled *CCNE1* amplified patients and decreased once the enrollment criteria were modified in the Zentalis-A2 cohort (22% - Supplementary Table 1). Given the totality of these observations, the prevalence of *CCNE1* amplification is expected to be around 20% in an interventional study of PROC patients using a sensitive diagnostic assay.

Cyclin E1 is highly expressed in a significant subset of non-amplified tumors

A subset of studies described above also evaluated *CCNE1* gene expression (via microarray or RNA-seq) or Cyclin E1 protein levels (via IHC) and observed high expression even in absence of copy number amplification in many cases. However, the definition of high expression across these studies is variable, sometimes referring to expression relative to normal tissue, or relative to housekeeping genes/proteins, and other times to raw measures such as staining intensity (IHC) or gene length-normalized number of sequencing reads (FPKM in RNA-seq). To compare the different studies and more extensively compare expression level with amplification status, we re-analyzed available datasets using a more universal classification of expression: positive^{TPA} (resp. negative^{TPA}) corresponds to an expression level equal or above (respectively below) the expression of the 10th percentile of copy number amplified tumors (referred to as the tenth percentile of amplified, or TPA, definition).

From the 6 datasets analyzed (4 published studies and 2 Zentalis cohorts), we determined that, in aggregate, the prevalence of Cyclin E1 positive^{TPA} tumors was 59% (675/1150), that the median prevalence was 58% (min:56%, max = 72%, Fig. 2A) and was consistent between datasets. The fraction of tumors both positive^{TPA} and amplified was however more variable (median 23%, min:7%, max:40%) and directly a consequence of the variability in *CCNE1* amplification prevalence reported above (Fig. 1). Consistent with the classification, the level of Cyclin E1 expression was 2 to 5-fold higher in positive^{TPA} vs negative^{TPA} tumors (Fig. 2B). In contrast, the Cyclin E1 expression level was only marginally higher in *CCNE1* amplified compared to non-amplified positive^{TPA} tumors (1.1 to 1.4-fold higher). Overall, the data suggests that Cyclin E1 is highly expressed in 45% (median) to 47% (390/829; aggregate) of non-amplified tumors and that characterizing *CCNE1* amplification status alone will miss many tumors for which Cyclin E1 is highly expressed and likely active.

Characterization of Cyclin E1 expression in tumor specimens

Using the Zentalis-A cohort, we were able to investigate whether any pre-analytical variables may impact the measurement of protein expression by IHC (Table 1, Fig. 3). The archived tissue specimens were collected from

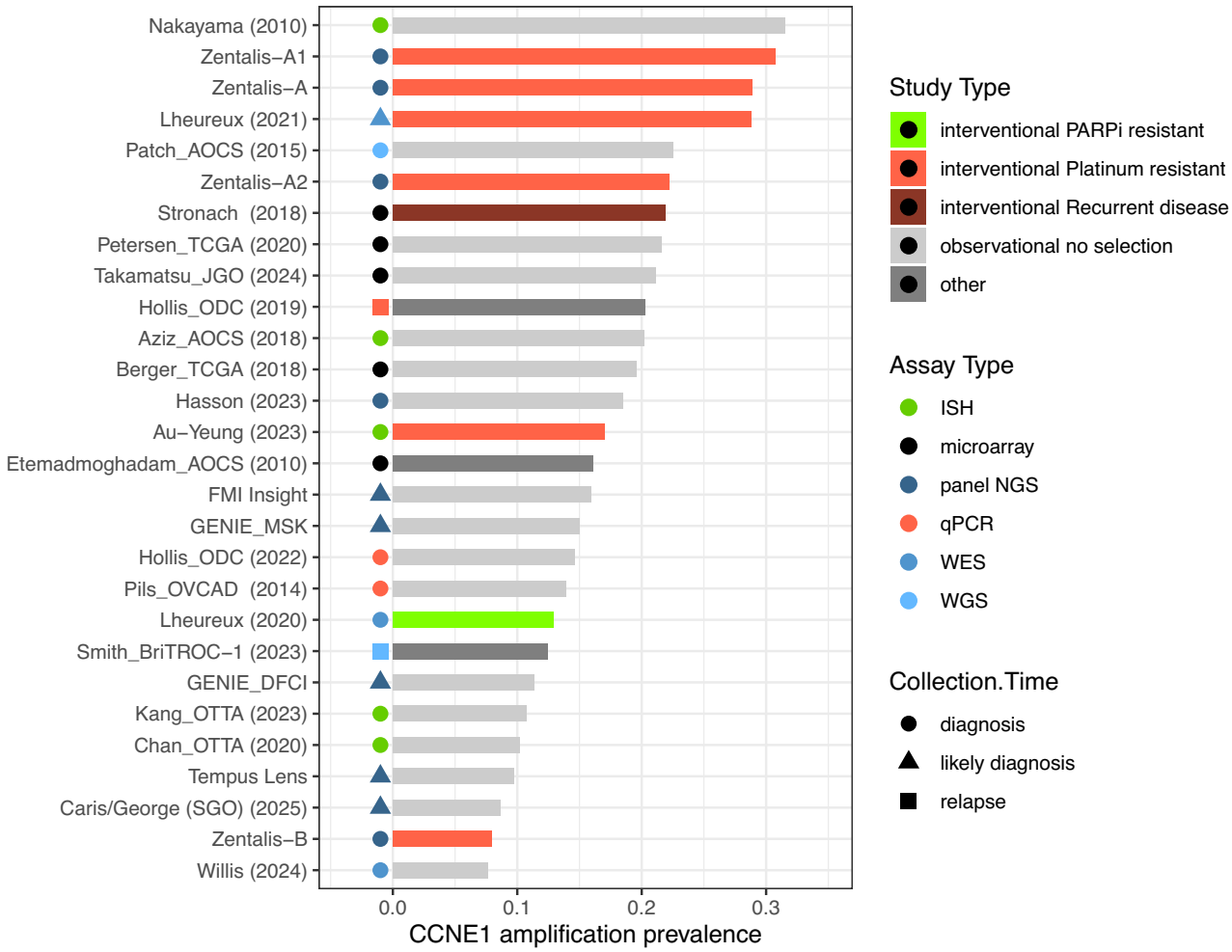

Fig. 1 | Prevalence of *CCNE1* amplification in selected studies. The studies (Supplementary Table 1) are sorted by decreasing *CCNE1* amplification prevalence and classified as interventional with patient selection, observational without selection, or other designs. A symbol on the left of each bar indicates the assay used (color) and the timing of the specimen collection (shape). The published studies are identified by the first author and year of publication. RWD are labelled after the diagnostics lab. Consortium data are referred to by the acronym of the consortium.

either the primary site (ovary, fallopian tube epithelium $N = 68$), from a lymph node metastasis ($N = 20$) or from metastatic spread to the omentum ($N = 35$), peritoneum ($N = 21$) or other locations ($N = 85$). The median Cyclin E1 expression H-score was between 100 and 180 across all sites, and the fraction of positive^{TPA} tumors (H-score ≥ 145) was between 33% and 69% (Table 1). The distribution of expression generally does not depend on the site of collection, except for peritoneal specimens which appear to have lower expression (median H-score 100 vs 165 in non-peritoneal specimen, $p < 0.008$, Wilcoxon test). The tissue specimens shared by the study site were obtained as FFPE blocks ($N = 84$) or freshly cut FFPE sections ($N = 125$). While Cyclin E1 expression was slightly higher in tumors with a block specimen (median H-score 172 vs 155, respectively, $p = 0.046$), the fraction of positive^{TPA} was not significantly affected (65% vs. 58%, respectively, $p = 0.376$). HGSOC specimens are typically collected during debulking surgery and re-biopsies are rarely performed. The age of the specimen at the time of consent varied greatly, and older specimens had lower Cyclin E1 expression (Spearman rho = −0.18, $p = 0.002$). The difference was the most pronounced in specimens older than 5 years (median H-score 115 vs. 155 or higher for more recent categories, $p = 0.013$, Table 1), which concordantly impacted the fraction of positive^{TPA} specimens (39% vs. 56% or higher, $p = 0.038$). These observations illustrate the robustness of the epitope and quality of the antibody and immune-histochemical staining process used. The findings highlight critical pre-analytical variables that may skew the results and suggest that diagnostic analysis may be best performed on samples collected within 5 years. Furthermore, while Cyclin E1 expression or status did not seem to be associated with the patient's race - albeit limited by the scarcity of non-white patients - we observed that Cyclin E1 expression was lower in younger patients, leading to a lower prevalence of CyclinE1 positive^{TPA} in patients younger than 55 (54%), and highest for patients older than 75 (80%, $p < 0.03$, Table 1, Fig. 3D). Additional correlations between Cyclin E1 expression and molecular markers or prognosis are presented below

Molecular characteristics of Cyclin E1 positive^{TPA} tumors

Using comprehensive genomic profiling, we determined if the Cyclin E1 expression status was associated with specific genetic features in 211 tumors from Zentalis-A patients. Of the genes included in the assay, 106 were mutated in at least 1 tumor and 7 were mutated in 10% or more, consistent with previous reports of HGSOC molecular landscape (Fig. 4A). As determined from other cohorts and studies, *CCNE1* amplification was mutually exclusive with *BRCA1/2* alterations (OR = 8.9, $p < 3E\text{-}4$, Fig. 4B). As *BRCA1/2* alterations are more frequent in younger patients (Supplementary Table 2A), such mutual exclusivity likely underlies the lower Cyclin E1 positive^{TPA} prevalence observed above (Table 1). There was a mild enrichment of *AKT2* amplification in *CCNE1*-amplified tumors (OR = 0.18, $p < 2E\text{-}3$), but no other genes alterations showed significant association with

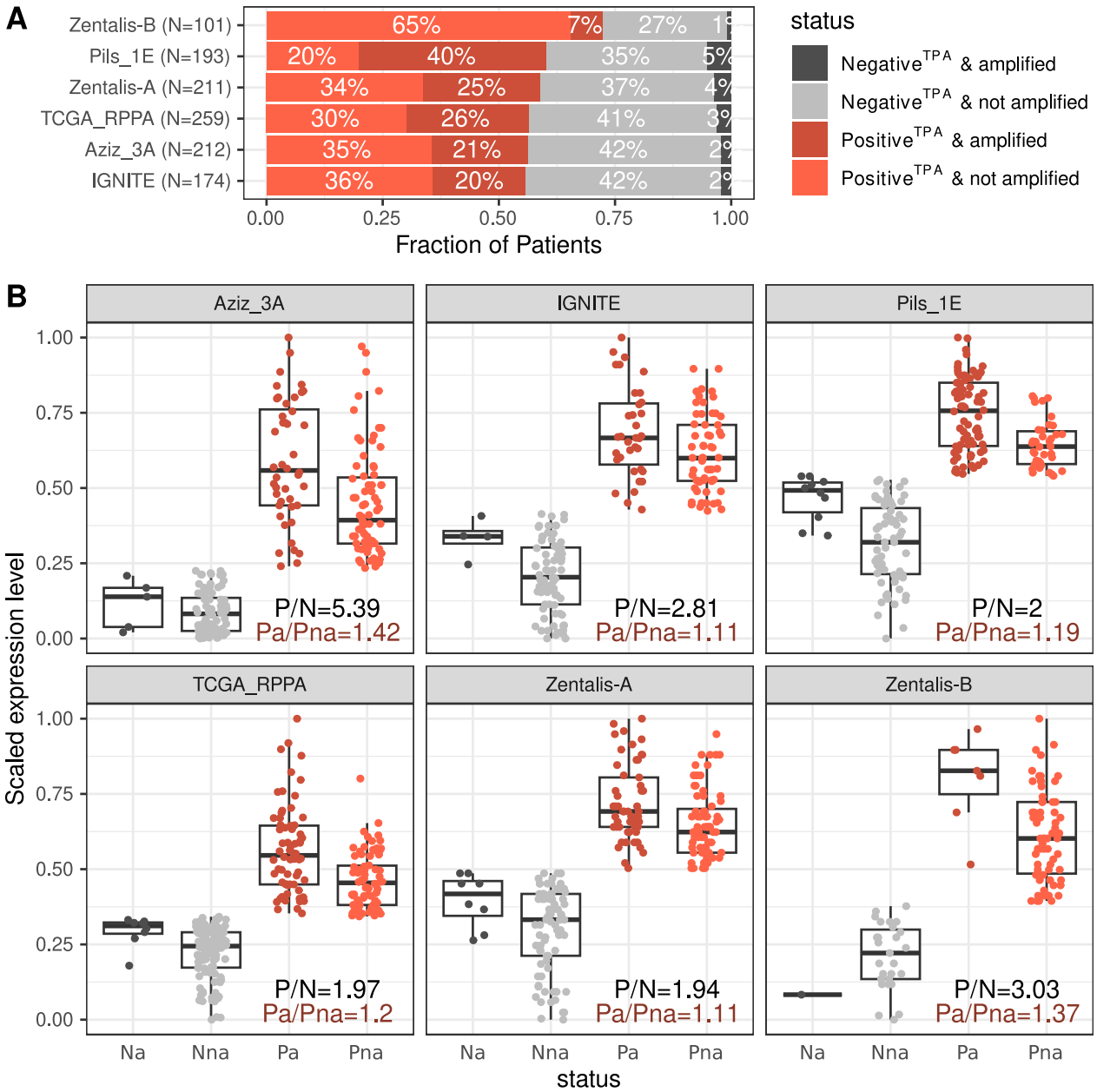

Fig. 2 | Cyclin E1 protein expression status according to expression and amplification in 6 PROC cohorts. A Distribution of tumors according to Cyclin E1 and *CCNE1* status. **B** Scaled Cyclin E1 expression levels according to Cyclin E1/*CCNE1* status (Pa Positive^{TPA} & amplified, Pna Positive^{TPA} & not-amplified, Na Negative^{TPA} & amplified, Nna Negative^{TPA} and not-amplified). Expression levels were scaled for each cohort between minimal (0) and maximal (1) value observed. The ratio of median expression between Positive^{TPA} and Negative^{TPA} (P/N) or between Positive^{TPA} & amplified and Positive^{TPA} & not amplified (Pa/Pna) is indicated in black and red respectively.

CCNE1 amplification status. Similarly, apart from *CCNE1* amplification, no other gene alterations were associated with the Cyclin E1 expression status, irrespective of whether tumors with *CCNE1* amplifications were included in the analysis (Fig. 4C, D). This suggests that the mutual exclusivity between *BRCA* mutations and *CCNE1* amplification does not extend to Cyclin E1 expression status. Consistently, prevalence of Cyclin E1 positivity in *CCNE1* non-amplified tumors was similar for *BRCA* mutated (19/35 – 54%), and *BRCA* wildtype patients (56/115 - 47%). Finally, after excluding *CCNE1* amplified tumors, we did not identify any significant correlations between Cyclin E1 expression status with molecular scores such as number of mutations, number of genes with copy number alteration, loss of heterogeneity score, or homologous recombination deficiency (Supplementary Table 2B).

Expression of ADC targets in Cyclin E1 positive^{TPA} tumors

Protein-based biomarkers play a central role in guiding the use of antibody-drug conjugates (ADCs) in ovarian cancer, with folate receptor alpha (FRα) being the most prominent clinically validated marker. Ongoing clinical studies investigate ADCs against additional proteins such as cadherin-6 (CDH6) and sodium-dependent phosphate transport protein 2B (NaPi2b), but these are investigational and lack validated companion diagnostics thus far[20]. Although the expression of protein-based biomarkers is typically measured using immunohistochemistry, there are currently no HGSOC cohorts for which all four expression biomarkers used in approved or investigational treatment – Cyclin E1, FRα, NaPi2b, and CDH6 – have been measured together. As a surrogate, we used the mass spectrometric measurement of protein expression in a subset of the TCGA samples to study the

Table 1 | Pre-analytical variables associated with Cyclin E1 expression and status

	mean H-score	expression *p*-value (chi-square)	N Positive ^{TPA} (%)	status *p*-value (Kruskal-Wallis)
Specimen format				
Block	172	0.046	55/84 (65.5%)	0.376
Slide	155		73/125 (58.4%)	
Anatomic site				
LN	180	0.164	12/20 (60%)	0.072
Omentum	155		22/35 (62.9%)	
Other	170		59/85 (69.4%)	
Ovary/FE	162		40/68 (58.8%)	
Peritoneum	100		7/21 (33.3%)	
unknown	155		12/23 (52.2%)	
Time since collection				
<6 m	160	0.013	36/57 (63.2%)	0.038
6m–2y	175		54/77 (70.1%)	
2y–5y	155		53/95 (55.8%)	
>5 y	115		9/23 (39.1%)	
Patient age at collection				
< 55	150	0.195	27/50 (54%)	0.027
55–74	160		113/187 (60%)	
75+	195		12/15 (80%)	
Race				
White	170	0.127	96/143 (67.1%)	0.149
Black	140		8/16 (50%)	
Asian	185		4/6 (66.7%)	
Other	155		4/7 (57.1%)	
Not-Reported	142		40/80 (50%)	

correlation of expression of these biomarkers at the protein level[21]. The expression of all four biomarkers was evaluable in 46 patients. Cyclin E1 expression was not significantly correlated with the expression of the other three ADC-targets and, from all pairwise comparisons, only NaPi2b and CDH6 showed significant positive correlation (Fig. 5A), hence suggesting that Cyclin E1 positive tumors do not significantly overlap with tumors expressing high levels of ADC targets. To quantify such overlap between Cyclin E1-positive and FRα-high tumors, we first determined that 33/46 patients were classified as Cyclin E1 positive^{TPA} based on their tumor Cyclin E1 expression measurement in the RPPA analysis presented in Fig. 2A. In parallel, 16 of the 46 patients were classified as FRα–high^{MS}, corresponding to the top 35% highest FRα expression measured by mass spectrometry, following the 35% prevalence estimated from mirvetuximab-soravtansine studies[19,22]. When the status of both biomarkers was considered together, we determined that 64% (21/33) of the Cyclin E1 positive^{TPA} patients were not FRα–high^{RPPA}, therefore suggesting that a substantial fraction of patients may not be eligible for mirvetuximab treatment and may benefit from therapies leveraging Cyclin E1-induced vulnerabilities such as WEE1 inhibitors.

To validate the observation above in the context of clinically relevant diagnostic assays, Cyclin E1 and FRα expression levels were measured in 150 commercially procured HGSOC FFPE tissue specimens using clinical grade immunohistochemical staining (method) for each biomarker. The expression of the two biomarkers was not correlated (Fig. 5B, rho = 0.06 $p = 0.44$ Spearman correlation between Cyclin E1 H-score and FRα PS2 +). By applying the FDA approved clinical cutoff for FRα high (PS2 + ≥ 75%), we determined 29% (44/150) were FRα-high in the assembled cohort, consistent with the observed clinical prevalence in the PROC patient population[19,22]. We further determined that 67% (56/84) of the Cyclin E1 positive^{TPA} tumors (H-score≥145) were not FRα–high and therefore would not be eligible for mirvetuximab treatment. Of note, the PROC status of both the TCGA and the commercially procured cohort is unknown, and these estimates may be different in a PROC restricted population.

Prognostic significance of Cyclin E1 expression

Previous reports have determined that patients whose tumors expressed high levels of Cyclin E1 had shorter progression free survival, an effect primarily driven by *CCNE1* copy number amplification[3,23]. To characterize the prognostic value of Cyclin E1 IHC status in the Zentalis-A cohort, we retrospectively evaluated the time to next therapy (TTN) following first line adjuvant treatment, which is used as a surrogate endpoint for progression free survival. We specifically compared the time between the end of the adjuvant treatment and start of the second line of treatment (excluding maintenance therapy, see methods). This analysis was restricted to patients whose tumor specimen was collected around the first line of treatment to limit the impact of non-histopathological factors on Cyclin E1 prognostic evaluation (Supplementary Figure 1, $N = 107$ evaluable patients). Patients with Cyclin E1 positive^{TPA} tumors by IHC had shorter TTN (8.8 m vs. 14.1 m, HR = 1.9, $p < 2E\text{-}3$, log rank test, Fig. 6A). This observation held true after excluding patients with BRCA mutated tumors, who typically derive greater benefit from platinum therapy and subsequent PARPi maintenance (Fig. 6B). Accordingly, 83% (20/24) of patients with *BRCA* wildtype tumors who needed treatment within 6 months were Cyclin E1

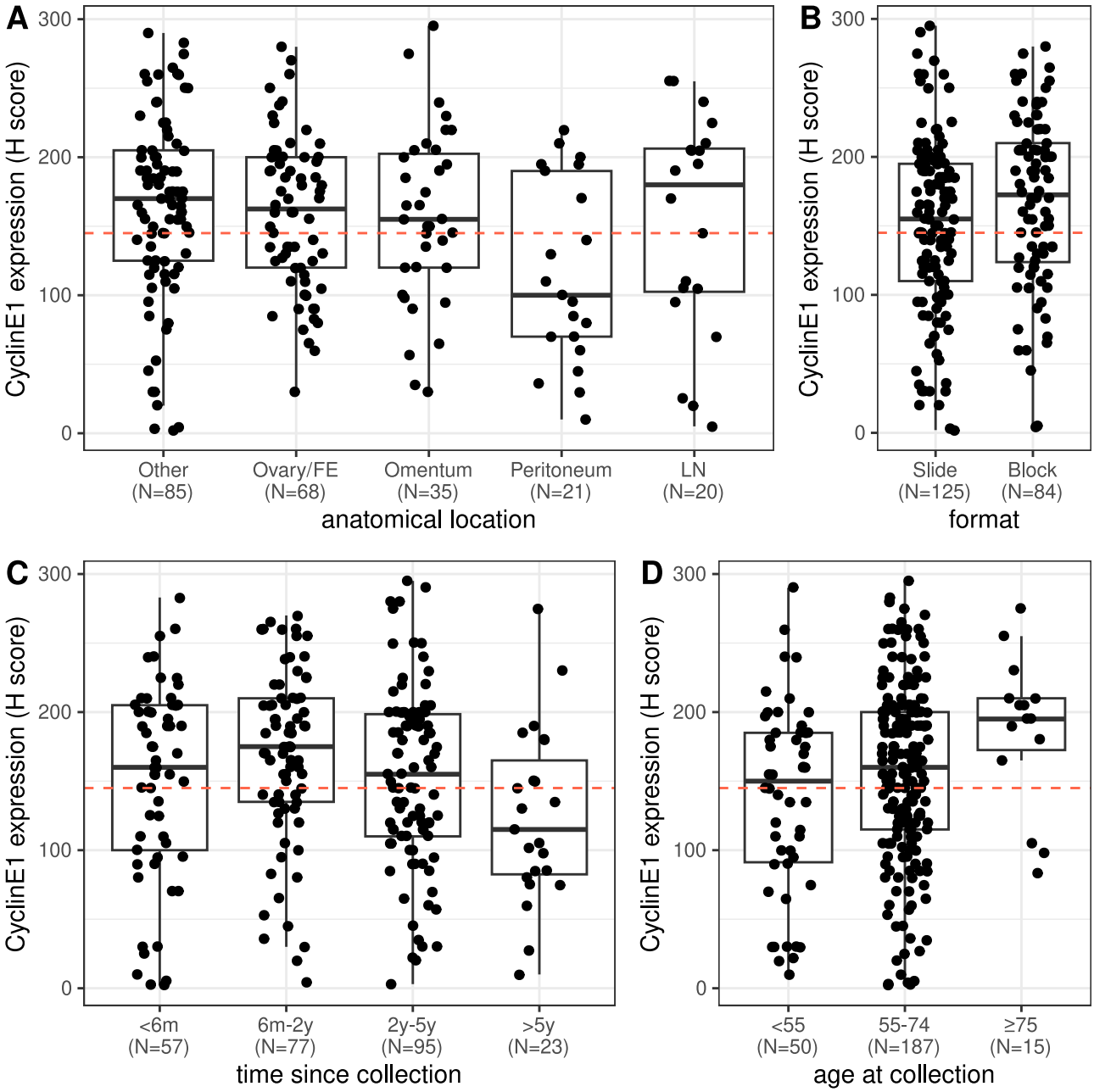

Fig. 3 | Expression of Cyclin E1 according to pre-analytical variables. Including anatomic location (**A**), specimen format (**B**) and time since collection (**C**) or age at collection (**D**). TPA tenth percentile of amplified specimen, cutoff indicated in dashed red line LN Lymph Node. FE Fallopian Epithelium.

positive^{TPA}, compared to 60% (39/64) of patients with TTN longer than 6 months. The poorest prognostic group were Cyclin E1 positive patients whose tumors display amplification of the *CCNE1* gene (median TTN = 6.5 m, $N = 33$, Fig. 6C). However, Cyclin E1 expression status remained prognostic after accounting for *CCNE1* amplification as confirmed by multivariate analysis (Table 2).

To further validate the prognostic value of Cyclin E1 expression and better understand the impact of treatment on Cyclin E1 expression, we used the same clinical grade IHC assay to measure Cyclin E1 expression in tumor tissue-microarrays collected prior (biopsy, $N = 111$) and after (surgery $N = 102$) platinum-based neo-adjuvant chemotherapy (NACT) treatment (GINECO cohort - see method). We first verified that the expression estimated from tissue-microarray was consistent with the one measured from whole slide (rho = 0.82, $p = 0.01$, $N = 8$ tumor specimen, Supplementary Figure 2). The median Cyclin E1 expression (H-score) was 102 and 124 in pre- and post-NACT respectively (1.2-fold increase, $p < 0.05$ Wilcoxon-test). Using the protein expression cutoff established in Zentalis-A cohort analyzed with the same IHC protocol (positive^{TPA}: H-score≥145), we determined that 20% (22/111) and 38% (39/102) of pre- and post-NACT tumors were Cyclin E1 positive^{TPA} with 18/76 (24%) of patients with paired specimen reclassified as positive^{TPA} post-NACT. This observation suggests that Cyclin E1 expression may increase in some patients following platinum treatment. Interestingly, and akin to the Zentalis-A cohort observation, patients with Cyclin E1 positive tumors had short progression free survival (12.7 vs 16.7 median time to progression, HR = 1.6, $p = 0.04$ log rank test - Fig. 6D).

Discussion

The work presented here uses a compendium of published, real world and clinical studies to characterize the level of expression of Cyclin E1 and its relationship to clinical outcomes on standard of care therapies and overlap with other important biomarkers in HGSOC. While numerous studies have

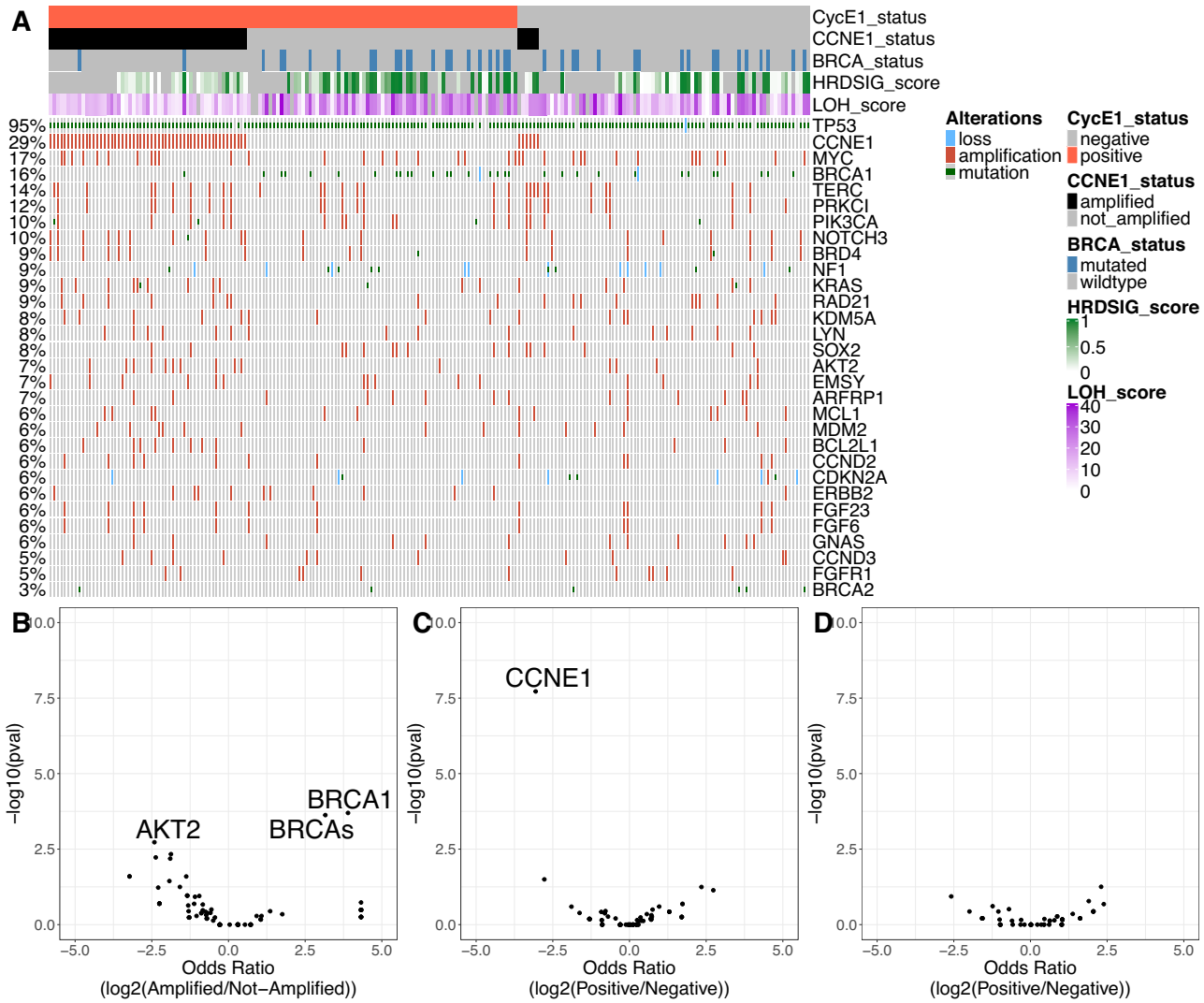

Fig. 4 | Molecular characterization of Cyclin E1 positive^{TPA} tumors. A Oncoprint diagram indicating the distribution of genetic alterations in the Zentalis-A cohort evaluable through comprehensive genomic profiling ($N = 211$), for all genes altered in more than 5% of tumors and *BRCA2*. Tumors are sorted according to *CCNE1* amplification and Cyclin E1 expression status. **B**–**D** Volcano plots indicating the enrichment of gene alterations (x-axis, log2 odds ratio) and their significance (y-axis, -log10 *p*-value, Fisher Exact Test) in *CCNE1* amplified tumors (**A**), Cyclin E1 Positive^{TPA} tumors (**B**) or Cyclin E1 Positive^{TPA}, *CCNE1* non-amplified tumors (**C**). Genes mutated in 3 or more tumors are displayed as points. Genes with corrected *p*-value lower than 0.05 (Benjamini-Hochberg) are labeled. Absolute value of odds ratio was capped at 20 for visualization purposes.

assessed the prognostic significance of Cyclin E1, the novelty of this study is the real-world, multi-cohort focus, systematic quantification of discordance between protein and gene markers, dynamic measurement of Cyclin E1 modulation by chemotherapy, and the direct demonstration that this patient population is particularly underserved by current targeted therapies. A key finding is that 59% of HGSOC patients have levels of Cyclin E1 protein expression equivalent to those with *CCNE1* amplification (TPA definition). The prevalence of patients with platinum-resistant disease with *CCNE1* amplified tumors was lower – at about 20% - consistent with the hypothesis that Cyclin E1 expression can also be deregulated at the transcriptional and translational level. This suggests that Cyclin E1 may be a much more prevalent oncogenic driver than previously thought, highlighting the utility of the Cyclin E1 IHC assay to capture disparate mechanisms of Cyclin E1 activation. Importantly, the majority of Cyclin E1 positive tumors are *BRCA* wildtype and do not express high levels of ADC target proteins. Given their poor prognosis, patients with Cyclin E1-positive tumors therefore represent a sizeable portion of the PROC population lacking effective therapeutic options.

The mutual exclusivity of *BRCA* mutations and *CCNE1* amplification is known and typically attributed to synthetic lethality of the two oncogenic drivers, which brings unsustainable level of chromosomal instability in early oncogenesis. However, the lack of such exclusivity in *CCNE1* wildtype, Cyclin E1 positive tumors is intriguing and questions this model. Since Cyclin E1 is overexpressed in 77% of Serous Tubal Intraepithelial Carcinoma, which are recognized as HGSOC precursor lesions[24], this suggests high expression contributes to oncogenesis but the strength of the synthetic lethality with *BRCA* mutations may be dose dependent. Indeed, *CCNE1* amplification is not mutually exclusive with HRD tumors with non-*BRCA* gene mutations believed to yield a milder disruption of the homologous recombination[25]. Similarly, since among Cyclin E1 positive tumors, *CCNE1* non-amplified tumors have marginally lower expression level of Cyclin E1 (Fig. 2), this may suggest that the high expression can co-exist with *BRCA* mutations. Additional variables to consider in a dynamically evolving tumor would be the plasticity of Cyclin E1 expression or the clonal heterogeneity of *CCNE1* amplification documented elsewhere[26]. Hence, additional experiments measuring the relative fitness and oncogenic potential of various

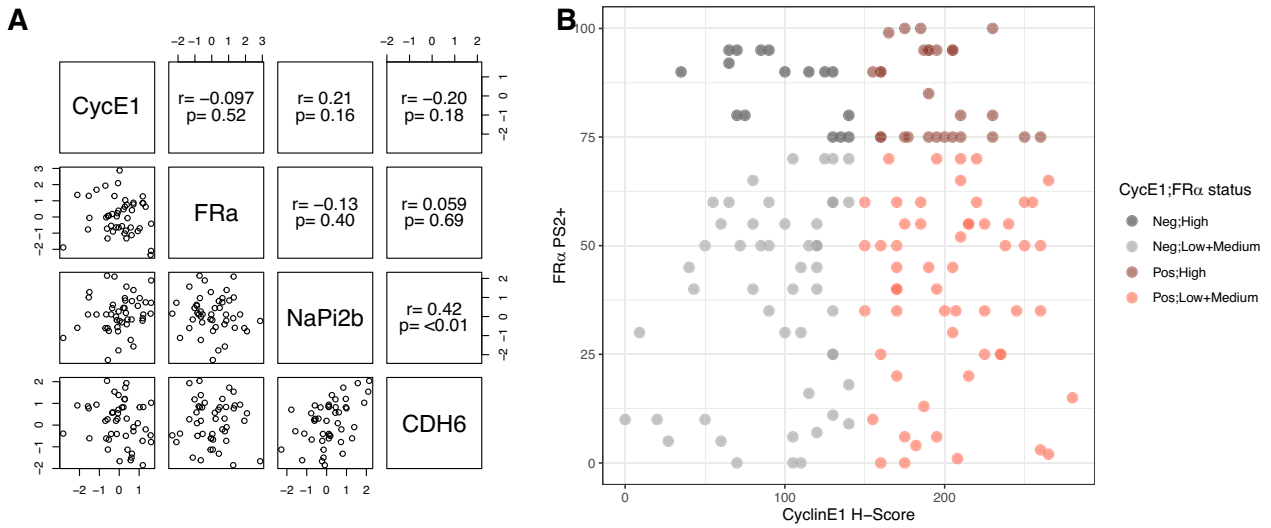

Fig. 5 | Biomarker expression correlation. A Scatter plots (lower triangle) and corresponding spearman correlation rho and *p*-value (upper triangle) for the pairwise correlation of the expression (Mass Spectrometry Z scores) of 4 biomarkers of interest (diagonal) in tumors of 46 TCGA/CPTAC patients. **B** Scatter plot comparing the expression levels of Cyclin E1 (H-score, x-axis) and FRα (PS2+ y-axis) in 150 commercially procured HGSOC specimen. Colors indicate the Cyclin E1 status (Positive^{TPA}/Negative^{TPA}, in red and grey) and FRα status (High in dark and Medium/Low in light).

mutations and Cyclin E1 expression level will be needed to better interpret this result.

The large variation in *CCNE1* amplification prevalence observed was not entirely surprising. Indeed, gene amplification is commonly identified by high-throughput sequencing, but the calculation of number of copies (using diploid or overall ploidy as reference) as well as the threshold needed to call for amplification (absolute, or relative to ploidy) may vary. Methods that can better account for intra-tumor heterogeneity – such as FISH – are not as common and more sensitive to spatial heterogeneity[26]. The FISH definition of amplification itself may vary, using absolute count or relative to the number of copies of centromeric or control gene probes. Similarly, the reported prevalence of HGSOC tumors with Cyclin E1 positive expression in previous studies has been highly variable – likely due to differences in assays and the use of arbitrary expression cutoff for classification[27]. In this report, we chose to use a definition of Cyclin E1 expression positivity relative to tumors with *CCNE1* amplification. We considered this definition sufficiently general to be applied to any cohorts, relatively independent of how expression and amplification are measured. Importantly, this definition is based on molecular features and the predictive value of the resulting Cyclin E1 status in respect to current or experimental therapeutics has not been demonstrated. We recently reported the results of a retrospective analysis supporting the predictive value of Cyclin E1 expression for azenosertib treatment in PROC[28]. This clinical definition uses a slightly different expression cutoff that was determined using the area under the receiver-operator curve (ROC-AUC) established from tumor Cyclin E1 protein level and best overall response observed in HGSOC patients treated with azenosertib. While the clinical and TPA definition for Cyclin E1 status are different, the prevalence of Cyclin E1 positive were similar (51% in Simpkins et al using clinical definition vs 59% in Zentalis-A cohort using TPA definition), suggesting that the observations reported here will likely apply to patients stratified according to the clinical definition used in the ongoing registrational study (NCT05128825 "DENALI" part 2).

Discrepancies between reports of prevalence may also come from ascertainment bias or variable inclusion criteria that are not always clearly captured. In HGSOC, most tissue specimens come from surgical resections at or around the time of diagnosis, from patients who are treatment-naïve or receiving NACT. At this stage of the treatment, it is estimated that 70% of the patients are platinum-sensitive and the analysis of the GINECO cohort, which was mostly comprised of platinum-sensitive patients, suggested that 38% of tumor specimens collected at the time of platinum sensitivity are positive for Cyclin E1. While a previous report suggested that Cyclin E1 expression did not change after chemotherapy[29], our own observations, using a larger number of patients and more validated assay and antibody, demonstrated a modest overall increase in expression (1.2-fold) that can result in a status change for 24% of patients. Importantly, it is unclear if this change is driven by regulatory changes in Cyclin E1 expression or by selection of tumor clones expressing higher levels of Cyclin E1. Phylogenetic analysis has shown that clonal expansion upon chemotherapy was associated with poor outcome, but the contribution of Cyclin E1 was not investigated[30]. Elsewhere, specific case reports suggested that *CCNE1* amplification was not sufficient for chemoresistance as clones with *CCNE1* amplification did not always expand[31], Such lineage tracing or clonal analysis are however restricted to genetic features such as mutations and amplifications, and clinically compatible methods are lacking to investigate the heterogeneity of changes in gene or protein expression upon treatment and understand intra-tumoral factors underlying the change in Cyclin E1 expression observed after NACT. Irrespective of the mechanism for the increase in Cyclin E1 expression, our observation implies that the proportion of patients with Cyclin E1 positive tumors would increase after each line of therapy and, as patients become increasingly PROC (59% Cyclin E1 positive prevalence in Zentalis-A cohort). However, the difference in prevalence observed between the GINECO and Zentalis-A cohorts cannot be explained by the induction of Cyclin E1 – or selection of Cyclin E1 high clones - by platinum treatment since Cyclin E1 status is typically determined from a treatment-naive archived surgical specimen collected during the initial debulking, and longitudinal samples are typically not collected. Instead, the difference in prevalence observed between platinum-sensitive and platinum-resistant cohorts is likely due to survivor bias introduced in the retrospective analysis of the Zentalis-A cohort, which selected patients who survived and met eligibility criteria of the azenosertib study after becoming platinum-resistant. Consequently, it is likely that cross-sectional studies enrolling patients from different treatment stages will observe different prevalences including higher prevalence in interventional platinum-resistant cohorts. There is however, a corollary to such stage-dependent landscape of Cyclin E1 expression. Indeed, given the poor prognostic value of Cyclin E1 expression and despite the slightly lower prevalence of Cyclin E1, patients with earlier-stage ovarian cancer might also benefit from therapies known to exploit Cyclin E1-driven vulnerability, such as inhibitors of WEE1, CDK2, or PKMYT1.

Our findings have important therapeutic implications for patients with Cyclin E1 positive HGSOC. First, we find that patients with Cyclin E1-

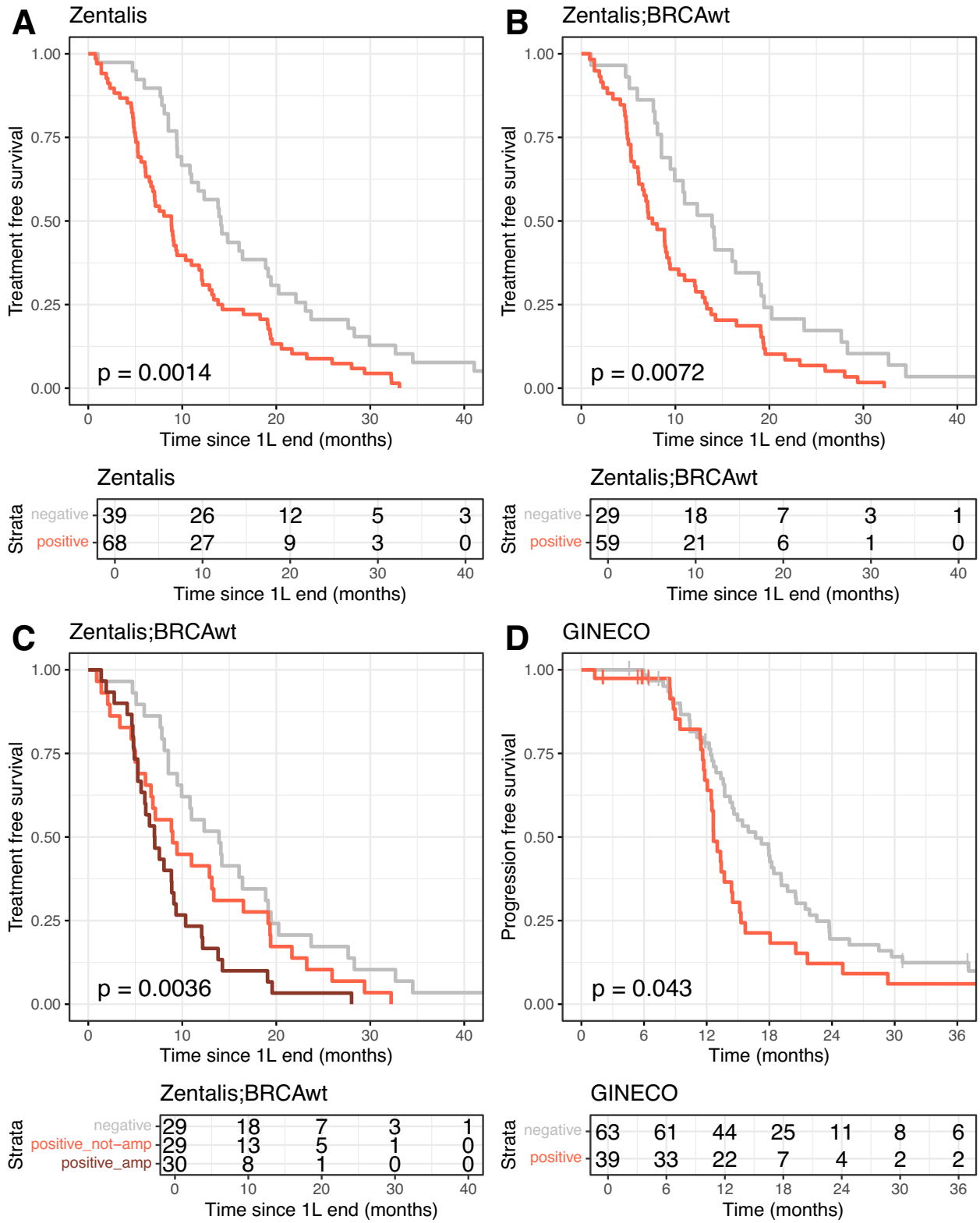

Fig. 6 | Prognostic significance of Cyclin E1 status. A–**C** Kaplan-Meyer analysis of the treatment-free survival (y axis) as a function of time (x axis, months), stratified by Cyclin E1 status (**A**–**C**) and *CCNE1* amplification status (**C** only) in all evaluable patients (**A**) and BRCAwt patients (**B**, **C**) from the Zentalis-A cohort. Time to next treatment is defined as the time between the end of 1 L adjuvant chemotherapy and the beginning of the 2 L treatment – maintenance treatments excluded. **D** Kaplan-Meyer analysis of the post-NACT progression-free survival (y axis) as a function of time (x axis, months), stratified by Cyclin E1 status in patients from the GINECO cohort. The number of patients at risk and color legend are indicated in the risk-table below the plot. The *p*-value was calculated using log-rank test statistics.

Table 2 | Results of the univariate and multivariate Cox regression analysis of the prognostic value of CyclinE1 expression status and *CCNE1* amplification in Zentalis-A cohort

Model	Strata	HR	CI95	*p*-value
Univariate	Cyclin E1 expression status	1.88	1.18–2.99	0.00824
Univariate	CCNE1 amplification	1.58	1.01–2.46	0.04
Multivariate	Cyclin E1 expression status	1.72	1.05–2.83	0.03
	CCNE1 amplification	1.31	0.92–2.1	0.26

positive tumors show a low prevalence of predictive biomarkers relevant for approved agents such as PARP inhibitors and the FRα-targeting ADC mirvetuximab. The overlap is also limited for other ADC targets, although the finding from RPPA-measured protein expression will need to be confirmed using clinically validated IHC assays once available. We also showed patients with Cyclin E1-positive ovarian cancer have poorer outcome on standard of care. These results suggest that Cyclin E1 overexpression defines a population of unmet need. Emerging data suggest Cyclin E1 IHC may serve as a robust predictive biomarker for sensitivity to WEE1 and PKMYT1 inhibitors in ovarian cancer. Multiple preclinical and early clinical studies have demonstrated that ovarian cancer cell lines and xenograft models with high Cyclin E1 expression are significantly more sensitive to inhibitors of WEE1 or PKMYT1 or CDK2, compared to those with low expression[12,32,33]. This heightened sensitivity is attributed to the increased replication stress and genomic instability driven by Cyclin E1/CDK2 activation, which leaves Cyclin E1-positive cells more reliant on checkpoint kinases for survival. Importantly, high Cyclin E1 protein levels, regardless of *CCNE1* gene amplification status, predict response to WEE1 inhibition across diverse ovarian cancer subtypes. These findings provide a strong rationale for incorporating Cyclin E1 testing into patient selection criteria for clinical trials and therapeutic strategies utilizing WEE1 and PKMYT1 inhibitors, paving the way for more targeted and effective treatments for this molecularly defined ovarian cancer subset. Second, our findings have important implications for Companion Diagnostic development. Cyclin E1 IHC captures a broader spectrum of patients (59% of HGSOC) with functional Cyclin E1 activation—including those without *CCNE1* amplification—and likely confers a good predictive value for WEE1 inhibitor sensitivity in both preclinical and clinical settings, currently undergoing prospective validation for the WEE1 and CDK2 inhibitors (NCT05128825 and NCT07023627, respectively). *CCNE1* amplification is a more specific but less sensitive marker, identifying genomically driven cases but missing other mechanisms of upregulation; its predictive value may be incomplete for broader patient selection, a question that will be explored in the ongoing studies referred above as well as matching randomized Phase 3 studies that are commencing (ASPENOVA and MAESTRA-2, respectively). For patient selection in WEE1 inhibitor trials, Cyclin E1 IHC offers a more inclusive and clinically actionable biomarker, while *CCNE1* amplification remains valuable for confirming gene-driven cases and possibly for capturing additional patients tested on broadly used NGS platforms.

Finally, Cyclin E1 is highly expressed in certain tumor types other than HGSOC, as driver of proliferation or mediator of therapeutic resistance[10,34]. Additional studies should seek to understand the prevalence, prognostic significance and predictive value of Cyclin E1 expression in these other tumor types and molecular contexts.

Methods

Public and Real-World Evidence Data sets

CCNE1 Amplification. Datasets are listed in Supplementary Table 1. For published datasets, the size of the cohort and number of patients with amplification were directly obtained from the report or conference presentation[3,8,9,15,35–49]. Data from the AACR GENIE cohort was derived from version 16.1, selecting tumors with HGSOC based on oncotree code, and further restricting to two consistent datasets: GENIE_MSK includes all tumors profiled with any version of the MSK-IMPACT solid tumor assay; GENIE_DFCI includes all tumors profiled with any version of the ONCOPANEL assay[50]. Amplification status in the TCGA OVCA cohort was obtained from the cBioPortal using the results of the PanGyn analysis[51]. The data from Foundation Medicine Inc. was obtained via their Insight™ portal using the database from 2024/05/07 and selecting profiles from "High Grade Serous Carcinoma of the Ovary." The data from Tempus AI was obtained using the Lens™ portal on 2024/11/07, selecting high grade serous ovarian cancer profiles with the xT solid tumor assay.

Cyclin E1 expression. A subset of cohorts identified for the amplification datasets was also characterized for Cyclin E1 expression (Supplementary Table 1). Of those, three had data available, either as supplementary data or as a report figure. Datapoints from the figures were digitized using the digitizeit software (http://www.digitizeit.de/).

Patient cohorts

Zentalis cohorts were assembled from four ongoing clinical studies of azenosertib, including ZN-c3-001 (NCT04158336), ZN-c3-002 (NCT04516447), ZN-c3-005 (NCT05128825 "DENALI"), and ZN-c3-006 (NCT05198804 "MAMMOTH") clinical trials. All studies were conducted in accordance with the Declaration of Helsinki, following approval by the Institutional Review Boards (or equivalent) at the applicable sites and under an FDA-approved investigational new drug application in the United States and ex-U.S. equivalents, where applicable. All patients enrolled in the clinical trials provided written informed consent. All enrolled and consented patients with successful central immunohistochemistry measurement of Cyclin E1 expression in their tumor specimens were included in the report. The *Zentalis-A* cohort included patients whose genomic profile was characterized using the FoundationOne CDx assay (Foundation Medicine, Inc.) while the *Zentalis-B* included patients whose tumor genome was profiled using the MI Profile assay (Caris Life Sciences). The *Zentalis-A* cohort was further split between *Zentalis-A1* (patients consented prior 4/1/2024) and *Zentalis-A2* (patients consented after 4/1/2024) to account for modification in the inclusion criteria, notably the enrichment of patients whose tumors showed *CCNE1* amplification in early versions of the NCT05198804 study protocol.

The GINECO cohort was assembled from patients enrolled in two clinical trials (NCT01583322 "CHIVA" and NCT03249142 "Ineov", sponsored by ARCAGY-GINECO, led by the GINECO group) with inoperable FIGO IIIC/IV epithelial ovarian cancer who were eligible to receive neoadjuvant chemotherapy (NACT). Patients received 3 to 6 cycles of platinum-based chemotherapy regimen before debulking surgery. Formalin-fixed paraffin-embedded (FFPE) tumor samples from diagnostic laparoscopy and interval cytoreductive surgery, were prospectively collected. Samples were selected based on their tumor cellularity, excluding necrotic samples. The most representative regions of each tumor were chosen by a pathologist on an H&E-stained slide to construct the different tissue microarray (TMA) used in this study. All patients provided written informed consent authorizing the use of biological samples obtained during their routine diagnosis and treatment as part of the prospective research study.

FRα/Cyclin E1 co-expression cohort: A set of $N = 150$ commercially procured HGSOC tissue samples (Discovery Life Science, Huntsville AL and BioIVT, Westbury NY). Tissue blocks were sectioned (4 μm) and processed for staining as described below.

Immunohistochemistry

Cyclin E1 protein expression levels in FFPE tissue specimens were measured through a CLIA/CAP validated immunohistochemistry assay using anti-Cyclin E1 mouse monoclonal antibody (Abcam Cat# ab238081, RRID:AB_3096040). Cyclin E1 reactivity in the nucleus of viable tumor cells was assessed by board-certified pathologists using H-score and tumor

proportion score methods. H-score was calculated by the following formula: H-score = [(% cells at null x 0] + [(% cells at 1+ staining intensity) x 1] + [(% cells at 2+ staining intensity) x 2] + [(% cells at 3+ staining intensity) x 3], with values ranging from 0 to 300. The resulting H-scores were highly correlated between two readings from the same pathologist (Spearman rho=0.99, 93% concordance in TPA status) and between two pathologists (Spearman rho=0.95, 90% concordance in TPA status), as shown from 30 specimens (Supplementary Figure 3).

FRα IHC staining of FFPE tissue samples was performed using VENTANA FOLR1 (FOLR1-2.1) RxDx Assay (cat#:740-5065) with OptiView DAB IHC detection kit. Percent positivity of all viable tumor cells was scored based on membranous FRα staining intensity, which was scored 0–3+ for none (0), weak (1 +), moderate (2 +), or strong staining (3 +), respectively. The cut-off threshold for FRα positivity (FRα-high) was ≥ 75% of tumor cells with ≥ 2+ staining intensity (PS2 + ≥ 75%).

Molecular Profiling

Molecular Alterations. Molecular alterations in tumors of the Zentalis-A and -B cohorts, including *CCNE1* amplification and mutations in HRD genes, were identified using the FoundationOne CDx assay (Foundation Medicine Inc) and MI Profile assay (Caris Life Sciences), respectively.

Expression classification. For both Zentalis and public expression datasets, the Cyclin E1 protein expression status was determined as positive for all samples with an expression equal to or higher than the tenth percentile of expression of amplified samples. This definition assumes that *CCNE1* gene amplification results in protein overexpression in 90% of cases, allowing for 10% of amplified cases to be false-negative calls or subjected to histological sampling bias resulting from intra-tumoral heterogeneity. The study cohort specific definition of amplification was used without standardization. The resulting classification is labelled TPA for "Tenth Percentile of Amplified" to prevent confusion with other definitions of positivity that may have been used elsewhere.

Retrospective Analysis of Early Treatment Outcomes

For each drug prescribed prior to enrollment, the class, regimen ID – organized by lines (1 L, 2 L, etc.), intent (neoadjuvant, adjuvant, maintenance, recurrence), start date, and end dates were collected per study protocol. These records of prior systemic therapy were then used to select eligible patients from the Zentalis-A cohort and characterize the lines of treatment as follows:

For each patient, the treatment start and end dates and their characteristics were used to identify three distinct lines of therapy (Supplementary Figure 1): first line (1 L), first line maintenance (1Lm) and second line (2 L). For each line of therapy, the start date was defined as the initiation of the earliest treatment and the end date as the completion of the latest treatment, excluding bevacizumab, which is frequently continued as maintenance monotherapy after completion of adjuvant chemotherapy for up to 12–15 months[52].

The following rules were used for specific line assignment:

- Any treatments ending more than 60 days prior to the specimen collection were excluded from the analysis as the intent is to understand the predictive and prognostic value of a tissue biomarker.
- Treatment starting within 42 days of sample collection and not given with maintenance intent was assigned to a reference line, which corresponded to 1 L for all patients, consistent with standard collection of tissue at debulking surgery.
- Treatment with maintenance intent and indicated as first line was assigned to the 1 Lm line.
- Treatments initiated more than 42 days after specimen collection, not already captured in the 1 L or 1Lm lines and characterized with the same line and treatment intent were assigned to 2 L.

The time-to-next treatment (TTN) was defined as the time from the end of 1 L to the start of 2 L, ignoring the intervening 1Lm line, if any.

Data Availability

Most of the data used in this study is from published studies or publicly available resources (Supplementary Table 1). Data from the Zentalis cohorts is owned by Zentalis Pharmaceuticals, used in the ongoing development of azenosertib and not available publicly at the time of this manuscript publication.

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Author contributions

D.K.: Conceptualization, Methodology, Writing; H.C.: Investigation, Methodology; M.A.: Investigation, Conceptualization, Writing; J.M., N.M., C.S., D.R., J.Y.: Investigation; J.J., A. Levy: Formal Analysis, Data Curation; E.Y.G.: Formal Analysis, Data Curation; F.B.D.: Writing, Conceptualization; C.D., P.F., N.B.-L., S.O., V.D., C.G.: Investigation; F.M.-B., J.F.L., F.S., L.M.R.: Investigation; D.J.: Project Administration, Conceptualization; A. Leary: Investigation, Methodology, Conceptualization; M.R.L.:

Conceptualization, Supervision, Writing; OH: Conceptualization, Methodology, Visualization, Writing.

Competing interests

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Additional information

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