European Journal of Cancer 191 (2023) 112948

Available online at www.sciencedirect.com

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journal homepage: www.ejcancer.com

Original Research

Faecal microbiota composition is related to response to CDK4/6-inhibitors in metastatic breast cancer: A prospective cross-sectional exploratory study

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Received 31 March 2023; Received in revised form 10 June 2023; Accepted 12 June 2023
Available online 20 June 2023

KEYWORDS
Biomarker;
Cyclin-dependent kinase (CDK)4/6-inhibitors;
Metastatic breast cancer;

Abstract ***Background:*** Cyclin-dependent kinase (CDK)4/6-inhibitors with endocrine therapy represent the standard of treatment of hormone receptor-positive(HR+)/human epidermal growth factor receptor 2 (HER2)-negative metastatic breast cancer (MBC). Gut microbiota seems to predict treatment response in several tumour types, being directly implied in chemotherapy resistance and development of adverse effects. No evidence is available on gut microbiota impact on efficacy of HR+ breast cancer treatment.

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https://doi.org/10.1016/j.ejca.2023.112948

Metagenomic DNA;
Faecal microbiota;
16S targeted
sequencing

Patients and methods: We assessed the potential association among faecal microbiota and therapeutic efficacy of CDK4/6-inhibitors on 14 MBC patients classified as responders (R) and non-responders (NR) according to progression-free survival. A stool sample was collected at baseline and V3–V4 16S targeted sequencing was employed to assess its bacterial composition. Statistical associations with R and NR were studied.

Results: No significant differences were observed between R and NR in terms of α-/β-diversity at the phylum and species level. Machine-learning (ML) algorithms evidenced four bacterial species as a discriminant for R (*Bifidobacterium longum*, *Ruminococcus callidus*) and NR (*Clostridium innocuum*, *Schaalia odontolytica*), and an area under curve (AUC) of 0.946 after Random Forest modelling. Network analysis evidenced two major clusters of bacterial species, named Species Interacting Groups (SIG)1–2, with SIG1 harbouring 75% of NR-related bacterial species, and SIG2 regrouping 76% of R-related species ($p < 0.001$). Cross-correlations among several patients' circulating immune cells or biomarkers and bacterial species' relative abundances showed associations with potential prognostic implications.

Conclusions: Our results provide initial insights into the gut microbiota involvement in sensitivity and/or resistance to CDK4/6-inhibitors + endocrine therapy in MBC. If confirmed in larger trials, several microbiota manipulation strategies might be hypothesised to improve response to CDK4/6-inhibitors.

1. Introduction

The combination of cyclin-dependent kinase (CDK)4/6-inhibitors with endocrine therapy (ET), either an aromatase inhibitor (AI) or fulvestrant, represents one of the major advances of the last decades for the treatment of hormone receptor-positive (HR+)/human epidermal growth factor receptor 2 (HER2)-negative metastatic breast cancer (MBC). Despite the impressive progression-free survival (PFS) and overall survival (OS) improvements observed in pivotal trials in comparison to single-agent ET, not all patients benefit from the combination [1–3]. At present, no selective biomarkers of response to CDK4/6-inhibitors have been clearly identified, hence patient selection for such therapeutic strategy is only based on classical endocrine receptors and HER2 status, along with clinical conditions [1–3].

Gut microbiota, composed of more than 100,000 billion bacteria, fungi, archaea and viruses, plays an important role in many physiological functions, including nutrient absorption, immune system correct functioning, and various metabolic processes, like oestrogen metabolism, which is strongly correlated to HR+/HER2-negative breast cancer development [4–6]. Importantly, host-microbe interactions in physiological conditions help counteract invading pathogens and prevent tumorigenesis. As a consequence, imbalances in the local microbial environment (dysbiosis) could modulate the host immune responses and inflammation favoring disease pathogenesis and progression [4–6]. Nevertheless, there is emerging evidence regarding the capability of faecal microbiota to predict treatment response in several tumour types (e.g. to ipilimumab in melanoma, to 5-fluorouracil in colorectal cancer etc.) and being directly implied in chemotherapy resistance and development of side-effects [7–10]. Still, very little is known regarding faecal microbiome impact on breast cancer treatment efficacy and only preliminary data are currently available [7,11,12].

Finally, neutrophil-to-lymphocyte ratio (NLR) is an inexpensive blood biomarker representative of the systemic immune-inflammatory status [13]. High levels of NLR have been associated with poor prognosis in malignant tumours, including breast cancer [14,15]. Interestingly, recent data suggest a potential relationship between inflammatory systemic status as detected via NLR and gut microbiome composition [16].

In this prospective observational cross-sectional study, we preliminarily explored the potential association among faecal microbiome, immune circulating cells with a focus on NLR, and therapeutic efficacy of first/second-line CDK4/6-inhibitors + ET on a series of patients affected by HR+/HER2-negative MBC treated at the Breast Unit of the Cremona Hospital in Italy between March 2019 and March 2021.

2. Materials and methods

2.1. Patient selection and study procedures

We included in this exploratory prospective cross-sectional observational study patients affected by HR+/HER2-negative MBC, with endocrine-sensitive tumours [17] and candidate to receive a first/second-line with CDK4/6-inhibitor-based regimen as per standard clinical practice. Full inclusion/exclusion criteria are reported in Supplementary materials.

All patients were treated with either palbociclib, ribociclib or abemaciclib with an AI or fulvestrant, according to standard treatment schedules. A mandatory

stool sample was collected at baseline before treatment started from each patient.

For the purpose of this study, patients were divided into responders (R) and non-responders (NR) to CDK4/6-inhibitors if disease progression happened after or before 10 months, respectively. This time point was selected since 9.5 months was the lowest median PFS obtained with a CDK4/6-inhibitor-based regimen in pivotal trials [3].

2.2. Sample collections

Before starting treatment, a blood sample was collected, as well as a stool sample, which was collected using eNAT Copan Kit at baseline before treatment. Approximately 5 g of the fresh stool was stored at +4 °C until processing and then kept at −70 °C until analysis. Blood samples were processed as elsewhere described to obtain blood cells counts [15].

2.3. DNA extraction, 16S rRNA gene amplification and sequencing

Total bacterial DNA was extracted from 50 mg of faecal material using the FastDNA SPIN Kit for Soil (MP Biomedicals, Eschwege, Germany). The genomic DNA was then quantified using the Qubit HS dsDNA fluorescence assay (Life Technologies, Carlsbad, California, United States of America). Amplification was carried out as described elsewhere [18], using Illumina's MiSeq v3 platform with 2 × 300 bp mode.

2.4. Bioinformatic analyses

Raw FASTQ files were analysed with DADA2 pipeline v.1.14 for quality check and filtering (sequencing errors, denoising, chimera detection). Raw reads were filtered and 2503 Amplicon Sequence Variants (ASV) were found. Analyses were performed with Python v.3.8.2. A matrix of bacterial species relative abundances and prevalence was built. Only bacterial species having a prevalence equal or higher than 20% were taken into account.

Data matrices were first transformed with pseudo count and centred-log-ratio (CLR), then normalised and standardised. For microbiota analysis, measurements of α-diversity (within sample diversity) were calculated at species level. Exploratory analysis of β-diversity (between sample diversity) was performed using the Bray-Curtis measure of dissimilarity and represented in Principal Coordinate Analyses (PCoA), along with methods to compare groups of multivariate sample units (analysis of similarities [ANOSIM], permutational multivariate analysis of variance [PERMANOVA]). We implemented Partial Least Square Discriminant Analysis (PLS-DA) and the subsequent Variable Importance Plot (VIP) as a supervised analysis wherein the VIP values (order of magnitude) were used to identify the most discriminant bacterial species among the cohorts.

Pearson matrices for network analysis were generated on normalised and standardised data with in-house scripts (Python v3.8.2). Intra-network communities (Species Interacting Groups, SIGs) were retrieved using the Blondel community detection algorithm [19].

2.5. Statistical analyses

The study was exploratory and the sample size was not based on a formal statistical assumption. Standard descriptive statistics were used, along with χ^2 test, Mann-Whitney U test and Kruskal-Wallis test, when appropriate. Survival differences between R and NR were estimated by the Kaplan–Meier method and the log-rank test. Exploratory univariate cox regression models were used to estimate PFS and OS hazard ratios (HR) with 95% confidence intervals (CI). Analyses were conducted with R version 3.6.1 for MacOSX. Significance was generally set at $p \leq 0.05$. More detailed bioinformatic and statistical methods are reported in Supplementary Materials.

3. Results

Fourteen MBC patients complied with inclusion criteria and accepted entering the study (Fig. 1). The median follow-up at the time of the analysis was 32.5 months (95% CI: 31.6–not estimable [NE]). Seven (50%) patients were considered as R, while other seven were considered as NR. Median PFS and OS for R were not reached at the time of the analysis. For NR, median PFS was 6.2 months (95% CI: 3.8–NE) and median OS was 14.7 months (95% CI: 7.7–NE). R to CDK4/6-inhibitors showed a significantly improved OS compared to NR (HR: 19.81, 95% CI: 2.30–170.78; $p = 0.007$) (Supplementary Fig. 1). Population characteristics and treatment details are fully reported in Table 1.

Clinicopathological characteristics and circulating immune cells were not associated with PFS and OS (not shown), with the exception of NLR. Higher levels of NLR were significantly associated with worse PFS (HR: 4.13, 95% CI: 1.08–15.74; $p = 0.038$), with a tendency towards a significantly worse OS (HR: 3.17, 95% CI: 0.87–11.72; $p = 0.081$). Consistently, R showed significantly lower levels of NLR (2.23 vs. 2.57; $p = 0.026$) compared to NR. No other significant differences were observed, except for a slightly lower body mass index (BMI) (median 21.2 vs. 23.8; $p = 0.016$) in the group of R, with no significant association with both PFS ($p = 0.912$) and OS ($p = 0.769$).

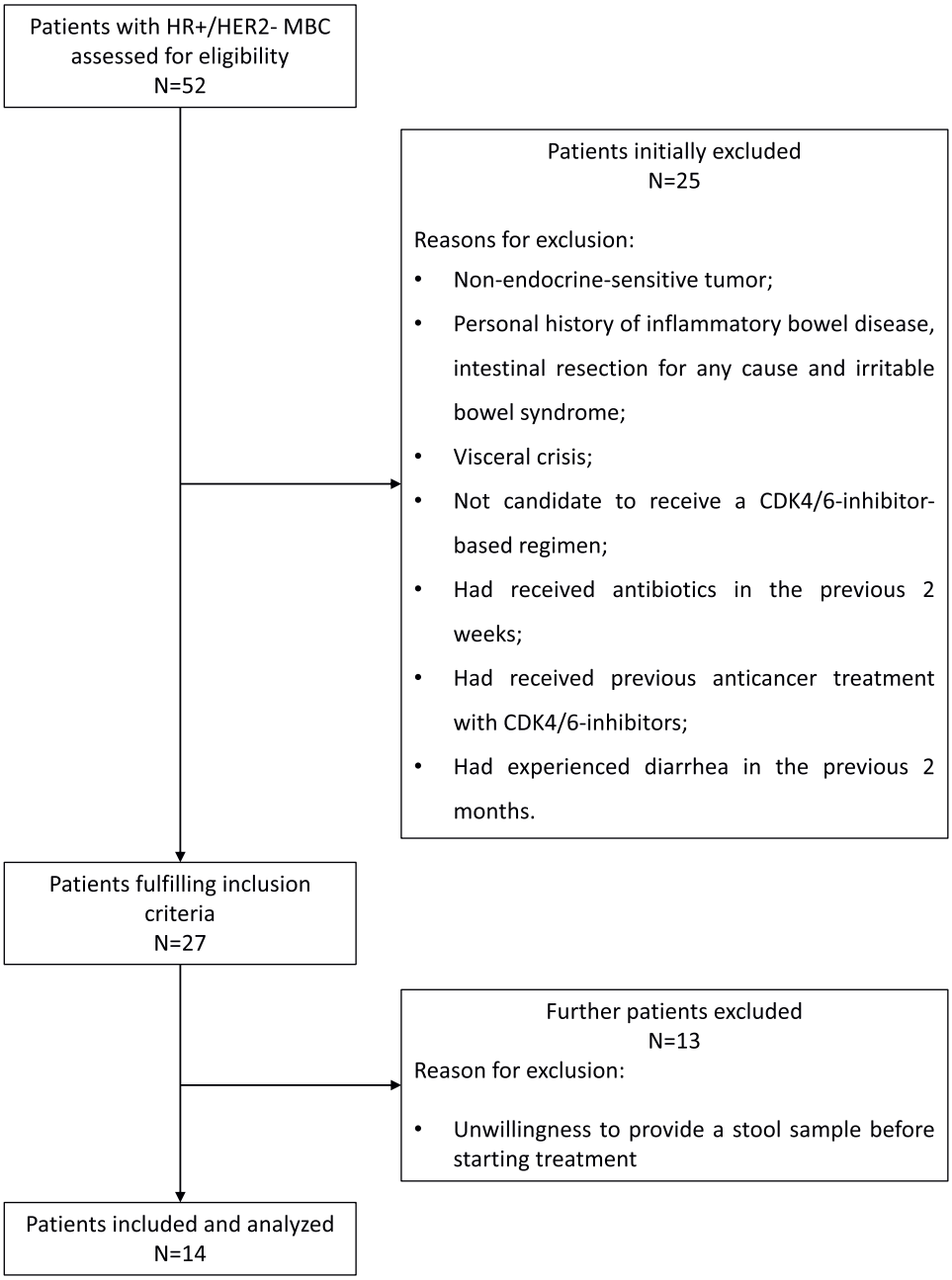

Fig. 1. STROBE flow chart for patients selection. CDK, cyclin-dependent kinase; HR+, hormone receptor positive; HER2−, human epidermal growth factor receptor 2-negative; MBC, metastatic breast cancer.

Regarding faecal microbiota composition, at phylum level was observed a higher relative abundance of *Actinobacteriota* in R patients, even if not significant (fold change = 1.8, $p = 0.125$) (Table 2). Employing 118 bacterial species, after a cut-off of prevalence ≥20%, we found no significant differences among NR and R in terms of α-diversity (richness $p = 0.337$, biodiversity $p = 0.655$, Fig. 2A) and β-diversity (PCoA, ANOSIM = 0.048, $p = 0.267$; PERMANOVA = 1.131, $p = 0.299$, Fig. 2B). PLS-DA and the derived VIP showed that four bacterial species were significant in dividing NR from R, namely *Bifidobacterium longum*, *Ruminococcus callidus*, *Clostridium innocuum* and *Schaalia odontolytica* (Fig. 2C). By using the relative abundances of these species in a random forest model, a good prediction was provided, even if limited by the sample number (area under curve [AUC] = 0.946, Specificity = 0.990, Sensitivity = 0.804) (Fig. 2D). *B. Longum* and *R. Callidus* were significantly more abundant ($p = 0.012$ and $p = 0.017$, respectively) in R, with the former being also significantly more prevalent ($p = 0.033$), while *C. Innocuum* and *S. Odontolytica* were present exclusively in NR (relative abundance $p = 0.025$ both) (Fig. 2E). Other differences in relative abundance and prevalence for all other species were not significant (all $p > 0.05$).

Table 1
Population clinicopathological characteristics and circulating immune cells levels.

Demographics	Responders		Non-responders		Overall patients		*p* Values*
	N	%	N	%	N	%	
	7	50.0	7	50.0	14	100.0	
Age							
Median	70	-	65	-	67.5	-	1.00
IQR	47.0–74.5	-	58.5–72.0	-	56.0–73.5	-	
BMI							
Median	21.2	-	23.8	-	23.5	-	*0.016*
IQR	20.0–29.4	-	23.1–28.4	-	20.9–29.3	-	
Oestrogen receptor							
Positive (> 1%)	7	100.0	7	100.0	14	100.0	-
Negative	0	0.0	0	0.0	0	0.0	
Progesterone receptor							
Positive (> 1%)	4	57.1	5	71.4	9	64.3	0.577
Negative	3	42.9	2	28.6	5	35.7	
Ki67%							
≤20	4	57.1	4	57.1	8	57.1	-
> 20%	3	42.9	3	42.9	6	42.9	
HER2							
0	5	71.4	7	100.0	12	85.7	0.311
1+	0	0.0	0	0.0	0	0.0	
2 + FISH neg.	2	28.6	0	0.0	2	14.3	
Menopausal Status							
Pre/Perimenopausal	2	28.6	1	14.3	3	21.4	0.515
Postmenopausal	5	71.4	6	85.7	11	78.6	
Treatment Line							
1st	6	85.7	4	57.1	10	71.4	0.237
2nd	1	14.3	3	42.9	4	28.6	
Endocrine therapy							
Aromatase Inhibitor	5	71.4	5	71.4	10	71.4	-
Fulvestrant	2	28.6	2	28.6	4	28.6	
CDK4/6-inhibitor							
Palbociclib	2	28.6	0	0	2	14.3	
Ribociclib	4	57.1	4	57.1	8	57.1	0.223
Abemaciclib	1	14.3	3	42.9	4	28.6	
ECOG							
0–1	7	100.0	7	100.0	14	100.0	-
≥2	0	0.0	0	0.0	0	0.0	
Visceral metastases							
Yes	2	28.6	5	71.4	7	50.0	0.109
No	5	71.4	2	28.6	7	50.0	
Number of metastases							
< 3	6	85.7	5	71.4	11	78.6	0.515
≥3	1	14.3	2	28.6	3	21.4	
Best response							
Complete response	0	0.0	0	0.0	0	0.0	0.213
Partial response	1	14.3	0	0.0	1	7.1	
Stable disease	6	85.7	5	71.4	11	78.6	
Progressive disease	0	0.0	2	28.6	2	14.3	
CD3+ CD4+ Lymphocytes (cells/UL)							
Median	633.0	-	722.7	-	677.8	-	0.710
IQR	559.3–784.1	-	601.1–877.6	-	592.0–803.4	-	
CD3+ CD8+ Lymphocytes (cells/UL)							
Median	463.0	-	361.0	-	442.5	-	0.456
IQR	402.0–647.0	-	253.0–633.5	-	295.8–688.8	-	
NK Lymphocytes (cells/UL)							
Median	381.0	-	258.0	-	296.0	-	0.805
IQR	157.0–470.0	-	194.0–355.5	-	183.2–429.0	-	
Tregs (cells/UL)							
Median	59.00	-	69.0	-	62.0	-	0.565
IQR	39.0–67.0	-	45.5–82.0	-	42.8–75.0	-	
CD4/CD8 Ratio							
Median	1.21	-	1.83	-	1.43	-	0.289
IQR	0.99–1.56	-	1.24–2.66	-	1.045–2.145	-	

(*continued on next page*)

Table 1 (*continued*)

Demographics	Responders		Non-responders		Overall patients		*p* Values*
	N	%	N	%	N	%	
	7	50.0	7	50.0	14	100.0	
NLR							
Median	2.23	-	2.57	-	2.39	-	*0.026*
IQR	1.52–2.27	-	2.53–3.01	-	1.87–2.57	-	
PLR							
Median	111.11	-	179.88	-	140.97	-	0.128
IQR	100.38–140.97	-	152.79–193.89	-	108.69–182.66	-	

BMI, body mass index; HER2, human epidermal growth factor receptor 2; IQR, interquartile range; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio.
Italic values are statistically significant.
* Exploratory, unadjusted.

With the aim of finding other bacterial species within the faecal microbiota of our MBC patients that would better describe the separation of NR and R, we employed a co-abundance network analysis of all the 118 species (Fig. 3A). Network analysis evidenced two major clusters of interacting bacterial species, named SIGs, in which a SIG1 group harboured 75% of NR-related species, while a SIG2 group harboured 76% of species with higher relative abundance in R (Fig. 3A). This topological distribution was highly significant (Chi-square $p < 0.001$), thus meaning that these two communities could have an opposite role in responsiveness to CDK4/6-inhibitors.

A cross-correlation analysis of all the 118 bacterial species with all of the 14 patients' immunological features and BMI was carried out. Two different clusters were formed by the correlation of the species with CD8 + , CD4 + , NK and Tregs lymphocytes, CD4/CD8 ratio, platelet-to-lymphocytes ratio (PLR), NLR and/or BMI (Fig. 3B). Among the four species evidenced by the VIP plot (Fig. 2C), only *C. Innocuum* showed a positive association with NLR ($r = 0.53$, $p = 0.049$) (Table 3), falling within the cluster1 (Fig. 3B). Interestingly, a bunch of species falling within the cluster1 were positively related to NLR, CD4/CD8 and PLR, and, at the same extent, negatively related to CD8 + , CD4 + and Tregs lymphocytes. When examining the species distribution among the correlogram and the network together, we found a correspondence among SIG1 and cluster1, and among SIG2 and cluster2, evidencing four species in common for SIG1/cluster1 and six species in common for SIG2/cluster2 (Fig. 3C).

4. Discussion

In a small population of 14 patients affected by HR +/HER2− MBC treated with CDK4/6-inhibitor + ET, we preliminarily observed some differential features of baseline faecal microbiome, especially in terms of phylum and species, which might be associated to poorer or better responses.

CDK4/6-inhibitors represent one of the major therapeutic advances in breast cancer of the decade. Also in our patients' cohort, although very small, women achieving the most prominent PFS results, presented a clear advantage in OS over women experiencing poorer responses. However, since a proportion of patients does not adequately respond to treatment, biomarkers for accurate patient selection are needed to maximise benefits and spare unnecessary toxicity and high treatment costs [20,21]. In the last couple of decades it has been extensively investigated the role of the immune system in cancer development and progression [22], as well as its complex interplay with chemotherapies and several targeted anticancer agents, with prognostic and predictive implications [15,23], leading also to the development of immune-checkpoint inhibitors (ICI) for the treatment of cancer [24]. To note, there is accumulating

Table 2
Relative abundances of Phyla.

Phylum	NR	R	*p* values*
	Mean ± SEM (%)	Mean ± SEM (%)	
Firmicutes	61.75 ± 4.95	55.80 ± 1.92	0.701
Bacteroidota	22.85 ± 3.60	26.61 ± 1.47	0.443
Actinobacteriota	4.14 ± 0.80	11.58 ± 3.35	0.125
Proteobacteria	5.97 ± 2.73	4.53 ± 2.17	1.000
Verrucomicrobiota	2.38 ± 1.69	1.07 ± 0.58	0.891
Desulfobacterota	0.37 ± 0.13	0.38 ± 0.10	1.000
Euryarchaeota	1.53 ± 1.02	0.02 ± 0.02	0.551

FDR, false discovery rate; NR, non-responders; R, responders; SEM, standard error mean.
* Two-sided Mann-Whitney *U* test, no FDR.

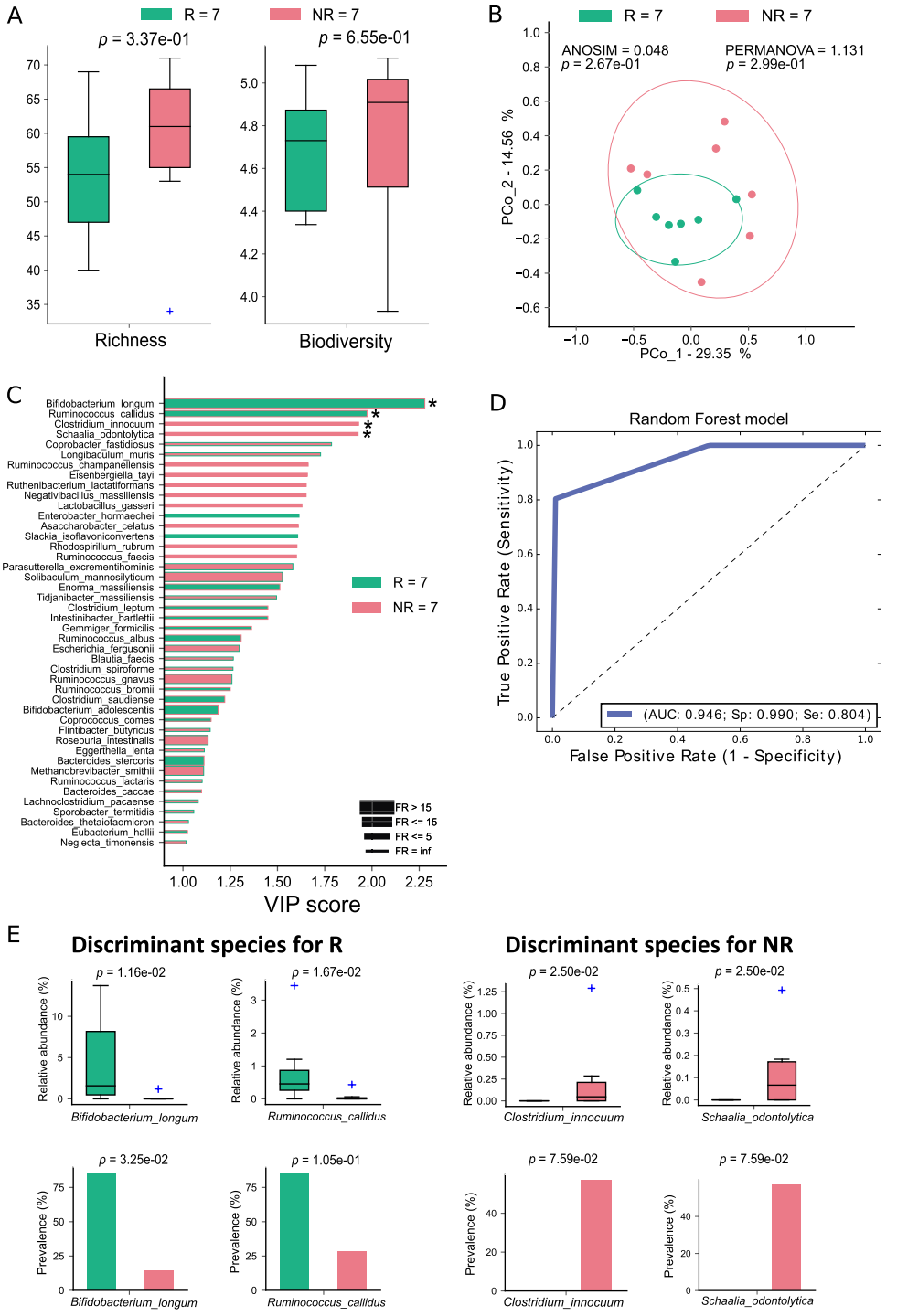

Fig. 2. Microbiota compositional shifts in responders and non-responders. Alpha- (A) and β-diversity (B) of responders (R, green, n = 7) and non-responders patients (NR, red, n = 7). (C) Variable Importance Plot (VIP). It shows: (i) discriminant species after Partial Least Square Discriminant Analysis (PLS-DA) in descending order of VIP score (bar length); (ii) the highest relative abundance depending on the cohort (central bar colour) and the lowest one (edge bar colour); (iii) fold ratio (FR) of the highest versus the lowest relative abundance (bar thickness), and (iv) significant difference after Mann–Whitney *U* test (non-false discovery rate [non-FDR], $*p \leq 0.05$). Absent borders indicate mean relative abundance of zero in the compared cohort. (D) Receiver Operating Characteristic (ROC) curve with fivefold cross-validation following a Random Forest model on the four species (retrieved by the VIP plot). Area Under Curve (AUC), sensitivity (Se) and specificity (Sp) are reported. (E) Pairwise analysis of the selected four species depicts significant differences in terms of relative abundance (box plots) and prevalence (bar plots). In each sub-graph are reported the *p*-value (from Mann–Whitney *U* test) among R and NR. Blue crosses are outliers.

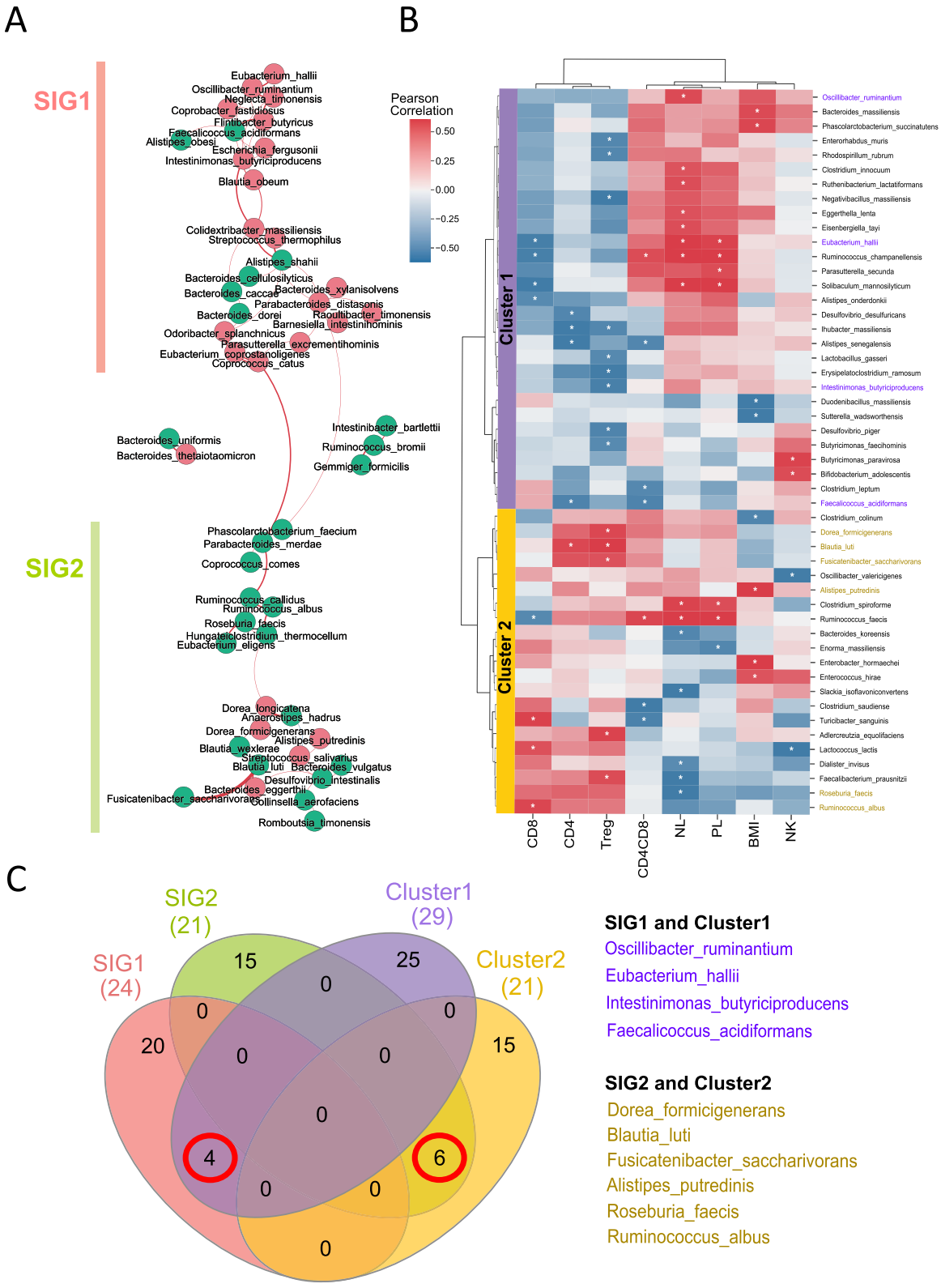

Fig. 3. Network analysis and correlation of bacterial species with immunological parameters. (A) Network analysis showing communities of bacterial species (namely, SIGs) and their positive (red Pearson coefficient) or negative (blue Pearson coefficient) relative abundances correlation. Nodes are coloured according to the cohort harbouring the higher relative abundance for a definite species, as NR (red) or R (green). Edge thickness is inversely proportional to the Pearson *p*-value after 10% Benjamini–Hochberg two-stages FDR, and it is coloured according to positive (red) or negative (blue) Pearson coefficient. (B) Correlogram of bacterial species and immunological

parameters shows positive (red) or negative (blue) Pearson correlation on CLR-transformed, normalised and standardised abundances. Significant correlation is marked with an asterisk inside each square: only species or parameters having at least one significant correlation were reported. Dendrograms on the x and y axes were generated following Bray–Curtis similarity, evidencing two different clusters for bacterial species (cluster1 and cluster2, shown here within white boxes). (C) Venn diagram showing species distribution among the two network SIGs and the two correlogram cluster, colours are as in panels (A) and (B). Highlighted in red the four and six species in common among SIGs and clusters. BMI, body mass index; CLR, centred-log-ratio; FDR, false discovery rate; NR, non-responders; NL, neutrophil-to-lymphocyte ratio; PL, platelet-to-lymphocyte ratio; R, responders; SIG, species-interacting group.

evidence suggesting that gut microbiota, especially bacteria, can trigger both innate and adaptive immune responses by eliciting the expression and secretion of immunomodulatory cytokines and chemokines that enter the systemic circulation and affect cancer pathogenesis, with the creation of tumour-promoting or tumour-antagonising immune microenvironments, as well as response to anticancer therapies [22,25,26]. In this perspective, the NLR indicates the dynamic relationship between innate (via neutrophil count) and adaptive (via lymphocyte count) immune responses and has been extensively investigated for the purpose of evaluating ongoing systemic inflammation during cancer development, severity stratification and prognosis of cancer disease in multiple cancer types, including breast cancer [27]. We observed in our exploratory analysis that patients experiencing more prolonged responses to CDK4/6-inhibitors-based regimens showed lower basal levels of NLR compared to poor CDK4/6-inhibitor responders. In addition, lower levels of NLR, as also observed elsewhere [27], showed an association with a better prognosis. Our results showed that some bacterial species, such as *C. Innocuum*, *Oscillibacter ruminantium* and *Eubacterium hallii*, seem to be positively related to NLR, thus probably exerting a negative effect on response to CDK4/6 inhibition. In fact, *C. Innocuum* showed higher relative abundance and prevalence in NR. On the contrary, *Roseburia fecis*, being negatively related to NLR, could have a favourable prognostic impact. These findings add to the previously mentioned postulated relationship between gut microbiota and systemic immunity [22,25,26].

Several members of the *Actinobacteria* phylum, such as *Bifidobacteria*, can be administered via probiotics [28]. Importantly, this phylum has shown to increase the efficacy of anti-PD-L1 ICI in breast and other tumours mouse models [29,30]. Intriguingly, an immunomodulatory effect for CDK4/6-inhibitors has been recently theorised after showing to promote tumour infiltration and activation of effector T cells and cytotoxic T cell-mediated clearance of tumour cells in preclinical studies [31,32]. In fact, a first phase I/II trial of palbociclib + letrozole and the ICI pembrolizumab already proved the combination to be well tolerated and active in the first-line setting of patients with HR+ MBC [33]. Here we found that *B. Longum* was more abundant in R, compared to NR. If *Actinobacteria* such as *Bifidobacteria* were effectively able to both improve response to CDK4/6-inhibitors and anti-PD-L1 agents, they could be easily provided to patients via probiotics as a strategy to boost therapeutic efficacy of regimens including both or only one of the two drug classes, with the advantage of being safe and likely cost-effective.

A higher abundance of *R. Callidus* was also observed in R, as well. Although there is no specific study associating this species with breast cancer, it has been reported to be negatively associated with colorectal cancer [34].

Finally, a clear and statistically significant differential distribution of faecal bacterial species in SIGs according to response to CDK4/6-inhibitors was observed in the network analysis. Noteworthy, species associated with better responses seemed to interact more than species associated with worse responses.

Although numbers are too small to provide in-depth reliable conclusions, the tendencies observed should be further explored. Due to obvious technical limitations related to the study of gut microbiota, faeces could be considered as a potential proxy for its assessment, although reliable methodologies that adequately correlate the activity of faecal microbiota (composed of species that mostly reside in the transient luminal compartment) with the mucosa-associated microbiota (MAM) are currently missing [35].

Our study has several limitations worth nothing. First, results significance has been surely hampered by the reduced population. Nevertheless, larger studies are costly and require more education and sensibilization of both patients and operators regarding optimal collection and management of faecal samples. Furthermore, there is no current evidence regarding faecal microbiota role as a predictive factor of response to CDK4/6-inhibitors, and this study was intended to be a preliminary effort to provide translational hypothesis-generating data. Secondly, the lack of a control group that only received ET was not available, considering that, apart from specific cases, the standard of care is now represented by the combination of ET with a CDK4/6-inhibitor as upfront therapy [17]. At the same time, a recent small study in early-stage disease showed a significant difference in the mean microbial abundance between endocrine-resistant and endocrine-sensitive cases in terms of the taxonomic rank of order and family [36]. Thirdly, we had no validation cohort to make more robust the putative biomarkers retrieved by machine learning algorithms. Finally, patients could have

Table 3
Pearson correlation coefficients and respective *p*-values.

Faecal bacterial species	Pearson coefficients								*p* values							
	BMI	NLR	PLR	CD8+	CD4+	CD4+/CD8+	NK	Treg	BMI	NLR	PLR	CD8+	CD4+	CD4+/CD8+	NK	Treg
Adlercreutzia equolifaciens	0.12	−0.03−	0.20	0.21	0.28	−0.26	0.08	**0.59**	0.675	0.925	0.484	0.480	0.333	0.371	0.788	**0.027**
Alistipes onderdonkii	−0.24	0.35	0.48	**−0.54**	−0.47	0.18	0.17	−0.37	0.401	0.219	0.083	**0.048**	0.093	0.549	0.567	0.188
Alistipes putredinis	**0.72**	0.28	−0.02	−0.05	0.35	0.33	0.17	0.02	**0.004**	0.333	0.948	0.862	0.221	0.255	0.552	0.947
Alistipes senegalensis	0.10	0.16	0.11	−0.06	**−0.61**	**−0.56**	0.16	−0.40	0.745	0.588	0.715	0.849	**0.020**	**0.039**	0.575	0.156
Bacteroides koreensis	−0.11	**−0.56**	−0.43	0.14	0.10	0.00	0.05	−0.25	0.711	**0.038**	0.129	0.621	0.721	1.000	0.852	0.394
Bacteroides massiliensis	**0.56**	0.28	0.28	−0.44	−0.07	0.42	0.41	−0.38	**0.035**	0.330	0.325	0.119	0.818	0.136	0.151	0.179
Bifidobacterium adolescentis	−0.31	−0.07	0.01	−0.10	−0.43	−0.34	**0.56**	−0.10	0.289	0.822	0.976	0.725	0.121	0.232	**0.038**	0.733
Blautia luti	−0.32	−0.07	0.15	0.05	**0.57**	0.27	−0.11	**0.61**	0.260	0.815	0.600	0.860	**0.033**	0.349	0.704	**0.020**
Butyricimonas faecihominis	0.10	−0.01	−0.26	−0.14	−0.10	0.12	0.44	**−0.54**	0.731	0.978	0.373	0.630	0.745	0.690	0.119	**0.047**
Butyricimonas paravirosa	−0.03	−0.09	−0.32	−0.12	−0.10	0.10	**0.59**	−0.37	0.929	0.751	0.262	0.680	0.732	0.728	**0.028**	0.194
Clostridium colinum	**−0.56**	0.20	0.25	−0.39	0.20	0.38	0.22	0.21	**0.037**	0.497	0.389	0.172	0.486	0.182	0.453	0.461
Clostridium innocuum	0.14	**0.53**	0.49	−0.34	−0.08	0.28	−0.08	−0.21	0.645	**0.049**	0.076	0.232	0.797	0.335	0.783	0.477
Clostridium leptum	0.05	−0.17	−0.41	0.24	−0.43	**−0.54**	0.25	−0.04	0.862	0.570	0.143	0.418	0.128	**0.044**	0.387	0.894
Clostridium saudiense	0.30	−0.22	−0.08	0.44	−0.12	**−0.63**	−0.22	0.27	0.302	0.456	0.791	0.118	0.676	**0.015**	0.454	0.349
Clostridium spiroforme	0.12	**0.59**	**0.55**	−0.19	0.15	0.09	−0.36	0.21	0.693	**0.027**	**0.041**	0.520	0.615	0.757	0.208	0.475
Desulfovibrio desulfuricans	0.05	0.34	0.36	−0.37	**−0.56**	−0.22	0.14	−0.16	0.861	0.228	0.204	0.192	**0.038**	0.456	0.631	0.573
Desulfovibrio piger	−0.06	−0.22	−0.01	−0.08	−0.16	0.07	0.26	**−0.56**	0.851	0.451	0.984	0.776	0.573	0.811	0.376	**0.036**
Dialister invisus	−0.19	**−0.53**	−0.15	0.42	0.08	−0.09	−0.45	0.13	0.507	**0.050**	0.599	0.137	0.786	0.769	0.108	0.647
Dorea formicigenerans	−0.23	0.26	0.30	−0.16	0.47	0.42	−0.11	**0.57**	0.438	0.373	0.291	0.587	0.092	0.136	0.703	**0.032**
Duodenibacillus massiliensis	**−0.72**	−0.43	−0.10	0.16	−0.09	−0.13	−0.19	−0.13	**0.004**	0.127	0.737	0.583	0.768	0.655	0.512	0.659
Eggerthella lenta	0.44	**0.54**	0.50	−0.44	−0.11	0.39	−0.02	−0.47	0.113	**0.046**	0.068	0.118	0.701	0.165	0.933	0.091
Eisenbergiella tayi	0.20	**0.56**	0.46	−0.39	−0.13	0.32	−0.01	−0.51	0.500	**0.039**	0.096	0.166	0.667	0.269	0.975	0.065
Enorma massiliensis	−0.10	−0.49	**−0.55**	0.38	0.15	−0.04	0.10	0.14	0.734	0.073	**0.041**	0.185	0.619	0.886	0.746	0.632
Enterobacter hormaechei	**0.61**	−0.11	−0.12	0.27	0.07	0.00	0.03	0.04	**0.021**	0.708	0.685	0.344	0.807	0.999	0.913	0.899
Enterococcus hirae	**0.53**	−0.17	−0.38	−0.01	0.12	0.09	0.48	−0.03	**0.050**	0.554	0.184	0.969	0.686	0.759	0.079	0.911
Enterorhabdus muris	0.09	0.17	0.40	−0.37	−0.08	0.39	−0.08	**−0.55**	0.764	0.551	0.158	0.189	0.784	0.168	0.792	**0.041**
Erysipelatoclostridium ramosum	0.14	0.20	0.25	−0.18	−0.53	−0.25	−0.03	**−0.61**	0.621	0.482	0.387	0.546	0.053	0.381	0.913	**0.021**
Eubacterium hallii	0.06	**0.89**	**0.58**	**−0.60**	−0.22	0.44	0.09	−0.29	0.833	**0.000**	**0.028**	**0.024**	0.454	0.114	0.772	0.319
Faecalibacterium prausnitzii	−0.28	**−0.62**	−0.27	0.32	0.36	−0.05	−0.15	0.56	0.331	**0.018**	0.353	0.266	0.207	0.853	0.620	**0.038**
Faecalicoccus acidiformans	0.06	−0.06	−0.32	0.24	**−0.57**	**−0.57**	0.14	−0.38	0.831	0.834	0.260	0.412	**0.034**	**0.034**	0.637	0.186
Fusicatenibacter saccharivorans	−0.35	−0.08	0.15	−0.03	**0.53**	0.34	−0.09	**0.54**	0.221	0.774	0.601	0.932	**0.050**	0.236	0.766	**0.047**
Ihubacter massiliensis	0.11	0.43	0.43	−0.37	**−0.67**	−0.23	0.05	**−0.55**	0.704	0.121	0.125	0.191	**0.009**	0.434	0.864	**0.042**
Intestinimonas butyriciproducens	0.18	0.35	0.08	−0.18	−0.41	0.02	0.09	**−0.59**	0.529	0.226	0.791	0.537	0.147	0.951	0.750	**0.027**
Lactobacillus gasseri	0.08	0.22	0.11	−0.03	−0.18	−0.05	−0.03	**−0.54**	0.790	0.440	0.713	0.925	0.529	0.876	0.930	**0.045**
Lactococcus lactis	−0.23	−0.06	−0.13	**0.54**	0.34	−0.11	**−0.62**	0.42	0.425	0.847	0.665	**0.044**	0.231	0.704	**0.018**	0.139
Negativibacillus massiliensis	0.11	0.51	0.45	−0.43	−0.19	0.38	−0.06	**−0.62**	0.697	0.060	0.111	0.123	0.508	0.185	0.832	**0.017**
Oscillibacter ruminantium	0.51	**0.56**	0.24	−0.32	−0.35	0.16	0.22	−0.35	0.061	**0.036**	0.402	0.262	0.213	0.579	0.460	0.220
Oscillibacter valericigenes	0.11	0.29	0.16	0.26	0.03	0.15	**−0.61**	0.08	0.716	0.322	0.584	0.373	0.906	0.618	**0.021**	0.776
Parasutterella secunda	0.10	0.50	**0.56**	−0.53	−0.02	0.53	−0.06	−0.13	0.730	0.067	**0.038**	0.051	0.944	0.051	0.832	0.655
Phascolarctobacterium succinatutens	**0.58**	0.28	0.33	−0.44	0.03	0.45	0.37	−0.08	**0.031**	0.334	0.245	0.119	0.924	0.105	0.197	0.786
Rhodospirillum rubrum	0.19	0.42	0.13	−0.40	−0.05	0.40	0.06	**−0.55**	0.525	0.139	0.655	0.158	0.852	0.154	0.836	**0.041**
Roseburia faecis	−0.45	**−0.62**	−0.40	0.44	0.48	−0.02	−0.40	0.47	0.108	**0.018**	0.158	0.113	0.084	0.935	0.158	0.092
Ruminococcus albus	−0.04	−0.50	−0.44	**0.58**	0.41	−0.04	−0.34	0.40	0.890	0.066	0.120	**0.031**	0.146	0.901	0.229	0.154

(*continued on next page*)

Table 3 (*continued*)

Faecal bacterial species	Pearson coefficients							*p* values						
	BMI	NLR	PLR	CD8+	CD4+	CD4+/CD8+	NK	Treg	BMI	NLR	PLR	CD8+	CD4+	CD4+/CD8+	NK	Treg
Ruminococcus champanellensis	0.10	**0.66**	**0.61**	**−0.59**	−0.13	**0.56**	−0.07	−0.43	0.743	**0.010**	**0.021**	**0.025**	0.648	**0.038**	0.812	0.122
Ruminococcus faecis	0.09	**0.67**	**0.61**	**−0.54**	0.36	**0.58**	−0.01	0.37	0.750	**0.008**	**0.021**	**0.047**	0.202	**0.030**	0.986	0.191
Ruthenibacterium lactatiformans	0.16	**0.56**	0.45	−0.34	−0.05	0.28	0.01	−0.24	0.586	**0.039**	0.105	0.233	0.856	0.336	0.986	0.401
Slackia isoflavoniconvertens	0.17	**−0.63**	−0.13	0.07	0.11	0.05	0.29	0.00	0.559	**0.016**	0.646	0.817	0.705	0.855	0.317	0.995
Solibaculum mannosilyticum	0.15	**0.70**	**0.74**	**−0.68**	−0.05	0.40	−0.05	−0.05	0.602	**0.005**	**0.002**	**0.008**	0.874	0.155	0.852	0.877
Sutterella wadsworthensis	**−0.63**	−0.07	0.03	−0.02	−0.13	−0.03	−0.15	−0.06	**0.017**	0.821	0.917	0.940	0.646	0.932	0.608	0.851
Turicibacter sanguinis	0.12	0.04	−0.10	**0.59**	−0.29	**−0.58**	−0.48	0.07	0.694	0.903	0.740	**0.026**	0.315	**0.030**	0.083	0.805

BMI, body mass index; NLR, neutrophil-to-lymphocytes ratio; PLR, platelet-to-lymphocytes ratio.
In bold, significant coefficients and respective *p*-values.

received any CDK4/6-inhibitor, thus it is hard to know if different molecules might have exerted different immunomodulatory effects and differential indirect interactions with gut microbiome. In any case, evidences in this perspective lacks and most patients in both cohorts had received ribociclib (57.1% in both cohorts).

In conclusion, although small and exploratory, and requiring validation in different cohorts, our study suggests that stool microbiota might be able to early-predict responses to CDK4/6-inhibitors. Moreover, targeted antibiotic-based depletions of specific bacteria and oral administration of probiotics like *Bifidobacteria* might improve response to CDK4/6 inhibition, in a relatively safe and cost-effective fashion. Faecal microbiota transplantation, a strategy effectively adopted in other diseases [37–39], might be also explored for the purpose.

Ethical declaration

The study was approved by the Ethic Committee of Val Padana (IRB n. 440/11/10/2019) and was conducted following the ethic principles of the Declaration of Helsinki, in compliance with all international, national and local regulatory laws and consistently with Good Clinical Practices guidelines. All patients signed a written informed consent before entering the study.

Funding

This research was supported by Mednote, spin-off–University of Trieste–Mozart Programme.

Author contributions

DG conceived the study. FS and AF performed the statistical analysis. VI performed bioinformatic analysis. FS and DG interpreted study results. FS, AF, VI and DG wrote the first manuscript draft. All authors except for FS, AF and VI participated in patients' recruitment and management. All authors participated in revising and writing the final manuscript. All authors approved the final manuscript version.

Data availability statement

All raw data (fastq.gz files) and clinical metadata, complying with FAIR principles (https://www.go-fair.org/fair-principles/), are available at NCBI SRA portal under PRJNA946762 Bioproject.

Declaration of Competing Interest

FS declares travel expenses by Novartis and Gilead and personal fees for educational events by Novartis, Daiichy-Sankyo and Gilead. DG declares personal fees for educational events by Novartis, Lilly, Pfizer,

Daiichy-Sankyo, Roche; research funds from Astrazeneca, Novartis and LILT. GC declares reports advisory/consulting fees from Seagen, Roche, Novartis, Eli Lilly, Daiichi Sankyo, AstraZeneca, Pfizer, Sanofi, Pierre Fabre and Gilead, and fees for non-CME services (e.g. speakers' bureaus) from Eli Lilly, Pfizer Inc and Daiichi Sankyo. The other authors have nothing to declare.

Acknowledgements

The authors want to gratefully thank all patients and their respective families involved in the present study. FS is supported by a Rio Hortega contract from the Instituto de Salud Carlos III (ISCIII) and an ESMO Fellowship–Translational. However, findings and hypothesis generated are solely of the article's authors.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejca.2023.112948.

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