Final Study Report

Full title of the trial:	Neoadjuvant Biomarker ResearcH Study of Palbociclib combined with Endocrine Therapy in Estrogen Receptor Positive/HER2 Negative Breast CAncer
Short title of the trial:	NeoRHEA
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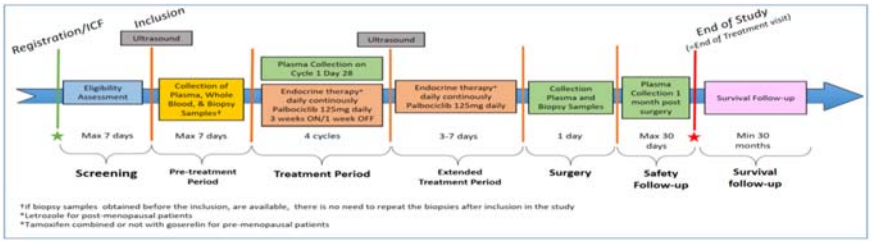
TRIAL INFORMATION

PHASE	Phase II
STUDY DESIGN	This was an open-label, single arm, Phase 2 study that included female subjects with ER-positive, HER2-negative early breast cancer who were candidates for neoadjuvant therapy. Subject received 4 cycles of palbociclib 125 mg (each cycle of palbociclib consists of treatment from D1 to D21 followed by a week of rest) combined with endocrine therapy given continuously (each cycle of endocrine therapy consists of treatment from D1 to D28). Endocrine therapy consisted of: - Post-menopausal subjects: All subjects received letrozole 2.5 mg continuously during palbociclib treatment (which consists of 4 cycles of 28 days), with an added 3 to 7 days as necessary, orally once a day every day until the day before curative intent surgery. There were no rest periods for letrozole (unless if toxicity occurs). - Pre-menopausal subjects: tamoxifen 20 mg continuously during palbociclib treatment (which consists of 4 cycles of 28 days), with an added 3 to 7 days as necessary, orally once a day every day until the day before curative intent surgery. They may also receive goserelin, at local investigator's discretion, 3.6 mg monthly during the same period. There were no rest periods for this regimen (unless if toxicity occurs). Subject's response to therapy were evaluated before and after the 4 cycles of treatment by ultrasound to determine response to therapy. Pre-treatment ultrasound had to be performed up to 7 days before the start of treatment. Post-treatment ultrasound had to be performed within a maximum of 14 days before surgery. If case local guidelines mandate MRI follow-up for neoadjuvant subjects, these could be performed at the same time points in addition (and not instead off) ultrasound. Surgery was performed 4 to 8 days after the end of the 4th cycle of study treatment. Between the end of the 4th cycle and surgery, subjects continued to receive palbociclib and endocrine therapy, at the same doses, with the last dose of endocrine therapy and palbociclib administered the day before surgery with a maximum of 7 additional treatment days. In case of delays due to toxicity, the surgery had to be performed no later than 4 months + 8 days from beginning of study treatment. Biopsy samples had to be collected after inclusion and prior to Day 1 Cycle 1 of study treatment and consisted of collection of 4 core biopsies (2 FFPE and 2 Frozen). However, if 4 biopsies were already available for submission (2 FFPE and 2 Frozen), which were obtained outside the study due to the Site's standard clinical practice, there was no need to repeat these biopsies after the subject's inclusion in the study. These biopsies had to be obtained up to 6 weeks before the beginning of treatment. FFPE and frozen material were collected from surgical material after sufficient and relevant part had been retained for the diagnosis or treatment of the subject. One whole blood sample (1x10 mL) were collected after inclusion and prior to Day 1 Cycle 1 of study treatment. Blood samples for plasma processing (4x10 mL per time point) were collected after inclusion and prior to Day 1 Cycle 1, prior to Day 1 Cycle 2 of study treatment, at the time of surgery and one month after surgery. All the biological samples collected (pre-treatment biopsy, material from surgery and all blood samples) within the study were mandatory. Pre-inclusion imaging assessment (ultrasound of the breast) was not admissible for assessment of study end-points.

	Information on subject survival and disease recurrence were collected at least 30 months from the date of the end of study treatment (i.e., last visit of the last subject one month after surgery day). Data collection were made based on medical charts if a follow up visit was performed not earlier than February 2022. If the data was missing from medical charts (i.e. loss of follow up), a phone call to the subject's general practitioner was made. 
STUDY RATIONALE	Palbociclib is a CDK4 and CDK6 inhibitor that received accelerated approval by the FDA to be used in combination with endocrine therapy in metastatic ER+/HER2- breast cancer based on the results of the PALOMA 1 trial. In this phase 2 trial, palbociclib significantly improved median progression-free survival (PFS) when added to letrozole in previously untreated women with estrogen receptor positive, HER2-negative breast cancer. The PALOMA-2 results, recently presented at ASCO confirm this PFs benefit. OS data is eagerly anticipated. Moreover, in the PALOMA 3 trial, palbociclib improved PFS when added to fulvestrant in subjects with hormone receptor positive metastatic breast cancer who progressed on previous endocrine treatment. In both trials toxicity was significantly more frequent in the palbociclib arm, with neutropenia being of special concern, even if few subjects had infections. The role of palbociclib in different settings of breast cancer treatment is being explored in several trials, such as the PALLAS trial (NCT02513394) and the PENELOPE-B trial (NCT01864746), and it is likely that its use will be expanded in the coming years. Biomarkers that help us predict response to palbociclib may lead to better subject selection, and thus avoid unnecessary toxicity and reduce costs. In the present study, we will use RNA-sequencing of the baseline tumour biopsy to identify biomarkers of resistance to a 4 month preoperative treatment of palbociclib and endocrine therapy given prior to surgery. Resistance will be defined as lack of response (stable disease) at ultrasound after the 4-month treatment period, as well as progression anytime during the 4-month treatment period by ultrasound (response evaluation will be defined according to WHO criteria). Resistance will be also defined as residual cancer burden (RCB) of 3 that has been associated with poor outcome after neoadjuvant treatment or high tumour proliferation as evaluated by Genomic Grade Index (GGI) from RNA-seq data at surgery. Recently, it has been demonstrated in breast cancer cell lines that the CDK4 T172 phosphorylation is associated with sensitivity to palbociclib and a signature of an 11-gene expression has been developed that can faithfully predict the CDK4 modification profile in breast tumours and breast cancer cell lines.[1] This 11-gene expression signature has been associated with sensitivity to CDK 4/6 inhibitors e.g. palbociclib in breast cancer cell lines (Patent: Method For determining sensitivity to a CDK4/6 inhibitor, filed the 18.05.16, European Patent Application No/patent No EP16170146.1-1403, Applicant: Université Libre de Bruxelles, Belgium.)
OBJECTIVES	Primary objective: • To identify biomarkers of resistance to a 4-month preoperative treatment of palbociclib plus endocrine therapy defined as stable or

	progressive disease by ultrasound (based on WHO criteria) using RNA-seq of the baseline tumour biopsy. Secondary objectives: • To identify biomarkers of resistance to a 4-month preoperative treatment of endocrine therapy and palbociclib by correlating tumour response by ultrasound (mandatory) or magnetic resonance (optional) imaging (response will be assessed as continuous or categorical variable) with RNA-seq and whole genome sequencing (WGS) of the baseline tumour biopsy • To identify biomarkers of resistance to a 4-month preoperative treatment of palbociclib plus endocrine therapy defined as residual disease burden, RCB of 3 using RNA-seq and WGS of the baseline tumour biopsy • To identify biomarkers of resistance to a 4-month preoperative treatment of endocrine therapy and palbociclib defined as GGI high by RNA-seq of the left over tumour tissue at surgery using RNA-seq and WGS of the baseline tumour biopsy • To identify genomic and transcriptomic biomarkers of resistance to a 4-month preoperative treatment of palbociclib plus endocrine therapy defined as absence of complete cell cycle arrest (CCA, centrally reviewed Ki67 ≤2.7%) at surgery. • To understand mechanisms of resistance to the combination of endocrine therapy and palbociclib by comparing the genome and transcriptome of tumours at baseline and at surgery using WGS and RNA-seq • To evaluate the safety of the combination of palbociclib plus endocrine therapy • To evaluate the role of plasma ctDNA in monitoring response/resistance to pre-operative treatment with endocrine therapy and palbociclib • To validate/further refine an 11-gene expression signature associated with response/resistance to palbociclib and endocrine treatment • To determine the effect of a pre-operative treatment with endocrine therapy and palbociclib on anti-tumour immune response • To determine the effect of a pre-operative treatment with endocrine therapy and palbociclib on tumour senescence. • To determine the effect of a pre-operative treatment with endocrine therapy and palbociclib on tumour proliferation, as measured by the Ki67 score. Exploratory objectives: • To compare changes in clonal composition, transcriptomic changes and changes in the open chromatin state of tumour cells using a combined genomic and transcriptomic (G&T) single tumour cell analysis and chromatin accessibility single tumour cell analysis of exceptional responders and exceptional non-responders. Based on the above results, additional bulk or single cell analyses in the entire study cohort could be performed. • To evaluate associations between l survival (distant relapse-free survival, relapse-free survival, invasive disease-free survival, overall survival) and the following: - Tumor clinicopathological characteristics and other biomarkers at baseline and/or surgery and/or their changes - Plasma ctDNA monitoring.
ENDPOINTS	Primary endpoint:

	• Baseline transcriptomic profile of resistance to 4 months of palbociclib and endocrine therapy defined as stable or progressive disease by ultrasound based on WHO criteria Secondary endpoints: • Baseline genomic and transcriptomic profile of resistance to 4 months of palbociclib and endocrine therapy assessed by ultrasound (mandatory) or magnetic resonance (optional) imaging (response will be assessed as continuous or categorical variable) • Baseline genomic and transcriptomic profile of resistance to 4 months of palbociclib and endocrine therapy, defined as an RCB of 3. • Baseline genomic and transcriptomic profile of resistance to 4 months of palbociclib and endocrine therapy, defined as an GGI high at surgery • Baseline genomic and transcriptomic profile of resistance to 4 months of palbociclib and endocrine therapy, defined as absence of CCA at surgery • Genomic and transcriptomic changes between pre-treatment and post-treatment tumour samples • Safety • Plasma ctDNA analysis to monitor response/resistance to pre-operative treatment with endocrine therapy and palbociclib • Validation of 11-gene expression signature associated with response/resistance to palbociclib and endocrine treatment • Changes in anti-tumour immune response between pre- and post-treatment tumour samples • Changes in tumour senescence between pre- and post-treatment tumour samples • Changes in Ki67 scores between pre- and post-treatment tumour samples. Exploratory endpoints: • Changes in clonal composition, transcriptomic changes and changes in the open chromatin state of tumour cells using a combined genomic and transcriptomic (G&T) single tumour cell analysis and chromatin accessibility single tumour cell analysis of exceptional responders and exceptional non-responders. Depending on the results of these analyses, additional bulk or single cell analyses in the entire study cohort could be performed • Distant relapse-free survival (DRFS) defined as the time between breast surgery and the date of diagnosis of distant recurrence or death from any cause • Relapse-free survival (RFS) defined as the time between breast surgery and the date of diagnosis of distant or locoregional invasive recurrence or death from any cause • Invasive disease-free survival (iDFS) defined as the time between breast surgery and the date of diagnosis of local or distant invasive recurrence, secondary malignancy or death due to any causes • Overall survival (OS) is defined as the time between surgery and the day of death (due to any causes) or day of last follow-up
INCLUSION CRITERIA	Subjects will only be eligible for study participation if they meet all the following inclusion criteria: 1. Female 2. Age ≥ 18 years 3. Histological diagnosis of breast adenocarcinoma that is estrogen receptor-positive, and HER2- negative as per the updated American Society of Clinical

	Oncology (ASCO) - College of American Pathologists (CAP) guidelines according to local testing. 4. Multifocal unilateral or bilateral breast adenocarcinoma tumours are allowed provided that all tested foci are ER-positive and HER2-negative. - ER-positive (ER+ is defined as having an IHC of 1% or more and/or an Allred of 3 or more and HER2-negative). - HER2 negative (HER2 negative is defined as having an IHC of 0 or 1+ without ISH OR IHC 2+ and ISH non-amplified with ratio less than 2.0 and if reported, average HER2 copy number < 4 signals/cells OR ISH non-amplified with ratio less than 2.0 and if reported, average HER2 copy number < 4 signals/cells [without IHC]); o Note: a IHC of 3+ is always considered HER2 positive, independently of the ISH result. o Note: in discrepant ISH cases (e.g. ratio >2.0 and average HER2 copy number < 4 signals/cells), ASCO/CAP guidelines should be followed and tumours may still be considered HER2-negative, if concordant with the ASCO/CAP guidelines. 5. A primary non metastatic or locally advanced tumour of ≥15 mm or more, determined by ultrasound, N0 or N1 without prior treatment candidate for preoperative treatment 6. ECOG Performance Status (PS) 0 or 1. 7. Adequate Bone Marrow Function including: a. Absolute Neutrophil Count (ANC) ≥1500/μL or ≥1.5 x10^9/L; b. Platelets ≥100000/μL or ≥100 x 10^9/L; c. Hemoglobin ≥ 9 g/dL. 8. Adequate Renal Function including: Serum creatinine ≤ 1.5 x upper limit of normal (ULN) or estimated creatinine clearance ≥ 60 ml/min as calculated using the method standard for the institution. 9. Adequate Liver Function, including all of the following parameters: a. Total serum bilirubin ≤ 1.0 x ULN unless the subject has documented Gilbert syndrome (in which case up to 3 x ULN is acceptable) ; b. Aspartate and Alanine Aminotransferase (AST and ALT) ≤ 1.5 x ULN; c. Alkaline phosphatase ≤ 2.5 x ULN. 10. Signed consent form 11. Willingness and ability to comply with the study scheduled visits, treatment plans, laboratory tests, radiological exams, tumour and blood specimen collection and other procedures. 12. Women who are not postmenopausal or have not undergone hysterectomy must have documented negative pregnancy test (serum) prior to inclusion. 13. Female subjects of child bearing potential and their partners, who are sexually active, must agree to the use of one highly effective form of contraception throughout the period of taking study treatment and for at least 90 days after last dose of study drug, or they must totally/truly abstain from any form of sexual intercourse. Use of oral hormonal contraceptive agents in this study is not permitted
EXCLUSION CRITERIA	Participants who exhibit any of the following conditions at screening will not be eligible for admission into the study. 1. Clinical T4 disease including inflammatory breast cancer. 2. Prior history of invasive cancer including breast cancer except basal or squamous cell carcinoma of skin that has been definitively treated. 3. Known hypersensitivity to the study drugs or excipients. 4. Any illness or medical condition that is unstable or could jeopardize the safety of the subject or her compliance with study requirements. 5. Subjects unable to swallow oral medications. 6. Prior intake of letrozole, tamoxifen, or any CDK inhibitor or anti-cancer therapy.

	7. Concurrent treatment with any of the drugs not permitted, i.e. strong CYP3A inhibitors/inducers and drugs known to cause QTc interval prolongation (see section 6.7 for specific instructions). 8. QTc exceeding 480 msec, family or personal history of long or short QT syndrome, Brugada syndrome or known history of QTc prolongation, or Torsade de Pointes (TdP). 9. Uncontrolled diabetes, according to investigator's clinical judgment. 10. Pregnant or lactating women
INVESTIGATIONAL MEDICINAL PRODUCTS	All subjects will receive palbociclib 125 mg for 4 cycles, orally, once a day, for 21 days followed by 7 days of rest (4 cycles of 28 day long). Each cycle begins (D1) at the first day of medication use, and finishes at the last day of medication rest (D28). After the final rest week (therefore post cycle 4), subjects will receive 3 to 7 additional days of palbociclib, as necessary, at the same dose and posology, until the day before curative intent surgery. - Post-menopausal subjects will receive letrozole 2.5 mg continuously during palbociclib treatment (which consists of 4 cycles of 28 days), with an added 3 to 7 days as necessary, orally once a day until the day before curative intent surgery. There are no rest periods for letrozole (unless if toxicity occurs). - Pre-menopausal subjects will receive tamoxifen 20 mg continuously during palbociclib treatment (which consists of 4 cycles of 28 days), with an added 3 to 7 days as necessary, orally once a day until the day before curative intent surgery. They may also receive goserelin, at the local investigator's discretion, 3.6 mg every 4 weeks during the same period. There are no rest periods for this regimen (unless if toxicity occurs). In case of delays due to toxicity, the surgery should be performed no later than 4 months + 8 days from beginning of study treatment. Should any adaptations to palbociclib schedule be necessary due to toxicity or other reasons, these adaptations are left at the investigator's discretion, as long as the subject receives palbociclib for 3 days before surgery. The last dose of endocrine therapy and palbociclib will be given the day (evening) before surgery.
NON-MEDICINAL STUDY TREATMENT	Curative breast surgery to be performed according to local guidelines, 4 to 8 days after the end of fourth cycle of study treatment and no later than 4 months + 8 days from the beginning of study treatment.
INDICATION	Primary breast cancer
PARTICIPATING COUNTRY	Belgium
PARTICIPATING SITES NUMBER	5
START DATE OF THE TRIAL	15/02/2017
LENGTH OF THE STUDY	• Actual start date of recruitment to the protocol: 05/07/2017 • Actual date stop date of recruitment to the protocol: 28/03/2019 • Long term follow-up planned? Yes for efficacy– Duration: 30 months after the last subject's EOS visit or last treatment dose for subjects for whom no surgery was performed.
INDEPENDENT DATA	No

MONITORING COMMITTEE	
PROTECTION OF TRIAL SUBJECTS	Only patients meeting eligibility criteria were enrolled in the trial. Doses reduction and/or discontinuation of study treatment due to toxicity were managed according to international recommendations and as detailed in the study protocol. Investigators were expected in all moments to treat subjects according to their best clinical judgment, based on the clinical situation.
ANALYSIS STAGE & DATE	Final Date of final analysis: 01/12/2023
GLOBAL END OF TRIAL DATE	• Global end of trial reached? Yes • Global end of trial date: 01/02/2023
PREMATURE END OF TRIAL	No

1 SUBJECT INFORMATION

1.1 General information

105 female subjects were registered in the NeoRHEA trial. The actual number of subjects registered in each age range for the whole trial is specified in the table 1.

Age categorical characteristic	Number of subjects
In Utero	0
Preterm newborn-gestational age>37 week	0
Newborns (0-27 days)	0
Infants and toddlers (28 days – 23 months)	0
Children (2 – 11 years)	0
Adolescents (12 – 17 years)	0
Between 18 and 65 years	69
From 65 years to 84 years	36
85 years and over	0
TOTAL	105

Table 1: Age range for the whole trial

The subjects' median age was 59 years (full range 34 - 83).

1.2 Subject disposition

105 subjects were registered in the trial and 100 subjects were eligible to receive the IMPs.

97 subjects completed the trial and 3 subjects did not complete the trial.

The reasons why some subjects did not complete the trial with the corresponding subjects' number are specified in the table 2.

Non-completion reasons	Number of subjects
Protocol deviation	1
Consent withdrawn by subject	2

Table 2: Non-completion reasons with corresponding subjects' number.

The reasons for earlier stopping of study treatment for 21 subjects who completed the trial are listed in the below table 3.

Reason for stop	Number of subjects
Adverse event	10
Non-compliance with study drug	4
Physician decision	5
Progressive disease	1
Other	1

Table 3: Reasons for stopping study treatments

2 OVERALL ANALYSIS

2.1 Statistical analysis

To assess differences in categorical variables, the likelihood Chi-square, Mantel-Haenszel Chi-square of Fisher Exact tests were used. To assess differences in continuous variables, the t-test or the Wilcoxon test were used. Statistical analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). No adjustment for multiple testing was performed.

2.2 Clinical characteristics at inclusion

Of the 100 subjects enrolled in the study, 97 subjects were evaluable and included in the present analysis. Of those, there were 24 subjects with insufficient tumour cellularity at baseline frozen samples (tumour cellularity cut-off ≥25% was required for RNA-sequencing [RNA-seq] – see details in section 4.1) thus leaving us with 73 subjects with available RNA-seq data at baseline. Their clinical characteristics at inclusion are as follow:

	All evaluable (N = 97)		RNA-seq not available (N = 24)		RNA-seq available (N = 73)		P-value*
Age at inclusion (years)							
Mean ± std	60 ± 11		60 ± 10		60 ± 12		0.83
Median (min-max)	59 (34 to 83)		59 (46 to 83)		62 (34 to 82)		
<45	8	8%	-	-	8	11%	0.04
45-54	26	27%	8	33%	18	25%	
55-64	28	29%	11	46%	17	23%	
≥65	35	36%	5	21%	30	41%	
Menopausal status							
Postmenopausal	66	68%	15	63%	51	70%	0.51
Premenopausal	31	32%	9	38%	22	30%	
BMI (kg/m²)							
Mean ± std	26.7 ± 4.5		26.1 ± 4.1		27.0 ± 4.6		0.32
Median (min-max)	26.0 (17.1 to 39.4)		24.8 (19.7 to 35.5)		26.3 (17.1 to 39.4)		
Underweight (<18.5)	2	2%	-	-	2	3%	0.34
Normal (18.5-24.9)	36	38%	13	54%	23	33%	
Overweight (25-29.9)	36	38%	7	29%	29	41%	
Obese (≥30)	20	21%	4	17%	16	23%	
Missing info	*3*				*3*		
Tumour laterality							
Bilateral	2	2%	-	-	2	3%	0.54
Left	54	56%	13	54%	41	56%	
Right	41	42%	11	46%	30	41%	
Multifocal/multicentric							
No	81	84%	22	91.7%	59	80.8%	0.34

	All evaluable (N = 97)		RNA-seq not available (N = 24)		RNA-seq available (N = 73)		P-value*
Yes	16	16%	2	8.3%	14	19.2%	
Clinical tumour size (mm)							
Mean ± std	35 ± 14		35 ± 14		35 ± 14		0.96
Median (min-max)	30 (16 to 80)		31 (20 to 65)		30 (16 to 80)		
Histology							
Ductal	71	73%	16	67%	55	75%	0.40¥
Lobular	22	23%	7	29%	15	21%	
Apocrine	1	1%	-	-	1	1%	
Invasive NOS	1	1%	1	4%	-	-	
Mucinous (colloid)	2	2%	-	-	2	3%	
Grade							
1	16	17%	6	26%	10	14%	0.19
2	62	65%	14	61%	48	67%	
3	17	18%	3	13%	14	19%	
Missing info	*2*		*1*		*1*		
cT							
T1c	9	9%	4	17%	5	7%	0.62
T2	75	77%	14	58%	61	84%	
T3	13	13%	6	25%	7	10%	
cN							
N0	65	67%	14	58%	51	70%	0.30
N1	32	33%	10	42%	22	30%	
cM							
M0	97	100%	24	100%	73	100%	--
TNM							
IA	5	5.2%	2	8%	3	4%	0.29
IIA	57	58.8%	11	46%	46	63%	
IIB	29	29.9%	8	33%	21	29%	
IIIA	6	6.2%	3	13%	3	4%	

Table 4: Clinical characteristics at inclusion of the evaluable subjects (n=97), those without available RNA-seq data at baseline (n=24) and those with available RNA-seq data at baseline (n=73).
NOS: not otherwise specified; std: standard deviation.
*For the comparison of subjects without available RNA-seq data *vs* those with available RNA-seq data at baseline
¥Comparison of ductal *vs* lobular histology.

Among the 97 evaluable subjects, median age at inclusion was 60 years of age, 68% were postmenopausal, 76% were normal or overweight, 98% had a unilateral breast tumour (56% left and 42% right), and 16% had a multifocal or multicentric tumour. Median tumour size was 30 mm. As expected, most subjects (73%) had invasive ductal carcinoma and 65% histological grade 2 tumours. The majority of subjects (59%) presented with stage IIA. There were no significant differences in the distribution of baseline clinical characteristics between subjects with available RNA-seq and those without RNA-seq not available.

2.3 Systemic treatment

Regarding the type of hormone therapy (HT) received, most subjects received letrozole, given that they were post-menopausal. There were no significant differences in terms of HT received between subjects with available RNA-seq and those with RNA-seq not available. Most subjects completed the four months of therapy with palbociclib (71% among all evaluable subjects). However, among subjects without available RNA-seq, the proportion of subjects who completed the four cycles of therapy with palbociclib was significantly lower compared to subjects with available RNA-seq at baseline (50% *vs* 78%, p=0.02).

	All evaluable (N = 97)		RNA-seq not available (N = 24)		RNA-seq available (N = 73)		P-value*
Hormone therapy							
Letrozole	71	73%	18	75%	53	73%	1¶
Tamoxifen only	3	3%	-	-	3	4%	
Goserelin + letrozole¥	1	1%	1	4%	-	-	
Tamoxifen + Goserelin	22	23%	5	21%	17	23%	
Palbociclib (number of cycles)							
2	1	1%	-	-	1	1%	
3	2	2%	1	4%	1	1%	
3+ bridge	3	3%	1	4%	2	3%	
4	22	23%	10	42%	12	16%	
4+ bridge	69	71%	12	50%	57	78%	0.02§

Table 5: Systemic treatments received by all evaluable subjects (n=97), those without available RNA-seq data at baseline (n=24) and those with available RNA-seq data at baseline (n=73).
* For the comparison of subjects without available RNA-seq data *vs* those with available RNA-seq data at baseline
¶ For the comparison of subjects receiving Letrozole *vs* Tamoxifen + Goserelin
¥ Considered as a major protocol deviation
§ Comparing the proportion of subjects receiving 4 cycles + bridge in each group (with *vs* without available RNA at baseline)

2.4 Ultrasound response

In terms of the clinical response to treatment, assessed by ultrasound (US) at the bridge period (see details in section 4.1), the results are as follow:

	All evaluable (N = 97)		RNA-seq not available (N = 24)		RNA-seq available (N = 73)		P-value*
US response							
Complete response	2	2%	-	-	2	3%	0.91
Partial response	51	53%	12	50%	39	54%	
Stable disease	39	41%	11	46%	28	39%	
Progressive disease	4	4%	1	4%	3	4%	
Missing	*1*				*1*		

Table 6: Clinical response to treatment assessed by ultrasound at the bridge period in all evaluable subjects (n=97), those without available RNA-seq data at baseline (n=24) and those with available RNA-seq data at baseline (n=73).

US: ultrasound.
*For the comparison of subjects without available RNA-seq data *vs* those with available RNA-seq data at baseline

The proportion of stable disease (SD) and progressive disease (PD) were 41% and 4% among the 97 evaluable subjects, with no significant differences among the two subcohorts (with and without available RNA-seq at baseline).

2.5 Surgery

Among the 97 evaluable subjects, there was one subject withdrawing consent just before surgery. Thus, among the 96 subjects that were operated and for whom there is available information regarding the type of surgery and characteristics of the tumour at surgery, their details are described below:

	All evaluable (N = 96)		RNA-seq not available (N = 23)		RNA-seq available (N = 73)		P-value*
Mastectomy							
No	43	45%	11	48%	32	44%	0.81
Yes	53	55%	12	52%	41	56%	
Axillary node dissection							
No	36	38%	8	35%	28	38%	0.81
Yes	60	63%	15	65%	45	62%	
pT							
T0	1	1%	-	-	1	1%	0.65
T1a	3	3%	-	-	3	4%	
T1b	2	2%	1	4%	1	1%	
T1c	37	39%	11	48%	26	36%	
T2	43	45%	8	35%	35	48%	
T3	9	9%	3	13%	6	8%	
T4a	1	1%	-	-	1	1%	
pN							
N0	49	51%	11	48%	38	52%	0.32
N1a	32	33%	6	26%	26	36%	
N1c	1	1%	1	4%	-	-	
$N1_{mi}$	4	4%	1	4%	3	4%	
N2a	7	7%	3	13%	4	5%	
N3a	3	3%	1	4%	2	3%	
RCB							
0	1	1%	-	-	1	1%	0.18
I	6	6%	3	13%	3	4%	
II	56	58%	10	43%	46	63%	
III	33	34%	10	43%	23	32%	

Table 7: Type of surgery and characteristics of the tumour at surgery among all evaluable subjects (n=97), those without available RNA-seq data at baseline (n=24) and those with available RNA-seq data at baseline (n=73).
pN: pathological nodal status; pT: pathological tumour status; RCB: residual cancer burden.
*For the comparison of subjects without available RNA-seq data *vs* those with available RNA-seq data at baseline

2.6 Survival data

Overall, 8 subjects developed distant and 1 subject locoregional recurrences. Four subjects died, of whom one due to non-breast cancer cause, in absence of disease relapse.

3 SAFETY ANALYSIS

3.1 General information

Serious adverse events (SAEs) and non-serious adverse events (AEs) were collected from the 1st administration of the study medications until 30 days after the protocol surgery. After this period, only SAEs which have a reasonable possibility to be related to study treatments (study medications or protocol surgery) were collected.

The following adverse events that have been defined in the trial protocol as “exempt” from entry into the safety database are excluded from the tabulations:

Some hospitalisation scenarios do not require reporting as a SAE such as:

- Elective hospitalisation for pre-existing conditions that have not been exacerbated by study medication.
- Hospitalisation planned before the subject consented for study participation and where admission did not take longer than anticipated.
- Hospitalisations for reasons described in the protocol (*e.g.* hospitalisation for study medication administration, hospitalisation for study related procedures). However, event requiring hospitalisation or prolongation of hospitalisation as a result of a complication of study medication administration or study related procedures will be reported as SAE.
- Hospitalisation or prolonged hospitalisation in absence of an AE (social, technical, practical reason and/or convenience admission to a hospital, palliative care, rehabilitation).

Recurrence and/or progression of underlying malignancy is not reported as an AE or SAE if it is clearly consistent with the suspected recurrence or progression of the underlying cancer but will be reported on the CRF, except if subject died due to progression of underlying malignancy within the active reporting period.

Unrelated second primary malignancy (*i.e.* a cancer that is unrelated to the study treatment(s) and is not a metastasis from the initial malignancy) should not be reported as an AE or SAE but it has however to be reported on the CRF.

Hospitalisation due solely to the recurrence and/or progression of underlying malignancy should NOT be reported as an SAE.

Clinical symptoms of recurrence and/or confirmed progression of underlying malignancy should not be reported as AEs or SAEs unless the symptoms cannot be determined as exclusively due to the recurrence or confirmed progression of the underlying malignancy, or do not fit the expected pattern of recurrence or confirmed progression for the disease under study.

- 99 subjects were exposed to the IMPs
- 4 subjects were affected by serious adverse events.
- 96 subjects were affected by non-serious adverse events.

Notes:

1. The adverse event and serious adverse event assessment method was systematic.

2. The MedDRA version used for this report is the version 23.1.

3.2 Serious Adverse Events overview

Table 8 presents all serious adverse events sorted by MedDRA System Organ Class (SOC), MedDRA Preferred Terms (PT).

MedDRA SOC *MedDRA PT*	Number of subjects affected	All SAE occurrences	SAE occurrences causally related to palbociclib	SAE occurrences causally related to letrozole	SAE occurrences causally related to tamoxifen	SAE occurrences causally related to goserelin	SAE occurrences causally related to surgery	Number of fatalities
Hepatobiliary disorders								
Drug-induced liver injury	1	1	1					0
Infections and infestations								
Viral infection	1	1						0
Respiratory, thoracic and mediastinal disorders								
Pulmonary embolism	1	1					1	0
Vascular disorders								
Malignant hypertension	1	1						0

Table 8: All serious adverse events sorted by MedDRA SOC and MedRA PT.

3.3 Non-Serious Adverse Events

The frequency threshold for reporting non-serious adverse events is 0%. Table 9 presents all non-serious adverse events sorted by MedDRA System Organ Class (SOC), MedDRA Preferred Terms (PT).

MedDRA Primary SOC *MedDRA PT*	Number of subjects affected	All AE occurrences	AE occurrences causally related to palbociclib	AE occurrences causally related to letrozole	AE occurrences causally related to tamoxifen	AE occurrences causally related to goserelin	AE occurrences causally related to surgery
Blood and lymphatic system disorders							
Anaemia	11	12	12				
Neutropenia	54	99	99	1		1	2

MedDRA Primary SOC *MedDRA PT*	**Number of subjects affected**	**All AE occurrences**	**AE occurrences causally related to palbociclib**	**AE occurrences causally related to letrozole**	**AE occurrences causally related to tamoxifen**	**AE occurrences causally related to goserelin**	**AE occurrences causally related to surgery**
Thrombocytopenia	5	6	6				
Cardiac disorders							
Palpitations	2	2	1				
Sinus bradycardia	1	1	1				
Ear and labyrinth disorders							
Ear pain	1	1					
Vertigo	4	4	1		1		
Eye disorders							
Dry eye	2	2	1	1			
Eye irritation	1	1	1				
Lacrimation increased	3	3	2				
Vision blurred	1	2	2				
Gastrointestinal disorders							
Abdominal distension	1	1					
Abdominal pain	4	5	1				
Abdominal pain upper	3	3	3				
Anal fissure	2	2	1	1			
Aphthous ulcer	2	2	2				
Constipation	10	10	5		2		
Diarrhoea	9	12	11		1		

MedDRA Primary SOC *MedDRA PT*	**Number of subjects affected**	**All AE occurrences**	**AE occurrences causally related to palbociclib**	**AE occurrences causally related to letrozole**	**AE occurrences causally related to tamoxifen**	**AE occurrences causally related to goserelin**	**AE occurrences causally related to surgery**
Dry mouth	2	2	2				
Dyspepsia	4	4	4				
Flatulence	1	1					
Gastrointestinal disorder	1	2					
Gingival pain	1	1	1				
Haematochezia	1	1					
Haemorrhoids	2	3					
Lip dry	1	1	1				
Nausea	16	16	12		2		
Proctalgia	1	1			1		
Salivary hypersecretion	1	1					
Stomatitis	15	16	15		1		
Toothache	1	1					
Vomiting	3	3	3				
General disorders and administration site conditions							
Asthenia	23	23	21	2	2	2	
Axillary pain	*3*	*3*					*1*
Fatigue	*37*	*40*	*35*	*13*	*7*	*3*	*1*
Mucosal inflammation	*4*	*4*	*4*				
Oedema peripheral	*1*	*1*					
Pain	*2*	*2*					
Hepatobiliary disorders							

MedDRA Primary SOC *MedDRA PT*	**Number of subjects affected**	**All AE occurrences**	**AE occurrences causally related to palbociclib**	**AE occurrences causally related to letrozole**	**AE occurrences causally related to tamoxifen**	**AE occurrences causally related to goserelin**	**AE occurrences causally related to surgery**
Hepatic pain	1	1					
Immune system disorders							
Allergy to metals	1	1					
Hypersensitivity	1	1					
Infections and infestations							
Bartholinitis	1	1					
Bronchitis	2	2					
Conjunctivitis	1	2	2				
Cystitis	3	3			1	1	
Fungal infection	3	3	1				
Gastroenteritis	1	1					
Gingivitis	2	2	1				
Herpes zoster	1	1					
Localised infection	1	1	1				
Mastitis	1	1					1
Nasopharyngitis	3	3					
Oral herpes	1	1					
Pharyngitis	2	2					
Postoperative wound infection	2	2					2
Rhinitis	1	1					
Sinusitis	2	2					
Skin infection	2	2					1

MedDRA Primary SOC *MedDRA PT*	**Number of subjects affected**	**All AE occurrences**	**AE occurrences causally related to palbociclib**	**AE occurrences causally related to letrozole**	**AE occurrences causally related to tamoxifen**	**AE occurrences causally related to goserelin**	**AE occurrences causally related to surgery**
Tracheitis	1	1					
Viral infection	1	1					
Viral pharyngitis	1	1					
Vulvovaginitis	1	1	1				
Wound infection	1	1					1
Injury, poisoning and procedural complications							
Axillary web syndrome	1	1					1
Concussion	1	1					
Foot fracture	1	1					
Ligament sprain	1	1					
Postmastectomy lymphoedema syndrome	1	1					1
Procedural pain	2	2					1
Spinal fracture	1	1					
Investigations							
Alanine aminotransferase increased	3	3	2	1			
Aspartate aminotransferase increased	3	3	1	2			
Blood pressure decreased	1	1	1				
Platelet count decreased	2	2	2				
Transaminases increased	1	1					
Weight decreased	2	2					

MedDRA Primary SOC *MedDRA PT*	**Number of subjects affected**	**All AE occurrences**	**AE occurrences causally related to palbociclib**	**AE occurrences causally related to letrozole**	**AE occurrences causally related to tamoxifen**	**AE occurrences causally related to goserelin**	**AE occurrences causally related to surgery**
Metabolism and nutrition disorders							
Decreased appetite	9	9	8	1	1		
Fluid retention	1	1					
Hypercalcaemia	1	1					
Hyperkalaemia	1	1					
Hypocalcaemia	1	1					
Hypoglycaemia	1	1					
Hypomagnesaemia	2	2					
Musculoskeletal and connective tissue disorders							
Arthralgia	26	28	5	20	8	1	
Back pain	4	4		1			
Chest wall necrosis	1	1					1
Flank pain	1	1					
Intervertebral disc protrusion	1	1					
Mobility decreased	1	1					1
Muscle spasms	7	7	2	2	1		
Musculoskeletal pain	2	2					
Musculoskeletal stiffness	1	1	1				
Myalgia	6	6	2	1	2		
Neck pain	1	1					
Osteoarthritis	3	3			1		
Osteopenia	1	1					
Pain in extremity	1	1					

MedDRA Primary SOC *MedDRA PT*	**Number of subjects affected**	**All AE occurrences**	**AE occurrences causally related to palbociclib**	**AE occurrences causally related to letrozole**	**AE occurrences causally related to tamoxifen**	**AE occurrences causally related to goserelin**	**AE occurrences causally related to surgery**
Tendonitis	1	1					
Neoplasms benign, malignant and unspecified (including cysts and polyps)							
Lipoma	1	1					
Tumour pain	1	2		2			
Psychiatric disorders							
Anxiety	3	3					
Depression	2	2					
Insomnia	7	7	3	2			
Mood altered	2	2	1		1	1	
Stress	2	2					1
Nervous system disorders							
Disturbance in attention	1	1			1		
Dysgeusia	5	5	3		1		
Headache	11	11	4	1	1		
Hypoaesthesia	1	1					1
Migraine	1	1					
Syncope	2	2					
Renal failure and impairment							

MedDRA Primary SOC *MedDRA PT*	**Number of subjects affected**	**All AE occurrences**	**AE occurrences causally related to palbociclib**	**AE occurrences causally related to letrozole**	**AE occurrences causally related to tamoxifen**	**AE occurrences causally related to goserelin**	**AE occurrences causally related to surgery**
Acute kidney injury	1	1					
Reproductive system and breast disorders							
Amenorrhoea	1	1	1		1		
Breast haematoma	1	1					1
Breast pain	5	5	1				1
Breast swelling	1	1					
Vaginal discharge	1	1					
Vulvovaginal dryness	2	2		1	1	1	
Vulvovaginal inflammation	1	1	1				
Respiratory, thoracic and mediastinal disorders							
Cough	3	3	1				
Dyspnoea	2	2					
Epistaxis	8	10	8				
Nasal dryness	1	1	1				
Oropharyngeal pain	2	2	1				
Skin and subcutaneous tissue disorders							
Alopecia	17	17	14	4	4	3	
Dry skin	7	7	4	3	1		
Eczema	1	1			1		
Hair texture abnormal	1	1	1				

MedDRA Primary SOC *MedDRA PT*	**Number of subjects affected**	**All AE occurrences**	**AE occurrences causally related to palbociclib**	**AE occurrences causally related to letrozole**	**AE occurrences causally related to tamoxifen**	**AE occurrences causally related to goserelin**	**AE occurrences causally related to surgery**
Hyperhidrosis	1	1			1	1	
Pruritus	4	4	1		2		
Rash	3	3	2				
Rash pruritic	1	1	1				
Scar pain	1	1					1
Skin fissures	1	1					
Toxic skin eruption	2	2	1				
Urticaria	1	1	1				
Vascular disorders							
Haematoma	1	1					
Hot flush	39	39		18	15	15	
Hypertension	8	8					
Lymphocele	1	1					1
Lymphoedema	1	1					1
Phlebitis	1	1			1		

Table 9: All non-serious adverse events sorted by MedDRA System Organ Class (SOC) and MedDRA Preferred Terms (PT).

Table 10 presents the non-serious adverse reactions (all grades), *i.e.* related to IMPs, experienced by the subjects in NeoRHEA trial sorted by MedDRA SOC, frequency and MedDRA Preferred Terms (PT). Multiple occurrences of a specific adverse reaction for a subject through cycles have been counted only once.

MedDRA SOC *Frequency* MedDRA PT	**Number of subjects (%)**
Blood and lymphatic system disorders	
Very common	
Anaemia	11 (11%)
Neutropenia	54 (55%)
Common	
Thrombocytopenia (incl. Platelet count decreased)	7 (7%)
Cardiac disorders	
Uncommon	
Palpitations	1 (1%)
Sinus bradycardia	1 (1%)
Ear and labyrinth disorders	
Common	
Vertigo	2 (2%)
Eye disorders	
Common	
Lacrimation increased	2 (2%)
Vision blurred	2 (2%)
Uncommon	
Dry eye	1 (1%)
Eye irritation	1 (1%)
Gastrointestinal disorders	
Very common	
Nausea	13 (13%)
Stomatitis	14 (14%)
Common	
Abdominal pain upper	3 (3%)
Aphthous ulcer	2 (2%)
Constipation	7 (7%)
Diarrhoea	8 (8%)
Dry mouth	2 (2%)
Dyspepsia	4 (4%)
Vomiting	3 (3%)
Uncommon	
Abdominal pain	1 (1%)
Anal fissure	1 (1%)
Gingival pain	1 (1%)
Lip dry	1 (1%)
Proctalgia	1 (1%)
General disorders and administration site conditions	

MedDRA SOC *Frequency* MedDRA PT	**Number of subjects (%)**
Very common	
Asthenia	21 (21%)
Fatigue	34 (34%)
Common	
Mucosal inflammation	4 (4%)
Infections and infestations	
Uncommon	
Conjunctivitis	1 (1%)
Cystitis	1 (1%)
Fungal infection	1 (1%)
Gingivitis	1 (1%)
Localised infection	1 (1%)
Vulvovaginitis	1 (1%)
Investigations	
Common	
Alanine aminotransferase increased	3 (3%)
Aspartate aminotransferase increased	3 (3%)
Uncommon	
Blood pressure decreased	1 (1%)
Metabolism and nutrition disorders	
Common	
Decreased appetite	8 (8%)
Musculoskeletal and connective tissue disorders	
Very common	
Arthralgia	26 (26%)
Common	
Muscle spasms	5 (5%)
Myalgia	4 (4%)
Uncommon	
Back pain	1 (1%)
Musculoskeletal stiffness	1 (1%)
Osteoarthritis	1 (1%)
Neoplasms benign, malignant and unspecified (incl cysts and polyps)	
Uncommon	
Tumour pain	1 (1%)
Nervous system disorders	
Common	
Headache	5 (5%)
Dysgeusia	4 (4%)
Uncommon	
Disturbance in attention	1 (1%)
Psychiatric disorders	
Common	
Insomnia	4 (4%)
Mood altered	2 (2%)

MedDRA SOC *Frequency* MedDRA PT	**Number of subjects (%)**
Reproductive system and breast disorders	
Common	
Vulvovaginal dryness	2 (2%)
Uncommon	
Amenorrhoea	1 (1%)
Breast pain	1 (1%)
Vulvovaginal inflammation	1 (1%)
Respiratory, thoracic and mediastinal disorders	
Common	
Epistaxis	6 (6%)
Uncommon	
Cough	1 (1%)
Nasal dryness	1 (1%)
Oropharyngeal pain	1 (1%)
Skin and subcutaneous tissue disorders	
Very common	
Alopecia	17 (17%)
Common	
Dry skin	7 (7%)
Pruritus	3 (3%)
Rash	2 (2%)
Uncommon	
Eczema	1 (1%)
Hair texture abnormal	1 (1%)
Hyperhidrosis	1 (1%)
Rash pruritic	1 (1%)
Toxic skin eruption	1 (1%)
Urticaria	1 (1%)
Vascular disorders	
Very common	
Hot flush	37 (37%)
Uncommon	
Phlebitis	1 (1%)

Table 10: Non-serious adverse reactions (N=99) sorted by MedDRA SOC, frequency and MedDRA PT.
N=number of subjects who received the investigational medicinal products.
Frequencies are defined as= very common (≥ 1/10); common (≥ 1/100 to < 1/10); uncommon (≥ 1/1000 to < 1/100); rare (≥ 1/10,000 to < 1/1000).

The most frequent adverse reactions observed in the subjects exposed to IMPs in the NeoRHEA trial were neutropenia (55%), hot flush (37%), fatigue (34%), arthralgia (26%), asthenia (21%), alopecia (17%), stomatitis (14%), nausea (13%) and anaemia (11%).

4 TRANSLATIONAL RESEARCH ANALYSIS

4.1 Methodology

1. Ultrasound assessment

The main method for evaluation of US response was bi-dimensional (longest diameter x greatest perpendicular diameter of primary tumour) ultrasound, performed before and after study treatment. Response criteria were those of the World Health Organization as evaluated through US.[2] Possible outcomes were defined as follows:

- Complete Response: Disappearance of the primary tumour. Any pathological lymph nodes (whether target or non-target) having reduction in short axis to <10 mm.
- Partial Response: At least a 50% decrease in the surface of target lesion (primary tumour), taking as reference the baseline surface.
- Stable Disease: Neither sufficient shrinkage to qualify for partial response nor sufficient increase to qualify for progressive disease, taking as reference the starting surface.
- Progressive Disease: At least a 25% increase in the surface of target lesion (primary tumour), taking as reference the baseline surface on study. (Note: the appearance of one or more new lesions was also considered progression).

Though scheduled evaluations only included beginning and end of study treatment, any signs of progressions (signs or symptoms) found during routine clinical evaluation subjects were evaluated with imaging at investigator discretion according to local guidelines.

For the purpose of these analyses, lack of response (stable disease) at ultrasound after the 4-month treatment period, as well as progression (progressive disease) anytime during the 4-month treatment period by US were grouped together.

2. Residual cancer burden (RCB)

In order to calculate the RCB, the surgical material was used and analysed according to the following parameters:[3]

- Primary tumour bed dimensions
- Cellularity fraction of invasive cancer
- Size of largest positive lymph node
- Number of positive lymph nodes

For the purpose of these analyses, we compared subejcts with RCB 0-II at surgery vs those with RCB of 3, as this score has been associated with poor outcome after neoadjuvant treatment.[3]

3. Response by Ki67 at surgery

Although initially we have defined response based on breast US assessment, this has posed problems in terms of reproducibility and accuracy. Therefore, we have opted to use centrally reviewed Ki67 percentage at surgery, done by immunohistochemistry (IHC) in formalin-fixed paraffin-embedded (FFPE) samples. This proliferation marker is now considered to be a reliable, reproducible and validated way of measuring response to endocrine therapy, improving prediction of recurrence-free survival in the adjuvant setting.[4–7] As palbociclib predominately affects proliferation, suppression of Ki67 is a rational endpoint for estimating whether there is efficacy with the addition of palbociclib to endocrine therapy. Hence, tumours were classified as:

- Sensitive: Ki67 ≤2.7% in the surgical sample (definition of complete cell cycle arrest [CCCA]);
- Resistant: Ki67 >2.7% in the surgical sample.

FFPE samples devoid of any tumour content as evaluated by our pathologist (Dr. Denis Larsimont, Head of Department) were excluded from IHC staining. Ki67 and PanCytokeratine (PanCK) staining were then performed using the monoclonal mouse anti-Human Ki67 antigen Clone MIB-1 (Dako Omnis) and monoclonal mouse anti-human cytokeratin clone AE1/AE3 Ready-To-Use (Dako Autostainer/Autostainer Plus), respectively. Briefly, PanCytokeratine staining was used to select only the Ki67 staining within the tumour cells, excluding Ki67-expressing lymphocytes. In order to do so, adjacent sections stained with each antibody were scanned using a digital slide scanner Nanozoomer (Hamamatsu) that converts glass slides into high-resolution digital data by high-speed scanning, and subsequently aligned using Visiopharm (Visopharm®), an histopathology image analysis software for cancer diagnostics and research with AI and deep learning technologies in Augmented Pathology®. An example of a scanned and aligned biopsy tissue section, with a report on Ki67 positive nuclei in the whole tissue, as well as within the hotspots (areas specific Ki67 counting allowing to bypass tumour heterogeneity for a more reliable Ki67 evaluation), can be seen on **Figure 1**:

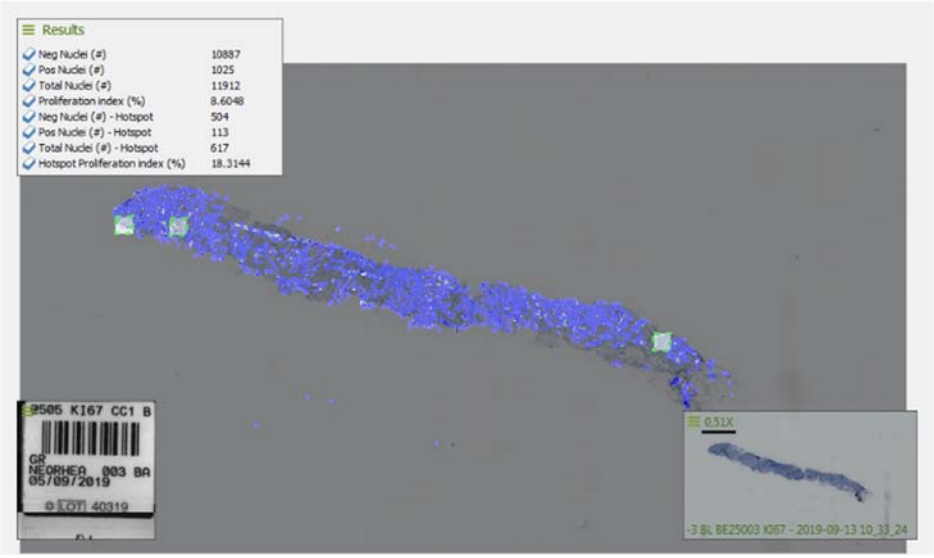


Figure 1: Adjacent biopsy tissue sections stained for Ki67 and PanCK were scanned, aligned and Ki67 Positive Nuclei counted by Visiopharm. Green squares correspond to hotspots area, and an average Ki67 score within these areas could be determined.

Of note, this procedure allows us to have a precise percentage (%) of Ki67 expression in a scale of 1-100% that is much more granular that the percentage (%) of Ki67 expression provided in the medical files (incremental bins of 10%). Therefore, it allowed to evaluate the presence or absence of CCCA in our subjects' tumours, defined as centrally reviewed Ki67 ≤2.7% at surgery.

4. Evaluation of tumour infiltrating lymphocytes (TILs) and PD-L1 expression by IHC in FFPE samples

FFPE samples devoid of any tumour content as evaluated by our pathologist were excluded from staining. Afterwards, evaluation of TILs was performed in H&E stained slides, according to the method described by Salgado et al.[8]

PD-L1 staining on tumour infiltrating immune cells was performed using the Ventana anti-PD-L1 SP142 antibody (Roche). Briefly, FFPE tissue sections were cut with a microtome (3.5µm), heated and incubated in a cell conditioning buffer, then stained with the pre-diluted antibody provided by Roche. Stained sections were counterstained with hematoxylin and bluing reagent. The whole procedure was performed on the automated Benchmark ULTRA IHC/ISH available within our pathology department. PD-L1 final scoring was done by the Head of the Pathology Department (Dr. Denis Larsimont). Positive samples were considered those with PD-L1 expression on tumour infiltrating immune cells of at least 1%.[9]

5. RNA extraction and sequencing (from frozen samples)

Frozen samples with <25% of tumour cellularity were excluded from this analysis. In the remaining samples, total RNA was extracted from 10µm-thick sections for all frozen biopsy and surgery tissue samples using an Allprep DNA/RNA/miRNA Universal Kit (Qiagen). RNA quality was evaluated using the Agilent 2100 Bioanalyzer (Agilent) and quantified using the RNA Integrity Number (RIN) – **Figure 2**.

For samples with an adequate RIN, strand specific cDNA libraries were constructed using the NEB Next Ultra directional RNA library Preparation Kit for Illumina paired-end sequencing on a NovaSeq 6000 instrument (Illumina) at the ULB BRIGHTcore sequencing facility with a targeted coverage of 40.10^6 reads.

6. RNA-Sequencing Data Processing

The transcriptome was indexed and reads were quantified at the transcript level using the alignment-free method Salmon (version 0.9.1).[10] Transcript-level abundances were converted to gene-level counts and imported into R using tximport,[11] and normalized into Transcripts per Million (TPM) or using the variance stabilizing transformation algorithm from

the DESeq2 package.[12] Results were plotted using the ggplot package,[13] and statistical significance computed using the non-parametric Wilcoxon statistical test. For this report, no multiple testing correction was applied, hence these analyses should be regarded as exploratory.

PAM50 subtypes (Luminal A, Luminal B, HER2-enriched, Basal-like and Normal-like) were calculated for each sample using the pam50 computation algorithm from the genefu package (version 2.22).[14] Briefly, the 104 NeoRhea samples, including baseline and surgery samples, were merged with 1084 TCGA breast cancer all comers samples and normalized identically for adequate algorithm calibration. The 11-gene signature profiles were calculated by Eric Raspé using the same methodology as described in Raspé et al.[1] and comprised the following:

- Profile A corresponding to “resistance to Palbociclib”, enriched in basal-like tumours;
- Profile H to “sensitivity to Palbociclib, highly proliferative”, enriched in HER2-enriched and Luminal B tumours;
- Profile L to “sensitivity to Palbociclib, low proliferation”, enriched in Luminal A tumours.

Changes in PAM50 subtypes and 11-gene signature profiles between baseline and surgery were represented using the networkD3 package (version 0.4).

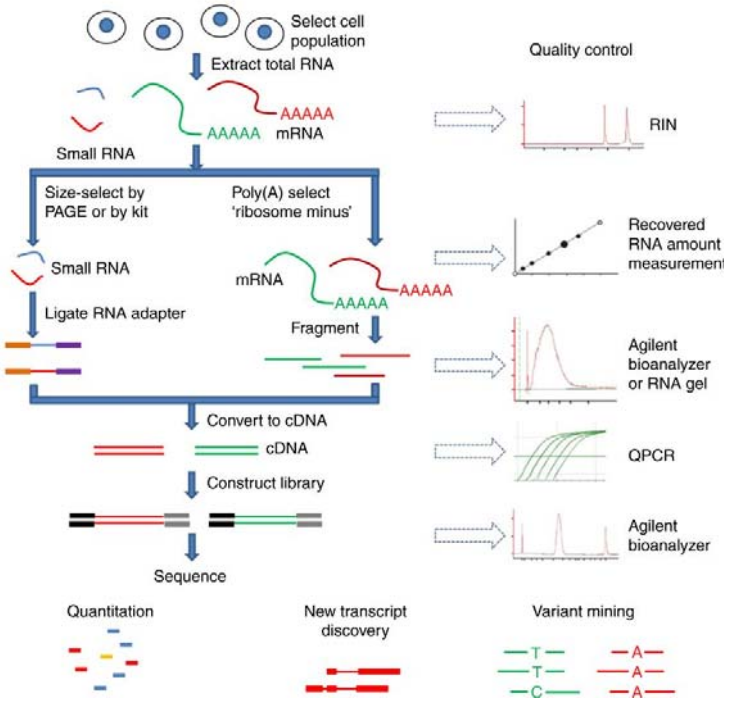


Figure 2: RNA-sequencing workflow

4.2 Results: baseline biomarkers of treatment outcome

From the 97 evaluable subjects, only 73 subjects had adequate tumour cellularity at a baseline frozen sample (a tumour cellularity cut-off ≥25% was required for RNA-seq) and were, therefore, included in the translational research (TR) analyses:

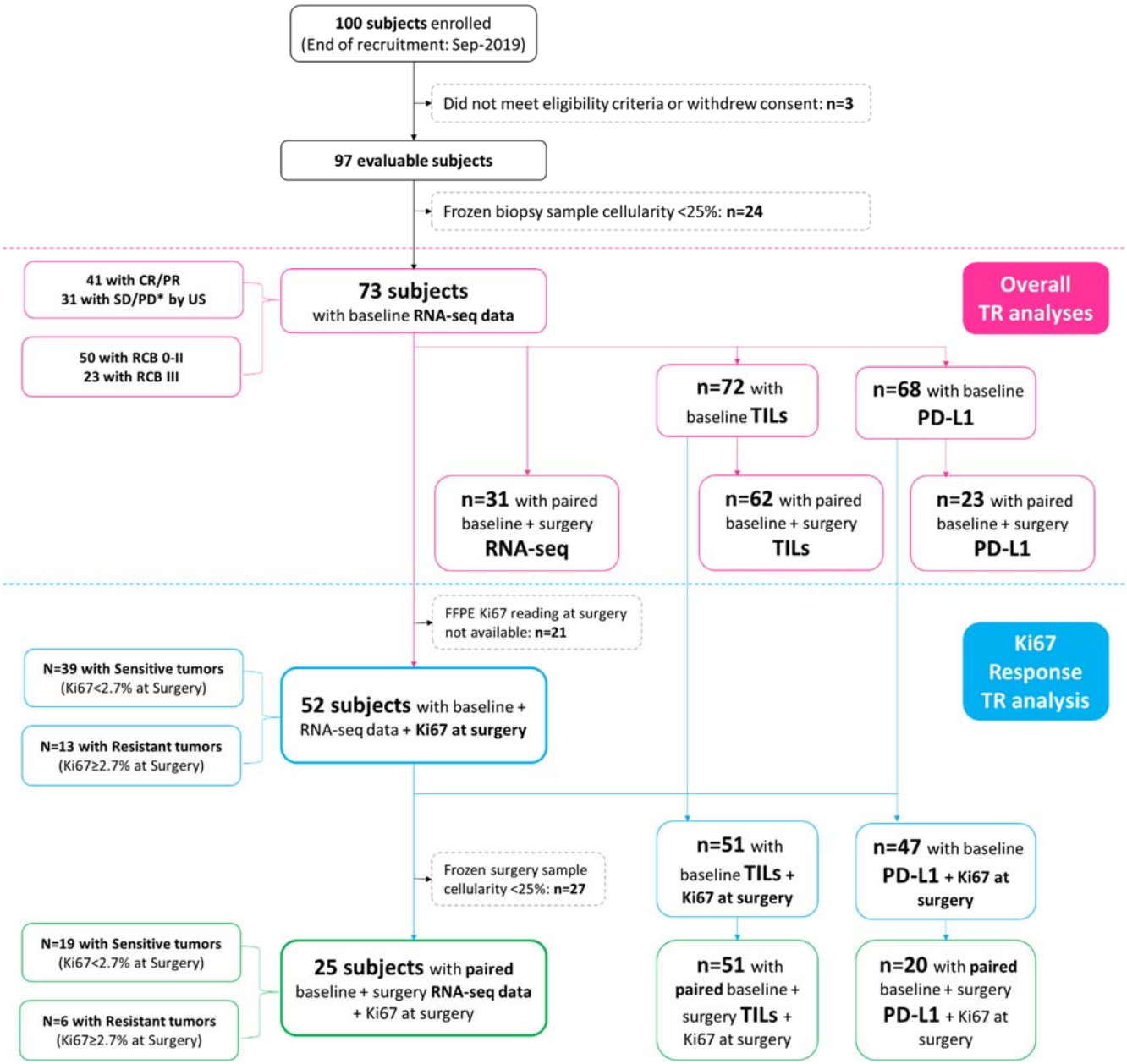


Figure 3: Flowchart of subjects included in the study.
CR: complete response; FFPE: formalin fixed paraffin-embedded; PD: progressive disease; PR: partial response; RCB: residual cancer burden; RNA-seq: RNA-sequencing; SD: stable disease; TILs: tumour infiltrating lymphocytes; TR: translational research; US: ultrasound.

First, we analysed TILs and PD-L1 by IHC *at baseline*:

1) Among all subjects included in the TR analysis (“all-comers”; n=73)

2) According to US assessment (n=72): subjects with CR/PR *vs* subjects with SD/PD

3) According to residual cancer burden (RCB) at surgery (n=73): subjects with RCB 0-II *vs* subjects with III

4) According to Ki67 response at surgery (n=52): sensitive (Ki67 ≤2.7%) *vs* resistant (Ki67 >2.7%) tumours.

Then, we analysed RNA-seq data *at baseline*:

1) According to US assessment (n=72): subjects with CR/PR *vs* subjects with SD/PD

2) According to residual cancer burden (RCB) at surgery (n=73): subjects with RCB 0-II *vs* subjects with III

3) According to Ki67 response at surgery (n=52): sensitive (Ki67 ≤2.7%) *vs* resistant (Ki67 >2.7%) tumours

TILs and PD-L1 by IHC at baseline

1) All-comers

Among the 73 subjects included in the TR analysis, there were 72 subjects with an available baseline value of stromal TILs. The proportion of TILs was low, with a mean (±standard deviation) value of 3.30% (±9.80) and a median value of 0% (min-max: 0-50). Similarly, among the 68 subjects with available baseline PD-L1, the mean score was 2.07% (± 7.44) and the median value was 0% (min-max: 0-40).

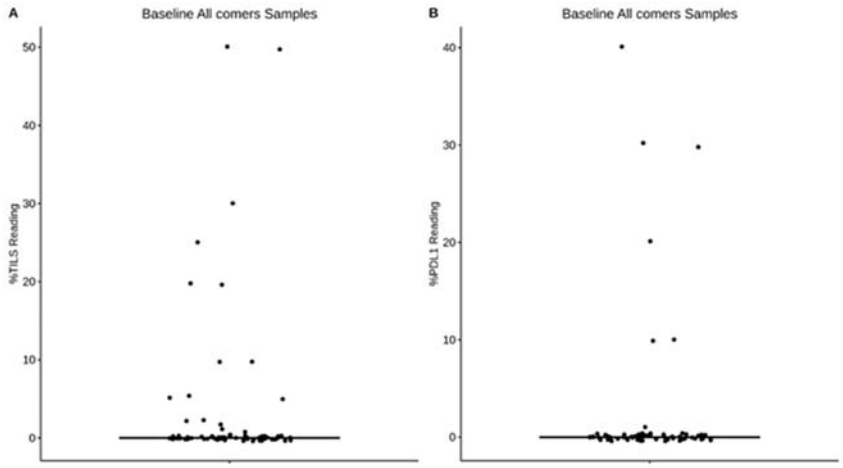


Figure 4: Distribution of the % of baseline tumour infiltrating lymphocytes (TILs; n=72; left panel) and PD-L1 (n=68; right panel) among subjects included in the translational research analysis.

2) According to US assessment

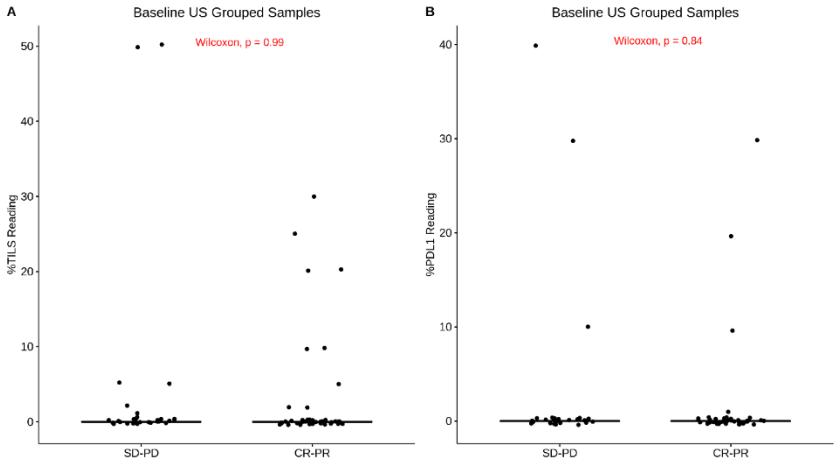


Figure 5: Distribution of the % of baseline tumour infiltrating lymphocytes (TILs; n=71; left panel) and PD-L1 (n=67; right panel) among subjects included in the translational research analysis according to Ultrasound assessment.

3) According to RCB

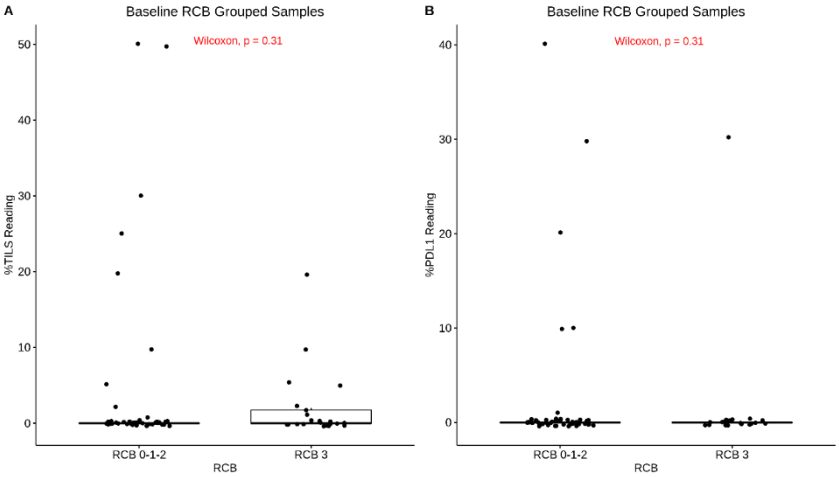


Figure 6: Distribution of the % of baseline tumour infiltrating lymphocytes (TILs; n=72; left panel) and PD-L1 (n=68; right panel) among subject included in the translational research analysis according to residual cancer burden (RCB) at surgery.

4) According to Ki67 response

Among the 73 subjects included in the TR analysis, 52 subjects had available central review of the tumour's Ki67 at surgery. As expected, most subjects' tumours (75%, n=39/52) exhibited a CCCA at surgery (Ki67 <2.7%) and were considered "sensitive". Of those 52 subjects, 42 subjects had completed the bridge period (*i.e.* received palbociclib plus HT until the day before surgery). Among them, 10 had resistant tumours (24%) and the other 32 had sensitive tumours (76%), which is similar to the proportion seen in the overall 52 subjects with Ki67 available at surgery.

In terms of clinical characteristics, there were no major differences at inclusion between subjects with sensitive *vs* resistant tumours, as detailed in table 11:

	All with Ki67 (N = 52)		Ki67 ≤2.7% (N = 39)		Ki-67 >2.7% (N = 13)		P-value*
Age (years)							
Mean ± std	60 ± 11		59 ± 10		63 ± 15		0.17
Median (min-max)	58 (34 to 82)		57 (40 to 81)		69 (34 to 82)		0.38
<45	8	8%	-	-	8	11%	0.62
45-54	26	27%	8	33%	18	25%	0.79
55-64	28	29%	11	46%	17	23%	
≥65	35	36%	5	21%	30	41%	
Menopausal status							
Postmenopausal	36	69%	26	67%	10	77%	0.73
Premenopausal	16	31%	13	33%	3	23%	0.69
BMI (kg/m²)							
Mean ± std	27.0 ± 5.0		26.8 ± 4.9		27.6 ± 5.4		0.57
Median (min-max)	26.1 (17.1 to 39.4)		25.5 (17.1 to 39.4)		27.5 (17.4 to 37.3)		0.40
Multifocal/multicentric							
No	41	79%	30	77%	11	85%	0.71
Yes	11	21%	9	23%	2	15%	0.65
Clinical tumour size (mm)							
Mean ± std	34 ± 14		34 ± 12		35 ± 19		0.69
Median (min-max)	31 (16 to 80)		31 (16 to 70)		30 (18 to 80)		0.88
Histology							
Ductal	40	77%	33	85%	7	54%	0.20¥
Lobular	10	19%	6	15%	4	31%	0.14¥
Apocrine	1	2%	-	-	1	8%	
Mucinous (colloid)	1	2%	-	-	1	8%	
Grade							
1	8	16%	6	15%	2	17%	0.08§
2	35	69%	29	74%	6	50%	0.61§
3	8	16%	4	10%	4	33%	
Missing	*1*						
cT							
T1c	4	8%	3	8%	1	8%	0.82
T2	43	83%	33	85%	10	77%	0.53
T3	5	10%	3	8%	2	15%	
cN							
N0	33	63%	24	62%	9	69%	0.75
N1	19	37%	15	38%	4	31%	0.45
TNM							
IA	2	4%	1	3%	1	8%	0.29
IIA	31	60%	25	64%	6	46%	0.51
IIB	16	31%	10	26%	6	46%	
IIIA	3	6%	3	8%	-	-	

Table 11: Clinical characteristics at inclusion of all subjects with available central review of the tumour's Ki67 at surgery (n=52), those with sensitive (Ki67 ≤2.7%, n=39), and those with resistant tumours (Ki67 >2.7%, n=13).
*For the comparison of subjects with sensitive (Ki67 ≤2.7%, n=39) *vs* resistant tumours (Ki67 >2.7%, n=13); P-values in green = when tested in subjects receiving bridge period, *i.e.* palbociclib plus

hormone therapy until the day before surgery (N = 41 subjects, of whom 10 with Ki67 at surgery >2.7%)
¥ For the comparison between ductal *vs* lobular
§For the comparison of grade 3 *vs* grade 1/2
BMI: body mass index; std: standard deviation;

There were no significant differences in the proportion of baseline TILs or PD-L1 between those with sensitive *vs* resistant tumours:

	All with Ki67 (N = 52)		**Ki67 at surgery ≤2.7% (N = 39)**		**Ki67 at surgery >2.7% (N = 13)**		**P-value***
Baseline TILs (%)							
N	51		39		12		0.76
Mean ± std	4.1 ± 11.2		2.7 ± 7.0		8.3 ± 19.5		0.89
Median (min-max)	0 (0 to 50)		0 (0 to 30)		0 (0 to 50)		
Baseline PD-L1 (%)							
0	44	94%	33	94%	11	92%	0.77
1	1	2%	1	3%	-	-	0.72
10	1	2%	-	-	1	8%	
20	1	2%	1	3%	-	-	
Missing	*5*						

Table 12: Proportion of baseline tumour infiltrating lymphocytes (TILs) or PD-L1 of all subjects with available central review of the tumour's Ki67 at surgery (n=52), those with sensitive (Ki67 ≤2.7%, n=39), and those with resistant tumours (Ki67 >2.7%, n=13).
*For the comparison of subjects with sensitive (Ki67 ≤2.7%, n=39) *vs* resistant tumours (Ki67 >2.7%, n=13); P-values in green = when tested in subjects receiving bridge period, *i.e.* palbociclib plus hormone therapy until the day before surgery (N = 41 subjects, of whom 10 with Ki67 at surgery >2.7%)

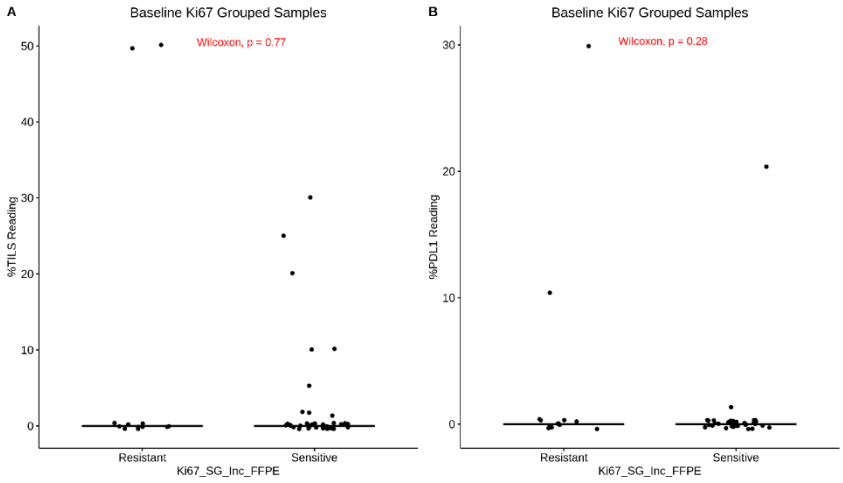


Figure 7: Distribution of the % of tumour infiltrating lymphocytes (TILs; left panel; n=51 subjects) and PD-L1 (right panel; n=47 subjects) among subjects with resistant tumours (Ki67 >2.7%) *vs* sensitive tumours (Ki67 ≤2.7%).

Results: RNA-seq at baseline

We have analysed several single genes and gene expression signatures, involving two broad biological categories: i) cell cycle / proliferation and ii) immunity. In addition, we also classified tumours according to the PAM50 algorithm and according to the 11-gene signature profile A *vs* L+H. The specific gene and gene signatures are detailed in the following table:

	Sub-categories	Single Genes	Gene Signatures
Cell cycle / proliferation	Cell cycle	*CCNE1, RB1, CDK6, E2F3*	
	Proliferation	*MKI67, FAT1*	GGI
	Oestrogen signalling	*ESR1*	
	Growth factor receptors and cytoplasmic signalling	*PTEN, ERBB2*	PIK3CA_GS, AKTmTOR, MAPK
	11-gene signature[1]	*CCNE1, CDKN2A, NUP155, CCDC99 (*also named *SPDL1), TIMM17A, TAGLN2, RAB31, GSN, TP53TG1, FBXL5, PPP1R3C*	
Immunity	Immune-related (general)	*STAT1*	TLS, TGFB, IL12
	Adaptive immune cells		TCell, CD8 TCell, Cytotoxic Cells, TFH, Th1, Th2, Th17, BCell
	- CD8+ T-cell cytolytic activity	*PRF1, GZMA, GZMB*	
	- T-regulatory cells	*FOXP3*	
	- Immune checkpoints	*CTLA4, PDL1*	
	- T-helper phenotype 1		IFNG, IL4
	Innate immune cells	*ARG1, CCL2*	Dendritic cells, Neutrophils, Macrophages, NK cells, Eosinophils, Mast cells

Table 13: Single genes and gene signatures expression assessed by RNA-sequencing in the translational research analysis.

Among “all-comers” (n=73), the distribution of PAM50 subtypes at baseline was the following: 56% of the subjects had Luminal A, 37% had Luminal B, 4% had Normal-like, 1% had HER2-enriched and 1% had Basal-like tumours.

In terms of the 11-gene signature profiles, the majority (89%) of tumours were classified as Profile L (“sensitivity to Palbociclib, low proliferation”), 7% were Profile H (“sensitivity to Palbociclib, highly proliferative”) and only 4% were Profile A (“resistance to Palbociclib”).

1) According to US assessment

Among the 72 subjects evaluable by US and with available baseline RNA-seq data, higher *CDK6* expression was significantly associated with SD/PD by US, while higher *NUP155* expression was associated with CR/PR by US:

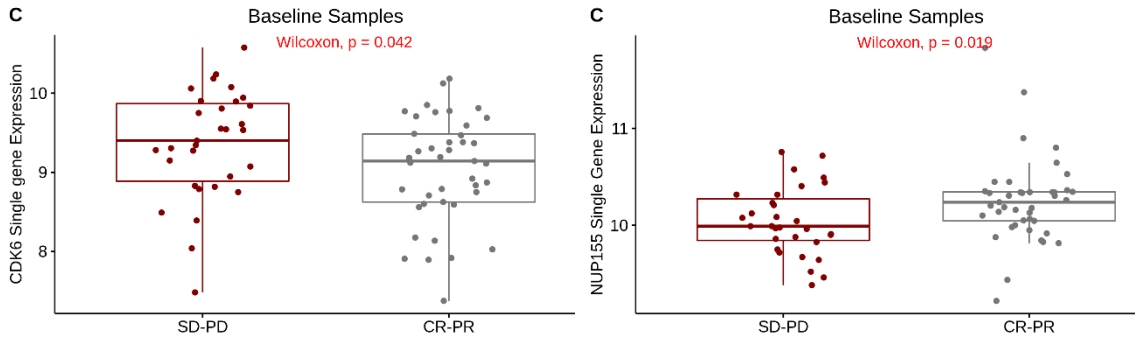


Figure 8: *CDK6* (left panel) and *NUP155* (right panel) gene expression at baseline (n=72) according to US assessment (stable disease [SD]/progressive disease [PD] *vs* complete response [CR]/partial response [PR]).

2) According to RCB

Among the 73 subjects evaluable for RCB and with available baseline RNA-seq data, higher *PIK3CA* gene signature and *Th17* gene signature expression were significantly associated with RCB III:

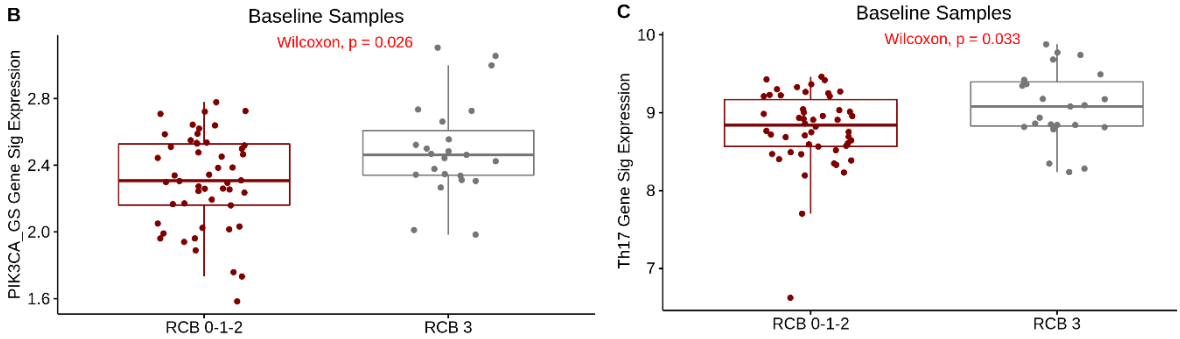


Figure 9: PIK3CA gene signature (left panel) and Th17 gene signature (right panel) expression at baseline (n=73) according to RCB 0-II *vs* III.

On the other hand, a higher *SPDL1* single gene expression was significantly associated with RCB 0-II:

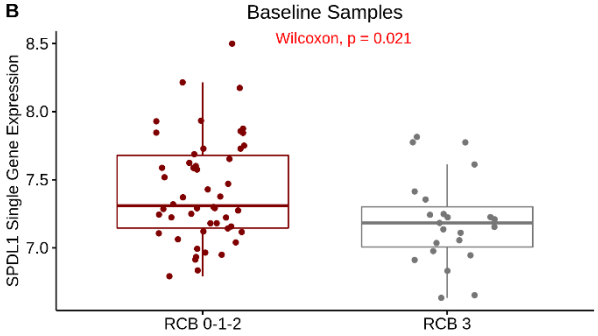


Figure 10: *SPDL1* single gene expression at baseline (n=73) according to RCB 0-II *vs* III.

3) According to Ki67 response

At baseline, there were no significant differences between the proportion PAM50 subtypes or of 11-gene signature profiles according to Ki67 response:

	All with Ki67 (N = 52)		Ki67 at surgery ≤2.7% (N = 39)		Ki67 at surgery >2.7% (N = 13)		P-value*
PAM50 subtypes							
HER2-enriched	1	2%	-	-	1	8%	0.06
Luminal A	31	60%	26	67%	5	38%	0.11
Luminal B	18	35%	11	28%	7	54%	
Normal	2	4%	2	5%	-	-	
11-gene signature profiles							
A	1	2%	-	-	1	8%	0.15
H	2	4%	1	3%	1	8%	**0.05**
L	49	94%	38	97%	11	85%	

Table 14: Proportion of baseline PAM50 subtypes and 11-gene signature profiles among subjects with available central review of the tumour's Ki67 at surgery (n=52), those with sensitive (Ki67 ≤2.7%, n=39), and those with resistant tumours (Ki67 >2.7%, n=13).
*For the comparison of subjects with sensitive (Ki67 ≤2.7%, n=39) *vs* resistant tumours (Ki67 >2.7%, n=13); P-values in green = when tested in subjectsreceiving bridge period, *i.e.* palbociclib plus hormone therapy until the day before surgery (N = 41 subjects, of whom 10 with Ki67 at surgery >2.7%)

Regarding the case of a subject with HER2-enriched subtype at baseline, the local HER2 score by IHC at baseline was 0. Therefore, we repeated the IHC analysis at the Pathology Department of Institut Jules Bordet, under CAP recommendations and ISO15189 certification. The results were the following:

- baseline tumor sample: confirmed HER2 IHC score of 0 (zero) in the entire sample;

- surgical tumor sample: most of the sample had a score of zero, but there was a very small focus of invasive tumor with 1.77 mm that had a HER IHC score of 2+. We have therefore performed a FISH analysis that revealed that this microfocus was indeed HER2-amplified (HER2/CEP17 ratio = 8.28; average HER2 copy number = 14.5 signals per cell). We have communicated these findings to the Site.

We then compared the expression of single genes and gene expression signatures at baseline (n=52 subjects) between resistant *vs* sensitive tumours by Ki67. Solely high *CDK6* expression was associated with resistance to neoadjuvant palbociclib plus HT:

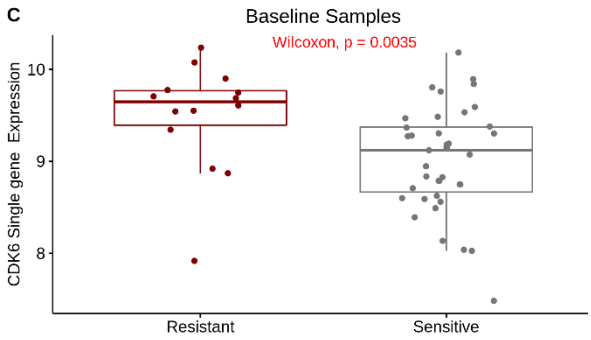


Figure 11: *CDK6* gene expression at baseline (n=52) among subjects with resistant (Ki67 >2.7%) *vs* sensitive tumours (Ki67 ≤2.7%).

The evaluation of the other genes and gene signatures is provided in the **Appendix**.

4.3 Results: changes in immune cells and tumor transcriptional programs after treatment

Afterwards, we analysed changes in TILs and PD-L1 by IHC and RNA-seq data between baseline and surgery: 1) among all subjects included in the TR analysis ("all-comers") and 2) according to Ki67 response at surgery.

Changes in TILs and PD-L1 between baseline and surgery

When looking for the evolution of TILs and PD-L1 by IHC between baseline and surgery among all subjects included in the TR analysis, no significant differences were observed:

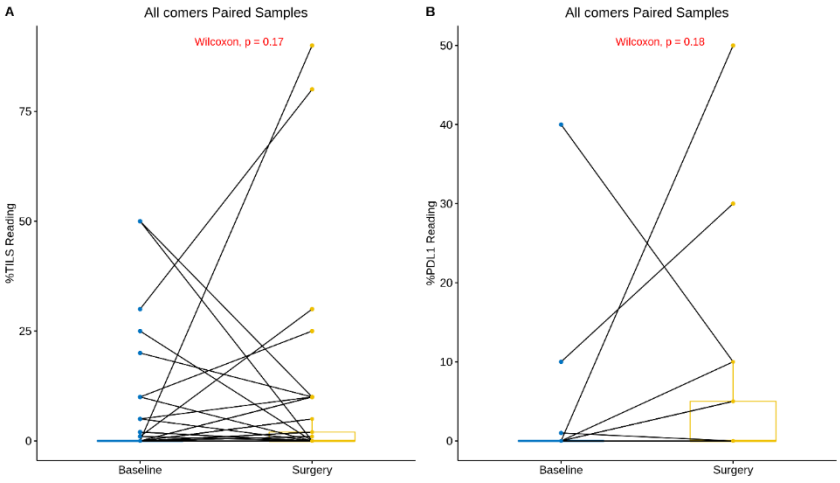


Figure 12: Changes in the % of tumour infiltrating lymphocytes (TILs; n=62, left panel) and in PD-L1 expression (n=23, right panel) between baseline and surgery among all-comers.

In terms of changes of the % of TILs or PD-L1 between baseline and surgery according to Ki67 response, there were no significant changes either in the sensitive or in the resistant groups:

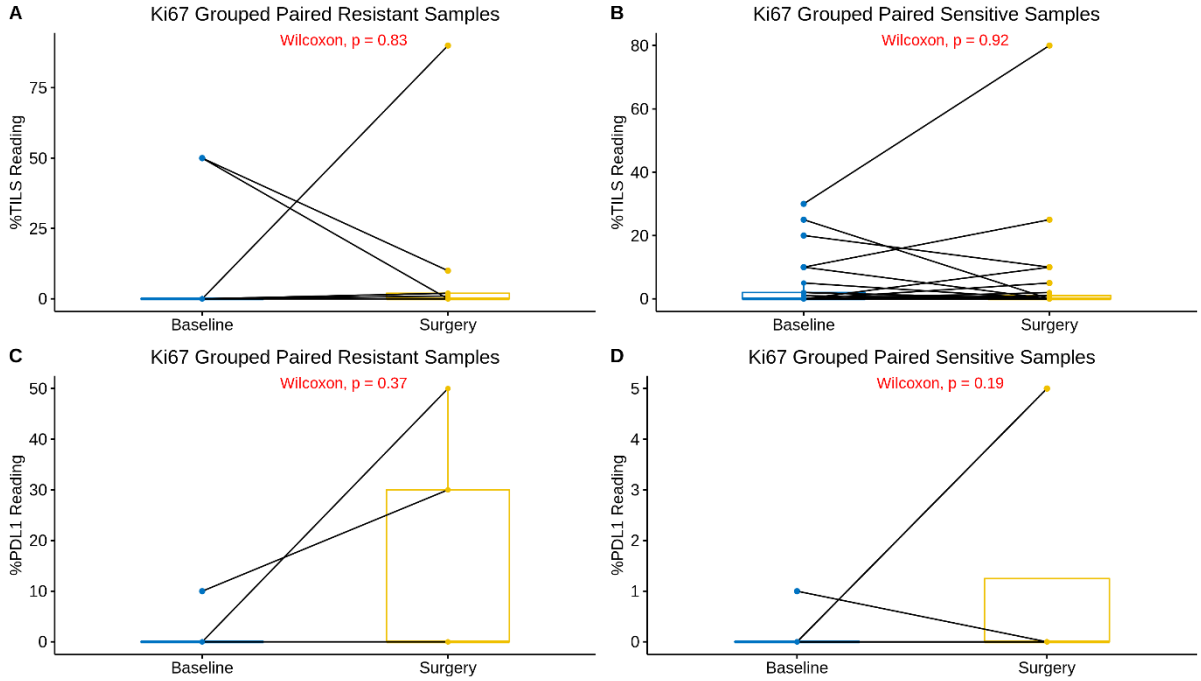


Figure 13: changes of the % of tumour infiltrating lymphocytes (TILs; n=51 subjects) between baseline and surgery in the resistant group (panel A) and in the sensitive group (panel B); and in PD-L1 expression (n=20) between baseline and surgery among all comers in the resistant group (panel C) and in the sensitive group (panel D)

Changes in RNA-seq between baseline and surgery

We then evaluated changes in gene expression from baseline to surgery in all subjects with available RNA-seq from paired baseline and surgery samples, regardless of the response (“all-comers”; n=31) and among subjects with sensitive tumours (Ki67 ≤2.7%; n=19).

Regarding PAM50 subtypes, we have seen an increase in the number of tumours classified as Luminal A, most of which were classified as Luminal B at baseline (subtype switch):

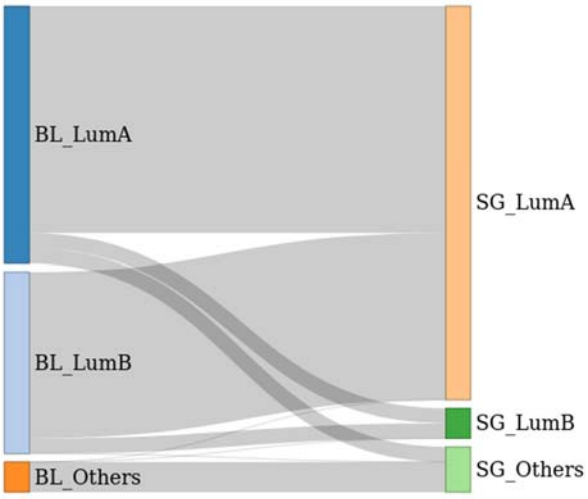


Figure 14: Changes in the distribution of PAM50 subtypes between baseline (BL) and surgery (SG) samples among “all-comers” (n=31). LumA: Luminal A subtype; LumB: Luminal B subtype.

Regarding the 11-gene signature profiles, most tumours conserved the profile L from baseline to surgery (*i.e.* a sensitivity profile), but there were two tumours that switched from profile L to profile A (*i.e.* a resistance profile):

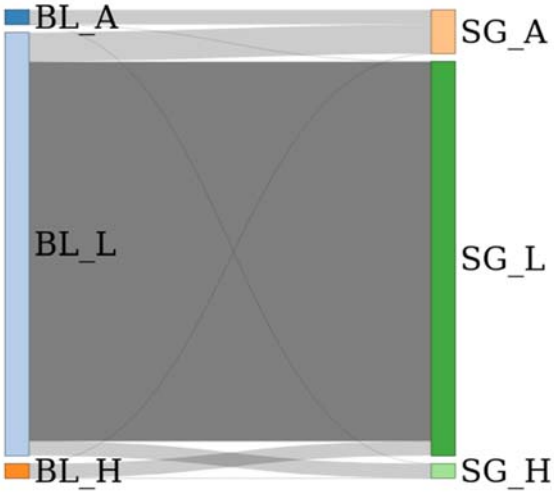


Figure 15: Changes in the distribution of the 11-gene signature profiles between baseline and surgery samples among “all-comers” (n=31)

We have also verified that several genes and gene signatures appeared to have significantly increased or decreased expression between baseline and surgery, both among “all-comers” and among sensitive subjects. The summary of these changes is provided in the following table:

Changes in expression	Among “all-comers” (n=31)	Among sensitive subjects (n=19)	Gene/gene signature function
Cell cycle / proliferation-related genes and gene signatures			
Increased	*PTEN* *GSN**	*PTEN* *GSN**	Cytoplasmic signaling Cell motility

	*CDKN2A**	*CDKN2A**	Cell cycle
Decreased	*CCNE1* *E2F3* *ESR1* *MIK67* *NUP155** *RAB31** *SPDL1** Genomic grade index (GGI) GS PIK3CA GS	*CCNE1* *E2F3* *ESR1* *MKI67* *NUP155** *TAGLN2** *SPDL1** Genomic grade index (GGI) GS AKTmTOR GS	Cell cycle Cell cycle Estrogen signaling Proliferation Intracellular signaling Cell cycle Cell cycle Cell cycle Proliferation Cytoplasmic signaling Cytoplasmic signaling
Immune-related genes and gene signatures			
Increased	*CCL2* *GZMA* *GZMB* Cytotoxic cells GS T cells GS CD8 T cell GS IL12 GS Tertiary lymphoid structures (TLS) GS Th1 GS B cells GS Dendritic cells GS Eosinophils GS Mast cells GS	*CCL2* *GZMB* Cytotoxic cells GS CD8 T cell GS IL12 GS Tertiary lymphoid structures (TLS) GS Th1 GS B cells GS Dendritic cells GS Eosinophils GS Mast cells GS NK cells GS *PD-L1* Follicular B helper T cells (TFH) GS	Innate immune cells CD8+ T-cell cytolytic activity CD8+ T-cell cytolytic activity Adaptive immune cells Adaptive immune cells Adaptive immune cells Immune-related (general) Immune-related (general) Adaptive immune cells Adaptive immune cells Innate immune cells Innate immune cells Innate immune cells Innate immune cells Immune checkpoints Adaptive immune cells
Decreased	Th2 GS	Th2 GS	Adaptive immune cells

Table 15: Description of significant changes in the expression of genes and gene-signatures between baseline and surgery among “all-comers” (n=31) and among subjects with sensitive tumours (n=19).
*Single genes that are included in the 11-gene signature.
GS: gene signature

The graphs corresponding to these significant increases or decreases from baseline to surgery among “all-comers” are hereby reported:

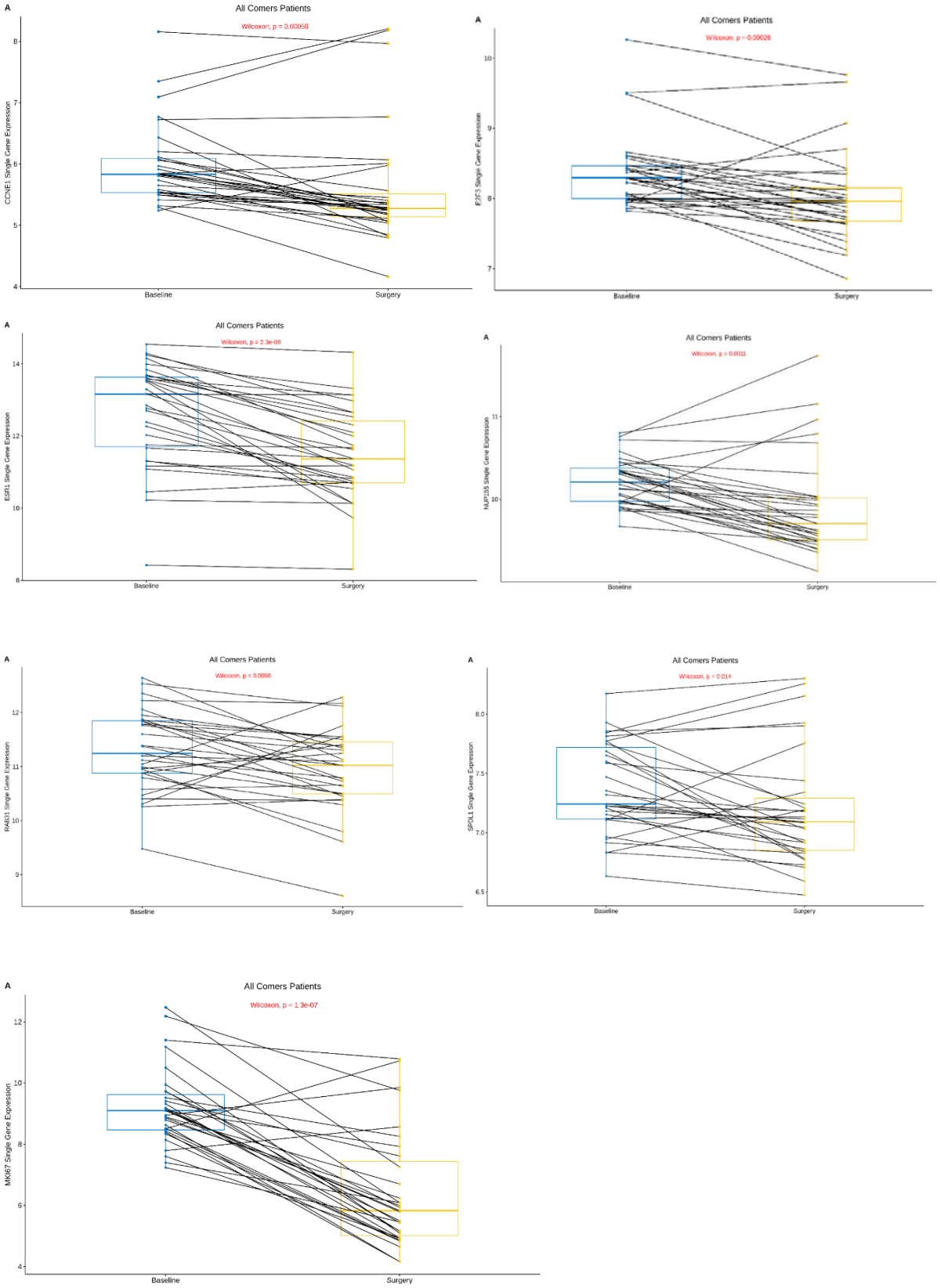


Figure 16: Changes in the *CCNE1*, *E2F3*, *ESR1*, *NUP155*, *RAB31*, *SPDL1* and *MIK67* single gene expression between baseline and surgery among “all-comers” (n=31).

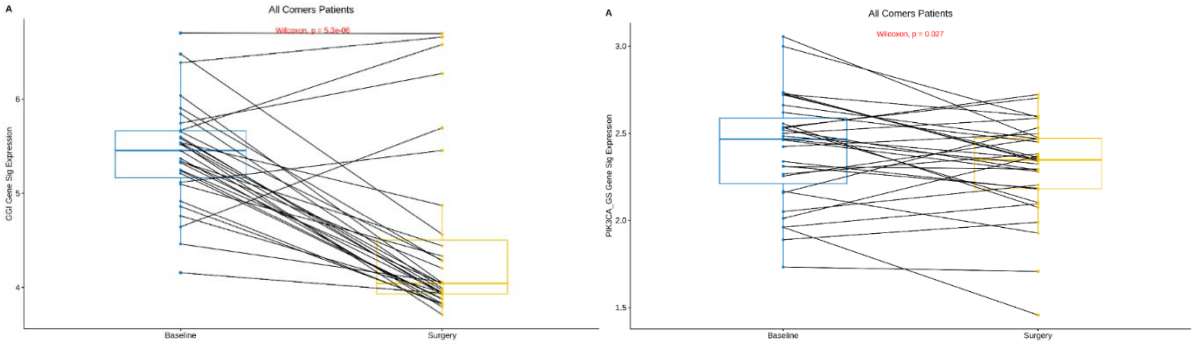


Figure 17: Changes in the GGI and PIK3CA gene signature expression between baseline and surgery among “all-comers” (n=31).

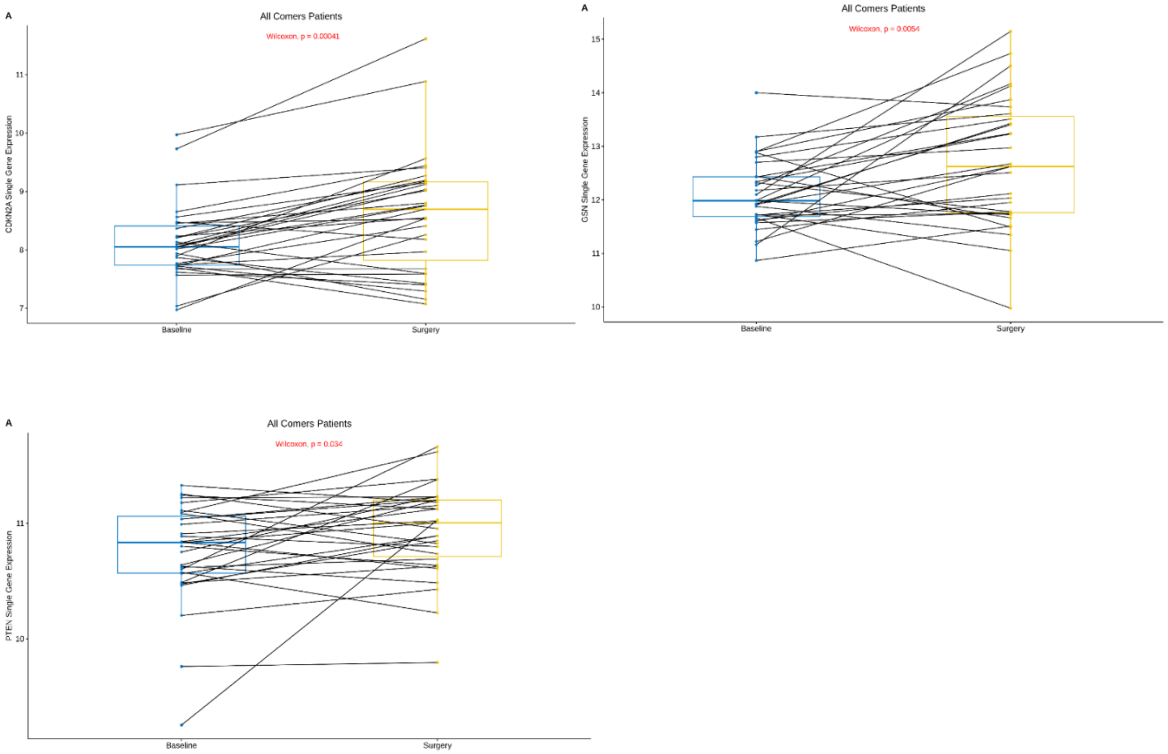


Figure 18: Changes in the *CDKN2A, GSN* and *PTEN* single gene expression between baseline and surgery among “all-comers” (n=31)

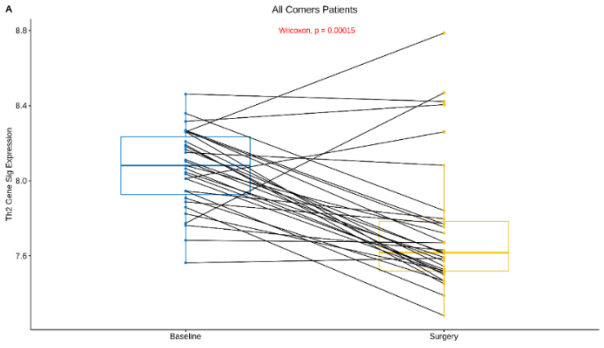


Figure 19: Changes in the Th2 gene signature expression between baseline and surgery among “all-comers” (n=31)

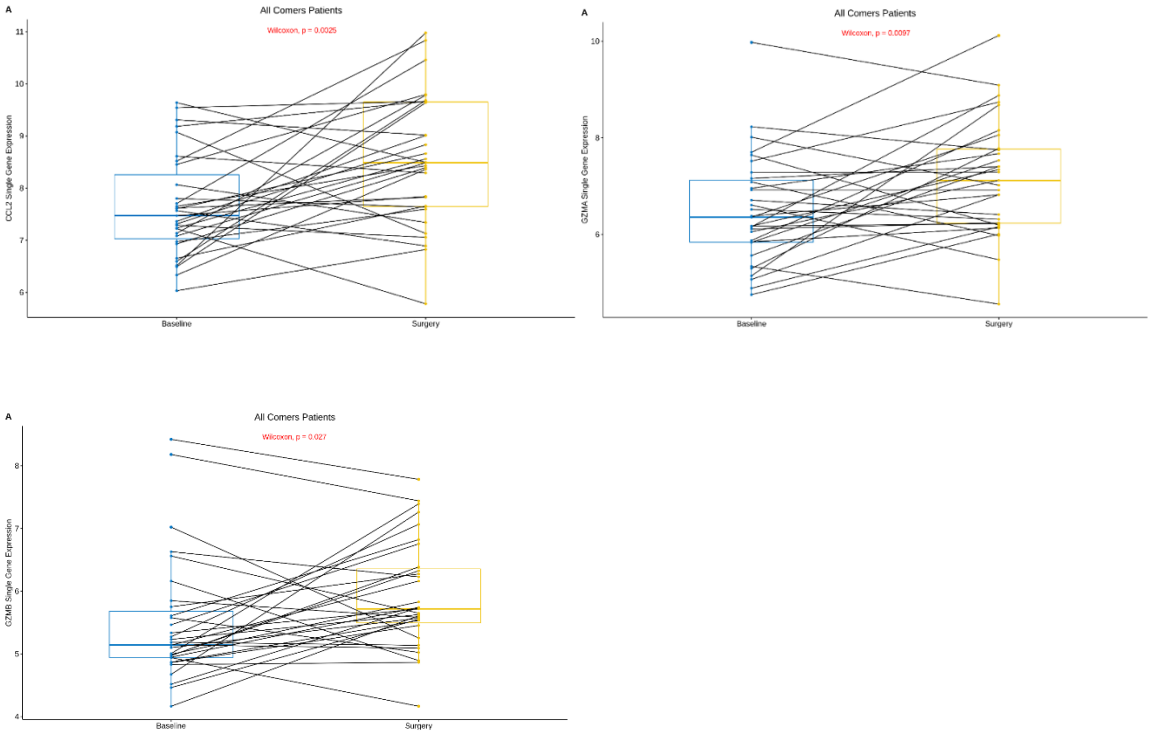
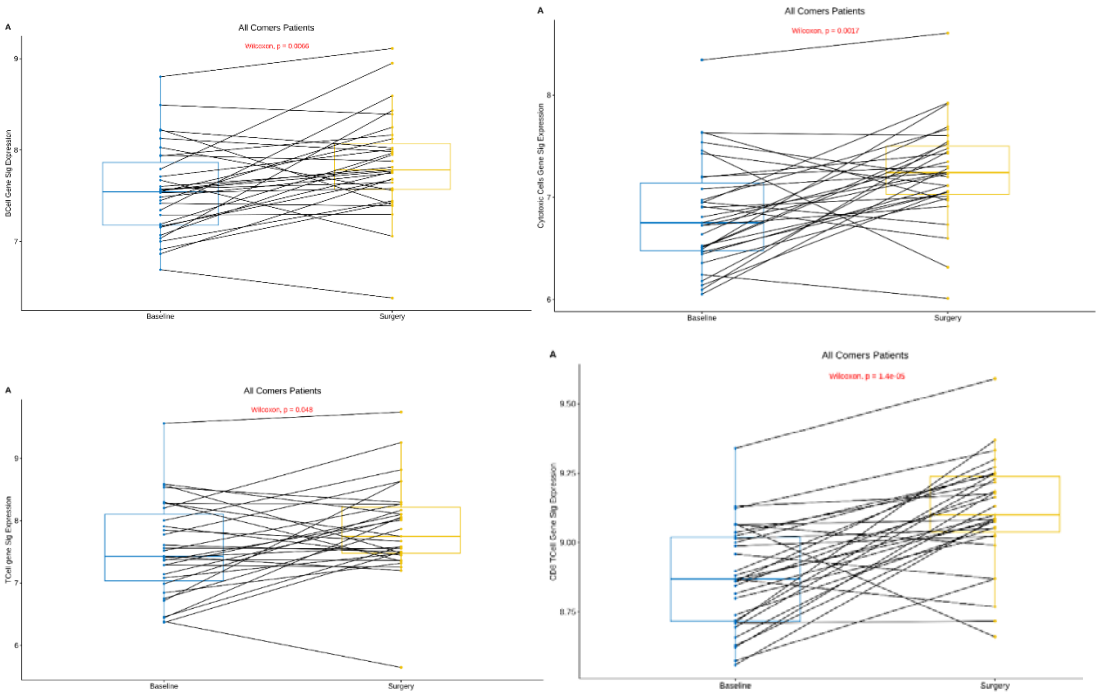


Figure 20: Changes in the *CCL2, GZMA* and *GZMB* single gene expression between baseline and surgery among “all-comers” (n=31)



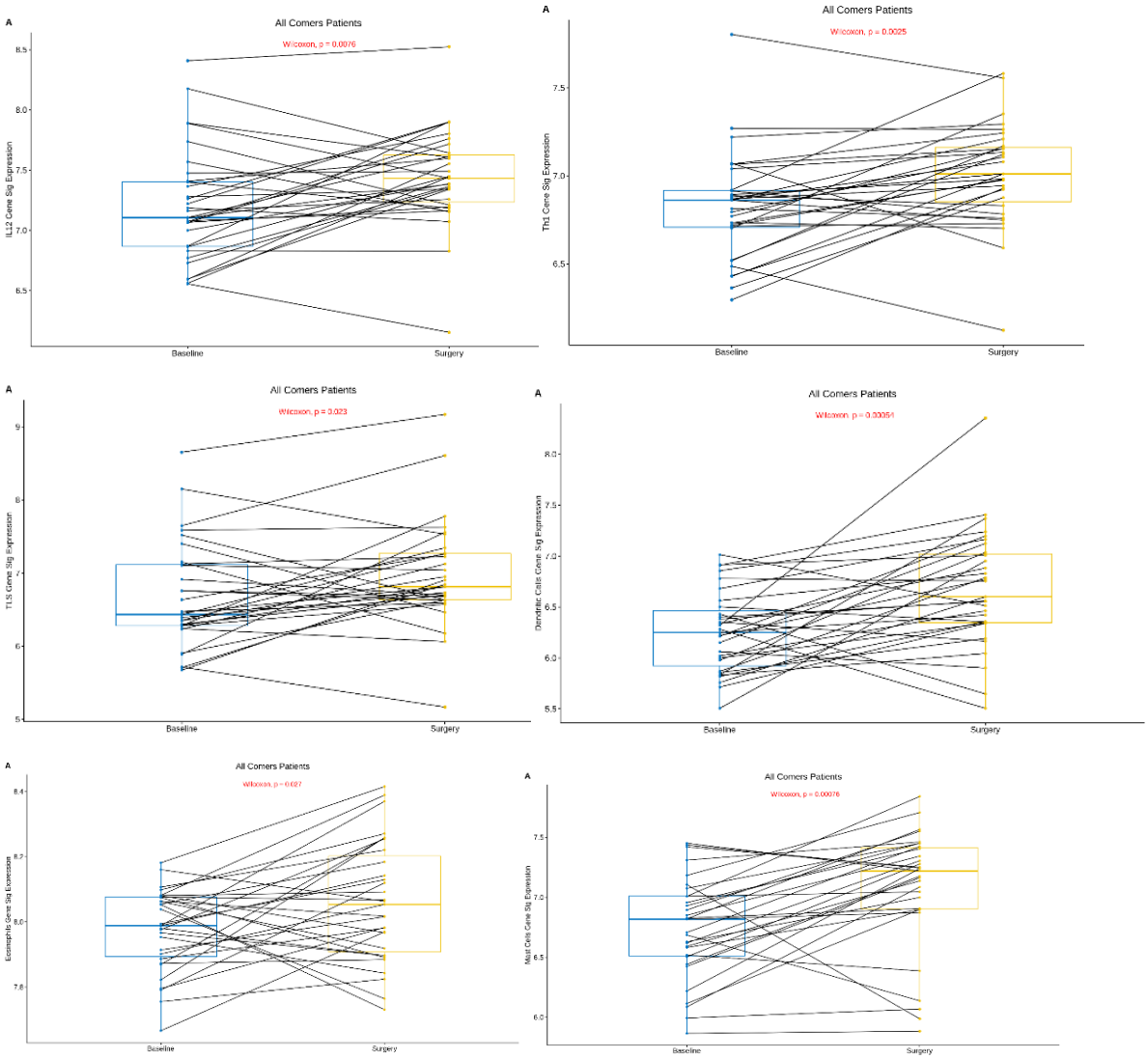


Figure 21: Changes in B cell, cytotoxic cells, T cells, CD8 T cell, Il12, Th1, TLS, dendritic cells, eosinophils and mast cells gene signature expression between baseline and surgery among “all-comers” (n=31).

All figures regarding significant changes in RNA-seq among sensitive subjects are reported in the **Appendix**.

4.4 Results: plasma circulating tumor DNA and association with treatment response/survival outcomes

Plasma samples were collected at four timepoints: baseline (BL), after treatment cycle (C1D28), before surgery (Surgery) and one month post Surgery (End of Study).

ctDNA detection was evaluated using the personalized RaDaR® assay by NeoGenomics Inc. Whole-exome sequencing (WES) was performed on BL tumor biopsies followed by a personalized assay development tracking up to 48-subject specific somatic variants in plasma cell-free DNA (cfDNA) using next generation sequencing.

Associations between ctDNA detection and clinicopathological characteristics at BL were investigated.

Moreover, associations between ctDNA detection at different time points and clinical outcome measures shown below were also investigated:

- Ultrasound response based on WHO criteria evaluation with responders defined as subjects with complete or partial response while non-responders as subjects with stable or progressive disease,
- Complete cell cycle arrest (CCCA) defined as Ki67 ≤2.7% at surgery (Ki67 at SG),
- Residual cancer burden (RCB) 0/I/II (Low) vs III (High) and
- Breast cancer free survival (BCFS) with events being locoregional or distant relapses

The NeoRHEA study enrolled 100 subjects, of which 80 subjects and 313 plasma samples were selected for RaDaR testing. Among these 80 subjects, **78 subjects and 302 plasma samples** met the QC thresholds and were successfully profiled using the RaDaR assay.

Out of the 78 subjects, 42 (53%**) were found to be ctDNA positive at BL**, 4 (5%) subjects were also ctDNA positive at C1D28 with 3 of them still ctDNA positive at SG. A fourth subject was ctDNA positive at SG, but not in the earlier C1D28 timepoint. However, none of the subjects were ctDNA positive at the end of the study.

34 subjects tested ctDNA negative at all timepoints (AlwaysNegative), 35 tested positive only at BL (BLpositiveThenNegative) and 5 tested positive both at BL and at one or more subsequent timepoints (BLpositiveThenPositive) .

RaDaR is calculating the estimated variant allele fraction (eVAF) using a proprietary algorithm; the %**eVAF range** for the positive ctDNA samples was between **9.93E-04 and 9.12E-01** with a median of 2.63E-02.

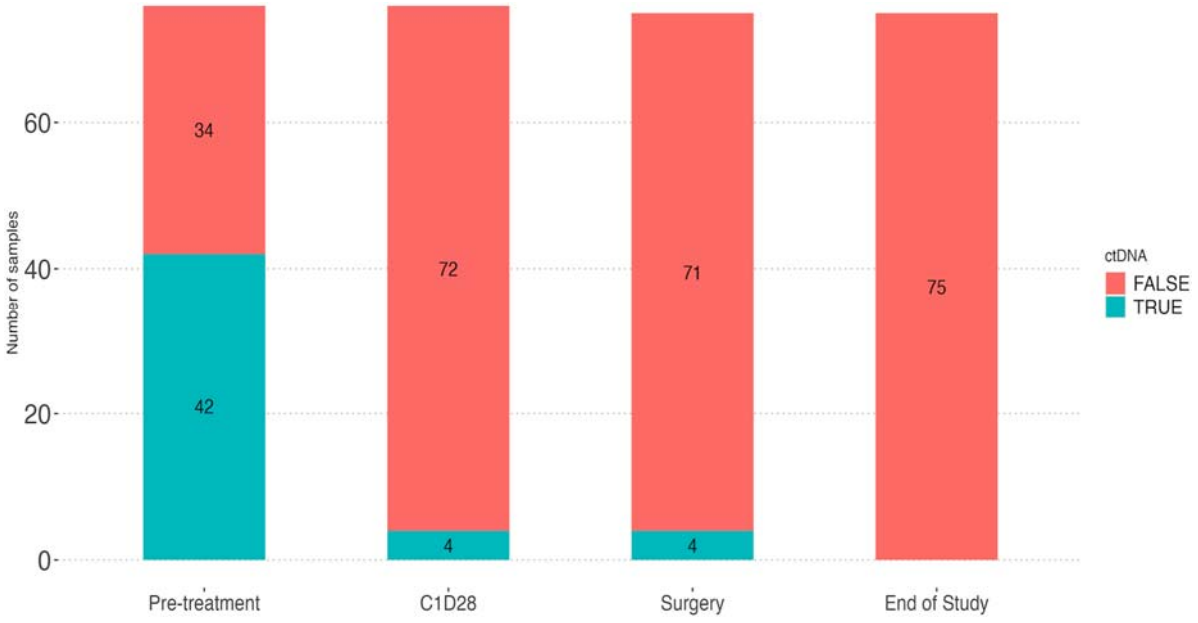


Figure 22. ctDNA detection by timepoints. Y-axis represents the number of subjects analysed and X-axis the four timepoints tested. Each number on the bars represents the absolute number of subjects in the specific category (TRUE - ctDNA detection / ctDNA FALSE - no ctDNA detection).

BL Clinicopathological characteristics and ctDNA detection at BL

In our cohort of 78 subjects, 67% were postmenopausal (52), 47% were Luminal A (based on PAM50 gene expression), 75 % had cT2 (59) and 69% cN0 tumors (54), 20% had multifocal/multicentric tumors, 16% had histological grade 3 tumors.

BL ctDNA detection rates were associated **with grade 3 tumors** (p=0.03). In contrast, multifocal/multicentric tumors had lower baseline detection rates (p=0.01).

No significant associations were found for menopausal status, clinical nodal status nor clinical tumor size.

We assessed the correlation between BL %eVAF percentage and clinical/pathological characteristics but did not observe any significant associations.

ctDNA detection and clinical outcome

Subjects grouped by ctDNA detection were tested for association with response (as defined previously) using Fisher's exact test.

Grouping subjects based on ctDNA monitoring is associated with Ultrasound response and RCB class, but not with Ki67 at SG.

Patients Group	RCB Low	RCB high	Ultrasound Responder	Ultrasound Non-responder	Ki67 at SG <=2.7%	Ki67 at SG >=7.4%	Ki67 at SG Indecisive
AlwaysNegative (n = 34)	28	6	17	17	20	3	5
BLpositiveThenNegative (n = 35)	20	15	22	13	21	3	0
BLpositiveThenPositive (n = 5)	2	3	0	5	2	2	0
Fisher's exact test p-value	0.0241		0.024		0.0508		

Figure 23. The table contains on the first column the subjects group definition and all the other columns contains the outcome measurements. On the rows, the number of subjects within each group and each response category is shown. The last row is presenting the Fisher's exact test p-values for all groups per clinical outcome.

With a median follow-up of 3.8 years (range 1-5 years), 4 subjects developed distant and one subject locoregional recurrences.

ctDNA detection after one month of treatment (logrank p=0.02) was associated with worse BCFS, but not at baseline nor at surgery (p=0.59,p=0.67).

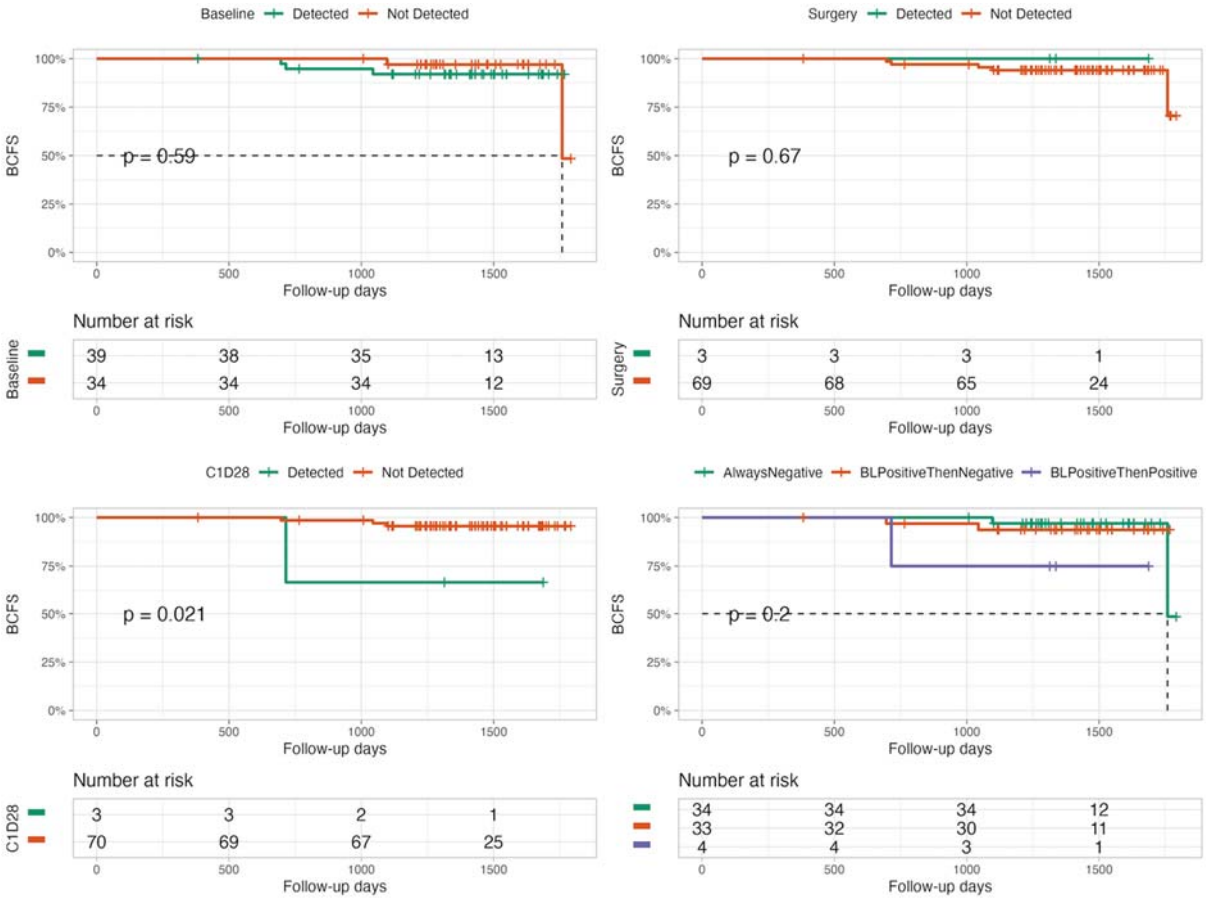


Figure 24. Kepler Meier curves with BCFS events for the three timepoints (BL, C1D28 and Surgery) and for the groups defined above.

In conclusion, our data suggests association of cDNA detection with pathological and clinical variables. CtDNA detection after one month of treatment with Palbociclib and ET was associated with worse outcome. Independent validation is needed.

4.5 Results: Baseline gene expression profiling according to treatment response

We observed an enrichment in inflammatory response, IFN-γ response, and in G2M checkpoint gene sets in subjects with US response (NES: 2.06, 2.30, 1.47; FDR: 7.04e-9, 5.48e-17, 0.0193 respectively); conversely, we observed an enrichment in early estrogen response gene set in subjects with absence of US response (NES: -1.78, FDR: 0.0006) (Table 16).

No signaling pathway was enriched in subjects without CCCA.

Gene Set	NES	FDR
HALLMARK_INFLAMMATORY_RESPONSE Genes defining inflammatory response	2.06	7.04e-9

HALLMARK_INTERFERON_GAMMA_RESPONSE Genes up-regulated in response to INF-gamma	2.30	5.48e-17
HALLMARK_G2M_CHECKPOINT Genes involved in the G2/M checkpoint, as in progression through the cell division cycle	1.47	0.0193
HALLMARK_ESTROGEN_RESPONSE_EARLY Genes defining early response to estrogen	-1.78	0.0006

Table 16. Gene set enrichment analysis according to US response. Abbreviations: NES: normalized enrichment score. FDR: false discovery rate.

Differential gene expression analysis of baseline samples between responders and non-responders revealed very few genes being differentially expressed (Figure S26). A gene set enrichment analysis using RCB 0, I and II as responders and Hallmark gene sets showed that responders were enriched in MYC targets, E2F targets, apoptotic pathways, and inflammatory response.

Using PDL2 staining as a biomarker of response proved to be predictive when response was defined by Ki67 by IHC at surgery as less or equal to 2.7 (Figure S27).

4.6 Single nuclei RNA & ATAC-seq between baseline and surgery samples

Furthermore, we analysed single nuclei data of 37 subjects from the NeoRHEA cohort out of which 26 were paired between baseline and surgery samples. We used the 10x Chromium Single Cell Multiome ATAC + Gene Expression kit. Frozen optimal cutting temperature (OCT) embedded baseline (pre-treatment) biopsy and surgery tumor samples with at least 25% tumor cellularity were selected for single nuclei RNA-seq and ATAC-seq analysis. Briefly, samples were processed into the single nuclei dissociation with protocol C “Multiome” (adapted version of “Nuclei Isolation from Complex Tissues for Single Cell Multiome ATAC + Gene Expression Sequencing”) and underwent single nuclei RNA-seq and ATAC-seq using Chromium Next GEM Single Cell. To arrive to count matrices for both RNA-seq and ATAC-seq data, various components of Cell Ranger ARC v2.0, provided by 10X Genomics, were used. More specifically, in order to demultiplex raw base call files generated by Illumina sequencers “cellranger-arc mkfastq” was used for both ATAC and RNA flow cells. Doing that resulted into a handful of fastq files for each sample which were then used as an input to “cellranger-arc count”. The latter trimmed and aligned all fastq files to the reference genome in use and then performed filtering, peak calling and counting for both the ATAC and RNA molecules. Doing these steps, we produced count matrices for both the RNA-seq and

ATAC-seq samples. Having arrived at count matrices for each cell, RNA counts were processed with the standard Seurat workflow (normalize data, find variable genes, scale data, find cell clusters and lastly run dimensionality reduction projections such as PCA, t-SNE and UMAP) while ATAC counts were processed again with Signac by finding the most frequently observed peaks, calculating singular value decompositions and dimensionality reduction projections. Before normalizing any of the produced count matrices we first applied some quality control filters to discard bad quality cells. More specifically, we discarded cells displaying less than 400 or more than 10,000 UMIs, less than 200 or more than 25,000 identified genes, more than 2% mitochondrial genes and more than 1% ribosomal genes concerning the RNA assay as well as cells with less than 1,000 or more than 100,000 UMIs concerning the ATAC assay. Having predicted cell type annotation for the RNA partition of our data, the single nuclei ATAC-seq had predicted annotations transferred from the single nuclei RNA-seq dataset. Cell types were identified by expression patterns of known in the literature biomarkers and tumor cells were identified by profiling the copy number aberrations of epithelial identified cells via infercnv. Concerning the ATAC single nuclei assay, peaks were called for all cells after quality control filtering via MACS2. After quantifying reads in the ATAC single nuclei assay we were able to assess the activity of transcription factor motifs by cell utilizing chromVAR using motifs from the JASPAR 2022 transcription factor database.

After this processing differential tests comparing genes and peaks between baseline and surgery conditions were performed. These included differential gene expression analyses, differential peak accessibility analyses, differential motif activation analyses as well as subsequent overexpression and gene set enrichment analyses. Lastly, we performed an analysis linking peaks with genes that were concurrently expressed and accessible.

Hallmark gene set ID	**NES**	**FDR**	**Description**
TNFA_SIGNALING_VIA_NFKB	2.44505681586732	1e-09	Genes regulated by NF-kB in response to TNF [GeneID=7124].
HYPOXIA	1.94950257333522	7.1750708198312e-09	Genes up-regulated in response to low oxygen levels (hypoxia).
EPITHELIAL_MESENCHYMAL_TRANSITION	1.86660826043666	1.6964441363896e-07	Genes defining epithelial-mesenchymal transition, as in wound healing, fibrosis and metastasis.
ALLOGRAFT_REJECTION	1.794473498581	9.7012543208994e-06	Genes up-regulated during transplant rejection.
INFLAMMATORY_RESPONSE	1.79183551720715	3.4366243317197e-06	Genes defining inflammatory response.
P53_PATHWAY	1.77064776632305	5.6215931171447e-06	Genes involved in p53 pathways and networks.
KRAS_SIGNALING_UP	1.76003042528516	9.7012543208994e-06	Genes up-regulated by KRAS activation.
MYOGENESIS	1.70975251342684	4.5770846865858e-05	Genes involved in development of skeletal muscle (myogenesis).
APOPTOSIS	1.70970511299188	7.7129793035669e-05	Genes mediating programmed cell death (apoptosis) by activation of caspases.
OXIDATIVE_PHOSPHORYLATION	1.70774515371057	9.7012543208994e-06	Genes encoding proteins involved in oxidative phosphorylation.

TGF_BETA_SIGNALING	1.669016410302 39	0.004280 9634890 3621	Genes up-regulated in response to TGFB1 [GeneID=7040].
UV_RESPONSE_DN	1.651954475067 98	0.000484 2961221 07375	Genes down-regulated in response to ultraviolet (UV) radiation.
INTERFERON_GAMMA_RESPONSE	1.591183288503 39	0.000532 4368595 79315	Genes up-regulated in response to IFNG [GeneID=3458].
UV_RESPONSE_UP	1.591041096522 69	0.001344 9170911 6859	Genes up-regulated in response to ultraviolet (UV) radiation.
IL6_JAK_STAT3_SIGNALING	1.589954116475 91	0.005889 9807417 8957	Genes up-regulated by IL6 [GeneID=3569] via STAT3 [GeneID=6774], e.g., during acute phase response.
ADIPOGENESIS	1.558707514368 51	0.001206 4203072 5029	Genes up-regulated during adipocyte differentiation (adipogenesis).
COMPLEMENT	1.479125840354 22	0.003983 1930018 1314	Genes encoding components of the complement system, which is part of the innate immune system.
XENOBIOTIC_METABOLISM	1.451559448904 13	0.017206 7599554 067	Genes encoding proteins involved in processing of drugs and other xenobiotics.
IL2_STAT5_SIGNALING	1.381654012500 26	0.029229 7085202 363	Genes up-regulated by STAT5 in response to IL2 stimulation.
SPERMATOGENESIS	-1.397872475395 94	0.032386 7544119 993	Genes up-regulated during production of male gametes (sperm), as in spermatogenesis.
MTORC1_SIGNALING	-1.435167982468 39	0.007285 0142002 2526	Genes up-regulated through activation of mTORC1 complex.
MYC_TARGETS_V2	-1.622730215628 4	0.017206 7599554 067	A subgroup of genes regulated by MYC - version 2 (v2).
MITOTIC_SPINDLE	-2.100341472961 92	1.017441 8625929 9e-08	Genes important for mitotic spindle assembly.
ESTROGEN_RESPONSE_LATE	-2.235477491476 73	1e-09	Genes defining late response to estrogen.
ESTROGEN_RESPONSE_EARLY	-2.475539054012 85	1e-09	Genes defining early response to estrogen.
G2M_CHECKPOINT	-2.890800703510 42	1e-09	Genes involved in the G2/M checkpoint, as in progression through the cell division cycle.
E2F_TARGETS	-2.896898109745 06	1e-09	Genes encoding cell cycle related targets of E2F transcription factors.

Table 16: Results of gene set enrichment analysis using hallmark gene sets of all single nucleis. NES: normalized enrichment score. Positive NES indicates upregulation in surgical samples while negative NES the contrary.

We observed that the majority of cells are labelled as tumor cells, second biggest population is stromal cells with most of these cells being fibroblasts, last immune cells are mostly T and myeloid cells with very few of them being B cells (Figure 25).

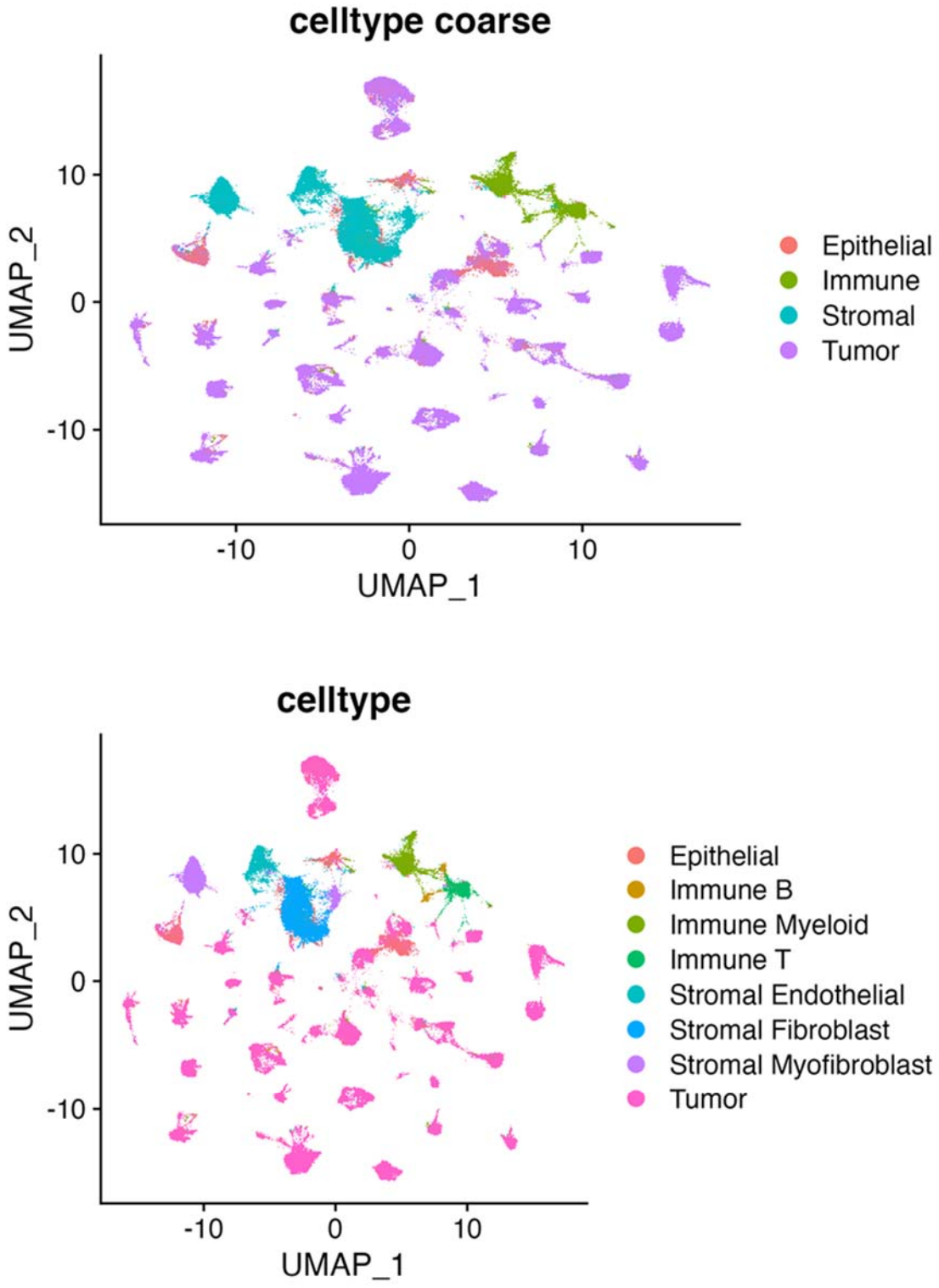


Figure 25. Representation of tumor, immune and stromal subsets.

Estrogen response downregulation is clearly driven by tumor cells only while immune cells upregulate TNFA signaling via NFKb as well as inflammatory response. Proofs of these mechanisms are depicted in both the GSEA of differentially expressed genes as well as the GSEA of differentially activated peaks (Supplementary Table). Peaks are mostly upregulated in all cell types with an exception of immune cells where the percentage of downregulated peaks is larger. Stromal cells seem to upregulate AP-1 related motifs.

5 DISCUSSION

Among all evaluable subjects in this study, the clinical response rate (CR/PR at US) was 55%, 7% achieved a RCB of 0-I and 1% a pathological complete response (pCR - *i.e.* a RCB of 0). Among those with Ki67 available at surgery (n=52), 75% had a CCCA.

Despite the fact that NeoRhea has some differences in terms of trial population (*e.g.* inclusion of pre-menopausal women), HT backbone (*e.g.* possibility of using tamoxifen) and CDK4/6 inhibitor duration (*i.e.* mandatory bridge period), our findings were similar to what has been observed in another trials testing neoadjuvant CDK4/6i plus HT among subjects with HR+/HER2- tumours. All demonstrated very low pCR rates, but with most tumours showing an effective proliferation shutdown (CCCA at surgery) under these agents:

Study	Design	Population	*N*[a]	Treatment arm(s)	pCR rate	Radiol. RR[b]	CCCA at surgery
Ma, 2017 (**NeoPalAna**)[15]	Ph II, single-arm	HR+/HER2-, stage II-III, pre/post-menopausal	39	Anastrozole x 4 weeks → Anastrozole + Palbociclib x 4 cycles	0%	41.0%	NA
Martin, 2017 & Hurvitz 2020 (**neo-MONARCH**)[16,17]	Ph II, rand.	HR+/HER2-, stage I (T≥1cm)-IIIB, post-menopausal	224	Anastrozole vs. Abemaciclib + Anastrozole vs. Abemaciclib x 2 weeks → all followed by Abemaciclib + Anastrozole x 14 weeks	3.7%	46.4%	NA
Chow, 2018 (**N007**)[18]	Ph II, single-arm	HR+/HER2-, T≥2cm, post-menopausal	20	Letrozole + Palbociclib x 4 cycles	5%	70%	40%
Cottu, 2018 (**NeoPAL**)[19]	Ph II, rand.	HR+/HER2-, Lum. A N+ and Lum. B (by PAM50), post-menopausal, stage II-III	52	Letrozole + Palbociclib x 19 weeks	3.8%; RCB 0/I: 7.7%	55.5%	NA
Johnston, 2018 (**PALLET**)[20]	Ph II, rand.	HR+/HER2-, T≥2cm, post-menopausal	204	Letrozole x 2 weeks → Palbociclib + Letrozole x 12 weeks vs. Palbociclib x 2 weeks → Palbociclib + Letrozole x 12 weeks vs. Palbociclib + Letrozole x 14 weeks	1.1%	54.3%	90.4% (overall)
NeoRhea trial	Ph II, single-arm	HR+/HER2-, T≥1.5cm (excluding T4 disease), N0-N1, pre/post-menopausal	97	Letrozole or Tamoxifen (±goserelin) + Palbociclib x 4 cycles → bridge period of 3-7 days	1%	55%	75%

Table 17: Summary of trials testing neoadjuvant CDK 4/6 inhibitors plus hormone therapy among subjects with hormone receptor (HR)-positive/HER2-negative tumours, only displaying the results of the CDK4/6 inhibitor plus hormone therapy arm(s).
CCCA: complete cell cycle arrest; Lum.: Luminal; N+: metastatic lymph nodes; NA: not available; pCR: pathological complete response (primary breast tumour and axillary lymph nodes – ypT0/is ypN0); RCB: residual cancer burden.
[a] Number of evaluable subjectsin the arm(s) containing a CDK4/6 inhibitor.
[b] Radiological response rate by ultrasound (according to RECIST criteria)

In terms of safety, the most frequent adverse reactions observed were neutropenia (55%), hot flushes (37%), fatigue (34%), arthralgia (26%) and asthenia (21%). These adverse reactions are in line with the known toxicity profile of palbociclib (*e.g.* neutropenia) and of HT (*e.g.* arthralgia, hot flushes), thus there were no new safety signs identified in this trial.

Baseline biomarkers of treatment outcome

No associations were observed between baseline clinico-pathological characteristics and CCCA, including the proportion of TILs or PD-L1 score. Yet, the majority of subjects had low TILs at baseline (92% with ≤10% of TILs). This is also in line with the knowledge that HR+/HER2- tumours have a lower levels TILs compared to HER2-positive or TNBC[21] and what has also been seen the NeoMonarch trial, in which the amount of TILs was very low at diagnosis.[17]

Most of the PD-L1 assays used in breast cancer, including the Ventana SP142 that we used, take into account the PD-L1 staining in the immune cells.[22] Therefore, given the low amount of TILs at baseline, it is not surprising that the PD-L1 score was also very low among most subjects (90% of them had a score of 0). Yet, this is the first trial assessing PD-L1 expression at baseline in HR+/HER2- tumours of subjects undergoing treatment with CDK4/6i plus HT, thus providing important information regarding its expression.

Besides these markers, we observed no significant difference in CCCA rates according to PAM50 subtype at baseline. In the NeoPalAna study, high rates of CCCA after 15 days of treatment were observed both in luminal A and B tumours, without a significant difference between each subtype.[15] Interestingly, there were only two subjects with non-luminal HR-positive tumours (one with a basal-like and another with a HER2-enriched tumour) and, in both cases, their tumours were resistant to palbociclib plus HT – a similar situation to what we also observed in NeoRhea. In the NeoPAL trial, more than 88% of tumours were luminal B and only 11% were luminal A (*i.e.* n=6 subjects with luminal A tumours, under palbociclib plus HT), thus preventing any conclusion in terms of association between response and intrinsic subtype at baseline. In the NeoMonarch, the intrinsic subtypes were determined in a small subset of subjects and there seemed to be no differences between luminal A *vs* B tumours in terms of CCCA achievement.[17]

Moreover, no significant differences were observed in Ki67 response according to the 11-gene signature.[1] The likely explanation for this is that, in NeoRhea, the 11-gene signature uncovered only one intrinsically “resistant” subjects (*i.e.* with profile A). This means that in our population of subjects with treatment-naïve early breast cancer, the proportion of subjects with intrinsically CDK4/6 inhibitor-resistant disease as identified by the 11-gene signature is very low.

On the contrary, among subjects with available baseline RNA-seq data, higher *CDK6* expression was significantly associated with lack of response to neoadjuvant palbociclib plus

HT, both by US assessment and by Ki67 at surgery. This is consistent with previous data, describing *CDK6* amplification as a potential mechanism of resistance to CDK4/6 inhibition.[23]

Another promising biomarker of resistance is the overexpression of *CCNE1.* In our analysis, *CCNE1* mRNA expression at baseline was not associated with US response, RCB or CCCA. Results from the PALOMA-3 study showed that palbociclib efficacy was lower in subjects with high (*i.e.* over the median) *vs* low (*i.e.* below the median) *CCNE1* mRNA expression (median progression-free survival: 7.6 *vs* 14.1 months in palbociclib arm; 4.0 *vs* 4.8 months in placebo arm, respectively; false discovery rate (FDR)–adjusted p=0.0238), suggesting that tumours with high levels of *CCNE1* expression had a more limited benefit from palbociclib.[24] One may argue that these findings come from a trial enrolling metastatic p subjects, most of them with hormone-resistant tumours, which is very different from the population enrolled in NeoRhea. Yet, those findings were validated in a cohort of 61 subjects from the POP trial, who received neoadjuvant palbociclib for 14 days (*without* HT) until surgery: high *CCNE1* mRNA expression was also associated with poor activity of palbociclib (Ki67 >2.7%).[25] In the neoadjuvant PALLET study, high baseline CCNE1 protein levels measured by IHC were associated with significantly less frequent CCCA in the palbociclib plus letrozole group.[20] However, in the NeoMonarch study, baseline mRNA expression of *CCNE1* was not associated with response to treatment (n=34 subjects).[17]

These apparent contradictions may be related to the fact that *CCNE1* mRNA levels might only partially reflect the real status of CCNE1 activity and other factors, such as different CCNE1 isoforms, localization or proteostasis, could have an important role as well.[26–28]

Moreover, other players might influence response to treatment, such as alterations in the FAT1/Hippo pathway. In a genomic analysis of HR+ breast cancers treated with CDK4/6i, Li et al. identified loss-of-function mutations affecting *FAT1* to be associated to drug resistance.[29] However, in our analysis, no significant differences were observed in *FAT1* expression between sensitive *vs* resistant tumours. Yet, our evaluation was based on mRNA expression, while Li's paper focused on genomic loss.

Changes in immune cells and tumor transcriptional programs after treatment

In terms of cell cycle/proliferation, we observed a decrease in the expression of *CCNE1, E2F3, ESR1, NUP155, RAB31, SPDL1* and *MIK67* single genes and of the GGI and PIK3CA gene signatures, from baseline to surgery, in all subjects with available RNA-seq ("all-comers"), which is consistent with findings from other studies. In the NeoMonarch study, *CCNE1* expression significantly decreased after treatment with abemaciclib, and anastrozole, suggesting again that the treatment combination can down-regulate genes associated with oestrogen signalling and cell cycle.[17] Similarly, in the NeoPalAna trial, among subjects resistant to palbociclib plus ET (Ki67>2.7%, 15 days after treatment), there was a persistently elevated expression of *CCNE1*, while among subjects with sensitive tumours, there was a marked decrease of *CCNE1* expression between baseline and day 15 of treatment.[15] Moreover, we have observed a PAM50 subtype switch from Luminal B to Luminal A in several tumours, which was most likely due to the shutdown in proliferation caused by palbociclib.

In the NeoRhea study, when we explored the evolution of TILs by IHC between baseline and surgery, there were no significant changes in the % of TILs among all-comers nor according to Ki67 response at surgery. This was also the case in the NeoMonarch study (abemaciclib plus HT),[17] in the POP trial (single-agent palbociclib)[25] or in the ABC-POP study (single-agent abemaciclib),[30] which did not find significant overall changes in the % of TILs after treatment.

NeoRhea is the first study to evaluate the evolution of PD-L1 expression by IHC between baseline and surgery among subjects under neoadjuvant CDK4/6i plus HT. Yet, similarly to what we have seen with TILs, there were no significant changes in PD-L1 expression neither in all comers, nor in sensitive and resistant tumours. However, we observed a clear upregulation of *PD-L1* single gene expression and of T cell-related and Th1 immune response-related genes and gene signatures (*CCL2*, *GZMA* and *GZMB* single genes, and B cell, cytotoxic cells, T cells, CD8 T cell, Il12, Th1 and TLS gene signatures), while the immunosuppressive Th2 gene signature decreased after treatment. Furthermore, we observed an increase in the expression of the innate immune cells (dendritic cells, eosinophils and mast cells gene signatures).

Preclinical data suggested that CDK4/6i increases tumour immunogenicity and ultimately promotes cytotoxic T-cell-mediated clearance of tumour cells by two mechanisms: 1. suppressing the proliferation of immunosuppressive regulatory T cells, and 2. activating tumour cell expression of endogenous retroviral double-stranded RNA, which enhances production of type III interferon and ultimately antigen presentation.[31] In the NeoMonarch trial, gene expression analysis using RNA-seq also revealed an upregulation of inflammatory and T cell-related pathways by gene set enrichment analysis in a limited subset of samples of subjects treated with abemaciclib plus HT.[17] More marked upregulation of IFN-gamma, APC-presentation and *PD-1* at the end of treatment were observed, although only among subjects who received the lead-in abemaciclib plus anastrozole (n=10), and not in the other arms, which is intriguing. Yet, no distinction was performed between sensitive and resistant tumours.

To our knowledge NeoRhea is the first study conducting such an extensive analysis on immune-related genes, including innate and adaptive immune cells, not only among “all-comers”, but also specifically among subjects with sensitive tumours by Ki67 response. Considering that no increase in TILs or PDL1 was observed between baseline and surgery, we believe that the increase in immune-related gene signature expression is less likely related to an activation of immune cells, and might rather refer to an activated immune-related transcriptional program of tumour cells. In other words, we hypothesize that the observed upregulation of immune-response related gene expression is tumour-intrinsic, instead of a reflection of immune-cells activity. This hypothesis, however, needs to be further explored. More translational analyses are ongoing in the NeoRhea study to further elucidate these findings.

ctDNA analysis

The results of our ctDNA analysis suggest that ctDNA detection after one month of treatment with Palbociclib and ET was associated with worse outcome, in line with literature data. Nonetheless, this observation should be regarded as exploratory, and independent validation is needed.

Single nuclei

Post treatment we consistently observe a proliferation decrease in all subjects. Investigating single nuclei patterns, we conclude that this decrease is driven by tumor cells. Parallel to this decrease we notice numerous immune pathways being upregulated in surgery samples as observed in both bulk and single nuclei sequencing techniques.

Limitations and strengths

Despite the efforts done by the research team to instruct and support the Sites in terms of collection of baseline and surgical samples, there was still an important proportion of subjects (25%) for whom the two baseline frozen biopsy cores had a low cellularity and, therefore, were of insufficient quality for RNA extraction. Moreover, among 21 of the 73 subjects with available RNA-seq at baseline, it was not possible to determine Ki67 at surgery, due to low cellularity of the FFPE surgical tissue. Unfortunately, in prospective translational research programs, between a third and a half of samples are excluded because of low quality.[17,20,24] No multiple testing correction was applied for the presented analyses, and our findings should be deemed as exploratory and need further validation.

Despite these difficulties, this study presented many advantages. It enrolled a cohort of newly diagnosed women, pre and postmenopausal, with treatment-naïve tumours, in which tissue was collected at the same timepoint, *i.e.* just before starting treatment with palbociclib and HT, thus eliminating the possible bias of having tissue from different timepoints of the subject's clinical course (like seen in the metastatic trials). The HT backbone consisted mostly of letrozole (and not fulvestrant), thus allowing the generalization of these findings for the early setting and also for the first-line advanced setting. Moreover, all subjects had a pre-defined treatment time (maximum of 4 cycles plus bridge period) and surgical tissue was thus collected around the same time for all subjects. However, part of the subjects (19%) did not receive palbociclib until the day before surgery – but the proportion of resistant tumours was similar between subjects receiving and not receiving palbociclib until the day before surgery (around 25%).

Conclusion

The NeoRhea study provided unique data about CDK4/6 inhibition in breast cancer, hinting that the combination of palbociclib plus HT may modulate immune-related pathways, namely cytotoxic T cell pathways, in the tumour cells themselves and/or in the tumour microenvironment. Therefore, these results represent an advance in the field of HR+/HER2- breast cancer, by generating clinical evidence of this immune modulation, which could be explored in the ongoing and future trials combining CDK4/6i with other immune modulating

drugs (*e.g.* immune checkpoint inhibitors). In addition, this study is a further step towards a better understanding of the mechanisms of resistance to CDK4/6i, namely regarding the baseline and dynamic expression of cell cycle-related genes and gene signatures. This generated knowledge is important to the ongoing effort of better selecting subjects who may derive the greatest benefit from these agents and to identify those who, on the contrary, are resistant to it. More translational analyses are ongoing in the NeoRhea study and will further contribute to improve our understanding about the effect of CDK 4/6 inhibition in HR+/HER2- tumours.

6 ADDITIONAL INFORMATION

6.1 Global substantial protocol amendments

The global substantial protocol amendments are summarised in the below table

Amendment date	Description
08/05/2017	• Inclusion of the additional hematology tests on day 14 of cycle 1 and day 14 of cycle 2 • Added Secondary Objectives and Secondary Endpoints • Prohibiting use of preparations containing St. John's wort during drug administration
29/03/2018	• Clarification of Allred score: positive is consider ≥3 • Clarification of Inclusion Criteria 5 • Change of required tumour size from 2 cm to 15 mm • Post treatment ultrasound changed from 7-10 days before surgery to up to 14 days before surgery. • Accept pre-study biopsies if available in the correct quantity • On study ultrasounds changed from 3D to 2D.
01/03/2019	• Addition of two secondary objectives and endpoints. • Addition of one exploratory objective and endpoint. • Note to IC 4 • Addition to Efficacy assessment • Interim analysis on first 65 subjects. • Addition of translational research assessments • Addition of GDPR information
21/03/2022	• Change in sponsor address • Addition of survival follow-up period • Addition of new exploratory objectives and related endpoints **Objectives:** To evaluate associations between patient survival (DRFS, RFS, IDFS, OS) and: - tumor clinicopathological characteristics and other biomarkers at baseline and/or surgery and/or their changes - plasma ctDNA monitoring **Enpoints:** distant relapse free survival, relapse free survival, invasive disease-free survival, overall survival • Addition of survival follow-up period in order to collect survival status, disease recurrence (if any) and/or secondary malignancy (if any) at least 30 months after last subject's end of study (EOS) visit. • Clarification of the study design • Clarification in study duration

6.2 Global interruptions and re-starts

There were no global interruptions to the trial.

6.3 Limitations, addressing sources of potential bias and imprecisions and caveats

There were no limitations and caveats.

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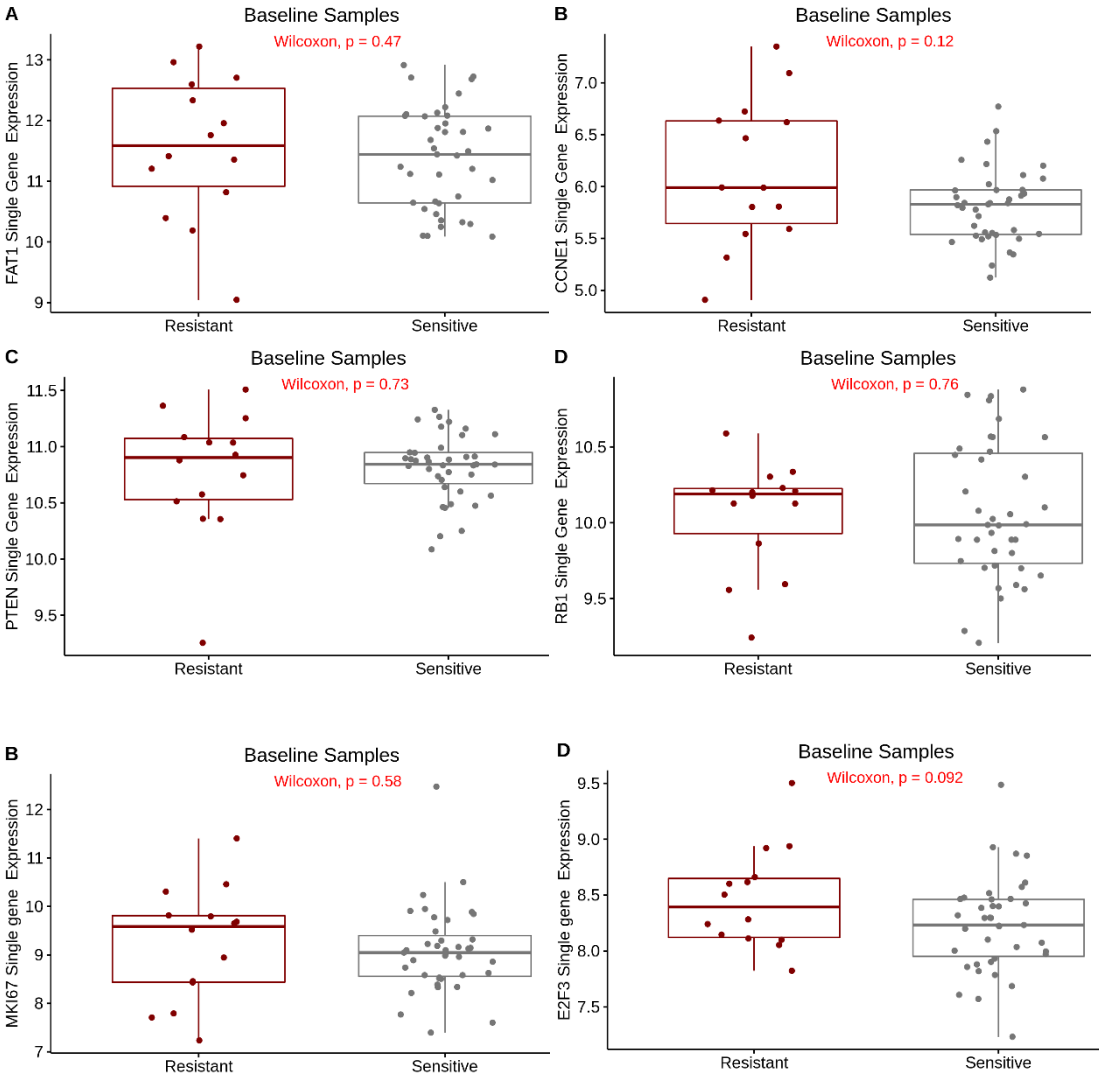
APPENDIX

RNA-sequencing at baseline according to Ki67 response

Cell cycle/proliferation-related genes and gene signatures

We provide below the graphs depicting the gene expression at baseline of the other cell-cycle / proliferation-related genes and gene-signatures at baseline, among subjects with resistant (Ki67 >2.7%) *vs* sensitive tumours (Ki67 ≤2.7%):



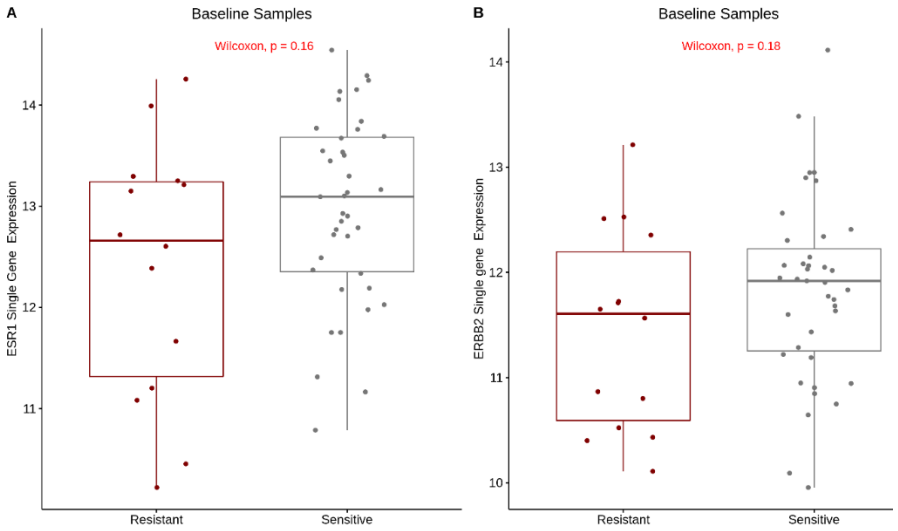
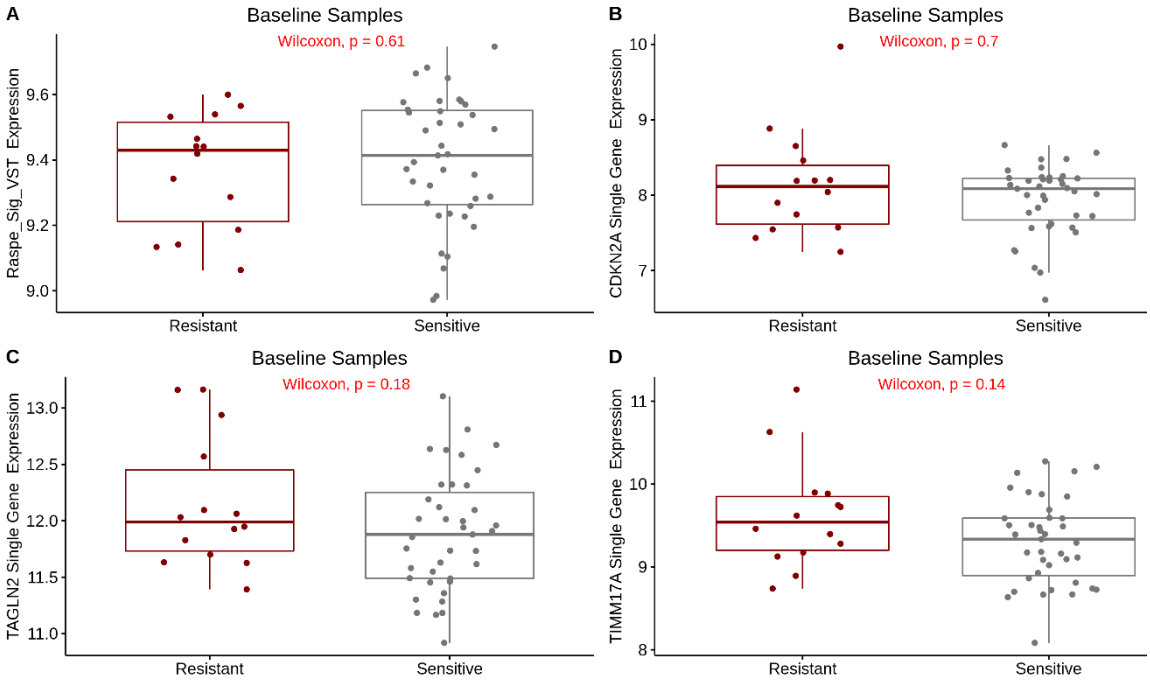


Figure S1. *FAT1, CCNE1, PTEN, RB1, MKI67 E2F3, ESR1* and *ERBB2* gene expression at baseline among subjects with resistant (Ki67 >2.7%) *vs* sensitive tumours (Ki67 ≤2.7%)



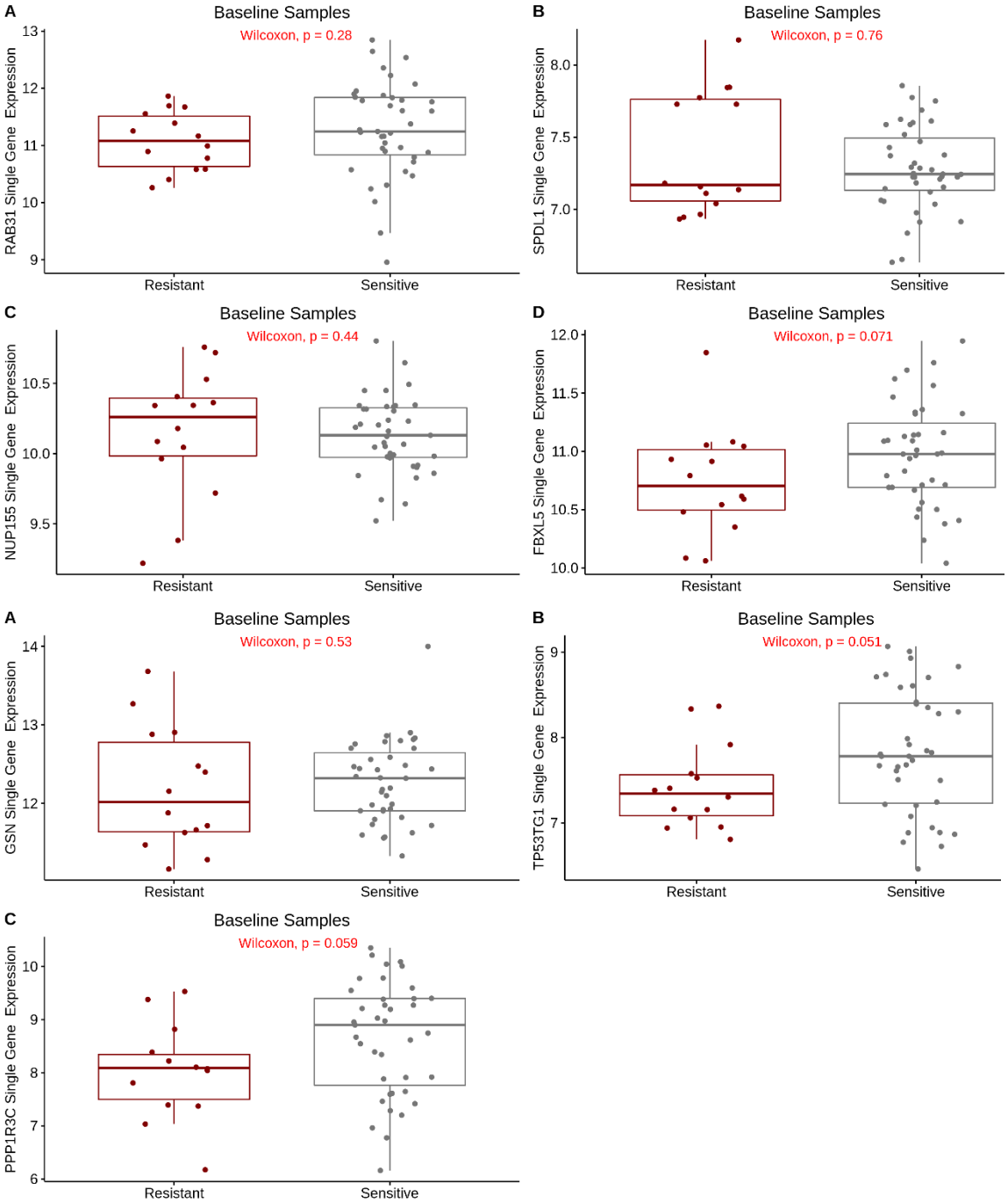


Figure S2. Expression at baseline of each of the 11 genes included in the 11-gene signature, among subjects with resistant (Ki67 >2.7%) *vs* sensitive tumours (Ki67 ≤2.7%)

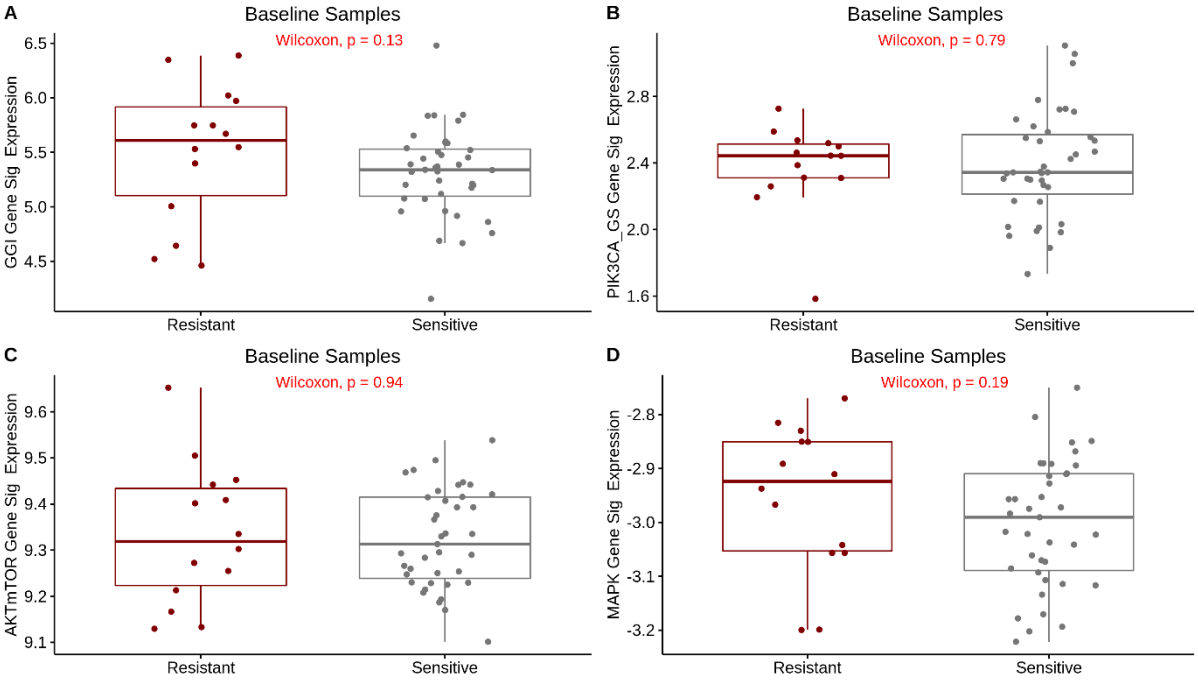
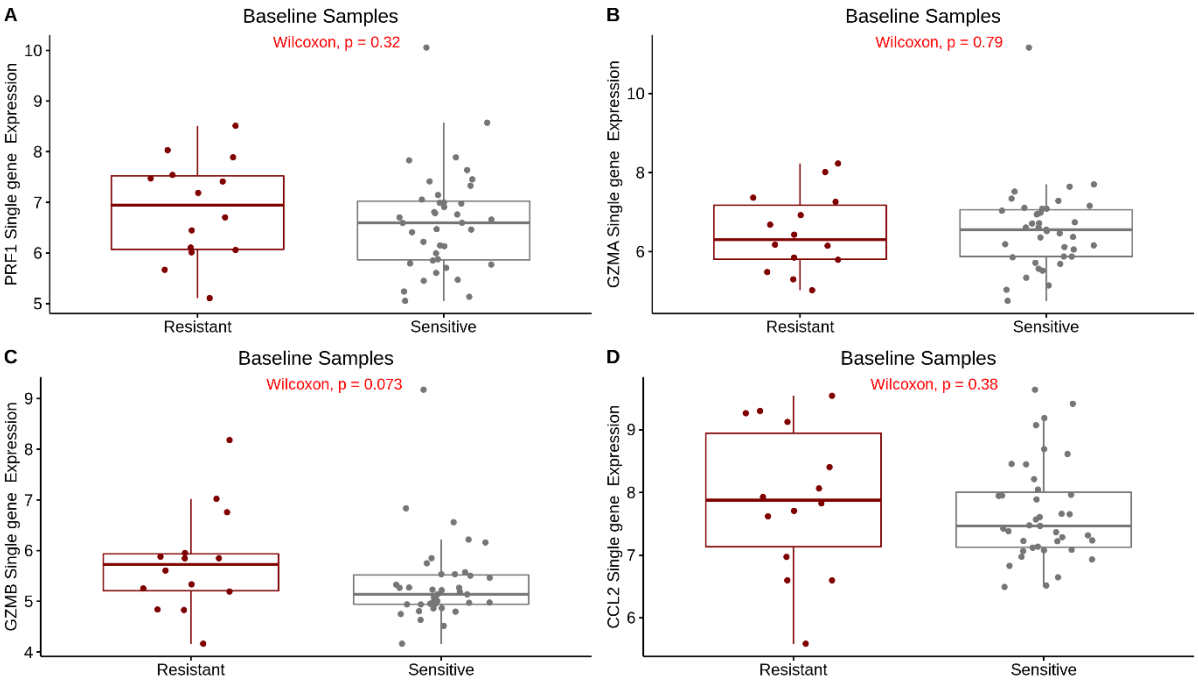
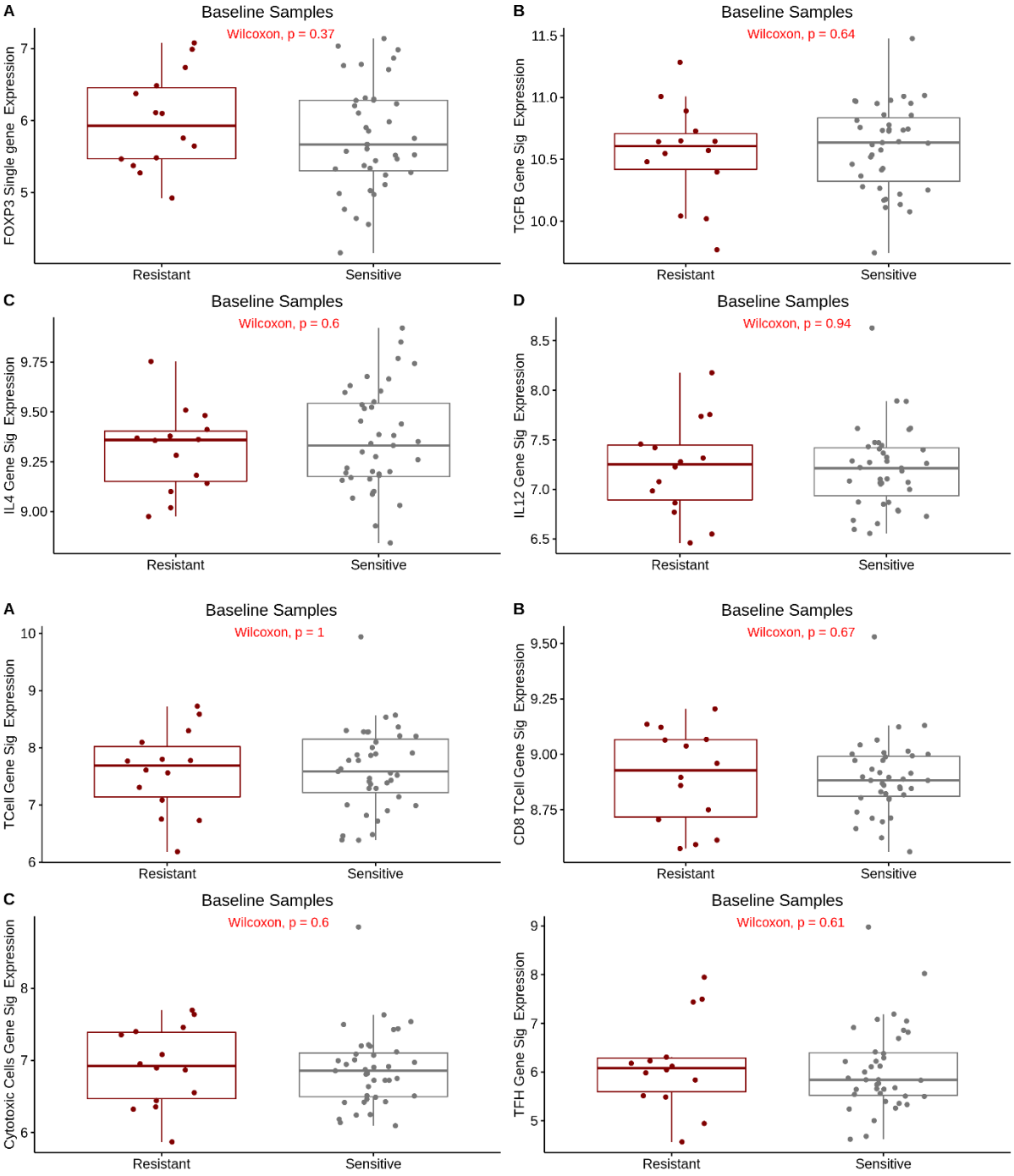


Figure S3. Genomic grade index (GGI), PIK3CA, AKTmTOR and MAPK gene signatures expression at baseline among subjects with resistant (Ki67 >2.7%) *vs* sensitive tumours (Ki67 ≤2.7%).

Immune-related genes and gene signatures

We provide below the graphs depicting the gene expression at baseline of the other immune genes and gene-signatures at baseline among subjects with resistant (Ki67 >2.7%) *vs* sensitive tumours (Ki67 ≤2.7%):




A
Baseline Samples
Wilcoxon, p = 0.37
FOXP3 Single gene Expression
Resistant
Sensitive
B
Baseline Samples
Wilcoxon, p = 0.64
TGFB Gene Sig Expression
Resistant
Sensitive
C
Baseline Samples
Wilcoxon, p = 0.6
IL4 Gene Sig Expression
Resistant
Sensitive
D
Baseline Samples
Wilcoxon, p = 0.94
IL12 Gene Sig Expression
Resistant
Sensitive
A
Baseline Samples
Wilcoxon, p = 1
TCell Gene Sig Expression
Resistant
Sensitive
B
Baseline Samples
Wilcoxon, p = 0.67
CD8 TCell Gene Sig Expression
Resistant
Sensitive
C
Baseline Samples
Wilcoxon, p = 0.6
Cytotoxic Cells Gene Sig Expression
Resistant
Sensitive
Baseline Samples
Wilcoxon, p = 0.61
TFH Gene Sig Expression
Resistant
Sensitive

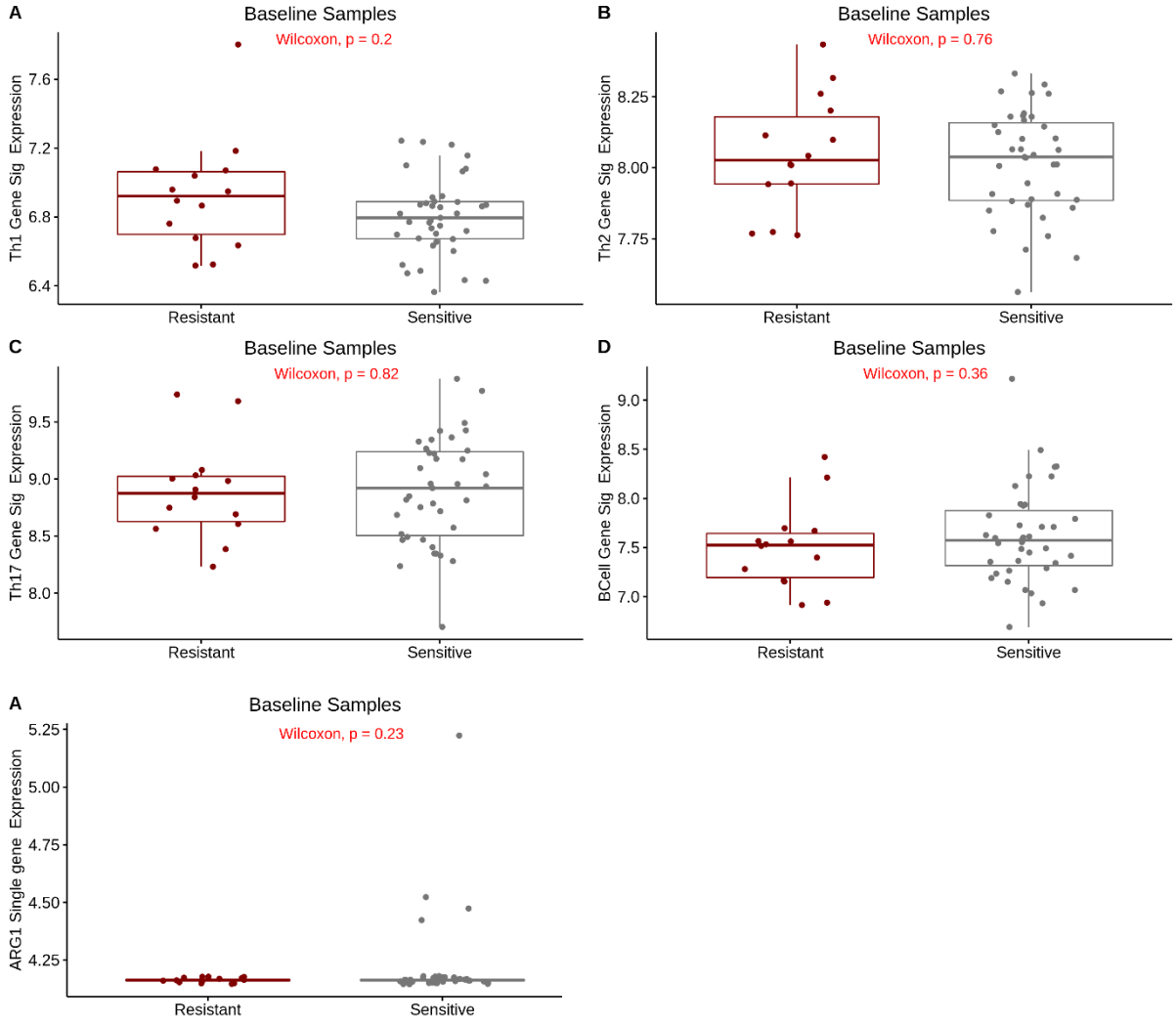


Figure S4. *PRF1, GZMA, GZMB, CCL2, FOXP3, TGFB, ARG1* genes expression and IL4, IL12, T Cell, CD8 TCell, Cytotoxic Cells, TFH, Th1, Th2, Th17, B Cell gene signature expression at baseline among subjects with resistant (Ki67 >2.7%) *vs* sensitive tumours (Ki67 ≤2.7%)

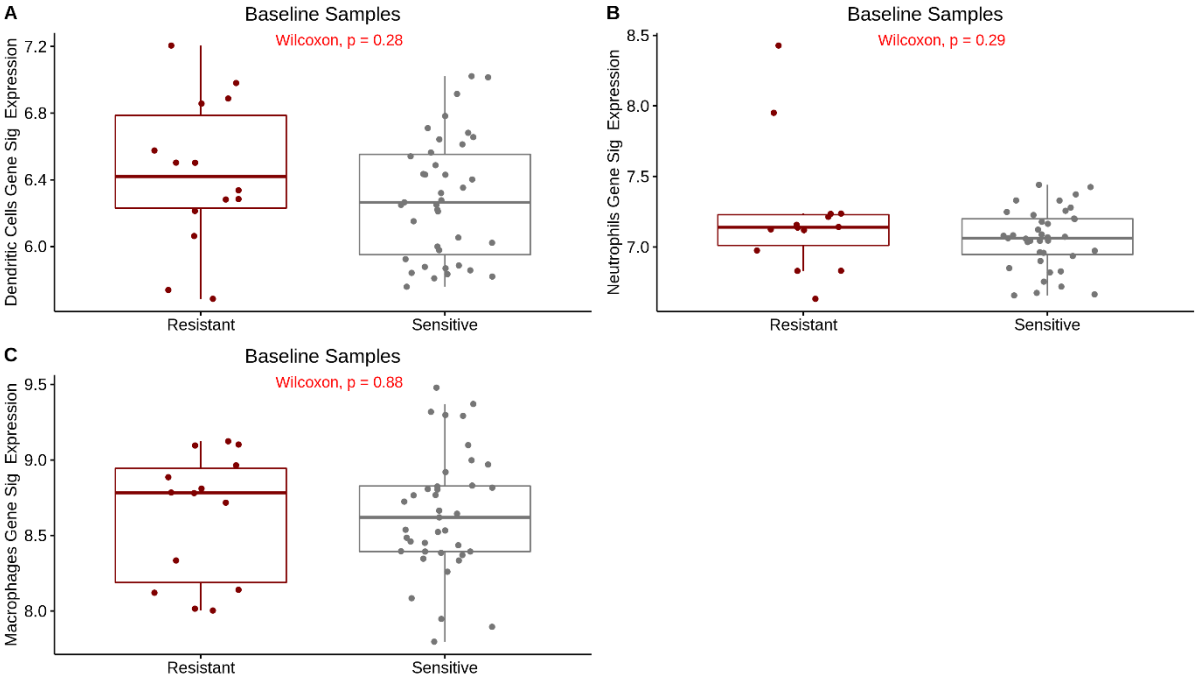


Figure S5. Dendritic cells, neutrophils and macrophages gene signature expression at baseline among subjects with resistant (Ki67 >2.7%) *vs* sensitive tumours (Ki67 ≤2.7%).

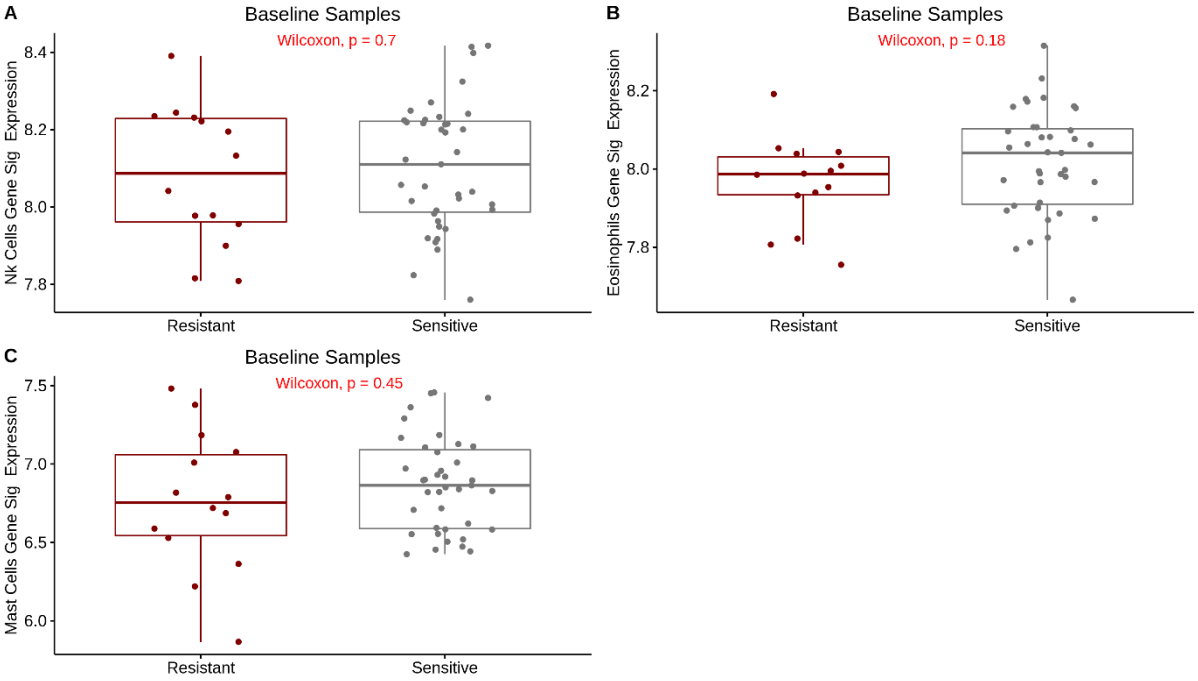


Figure S6. NK cells, eosinophils and mast cells gene signature expression at baseline among subjects with resistant (Ki67 >2.7%) *vs* sensitive tumours (Ki67 ≤2.7%)

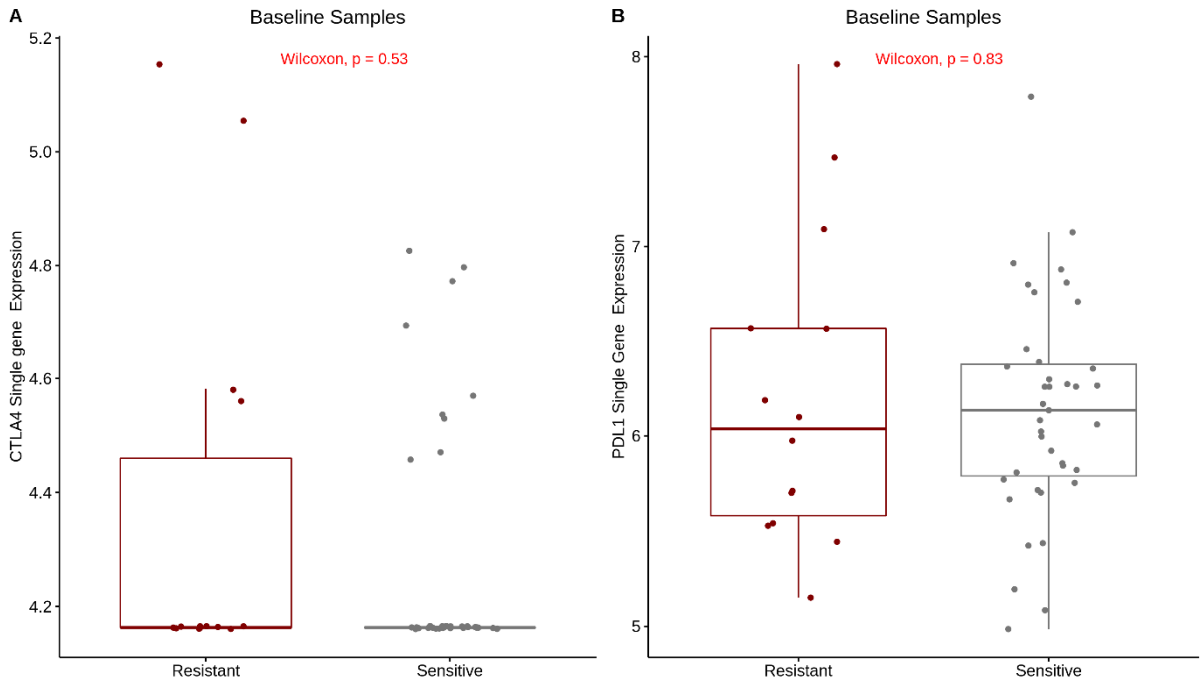


Figure S7. *CTLA4* and *PDL1* gene expression at baseline among subjects with resistant (Ki67 >2.7%) *vs* sensitive tumours (Ki67 ≤2.7%)

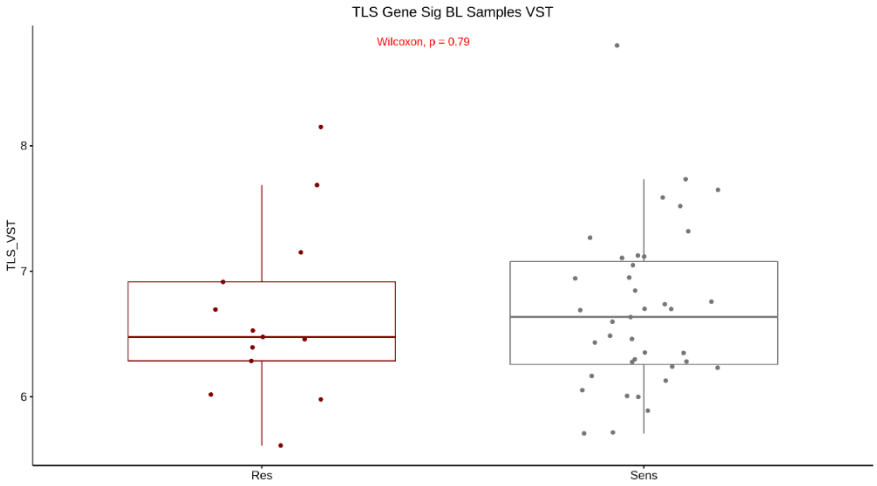


Figure S8. Tertiary lymphoid structures (TLS) gene signature expression at baseline among subjects with resistant (Ki67 >2.7%) *vs* sensitive tumours (Ki67 ≤2.7%).

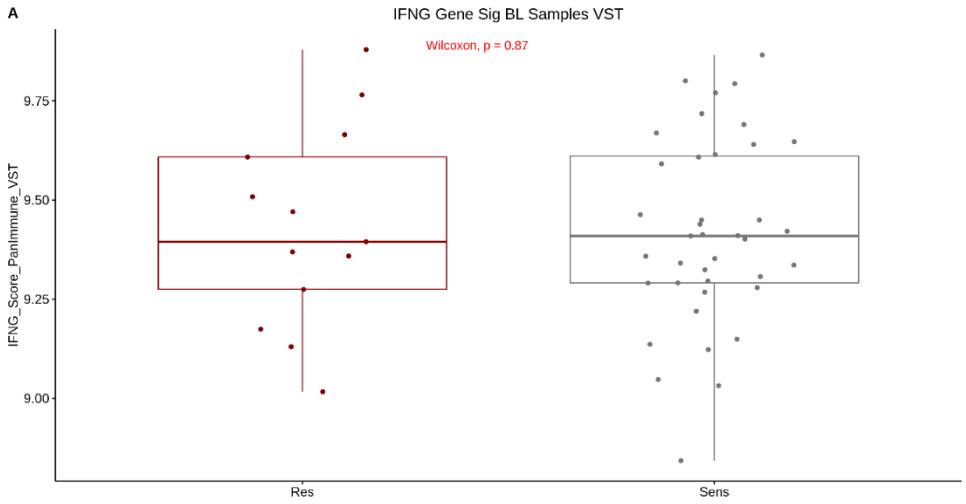


Figure S9. IFNG gene signature expression at baseline among subjects with resistant (Ki67 >2.7%) *vs* sensitive tumours (Ki67 ≤2.7%).

Changes in immune cells and tumor transcriptional programs after treatment

Cell cycle/proliferation-related genes and gene signatures

We provide below the graphs depicting the significant changes in the expression of cell cycle / proliferation-related genes or gene signatures from baseline to surgery among sensitive (n=19) and resistant (n=6) subjects:

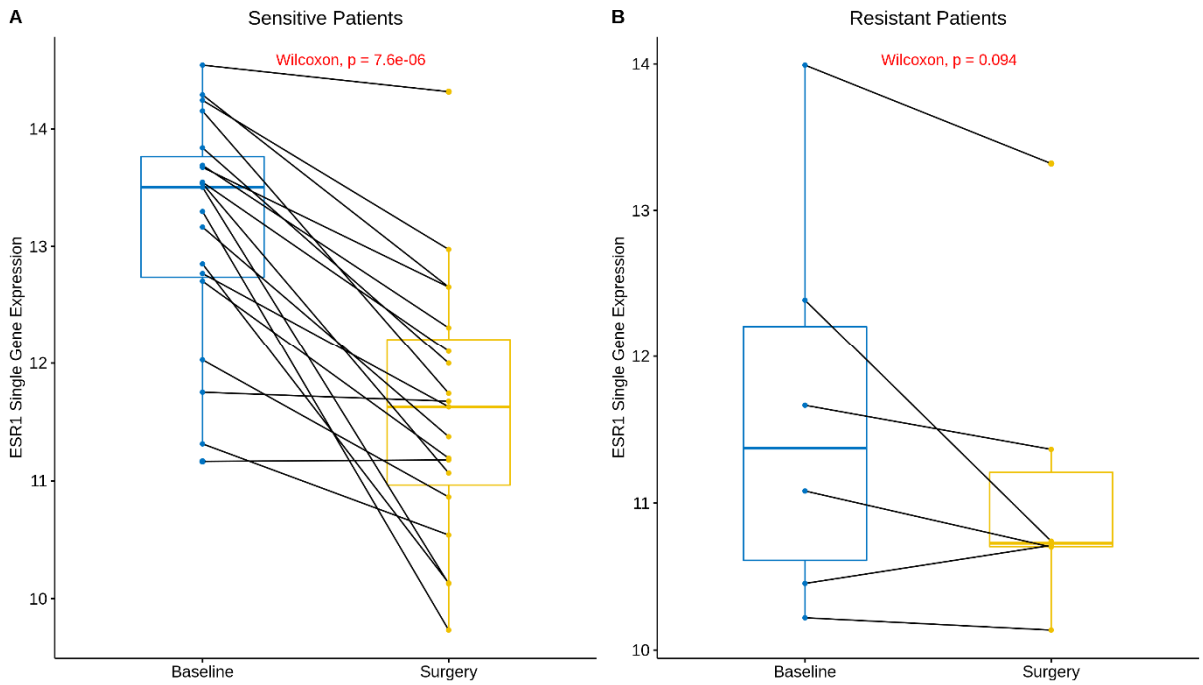


Figure S10: Changes in *ESR1* gene expression between baseline and surgery among sensitive subjects (n=19; left panel) and among resistant subjects (n=6; right panel).

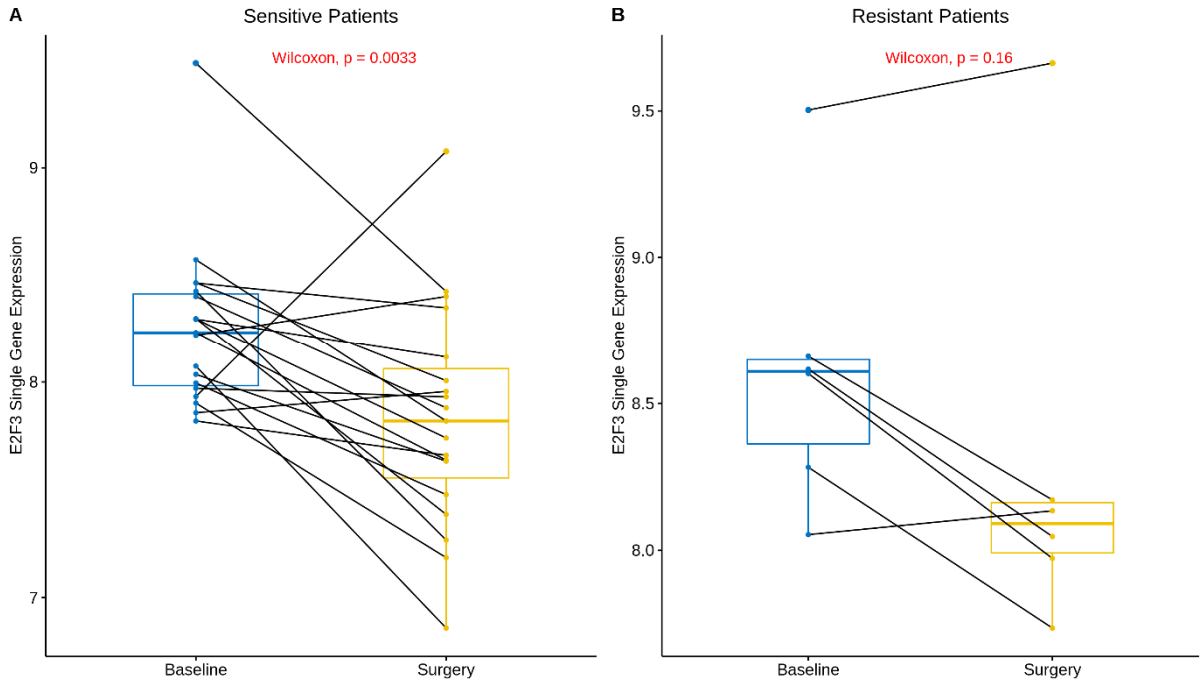


Figure S11: Changes in *E2F3* gene expression between baseline and surgery among sensitive subjects (n=19; left panel) and among resistant subjects (n=6; right panel).

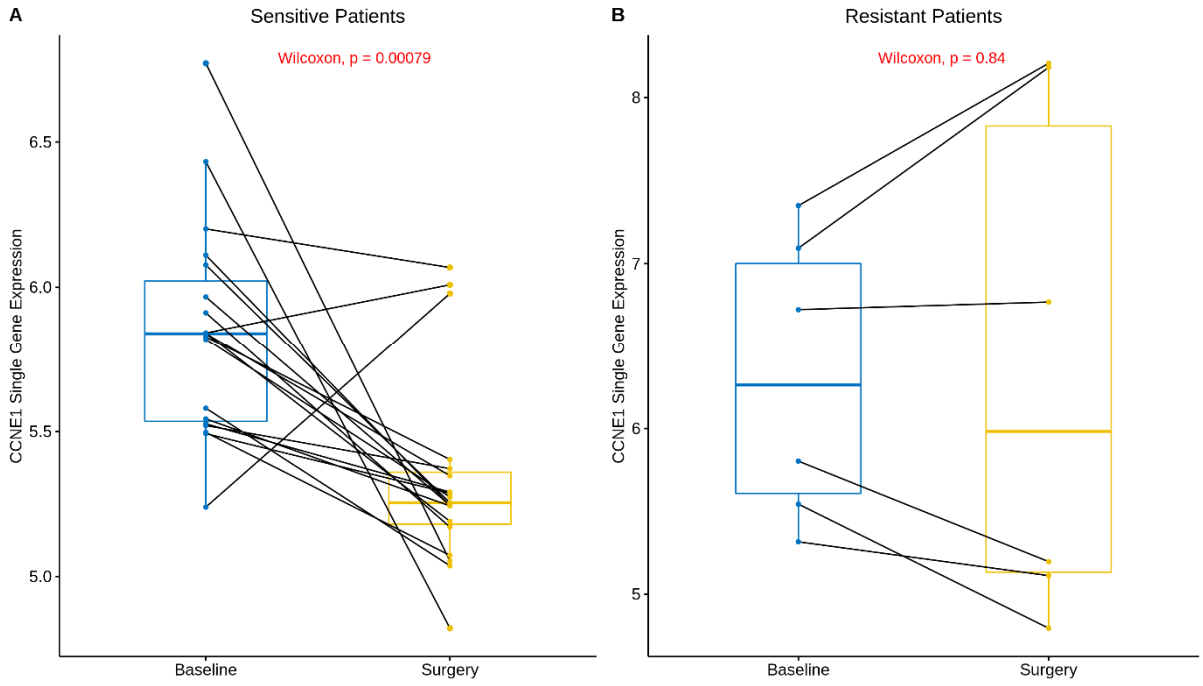


Figure S12: Changes in *CCNE1* gene expression between baseline and surgery among sensitive subjects (n=19; left panel) and among resistant subjects (n=6; right panel).

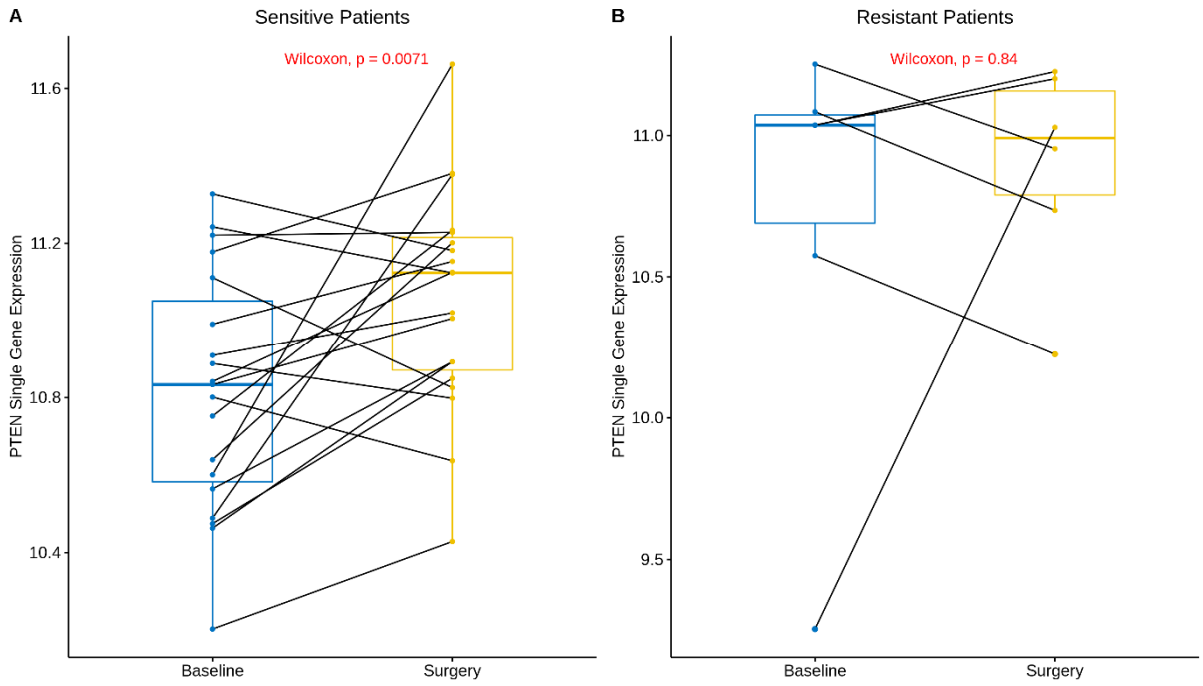


Figure S13: Changes in *PTEN* gene expression between baseline and surgery among sensitive subjects (n=19; left panel) and among resistant subjects (n=6; right panel).

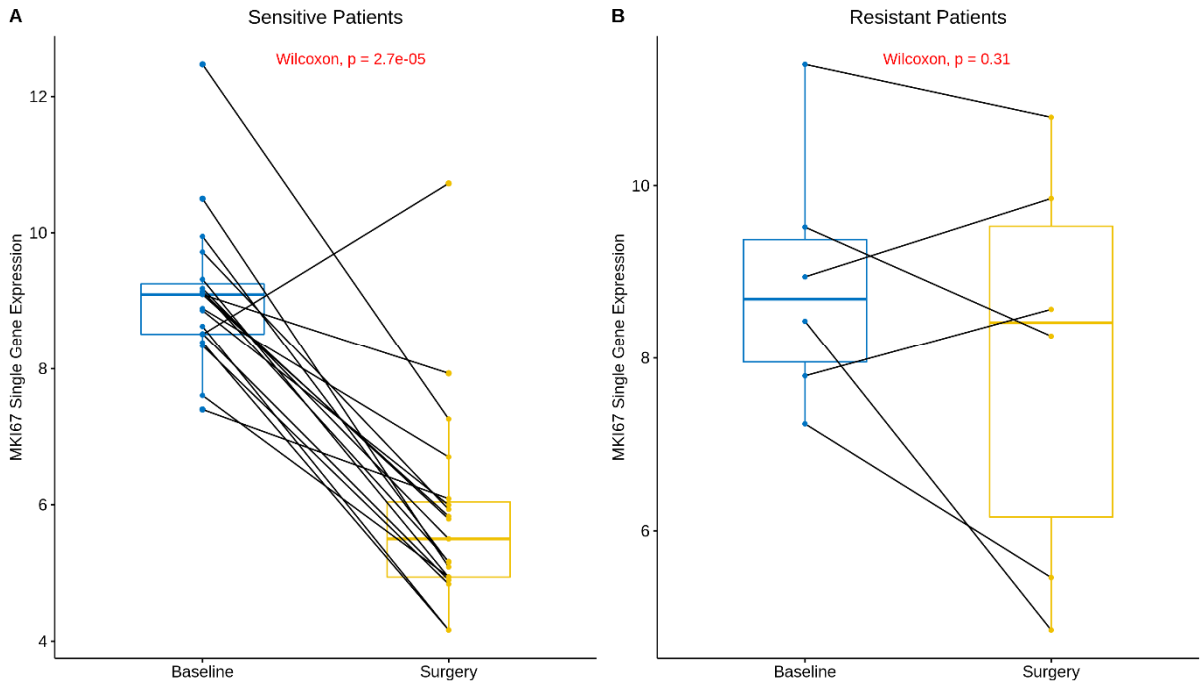


Figure S14: Changes in *MKI67* gene expression between baseline and surgery among sensitive subjects (n=19; left panel) and among resistant subjects (n=6; right panel).

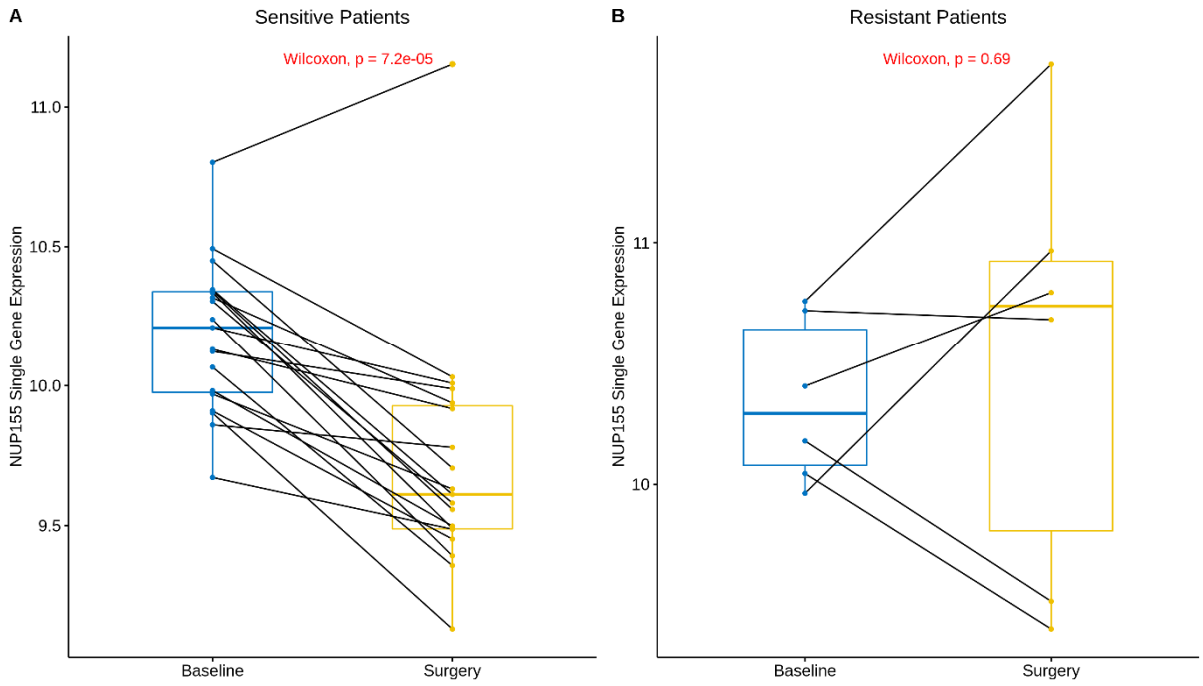


Figure S15: Changes in *NUP155* gene expression between baseline and surgery among sensitive subjects (n=19; left panel) and among resistant subjects (n=6; right panel).

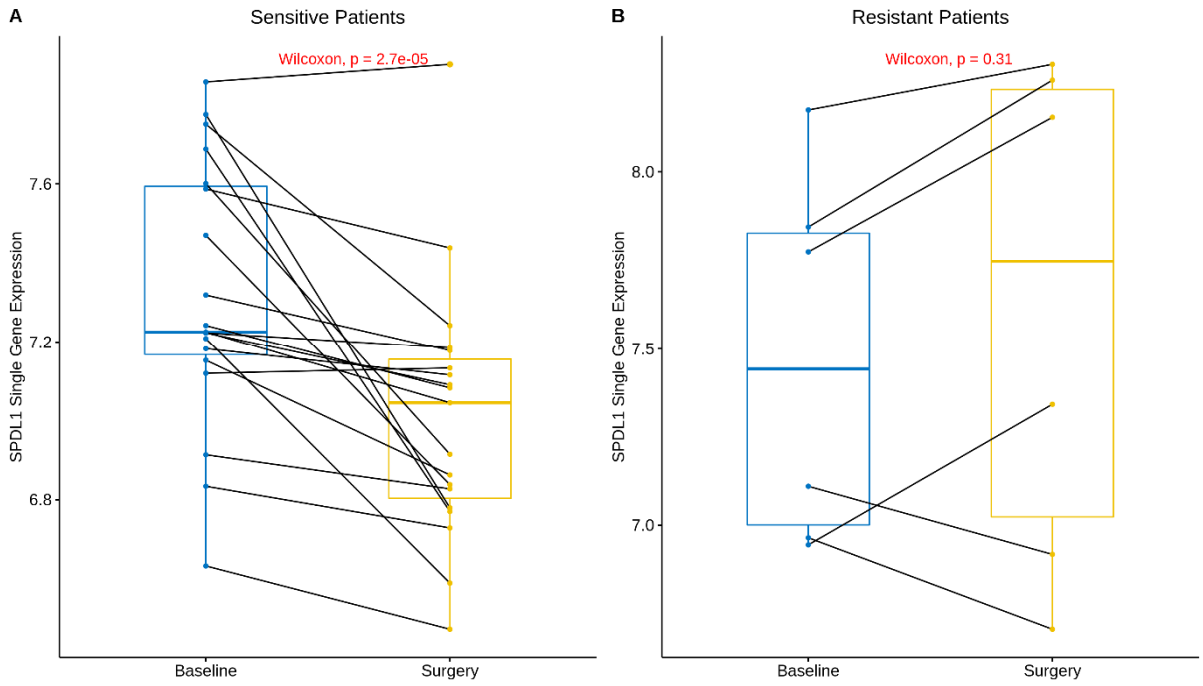


Figure S16: Changes in *SPDL1* gene expression between baseline and surgery among sensitive subjects (n=19; left panel) and among resistant subjects (n=6; right panel).

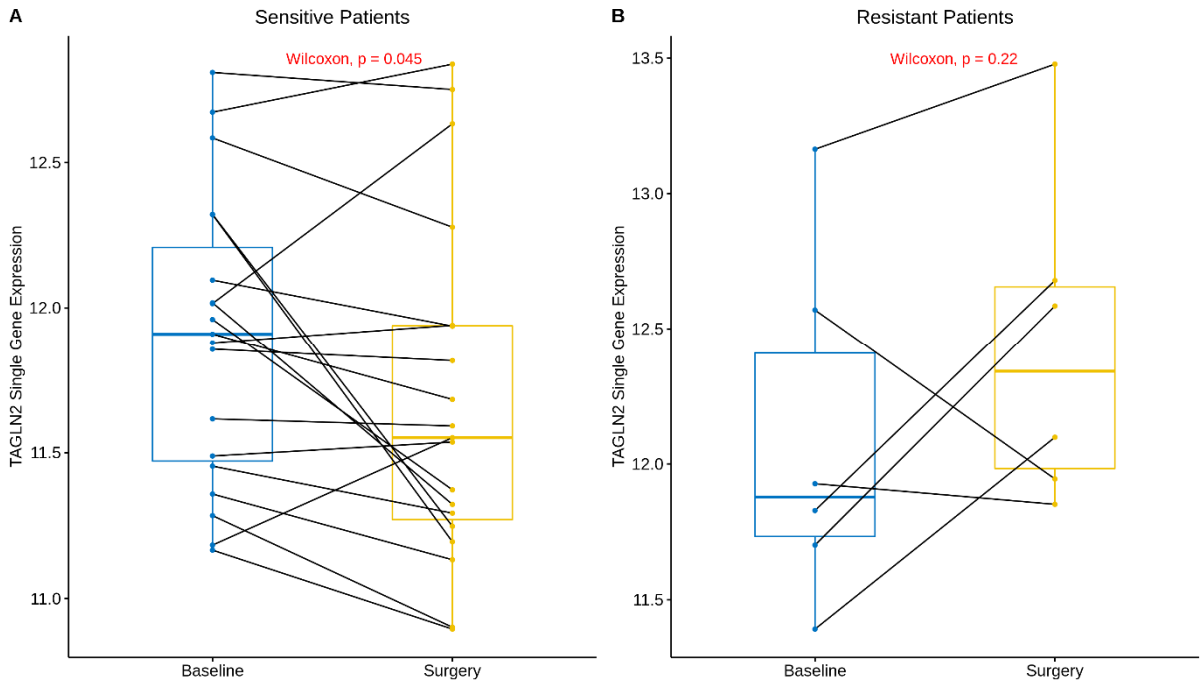


Figure S17: Changes in *TAGLN2* gene expression between baseline and surgery among sensitive subjects (n=19; left panel) and among resistant subjects (n=6; right panel).

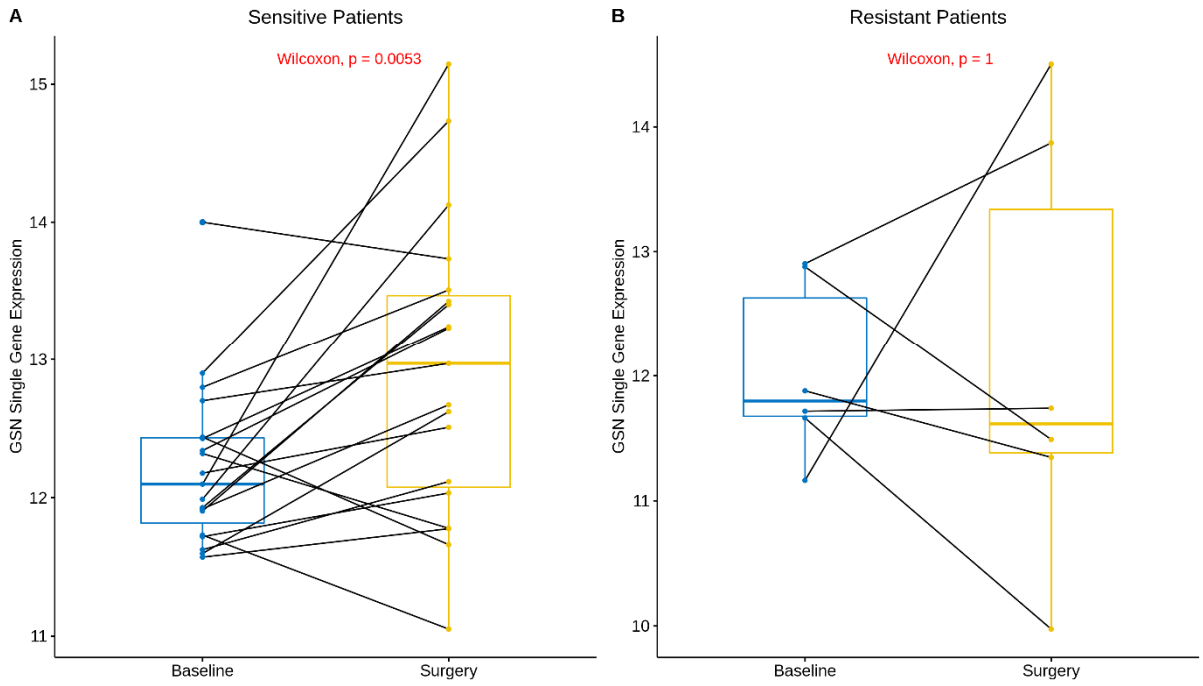


Figure S18: Changes in *GSN* expression between baseline and surgery among sensitive subjects (n=19; left panel) and among resistant subjects (n=6; right panel).

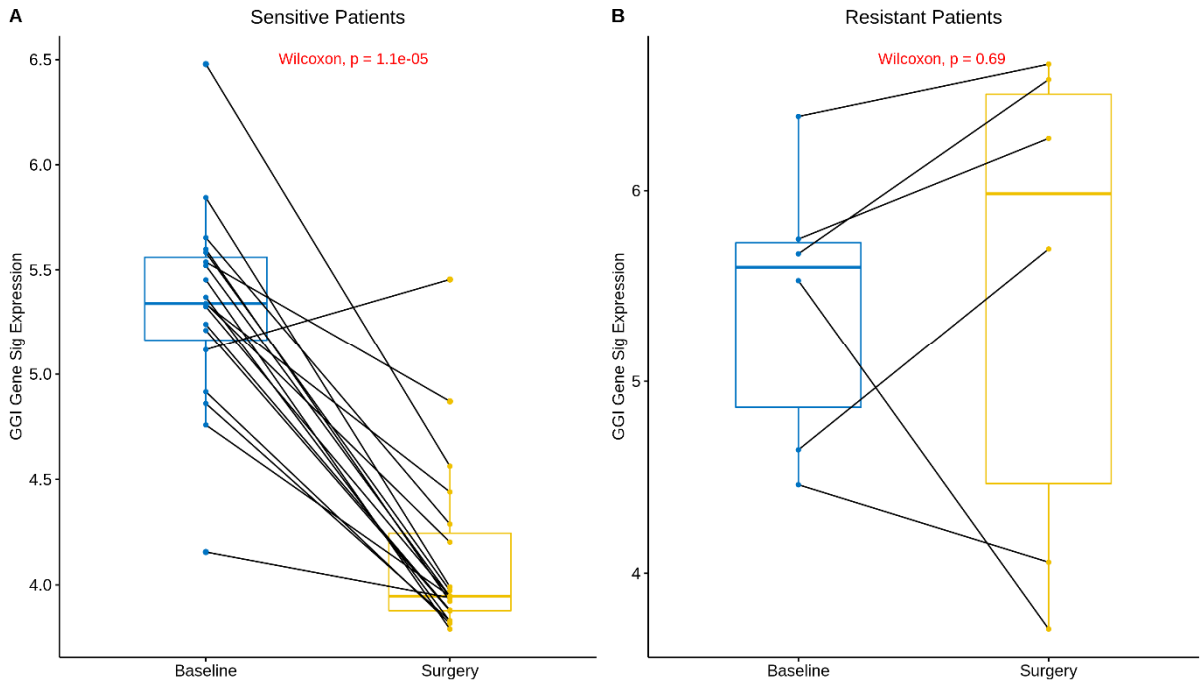


Figure S19: Changes in GGI gene signature between baseline and surgery among sensitive subjects (n=19; left panel) and among resistant subjects (n=6; right panel).

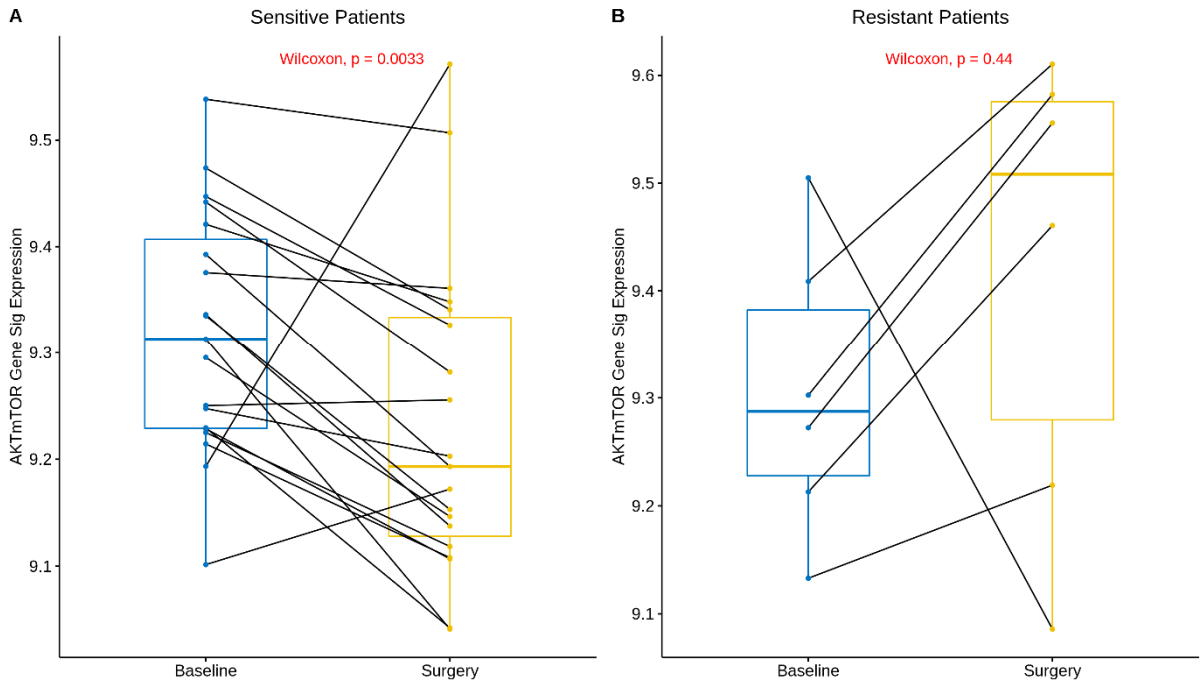


Figure S20: Changes in AKTmTOR gene signature expression between baseline and surgery among sensitive subjects (n=19; left panel) and among resistant subjects (n=6; right panel).

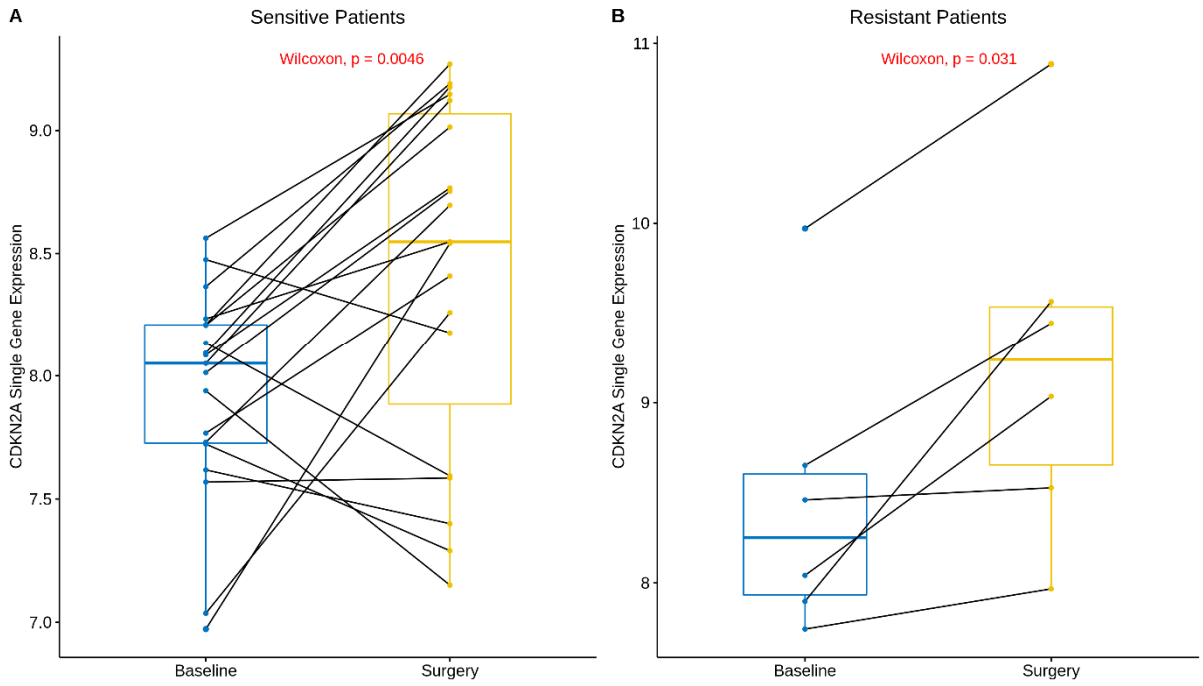
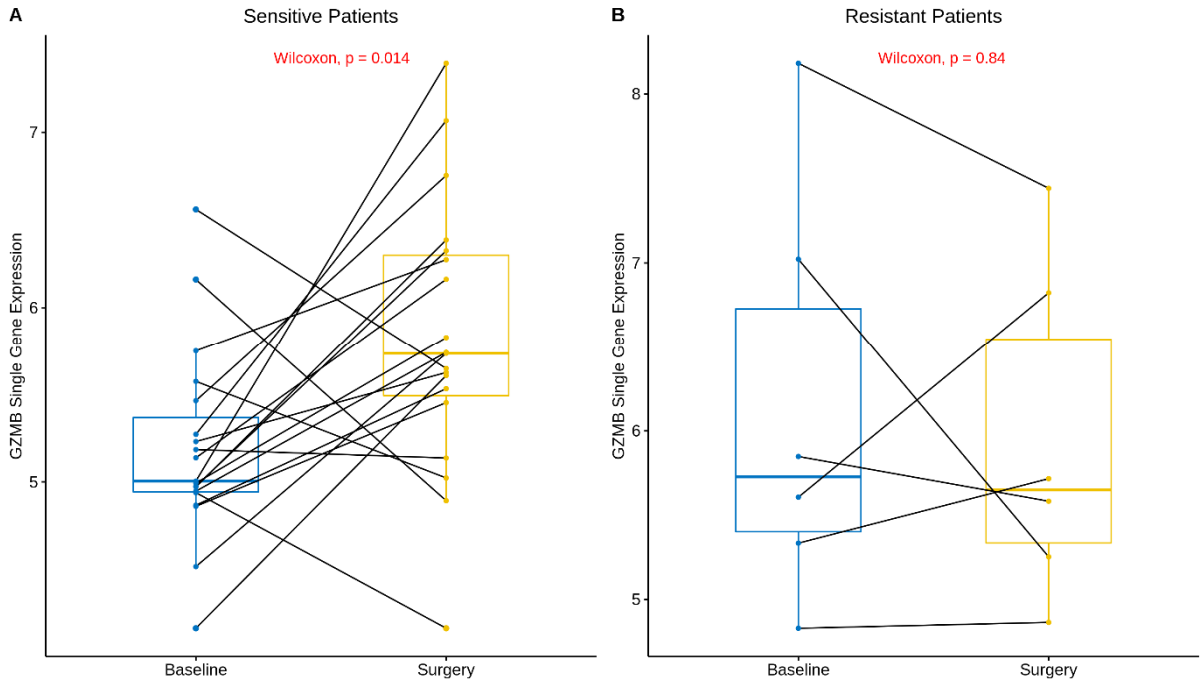
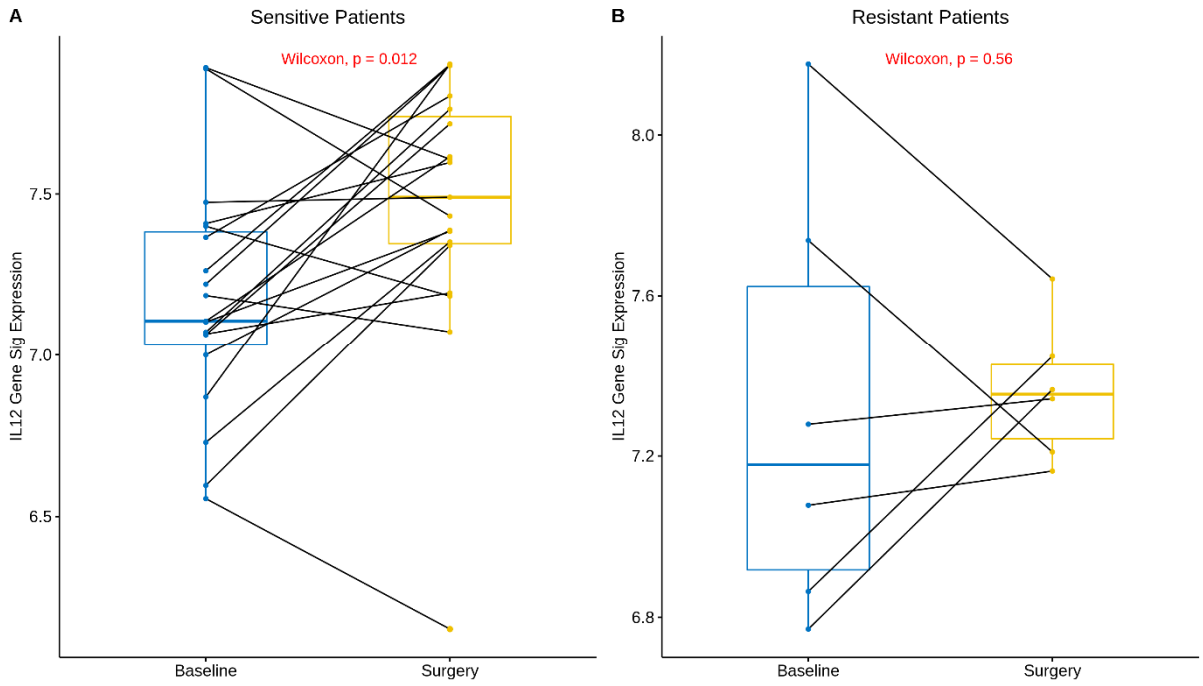


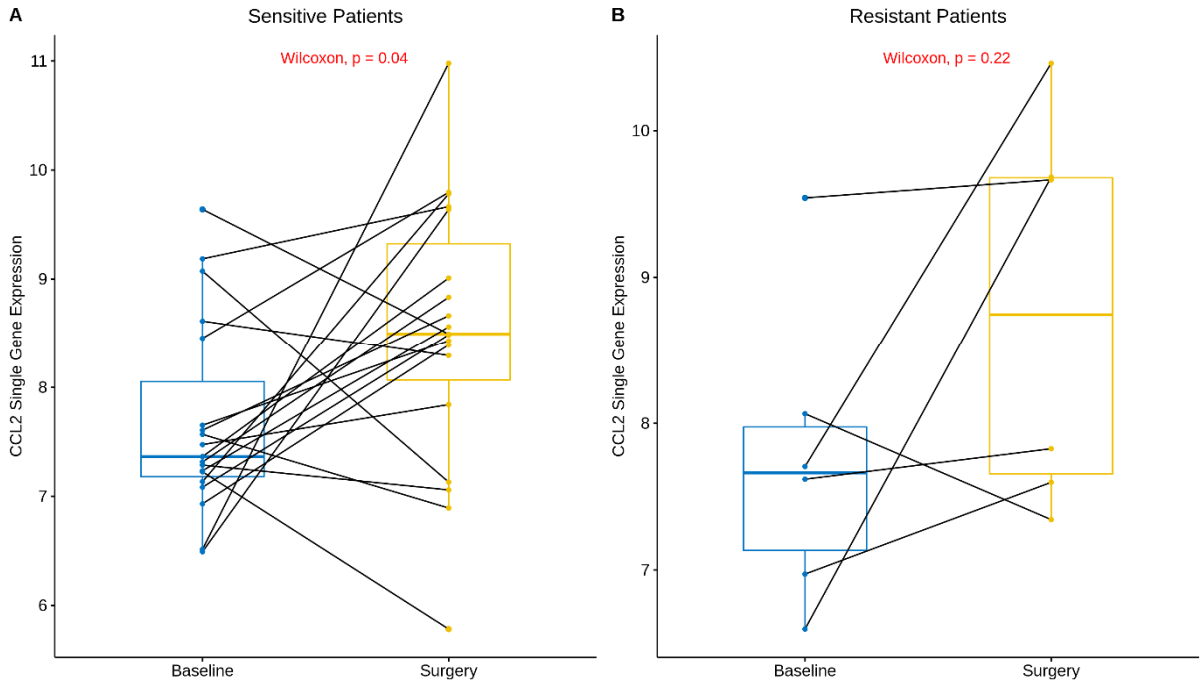
Figure S21: Changes in *CDKN2A* expression between baseline and surgery among sensitive subjects (n=19; left panel) and among resistant subjects (n=6; right panel).

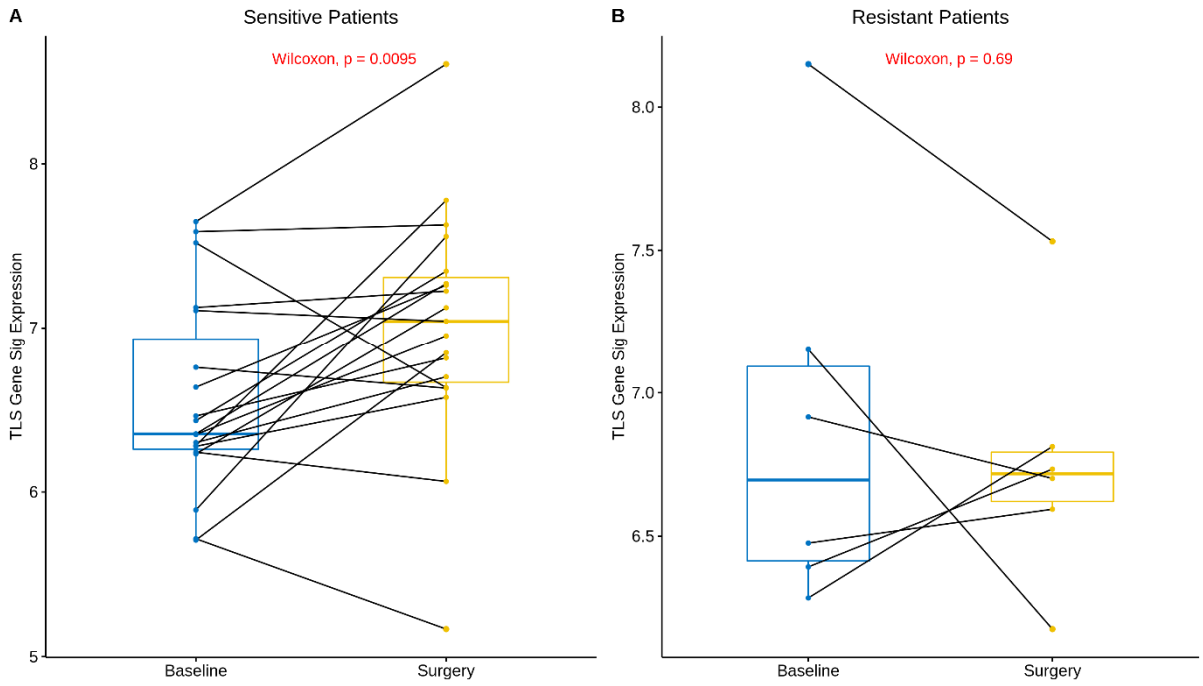
Immune-related genes and gene signatures

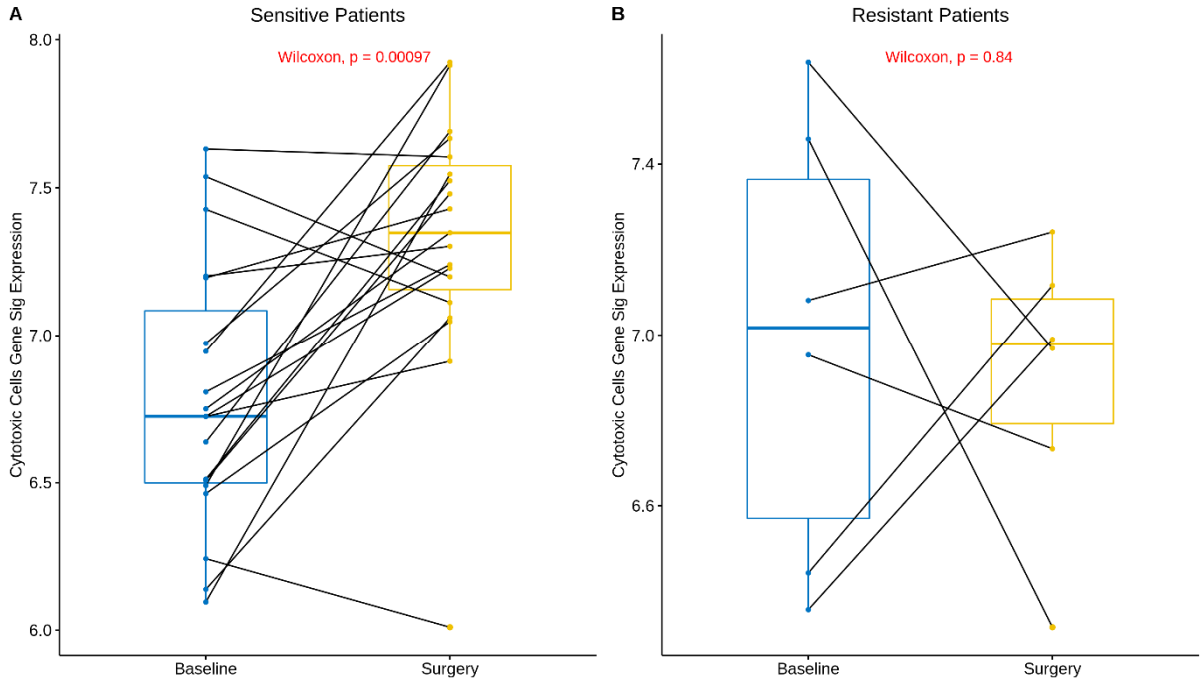
We provide below the graphs depicting the significant changes in the expression of immune-related genes or gene signatures from baseline to surgery among sensitive (n=19) and resistant (n=6) subjects:




A
Sensitive Patients
Wilcoxon, p = 0.012
IL12 Gene Sig Expression
7.5
7.0
6.5
Baseline
Surgery
B
Resistant Patients
Wilcoxon, p = 0.56
IL12 Gene Sig Expression
8.0
7.6
7.2
6.8
Baseline
Surgery


A
Sensitive Patients
Wilcoxon, p = 0.04
CCL2 Single Gene Expression
11
10
9
8
7
6
Baseline
Surgery
B
Resistant Patients
Wilcoxon, p = 0.22
CCL2 Single Gene Expression
10
9
8
7
Baseline
Surgery


A
Sensitive Patients
Wilcoxon, p = 0.0095
TLS Gene Sig Expression
8
7
6
5
Baseline
Surgery
B
Resistant Patients
Wilcoxon, p = 0.69
TLS Gene Sig Expression
8.0
7.5
7.0
6.5
Baseline
Surgery


A
Sensitive Patients
Wilcoxon, p = 0.00097
Cytotoxic Cells Gene Sig Expression
8.0
7.5
7.0
6.5
6.0
Baseline
Surgery
B
Resistant Patients
Wilcoxon, p = 0.84
Cytotoxic Cells Gene Sig Expression
7.4
7.0
6.6
Baseline
Surgery

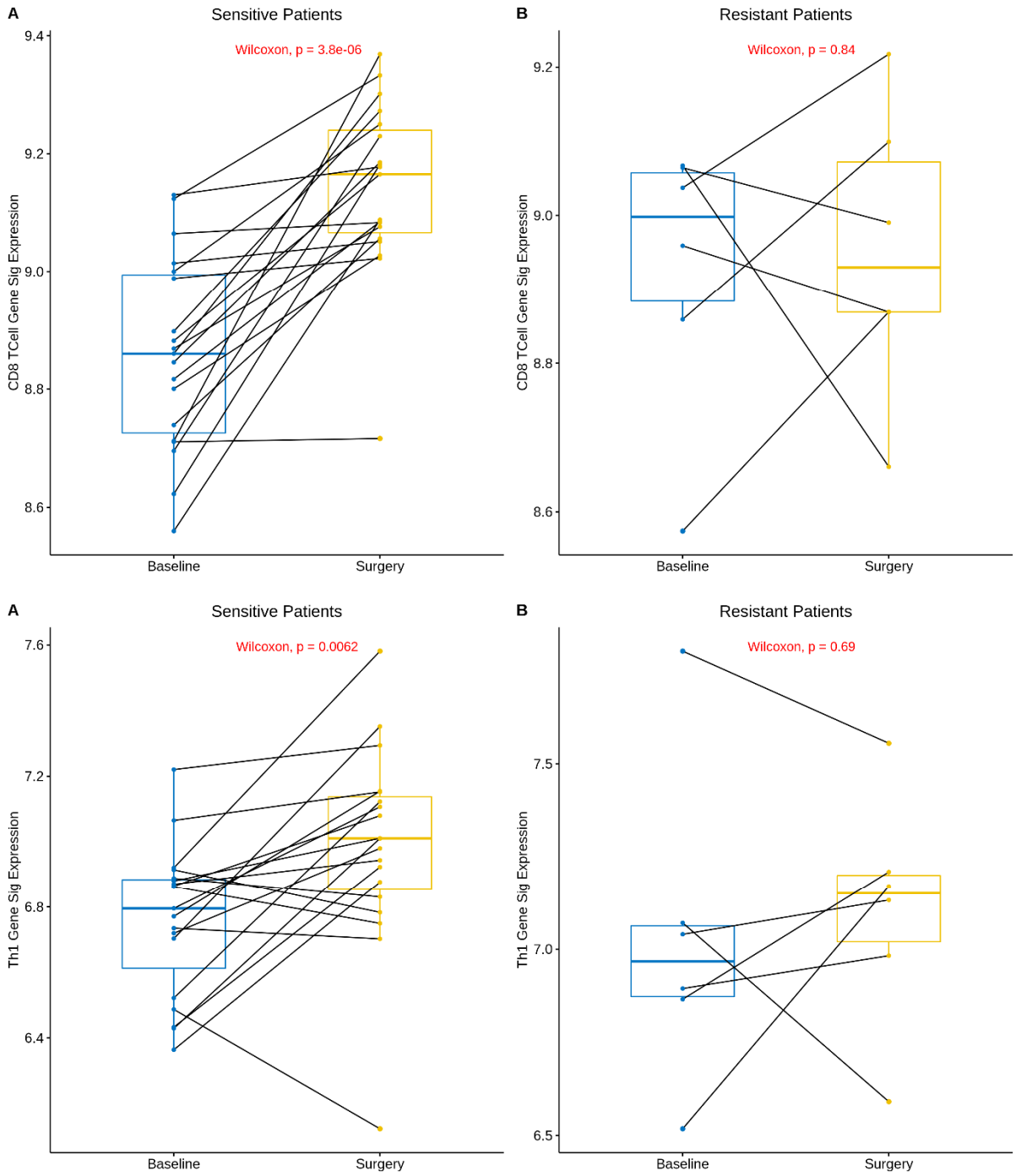


Figure S22: Changes in the expression of single genes (*GMZB, CCL2*) and gene signatures (IL12, TLS, cytotoxic cells, CD8 T-cells, Th1) between baseline and surgery among subjects with sensitive tumours (n=19; left panels) and among resistant subjects (n=6; right panels).

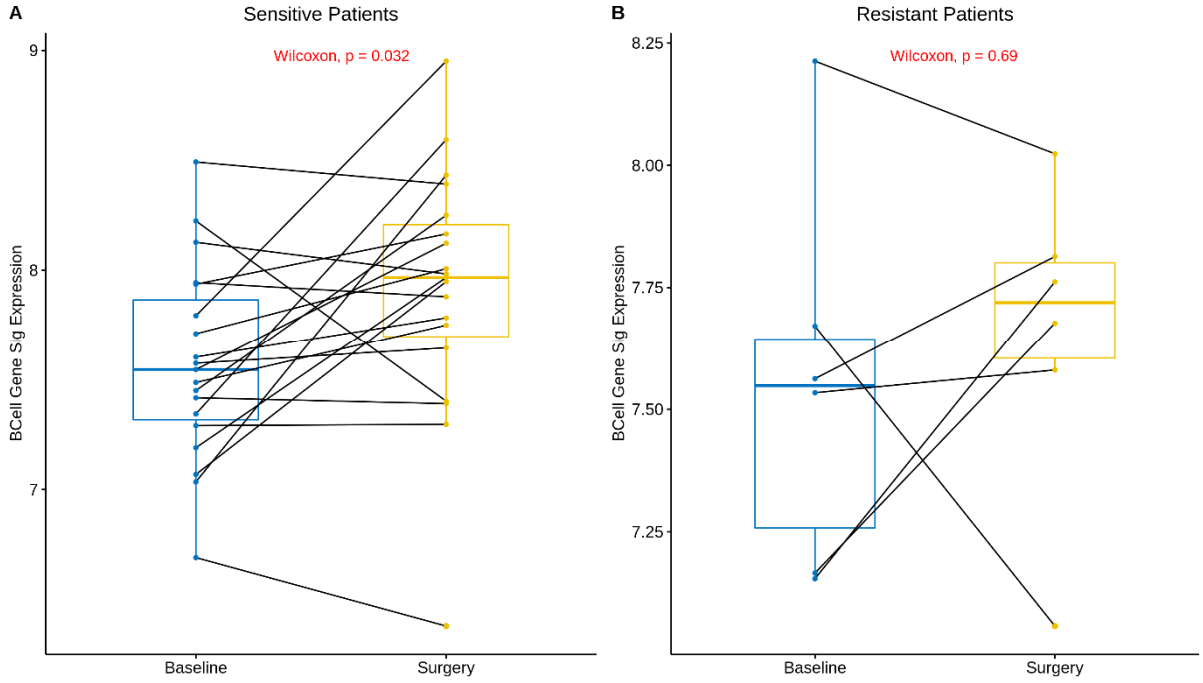
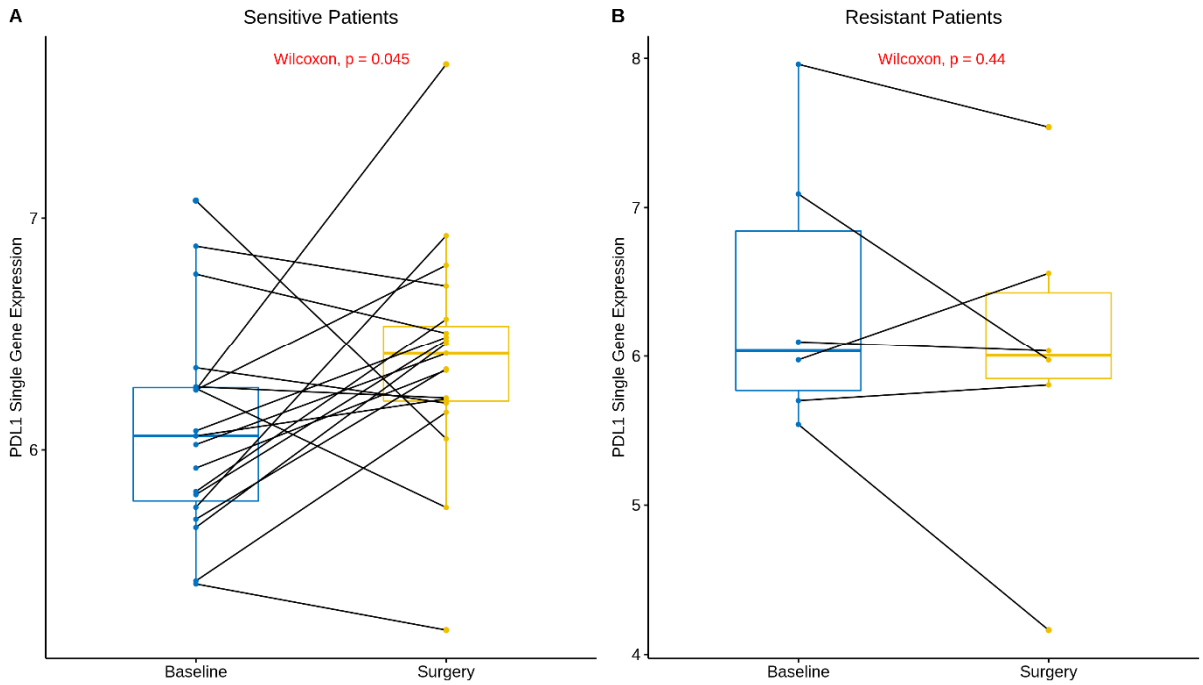


Figure S23: Changes in B cells gene signature expression between baseline and surgery among sensitive subjects (n=19; left panel) and among resistant subjects (n=6; right panel).



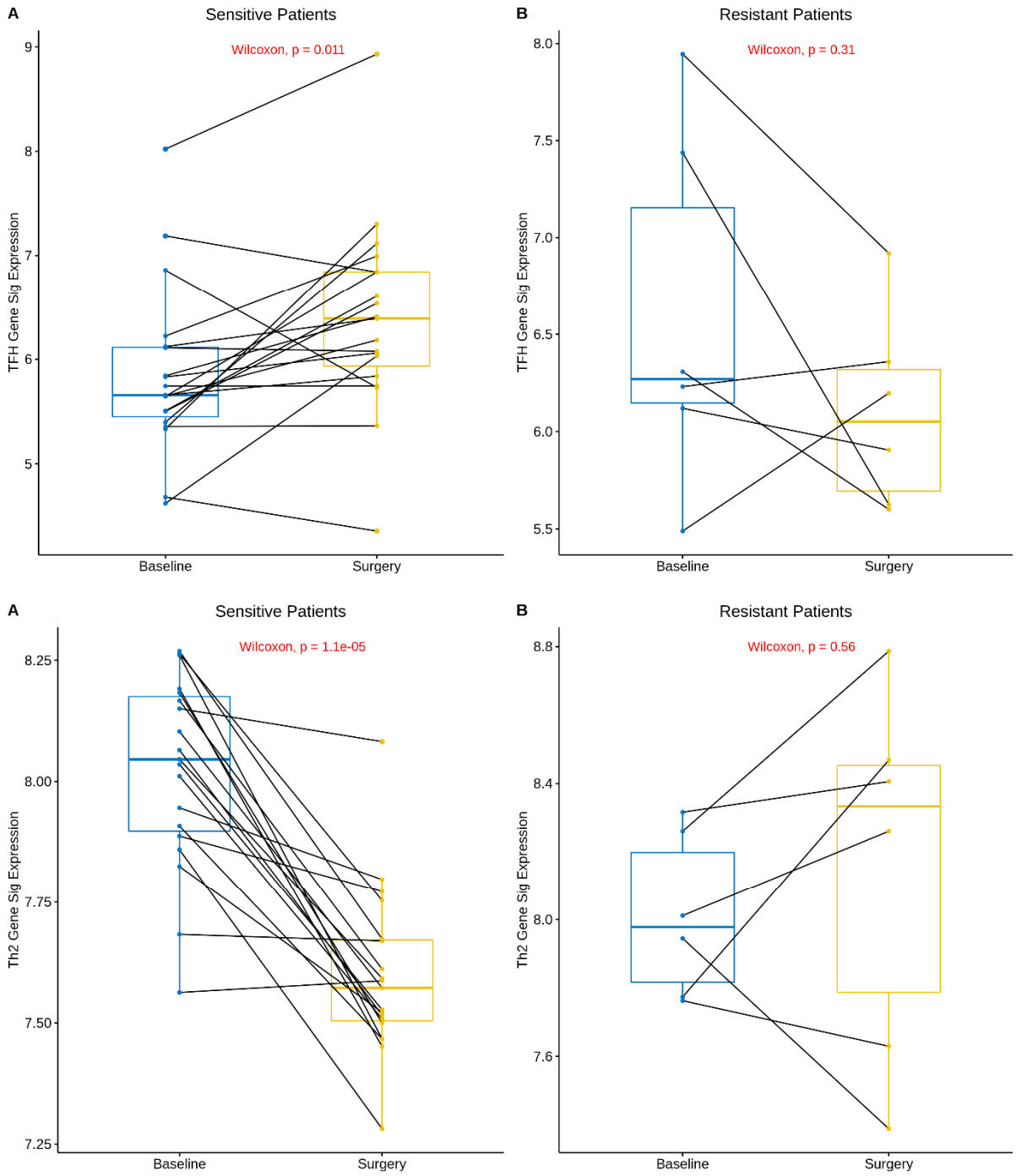
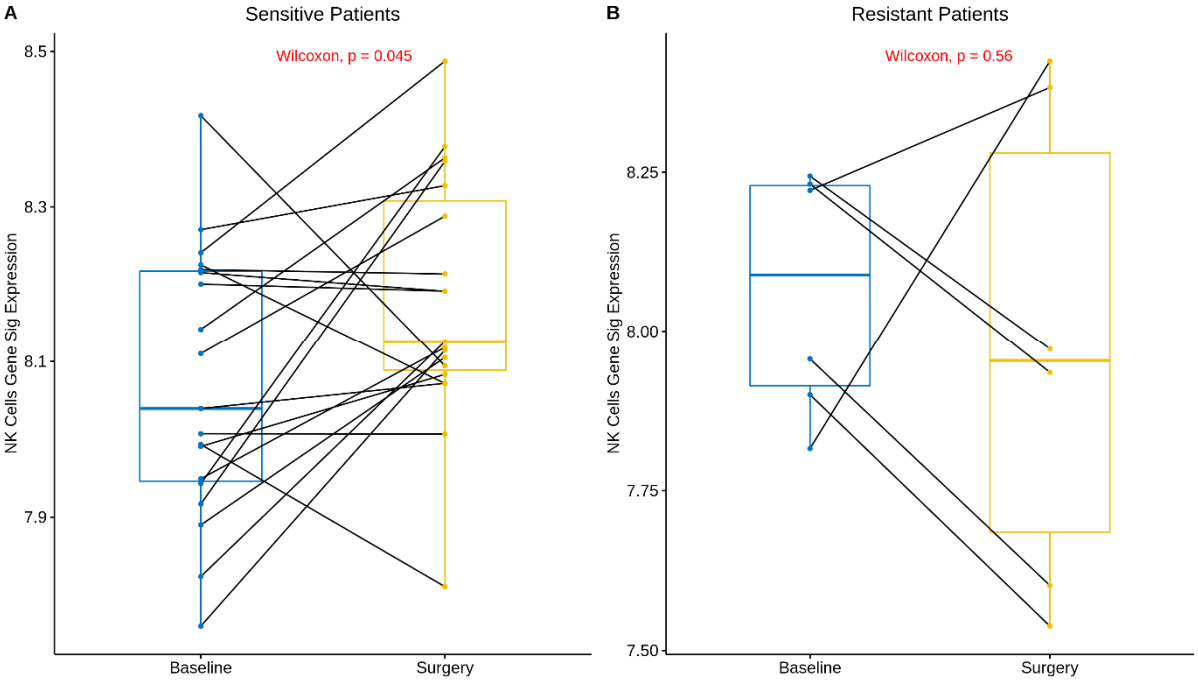
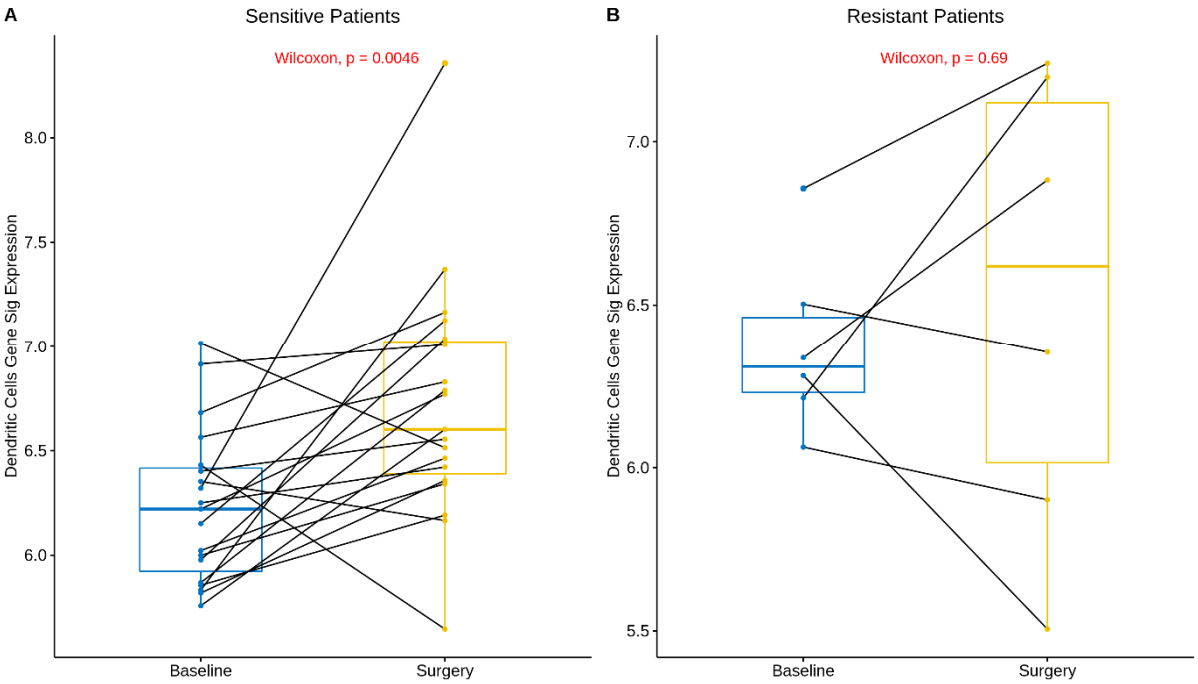


Figure S24: Changes in the *PD-L1* single gene, Follicular B helper T cells (TFH) and Th2 gene signature expression between baseline and surgery among subjects (n=19; left panels) and among resistant subjects (n=6; right panels)


A
Sensitive Patients
Wilcoxon, p = 0.045
NK Cells Gene Sig Expression
8.5
8.3
8.1
7.9
Baseline
Surgery
B
Resistant Patients
Wilcoxon, p = 0.56
NK Cells Gene Sig Expression
8.25
8.00
7.75
7.50
Baseline
Surgery


A
Sensitive Patients
Wilcoxon, p = 0.0046
Dendritic Cells Gene Sig Expression
8.0
7.5
7.0
6.5
6.0
Baseline
Surgery
B
Resistant Patients
Wilcoxon, p = 0.69
Dendritic Cells Gene Sig Expression
7.0
6.5
6.0
5.5
Baseline
Surgery

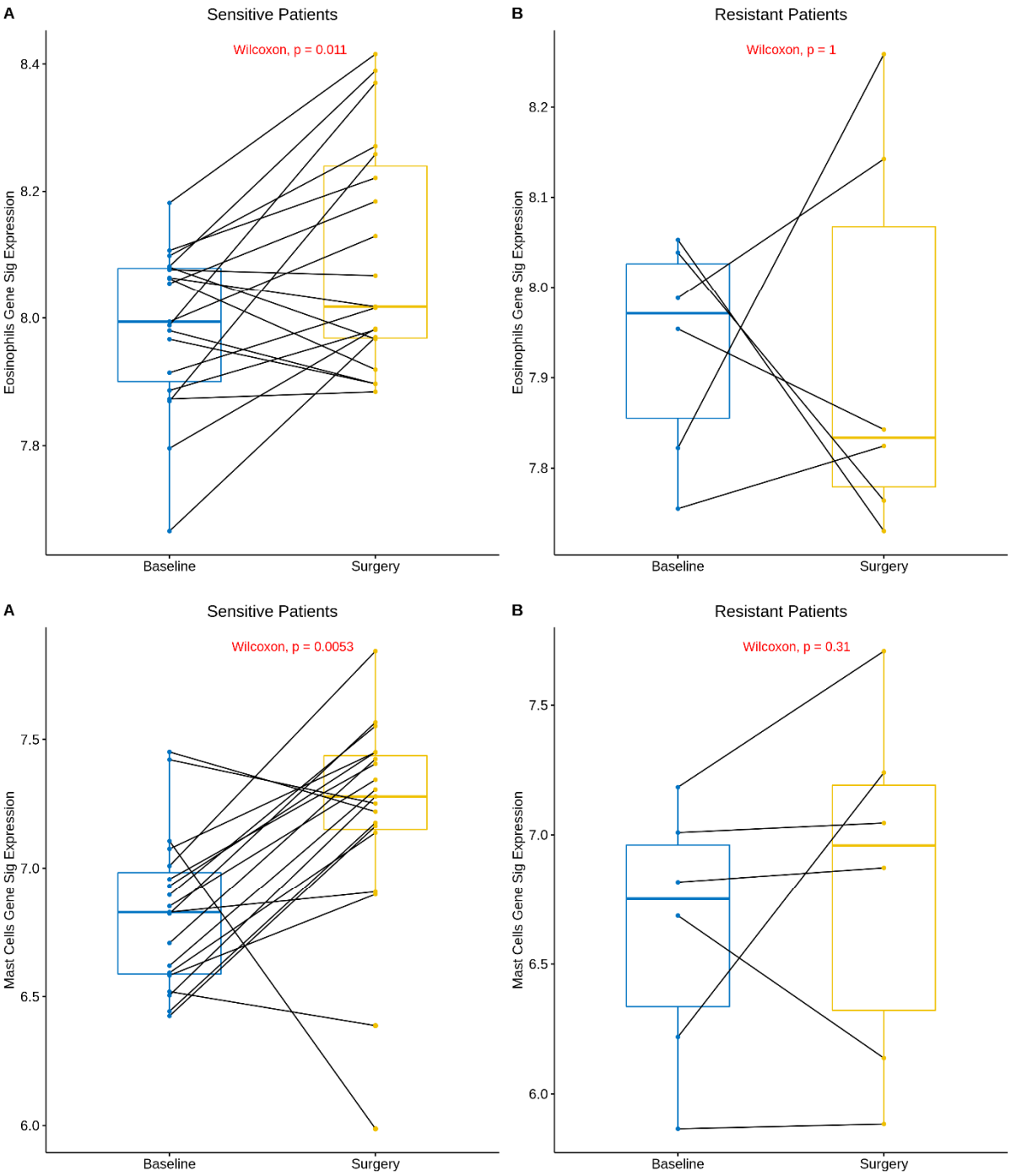


Figure S25: Changes in the gene signature expression of NK cells, dendritic cells, eosinophils and mast cells between baseline and surgery among sensitive subjects (n=19; left panels) and among resistant subjects (n=6; right panels).

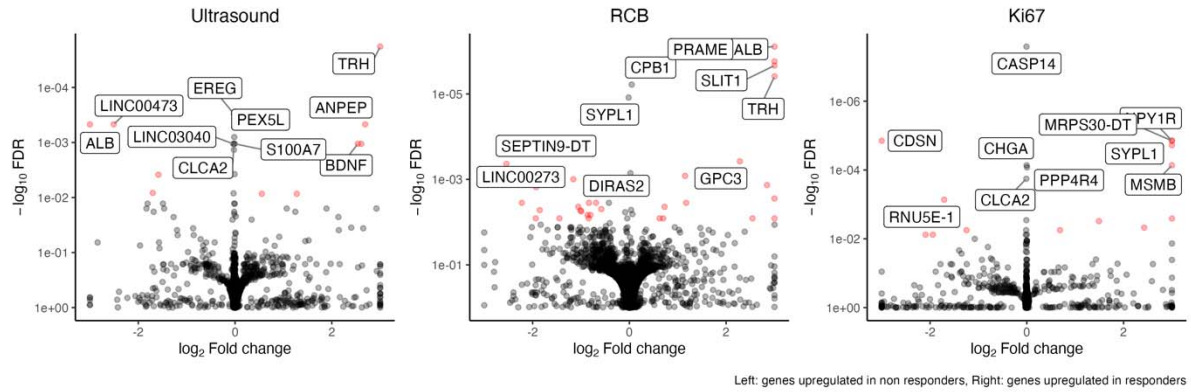


Figure S26: Volcano plot of differentially expressed genes between responders and non-responders of baseline samples, each of the three subfigures shows genes depending on the method (Ultrasound response, RCB status of Ki67 at surgery by IHC) defining responders and non-responders. When RCB class was used, subjects with RCB 0, I or II were considered as responders, when KI67 by IHC was used, subjects with a Ki67 quantification value of less or equal of 2.7 were considered responders, when Ultrasound response was used, subjects with complete or partial response were considered as responders. Each dot represents a gene and on the X axis the log 2 transformed fold change of the gene is displayed. Genes with negative fold change were upregulated in non-responders while genes with positive fold change were upregulated in responders. Y axis denotes the minus log 10 transformed FDR, genes with values close to 0 are not significantly differentially expressed. Genes coloured as red had FDR values less than 0.01 and absolute transformed fold changes greater that 0.5.

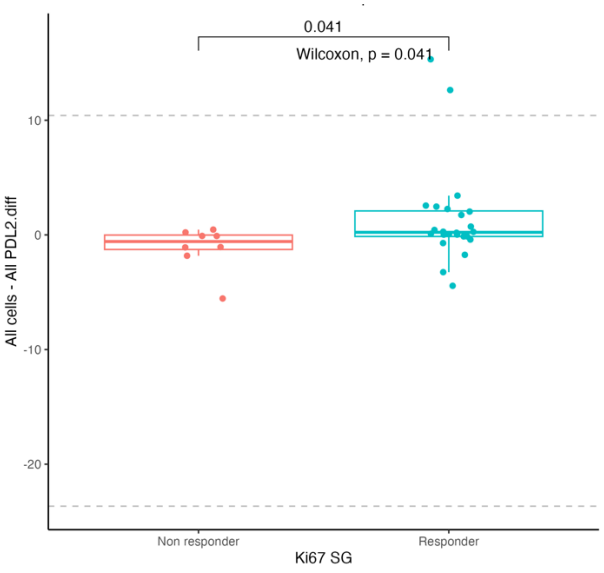


Figure S27: Responder vs non- responder boxplots. Each dot represents a baseline sample while Y axis denotes PDL2 quantification. Two separate boxplot split the subjects into responders and non-responders based on their Ki67 by IHC quantifications.

Supplementary Tables. DGEs

First 20 genes shown, sorted by FDR

All

Gene	P value	log2 Fold Change	Surgery %	Baseline %	FDR
PER3	0	0,561342	35,7	23,1	0
ERRFI1	0	0,864323	26,6	16,5	0
RERE	0	0,272863	79,9	76,3	0
CASZ1	0	0,375374	47,8	40,7	0
KAZN	0	0,928283	61,7	49,8	0
CYP4X1	0	0,257157	19	12,1	0
OSBPL9	0	0,401345	44,5	34,8	0
ECHDC2	0	0,347788	39,9	31,4	0
USP24	0	0,268602	45,4	37,7	0
NFIA	0	0,660729	64,9	53,5	0
PATJ	0	0,422926	71,7	65,5	0
PDE4B	0	0,45019	29,8	22,6	0
SLC44A5	0	0,974683	25,9	11,4	0
LINC01725	0	0,520472	25,8	15	0
SLC25A24	0	-0,41282	22,2	31,1	0
PRPF38B	0	-0,32547	22,5	29,5	0
SLC22A15	0	-0,26721	12,4	19,7	0
ATP1A1	0	0,350932	20,9	13,5	0
NBPF15	0	-0,2785	12	20,6	0
TXNIP	0	1,127371	32,5	19,4	0

Tumor

Gene	P value	log2 Fold Change	Surgery %	Baseline %	FDR
PER3	0	0,627088	41	25,1	0
RERE	0	0,297929	86,9	81	0
CASZ1	0	0,377828	64,7	55,1	0
KAZN	0	0,982658	73,8	58,7	0
MACF1	0	0,280405	80,9	74,2	0
CYP4X1	0	0,290773	26,6	16,8	0
CYP4Z1	0	1,065884	25,4	12,8	0
EPS15	0	0,323364	45,8	35,3	0
OSBPL9	0	0,46928	45	31,5	0
ECHDC2	0	0,365454	45,5	34,5	0
USP24	0	0,337642	49,5	37,6	0
NFIA	0	0,668953	70,9	58,1	0
PDE4B	0	0,62617	31	20	0
SLC44A5	0	1,149708	35,8	14,7	0
LINC01725	0	0,586708	32	17,3	0
PRKACB	0	0,46016	25,8	15,1	0
SLC25A24	0	-0,47829	24,3	33,9	0
FAM102B	0	-0,49486	14,1	22,4	0
SLC22A15	0	-0,41052	11,7	21,5	0
ATP1A1	0	0,402588	23,6	13,7	0

Immune

Gene	P value	log2 Fold Change	Surgery %	Baseline %	FDR
MTRNR2L12	0	-2,40512	21,5	49,2	0

FKBP5	0	2,256366	58,5	27,2	0
MT-CO1	6,4E-231	-0,904	42,4	62,6	2,3E-226
PLCG2	3,1E-211	-0,72482	57,9	74,3	1,1E-206
CYTIP	3E-187	1,128534	34,9	16	1,1E-182
NEAT1	2,5E-179	1,503478	84,3	77	9E-175
SMAP2	5,7E-168	0,939411	50,9	33,5	2,1E-163
ACSL1	6,4E-159	1,254372	28,9	12,7	2,3E-154
KLF6	5,9E-143	0,967076	29,2	13,6	2,2E-138
SH3BP5	1,1E-140	1,043091	26,6	11,6	4E-136
RASGEF1B	2,1E-127	1,101789	27,3	13,2	7,8E-123
MT-ND3	7,5E-126	-0,68536	31,1	46,9	2,7E-121
CELF2	1,7E-118	0,693503	71,3	62,6	6,3E-114
ANKRD28	3,3E-117	1,082535	39,3	24,7	1,2E-112
TXNIP	1,5E-114	1,215501	44,4	31,4	5,6E-110
FNIP2	6,5E-113	1,283004	32,2	18,7	2,4E-108
CBLB	4,1E-109	1,031889	55,5	44,5	1,5E-104
PIK3CD	8,3E-108	-0,57927	19,5	33,6	3E-103
MT-ND5	3,3E-103	-0,5901	17,3	30,3	1,19E-98
NAMPT	7,4E-102	1,270401	24	11,8	2,71E-97

Stromal

Gene	P value	log2 Fold Change	Surgery %	Baseline %	FDR
TGFBR3	0	1,028932	43,9	28,4	0
DNM3OS	0	-0,68772	17,1	32,9	0
KIF26B	0	-1,02796	26,6	50,3	0

MIR4435-2HG	0	-0,69662	32	55,7	0
FAP	0	-0,79746	27,2	48,9	0
COL3A1	0	-0,67541	39,1	59,2	0
FN1	0	-0,17182	33,8	56,4	0
COL6A3	0	-0,72128	41,9	60,7	0
GXYLT2	0	-0,72654	20,3	37,2	0
MTRNR2L12	0	-1,22098	17,8	33,1	0
SPARCL1	0	1,310157	52,1	33,8	0
LIFR	0	1,874597	39,1	18,6	0
ADAMTS6	0	-0,92053	20,4	37,2	0
PIK3R1	0	1,372218	54,8	39,8	0
VCAN	0	-1,02286	32,3	55,7	0
NREP	0	-0,81236	20,7	38,4	0
CHSY3	0	-0,63877	28,7	46,8	0
PDGFRB	0	-0,67546	18,7	35,6	0
SPARC	0	-0,70691	22,2	40	0
FKBP5	0	2,517205	44,4	11,6	0

GSEAs

First 10 gene sets shown, sorted by FDR, only hallmark genesets included, genes with dge FDR less than 0.05 and expressed in more than 10% of both baseline and surgery cells are included

All

Pathway	# of genes	NES	P value	FDR
HALLMARK_KRAS_SIGNALING_DN	24	2,09	0,000102	0,003867
HALLMARK_ESTROGEN_RESPONSE_EARLY	74	-1,86	0,000384	0,007284
HALLMARK_MYC_TARGETS_V1	71	-1,72	0,002249	0,028414

HALLMARK_P53_PATHWAY	48	1,77	0,004326	0,040948
HALLMARK_ESTROGEN_RESPONSE_LATE	56	-1,71	0,005403	0,040948
HALLMARK_HYPOXIA	43	1,74	0,009336	0,05693
HALLMARK_E2F_TARGETS	47	-1,68	0,010516	0,05693
HALLMARK_ANDROGEN_RESPONSE	55	1,62	0,015532	0,064933
HALLMARK_CHOLESTEROL_HOMEOSTASIS	20	1,59	0,016638	0,064933
HALLMARK_UV_RESPONSE_DN	74	1,55	0,017135	0,064933

Tumor

Pathway	# of genes	NES	P value	FDR
HALLMARK_ESTROGEN_RESPONSE_EARLY	88	-2,26	0	0,000004
HALLMARK_ESTROGEN_RESPONSE_LATE	62	-2,04	0,00003	0,000657
HALLMARK_E2F_TARGETS	49	-1,79	0,001807	0,019976
HALLMARK_MYC_TARGETS_V1	73	-1,77	0,00151	0,019976
HALLMARK_KRAS_SIGNALING_DN	23	1,85	0,002294	0,020288
HALLMARK_INTERFERON_GAMMA_RESPONSE	32	1,62	0,014989	0,09467
HALLMARK_UV_RESPONSE_DN	67	1,59	0,012954	0,09467
HALLMARK_CHOLESTEROL_HOMEOSTASIS	20	1,61	0,032299	0,178494
HALLMARK_ANGIOGENESIS	5	1,51	0,050096	0,246087
HALLMARK_P53_PATHWAY	46	1,41	0,06903	0,305185

Immune

Pathway	# of genes	NES	P value	FDR
HALLMARK_TNFA_SIGNALING_VIA_NFKB	27	2,61	0	0,000003
HALLMARK_ANDROGEN_RESPONSE	25	2,45	0,000001	0,000015

HALLMARK_ESTROGEN_RESPONSE_LATE	15	2,34	0,000008	0,000071
HALLMARK_ESTROGEN_RESPONSE_EARLY	19	2,28	0,000041	0,000282
HALLMARK_HYPOXIA	24	2,23	0,000057	0,000311
HALLMARK_HEME_METABOLISM	26	2,15	0,000358	0,001631
HALLMARK_CHOLESTEROL_HOMEOSTASIS	11	2,08	0,000824	0,002817
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	17	2,07	0,00074	0,002817
HALLMARK_INFLAMMATORY_RESPONSE	19	2,01	0,001254	0,003815
HALLMARK_COMPLEMENT	27	2,05	0,001702	0,004235

Stromal

Pathway	# of genes	NES	P value	FDR
HALLMARK_P53_PATHWAY	38	1,83	0,000688	0,018309
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	71	-1,69	0,000915	0,018309
HALLMARK_ANGIOGENESIS	12	-1,76	0,001554	0,020725
HALLMARK_ESTROGEN_RESPONSE_EARLY	41	1,81	0,00262	0,026203
HALLMARK_ANDROGEN_RESPONSE	41	1,67	0,011404	0,083166
HALLMARK_IL2_STAT5_SIGNALING	45	1,6	0,012475	0,083166
HALLMARK_KRAS_SIGNALING_DN	14	1,67	0,015472	0,084793
HALLMARK_ADIPOGENESIS	42	1,6	0,019078	0,084793
HALLMARK_GLYCOLYSIS	31	-1,51	0,018072	0,084793
HALLMARK_ESTROGEN_RESPONSE_LATE	27	1,58	0,035563	0,142251

Number of peaks

Peaks are filtered if they are accessible in less that 10% of both baseline and surgery cells, this applies to all subsequent tables (GSEAs, peak overrepresentation, motif overrepresentation)

Celltype	All	Upregulated	Downregulated	Upregulated %	Downregulated %
All	9085	7606	1479	83,72	16,28
Epithelial	9307	7823	1484	84,06	15,94
Immune	7396	2785	4611	37,66	62,34
Stromal	9122	5193	3929	56,93	43,07
Tumor	9733	8090	1643	83,12	16,88

GSEA of differentiated peaks

First 10 gene sets shown, sorted by FDR, only hallmark genesets included

All

Pathway	# of genes	NES	P value	FDR
HALLMARK_MYC_TARGETS_V1	84	-1,92	0,000059	0,002172
HALLMARK_ANDROGEN_RESPONSE	51	1,92	0,000331	0,006101
HALLMARK_APOPTOSIS	41	1,84	0,001152	0,012365
HALLMARK_E2F_TARGETS	57	-1,72	0,001342	0,012365
HALLMARK_ESTROGEN_RESPONSE_EARLY	69	-1,7	0,002039	0,015021
HALLMARK_KRAS_SIGNALING_DN	18	1,73	0,012051	0,061847
HALLMARK_HYPOXIA	39	1,72	0,011647	0,061847
HALLMARK_UV_RESPONSE_DN	73	1,51	0,01343	0,061847
HALLMARK_MYC_TARGETS_V2	12	-1,64	0,017895	0,073254
HALLMARK_TNFA_SIGNALING_VIA_NFKB	33	1,6	0,021467	0,079087

Tumor

Pathway	# of genes	NES	P value	FDR

HALLMARK_ESTROGEN_RESPONSE_EARLY	78	-2,19	0	0,000013
HALLMARK_MYC_TARGETS_V1	89	-1,84	0,000319	0,007052
HALLMARK_E2F_TARGETS	58	-1,84	0,000773	0,011388
HALLMARK_ANDROGEN_RESPONSE	51	1,76	0,002679	0,025105
HALLMARK_ESTROGEN_RESPONSE_LATE	61	-1,75	0,002839	0,025105
HALLMARK_MYC_TARGETS_V2	13	-1,65	0,016974	0,125069
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	47	-1,48	0,023176	0,146377
HALLMARK_KRAS_SIGNALING_DN	19	1,59	0,033059	0,182695
HALLMARK_CHOLESTEROL_HOMEOSTASIS	20	1,55	0,0504	0,247579
HALLMARK_UV_RESPONSE_DN	63	1,37	0,062783	0,277567

Immune

Pathway	# of genes	NES	P value	FDR
HALLMARK_TNFA_SIGNALING_VIA_NFKB	39	2,15	0,000001	0,000008
HALLMARK_ANDROGEN_RESPONSE	39	2,15	0,000001	0,000008
HALLMARK_HYPOXIA	39	1,99	0,000039	0,000231
HALLMARK_ESTROGEN_RESPONSE_EARLY	39	1,99	0,00004	0,000231
HALLMARK_UV_RESPONSE_UP	29	2,05	0,000079	0,000364
HALLMARK_HEME_METABOLISM	56	1,87	0,000254	0,000979
HALLMARK_ESTROGEN_RESPONSE_LATE	28	1,99	0,000399	0,001203
HALLMARK_COMPLEMENT	45	1,88	0,000416	0,001203
HALLMARK_INFLAMMATORY_RESPONSE	26	1,93	0,000497	0,001278
HALLMARK_CHOLESTEROL_HOMEOSTASIS	14	1,93	0,001493	0,003386

Stromal

Pathway	# of genes	NES	P value	FDR
HALLMARK_ANDROGEN_RESPONSE	36	1,95	0,000341	0,006645
HALLMARK_IL2_STAT5_SIGNALING	50	1,91	0,000303	0,006645
HALLMARK_ANGIOGENESIS	8	-1,8	0,002394	0,029619
HALLMARK_ESTROGEN_RESPONSE_EARLY	42	1,77	0,003802	0,029619
HALLMARK_ESTROGEN_RESPONSE_LATE	31	1,75	0,003121	0,029619
HALLMARK_APOPTOSIS	40	1,71	0,004982	0,032339
HALLMARK_ADIPOGENESIS	44	1,64	0,009274	0,051599
HALLMARK_HYPOXIA	42	1,68	0,010998	0,053541
HALLMARK_UV_RESPONSE_DN	74	1,52	0,023679	0,092222
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	51	-1,47	0,021834	0,092222

Overrepresentation of upregulated peaks

First 10 gene sets shown, sorted by FDR, only hallmark genesets included

All

ID	Mean TSS distance	Fold enrichment	P value	FDR
HALLMARK_MYC_TARGETS_V1	18161	2,153513	0	0
HALLMARK_E2F_TARGETS	16275	1,868046	1,18E-14	2,94E-13
HALLMARK_DNA_REPAIR	11854	1,994423	1,91E-11	3,18E-10
HALLMARK_OXIDATIVE_PHOSPHORYLATION	18266	1,678711	1,32E-09	1,65E-08
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	20673	1,875675	2,38E-09	2,38E-08

HALLMARK_P53_PATHWAY	61512	1,576324	3,38E-09	2,81E-08
HALLMARK_MTORC1_SIGNALING	35036	1,569251	8,94E-09	6,38E-08
HALLMARK_MYC_TARGETS_V2	6922	2,332492	5,31E-08	3,32E-07
HALLMARK_HEME_METABOLISM	32610	1,451568	3,56E-06	1,98E-05
HALLMARK_ADIPOGENESIS	16268	1,435852	8,68E-06	4,34E-05

Tumor

ID	Mean TSS distance	Fold enrichment	P value	FDR
HALLMARK_MYC_TARGETS_V1	19488	2,100465	0	0
HALLMARK_E2F_TARGETS	22788	1,805072	1,02E-13	2,55E-12
HALLMARK_P53_PATHWAY	65645	1,633406	3,27E-11	5,45E-10
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	28682	1,94646	4,47E-11	5,58E-10
HALLMARK_DNA_REPAIR	9459	1,891551	3,27E-10	3,27E-09
HALLMARK_OXIDATIVE_PHOSPHORYLATION	25896	1,639374	3,13E-09	2,6E-08
HALLMARK_MTORC1_SIGNALING	40894	1,549548	9,5E-09	6,79E-08
HALLMARK_MYC_TARGETS_V2	17963	2,235118	1,47E-07	9,21E-07
HALLMARK_ADIPOGENESIS	23494	1,469565	9,8E-07	5,44E-06
HALLMARK_HEME_METABOLISM	39214	1,456823	1,41E-06	7,04E-06

Immune

ID	Mean TSS distance	Fold enrichment	P value	FDR
HALLMARK_MYC_TARGETS_V1	16460	2,610448	1,95E-14	9,38E-13

HALLMARK_P53_PATHWAY	80750	2,059935	4,95E-10	1,19E-08
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	12483	2,56129	1,76E-09	2,82E-08
HALLMARK_E2F_TARGETS	17973	2,069041	1,45E-08	1,75E-07
HALLMARK_DNA_REPAIR	13500	2,293427	2,47E-07	2,37E-06
HALLMARK_G2M_CHECKPOINT	32246	1,683107	3,71E-06	2,97E-05
HALLMARK_MTORC1_SIGNALING	20226	1,747806	6,39E-06	3,69E-05
HALLMARK_ADIPOGENESIS	7130	1,762147	6,52E-06	3,69E-05
HALLMARK_OXIDATIVE_PHOSPHORYLATION	21307	1,833864	7,62E-06	3,69E-05
HALLMARK_UV_RESPONSE_UP	89596	1,795119	7,68E-06	3,69E-05

Stromal

ID	Mean TSS distance	Fold enrichment	P value	FDR
HALLMARK_MYC_TARGETS_V1	19803	2,327678	0	0
HALLMARK_E2F_TARGETS	21651	2,158447	2,22E-16	5,44E-15
HALLMARK_DNA_REPAIR	8601	2,229306	2,14E-11	3,04E-10
HALLMARK_P53_PATHWAY	55624	1,812272	2,48E-11	3,04E-10
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	33413	2,047467	6,77E-09	6,64E-08
HALLMARK_OXIDATIVE_PHOSPHORYLATION	9790	1,729055	7,83E-08	6,4E-07
HALLMARK_G2M_CHECKPOINT	36727	1,566666	1,23E-07	8,6E-07
HALLMARK_ADIPOGENESIS	19857	1,610557	5,67E-07	3,47E-06
HALLMARK_MTORC1_SIGNALING	33275	1,592205	7,24E-07	3,94E-06
HALLMARK_MYC_TARGETS_V2	17660	2,299444	9,5E-06	4,66E-05

Overrepresentation of downregulated peaks

First 10 gene sets shown, sorted by FDR, only hallmark genesets included

All

ID	Mean TSS distance	Fold enrichment	P value	FDR
HALLMARK_FATTY_ACID_METABOLISM	23251	1,586195	0,013333	0,441124
HALLMARK_E2F_TARGETS	27868	1,494381	0,026158	0,441124
HALLMARK_MTORC1_SIGNALING	49145	1,442705	0,028157	0,441124
HALLMARK_PROTEIN_SECRETION	66459	1,532863	0,039135	0,459841
HALLMARK_OXIDATIVE_PHOSPHORYLATION	13724	1,33673	0,097788	0,802219
HALLMARK_PEROXISOME	36265	1,385076	0,124124	0,802219
HALLMARK_XENOBIOTIC_METABOLISM	65884	1,282238	0,128681	0,802219
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	22099	1,364995	0,144345	0,802219
HALLMARK_DNA_REPAIR	14606	1,349559	0,153616	0,802219
HALLMARK_REACTIVE_OXYGEN_SPECIES_PATHWAY	31750	1,522017	0,205675	0,966673

Tumor

ID	Mean TSS distance	Fold enrichment	P value	FDR

HALLMARK_DNA_REPAIR	24961	2,105739	0,000441	0,020286
HALLMARK_OXIDATIVE_PHOSPHORYLATION	13127	1,704676	0,002425	0,040212
HALLMARK_E2F_TARGETS	17167	1,681519	0,002623	0,040212
HALLMARK_MYC_TARGETS_V2	25898	2,284169	0,010537	0,104472
HALLMARK_MTORC1_SIGNALING	51356	1,501619	0,011356	0,104472
HALLMARK_MYC_TARGETS_V1	28998	1,439423	0,041934	0,321492
HALLMARK_G2M_CHECKPOINT	34406	1,246131	0,102946	0,676506
HALLMARK_PROTEIN_SECRETION	61598	1,314149	0,136305	0,708771
HALLMARK_PEROXISOME	65572	1,324748	0,151851	0,708771
HALLMARK_WNT_BETA_CATENIN_SIGNALING	105650	1,383954	0,154081	0,708771

Immune

ID	Mean TSS distance	Fold enrichment	P value	FDR
HALLMARK_E2F_TARGETS	13610	1,883081	8,71E-10	4,12E-08
HALLMARK_MYC_TARGETS_V1	10880	1,918618	1,65E-09	4,12E-08
HALLMARK_OXIDATIVE_PHOSPHORYLATION	11229	1,732915	3,49E-07	5,81E-06
HALLMARK_MTORC1_SIGNALING	46888	1,576257	4,89E-06	6,12E-05
HALLMARK_DNA_REPAIR	8099	1,789227	1,65E-05	0,000165
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	11036	1,63456	0,000379	0,003054
HALLMARK_REACTIVE_OXYGEN_SPECIES_PATHWAY	33423	2,115508	0,000428	0,003054
HALLMARK_HEME_METABOLISM	29504	1,424895	0,000509	0,003183

HALLMARK_MYC_TARGETS_V2	1828	1,997753	0,00078	0,004336
HALLMARK_FATTY_ACID_METABOLISM	15123	1,30829	0,015545	0,077723

Stromal

ID	Mean TSS distance	Fold enrichment	P value	FDR
HALLMARK_MYC_TARGETS_V1	21502	1,560552	0,000282	0,014123
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	16169	1,609993	0,001407	0,035179
HALLMARK_OXIDATIVE_PHOSPHORYLATION	29789	1,425698	0,003089	0,051482
HALLMARK_DNA_REPAIR	19785	1,52405	0,004649	0,058118
HALLMARK_HEME_METABOLISM	49846	1,344679	0,006688	0,063561
HALLMARK_MITOTIC_SPINDLE	37107	1,30611	0,007627	0,063561
HALLMARK_E2F_TARGETS	12804	1,285788	0,028706	0,195105
HALLMARK_MTORC1_SIGNALING	40406	1,255872	0,031217	0,195105
HALLMARK_PROTEIN_SECRETION	56475	1,318899	0,036519	0,202882
HALLMARK_FATTY_ACID_METABOLISM	13268	1,215513	0,08219	0,410952

Motif overrepresentation of upregulated peaks

First 10 motifs shown, sorted by FDR

All

Motif	JASPAR ID	Peaks #	Peaks %	Fold enrichment	P value	FDR

CREB3L4	MA1475.1	891	11,71	6,189927	0	0
Atf1	MA0604.1	512	6,73	6,119571	0	0
ELK3	MA0759.2	1878	24,69	6,077793	0	0
ETV5	MA0765.3	1586	20,85	5,983345	0	0
ETV4	MA0764.3	1644	21,61	5,869522	0	0
ZBED1	MA0749.1	178	2,34	5,850644	0	0
GMEB2	MA0862.1	198	2,6	5,849906	0	0
ELK1	MA0028.2	1630	21,43	5,823492	0	0
ZBTB33	MA0527.1	1556	20,46	5,710407	0	0
FEV	MA0156.3	1634	21,48	5,690871	0	0

Tumor

Motif	JASPAR ID	Peaks #	Peaks %	Fold enrichment	P value	FDR
ETV5	MA0765.3	1626	20,1	6,049327	0	0
ELK3	MA0759.2	1923	23,77	6,033017	0	0
ZBED1	MA0749.1	180	2,22	5,973071	0	0
ELK1	MA0028.2	1669	20,63	5,885994	0	0
ETV4	MA0764.3	1676	20,72	5,860519	0	0
CREB3L4	MA1475.1	875	10,82	5,768438	0	0
Atf1	MA0604.1	509	6,29	5,75901	0	0
FEV	MA0156.3	1669	20,63	5,718755	0	0
ETV3	MA0763.1	1463	18,08	5,68681	0	0
ZBTB33	MA0527.1	1550	19,16	5,639281	0	0

Immune

Motif	JASPAR ID	Peaks #	Peaks %	Fold enrichment	P value	FDR
Nrf1	MA0506.2	1323	47,5	3,59542	0	0
KLF3	MA1516.1	1409	50,59	3,155619	0	0
KLF15	MA1513.1	2186	78,49	3,049414	0	0
KLF14	MA0740.2	2117	76,01	2,983881	0	0
KLF12	MA0742.2	2215	79,53	2,968488	0	0
ZNF610	MA1713.1	1608	57,74	2,885451	0	0
KLF6	MA1517.1	1486	53,36	2,854857	0	0
SP3	MA0746.2	1970	70,74	2,780233	0	0
SP9	MA1564.1	1975	70,92	2,779097	0	0
KLF2	MA1515.1	1930	69,3	2,743732	0	0

Stromal

Motif	JASPAR ID	Peaks #	Peaks %	Fold enrichment	P value	FDR
CREB3L4	MA1475.1	726	13,98	6,836361	0	0
FOSL1::JUND	MA1143.1	668	12,86	6,321116	0	0
FOSL1::JUN	MA1129.1	538	10,36	6,203653	0	0
ATF7	MA0834.1	661	12,73	5,619723	0	0
JUNB	MA1140.2	641	12,34	5,617083	0	0
JUN::JUNB	MA1133.1	699	13,46	5,438557	0	0
FOS	MA1951.1	668	12,86	5,261133	0	0
Creb5	MA0840.1	653	12,57	4,955515	0	0
CREM	MA0609.2	697	13,42	4,911954	0	0

NFYA	MA0060.3	1260	24,26	4,896757	0	0

Motif overrepresentation of downregulated peaks

First 10 motifs shown, sorted by FDR

All

Motif	JASPAR ID	Peaks #	Peaks %	Fold enrichment	P value	FDR
KLF15	MA1513.1	1084	73,29	2,770731	0	0
KLF12	MA0742.2	1083	73,23	2,656936	9,1E-304	3,8E-301
ZNF610	MA1713.1	915	61,87	3,069898	1,3E-280	3,6E-278
SP2	MA0516.3	1137	76,88	2,351136	9,3E-277	1,9E-274
KLF10	MA1511.2	1098	74,24	2,414288	5,2E-270	8,7E-268
KLF14	MA0740.2	1018	68,83	2,622606	7,8E-268	1,1E-265
ZNF93	MA1721.1	970	65,58	2,732418	1,7E-262	2E-260
Foxn1	MA1684.1	993	67,14	2,64878	1E-260	1,1E-258
KLF7	MA1959.1	1068	72,21	2,375751	4,9E-250	4,6E-248
PATZ1	MA1961.1	1141	77,15	2,195723	1,8E-248	1,5E-246

Tumor

Motif	JASPAR ID	Peaks #	Peaks %	Fold enrichment	P value	FDR
KLF15	MA1513.1	1097	66,77	2,892586	0	0
KLF12	MA0742.2	1102	67,07	2,772447	0	2,1E-307
SP2	MA0516.3	1181	71,88	2,485931	3,4E-297	9,5E-295

KLF14	MA0740.2	1035	62,99	2,718797	7,3E-273	1,5E-270
KLF10	MA1511.2	1119	68,11	2,486796	1E-270	1,7E-268
PATZ1	MA1961.1	1180	71,82	2,302102	5,5E-262	7,7E-260
KLF7	MA1959.1	1092	66,46	2,45571	1,2E-254	1,5E-252
SP1	MA0079.5	1120	68,17	2,352242	1,3E-247	1,3E-245
SP4	MA0685.2	1120	68,17	2,288476	3,4E-236	3,2E-234
Foxn1	MA1684.1	976	59,4	2,62007	6,8E-236	5,7E-234

Immune

Motif	JASPAR ID	Peaks #	Peaks %	Fold enrichment	P value	FDR
ZBTB33	MA0527.1	1063	23,05	5,003487	0	0
ELK3	MA0759.2	1199	26	4,738594	0	0
ETV5	MA0765.3	1000	21,69	4,653921	0	0
ELK1	MA0028.2	1039	22,53	4,63167	0	0
ETV4	MA0764.3	1046	22,68	4,603731	0	0
FEV	MA0156.3	1040	22,55	4,54504	0	0
ETV3	MA0763.1	908	19,69	4,537337	0	0
HINFP	MA0131.2	1068	23,16	4,473588	0	0
FLI1	MA0475.2	895	19,41	4,44676	0	0
Nrf1	MA0506.2	2321	50,34	4,391376	0	0

Stromal

Motif	JASPAR ID	Peaks #	Peaks %	Fold enrichment	P value	FDR

HINFP	MA0131.2	952	24,23	4,615254	0	0
ZBTB33	MA0527.1	834	21,23	4,347522	0	0
Nrf1	MA0506.2	1958	49,83	4,235832	0	0
TCFL5	MA0632.2	1245	31,69	4,209559	0	0
ZBTB14	MA1650.1	1617	41,16	3,967752	0	0
EGR2	MA0472.2	1211	30,82	3,59861	0	0
ZNF610	MA1713.1	2518	64,09	3,581811	0	0
TFDP1	MA1122.1	1359	34,59	3,500021	0	0
ELK4	MA0076.2	1195	30,41	3,414523	0	0
ZBTB7A	MA0750.2	1439	36,63	3,362414	0	0