5.2.1 PIK3CA mutation subtypes and response to study treatment:

Predefined subset analyses investigated potential interactions of PI3K mutations, luminal A/B subtypes, and baseline Ki-67 scores with Ki-67 response. Three major hotspots of mutations of the PIK3CA gene have been described; these are concentrated in the helical (E542K and E545K) and kinase (H1047R) domains, accounting to approximately 90% of all PIK3CA mutations. In the OPPORTUNE trial, PIK3CA mutations were tested using NGS. Given the limited power of these analyses, results must be considered exploratory and interpreted with caution.

At least one PIK3CA mutation was detected in 49 tumours (36.0%), including 19 helical domain and 29 kinase domain mutations. There was no significant correlation between PIK3CA mutation and added activity of pictilisib; the ratio (combination/anastrozole) of geometric mean Ki67 proportional change was 0.63 (0.39–1.0; p=0.05) for patients with PIK3CA-wildtype tumours and 0.72 (0.46–1.15; p=0.12) for patients with PIK3CA-mutated tumours.

A significant interaction was observed between PIK3CA mutation subtypes [helical domain mutations (HD), kinase domain mutations (KD), wildtype (WT)] and mean Ki67 suppression. The combination/anastrozole geometric mean ratio of Ki67 suppression was 0.48 (0.27-0.84; p=0.02) for patients with HD mutations and 0.63 (0.39–1.0; p=0.05) for patients with PIK3Ca WT, compared to 1.17 (0.57–2.41; p=0.64) for patients with KD mutations. This was largely due to patients with HD mutations showing a particularly poor response to anastrozole alone [mean Ki67 suppression 53.9% (9.5%-76.5%)], that was reversed by the addition of pictilisib [mean Ki-67 suppression 78.1% (71.0%-83.4%)]. On the other hand, patients with KD mutations responded well to anastrozole alone [mean Ki-67 suppression 77.7% (57.0%-88.4%)] and showed no benefit from the addition of pictilisib [mean Ki-67 suppression 73.9% (59.8%-83.0%)].

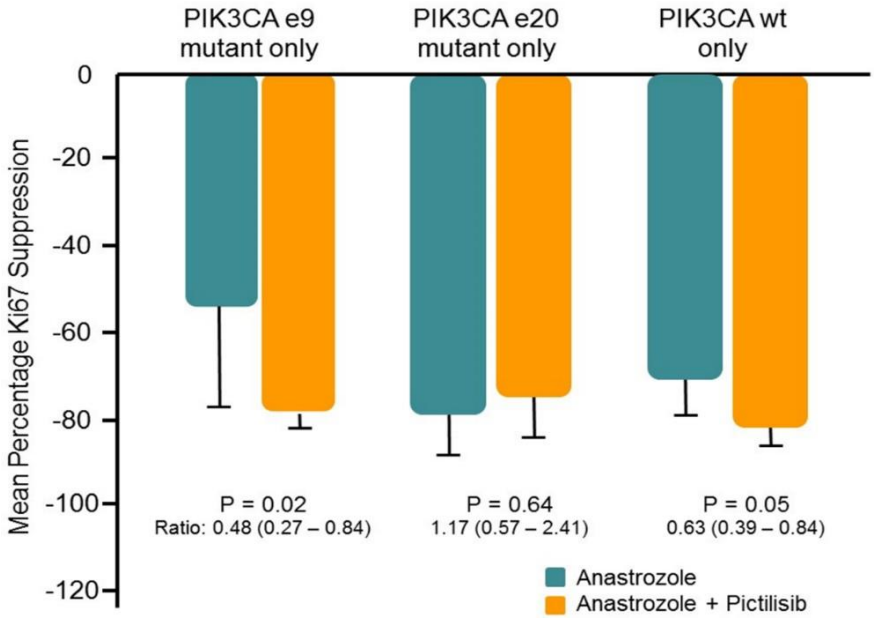


Figure 9: Anti-proliferative response to study treatment by PIK3CA mutation status; e9: exon 9 domain mutations (helical domain); e20: exon 20 domain mutations (kinase domain).

	Anastrozole (n = 46)	Pictilisib plus Anastrozole (n = 90)	Relative Risk (combination/ anastrozole)	p-Value
Geometric mean Ki67 suppression [% (95% CI)]				
PIK3Ca WT	69.9 (45.7 – 80.2)	81.1 (75.2 – 85.6)	0.63 (0.39–1.0)	0.05
HD mutations	53.9 (9.5 - 76.5)	78.0 (71.0 – 83.3)	0.48 (0.27-0.84)	0.02
KD mutations	77.7 (57.0-88.4)	73.9 (59.8 – 83.0)	1.17 (0.57–2.41	0.64
$R_{Ki67\text{-}Day15}$ response rate [% (95% CI)]				
PIK3Ca WT	59.3 (42.8-75.7)	84.9 (76.6-93.2)	1.43 (1.08-1.89)	0.01
HD mutations	75.0 (16.2-133.8)	84.6 (66.1-103.3)	1.13 (0.68 -1.88)	0.58
KD mutations	81.8 (59.7-103.9)	78.6 (58.4-98.7)	0.96 (0.69-1.33)	0.62

Table 5: PIK3CA status and anti-proliferative response to anastrozole or anastrozole plus pictilisib. Geometric mean Ki67 suppression is defined as Ln($Ki67_{Day15}$) - Ln($Ki67_{baseline}$); the ratio (combination/ anastrozole) of geometric mean Ki67 suppression is provided with 95% CI; individual EOT anti-proliferative response $R_{Ki67\text{-}Day15}$ is defined as Ln($Ki67_{Day15}$) ≤2.

Further NGS analysis demonstrated a range of somatic mutations in keeping with the expected mutational landscape of ER-positive early breast cancer. Figure 10 shows an overview of the somatic variants for each treatment group, divided by response to treatment. There was no specific mutational pattern associated with response to anastrozole or anastrozole plus pictilisib.

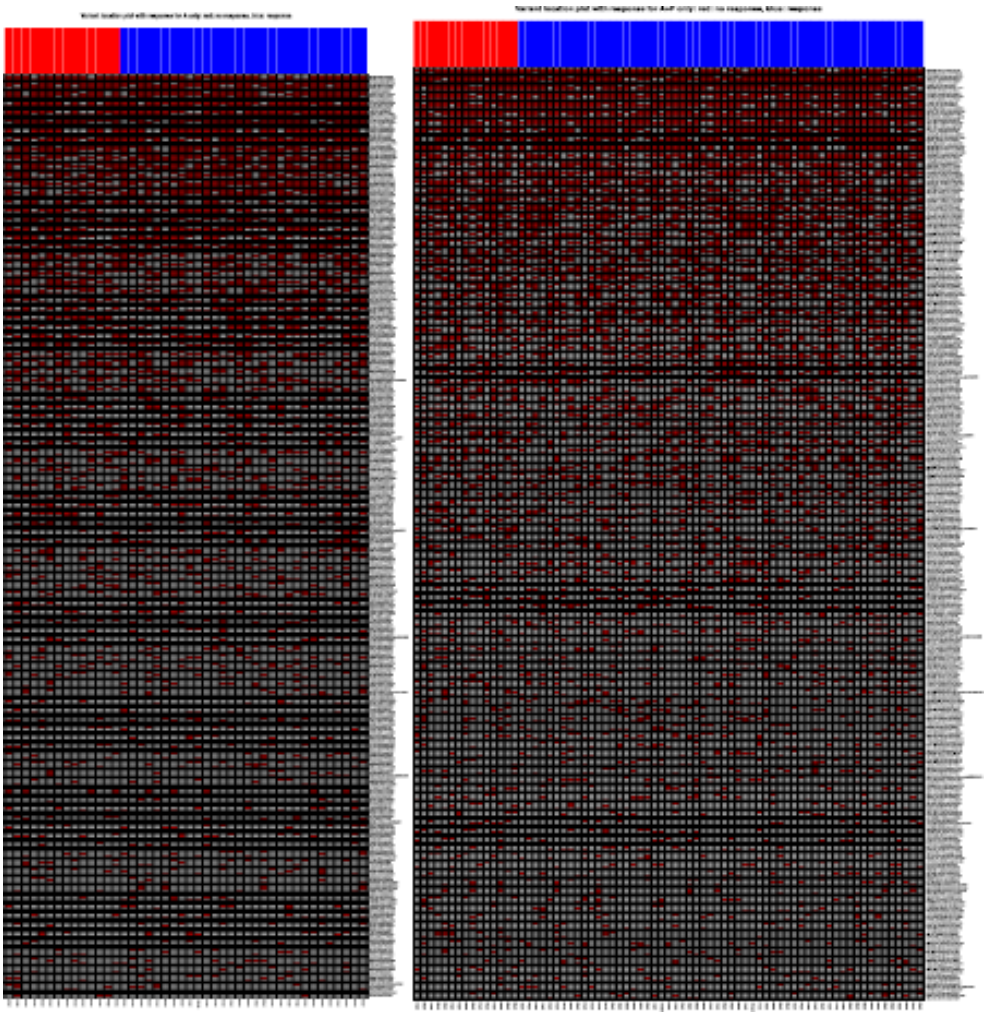


Figure 10: Somatic variant analysis and response to anastrozole (a) or anastrozole and pictilisib (b). Red demonstrates the presence of a somatic mutation; grey, no mutation detected.

5.2.2 PAM50 Luminal Status and treatment response:

NanoString PAM50 analysis was performed in a subset of patients (n=53) to assess luminal status. PAM50 results analysis showed that patients with Luminal B tumours had a significantly higher anti-proliferative response with the combination of anastrozole plus pictilisib compared to anastrozole alone [geometric mean Ki67 suppression, 86.5% versus 63.6%; ratio (combination/anastrozole) 0.37 (0.18-0.76; p=0.008)], whereas adding pictilisib to anastrozole had no apparent benefit for Luminal A tumours (ratio, 1.01; p=0.98).

5.2.3 Baseline Ki67 expression, PR, tumour grade and treatment response:

As it had been hypothesised that luminal B biology could be a determinant of suboptimal response to endocrine therapy alone and potentially therefore define a subgroup that might derive an increased benefit from combination therapy with pictilisib and anastrozole, the impact of several baseline characteristics that have been linked with luminal B phenotype were explored. These include baseline Ki67 expression, PR expression and tumour grade.

In an analysis involving all evaluable patients (n=136), luminal status was defined by baseline Ki67 expression in accordance to the St Gallen criteria using a Ki67 expression of 14% as the cut-off between luminal A and luminal B. In contrast to the PAM50 analysis, patients with Luminal A status (n=50) defined as baseline Ki67 of <14% had a significant benefit of the combination of anastrozole plus pictilisib compared to anastrozole alone [geometric mean Ki67

suppression, 74.1% versus 43.4%; ratio (combination/anastrozole) 0.46 (0.25 – 0.85); p=0.02)]. In patients with Luminal B tumours (n=86), defined as Ki67 >14%, geometric mean Ki67 suppression was 78.7% in the anastrozole alone group and 86.3% for patients treated with anastrozole plus pictilisib [ratio, 0.64 (0.43 – 0.97); p=0.04].

Using a Ki67 cut-off of 20%, mean geometric Ki67 suppression for Luminal A tumours was 61.6% in the anastrozole alone group and 77.6% for patients treated with anastrozole plus pictilisib [ratio, 0.58 (0.25 – 0.97); p=0.04]. For Luminal B tumours, geometric mean Ki67 suppression was 77.6% for patients treated with anastrozole alone and 86.7% for patients treated with anastrozole plus pictilisib [ratio, 0.59 (0.36 - 0.96); p=0.04].

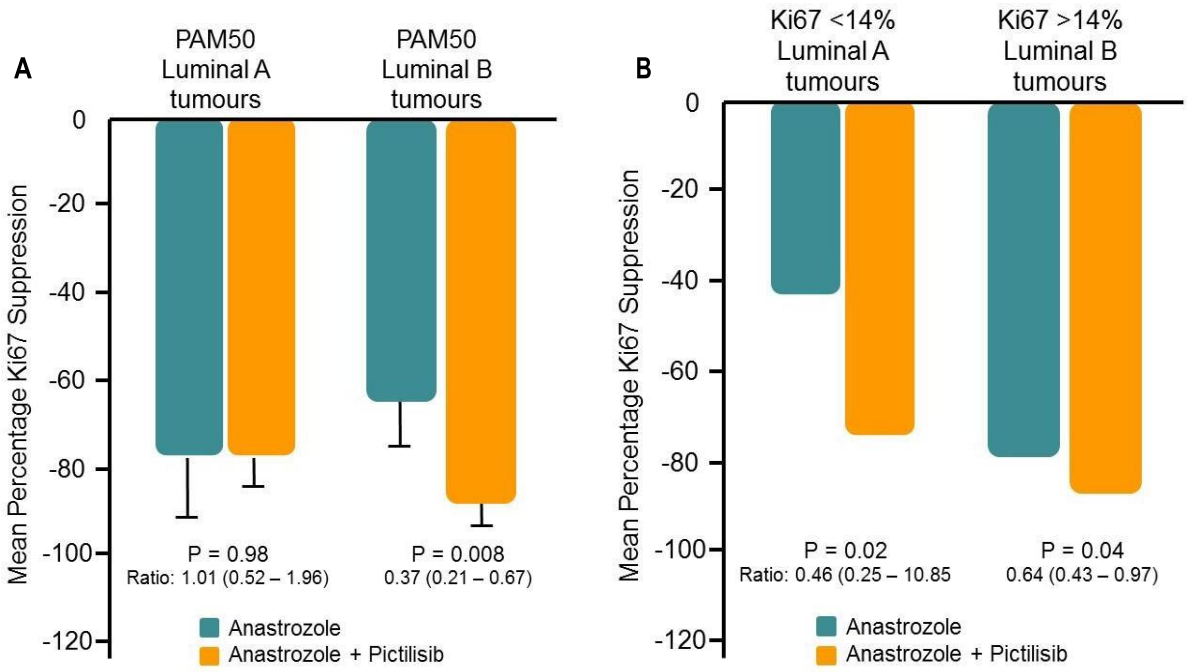


Figure 11: Anti-proliferative response to study treatment; a) anti-proliferative response by Luminal subtype defined by PAM50; c) anti-proliferative response by Luminal subtype defined by baseline Ki67 expression (cut-off 14%)

PR receptor status was available in 136 patients; the majority of tumours were classified as PR positive (84.6%), defined by an Allred score of 3 or higher. Only 21 tumours were PR negative (15.4%). The addition of pictilisib increased the anti-proliferative response in both subsets with a slightly more pronounced benefit in patients with PR negative tumours. In PR-positive tumours, the geometric mean Ki67 suppression was 72.1% with anastrozole compared to 81.7% with the combination [0.65 (0.43–0.98); p=0.04], whereas in PR-negative tumours the mean Ki67 suppression was 66.7% with anastrozole compared to 88.4% with the combination [0.35 (0.14–0.87); p=0.03].

Tumour grade was available for 135 patients; the majority of tumours were classified as Grade 1 or Grade 2 (n=115; 85.2%) with the remaining 21 tumours classified as Grade 3 (15.6%). Tumour grade was a strong predictor of response to anastrozole alone with a mean geometric Ki67 suppression of 73.2% (61.0%-81.6%) in patients with Grade 1 or Grade 2 tumours compared to 50% (19.4%-69.0%) in patients with G3 tumours. In contrast, patients responded to anastrozole plus pictilisib irrespective of the tumour grade with a mean geometric Ki67 suppression of 80.4% (74.8%-84.8%) for patients with Grade 1 or Grade 2 tumours and 90.3% (78.8%-95.5%) for patients with Grade 3 tumours.

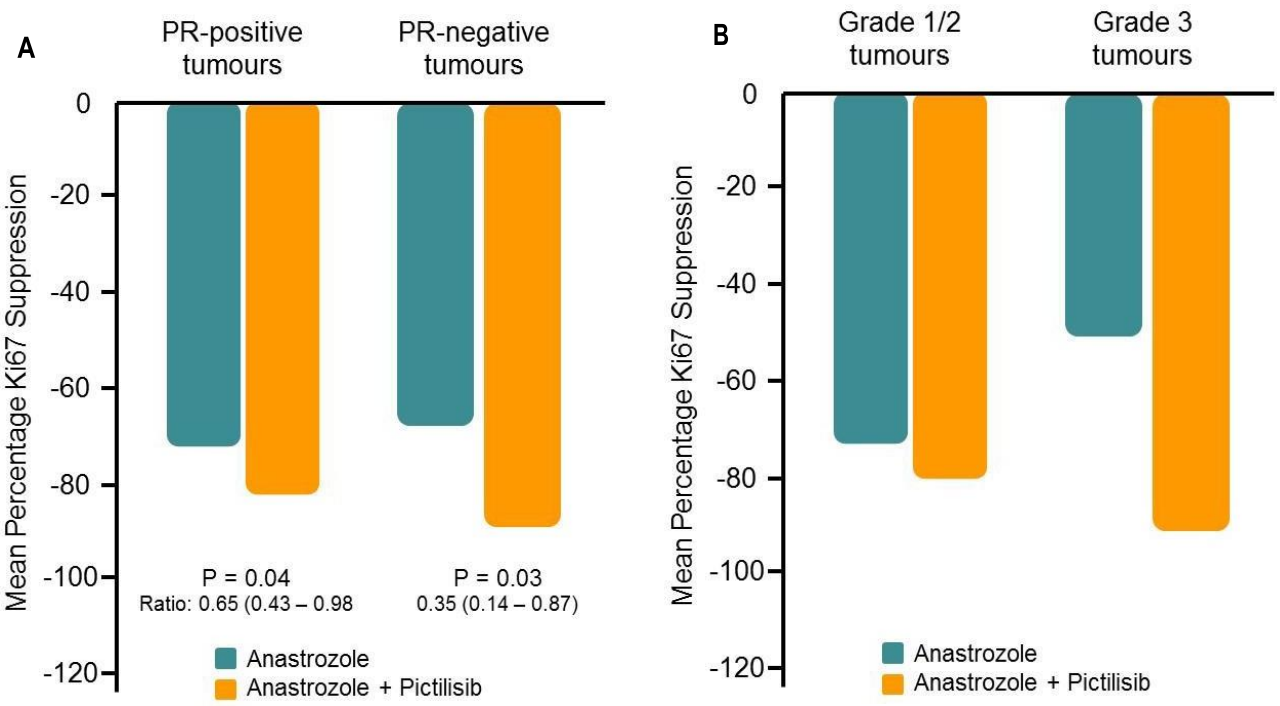


Figure 12: Anti-proliferative response to study treatment PR status (a) and tumour grade (b)

Multivariate linear regression analysis confirmed a significant interaction between treatment effect and molecular subtype by PAM50 (p=0.03), supporting the observation that the combination treatment is more effective than anastrozole alone for patients with Luminal B tumours irrespective of PR status or the baseline Ki67 expression. However, patients with PR-negative Luminal B cancers showed the greatest anti-proliferative effect from combination treatment (ratio=0.12). Furthermore, combined treatment also appeared to be more effective in PR-negative Luminal A cancers.

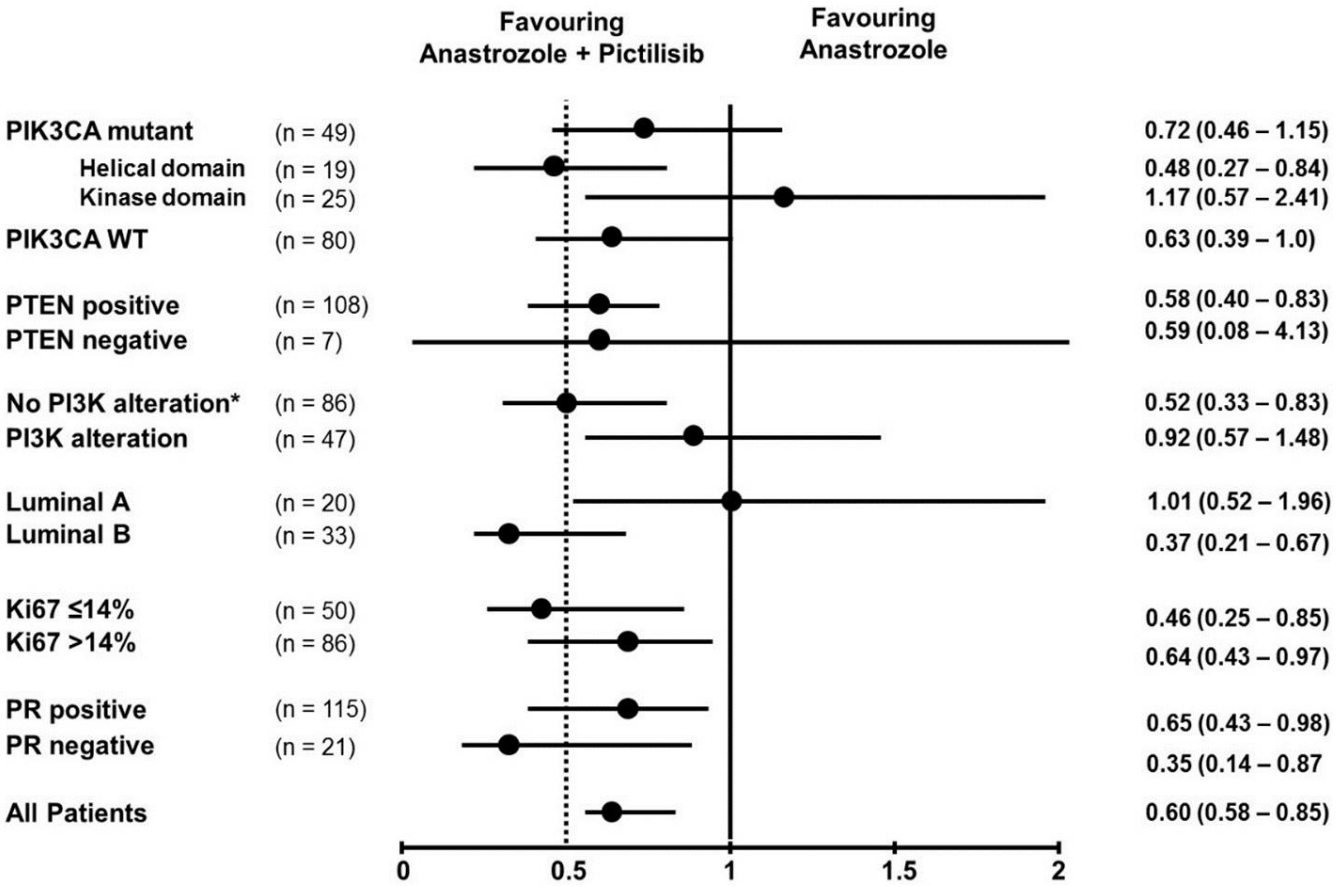


Figure 13: Ratio (combination/anastrozole) of geometric mean of Ki67 proportional changes in pre-specified subgroups

RPPA analysis focused on key genes involved in the activation of the PI3K pathway and cell cycle. There was substantial downregulation of cell cycle genes in both arms, associated with endocrine therapy. Phospho-AKT levels, pS6 levels or p4E-BP1 levels were comparable between both arms. Overall, the end-of-treatment profiles as well as the treatment-associated changes were largely comparable between both groups, suggesting a dominant anti-oestrogen effect. The effects on Cyclin D1 were more pronounced with the combination in keeping with the more substantial anti-proliferative effect as per Ki67 analysis.

5.2.4 PI3K kinase pathway activation and treatment benefit

To further assess the potential interaction of activation of the PI3K pathway and study treatment, an analysis of changes in gene/protein expression and phosphorylation of selected signalling markers was performed.

Two gene signatures (GS) were calculated as baseline and at the end of treatment and correlated with response to anastrozole and the combination therapy, respectively. The PIK3CA mutation associated GS [48] has previously been shown to negatively correlate with proliferation, AKT/mTOR activation and PTEN loss and strongly positively correlated with ESR1 and better outcome in ER-positive breast cancer. O'Brien et al identified a PIK3 inhibitor sensitivity GS, based on a number of genes that are differentially expressed between sensitive and resistant breast cancer cell lines; the PI3K inhibitor sensitivity GS [43] has been shown to correlate with activation of the PI3K pathway and can be used to characterise patients who are sensitive to PI3K inhibition.

The baseline PIK3 inhibitor sensitivity GS (O'Brien) score was associated with higher proliferation and Luminal B phenotype (Figure 14c). The baseline PIK3 inhibitor sensitivity (O'Brien) score was inversely associated with ΔKi67 in the anastrozole arm, characterising patients with partial endocrine resistance (Figure 14d). Post-treatment PIK3 inhibitor sensitivity GS (O'Brien) scores were significantly down-regulated in both arms, consistent with an attenuation of the flux through the PI3K pathway (Figure 14a).

In contrast, we observed no relevant modulation of the PIK3CA mutation-associated GS (Loi) with study treatment (Figure 14b). The PIK3CA mutation-associated GS (Loi) was not predictive of a treatment-induced change in Ki67 in either treatment arm.

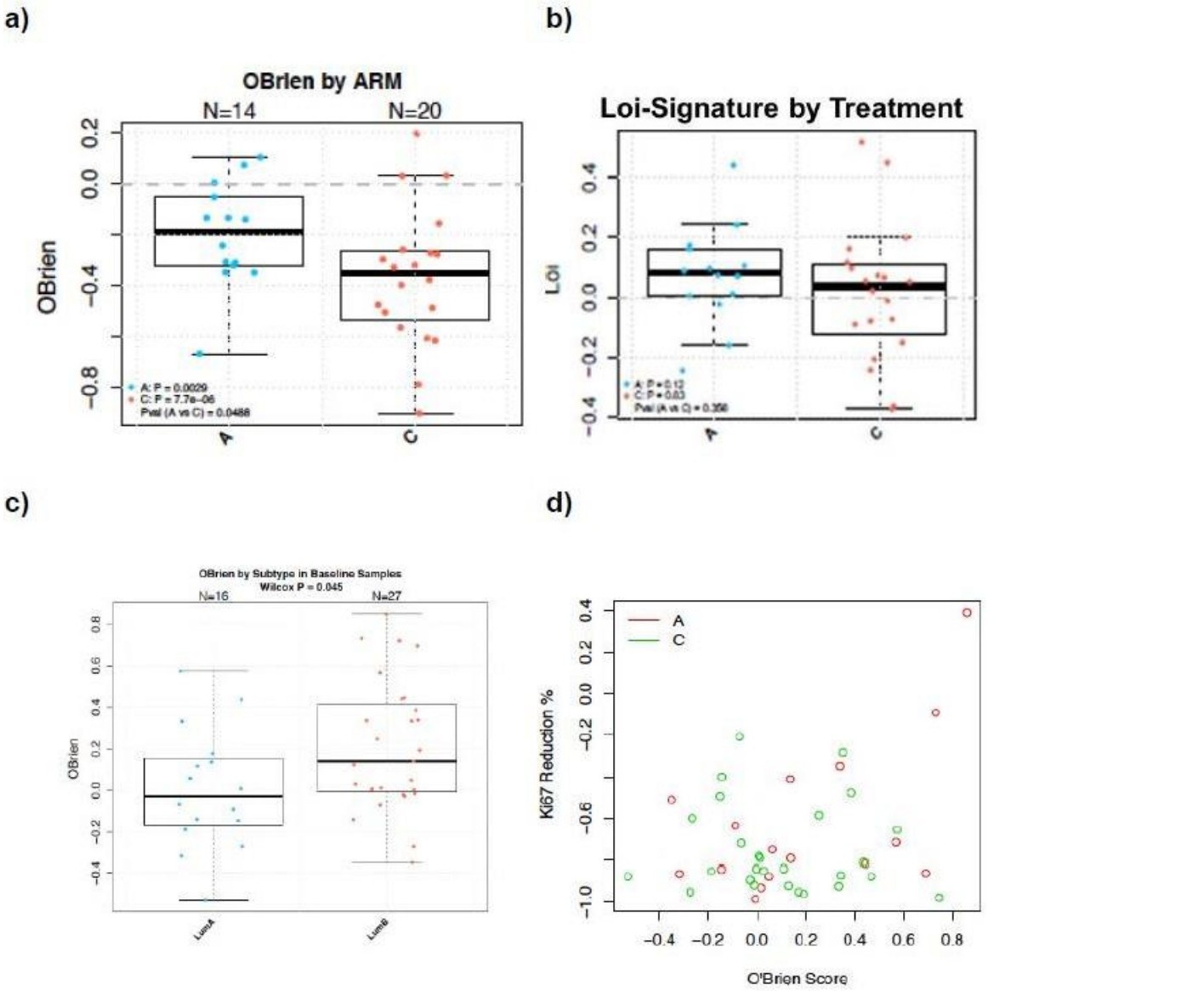


Figure 14: PI3K mutation and PI3K inhibitor sensitivity gene signatures: a) down-regulation of post-treatment PIK3 inhibitor sensitivity GS (O'Brien) scores in both treatment arms; b) post-treatment PIK3CA mutation-associated GS (Loi) in both treatment groups; c) association of baseline PIK3 inhibitor sensitivity GS (O'Brien) score and Luminal B phenotype; d) inverse association of baseline PIK3 inhibitor sensitivity GS (O'Brien) score with ΔKi67 in the anastrozole arm.

5.2.6 Treatment-induced changes in gene/protein expression and phosphorylation

Treatment-induced changes in protein expression and phosphorylation and gene expression were evaluated in subsets of patients, using RPPA (n=32) and Nanostring analysis (n=64). Figure 18a and 18b provide an overview of differentially expressed genes between pre- and post-treatment samples in the anastrozole and anastrozole and pictilisib groups, respectively. The top differentially expressed canonical pathways in the anastrozole group included cyclins and cell cycle regulation, oestrogen-dependent signalling and gene expression, ATM signalling, mitotic kinases, and aryl hydrocarbon receptor signalling. There was substantial upregulation of a number of genes associated with the immune system, whereas many of the most downregulated genes were involved in cell cycle control.

In the anastrozole plus pictilisib group, top upregulated canonical pathways included pancreatic adenocarcinoma signalling, aryl hydrocarbon receptor signalling, IL-8 signalling, bladder cancer signalling and GADD45 signalling. There was also substantial upregulation of genes associated with the immune system and downregulation of cell cycle genes.

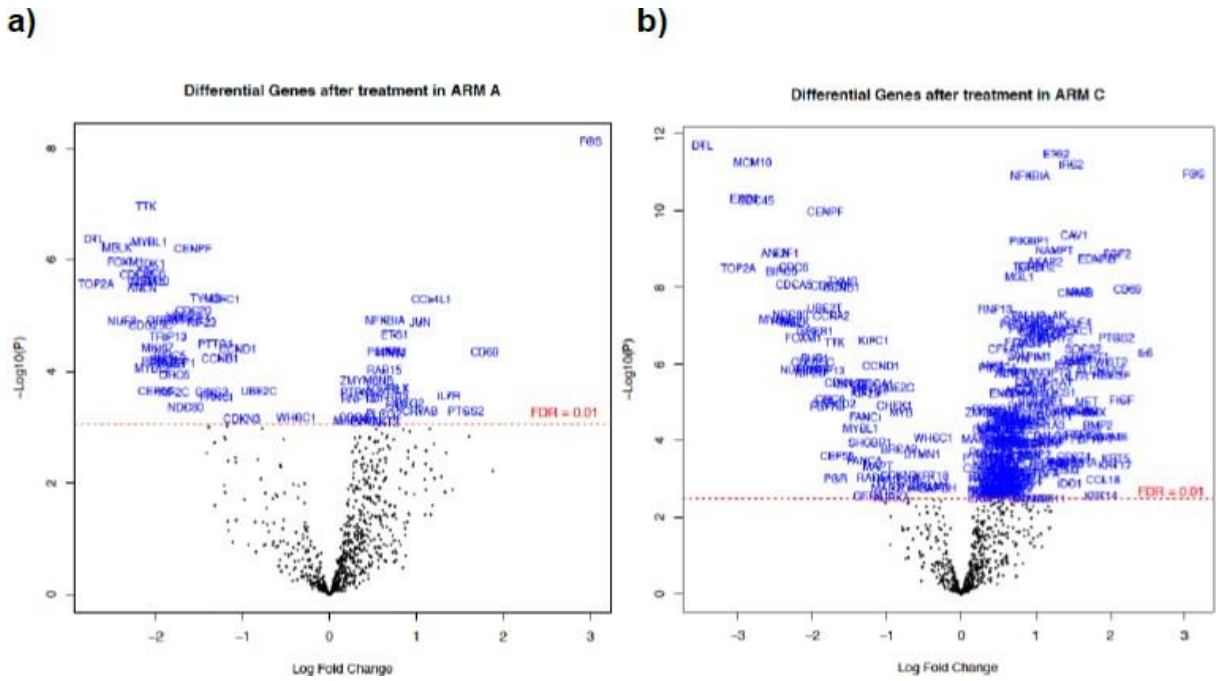


Figure 15: Differentially-expressed genes between pre- and post-treatment samples in the anastrozole arm (a) and anastrozole and pictilisib arm (b)

ER target genes: Previous data suggested that single agent PI3K inhibition up-regulates expression of ER target genes in vivo and in vitro [49]. In a preclinical study, treatment with the PI3K inhibitor BYL719 (p110a) was associated with substantially increased expression of ER-target genes. Furthermore, treatment with BYL719 upregulated ESR1 expression in tumour samples of treated patients.

The effect of treatment with pictilisib and anastrozole on ER target genes was therefore investigated, using Nanostring gene expression analysis. As illustrated in Figure 15 there was a significant treatment-associated reduction in the expression of ER target genes such as GREB1 or PR. No differences were observed between the 2 study arms, suggesting that induction of ER target genes by PI3K inhibition requires oestrogen.

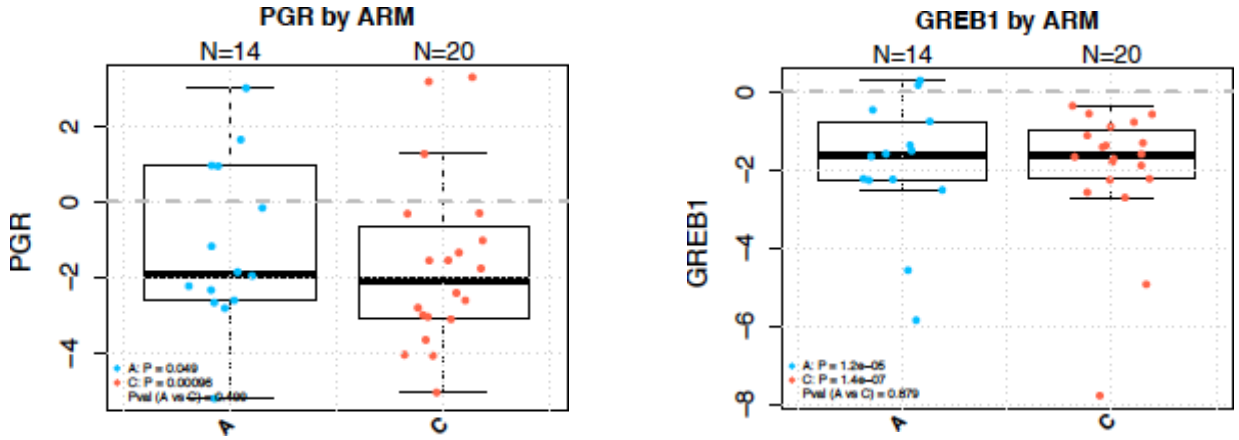


Figure 16: Treatment-induced changes in expression of ER target genes PR and GREB1; A, anastrozole alone; C, combination.

PI3K pathway and cell cycle. RPPA analysis focused on key genes involved in the activation of the PI3K pathway and cell cycle. Baseline protein expression and phosphorylation was comparable between both groups. There was substantial downregulation of cell cycle genes in both arms, associated with endocrine therapy. Phospho-AKT levels, pS6 levels or p4E-BP1 levels were comparable between both arms (Figure 18). Overall, the end-of-treatment profiles as well as the treatment-associated changes (Figure 17) were largely comparable between both groups, suggesting a dominant anti-oestrogen effect. The effects on Cyclin D1 were more pronounced with the combination in keeping with the more substantial anti-proliferative effect as per Ki67 analysis. Gene expression analysis in the anastrozole group demonstrated upregulation of genes involved in cell cycle arrest such as p21. In the combination group, gene expression analysis also demonstrated up-regulation of the PI3K-regulated genes IRS2 and PIK3IP1, confirming treatment-associated pathway inhibition.

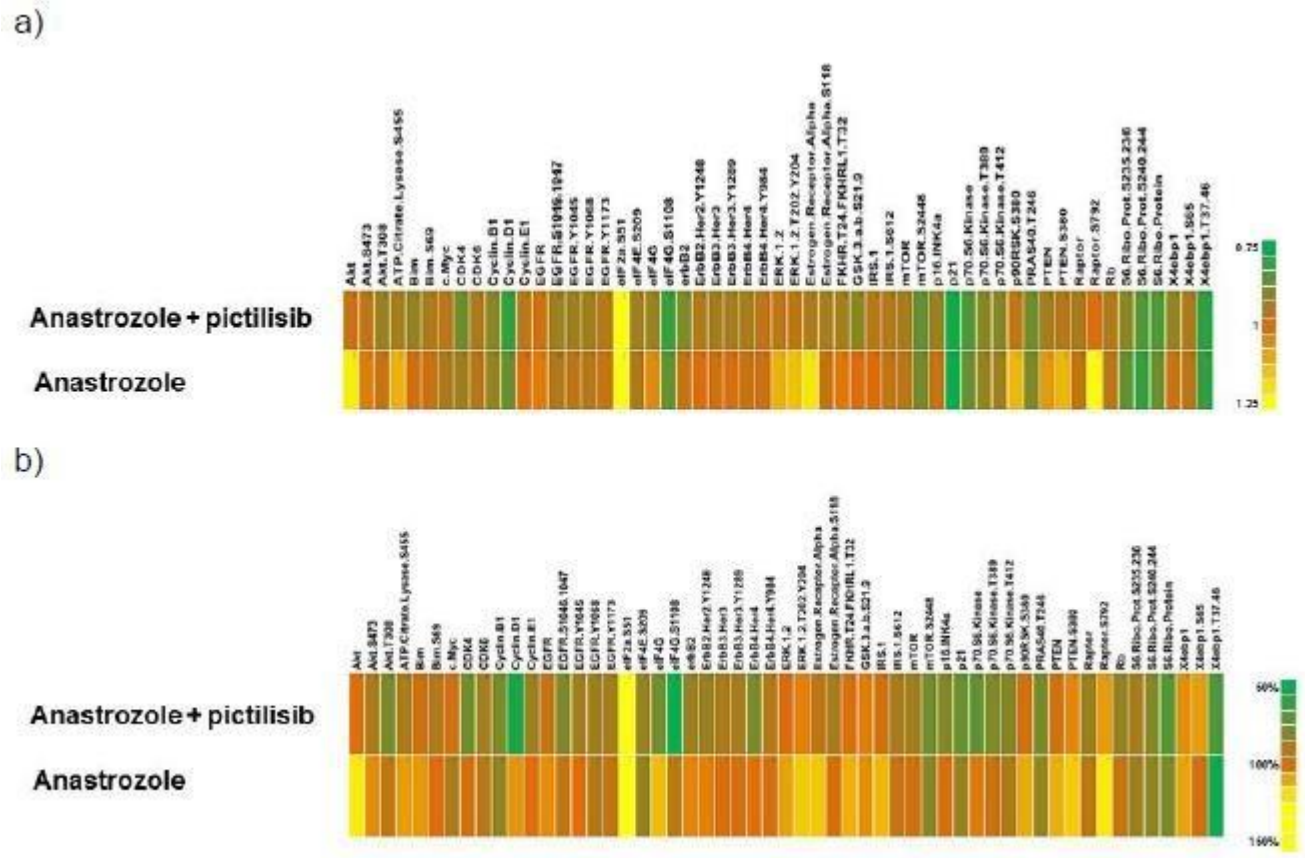


Figure 17: RPPA analysis focusing on key genes involved in the activation of the PI3K pathway and cell cycle. A) Mean end-of-treatment RPPA expression in the anastrozole and combination therapy groups; B) Mean treatment-associated changes in RPPA expression with anastrozole and anastrozole plus pictilisib.

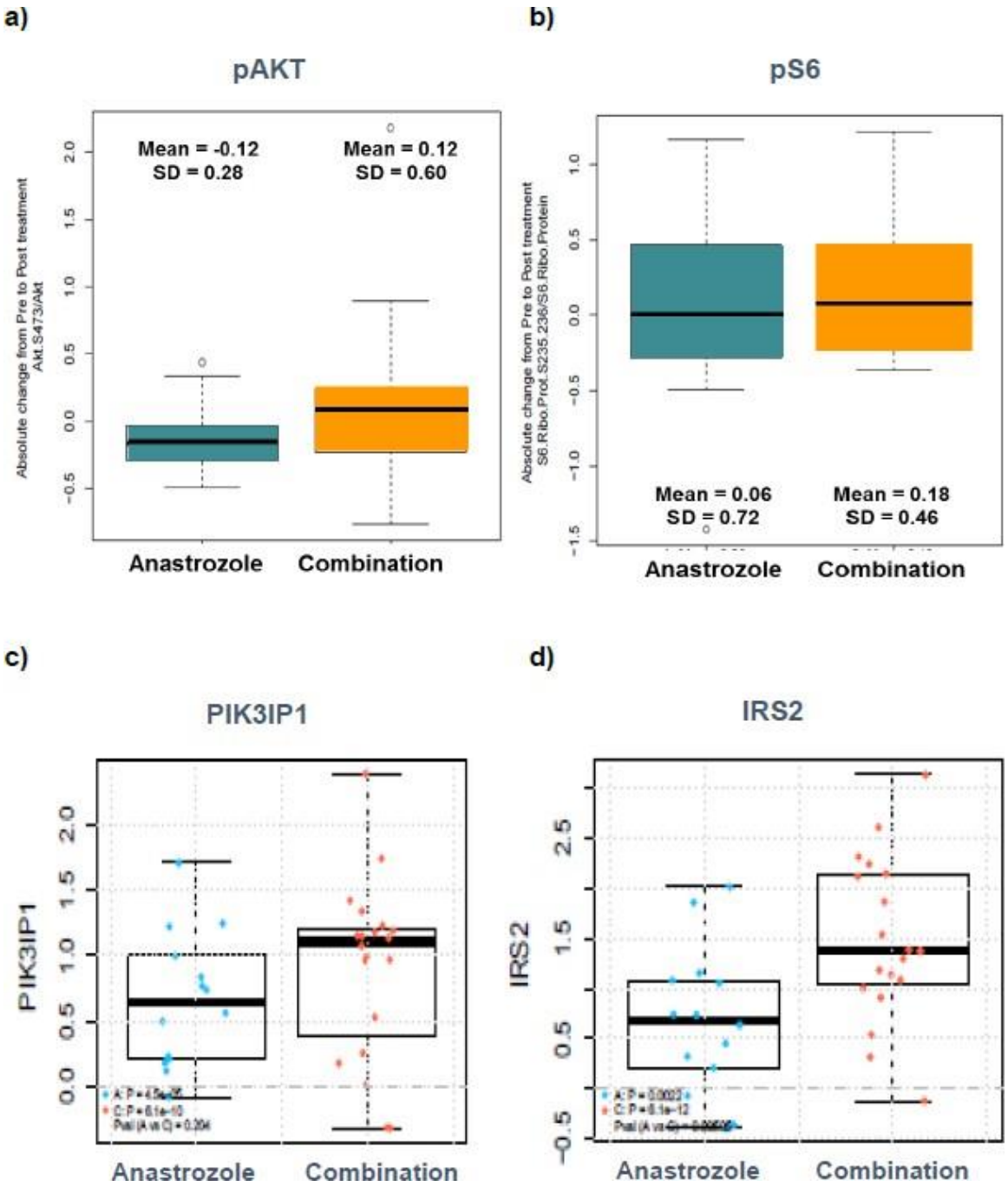


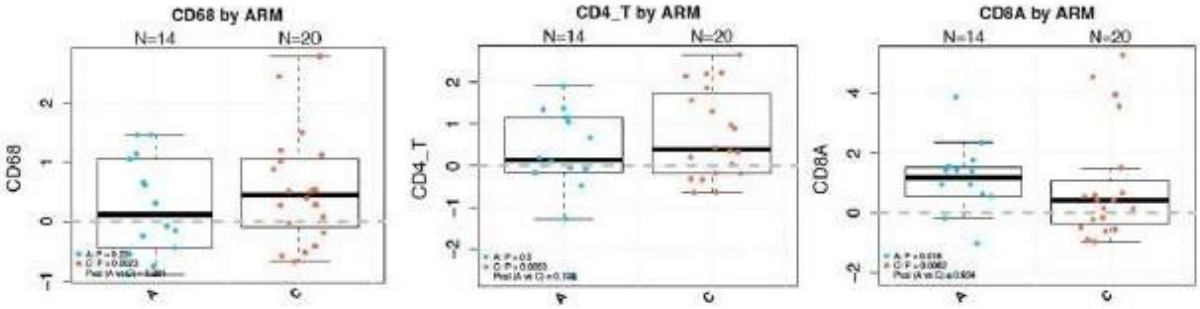
Figure 18: RPPA analysis of Phospho-AKT levels (a) and pS6 levels (b) after treatment with anastrozole or combination therapy; treatment-associated upregulation of PIK3CA-regulated genes PIK3IP1 and IRS2.

5.2.8 Effects of PI3K inhibition on the tumour microenvironment and immune system

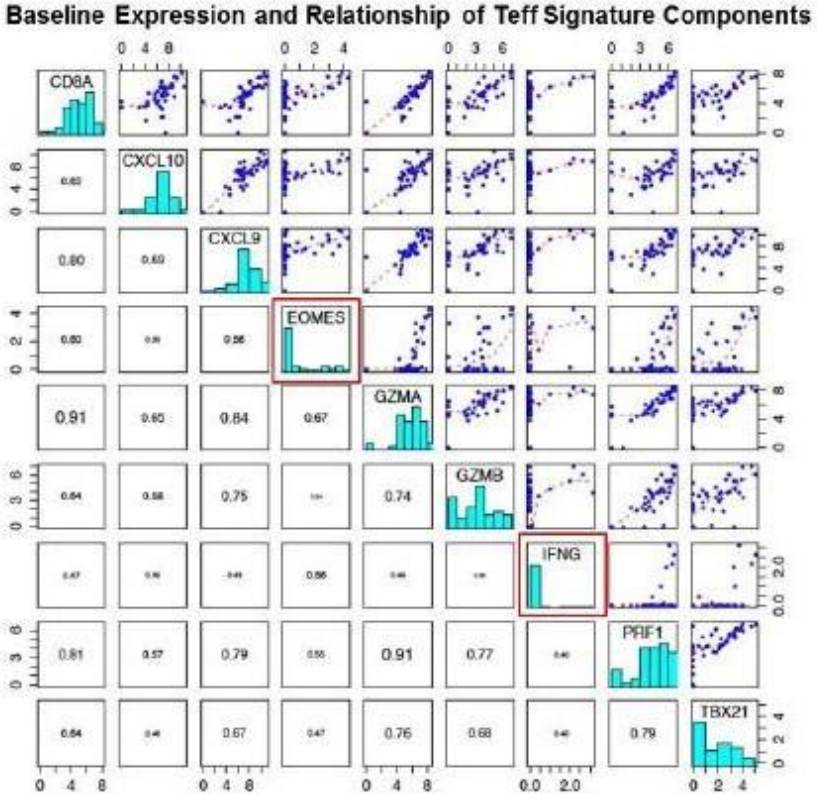
There is substantial evidence that oestradiol and/or ER signalling regulates the development and function of dendritic cells [50], B and T lymphocytes, NK cells, monocytes and macrophages [51, 52]. In addition, there is discussion around the role of PI3K signalling and the tumour microenvironment. We therefore performed gene expression analysis to assess the impact of anastrozole and the combination therapy on the tumour microenvironment and immune system.

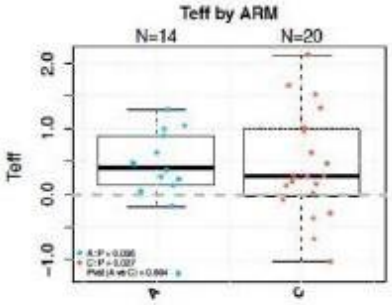
Analysis of pre- and post-treatment samples showed that a 2-week treatment of anastrozole and anastrozole plus pictilisib have a modest impact on the tumour immune microenvironment; the observed effects differed between the two treatment groups. Whilst in patients treated with anastrozole a modest increase in CD8A transcript was observed, combination therapy was associated with a modest increase in CD68, CD4 and CD8A transcripts. Study treatment had a minimal impact on Teff and Treg signatures and on Tcell immunosuppressive signature but a modest impact on APC immunosuppressive signature. In the tumour samples from patients that received combination therapy, there was an increase in the expression of markers indicative for macrophages, CD4 and CD8+ cell recruitment, as well as increase of immunosuppressive molecules such as PD-L1, PD-L2 and IDO. No significant increase in FOXP3+ effector cells was observed in either arm.

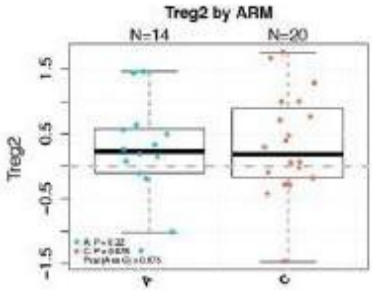
a)


CD68 by ARM
N=14
N=20
CD68
CD4_T by ARM
CD4_T
CD8A by ARM
CD8A
A
C

b)


Baseline Expression and Relationship of Teff Signature Components
CD8A
CXCL10
CXCL9
EOMES
GZMA
GZMB
IFNG
PRF1
TBX21


Teff by ARM
N=14
N=20
Teff
A
C


Treg2 by ARM
N=14
N=20
Treg2
A
C

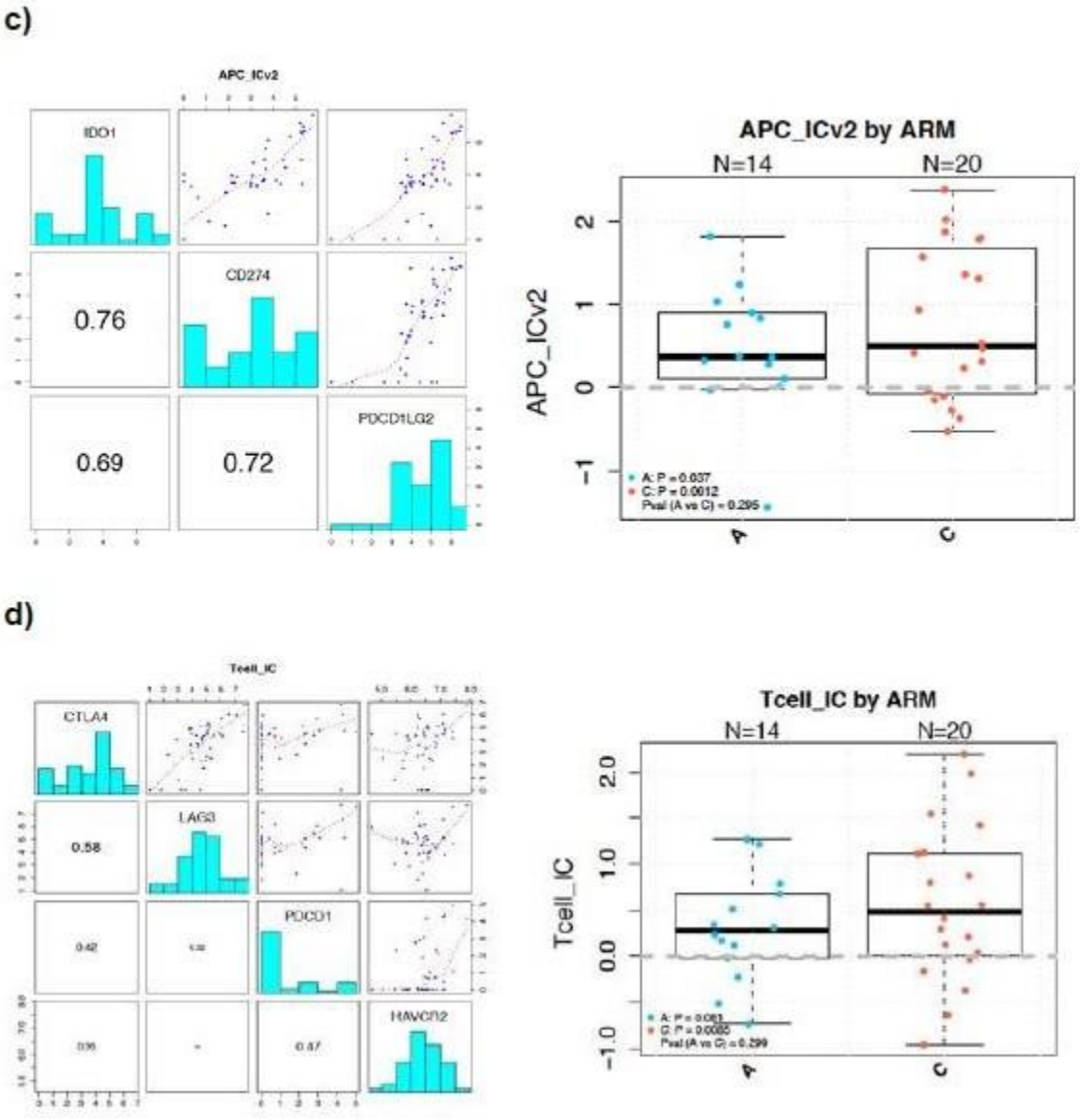


Figure 19: a) Impact on markers of immune cell populations (CD68, CD4, CD8A) in the post-treatment samples; b) Treatment effect on Teff and Treg signatures; c) APC immunosuppressive signature; d) Tcell immune-suppressive signature