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Urothelial Cancer

Final Results of Neoadjuvant Atezolizumab in Cisplatin-ineligible Patients with Muscle-invasive Urothelial Cancer of the Bladder

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Abstract

Background: Neoadjuvant immunotherapies hold promise in muscle-invasive bladder cancer (MIBC).
Objective: To report on 2-yr disease-free (DFS) and overall (OS) survival including novel tissue-based biomarkers and circulating tumor DNA (ctDNA) in the ABACUS trial.
Design, setting, and participants: ABACUS was a multicenter, single-arm, neoadjuvant, phase 2 trial, including patients with MIBC (T2-4aN0M0) who were ineligible for or refused neoadjuvant cisplatin-based chemotherapy.
Intervention: Two cycles of atezolizumab were given prior to radical cystectomy. Serial tissue and blood samples were collected.
Outcome measurements and statistical analysis: The primary endpoints of pathological complete response (pCR) rate and dynamic changes to T-cell biomarkers were published previously. Secondary outcomes were 2-yr DFS and OS. A biomarker analysis correlated with relapse-free survival (RFS) was performed, which includes FOXP3, major histocompatibility complex class I, CD8/CD39, and sequential ctDNA measurements.

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Disease-free survival
Overall survival

Results and limitations: The median follow-up time was 25 mo (95% confidence interval [CI] 25–26). Ninety-five patients received at least one cycle of atezolizumab. Eight patients did not undergo cystectomy (only one due to disease progression). The pCR rate was 31% (27/88; 95% CI 21–41). Two-year DFS and OS were 68% (95% CI 58–76) and 77% (95% CI 68–85), respectively. Two-year DFS in patients achieving a pCR was 85% (95% CI 65–94). Baseline PD-L1 and tumor mutational burden did not correlate with RFS (hazard ratio [HR] 0.60 [95% CI 0.24–1.5], $p = 0.26$, and 0.72 [95% CI 0.31–1.7], $p = 0.46$, respectively). RFS correlated with high baseline stromal CD8+ (HR 0.25 [95% CI 0.09–0.68], $p = 0.007$) and high post-treatment fibroblast activation protein (HR 4.1 [95% CI 1.3–13], $p = 0.01$). Circulating tumor DNA positivity values at baseline, after neoadjuvant therapy, and after surgery were 63% (25/40), 47% (14/30), and 14% (five/36), respectively. The ctDNA status was highly prognostic at all time points. No relapses were observed in ctDNA-negative patients at baseline and after neoadjuvant therapy. The lack of randomization and exploratory nature of the biomarker analysis are limitations of this work.
Conclusions: Neoadjuvant atezolizumab in MIBC is associated with clinical responses and high DFS. CD8+ expression and serial ctDNA levels correlated with outcomes, and may contribute to personalized therapy in the future.
Patient summary: We showed that bladder cancer patients receiving immunotherapy followed by cystectomy have good long-term outcomes. Furthermore, we found that certain biological features can predict patients who might have particular benefit from this therapy.

1. Introduction

Neoadjuvant cisplatin-based chemotherapy followed by radical cystectomy (RC) is the standard treatment for patients with muscle-invasive bladder cancer (MIBC). Up to 50% of patients are unfit to receive cisplatin-based chemotherapy [1,2] and undergo upfront RC. Survival of these patients is poor [3].

Both atezolizumab and pembrolizumab have been used in front-line metastatic, cisplatin-ineligible, biomarker-positive patients. Both agents have also been investigated in the neoadjuvant setting [4,5]. Neoadjuvant pembrolizumab showed a pathological complete response (pCR) rate of 38.5% (95% confidence interval [CI] 30.5–46.5) and 2-yr event-free survival of 71.7% (95% CI 62.7–82) [6]. Pathological response correlated with PD-L1–positive status and high tumor mutational burden (TMB) [5].

ABACUS was a single-arm, phase 2 study investigating two cycles of atezolizumab before RC in patients with MIBC. A primary endpoint analysis showed a pCR rate of 31% (95% CI 21–41) and correlated biomarkers with the pCR [4]. Here, we report the final analysis of this trial, including disease-free survival (DFS), overall survival (OS), and 2-yr survival rates. A correlation of previously established biomarkers (CD8+ T cells, PD-L1, TMB, fibroblast activation protein [FAP], and CD8+/GZMB+ double positive T cells) and new exploratory biomarkers (forkhead box P3 protein [FOXP3], major histocompatibility complex [MHC] class I molecules, and CD8+/CD39+ double positive T cells) with relapse and survival from sequential tissue was observed. FOXP3 and MHC class I have previously been associated with resistance to immune checkpoint inhibitors, thus justifying their exploration. Circulating tumor DNA (ctDNA) levels were explored in the adjuvant setting and shown to be highly prognostic and predictive. Furthermore, ctDNA responses have been seen in the neoadjuvant setting with chemotherapy [7]. Here, we aim to better characterize the clinical utility of ctDNA levels in patients treated with neoadjuvant atezolizumab.

2. Patients and methods

2.1. Study design and participants

This multicenter, single-arm, phase 2 trial investigated two cycles of neoadjuvant atezolizumab in patients with MIBC (NCT02662309). A detailed account of the methods has been published previously (Supplementary Fig. 1) [4]. Patients with histologically confirmed MIBC (T2-4aN0M0) who were either ineligible for or refused cisplatin-based neoadjuvant chemotherapy were recruited. An evaluable baseline tissue sample demonstrating MIBC was required for inclusion. The study protocol was approved by an independent institutional review board or ethics committee at each study site, and the trial was performed in compliance with Good Clinical Practice and the Declaration of Helsinki. All patients signed a written informed consent form before enrollment.

2.2. Study interventions

Participants were planned to receive two cycles of 1200 mg atezolizumab in 21-d cycles. Atezolizumab could be withheld temporarily or discontinued permanently if toxicity occurred, as per protocol-specified rules. Delays to surgery were discouraged, and patients developing treatment-related toxicity after the first cycle were encouraged to proceed to surgery after resolution of side effects. Cross-sectional imaging occurred at study entry and before cystectomy. Adverse events (AEs) were monitored at each study visit and graded using the Common Terminology Criteria for Adverse Events version 4.03. Patients were scheduled to undergo RC and pelvic lymph node dissection 4–8 wk following enrollment. Serial blood samples were collected at scheduled clinical visits. Follow-up visits occurred at 4, 12, and 24 wk after cystectomy, and patients were contacted to assess relapse and survival at 12 and 24 mo after surgery. An exploratory biomarker analysis was conducted on both baseline and matched cystectomy samples.

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2.3. Biomarker analysis

A central pathology review of all available tissue at baseline (n = 92) and cystectomy (n = 84) was performed. All immunohistochemistry analyses (PanCK/CD8, PD-L1, CD8/GZMB, FAP, MHC class I, PanCK/CD8/CD39, and CD8/FOXP3/GZMB) were performed at a central laboratory (CellCarta, Antwerp, Belgium). Antibodies to PD-L1 (SP142), PanCK (AE1/AE3/PCK26), CD8 (SP239), GZMB (EPR8260), FAP (SP325), MHC class I (EP1395Y), FOXP3 (Ab20034), and CD39 (EPR20627) were used for a biomarker analysis with established methods on the Ventana BenchmarkR ULTRA and Ventana BenchmarkR XT platforms. Immunohistochemistry analyses including PanCK/CD8, CD8/GZMB, PanCK/CD8/CD39, CD8/FOXP3/GZMB, MHC class I, and FAP were scored via a quantitative method using the image analysis software VisiopharmR in the total tumor area. In the PanCK/CD8 and PanCK/CD8/CD39 analyses, the values of CD8+ cells within the cytokeratin-positive tumor strands were used. Low, medium, and high FAP expression levels were measured in the tumor stroma area. PanCK/CD8, CD8/GZMB, CD8/CD39, FAP, and FOXP3 levels above and below the median were compared. MHC class I (H score) was calculated by multiplying the proportion score by the staining intensity, which was graded on a scale of 0–3, with 3 indicating the highest intensity. MHC class I loss was defined as an H score of $\leq$50. The standard definition of PD-L1 positivity for atezolizumab in bladder cancer was used ($\geq$5% of immune cell staining) [8]. TMB was assessed using the FoundationOne CDx assay (cutoff: 10 mut/Mb; Supplementary Fig. 2A).

A ctDNA analysis was performed at baseline, after the completion of neoadjuvant atezolizumab (PostNeo) and after radical cystectomy (PostCx; single time point between 1 and 6 mo), using Natera's Signatera assay (Supplementary Fig. 2B). Whole exome sequencing of tumor tissue from baseline and matched normal specimen from whole blood were performed [9,10]. This allowed identification of clonal somatic single nucleotide variants (SNVs), from which 16 SNVs were selected for inclusion in a multiplex PCR-NGS ctDNA assay. The designed assays were then used to assess ctDNA levels in plasma. This method has defined and validated ctDNA positivity based on the presence of two or more variants. The concentration of ctDNA was quantified in mean tumor molecules (MTMs) per milliliter of plasma [11]. Responses from baseline to pretreatment have been published previously [7].

2.4. Outcomes

The primary clinical endpoint of the study was pCR rate in all patients who received at least one cycle of atezolizumab and underwent RC, or withdrew from the study for disease progression prior to surgery. This was published previously [4]. Secondary endpoints included DFS (time from enrollment until relapse or death, whichever occurred first), OS (time from enrollment until death due to any cause), safety assessments, and surgical complication rates. Owing to the lack of follow–up, these were immature in a previous analysis. A comprehensive safety analysis was also reported previously [12]. Associations between response to treatment and biomarker expression, including but not limited to CD8 and PD-L1 and ctDNA levels, were also prespecified endpoints [4,7].

2.5. Statistical analysis

The primary endpoint analysis occurred when all patients underwent surgery and were assessed for the pCR [4]. The end of study analysis was defined as the completion of 2-yr postcystectomy follow-up. DFS and OS were secondary endpoints for the trial, which are reported here. All clinical efficacy endpoints were analyzed using STATA version 16.1 (Stata Corp., College Station, TX, USA). The Kaplan-Meier method was used to measure time to disease recurrence or death, and estimates are reported for the medians with 95% CIs.

Dynamic changes to CD8 expression with atezolizumab was the predefined biomarker endpoint. The relationship between PD-L1, TMB, and outcome was predefined. While performing RNA, DNA and protein analysis was predefined, the statistical analysis plan was not; therefore, these results are exploratory in nature. The p values of <0.05 are described as significant, but these should be interpreted with caution as these were not predefined. No adjustments were made for multiple comparisons. No multivariate analyses were performed due to sample size limitations. Relapse-free survival (RFS) was used for a biomarker analysis (time from enrollment until disease recurrence or death due to relapse, whichever occurred first). This sensors patient who died of noncancer causes, which would contaminate results. We assessed the association between protein expression and relapse using the two-sided Mann-Whitney U test. Correlations between biomarkers were measured by the Pearson product-moment correlation coefficient. Associations between ctDNA positivity and baseline prognostic factors were measured using the Kruskal-Wallis rank sum test for numeric variables and Fisher's exact test for categorical variables. All biomarker analyses were exploratory in nature and were performed in R (R Foundation for Statistical Computing, Vienna, Austria; https://www.R-project.org/).

3. Results

3.1. Patient characteristics and efficacy

Between May 2016 and June 2018, 95 patients were prospectively accrued and received study drug. Of these, 87 patients underwent RC. Eight patients did not have surgery (one patient had disease progression after neoadjuvant therapy, one refused surgery, one withdrew consent, and five became unfit for surgery). Baseline patient and tumor characteristics were in line with expectations, with 74% of patients having T2 disease and 75% with Eastern Cooperative Oncology Group performance status of 0 (Table 1) [4]. The pCR rates were 31% (27/88; 95% CI 21–41) in all patients and 37% (95% CI 22–55) in PD-L1–positive patients [4].

As of June 11, 2020, when the last patient underwent surgery, the median follow-up was 25 mo (95% CI 25–26). Of 95 patients, 22 (23%) relapsed or died due to relapse and 22 patients died due to any cause. Three non–cancer-related deaths occurred during the treatment and surgical period (one non–treatment-related aspiration pneumonia, one immune-related myocardial infarction, and one cardiogenic shock after RC). The 2-yr DFS rate was 68% (95% CI 58–76; Fig. 1A). The 2-yr DFS rate in patients with a pCR was 85% (95% CI 65–94; Supplementary Fig. 3). Higher T stage (T3–4) both at baseline (hazard ratio [HR] 2.4 [95% CI 1.0–5.6], p = 0.045) and at cystectomy (HR 13 [95% CI 3.7–43], p < 0.001), and node-positive disease at surgery (HR 6.6 [95% CI 2.4–18], p < 0.001) correlated with poor DFS. The 2-yr OS rate was 77% (95% CI 68–85; Fig. 1B).

3.2. Association of biomarker expression and clinical outcome

Pretreatment biomarkers showed a correlation between RFS and high baseline expression of stromal CD8+ (risk ratio [RR] 0.29 [95% CI 0.12–0.71], p = 0.01; Fig. 2A). There was no significant correlation between PD-L1 expression (RR 0.61 [95% CI 0.28–1.4], p = 0.22) or TMB (RR 0.80 [95% CI 0.38–1.7], p = 0.54) and relapse (Fig. 2A). Next, we

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Table 1 – Patient and tumor characteristics

	Full analysis set[a] (n = 95)	Relapsed patients[b] (n = 22)	ctDNA BEP (n=40)
Age (years), median (range)	73 (53–87)	73 (60–87)	73 (54–85)
Male gender, n (%)	81 (85)	18 (82)	35 (88)
Current or ex-smoker, n (%)	74 (78)	13 (59)	31 (78)
Previous BCG treatment, n (%)	11 (12)	1 (5)	3 (8)
ECOG performance status, n (%)			
0	71 (75)	15 (68)	34 (85)
1	24 (25)	7 (32)	6 (15)
cT stage at enrolment, n (%)			
cT2	70 (74)	13 (59)	31 (78)
cT3	17 (18)	7 (32)	6 (15)
cT4	8 (8)	2 (9)	3 (8)
Nodal stage 0, n (%)	95 (100)	22 (100)	40 (100)
Previous NMIBC, n (%)	14 (15)	3 (14)	2 (5)
PD-L1 positive (≥5%), n (%)	39 (41)	7 (32)	20 (50)
yT stage at surgery, n (%)[c]			
yT0	27 (31)	1 (5)	14 (35)
yT1	7 (8)	0	3 (8)
yT2	23 (26)	2 (11)	8 (20)
yT3	19 (22)	9 (47)	10 (25)
yT4	11 (13)	7 (37)	5 (13)
Upstaging of T stage[d]	19 (22)	11 (58)	10 (25)
yN stage at surgery, n (%)[c]			
yN0	62 (71)	6 (32)	30 (75)
yN1	12 (14)	4 (21)	5 (13)
yN2	8 (9)	6 (32)	3 (8)
yN3	1 (1)	0	0
yNX	4 (5)	3 (16)	2 (5)

BCG = Bacillus Calmette-Guerin. BEP = biomarker evaluable population. ctDNA = circulating tumor DNA. ECOG = Eastern Cooperative Oncology Group. NMIBC = Non muscle invasive bladder cancer.

[a] All patients enrolled into the trial who received at least one administration of study treatment.

[b] Recurrence of disease or death due to relapse.

[c] 87 patients had cystectomy in the full analysis set population and 19 had surgery in the relapsed population.

[d] From T2 to T3/T4 or T3 to T4.

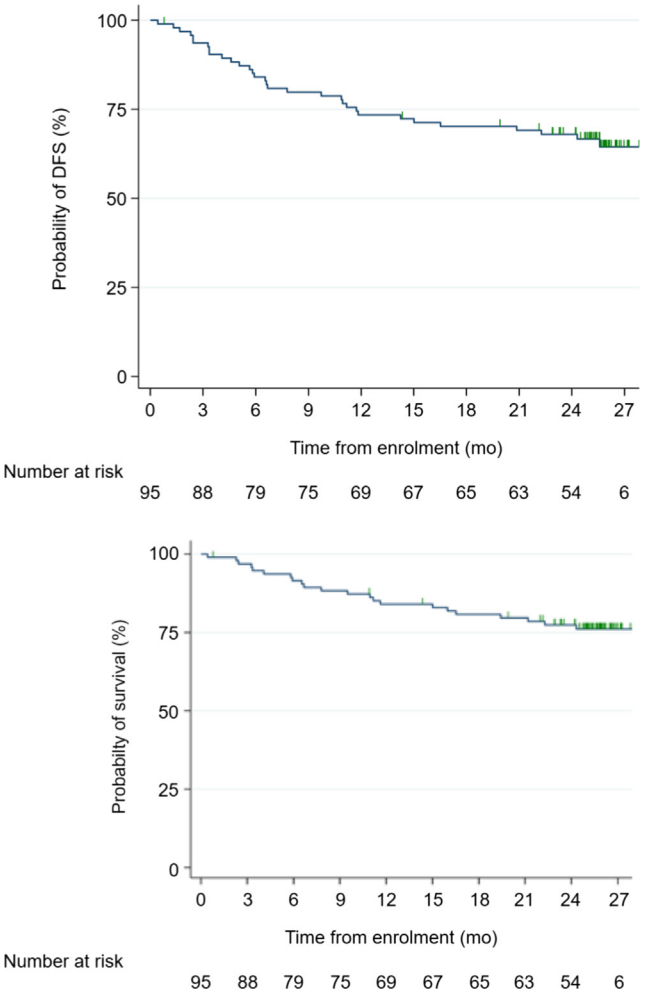

Fig. 1 – Kaplan-Meier survival analysis displaying (A) disease-free survival (DFS; time from enrollment until relapse or death, whichever occurred first) and (B) overall survival (time from enrollment until death due to any cause) in all patients who received at least one cycle of atezolizumab (full analysis set).

correlated relapse with biomarker expression after treatment. Results showed that the presence of FAP in the tumor microenvironment is associated with poor outcome (RR 3.3 [95% CI 1.2–9.3], $p = 0.02$; Fig. 2B).

There was no association between baseline, intraepithelial CD8+/CD39+ expression, and response (Fig. 3A) or RFS (Fig. 2A). However, in post-treatment samples, there was increased expression in CD39/CD8+ T cells in responding tumors ($p < 0.05$; Fig. 3A). Loss of MHC class I (H score <50) was seen in 11% (8/76) of samples. There was no statistically significant correlation between MHC class I loss and response (Fig. 3B) or relapse (Fig. 2A). High expression of MHC class I at baseline was not predictive of increased RFS (HR 2.3 [95% CI 0.30–17], $p = 0.424$; Supplementary Fig. 4A).

High FOXP3 expression correlated with response before and after treatment (Fig. 3C), but no association was seen between FOXP3 at baseline and relapse (RR 0.87 [95% CI 0.37–2.0], $p = 0.74$; Fig. 2A) or RFS (HR 0.86 [95% CI 0.33–2.2], $p = 0.75$; Supplementary Fig. 4A). We showed a positive correlation between baseline CD8 and FOXP3 expression ($r = 0.40$; Fig. 3D).

3.3. *Exploratory analysis of circulating tumor DNA and correlation with outcome*

At baseline, 63% (25/40) of patients were ctDNA positive (ctDNA+; Fig. 4A). Baseline ctDNA positivity was significantly associated with increased PD-L1 expression both in tumor infiltrating immune cell (≥5% of immune cells, $p = 0.008$) and tumor cell staining (≥5% of tumor cells, $p = 0.007$; Supplementary Fig. 5). At the postneoadjuvant time point (PostNeo), 47% (14/30) of patients were ctDNA + (Fig. 4A). PostNeo ctDNA status was significantly correlated with lymph node status and T stage at surgery ($p = 0.02$ and $p = 0.0005$, respectively). No correlation was observed between PostNeo ctDNA status and other clinical features at surgery including PD-L1 status. At the postcystectomy time point (PostCx), 14% (five/36) of patients were ctDNA+ (Fig. 4A). Overall, three patients with ctDNA+

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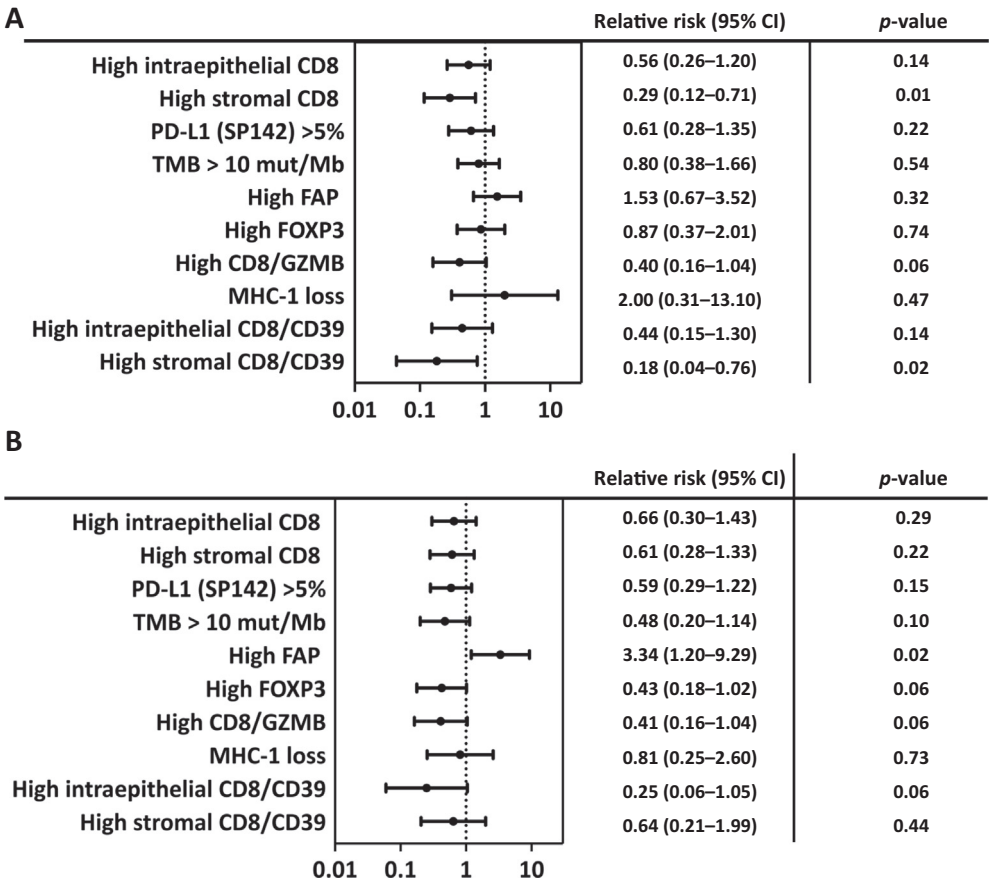

Fig. 2 – (A) Association between baseline protein expression levels and relapse. CD8 and CD8/CD39 were scored within both cytokeratin-positive tumor strands and stroma. FAP high was analyzed by the area stained for high FAP in the tumor region divided by the area of the tumor region (%). FOXP3 was measured in the total tumor area. For the above, expression levels above and below the median were compared and correlated with clinical outcome. PD-L1 positivity was determined using the standard cutoff of ≥5% of immune cell staining. Tumor mutational burden was assessed using the FoundationOne CDx assay (cutoff: 10 mut/Mb). MHC class I (H score) was calculated by multiplying the proportion score by the staining intensity, which was graded on a scale of 0–100, with 100 indicating the highest intensity. MHC class I loss was defined as a H score of ≤10. (B) Association between post-treatment protein expression levels and relapse. CD8 and CD8/CD39 were scored within both cytokeratin-positive tumor strands and stroma. FAP high was analyzed by area stained for high FAP in the tumor region divided by the area of the tumor region (%). FOXP3 was measured in the total tumor area. For the above, expression levels above and below the median were compared and correlated with clinical outcome. PD-L1 positivity was determined using the standard cutoff of ≥5% of immune cell staining [1]. Tumor mutational burden was assessed using the Foundation One CDx assay (cutoff: 10 mut/Mb). MHC class I (H score) was calculated by multiplying the proportion score by the staining intensity, which was graded on a scale of 0–100, with 100 indicating the highest intensity. MHC class I loss was defined as an H score of ≤10. CI = confidence interval; FAP = fibroblast activation protein; MHC = major histocompatibility complex; TMB = tumor mutational burden.

disease at baseline became ctDNA negative (ctDNA–) after neoadjuvant atezolizumab. These patients subsequently also achieved a pCR at surgery. Two other patients with ctDNA+ disease at baseline and PostNeo subsequently cleared ctDNA after surgery and achieved a pCR (Fig. 4A). PostNeo ctDNA status and other clinical features at surgery included PD-L1 status. At the postcystectomy time point (PostCx), 14% (five/36) of patients were ctDNA+ (Fig. 4A). At the PostCx time point, 100% of responders and 100% of stable disease patients were ctDNA–, while most relapsed patients were ctDNA+ (83% [five/six] ctDNA+).

Continuous metrics of ctDNA, as measured by the MTMs per milliliter of plasma, was also associated with time point and clinical response (Fig. 4B). In addition to associations between ctDNA and clinical response/relapse, strong associations between ctDNA status and RFS were shown (Fig. 4C), as described previously [7]. Notably, at the postsurgery time point, ctDNA+ patients exhibited a much higher rate of relapse than ctDNA– patients (RFS, HR 78, $p < 0.001$; Fig. 4C). No relapse events were observed in the ctDNA– patients at baseline and at the postneoadjuvant time point. PD-1–positive patients were more likely to be ctDNA+. The outcome was particularly poor in ctDNA+ PD-L1–negative patients (Supplementary Fig. 3).

Both Lund and The Cancer Genome Atlas (TCGA) molecular classifications were applied to baseline tumor transcriptomes and correlated with baseline ctDNA status (Fig. 5A) [13,14]. Patients who were ctDNA+ were enriched in the TCGA basal squamous and the Lund squamous cell carcinoma-like subgroups (Fig. 5A). Tumors from baseline ctDNA+ patients were enriched for immune transcripts, especially from the myeloid lineage (CD14, CD83, CD86, FCGR3B, CD163, and CXCL8/IL8) and the B/plasma cell lineage (TNFSF13B/BAFF, JCHAIN, and SLAMF7; Fig. 5B). This enrichment in myeloid signals in tumors from ctDNA+ patients was confirmed by the Reactome pathway

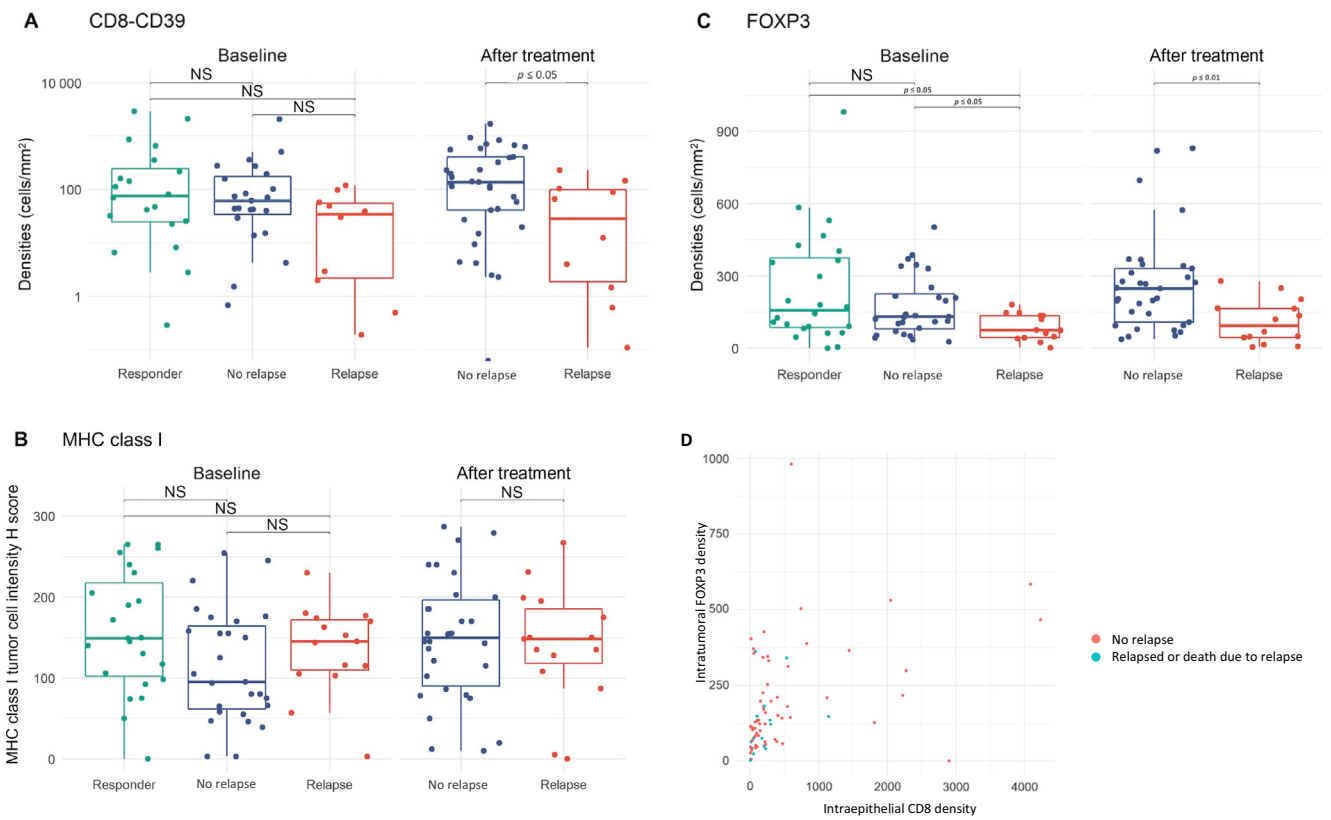

Fig. 3 – Biomarker expression of (A) CD8+/CD39+ double-positive T cells, (B) MHC class I molecule, and (C) FOXP3 by immunohistochemistry before and after therapy stratified by clinical outcome. (D) Correlation between baseline intraepithelial CD8+ T cells and FOXP3. A positive correlation between baseline CD8 and FOXP3 expression (r = 0.40) was observed, which was not predictive of relapse. MHC = major histocompatibility complex; NS = nonsignificant.

enrichment analysis (Fig. 5C). Deconvolution of bulk RNA sequencing data to quantify the relative frequency of cell subpopulations confirmed that tumors from ctDNA+ patients exhibited an increased global immune score mainly driven by an increase in several myeloid subsets, including monocytes, neutrophils, M1 macrophages, and dendritic cells (Fig. 5D).

4. Discussion

The standard treatment for cisplatin-ineligible patients is upfront RC resulting in a 2-yr DFS rate of 40–50% [15]. These data are generated from a large, neoadjuvant, randomized trial. The 2-yr DFS rate in ABACUS was 68%. Similar results were achieved using three cycles neoadjuvant pembrolizumab in the PURE-01 study (71.7%), with a median follow-up of 23 mo (interquartile range 15–29); however, it enrolled a majority of cisplatin-eligible patients (92%) [5]. Indirect comparisons should be avoided due to imbalances in patient populations (T2 stage for ABACUS was 74% vs 40% for the randomized trial). Nevertheless, these data support further exploration of neoadjuvant immune checkpoint inhibitors in this setting. Other recently reported neoadjuvant trials using PD-1/PD-L1 and CTLA-4 have not reported on 2-yr outcome yet [16,17].

These results are intriguing as recent data show that 1 yr of adjuvant atezolizumab is not associated with improved DFS in unselected patients (HR 0.89 [95% CI 0.74–1.08], p = 0.245) [18]. There are theoretical reasons why the neoadjuvant approach may be more attractive, including higher tumor and neoantigen load.

In previous work, we focused on correlating biomarker expression with pCR [4]. There are concerns around pCR as an endpoint as it has not been validated. Therefore, in this analysis, we correlated with cancer relapse and introduced novel biomarkers potentially associated with resistance. Results consistently show that existing active T-cell immunity is associated with outcome. T-cell activation as a biomarker for immune checkpoint inhibitors has not been studied as extensively in clinical trials, but exploratory results have been encouraging [19]. TMB and PD-L1 did not correlate with relapse in ABACUS. These findings are intriguing as neoadjuvant pembrolizumab showed a significant correlation between TMB, PD-L1, and outcome [5]. Different modes of action of the drugs, different assays for PD-L1, and different duration of therapy may account for these dissimilarities. Inconsistencies in results are hampering advances in biomarker development in urothelial cancer. Two factors driving this are the use of different methodologies for measuring PD-L1 and TMB, as well as the lack of randomized biomarker-driven studies. Recent robust data from advanced disease suggest that tumor cell expression of PD-L1 may be relevant in predicting response [20].

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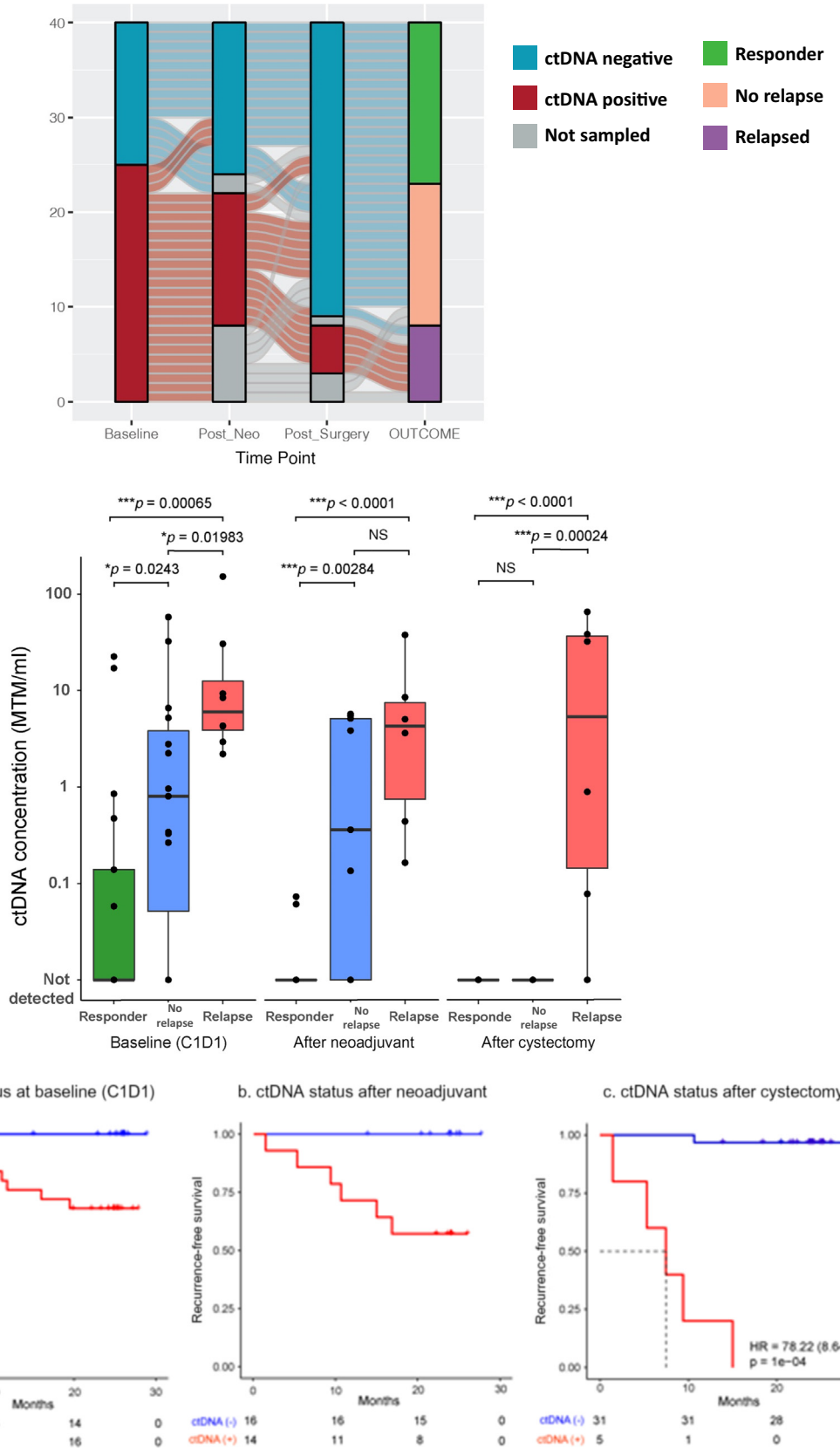

Fig. 4 – (A) Sankey plot showing ctDNA dynamics and its association with response and relapse. –(B) Levels of ctDNA are associated with clinical outcomes. (C) Positive ctDNA status is associated with higher rates of relapse and death: (a) Kaplan-Meier (KM) estimates comparing RFS of ctDNA-positive patients with ctDNA-negative patients as assessed at the baseline (C1D1) time point; (b) KM estimates for ctDNA-positive versus ctDNA-negative patients at the postneoadjuvant time point; and (c) KM estimates for ctDNA-positive versus ctDNA-negative patients at the postcystectomy time point. ctDNA = circulating tumor DNA; HR = hazard ratio; MTM = mean tumor molecule; NS = nonsignificant; RFS = relapse-free survival.

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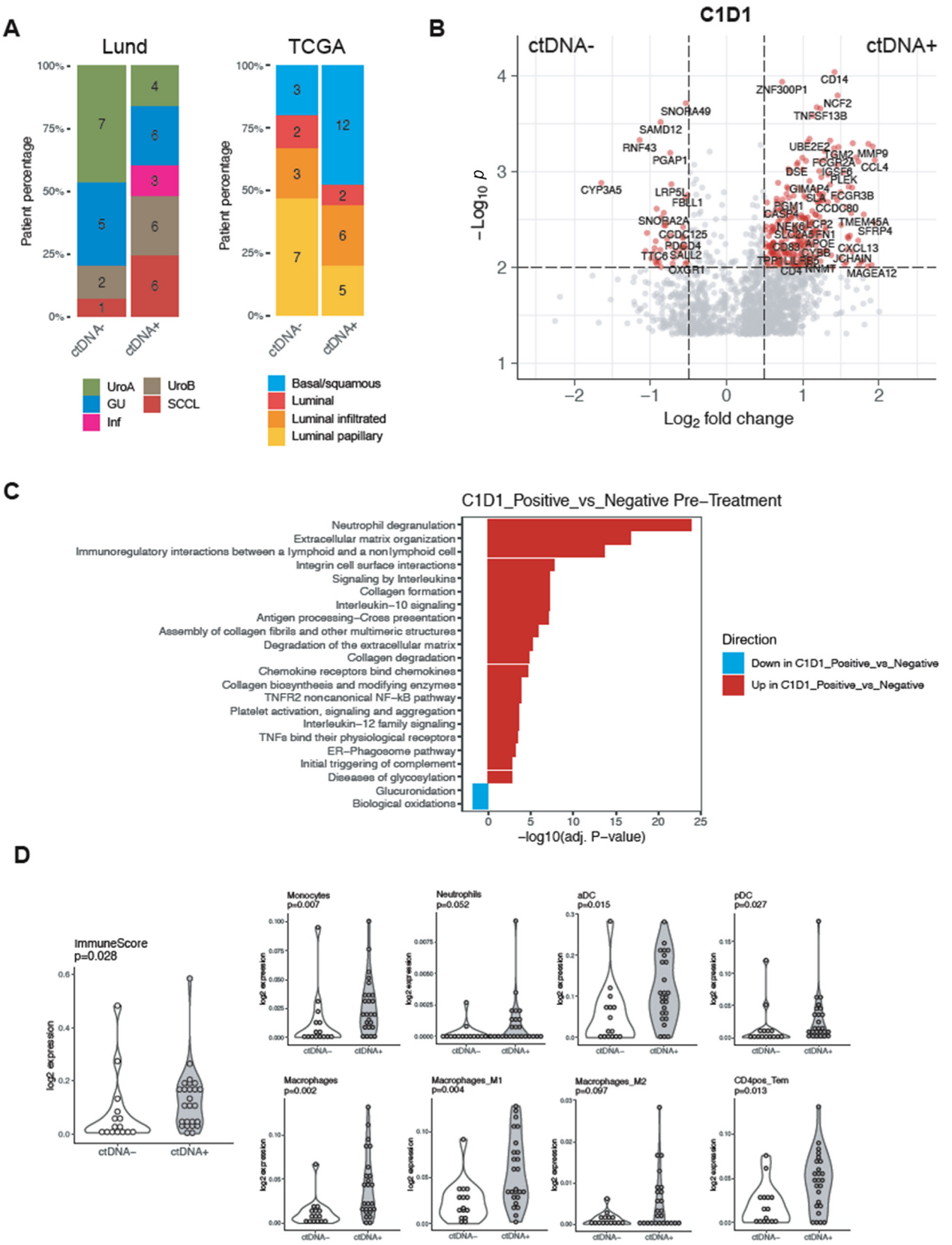

Fig. 5 – Increased myeloid signatures in tumors from ctDNA+ patients at baseline. (A) Bar charts representing the distribution of molecular subgroups, defined by the Lund classification (left panel) or the TCGA classification (right panel), by ctDNA status at baseline.(B) Volcano plot depicting genes differentially expressed (nominal $p < 0.01$, absolute log fold change ≥ 0.5) between tumors from ctDNA+ and ctDNA– patients at baseline (C1D1). (C) Bar chart representing reactome pathway enrichment analysis in genes identified in Figure 5B. (D) Violin plots depicting xCell deconvolution scores in pretreatment tumors from ctDNA+ and ctDNA– patients. ctDNA = circulating tumor DNA; GU = genomically unstable; SCCL = squamous cell carcinoma like; TCGA = The Cancer Genome Atlas; UroA = urobasal A; UroB = urobasal B.

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FAP was associated with stromal infiltration [21] and continues to be a promising marker of resistance in treated tissue. These results also point toward the importance of biomarkers that are not directly related to the immune action of atezolizumab. Instead, FAP may contribute to T-cell exclusion via its effect on the stroma [22].

The downregulation of MHC class I molecules is a frequent mechanism of tumor escape and has been associated with worse survival outcomes in PD-1/PD-L1 checkpoint inhibition [23,24]. It has not been described extensively in urothelial carcinoma.

Tumor infiltration by FOXP3 is thought to be associated with resistance to immune therapy and predictive of poor OS [25]. We found a positive correlation between baseline CD8 and FOXP3 expression, both of which increased with atezolizumab. These results show that FOXP3 (a Treg marker) is tracking other active T-cell biomarkers. Concurrent immune activation and inhibition with atezolizumab may in part be a mechanism of resistance to therapy, and justifies exploring CTLA-4 in combination, which targets Tregs.

Recent studies have highlighted the role of CD39 expression on tumor-infiltrating CD8+ T cells in cancer antigen specificity. CD39 is highly expressed by tumor-specific CD8+ TILs in lung and colorectal tumors, with low CD39 expression in bystander CD8+ T cells [26]. We observed similar results with increased CD39/CD8+ T cells in tumors responding after treatment. To our knowledge, CD39 has not previously been described as a potential biomarker in urothelial carcinoma, opening new avenues in this field.

Patients who were ctDNA– at baseline were more likely to achieve a pCR with neoadjuvant atezolizumab. The ctDNA– status prior to neoadjuvant treatment may reflect nonmetastatic disease, or alternatively baseline tumor resection with curative intent may have led to complete removal of the tumor. None of the patients who were ctDNA– at baseline became positive during the study, highlighting the good prognosis of baseline ctDNA– patients and potentially the safety of neoadjuvant immune therapy approaches. As a surrogate marker of response and relapse, ctDNA has been explored with chemotherapy in MIBC [7,11]. A relationship between baseline ctDNA status and PD-L1 status was established, suggesting that the use of a dual tissue–based and circulating biomarker may be important for the future. At baseline, ctDNA+/PD-L1–negative patients have a particularly poor outcome and require attention. Alternative to neoadjuvant immune therapy should be sought in this population, which may influence the results of clinical trials. Postsurgical ctDNA status was found to be highly prognostic of relapse. In this study, only ctDNA+ patients experienced relapse at the postsurgical time point, as described previously in this setting [11]. Recent randomized data suggest that ctDNA may be both prognostic and predictive for response to adjuvant atezolizumab [7].

It is becoming increasingly apparent that single-agent immune checkpoint inhibitors are effective only in selected patients. The neoadjuvant data sets show inconsistencies with the established biomarkers such as PD-L1 and TMB, which are therefore unlikely to yield positive results in randomized neoadjuvant trials. They should therefore be avoided as primary endpoints in the opinion of the authors. The data presented here also suggest that it is more likely that a combination of biomarkers, including existing T-cell immunity and ctDNA, may be a preferred method of patient selection. Our data also show that ctDNA may be useful in monitoring clinical benefit and selecting patients for adjuvant therapy after neoadjuvant treatment (only 3% of ctDNA– patients after surgery relapsed). Finally, these data extensively explored sequential tissue. While on treatment analysis identified (1) dynamic changes to key biomarkers, (2) an intriguing relationship between FOXP3 and CD8, and (3) FAP as a potential marker of resistance, sequential tissue does not appear to optimally select patients for therapy. This may be because host responses to immune therapy are complicating the results. Baseline tissue and circulating biomarkers appear to have a greater value for the future. New methods exploring "on treatment" tissue such as single-cell RNA sequencing or spatial transcriptomics are required.

Limitations of our study include the single-arm design, short period of therapy, exploratory nature of the biomarker analysis, and a large number of comparisons between groups.

5. Conclusions

Neoadjuvant atezolizumab in cisplatin-ineligible patients is associated with clinical responses and high DFS. Several randomized neoadjuvant trials using a backbone of immune checkpoint inhibitors are ongoing and supported by these data (NCT03732677 and NCT03924856). Exploratory pre- and post-treatment biomarker analyses including a serial ctDNA analysis correlated with outcomes and may inform the development of personalized therapy in the future.

Author contributions: Thomas Powles had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Szabados, Kockx, Assaf, van Dam, Rodriguez-Vida, Duran, Crabb, Van Der Heijden, Pous, Gravis, Herranz, Protheroe, Ravaud, Maillet, Mendez, Suarez, Linch, Prendergast, Tyson, Stanoeva, Daelemans, Rombouts, Mariathasan, Tea, Mousa, Banchereau, Castellano, Powles.

Acquisition of data: Szabados.

Analysis and interpretation of data: Szabados, Kockx, Assaf, van Dam, Rodriguez-Vida, Duran, Crabb, Van Der Heijden, Pous, Gravis, Herranz, Protheroe, Ravaud, Maillet, Mendez, Suarez, Linch, Prendergast, Tyson, Stanoeva, Daelemans, Rombouts, Mariathasan, Tea, Mousa, Banchereau, Castellano, Powles.

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Critical revision of the manuscript for important intellectual content: Szabados, Kockx, Assaf, van Dam, Rodriguez-Vida, Duran, Crabb, Van Der Heijden, Pous, Gravis, Herranz, Protheroe, Ravaud, Maillet, Mendez, Suarez, Linch, Prendergast, Tyson, Stanoeva, Daelemans, Rombouts, Mariathasan, Tea, Mousa, Banchereau, Castellano, Powles.

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Statistical analysis: Szabados, Kockx, Assaf, van Dam, Rodriguez-Vida, Duran, Crabb, Van Der Heijden, Pous, Gravis, Herranz, Protheroe, Ravaud, Maillet, Mendez, Suarez, Linch, Prendergast, Tyson, Stanoeva, Daelemans, Rombouts, Mariathasan, Tea, Mousa, Banchereau, Castellano, Powles.
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Appendix A Peer Review Summary

Peer Review Summary and Supplementary data to this article can be found online at https://doi.org/10.1016/j.eururo.2022.04.013.

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