Final results of neoadjuvant atezolizumab in cisplatin-ineligible patients with muscle-invasive urothelial cancer of the bladder

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**Background:** Neoadjuvant immunotherapies hold promise in muscle invasive bladder cancer (MIBC).

**Objective:** To report on two-year disease-free (DFS) and overall survival (OS) 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 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 two-year DFS and OS. Biomarker analysis correlated with relapse free survival (RFS) was performed, which includes FOXP3, MHC class I, CD8/CD39 and sequential ctDNA measurements.

**Results and Limitations:** Median follow-up was 25.3 months (95%CI 25.2–25.8). 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 68.0% (95%CI 57.5 – 76.4) and 77.4% (95%CI 67.5-84.6), respectively. Two-year DFS in patients achieving pCR was 85.0% (95%CI 64.9 – 94.1). Baseline PD-L1 and tumor mutational burden (TMB) did not correlate with RFS (hazard ratio [HR] 0.60 (95%CI 0.24–1.46), p=0.26 and 0.72 (95%CI 0.31–1.70), 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 (FAP) (HR 4.09 [95%CI 1.33-12.60], p=0.01). ctDNA positivity at baseline, post-neoadjuvant and post-surgery were 63% (25/40), 47% (14/30), and 14% (5/36), respectively. ctDNA status was highly prognostic at all timepoints. No relapses were observed in ctDNA negative patients at baseline and post-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.

**Key words:** muscle-invasive bladder cancer, neoadjuvant immunotherapy, circulating tumour DNA, CD8, disease-free survival, overall survival.

Take home message:

Neoadjuvant atezolizumab in MIBC is associated with clinical responses and high DFS/OS rates, supporting the randomised trials. CD8+ expression and serial ctDNA levels correlated with outcomes and may contribute to personalized immune therapy in the future.

**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%CI 30.5-46.5) and a 2-year 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. Primary endpoint analysis showed a pCR rate of 31% (95%CI 21–41) and correlated biomarkers with pCR[4]. Here, we report the final analysis of this trial, including disease-free survival (DFS), overall survival (OS) and 2-year survival rates. Correlation of previously established biomarkers (CD8+ T cells, PD-L1, TMB, fibroblast activation protein (FAP), 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 occurred. 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.

**Patients and methods**

*Study design and participants*

This multicenter, single-arm, phase 2 trial, investigated 2 cycles of neoadjuvant atezolizumab in patients with MIBC (NCT02662309). A detailed account of the methods has been published previously (**Suppl. 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 before enrolment.

*Study interventions*

Participants were planned to receive 2 cycles of 1200 mg atezolizumab in 21-day cycles. Atezolizumab could be temporarily withheld or permanently discontinued if toxicity occurred, 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 pre-cystectomy. Adverse events (AEs) were monitored at each study visit and graded using the Common Terminology Criteria for AEs version 4.03. Patients were scheduled to undergo RC and pelvic lymph node dissection 4-8 weeks following enrolment. Serial blood samples were collected at scheduled clinical visits. Follow-up visits occurred at 4-, 12- and 24-weeks post-cystectomy and patients were contacted to assess relapse and survival at 12- and 24-months post-surgery. Exploratory biomarker analysis occurred on both baseline and matched cystectomy samples.

*Biomarker analysis*

A central pathology review of all available tissue at baseline (n=92) and cystectomy (n=84) occurred. All immunohistochemistry analysis (PanCK/CD8, PD-L1, CD8/GZMB, FAP, MHC class I, PanCK/CD8/CD39, 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 biomarker analysis with established methods on the Ventana BenchmarkR ULTRA and Ventana BenchmarkR XT platform. Immunohistochemistry analysis 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 analysis, the values of CD8+ cells within the cytokeratin-positive tumor strands were used. Low, medium, and high FAP expression 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 to 3, with 3 indicating the highest intensity. MHC class I loss was defined as a H score ≤50.The standard definition of PD-L1 positivity for atezolizumab in bladder cancer was used (≥5% of immune cells staining)[8]. Tumor mutational burden was assessed using the FoundationOne CDx assay (cut-off: 10mut/Mb) (**Suppl. Fig. 2a**).

ctDNA analysis was performed at baseline, after the completion of neoadjuvant atezolizumab (PostNeo) and post radical cystectomy (PostCx) (single time point between 1-6 months) using Natera’s Signatera assay (**Suppl. Fig. 2b**). Whole exome sequencing (WES) 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. ctDNA concentration was quantified in mean tumor molecules (MTM)/mL of plasma[11]. Responses from baseline to pretreatment have been published previously [7].

*Outcomes*

The primary clinical endpoint of the study waspCR rate in all patients who received at least one cycle of atezolizumab and underwent RC or withdrew for disease progression prior to surgery. This was published previously[4]. Secondary endpoints included disease-free survival (DFS, time from enrolment until relapse or death, whichever occurred first), OS (time from enrolment until death due to any cause), safety assessments and surgical complication rates. Due to the lack of follow up these were immature in 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 pre-specified endpoints[4,7].

*Statistical Analysis*

Primary endpoint analysis occurred after 13.1 months (95%CI 9.5–13.5) of follow up when all patients undergoing surgery were assessed for pCR[4]. The end of study analysis was defined as completion of 2-year post-cystectomy follow-up. DFS and OS were secondary endpoints for the trial which are reported here. All clinical efficacy endpoints were analyzed using STATA v.16.1. The Kaplan-Meier method was used to measure time to disease recurrence or death and estimates are reported for the medians with 95% confidence intervals.

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. P-values < 0.05 are described as significant, but these should be interpreted with caution as they 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 biomarker analysis (time from enrolment until disease recurrence or death due to relapse, whichever occurred first). This sensors patient who died of non-cancer causes which would contaminate results. We assessed the association between protein expressions and relapse using the two-sided Mann–Whitney U-test. Correlations between biomarkers were measured by Pearson product–moment correlation coefficient. Association 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 (https://www.R-project.org/).

**Results**

*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 an ECOG performance status of 0 (**Table 1**)[4]. pCR rate was 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, the median follow-up was 25.3 months (95%CI 25.2–25.8). Twenty-three percent of patients (22/95) 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 (1 non-treatment related aspiration pneumonia, 1 immune-related myocardial infarction and 1 cardiogenic shock post-RC). Two-year DFS rate was 68.0% (95%CI 57.5 – 76.4) (**Fig. 1a**). Two-year DFS rate in patients with pCR was 85.0% (95%CI 64.9 – 94.1) (**Suppl. Fig. 3**). Higher T stage (T3-4) both at baseline (hazard ratio (HR) 2.39 (95%CI 1.02-5.59), p=0.045) and at cystectomy (HR 12.60 (95%CI 3.66-43.35), p<0.001) and node positive disease at surgery (HR 6.60 (95%CI 2.39-18.22), p<0.001) correlated with poor DFS. Two-year OS rate was 77.4% (95%CI 67.5–84.6) (**Fig. 1b**).

*Association of biomarker expression and clinical outcome*

Pre-treatment biomarkers showed acorrelation between RFS and high baseline expression of stromal CD8+ (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: 0.28-1.35], p=0.22) or TMB (RR: 0.80 [95%CI: 0.38-1.66], p=0.54) and relapse (**Fig. 2a**). Next, we correlated relapse with biomarker expression post-treatment. Results showed the presence of FAP in the tumor microenvironment is associated with poor outcome (RR: 3.34 [95%CI: 1.20-9.29], 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 an 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 2.28 (95%CI 0.30-17.10), p=0.424) (**Suppl. Fig. 4a**).

High FOXP3 expression correlated with response pre- and post-treatment (**Fig. 3c**), but no association was seen between FOXP3 at baseline and relapse (RR: 0.87 [95%CI: 0.37-2.01], p=0.74) (**Fig. 2a**) or RFS (HR 0.86 (95%CI 0.33-2.22), p=0.75) (**Suppl.** **Fig. 4a**). We showed a positive correlation between baseline CD8 and FOXP3 expression (r=0.40) (**Fig. 3d**).

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

At baseline, 63% (25/40) of patients were ctDNA positive (ctDNA+) (**Figure 4a**). Baseline ctDNA positivity was significantly associated with increased PD-L1 expression both in tumor infiltrating immune cell (≥5% of IC, p=0.008) and tumor cell staining (≥5% of TC, p=0.007) (**Suppl Fig 5**). At the post-neoadjuvant time point (PostNeo), 47% (14/30) of patients were ctDNA+ (**Figure 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 post-cystectomy time point (PostCx), 14% (5/36) of patients were ctDNA+ (**Figure 4a**). Overall, 3 patients with ctDNA+ disease at baseline became ctDNA- after neoadjuvant atezolizumab. These patients subsequently also achieved pCR at surgery. Two other patients with ctDNA+ disease at baseline and postNeo subsequently cleared ctDNA post-surgery and achieved pCR. (**Figure 4a**). PostNeo ctDNA status and other clinical features at surgery including PD-L1 status. At the post-cystectomy time point (PostCx), 14% (5/36) of patients were ctDNA+ (**Figure 4a**). At the PostCx time point, 100% of responders and 100% of SD patients were ctDNA- while most relapsed patients were ctDNA+ (83% (5/6) ctDNA+).

Continuous metrics of ctDNA, as measured by the mean tumor molecules (MTM) per mL of plasma, was also associated with time point and clinical response (**Figure 4b**). In addition to associations between ctDNA and clinical response/relapse, strong associations between ctDNA status and RFS were shown (**Figure 4c**), as described previously[7]. Notably, at the post-surgery time point, ctDNA+ patients exhibited a much higher rate of relapse compared to ctDNA- patients (RFS HR=78.2, p<0.001) (**Figure 4c**). No relapse events were observed in the ctDNA negative patients at baseline and post-neoadjuvant time point. PD-1 positive patients were more likely to be ctDNA positive. Outcome was particularly poor in ctDNA positive PD-L1 negative patients **(Suppl Fig 3).**

Both Lund and TCGA molecular classifications were applied to baseline tumor transcriptomes and correlated with baseline ctDNA status (**Figure 5a**)[13,14]. ctDNA+ patients were enriched in the TCGA basal squamous and the Lund squamous cell carcinoma-like (SCCL) subgroups (**Figure 5a**). Tumors from baseline ctDNA+ patients were enriched for immune transcripts, especially from the myeloid lineage (CD14, CD83, CD86, FCGR3B, CD163, CXCL8/IL8) and the B/plasma cell lineage (TNFSF13B/BAFF, JCHAIN, SLAMF7) (**Figure 5b**). This enrichment in myeloid signals in tumors from ctDNA+ patients was confirmed by Reactome pathway enrichment analysis (**Figure 5c**). Deconvolution of bulk RNA-seq data to quantify the relative frequency of cell subpopulations confirmed that tumors from ctDNA+ patients exhibited an increased global immune score mainly driven by increase in several myeloid subsets, including monocytes, neutrophils, M1 macrophages and dendritic cells (**Figure 5d**).

**Discussion**

The standard treatment for cisplatin-ineligible patients is upfront RC resulting in a 2-year DFS rate of 40-50%[15]. This data is generated from a large, neoadjuvant, randomized trial. The 2-year DFS rate in ABACUS was 68%. Similar results were achieved using 3 cycles neoadjuvant pembrolizumab in the PURE-01 study (71.7%), with a median follow-up of 23 months (IQR 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 the 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-year outcome yet [16,17].

These results are intriguing as recent data shows that one year 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 as extensively studied in clinical trials but exploratory results have been encouraging[19]. TMB and PD-L1 did not correlated 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 suggests tumor cell expression of PD-L1 may be relevant in predicting response [20].

FAP was associated with stromal infiltration[21] and continues to be a promising marker of resistance in treated tissue. These results also point towards the importance of biomarkers which 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 mechanisms 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 responding tumors post-treatment. To our knowledge, CD39 has not been previously described as a potential biomarker in urothelial carcinoma opening new avenues in this field.

ctDNA- patients at baseline were more likely to achieve pCR with neoadjuvant atezolizumab. ctDNA- status prior to neoadjuvant treatment may reflect non-metastatic disease, or alternatively the baseline tumor resection with curative intent may have led to the 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. ctDNA as a surrogate marker of response and relapse has been explored with chemotherapy in MIBC[7,11]. A relationship between baseline ctDNA status and PD-L1 status was established, suggesting the use of dual tissue based and circulating biomarker may be important for the future. ctDNA+ / PD-L1 negative patients at baseline have a particularly poor outcome and require attention. Alternative to neoadjuvant immune therapy should be sought in this population, they may influence the results of clinical trials. Post-surgical ctDNA status was found to be highly prognostic of relapse. In this study, only ctDNA+ patients experienced relapse at the post-surgical time point, as described previously in this setting[11]. Recent randomized data suggest 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 only effective 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 suggests 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 shows ctDNA may be useful in monitoring clinical benefit and selecting patients for adjuvant therapy after neoadjuvant treatment (only 3% of ctDNA negative 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, 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 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, non-randomized design, short period of therapy and the exploratory nature of the biomarker analysis.

**Conclusion**

The use of neoadjuvant atezolizumab in cisplatin-ineligible patients demonstrated attractive outcomes. Exploratory analysis of pre- and post-treatment biomarker analysis demonstrated that a subgroup of patients benefits from this approach. Serial ctDNA analysis correlated with outcomes and may inform the development of personalized therapy in the future. Further randomized neoadjuvant trials using a backbone of immune checkpoint inhibitors are ongoing and supported by this data (NCT03732677, NCT03924856).

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