Title: Viral targeting of non-muscle invasive bladder cancer and priming of anti-tumour immunity following intravesical Coxsackievirus A21

Running Title: Viral targeting of non-muscle invasive bladder cancer.

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Statement of translational relevance

This report describes the first 'window-of-opportunity' study of the tumour microenvironment of non-muscle invasive bladder cancer (NMIBC) following exposure to a novel oncolytic virus, CVA21. Post-treatment tissue was directly visualised and available for study after resection. No clear differences were seen in tumours receiving CVA21 versus prior low-dose mitomycin-C, an agent used to increase expression of the CVA21 entry molecule, ICAM-1.

Intravesical CVA21 therapy was extremely well-tolerated, was highly tumour-selective, and replicated in NMIBC causing inflammatory changes (immunological 'heat') in tumour but not normal bladder. Upregulation of interferon response genes and immune checkpoint genes (PD-L1 and LAG3) was evident, supporting combination studies with immune checkpoint inhibitor antibodies. NMIBC is regarded as a malignancy that responds well to immunotherapy. Oncolytic viral therapy, alone or in combination with immune checkpoint blockade may offer an alternative to the 40 year old standard of care, BCG therapy, but without its limiting toxicities.

Abstract:

Purpose

The CANON (**CA**VATAK in **NON**–muscle invasive bladder cancer) study evaluated a novel ICAM-1-targeted immunotherapeutic-coxsackievirus A21 as a novel oncolytic agent against bladder cancer.

Experimental Design

Fifteen patients enrolled on this 'window of opportunity' phase 1 study, exposing primary bladder cancers to CAVATAK prior to surgery. The first nine patients received intravesical administration of monotherapy CAVATAK; in the second stage, six patients received CAVATAK with a sub-therapeutic dose of mitomycin C, known to enhance expression of ICAM-1 on bladder cancer cells. The primary endpoint was to determine patient safety and maximum tolerated dose. Secondary endpoints were evidence of viral replication, induction of inflammatory cytokines, anti-tumour activity and viral-induced changes in resected tissue.

Results

Clinical activity of CAVATAK was demonstrated by induction of tumour inflammation and haemorrhage following either single or multiple administrations of CAVATAK in multiple patients, and a complete resolution of tumour in one patient. Whether used alone or in combination with mitomycin C, CAVATAK caused marked inflammatory changes within NMIBC tissue biopsies by up-regulating interferon-inducible genes including both immune checkpoint inhibitory genes (PD-L1 and LAG3) and Th1-associated chemokines as well as the induction of the innate activator RIG-I, compared to bladder cancer tissue from untreated patients. No significant toxicities were reported in any patient, from either virus or combination therapy.

Conclusions

The acceptable safety profile of CAVATAK, proof of viral targeting, replication and tumour cell death together with the virus-mediated increases in "immunological heat" within the tumour microenvironment all indicate that CAVATAK may be potentially considered as a novel therapeutic for NMIBC.

Introduction

Non-muscle invasive bladder cancer (NMIBC) is a highly prevalent cancer with lifelong risk of occurrence. Transurethral resection (TUR) of all visible lesions is a standard treatment for NMIBC (1), but is accompanied with a high tumor recurrence rate ranging from 50% to 70% as well as a high tumor progression rate between 10% and 20% over a period of 2–5 years (2, 3). Thus, guidelines recommend intravesical chemotherapy and immunotherapy in the management of NMIBC to reduce these risks of recurrence and progression (4). Immunotherapy with Bacille Calmette-Guerin (BCG) decreases frequency and delays time to cancer recurrence and progression in patients with NMIBC (1, 5). Unfortunately, one third of NMIBC patients experience serious side effects of local and systemic BCG infection and one third do not respond (6, 7). Treatment options for BCG-refractory patients are limited and patients often undergo cystectomy. Combined with a potential worldwide shortage of BCG (8) there is an urgent need to develop novel therapies for this disease.

The success of BCG treatment for NMIBC stems, uniquely, from the BCG-induced immune response. Antigen-presenting cells in the urothelium can phagocytize BCG, followed by the presentation of antigen to BCG-specific CD4+ T-cells. Pro-inflammatory cytokines are released, resulting in a predominant Th1-cell-induced immunity with an enhanced recognition of cancer cells through activated macrophages, CD8+ T-cells, natural killer cells and other effector cells (9,10). Oncolytic viruses are emerging immunotherapeutic agents for a broad range of malignancies. As well as their ability to preferentially replicate in and lyse cancer cells directly, it is their induction of host immunity which is increasingly recognized to be the major component of their anti-tumour efficacy (11). Coxsackievirus A21 (CVA21), a naturally

occurring common cold-producing enterovirus, is one such effective oncolytic agent against a range of solid tumours (12-15). CVA21 specifically targets and lytically infects susceptible cells expressing the CVA21 cellular receptors, intercellular adhesion molecule-1 (ICAM-1) and decay-accelerating factor (DAF) (16). Our group recently demonstrated the susceptibility of bladder cancer cell lines to CVA21, the ability to enhance oncolysis by modulating expression of the viral receptor ICAM-1 by low doses of mitomycin C treatment and the induction of immunogenic cell death in CVA21-treated cell lines capable of generating long-lasting protective anti-tumour immunity in the bladder mucosa (17). These results provided the rationale for a Phase I/II clinical trial (CANON) to investigate the therapeutic potential of CVA21 as a new immunotherapy approach for the treatment of NMIBC.

This trial determined safety, feasibility and biological effects of escalating intravesical doses of a novel bio-selected formulation of CVA21 (CAVATAK) administered alone or in combination with mitomycin C in 15 first-line NMIBC patients prior to TURBT surgery.

Materials and Methods

Participants

Eligible patients were ≥18 years of age, with a clinical diagnosis of NMIBC based on cystoscopic appearance and which was suitable for transurethral resection. Eastern Cooperative Oncology Group (ECOG) performance status (PS) ≤2, ANC >1500/mm³; Hb >9.0 g/dL; Platelet >100000/mm³ and serum creatinine ≤ 1.5 mg/dL were required. Patients with prior local or systemic treatments for NMIBC were excluded.

The study was conducted in accordance with UK recognized ethical guidelines and received approval from National Research Ethics Committee (Ref 14/LO/1804). The patients provided written informed consent for treatment and analysis of their biological samples. The trial is registered on ClinicalTrials.gov (identifier: NCT02316171). Supplementary Table 1 outlines the patients and treatment characteristics.

Study Design and outcome measures

CANON was a Phase I, two-part, open-label, dose-escalation study designed to evaluate the safety and clinical activity of intravesical CAVATAK™ (Coxsackievirus A21, CVA21) alone and in sequential combination with low-dose mitomycin C in 16 first line patients with non-muscle invasive bladder cancer (NMIBC) who were candidates for and were planning to undergo TUR for treatment and staging of their disease (See Supplementary Materials and Methods for inclusion and exclusion criteria). This gave a relatively homogeneous study population and facilitated collection of resected tumour tissue for histological, pharmacodynamics (PD) and pharmacokinetic (PK) analyses.

The study consisted of 2 sequential parts. Part 1 (VLA012A) was a study of the safety and tolerability of CVA21 administered via intravesical instillation as a single agent in subjects with NMIBC scheduled to undergo TURBT for treatment and staging of their disease. Three cohorts consisting of 3 subjects each received CVA21 on Day 1 in Cohorts A1 ($1x10^8$ $TCID_{50}$) and A2 ($3x10^8$ $TCID_{50}$) with subject in Cohorts A3 receiving to doses of CVA21 ($3x10^8$ $TCID_{50}$) on Days 1 and 2. As these doses were well tolerated, Part 2 (VLA012B) commenced. Part 2 (VLA012B) evaluated the safety and tolerability of CVA21 administered in sequential combination with low-dose mitomycin C in the same subject population. Two dose levels and two schedules of administration of CVA21 were evaluated with a fixed dose of mitomycin C (10 mg) in 2 cohorts of subjects. Mitomycin C was administered by intravesical instillation on Day 1 and intravesical instillation of CVA21 ($3x10^8$ $TCID_{50}$) occurred 4 hours after instillation of mitomycin C. In subjects scheduled to receive two instillations of CVA21 ($3x10^8$ $TCID_{50}$), the instillations were at approximately the same time each day. Both parts of the study were open-label, with ascending doses and increased frequency of CVA21 dosing in a standard 3+3 design. After the maximum tolerated dose (MTD) of the combination was established in VLA012B, up to 10 additional subjects were to be enrolled at the MTD to further explore the safety and PD of the combination. This part of the study was not executed due to the objectives of the study being met and the sponsor deciding to terminate the study. The maximum tolerated dose (MTD) was defined in the protocol as the highest intravesical CVA21 dose at which no more than one subject in each 3-subject cohort experienced a dose-limiting toxicity (DLT). The period of observation for DLTs was from the time of first administration to 7 days later

The highest dose of intravesical CVA21 used in this study ($3 x10^8$ $TCID_{50}$) on Days 1 and 2 was well tolerated and may be used in future studies. The safety and tolerability of the treatment of

CVA21 with and without low dose mitomycin C was assessed through daily physical examinations, vital signs, and review of hematology, serum chemistry and urinalysis data. Treatment-emergent adverse events were also reviewed (Supplementary Materials and Methocds and Sup Table 2). The mitomycin C dose (Supplementary Materials and Methods) was based on *in vitro* studies where ICAM-1 expression was upregulated without cytopathic effects on tumour cells. Cystoscopy photography was performed before and after treatment. Intravesical treatment was followed by TURBT surgery after 8-11 days which allowed tissues to be analysed for virus replication, apoptosis, evidence of viral-induced changes in immune cell infiltrates and immune checkpoint molecules. Serum and urine was collected on day 1 (before virus instillation), 3, 5 and 8 post-virus treatment.

RNA extraction and Viral RNA detection

Aliquots of urine were clarified by centrifugation (1 min, 10,000 RPM) and viral RNA extracted from 140 µL of supernatant using Qiagen QIAmp Viral RNA Mini Kit (Qiagen). Control samples spiked with $1x10^3$ and $1x10^6$ copies were also extracted as internal controls. For detection of viral RNA 5 µL extracted RNA was tested in triplicate with 20 µL of Qiagen's Quantifast® pathogen RT-PCR + IC kit. Primer and Probe sequences are as follows: Forward primer (KKVP3fwd): 5'-GAGCTAAACCACCAACCAATCG-'3'; Reverse primer (KKVP3rev): 5'-CGGTGCAACCATGGAACAA-'3'; Probe (KKVP3): 6FAM-CACACACATCATCTGGGA-MGB. Samples that tested positive were further tested by TCID50 assay to confirm replication competent virus.

TCID50 assay

Aliquots of urine samples were thawed, vortexed and centrifuged (1 min, 10,000 RPM) and supernatant serially diluted (DMEM, 2% FBS) and incubated on 70-90% confluent monolayers

of SKMEL-28 cells, prepared in 96-well plates, 24 hours previously. Plates were incubated for 5 days before scoring wells positive/negative for cytopathic effect (CPE) and calculating the viral titre using the Karber method.

Neutralising Anti-Coxsackievirus Antibody Assay

SK-MEL-28 cells were plated 24 hours prior to the assay. Test serum was serially diluted in the range 1:4 to 1:32768 and 100ul of each dilution mixed with 100ul of media containing 100 TCID50 CVA21 and incubated at 37°C for 1 hour. Following incubation, the sera were added to SK-MEL-28 cells and incubated for 72 hours with cytopathic effect scored visually at the endpoint and 50% neutralising titre calculated. Positive and negative control serum was also tested and viral titre confirmed by TCID50 with each assay.

Immunohistochemistry

4 µm sections were deparaffinised before antigen retrieval and endogenous peroxidase blocking was performed. Slides were incubated with mouse anti-enterovirus antibody (DakoCytomation, Glostrup, Denmark), rabbit anti-cleaved Caspase 3 (Cell Signaling Technology), rabbit anti-HMGB1 antibody (Abcam, Cambridge, UK), and mouse anti-ICAM1 antibody (SantaCruz). Subsequently, slides were incubated with an HRP-labelled secondary antibody (DakoCytomation, Glostrup, Denmark), followed by detection with diaminobenzidine solution and counterstained with hematoxylin (VectorLabs).

Total RNA extraction from FFPE-tumour tissues

Total RNA was isolated from paraffin-embedded tumour tissues using Norgen FFPE RNA Purification kit (Norgen Biotek, ON, Canada) as per the manufacturer's instructions. RNA concentration and purity was measured using an Agilent 2100 BioAnalyzer.

Human Cell Death PathwayFinder RT² Profiler PCR Array

cDNAs were reverse transcribed from RNA extracted from CAVATAK-treated bladder tumour tissues (see above) using the RT² First Strand Kit (SABiosciences). Comparison of the relative expression of 12 cell-death-related genes between the samples was made with PCR Array: the cDNAs from those samples were characterized using a custom-designed Cell Death PathwayFinder RT² Profiler PCR Array (SABiosciences) and the RT2 SYBR Green/Rox PCR master mix (SABiosciences) on a Stratagene Mx3000P Thermal Cycler. ATP5B and GAPDH housekeeping genes were used for normalization (18) and data were analyzed with $\Delta\Delta C_t$method.

Multispectral immunohistochemical analysis

Tissue sections were cut at 4 um from formalin-fixed paraffin-embedded blocks. All sections were deparaffinized and subjected to heat-induced epitope retrieval in citrate buffer pH6.0 (Biogenex). 6-plex panel immunohistochemistry was performed for each tissue slide using the following antibodies: anti-FoxP3 (clone 236A/E7, Abcam), anti-PD-L1 (clone E1L3N, Cell signaling), anti-CD8 (clone SP16, Spring Bioscience), anti-CD3 (clone SP7, Spring Bioscience), anti-CD163 (clone MRQ26, Ventana), anti-Cytokeratin (clone AE1/AE3, DAKO). Antigen-antibody binding was visualized with TSA-Cy5, TSA-Cy3, TSA-FITC, TSA-Cy5.5, TSA-Coumarin (PerkinElmer) and TSA-Alexa594 (Life Technologies). Microwave treatment in citrate buffer pH6.0 was performed between antibody detection to prevent crossreactivity. Tissue slides were counterstained with DAPI and coverslipped with VectaShield mounting media (Vector Labs). Control tissue samples were stained for each different marker. Hematoxylin and eosin staining was performed for each sample and reviewed by a pathologist to ensure the representativity of the tissue sample. For a detailed immunohistochemistry protocol for multispectral analysis see Feng Z et al. (19).

Microscopy and image analysis of multiplexed IHC: Phenotype cell quantification in high-resolution images

Digital images were captured with PerkinElmer Vectra 2.0 platform following hot spot lymphocyte assessment: Tumor areas with the highest immune cell (CD3+CD8+) infiltrates were scanned at 20X and selected for analysis. Three images of 0.36 mm^2 each per tissue sample were analyzed with InForm Software (PerkinElmer). The total number of cells were enumerated for the following phenotypes: PD-L1+ tumor cells+, PD-L1+ other cells, CD3+PD-L1+, CD3+PD-L1- FoxP3+, CD3+CD8+PD-L1+, CD3+CD8+PD-L1-, CD163+PD-L1+, CD163+PD-L1- in the stroma and tumor compartment.

Detection of urinary HMGB1

Urine samples of patients and controls were tested using a commercially available HMGB1 ELISA kit (ST51011; IBL International GmbH). The assay was conducted according to the manufacturer's instructions. Urinary HMGB1 was expressed as HMGB1/Cr ratio (mg/μmolCr) to correct for differences in dilution.

Detection of urinary cytokine levels

Quansys Biosciences (Logan, UT) were contracted to test the urinary cytokine levels in 12 CANON patients, 15 untreated NMIBC patients and 13 healthy controls using their Q-Plex™ Human Cytokine multiplexed ELISA array. Thawed samples were diluted with the appropriate Quansys sample dilution buffer at a ratio of (sample:buffer) of 1:2 (50%). Polypropylene low-binding 96-well plates were used to prepare the samples and standards prior to loading the Q-Plex ™ plate. Each dilution was measured in triplicate, a total of 3 wells per sample. The intensity of chemiluminescence from each array was measured using the Q-View

chemiluminescent imager (Quansys Biosciences) and quantified using the Q-View software (Quansys Biosciences).

Nanostring

All RNA samples included in the study passed quality control requirements (as assessed by the RNA integrity number) of the platform. Digital multiplexed NanoString nCounter analysis system (NanoString Technologies, Seattle, WA, USA)-based gene expression profiling was performed on 100ng total RNA from each sample as input material according to the manufacturer's instructions. Nanostring RNA analysis of 700 immune-related genes was performed using the nCounter GX Human PanCancer Immune profiling Kit (XT) on the nCounter® Analysis System. Analysis and normalization of the raw Nanostring data was performed using nSolver Analysis Software v1.1 (Nanostring Technologies).

Statistical analysis

Correlations were evaluated by the Pearson tests. All P values were calculated using two-tailed test. P values < 0.05 were considered statistically significant. Analyses were performed using GraphPad Prism.

Results

Patients, study design, and toxicity of the trial

Fifteen patients were recruited into this study. All patients presented with cytoscopically visible bladder tumours positive for urine cytology and were scheduled to undergo a transurethral resection of their bladder tumour (TURBT) as part of their standard clinical care. The patients' clinical characteristics are shown in Sup Table 1, and the design of the clinical trial (CANON) is illustrated in Fig 1A and in supplementary materials and methods. Intravesical administration of CAVATAK either as a single agent or in combination with mitomycin C was generally well tolerated with no grade 2 or higher product-related adverse events observed (Sup Table 2). Six patients developed urinary tract infections which were attributed to displacement of bacteria from the urethra into the bladder during catherization. This was prevented in further patients by a 5 day course of oral amoxicillin commencing the day of first catheter insertion. The primary endpoint of this trial was to determine patient safety and maximum tolerated dose. Secondary objectives were assessment of viral replication, anti-tumour activity and viral-induced changes in immune cell infiltrates.

Tumour Response: Pre and post treatment cystoscopy

The clinical trial design of CANON (Fig 1A), administering CAVATAK a week before the patients underwent a TURBT from which tissue was then available to study, gave a unique window of opportunity to study the effect of this oncolytic virus on bladder cancer. Response was defined as complete (disappearance of tumour i.e. pathological complete response) or macroscopic increase of haemorrhage compared to pre-treatment. Due to the heterogenous size, shape and pre-existing haemorrhage/necrosis, only observational comparison was possible. Cystoscopic photography was performed in all subjects to record the appearance of their tumour

before and after intravesical CAVATAK treatment. As shown in Figure 1B, 8-11 days following intravesical CAVATAK treatment surface haemorrhage and inflammation of tumours was observed in a number of the patients and one complete response confirmed by histology. Sup Fig 1 shows the urine cytology slide from this complete responder (patient B008) confirming he did have bladder cancer prior to the intravesical CAVATAK treatment.

Viral-induced anti-tumour activity in transurethral resection tissue

All patients underwent surgery 8-11 days after treatment with CAVATAK monotherapy or in combination with mitomycin C, which allowed us to evaluate specific tumour targeting of the virus in the resected tumours. Immunohistochemical staining for enterovirus protein indicated viral infection in 12 of 14 tissues available for study with varying amounts of positive tumour staining observed (Fig 2A). No viral staining was detected in surrounding stromal areas or in areas of tumour tissues displaying normal glandular change within urothelium (Fig 2B) consistent with selective CAVATAK targeting to, and/or replication in, malignant cells. Viral protein positivity in the tumour appeared to be correlated with those tumours that displayed at least localized areas of ICAM-1 positivity, in keeping with our previous work showing the necessity of ICAM-1 for CVA21 infection of bladder cancer cells (17). One patient (B004) displayed extensive homogeneous ICAM-1 expression by the tumour cells, n=5 displayed <30%, n=4 <10% ICAM-1 tumour positivity and n=4 no ICAM-1 tumour positivity was detected. Immunohistochemical analysis of the other CVA21 cellular receptor, decay-accelerating factor (DAF), showed strong homogeneous DAF expression by the tumour cells in all patient cases (Sup Fig 2). Haematoxylin and eosin-stained sections of the viral-positive tissues showed areas of non-viable tumour and apoptotic bodies which was further confirmed by staining for cleaved caspase 3. Tissues that demonstrated minimal to no viral infection also displayed little to no

cleaved caspase 3 positivity and were confirmed as viable tumour from the haematoxylin and eosin stains. The detection of significant areas of non-viable tumour based on haematoxylin and eosin staining as well as cleaved caspase 3 was not observed in untreated historical bladder cancer control tissues (data not shown) providing further evidence that the increased tumour cell apoptosis observed in the viral positive tumours was due to CAVATAK induced cell death.

Previous preclinical work from our group showed that the mode of CAVATAK-induced cell death in bladder cancer cell lines was predominantly by apoptosis (17). To ascertain the cell death route taken by bladder cancer cells in response to CAVATAK infection in this human clinical setting, we analysed the total RNA extracted from the CAVATAK-treated tumours using a custom Human Cell Death PathwayFinder RT2 Profiler PCR Array. Using this array the expression of key genes important for the central mechanisms of cellular death: apoptosis, autophagy, and necrosis was profiled and their expression levels compared to archival untreated bladder cancers. As shown in Table 1 genes encoding the intrinsic apoptotic cell death pathway (BCL2L1, BAK1, and caspase-9) were predominantly upregulated compared to untreated bladder cancer in the majority of patient tumours studied. Three of the tumours also displayed upregulation of one of the main players in programmed necrotic cell death, the serine/threonine kinase receptor-interacting protein 3 (RIPK3). In addition, seven of the tumours displayed a significant increase in the immunogenic cell death marker, Calreticulin.

Increases in infectious virus in patient urine following intravesical CVA21 administration

To measure viral replication within the patients' tumours, levels of viral shedding into the urine was assessed by analysis of viral copy number by RT-PCR and retrieval of replication-competent virus was determined by adding urine to CVA21-sensitive SKMEL-28 cells in a tissue culture infectious dose ($TCID_{50}$) assay. Between days 2 and 5 following the initial instillation of

CAVATAK all patients from both the monotherapy and combination cohorts showed an increase in viral levels with several patients (4,5,6,7,8,11 and 15) demonstrating a second peak at later time points suggesting more than one cycle of viral replication within the bladder of these patients (Figure 3).

Urinary cytokine profile in CAVATAK-treated patients

We investigated the ability of CAVATAK to modulate the immune response in the NMIBC microenvironment as reflected by shed urinary cytokines in 12 CANON patients, and compared to 15 untreated NMIBC patients and 13 age-matched healthy controls. A 17-plex quantitative ELISA-based chemiluminescent assay was used to screen the urines of CANON trial patients pre-treatment at day 1 and then post-treatment on days 3, 5 and 8 (with the exception of patient B005 where no d8 urine sample was obtained). Figure 4A shows the kinetics of the CAVATAK-induced cytokine response to 8 of the 17 cytokines studied in six representative patients. Whilst the cytokine levels did not vary significantly over the treatment period in the majority of patients, patient B004 was the exception displaying a peak in the level of virally-induced cytokines (IL-6, IL-1a, IL-1b and TNFα) at day 3 post-infection which then declined with a second increase from day 5. Notably, four of the virus-treated patients (B001, B005, B008 and B010) including the patient (B008) that demonstrated a complete response to CAVATAK, displayed increased levels of the pro-inflammatory cytokine IL-23 following treatment which was consistently undetectable in the untreated NMIBC patients and healthy controls (data not shown).

Increases in urinary HMGB1 levels in CAVATAK-treated patients

Having previously shown the release of HMGB1 by CVA21- treated bladder cancer cells *in vitro* and the importance of this potent cytokine for directing an immune response (17,20), HMGB1

levels were assessed in the urine of the virus-treated patients. Figure 4B shows the increases in urinary levels of HMGB1 in selected patients (6 out of 11 assessed) after CAVATAK treatment using creatinine normalized values of HMGB1 (pg HMGB1/mg creatinine).

Interestingly, immunohistochemical analysis of this cytokine showed increased expression of cytoplasmic HMGB1 in CAVATAK-treated tumour tissues when compared to untreated bladder cancer controls (Fig 4C). The untreated bladder cancer control tissues displayed a predominantly nuclear localization of HMGB1 consistent with that seen in the positive control tissue, normal kidney. This result suggests that CAVATAK infection may trigger the translocation of HMGB1 to the cell cytoplasm whence, upon cell death, it can be passively released to the extracellular environment, in keeping with the increased levels of urinary HMGB1 in virus-treated patients.

CAVATAK induces upregulation of PD-L1 in the NMIBC microenvironment

A multispectral immunohistochemistry method which allowed the simultaneous detection of 7 markers was employed to investigate changes in the immune microenvironment of CAVATAK treated NMIBCs compared to control untreated archival cases of NMIBC and normal bladder tissue. Quantitation of the CD8+ T-cell infiltration revealed no significant differences between the virus-treated tumours and untreated bladder cancer or normal bladder tissue in either the stromal regions or intraepithelial regions (Fig S3). In addition, despite having shown in a previous preclinical study an enhanced uptake of the virus in mitomycin C treated bladder cancer cell lines, this treatment did not result in an increased CD8+ T-cell infiltration in the patient tumours compared to control tumour tissues. However, notably, the patient (B008) who experienced a complete response to CAVATAK treatment showed a considerable immune infiltrate within the biopsy taken from the bladder area where the tumour had previously been identified (Fig S4). The 7-marker multispectral analysis also revealed an increase in PD-L1

within the stromal areas of the CAVATAK-treated tumours compared to the control bladder tissues although this did not reach statistical significance (Fig S3).

CAVATAK induces upregulation of immune response genes

To further explore the immune response to CAVATAK, Nanostring Pan-Cancer Immune profiling was performed on RNA derived from 12 CAVATAK-treated and 7 untreated NMIBCs. CAVATAK led to elevated expression of the interferon-inducible genes *IFIT1*, *IFIH1, OAS3*, and *MX1*, compared with levels seen in untreated NMIBC control tissues. Furthermore, CAVATAK led to higher expression of the IFN-γ-induced chemokine genes encoding *CXCL9*, *CXCL10*, and *CXCL11* (Fig 5A) associated with a Th1-mediated immune response.

Due to the known barriers that hamper oncolytic virus-induced anti-tumour immune responses we were interested to explore the expression levels of immune checkpoint molecules and immunosuppressive enzymes within the CAVATAK treated tumours compared to untreated archival NMIBCs. As shown by the histograms in Fig 5A, in CAVATAK-treated tumours there was an increase in the expression level of the immune checkpoint molecules PD-L1 and LAG3 as well as the amino acid-depleting enzyme, indoleamine 2,3-dioxygenase (IDO). These immunosuppressive mechanisms could be dampening T-cell responses and thus limiting the efficacy of CAVATAK thus providing a rationale for future combination therapies using immune checkpoint inhibition or IDO-1 inhibition.

Another notable result from the Nanostring immune profiling was the significant upregulation in CAVATAK-treated tumours of the gene DDX58 which encodes the RIG-I-like receptor dsRNA helicase enzyme (Fig 5B). This specific upregulation of DDX58 further confirms the infection by

and sensing of CAVATAK within these tumours and is in keeping with the induction of a type I interferon response.

ICAM-1 expression is essential for productive CAVATAK infection

Previous work from our group has clearly shown the importance of ICAM-1 expression levels on bladder cancer cell lines in order to obtain a sufficient level of CAVATAK infection and subsequent oncolytic effect (17). This finding is further exemplified in this clinical study by one particular patient (B004) who, despite receiving the lowest dose of virus (single CAVATAK dose of $1x10^8$), demonstrated significant viral infection and viral-induced changes in their bladder tumour. Whilst all the patients' tumours expressed DAF, many of the patients' tumours only showed focal regions of ICAM-1 tumour positivity. Notably, patient B004's resected CAVATAK-treated tumour displayed a high level of ICAM-1 expression throughout the tumour, enabling a high level of virus infection and apoptotic tumour cell death (Fig S5B). Furthermore, the virus-treated tumour displayed cytoplasmic HMGB1 expression, a high level of urinary cytokines (Fig 4A), immune infiltration, increased levels of stromal PD-L1 and a high level of perforin expression indicating a clear immune response to this productive infection (Fig S5B). Perforin positive cells were also detected in four other CANON patients (B006, B007, B008 and B010), but not at the same high prevalence (Fig S5A). Such perforin positive cells were not detected in untreated bladder cancer (n=10 tissues studied) and normal bladder tissues (n=10 tissues studied).

Discussion

NMIBC remains a highly prevalent, and significant health problem worldwide, requiring intrusive surveillance and high health economic costs. It is the prototypical example of successful immunotherapy with high primary response rates to BCG likely involving a combination of innate and adaptive immune responses initially to BCG, followed by tumour-specific T-cell responses (9). The unrestricted BCG-induced vigorous inflammatory response in the entire bladder mucosa is highly problematic and often results in incomplete treatment programs.

Oncolytic viruses are emerging as highly potent cancer immunotherapeutics, which in many ways have similar properties to BCG (21). They target cancers through direct cytotoxicity and also induce cancer-specific adaptive immunity. Their ability to convert 'cold' tumours into 'hot' tumours through inflammation make them potential partners for combination with immune checkpoint inhibitors (22), and there are currently a large number of clinical trials ongoing with this combination. This includes delivery of CVA21 into metastatic melanoma deposits alone (CALM, ClinicalTrials.gov Identifier: NCT01227551) or combined with anti-CTLA4 antibody (MITCI ClinicalTrials.gov Identifier: NCT02307149) and anti-PD1 (CAPRA, ClinicalTrials.gov Identifier: NCT02565992) (23, 24). These studies confirmed that CVA21 exposure causes a marked immune inflammatory and very durable local and abscopal anti-tumour response. NMIBC is an ideal model for oncolytic immunotherapy with easy and direct access to bladder cancer, their visualisation, delivery of high doses of virus, no risk of neutralising antibody limitation, complete control of length of exposure through catheterization and sequencing of repeat doses and/or combination treatments based on existing schedules used for BCG therapy.

Our preclinical study had indicated ICAM-1-targeted cytotoxicity after CVA21 exposure and the induction of immunogenic cell death in bladder cancer cell lines, enhanced by exposure to low, non-cytotoxic doses of mitomycin-C through induction of ICAM-1 expression (17). DAF expression was not dysregulated across numerous cell lines and was not predictive of CAVATAK infection. In the current neoadjuvant clinical study, 15 patients received intravesical CVA21 +/- low dose mitomycin C prior to tumour resection. The purpose of the study was to determine safety and tolerability for this approach, but also biological endpoints as a 'window of opportunity' approach including tumour response, changes in the immune environment and evidence of virus replication. Importantly, the study was not attempting to compare efficacy or immunogenicity with BCG, but aimed to provide a clear picture of immunomodulatory effects of CAVATAK in treatment naive tissue. All patients had detailed prior imaging to exclude locally advanced and metastatic disease. All patients had positive urine cytology, but evidence of muscle invasion would have only been possible after TURBT: this was not evident in any patient post-TURBT, and despite the cohort all being urine cytology positive, not all were high grade tumours. The treatment was extremely well-tolerated with minor toxicities reported. Objectively, clinical photography indicated virus-induced haemorrhagic and inflammatory changes in 3 patients, but notably there was no evidence of a general inflammation of the normal bladder, in contrast to what would be expected with BCG therapy. The patient with complete response showed clear evidence of acute inflammation within the pre-existing area of tumour within the bladder providing further evidence for the role of the immune response in the therapeutic outcome of this viral treatment.

We found evidence of secondary viral replication in most patients in urine samples between 2 and 5 days post-CVA21 treatment by both viral copy number by RT-PCR and TCID50,

representing replication competent virus production. Immunohistochemical staining for enteroviral protein was shown to be specifically associated with cancer cells and not normal urothelium in all but 2 patients (in whom the tissue was non-evaluable due to damage at resection).

After intravesical delivery of CVA21, no virus was detected in serum in any patient, nor was there evidence of induction of a systemic neutralising antibody response in either cohort (Sup Table 3). After systemic delivery of CVA21 in an ongoing study (STORM, https://clinicaltrials.gov/ct2/show/NCT02043665) approximately 80% of patients demonstrated a neutralising antibody response at day 8 post treatment (personal communication with Viralytics Ltd.).

We previously demonstrated the importance of ICAM-1 expression for CVA21 uptake in bladder cancer cell lines (17). In this study, ICAM-1 expression was mostly focal, in 8 out of the 14 patient tumours studied. This proportion was lower than expected and may be explained by the paucity and quality of tissue after TURBT, and heat artefacts to adjacent tissue. Indeed, the importance of ICAM-1 expression for a productive CVA21 infection was clearly demonstrated by one particular patient (B004) whose tumour displayed high, widespread ICAM-1 expression which was associated with high levels of virus protein expression, apoptosis, HMGB-1 in urine, urinary cytokines and immune infiltration with PD-L1 induction.

Virus infection was associated with apoptosis, determined by cleaved caspase 3 expression in patient tumour tissues. In preclinical studies, we found the mode of cell death in bladder cancer lines was through the intrinsic apoptosis pathway; in patient tissue we found this was also the case in the majority of cases with additional evidence of necroptosis in 3 patients. Using bladder cancer cell lines we had previously determined that CVA21 induces a type of cancer cell death

that is immunogenic (17,25). In this clinical study 6 of 11 patients, despite wide intra-and inter-patient variations in urine pH, specific gravity and metabolite content, showed increased levels of one of the hallmarks of immunogenic cell death, high mobility group box 1 (HMGB1) in the urine post-CVA21 treatment. Furthermore, immunohistochemical analysis of HMGB1 expression in the tumour tissues of the study patients revealed a greater degree of cytoplasmic expression (24). Unfortunately, we were unable to correlate the cytoplasmic HMGB1 expression revealed in tissue with peaks in urinary HMGB1 as we excluded any patient urine samples that displayed haematuria to prevent false positive results. HMGB-1 is one of the most abundantly secreted DAMPs following oncolytic virus infection (26-30) and is thought to aid the therapeutic efficacy of oncolytic viral therapy by acting as a potent immunostimulatory molecule and chemoattractant for monocytic cellular infiltration during virus infection (31).

In addition to levels of HMGB1 in the urine, the levels of inflammatory cytokines were also evaluated to see whether they reflected local immunomodulation caused by CVA21 infection. Such an analysis of urinary cytokine profiles has previously been used to determine responders and nonresponders to BCG therapy (32). Looking at the kinetics of the cytokine responses in individual patients, there were increases in classical virally-induced cytokines (33) on day 3 post-infection, often with a second peak at day 8. Interestingly, one cytokine that did differ between bladder cancer controls and CVA21-treated patients, was the levels of IL-23 which was consistently undetectable in the untreated NMIBC patients, but was elevated in four of the virus-treated patients, including the patient that demonstrated a complete response to CAVATAK. IL-23 has been shown to have significant antitumour effects in various models of cancer and is associated with the promotion of cell-mediated immune responses and activation of CTLs or NK cells (34-37).

One of the key aims of the study was to induce immunological 'heat' into the NMIBC tumour microenvironment we have previously observed as relatively 'cold'. Multispectral IHC analysis did not show significant differences in the quantitation of CD8+ T-cells in the stromal or intraepithelial regions between the CAVATAK-treated and untreated bladder tumours. Although CAVATAK treatment did not appear to result in increased TILs, further analysis revealed that the CD8+ TILs that were present in the virus-treated tumours displayed a more activated phenotype based on perforin expression and several cases revealed higher PD-L1 expression in the stroma. Interestingly, whilst natural killer (NK) cell activation has been shown to be a prominent feature of the immune response to BCG (38,39), CD56 immunohistochemical staining of these virus-treated tumours did not show any evidence for significant NK cell involvement in the immune response to CAVATAK in the bladder tumour microenvironment.

An even more comprehensive analysis of the expression of immune response genes in patient tissue compared with tissue from untreated, surgically resected NMIBC of similar tumour stage and grade was performed using Nanostring analysis. Untreated NMIBC had significant expression of genes associated with local immunosuppression such as IDO and ARG-2. Data have already indicated that such IDO gene expression is a feature of more aggressive NMIBC, emphasizing the potential immunosuppressive role of IDO (38). As expected, as a result of virus infection/replication the induction of interferon-inducible genes (IFIT1, IFIH1, MX1) and IFN-g induced chemokine genes was observed. In keeping with CVA21-associated induction of interferon, we also found marked increases in expression of immune checkpoint molecules PD-L1 and LAG-3 as well as the amino acid-depleting enzyme, indolamine 2,3-dioxygenase (IDO) presumably triggered to dampen down the immune response to CVA21. The pattern of response is very similar to changes observed after intratumoral injection of metastatic malignant

melanoma in the studies mentioned earlier (CALM, and CAPRA) (23, 24). We demonstrated activation of innate immunity by CVA21-infected tumours by the induction of RIG-I gene (retinoic acid-inducible gene I) expression. RIG- is a RIG-I-like receptor dsRNA helicase enzyme that is encoded by the *DDX58* gene. RIG-I is part of the RIG-I-like receptor family, which also includes MDA5 and LGP2, and functions as a pattern recognition receptor which is a sensor for a number of viruses. (40)

Although this study has obvious limitations in terms of size, some heterogeneity of patient tumour size and distribution, it does provide valuable insights into the potential efficacy and utility of oncolytic immunotherapy for this condition. The study ended at the point of TURBT resection so although no safety concerns arose from any patient at any time after the study, formal follow up tumour evaluations were not possible within the scope of the study. The scheduling of mitomycin-C/CAVATAK was based on *in vitro* demonstration of ICAM-1 upregulation following mitomycin C (within 24 hours), but may have been suboptimal in the trial context. The circa 11 days was judged by the ethics committee as the limit of an acceptable temporary delay of the TURBT procedure. The translational aspects were limited by tissue quality following surgery and measuring urinary markers such as cytokines and HMBG-1 is challenging. We were unable to take distant random bladder biopsies for comparison at surgery due to the risk of tumour seeding. Although Nanostring allowed evaluation of all of the available tumour, multispectral analysis is limited by its selectivity and focus on very small areas within the whole tumour sample. The role and value of mitomycin C for ICAM-1 modulation was still unclear.

Previously, both replicating and non-replicating viruses have been evaluated in preclinical models of bladder cancer (41-45). In human trials, intravesical vaccinia (Dryvax) resulted in

immune infiltration of both malignant and normal tissue (46), and an oncolytic adenovirus CG0070 expressing granulocyte-macrophage colony-stimulating factor (GM-CSF) is being evaluated in a randomized study phase II/III study after demonstrating objectives responses of 48-63% in different dosing regimens (47, and www.clinicaltrials.gov, NCT01438112). These studies all indicate the approach is feasible but have focused mainly on recurrent disease after BCG failure, where the natural history of the cancer has been altered. None of the studies has provided detailed, mechanistic evaluation of virus protein expression, viral replication kinetics and the effect on the immune microenvironment.

The lack of significant toxicity in any patient gives us great scope to design more prolonged dosing schedules for future studies. The study suggested no useful role for low dose mitomycin-C in the context of potential ICAM-1 upregulation. In keeping with evolving data from clinical trials of CVA21 in malignant melanoma, the virus-induced immune checkpoint molecules, presumably as a result of interferon induction, would suggest obvious combination therapy with a checkpoint inhibitor (48). These agents are already under evaluation, as single treatment, in patients with NMIBC failing BCG therapy who are at high risk of relapse. Therefore, the next stage of clinical evaluation of CVA21 in NMIBC would logically move to combination therapy, sequencing the checkpoint inhibitor after CVA21 therapy to provide a potentially alternative effective treatment for this disease to BCG.

Acknowledgements: None

List of Supplementary Materials:

Sup Table 1. Patients and treatment characteristics

Sup Table 2. Summary of treatment emergent adverse events in study VLA-012

Sup Table 3. Neutralising anti-coxsackievirus antibody titres

Fig S1. Urine cytology slide from complete responder patient B008

FigS2. Homogeneous DAF expression in NMIBC tissues

FigS3. Quantitation of intratumoral immune cell and PDL1+ immune cell densities

FigS4. Opal seven colour multiplex analysis of biopsy from patient B008 identifies significant immune infiltration

Fig S5A. Perforin positive cells detected in CAVATAK-treated bladder cancer

FigS5B. ICAM-1 positive tumour from patient B004 shows productive CAVATAK infection

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Cohort	Canon Patient	Fold Increase in Cell Death Pathway genes compares to untreated bladder cancer									
		Caspase8	BCL2L1	Caspase9	BAX	BAK1	Caspase3	Caspase7	RIPK3	HMGB1	Calreticulin
A1 1x10^{8} CVA21	B001	20.1	24547.43	20217.04	6.9	23062.87	23959.07	65231.64	63009.61	11.6	7848.95
	B003	1.05	10	3198.73	0.81	1811.9	ND	ND	ND	2.21	ND
	B004	2.4	ND	ND	2.4	6453.13	ND	ND	ND	11.26	ND
A2 3x10^{8} CVA21	B005	2.38	7260.15	2462.29	0.85	1433.96	ND	ND	ND	0.83	4198.87
	B006	1.21	3630.08	ND	1.12	1765.41	ND	ND	ND	0.49	4707.63
	B007	1.38	2648.18	10	0.69	2938.34	ND	ND	ND	1.11	2027.92
A3 (3x10^{8})x2 CVA21	B010	1.45	ND	ND	0.72	1630.14	ND	ND	ND	0.69	ND
	B011	.187	ND	ND	1.24	ND	ND	ND	ND	ND	ND
B1 (1x10*)x2 CVA21+MitC	B012	1.56	ND	ND	0.63	1424.05	ND	ND	ND	0.59	ND
	B013	6.18	4531.54	3294.36	3.07	2979.35	ND	12466.63	25723.23	2.33	3470.15
B2 (3x10^{8}) x2 CVA21+MitC	B015	3.95	6375.33	5001.98	2.27	4294.53	12188.93	4523.69	ND	3.3	5036.77
	B016	137.75	43036.83	56590.97	22.49	180705.9	29191.98	106706.2	476885.8	31.04	3870.45

Table 1. CAVATAK induces the intrinsic apoptotic cell death pathway in bladder tumours
Total RNA was isolated from the CANON trial patient paraffin-embedded tumour tissues and from archival untreated non-muscle invasive bladder tumour blocks (n=6). The cDNAs reverse transcribed from the RNA were characterized using a custom-designed Cell Death PathwayFinder RT^{2} Profiler PCR Array (SABiosciences). The results are displayed as the fold increase in each gene compared to the average expression for that gene from n=6 untreated bladder cancers. ND= not determined.

Figure Legends

Fig 1. Tumour response: pre- and post-treatment cystoscopy -Cystoscopic photography was performed in all subjects to record the appearance of their tumour before and after intravesical CAVATAK treatment. Representative images pre- and post-CAVATAK treatment are shown for 2 patients (B001 and B008).

Fig 2. Intravesical CAVATAK selectively targets tumour cells within the bladder (A) Immunohistochemical images showing representative cases displaying ICAM-1 positivity, enterovirus protein (VP-1) and cleaved caspase 3 (positive staining shown in brown). The haematoxyin and eosin images of the same cases show the extent of tumour necrosis/apoptotic bodies outlined by the broken black line. Magnification x20. **(B)** Left image (Patient B006): Anti-enterovirus positivity by tumour cells compared to absence of anti-enterovirus staining on normal urothelium (indicated by broken line). Right image (Patient B004): Anti-enterovirus staining (brown) only present on tumour cells and not on stromal cells and lymphocytic infiltrate.

Fig 3. Increased levels of CAVATAK in patient urine indicates secondary viral replication within the bladder

Viral shedding was assessed in the urines of patients before and between days 2 and 5 after initial intravesical treatment with CAVATAK. The blue line represents the analysis of viral copy

number by RT-PCR, the red line depicts the levels of replication competent virus as determined by a $TCID_{50}$ assay. Indications of secondary viral replication were evident in patients 4,5,6,7,8,11 and 15.

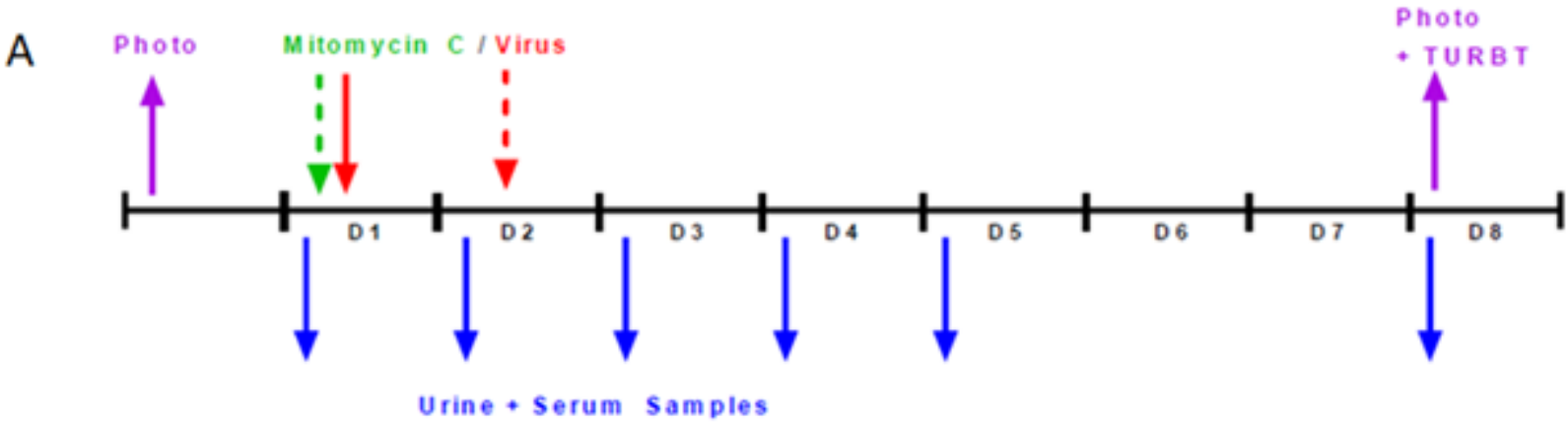
Fig 4A Urinary cytokine levels in patients following CAVATAK treatment

The Q-Plex™ Human Cytokine multiplexed ELISA array (Quansys Biosciences) was used to assess the level and kinetics of the CAVATAK-induced cytokine response in the urine of CAVATAK-treated patients on days 1, 3, 5 and 8 post viral treatment. Representative results are shown for 6 patients. **Fig 4 B and C - CAVATAK-specific induction of HMGB1 (B**) Urinary HMGB1 levels were measured in the trial patients on day 1 (before CAVATAK treatment) and then on days 3, 5 and 8/9 post-viral treatment using an HMGB1 ELISA kit (ST51011; IBL International GmbH). Urinary HMGB1 was expressed as HMGB1/Cr ratio (mg/μmolCr) to correct for differences in dilution. Six out of the 11 patients tested showed a marked increase in HMGB1 levels on day 3, 48hrs post-viral treatment. **(C**)Screening of HMGB1 expression (brown staining) in untreated non-muscle invasive bladder cancer (3 representative cases shown, control 1-3) and CAVATAK-treated bladder cancer (3 representative cases shown, B004, B003 and B007) by immunohistochemistry. The untreated bladder cancer cases showed nuclear localisation of HMGB1 consistent with that seen in the control tissue, normal human kidney. In contrast the CAVATAK-treated bladder cancers expressed HMGB1 within the cytoplasm. (Magnification x20, inset images x40)

Fig 5. CAVATAK treatment up-regulates interferon-induced genes and immune checkpoint molecules within the microenvironment of NMIBC tissue

Nanostring Pan-Cancer Immune profiling was performed on RNA derived from 12 CAVATAK-treated and 7 untreated NMIBCs. **(A)** CAVATAK treatment induced elevated expression of the viral RNA response genes *IFIT1*, *IFIH1*, *OAS3*, and *MX1*, and IFN-γ

induced chemokine genes encoding *CXCL9*, *CXCL10*, and *CXCL11* compared with levels seen in untreated NMIBC control tissues. Of the immune checkpoint inhibitory and immunosuppressive genes, PD-L1, PD-L2 and LAG3 were upregulated in the CAVATAK-treated tumours compared to untreated NMIBC controls. **(B)** CAVATAK treatment induced elevated expression of the RIG-I-like receptor dsRNA helicase enzyme that is encoded by the DDX58 gene. The levels of significance as determined by an unpaired t-test refer to the average expression of upregulated genes in the CAVATAK-treated patients compared to the average expression of untreated NMIBC control tissues are indicated: *=$p<0.05$; **=$p<0.01$; ***=$p<0.001$.



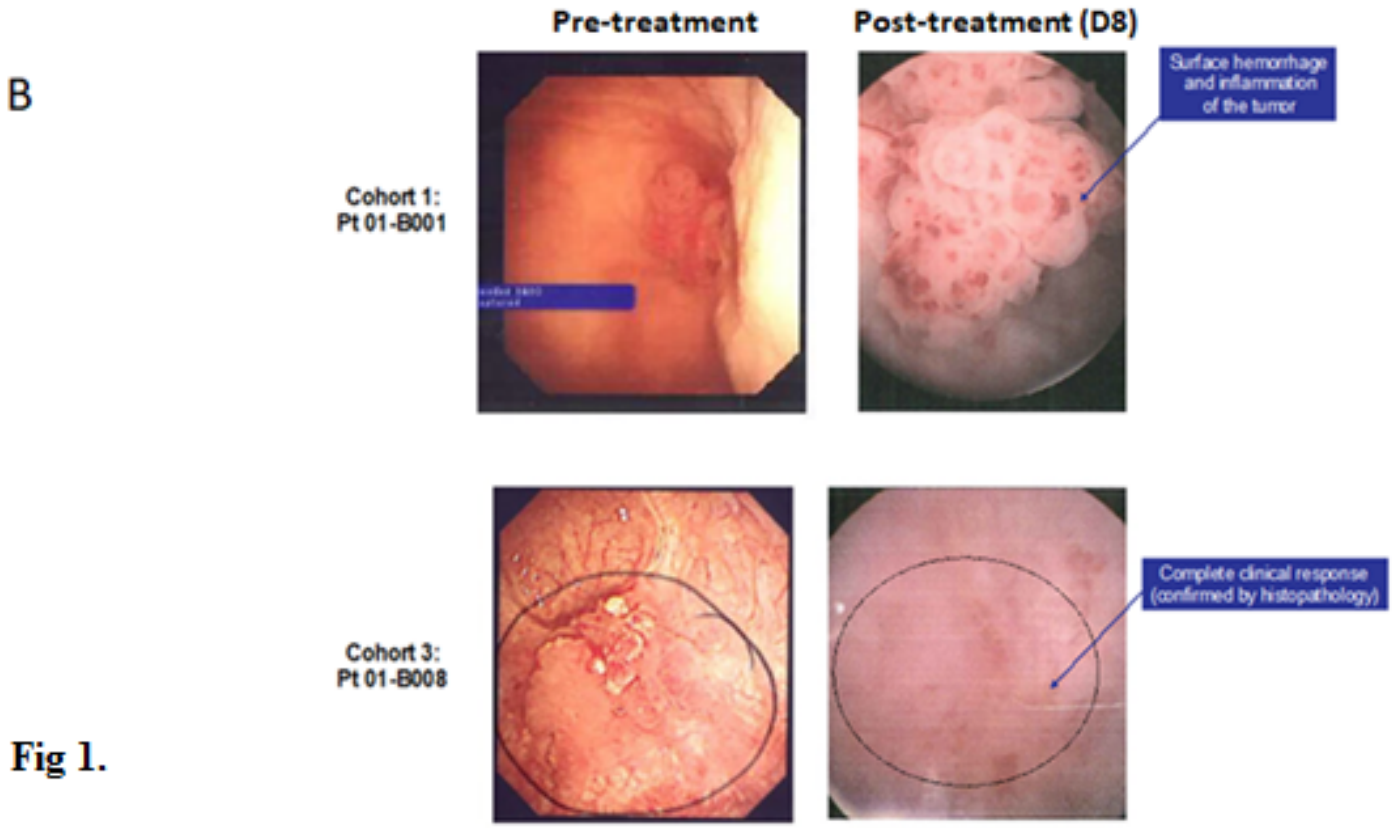
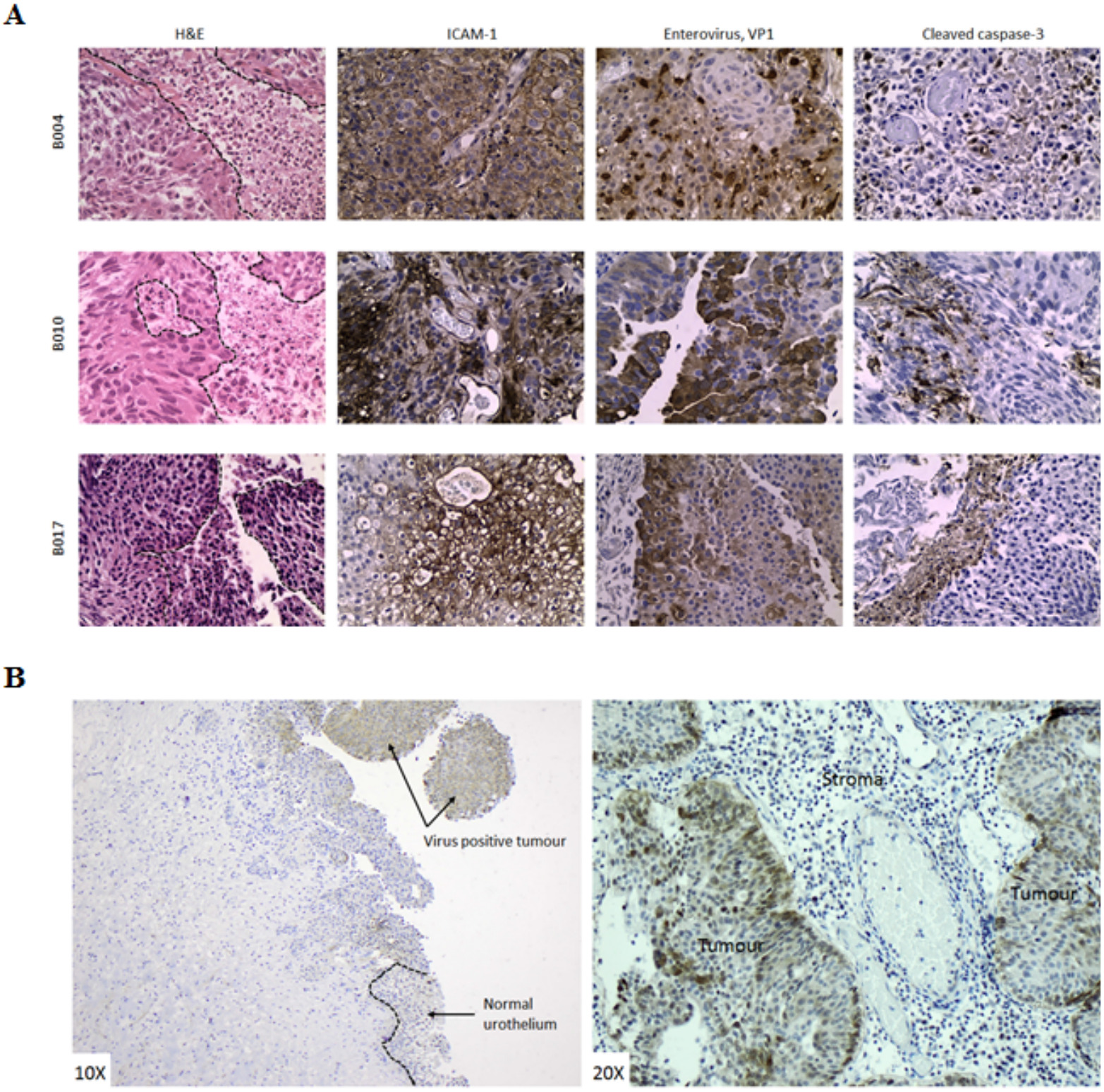


Fig 1.

Fig 2.



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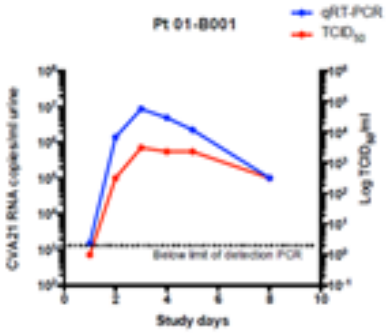
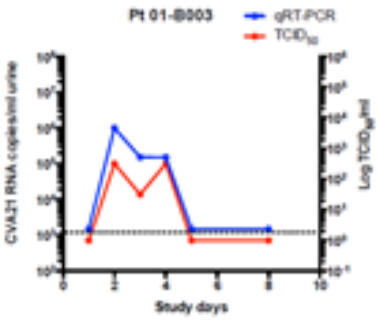
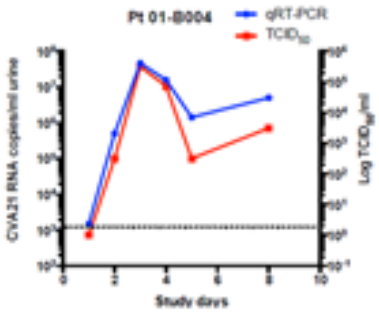
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Fig.3

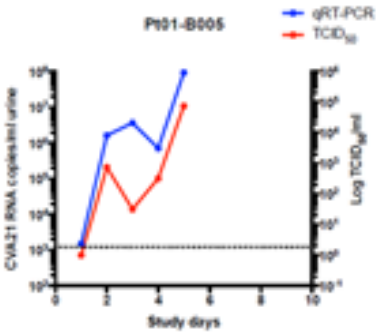
Cohort A1

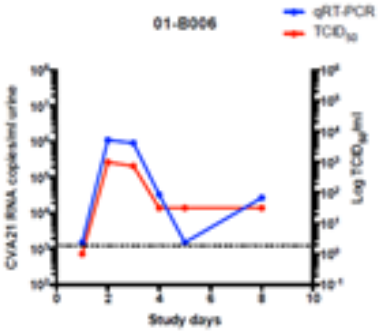


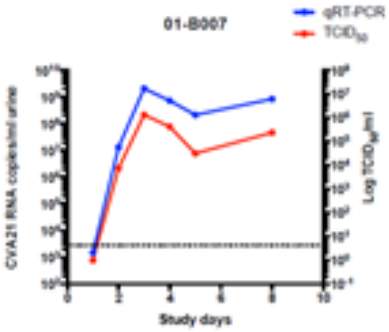




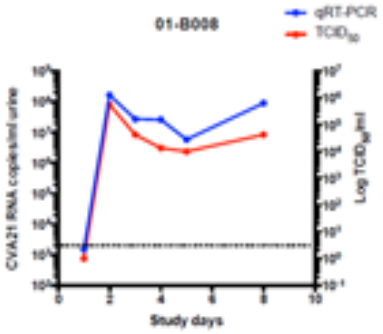
Cohort A2

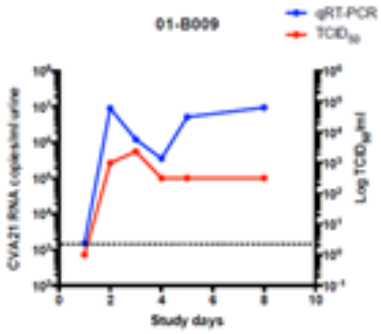


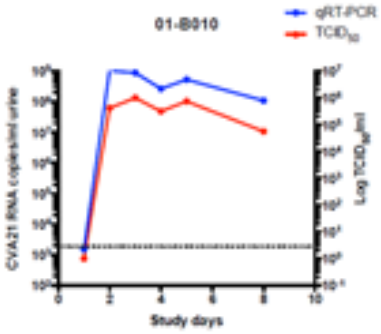




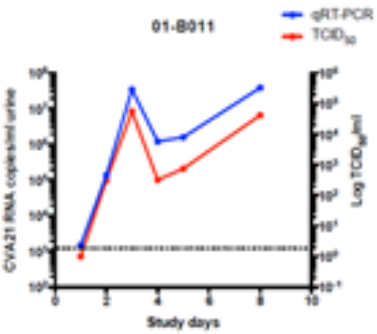
Cohort A3

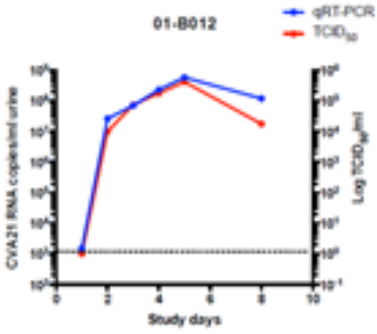


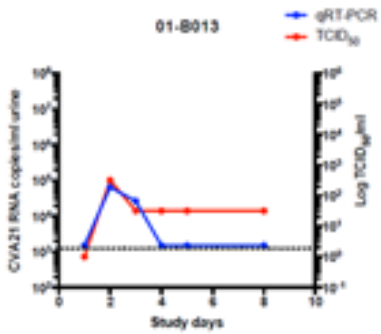




Cohort B1







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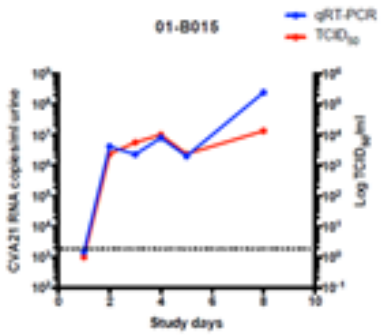
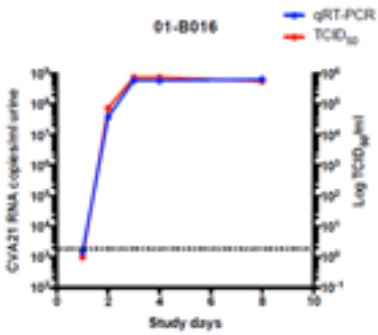




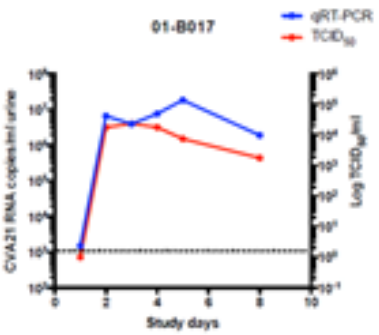
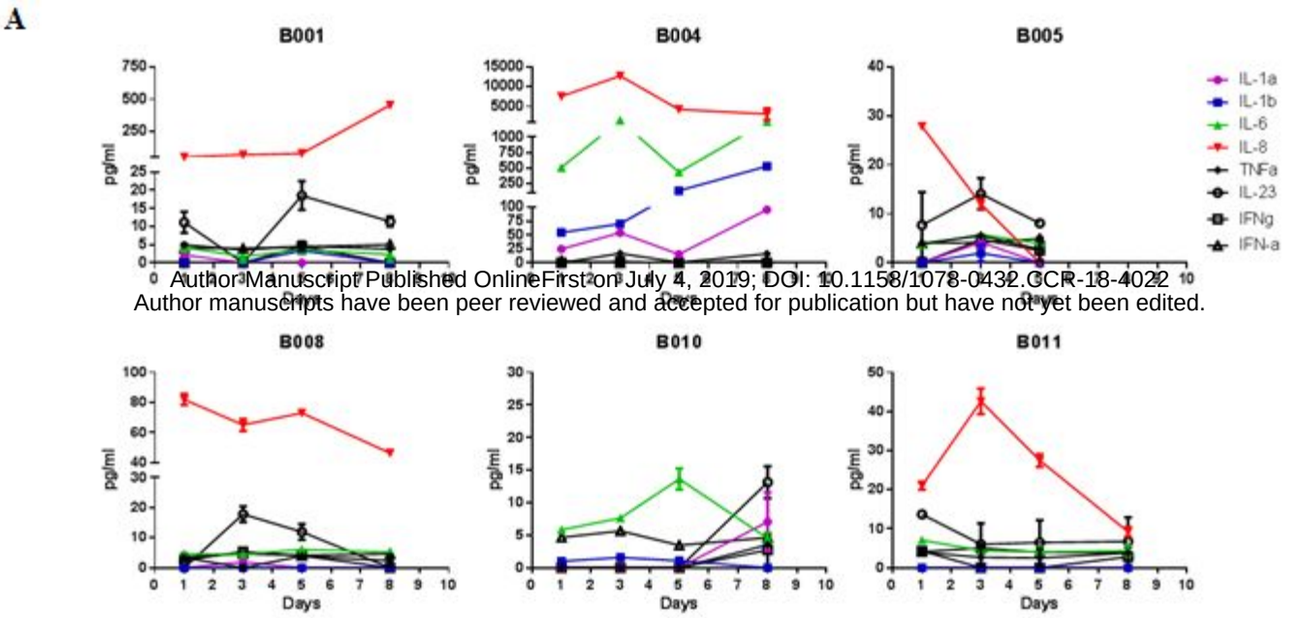
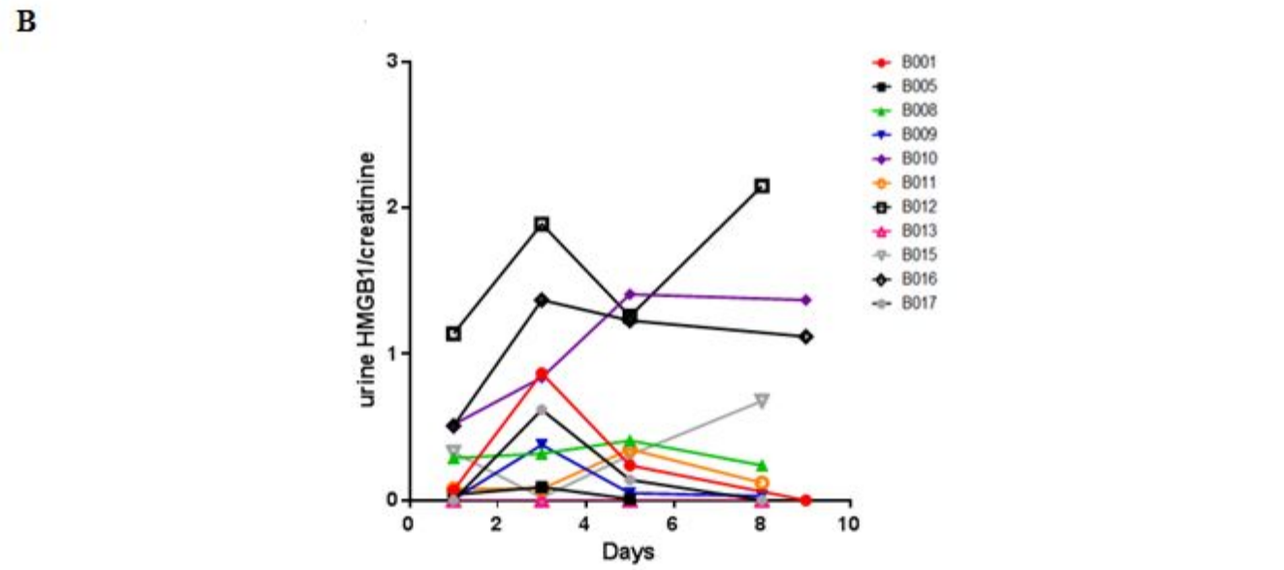


Fig. 4





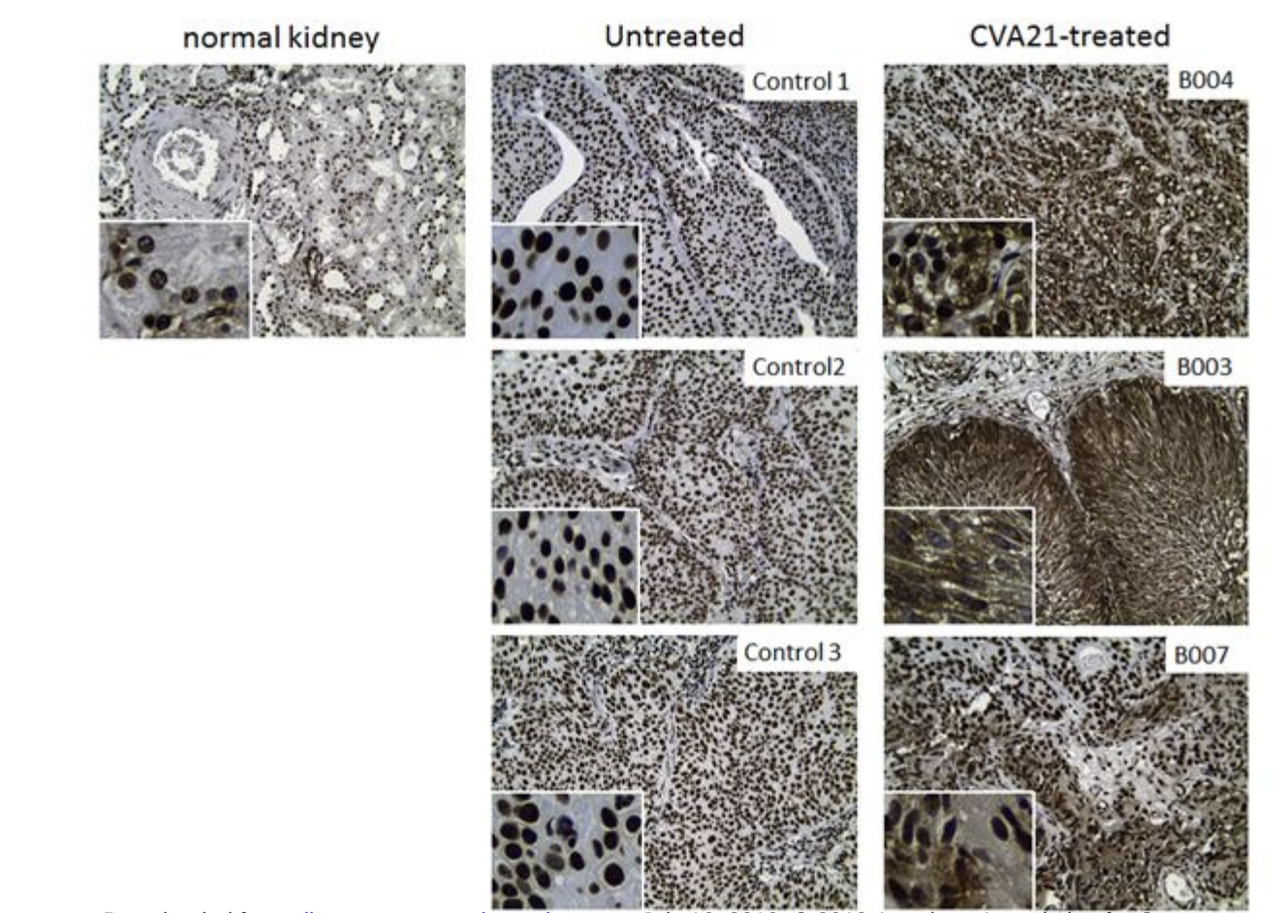
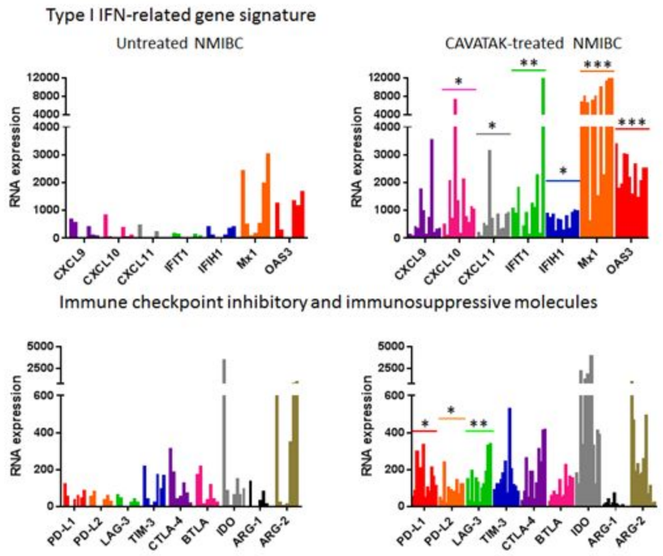
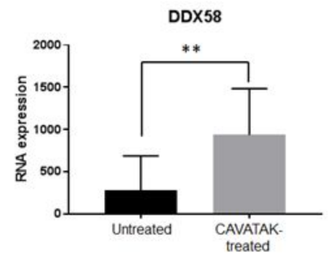


Fig. 5

A



B



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AACR American Association for Cancer Research

Clinical Cancer Research

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