

Neoadjuvant intratumoral influenza vaccine treatment in patients with proficient mismatch repair colorectal cancer leads to increased tumor infiltration of CD8+ T cells and upregulation of PD-L1: a phase 1/2 clinical trial

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ABSTRACT

Background In colorectal cancer, the effects of immune checkpoint inhibitors are mostly limited to patients with deficient mismatch repair tumors, characterized by a high grade infiltration of CD8+T cells. Interventions aimed at increasing intratumoral CD8+T-cell infiltration in proficient mismatch repair tumors are lacking.

Methods We conducted a proof of concept phase 1/2 clinical trial, where patients with non-metastasizing sigmoid or rectal cancer, scheduled for curative intended surgery, were treated with an endoscopic intratumorally administered neoadjuvant influenza vaccine. Blood and tumor samples were collected before the injection and at the time of surgery. The primary outcome was safety of the intervention. Evaluation of pathological tumor regression grade, immunohistochemistry, flow cytometry of blood, tissue bulk transcriptional analyses, and spatial protein profiling of tumor regions were all secondary outcomes.

Results A total of 10 patients were included in the trial. Median patient age was 70 years (range 54–78), with 30% women. All patients had proficient mismatch repair Union of International Cancer Control stage I–III tumors. No endoscopic safety events occurred, with all patients undergoing curative surgery as scheduled (median 9 days after intervention). Increased CD8+T-cell tumor infiltration was evident after vaccination (median 73 vs 315 cells/mm², $p<0.05$), along with significant downregulation of messenger RNA gene expression related to neutrophils and upregulation of transcripts encoding cytotoxic functions. Spatial protein analysis showed significant local upregulation of programmed death-ligand 1 (PD-L1) (adjusted p value <0.05) and downregulation of FOXP3 (adjusted p value <0.05).

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ An intervention to increase T-cell infiltration in proficient mismatch repair tumors (pMMR) is needed for this major group of patients to benefit from immunotherapy.

WHAT THIS STUDY ADDS

⇒ Intratumoral administration of the seasonal influenza vaccine before curative intended colorectal cancer surgery was found to be safe, and resulted in an increased intratumoral CD8+ infiltration, a shift in gene signatures in CD8+T cells versus neutrophils, and intratumorally increased programmed death-ligand 1 protein expression and decreased FOXP3 protein expression.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The influenza vaccine is unparalleled in terms of its safety profile and is used across all patient groups and ages. If the combination of the influenza vaccine and immune checkpoint inhibitor treatment can be proven successful in future studies, the large group of patients with pMMR tumors can benefit from immune checkpoint inhibitor treatment.

Conclusions Neoadjuvant intratumoral influenza vaccine treatment in this cohort was demonstrated to be safe and feasible, and to induce CD8+T-cell infiltration and upregulation of PD-L1 proficient mismatch repair sigmoid and rectal tumors. Definitive conclusions regarding safety and efficacy can only be made in larger cohorts.

INTRODUCTION

Colorectal cancer (CRC) is the third most diagnosed cancer and accounts for the second most deaths due to cancer.¹ The prognosis is excellent if it is diagnosed and treated while the disease is localized to the bowel wall and is worse if the tumor has regionally spread to lymph nodes. Unfortunately, many more patients tend to be diagnosed with CRC before turning 50 years of age and these patients may even have a worse prognosis.²

Treatment with immune checkpoints inhibitors (ICI) has led to long-term tumor regression in selected patients, and it has been demonstrated that increased intratumoral (IT) T-cell infiltration before ICI administration correlates with the probability of response to ICIs in several tumor types.^{3,4} In CRC, ICIs are highly effective in deficient mismatch repair (dMMR) tumors.^{5,6} Most proficient MMR (pMMR) tumors, have, unlike dMMR tumors, little-to-none IT T-cell infiltration.^{7,8} Interventions to induce T-cell infiltration in pMMR tumors are thus warranted.

In preclinical models, repurposing of infectious disease vaccines have produced encouraging results in terms of increasing IT immune infiltration.⁹ In a murine study, IT injection with an inactivated, non-adjuvanted seasonal influenza vaccine reduced tumor size and increased infiltration of antitumor CD8+T cells within the tumor, while application of a squalene-based adjuvanted influenza vaccine induced an increase in regulatory B cells that hindered the antitumor activity.¹⁰ We have previously performed a registry-based cohort study that indicated that patients, who underwent curative surgery for a solid tumor and received an inactivated trivalent influenza vaccine in the postoperative period, had a decreased overall mortality and cancer-related mortality.¹¹ In addition, systemic influenza vaccine administration 6–12 months before surgery for CRC was associated with a reduced risk of recurrence.¹² The preclinical and epidemiological data suggest that repurposing the influenza vaccine for cancer treatment could produce encouraging results.

In this proof of concept phase 1/2 study, we aimed to investigate if neoadjuvant IT influenza vaccine treatment is safe and feasible and to explore the potential tumor microenvironment (TME) changes following the treatment.

METHODS

Patients

The study was open to all patients adhering to the following inclusion criteria: above age of 18 years, non-metastatic clinically suspected or histologically verified sigmoid or rectal adenocarcinoma, and scheduled for curative-intended surgery. The tumor needed to be described as non-obstructive at the index endoscopy and

the patient cases were reviewed by a multidisciplinary team (MDT). Exclusion criteria included: intraluminal ulceration or bleeding before the intervention, ongoing immunosuppressive treatment, concurrent treatment with an investigational intervention, indication for neoadjuvant therapy, acute febrile illness, pregnancy, any previous allergic reactions to an influenza vaccine or its component, and influenza vaccine administration within 30 days of study inclusion. All study participants provided written informed consent. The study was registered at ClinicalTrials.gov.

Study design and treatment

This study was an investigator-initiated, multicenter, proof of concept, phase 1/2 clinical trial with the aim of investigating the safety and efficacy of neoadjuvant IT influenza vaccine treatment before intended curative surgery in patients with early stage sigmoid or rectal cancer. Inclusion was planned at two additional centers for a total number of 30 patients, but COVID-19 restrictions hindered this.

The study was conducted in two phases; the first phase was conducted as a pilot study including six patients. Patients were recruited from the Department of Surgery, Zealand University Hospital, after their cases were reviewed by the MDT from March to April 2021. The pilot study was conducted to ensure that no stop rules were violated.

Standard treatment involves intended curative surgery within 2 weeks after the diagnosis. Administration of the IT influenza vaccine was conducted within a few days after diagnosis and it was ensured that the experimental treatment did not lead to any significant delay in the intended curative surgery.

As the pilot study was completed without violation of any stopping rules or any serious adverse events (AEs) the second phase of the study was initiated.

The primary outcome was safety of the treatment with predefined specific stopping rules for the trial. Secondary outcomes included assessment of pathological tumor regression grade (TRG), evaluation of pathological TRG, immunohistochemistry (IHC), flow cytometry in blood, tissue bulk transcriptional analyses, spatial protein profiling of tumor regions, and any difference in the quality of recovery 15 (QoR-15) questionnaires¹³ before and after the intervention.

Patients were excluded from the study if they withdrew their consent, if the disease progressed such that the patient needed another treatment, or if the investigator deemed that withdrawal was in the patient's best interest.

Intervention

Every patient received one vial (0.5 mL) of the 2021 seasonal Influvac Tetra (Viartis, USA). Influvac Tetra is a non-adjuvanted quadrivalent (subunit) influenza vaccine with inactivated fragments from four influenza viruses. A non-adjuvanted influenza vaccine was chosen based on results from the preclinical study, demonstrating that

the presence of a squalene-based adjuvant hampered the antitumor immune response.¹⁰

Tumors in the colon can be fibrous and rigid, restricting the possibility of injecting liquids into those parts of the tumor and with a risk of spilling part of the injection fluid into the lumen. To ensure sufficient injection volume, the vaccine was mixed with 1.5 mL saline to a total volume of 2.0 mL before administration. This mixing procedure was applied for all patients.

Included patients were scheduled for an additional endoscopy to apply the intervention. For all patients, Endoscopes GIF-H190 Olympus Exera System (Olympus, Japan) was used. Board-certified gastroenterology surgeons performed all endoscopic procedures. Before the intervention, blood samples were drawn, and a QoR-15 questionnaire was filled. During the endoscopy, the tumor was visualized and up to eight biopsies were taken, formalin fixated, and paraffin embedded (FFPE) for later analysis. After the biopsy collection, the vaccine was applied in distinct quadrants of the tumor to ensure distribution to the complete tumor area. For all patients, the vaccine was injected with a 23G, 3 mm injection needle (Jiuhong Medical Instrument, China). Any spilling of injection fluid was noted during the procedure. The patients underwent standard surgical procedures for sigmoid and rectal cancer a minimum of 7 days after the vaccine treatment, as scheduled and outlined by the MDT. On the day of admission, before surgery, blood samples were drawn, and the QoR-15 questionnaire was repeated. The surgical specimen was evaluated at the department of pathology according to the tumor, node, metastases classification and sampled for further biomarker analyses.¹⁴

Primary outcome

AEs and stopping rules

The primary outcome was safety of the intervention. Stopping rules were defined as perforation at the tumor site during study treatment, anaphylactic shock, and unexpected, significant or unacceptable risks to patients. Other AEs and adverse reactions were recorded from day of treatment (Day 0) until the surgery. AEs were classified according to the Common Terminology Criteria for Adverse Events V.4.0.

Secondary outcomes

TRG

All slides from the surgical specimen were evaluated by two gastrointestinal pathologists regarding TRG according to the Mandard *et al* scoring system which includes five categories with TRG1 corresponding to complete regression and TRG5 corresponding to no regression.¹⁵

QoR-15 questionnaire

QoR-15 is a questionnaire with 15 items that covers five different domains of recovery. To answer each item, a numerical rating of 0–10 with a composite score of 0–150 is used. Higher scores indicate better recovery, with 0–89 being rated as ‘Poor’; 90–121 as ‘Moderate’; 122–135

as ‘Good’, and 136–150 as ‘Excellent’. The QoR-15 has been validated for use in Danish with a minimally clinical important difference of 8.0 points.^{16 17}

IHC and digital counting of tumor-infiltrating lymphocytes

Biopsies taken at the time of vaccination that displayed presence of invasive tumor were selected for the following IHC staining. From the surgical specimen one slide with presence of both the central part of the tumor and invasive margin was selected.

Sections with a thickness of 4 µm were cut from the FFPE tissue blocks. Staining procedure is described in online supplemental materials.

Slides were scanned using a NanoZoomer S60 slide scanner (Hamamatsu, Japan). Digital images were processed using Visiopharm Quantitative Digital Pathology software (Visiopharm, Denmark, V.2021.02) and previously developed application protocol packages were used to generate automated CD3+ and CD8+ lymphocyte counts separately for the central tumor and the invasive margin. The process has been described in detail in a previously published paper.¹⁸ We only compared tumor regions of baseline samples with central tumor regions of post vaccination samples within each individual patient.

Flow cytometry

Peripheral EDTA-anticoagulated blood samples were used to determine lymphocyte subpopulations (T, B and natural killer (NK) cells, the T cells further subdivided in CD4+ and CD8+ T cells) using the single platform method with BD Multitest 6-color TBNK kit (Becton Dickinson, California, USA) in BD TruCount tubes on FACSCanto II flow cytometers (Becton Dickinson, Belgium). Gating followed manufacturer’s instructions and data analysis was performed using BD FACSDiva software V.8.0.1.

NanoString expression panels

Tissue samples from the same tissue block were used for the IHC analysis. RNA isolation and panel preparation is described in online supplemental materials. We used the used the nCounter IO360 panel of 750 endogenous human transcripts; for T-cell receptor (TCR) expression analysis—the nCounter TCR diversity panel of 119 TCR variable and constant regions and lymphocyte transcripts (NanoString, USA).

Gene expression analysis

Raw data generated with the nCounter platform was preprocessed using an iterative quality control (QC) and normalization framework as described in Bhattacharya *et al*¹⁹ and summarized in online supplemental materials. No samples were removed after QC and normalization. Generation of principal component analysis (PCA) plots, heatmap and volcano plots are described in online supplemental materials along with description of the enrichment analysis. Differentially expressed (DE) genes between groups were identified using the Wald significance test and adjusting for multiple testing with the Benjamini-Hochberg approach.²⁰ Genes were considered

DE if they met threshold requirements of adjusted p value < 0.05 and log2 fold change (logFC) ≥ 0.5 . DE analysis was performed using a function from DESeq2 package (V.4.2.1) 'DESeq' with unwanted variation vectors ($n=5$) included in the design formula.

TCR expression analysis is described in online supplemental materials.

GeoMx digital spatial profiling

Slide preparation and sample collection

For slide preparation, FFPE tissue sections of 4 μ m in thickness and 2mm in diameter were mounted on histology slides by grouping baseline samples on one slide, and post-vaccination samples on two slides. Six to eight regions of interest (ROI) on the before and after vaccination tumor samples were chosen; one ROI from normal, highly immune-infiltrated, and low-infiltrated tumor areas from samples before vaccination; and one ROI from normal, highly immune-infiltrated tumor, low-infiltrated tumor, invasive margin with high immune cell infiltration, and low-infiltrated invasive margin areas from after vaccination samples. A specific focus in drawing ROIs in tumor and invasive margin sections was to target regions with immune infiltration guided by anti-CD45 and anti-CD8 as morphological markers. This strategy aimed to ensure that changes in protein expression were related to the intervention and not to a bias in ROI selection. A dedicated gastrointestinal pathologist drew the ROIs. This person was not blinded to the sampling time points.

The GeoMx digital spatial profiler (DSP) slide preparation user manual (MAN-10100-03) was followed to prepare slides for data collection. For visualization of the composition of the TME, we used the following morphology markers: anti-CD45 (1:40, immune cells), anti-CD8 (1:100, T cells), anti-Pan-CK (1:40, epithelial cells), and anti-SYTO13 (1:10, DNA). All of the applied antibodies were conjugated monoclonal antibodies unless otherwise stated. A previous description of this method has been provided in Merritt *et al.*²¹ We used five different panels (immune cell profiling-panel, pan-tumor-panel, cell death-panel, immune activation status-panel, immune cell typing-panel), that cover 52 antibodies and six internal reference controls.

Spatial expression data analysis

After data collection, RCC files were loaded into the GeoMx DSP analysis suite (V.2.4.2.2) where QC and scaling of data was performed (see online supplemental materials). No ROIs failed QC criteria. Following this, data was scaled to the geometric mean of the number of nuclei, and then exported to R for further analyses. The same iterative QC and normalization framework was used as for the NanoString panels.¹⁹ We based our choice on previously published applications of this framework on GeoMx data^{22–24} and assumption that unwanted variation estimation step will correct for ROI-related variation. After iterative QC and normalization, we removed $n=1$ vectors of unwanted variation from the data set. DE analyses were

performed in R using the same packages and parameters as for NanoString expression panel analysis.

Descriptive statistics

Statistical analyses were performed using R. Summary statistics were generated based on baseline patient characteristics. To evaluate if numerical variables in all gene expression panels, GeoMx spatial profiling data, IHC data and flow data displayed normal distributions, we generated distribution histograms or QQ plots. Wald significance test was used to compute DE genes and proteins between the respective pairwise comparisons. For all other comparisons (IHC and flow data), depending on distribution, Wilcoxon rank-sum test or t-test were applied. All box plots were presented as the median and IQRs. In PCA, model group differences were assessed by permutational multivariate analysis of variance using distance matrices (PERMANOVA). In this study, p values and adjusted p values below 0.05 were considered significant unless stated otherwise. The CI for AEs was calculated as described previously.²⁵

RESULTS

Patient characteristics

Ten patients were treated with the neoadjuvant IT influenza vaccine between March 10, 2021, and August 25, 2021, (see flow chart of patient inclusion in online supplemental figure S1). The study design is shown in figure 1A. All patients had pMMR tumors that were located in the sigmoid colon ($n=4$), mid rectum ($n=2$), or upper rectum ($n=4$). Based on pretreatment radiological assessment, patients had cT1–cT3 tumors with no distant metastases. Baseline QoR15 was 144 (range 112–150). Baseline characteristics are shown in table 1.

Patient inclusion was halted before the planned inclusion of 30 patients due to no inclusion of patients at two out of three centers as a result of COVID-19 restrictions and the expiration of the seasonal influenza vaccine (expiry date September 02, 2021) used in this trial.

Study intervention and safety

No procedural AEs were recorded for any patient. Endoscopic visualization of the quadrants (yellow arrows) of the tumor and injection of influenza vaccine are depicted in figure 1B. Endoscopic procedure time from start to end of endoscopy, including biopsy collection, and final injection of treatment was 23min (range 10–40min). All patients had a successful injection of the influenza vaccine suspension, but for three patients, a small, non-quantifiable, volume of the injection fluid was spilled into the lumen due to fibrous tumors.

The curative intended surgery was scheduled 9 days (range 7–13 days) from the time of treatment. In this period, one grade 1 AE (a mild fever that subsided without intervention) (10% of patients (95% CI 0 to 30). was recorded for a patient that was resolved before the scheduled surgery. The remaining patients tolerated

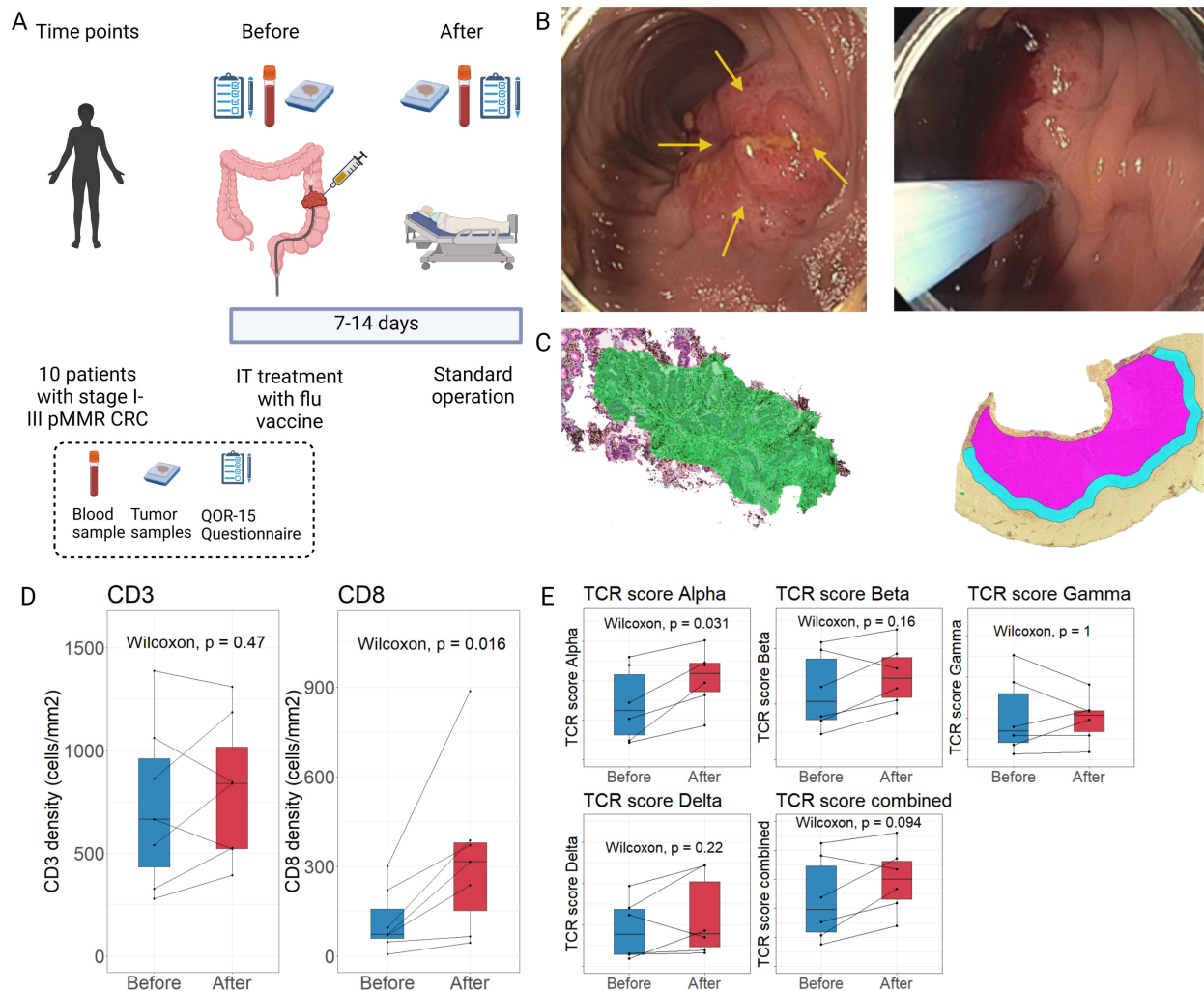


Figure 1 Neoadjuvant intratumoral influenza vaccine treatment increases CD8+T-cell infiltration and TCR alpha chain diversity. (A) Overview of the study design and sample time points. At each time point, blood and tumor samples were taken and a QoR-15 questionnaire was filled. (B) Representative pictures showing the quadrant visualization (yellow arrows, left picture) and intratumoral (IT) injection of the influenza vaccine (right picture). (C) Representative tumor tissue slides from immunohistochemistry (IHC) staining with anti-CD3/cytokeratin for digital analysis of T-cell infiltration. Tumor slides with IHC staining of anti-CD8/cytokeratin are not shown. Left picture shows a representative sample from before vaccination. Right picture shows a sample after the vaccination with the central tumor (pink) and invasive margins (light blue). (D) Comparison of the IHC staining density of CD3+ and CD8+ T cells before (green area) and after vaccination (pink area) samples ($n=7$). (E) Comparison of alpha, beta, gamma, and delta variable chains, and combined variable chain score between time points ($n=6$). (D, E) CD3+ and CD8+ T-cell densities and normalized expression of TCR variable chain expression depicted as boxplot showing median, upper and lower quartiles. Whiskers extend into a max of 1.5 times the IQR. CRC, colorectal cancer; pMMR, proficient mismatch repair; QoR-15, quality of recovery 15 questionnaire; TCR, T-cell receptor.

the treatment well, with all surgeries performed without delay. There was no significant change in QoR-15 between the day of treatment and the day of surgery (difference between groups 2.0 (95% CI -11.16 to 11.38)). All endoscopic procedural data is presented in table 2. All but one patient was deemed as Mandard TRG 5 by both pathologists, with one deemed TRG 4 by one pathologist.

Neoadjuvant IT influenza vaccine treatment increases CD8+ T-cell infiltration in pMMR tumors

In seven patients, invasive tumor tissue was present in biopsies taken before vaccination, while the biopsies from three patients only contained adenoma tissue. These three patients were therefore excluded from all tumor

analyses. In figure 1C, the digital assessment of CD3+ and CD8+ T cells is depicted. When comparing before (green area, left picture in figure 1C) and after vaccination (pink area, right picture in figure 1C) tumor tissue samples, data showed no significant changes in overall density of CD3+T cells (paired Wilcoxon test, $p=0.47$) (figure 1D), but we found the density of the proportion of CD8+T cells to be significantly increased within the invasive tumor region after vaccination (paired Wilcoxon test, $p=0.016$) (figure 1D). It did not affect the results if patients previously had received a systemic influenza vaccine or if any spilling of the injection fluid occurred during the intervention (data not shown).

Table 1 Baseline characteristics of enrolled patients

Number of patients	10
Sex (%)	Female: 3 (30) Male: 7 (70)
Age (median (min–max))	70 (54–78)
BMI (median (min–max))	24.0 (19.6–33.1)
ASA score (%)	1: 1 (10) 2: 6 (60) 3: 3 (30)
WHO performance score (%)	0: 7 (70) 1: 3 (30)
Clinical T stage	1: 1 (10%) 2: 5 (50%) 3: 4 (40%)
Clinical N stage	0: 9 (90%) 1: 1 (10%)
MMR status	pMMR: 10 (100%)
QoR15 (median (min–max))	144 (112–150)
QoR15 group	Moderate: 1 (10%) Good: 1 (10%) Excellent: 8 (80%)

ASA, American Society of Anesthesiologists; BMI, body mass index; MMR, mismatch repair; QoR15, quality of recovery 15 questionnaire.

A statistically significant diversity was seen in the TCR score for alpha variable chains between the time points (paired Wilcoxon $p=0.031$, [figure 1E](#)). In contrast, no significant differences were seen for the remaining three variable chain types (paired Wilcoxon $p>0.05$, [figure 1E](#)). Combining all variable chains in one TCR score showed an increase that was not statistically significant between time points (paired Wilcoxon, $p=0.094$).

Genes encoding cytotoxic responses are generally enhanced within the TME following IT influenza vaccine treatment

We next investigated the expression of genes in tumor tissue excised before and after the IT influenza vaccine

Table 2 Procedural data

Minutes from start of procedure to intratumoral influenza vaccine treatment (median (min–max))	11 (5–31)
Minutes from intratumoral influenza vaccine treatment to end of procedure (median (min–max))	9 (3–22)
Minutes from start to end of procedure (median (range))	23 (10–40)
Spilling of injection fluid	No: 7 (70%) Yes: 3 (30%)
Days to surgery (median (range))	9 (7–13)

treatment. This was performed using the nCounter IO360 panel, comprised of 750 TME biology associated transcripts on tissue slides from before and after the vaccination. Two-dimensional data representation using PCA showed a clear difference in tumor gene expression before and after treatment ([figure 2A](#), paired PERMANOVA: $F=3.66$, $p=0.003$). A total of 72 DE genes ($\log_{2}FC>0.5$, adjusted p value <0.05) were identified before and after vaccination, with 27 genes being significantly upregulated and 45 genes significantly downregulated after treatment ([figure 2B](#); distribution, correlation, p value distribution, volcano plot, and individual gene expressions plots are available in online supplemental figures S2–S5). A significantly increased expression of several cytotoxicity associated genes, such as *GZMA*, *CD8A*, *KLRB1*, and *KLRK1*, as well as the transcript encoding the co-stimulatory molecule CD27 (*CD27*) was observed. A significantly decreased expression of the genes encoding the interleukins (*IL1B*, *IL6*, and *IL24*, the chemokines *CXCL1*, *CXCL2*, and *CXCL8* (*IL8*) and the chemokine receptor *CXCR2* was evident. The latter are all relevant for neutrophil-based immune responses. Moreover, we identified concomitantly decreased expression of transcripts encoding innate immunity associated proteins such as *CXCL5*, *CXCL6*, and *TLR1*, and importantly of transforming growth factor (*TGF*) β 1, encoding the anti-inflammatory protein TGF- β 1 that is normally highly upregulated in the TME, and is one of the most critical regulators of a non-effective anticancer immunity.²⁶ The genes encoding cyclooxygenase 2 (*PTGS2*) and matrix metalloproteinase 1, enzymes secreted from, for example, tumor associated macrophages that increase angiogenesis through matrix degradation and endothelial cell invasion were also downregulated.²⁷ In the preclinical study that investigated IT influenza vaccine, a significant change in IT regulatory B cells was evident in the design that used a squalene-based adjuvanted influenza vaccine.¹⁰ We did not find that any of the significantly expressed genes were related to regulatory B-cell function. An enrichment analysis was performed to test for cell type specific gene signature enrichment and over-representation of functional pathways between the time points that showed a disruption of neutrophil-associated pathways being affected (online supplemental file 2). Guided by the results of initial enrichment analysis, we calculated a functional enrichment score, where normalized expression of cell type and functional pathway specific genes identified or associated with the enrichment analysis as well as additional NanoString validated pathways (NS)²⁸ were summarized and compared ([figure 2C,D](#)). We identified that neutrophil-associated pathways were downregulated after vaccination, with significant downregulation of the Gene Ontology-based neutrophil mediated immunity and NS-neutrophils pathways ([figure 2C](#), paired t-test, $p=0.0069$, and $p=0.023$, respectively). We found enrichment of T-cell associated pathways after vaccination ([figure 2D](#)) with significant upregulation of the NS-Th1 cell pathway and the NS-tumor-infiltrating lymphocyte

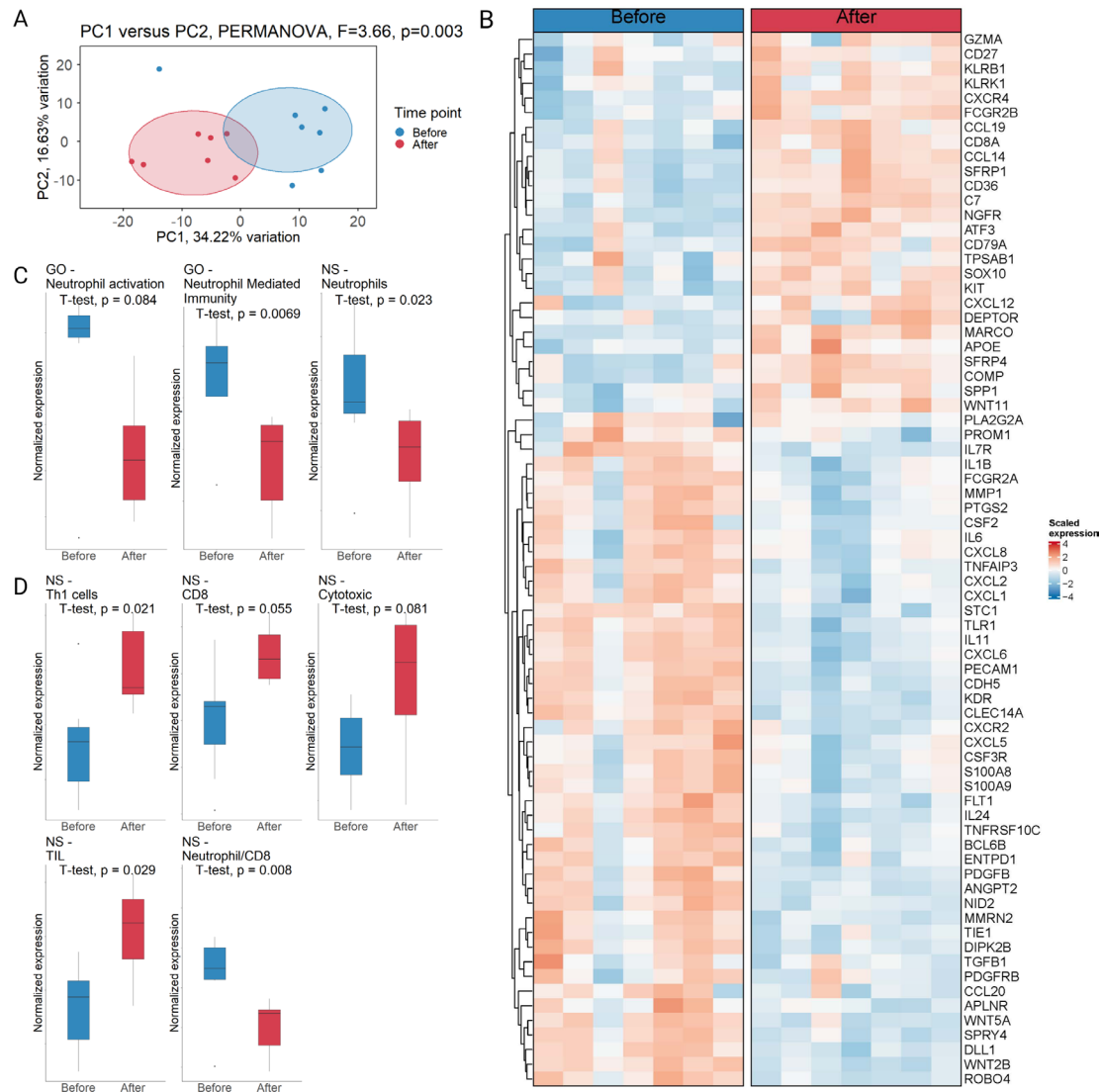


Figure 2 Changes in the tumor microenvironment after intratumoral influenza vaccine treatment. (A) Principal component analysis based on the top 400 most variable genes from nCounter IO360 panel, which includes 750 genes typically associated with tumor microenvironment biology. The statistical significance was tested using a PERMANOVA on the centroid differences between time points ($n=7$). (B) Heatmap of paired differentially expressed genes compared between time points ($n=7$, same order of patients before and after). (C, D) Significant pathways identified using functional enrichment score analysis. Here, the normalized expression of all represented genes in a pathway are analyzed via a paired t-test comparing before and after vaccination tumor samples. Normalized expression depicted as boxplot showing median, upper and lower quartiles ($n=7$) Whiskers extend into a max of 1.5 times the IQR. EMT, epithelial to mesenchymal transition; GO, Gene Ontology; NS, NanoString; PERMANOVA, permutational multivariate analysis of variance using distance matrices; TIL, tumor-infiltrating lymphocytes.

(TIL) pathway (paired t-test, $p=0.021$, and $p=0.029$, respectively). Importantly, we found a significant shift in the neutrophil/CD8+T-cell ratio, suggesting a shift in the immune phenotype after vaccination (paired t-test, $p=0.008$).

Spatial analysis of protein expression in immune-infiltrated tumor regions reveals increased local programmed death-ligand 1 and decreased FOXP3 protein expression following IT influenza vaccine treatment

To investigate the effect of IT influenza vaccine treatment on immune-infiltrated regions of tumor samples, we performed spatial protein expression analysis using

the NS GeoMx platform. Representative ROIs with selection of immune-infiltrated tumor areas before and after vaccination were identified within the same patient by a gastrointestinal pathologist (figure 3A,B). A comparison of adjacent normal tissue in the samples before and after vaccination revealed no significant changes in protein expression besides the proliferation marker Ki-67 (MKI67) (online supplemental figure S6). When comparing protein expression in immune-infiltrated tumor regions before and after IT influenza vaccine treatment, we found the cellular markers programmed death-ligand 1 (PD-L1), CD3G (T cells), Human Leukocyte

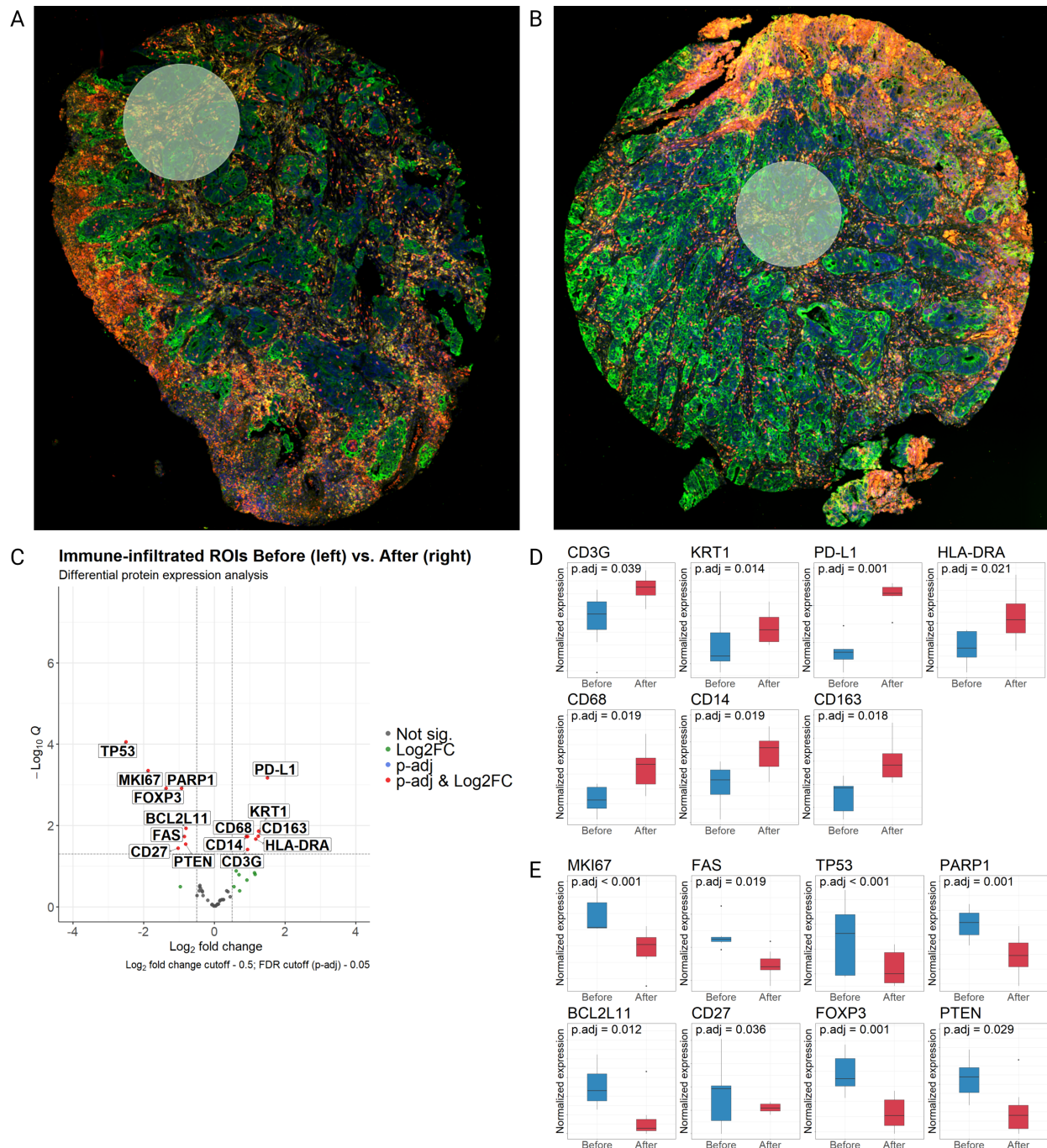


Figure 3 Spatial protein analysis within immune-infiltrated regions of tumors before and after intratumoral influenza vaccine treatment. (A, B) Picture of region of interest (ROI) selection in a patient before (A) and after (B) IT influenza vaccination. ROIs were drawn by a gastrointestinal pathologist and based on infiltration of CD45+ (yellow) and CD8+ (red) cells in areas of Pan-CK (green) and DNA (blue) positive regions. (C) Volcano plot of differentially expressed proteins in ROIs of immune-infiltrated regions of tumors before versus after vaccination (n=7). (D, E) Box plots of differentially expressed proteins upregulated (D), and downregulated (E) after vaccination (n=7). Differential expression of proteins depicted as boxplots showing median, upper and lower quartiles. Whiskers extend into a max of 1.5 times the IQR. FDR: False Discovery Rate; HLA-DRA, Human Leukocyte Antigen DR alpha chain; IT, intratumoral; KRT1, keratin 1; logFC, log₂ fold change; MKI67, marker Ki-67; PD-L1, programmed death-ligand 1.

Antigen DR alpha chain (HLA-DRA, antigen presenting cells), and keratin 1 to be significantly upregulated (logFC>0.5, adjusted p value<0.05) after treatment, along with the monocyte and macrophage markers CD14, CD68, and CD163 (figure 3C,D). We found FOXP3, the

transcriptional regulator of regulatory T cells (Tregs), a cell type, that is, typically abundant in TME, to be downregulated on treatment (figure 3E). The DNA-repair proteins and tumor suppressors TP53, PTEN, and PARP1 were likewise significantly downregulated, along with

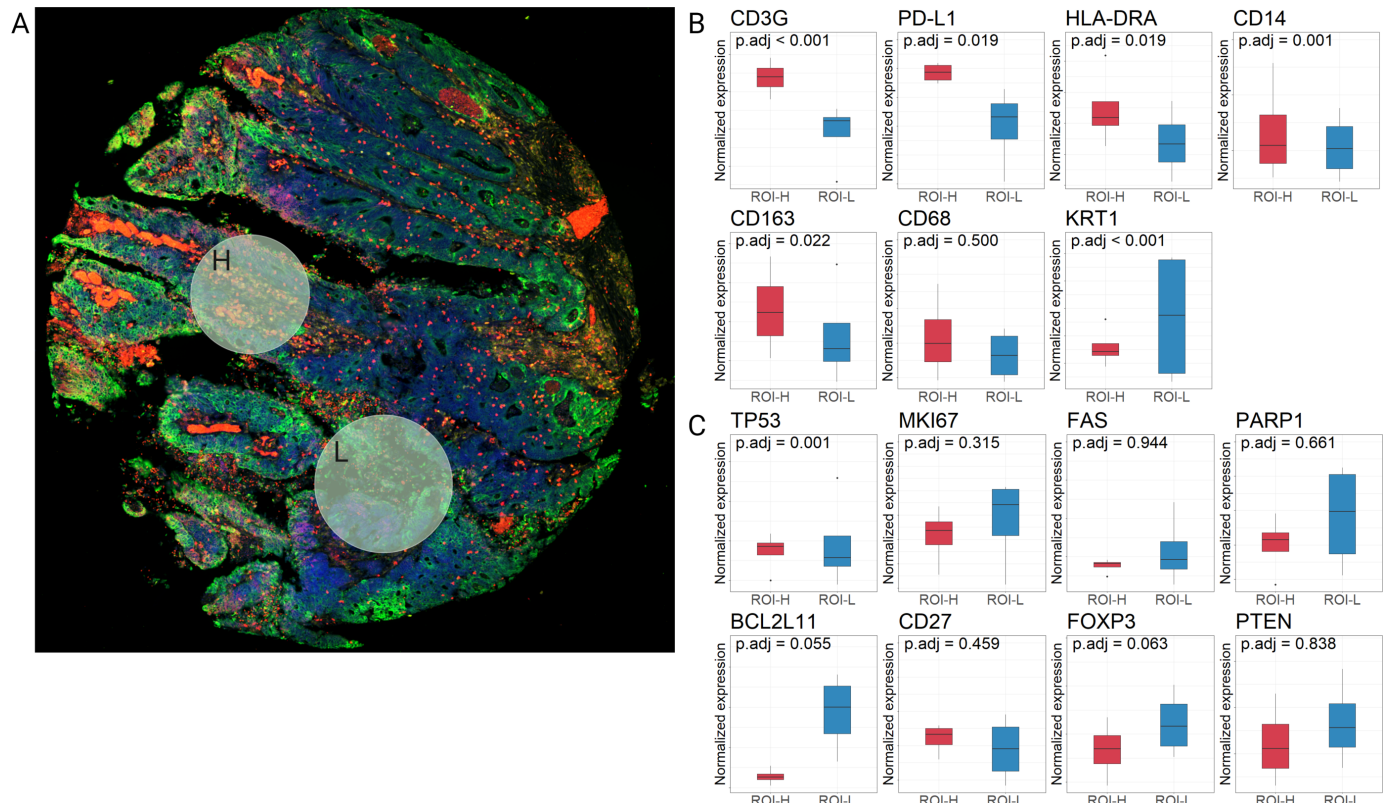


Figure 4 Spatial analysis of high immune-infiltrated versus low immune-infiltrated regions of tumors after vaccination. (A) Pictures of region of interest (ROI) selection in a patient after vaccination. Upper ROI designates a tumor area with high immune-infiltration (ROI-H) while the lower ROI designates a tumor area with low immune-infiltration (ROI-L). (B) Box plots of differentially expressed (DE) proteins upregulated on vaccination (from figure 3 (n=4)). (C) Box plots of the downregulated DE proteins on vaccination (from figure 3 (n=4)). Differential expression of proteins depicted as boxplots showing median, upper and lower quartiles. Whiskers extend into a max of 1.5 times the IQR. HLA-DRA, Human Leukocyte Antigen DR alpha chain; KRT1, keratin 1; MKI67, marker Ki-67; PD-L1, programmed death-ligand 1.

the proliferation MKI67, and the apoptosis-regulating proteins FAS (CD95) and BCL2L11. The co-stimulatory molecule CD27 was found to be downregulated at the protein level within the immune-infiltrated tumor regions (figure 3E), while being upregulated at protein level across the general tumor tissue that may also contain normal tissue (figure 2B). No significant difference in the B-cell marker CD20 was found.

Spatial analysis of protein expression in high immune-infiltrated versus low immune-infiltrated regions of tumors after vaccination

When comparing paired samples of high immune-infiltrated and low immune-infiltrated tumor areas from the same patients after vaccination (representative ROIs in figure 4A), we identified that PD-L1, CD3G and HLA-DRA protein expression were significantly upregulated ($\log_{2}FC > 0.05$, adjusted p value < 0.05) in the high immune-infiltrated ROIs (ROI-H) compared with the low immune-infiltrated ROIs (ROI-L), suggesting that PD-L1 protein expression in the tumor cells is linked to presence of immune cells (figure 4B). There was no difference in CD68 expression, while CD163 and CD14 upregulation were seen in ROI-H only. FOXP3 expression did not differ significantly between ROI-H and

ROI-L (figure 4C). Only TP53 remained downregulated in high immune-infiltrated tumor ROIs compared with low immune-infiltrated tumor ROIs.

No changes in the circulating levels of CD8+ T cells and C-reactive protein but enhanced systemic B-cell levels on IT influenza vaccination

Despite the change in tumor-infiltrating CD8+T cells in the invasive region of tumors on IT influenza vaccination (figure 1D), we identified no increases in systemic levels of general CD3+T cells nor in CD8+T cells in circulating blood (figure 5A,B). This lack of difference was also the case for general leukocytes, lymphocytes, neutrophils, thrombocytes, CD4+T cells, the ratio between CD8+ and CD4+ cells, CD56+cells (NK cells), and for the acute phase protein C-reactive protein. However, we noticed a significant increase in circulating CD19+cells (B cells) on IT influenza vaccination ($p=0.024$).

DISCUSSION

In the present study, we show that neoadjuvant IT influenza vaccine treatment is a safe intervention in patients with pMMR early stage CRC, causing no delay of surgery, and that it increases the CD8+T-cell infiltration of the

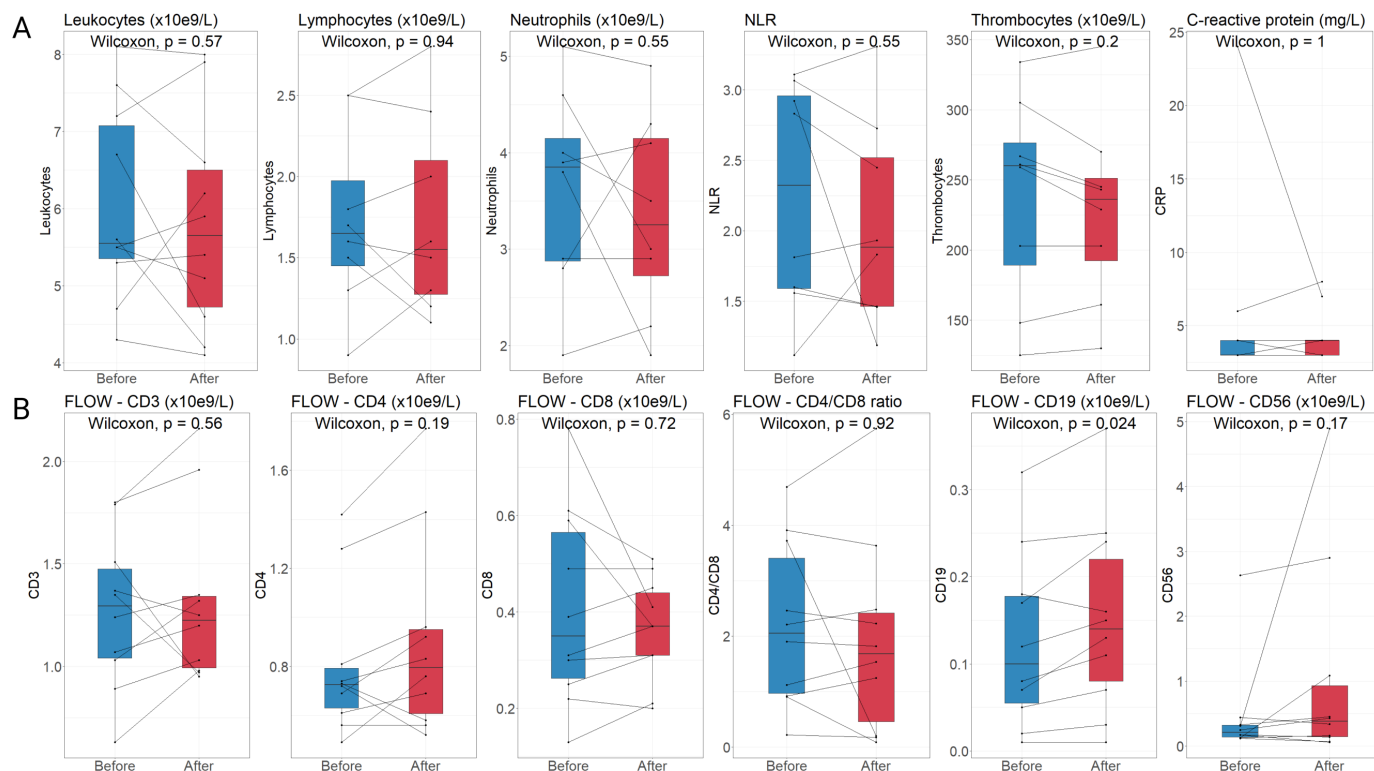


Figure 5 Circulating levels of immune cells and C-reactive protein after vaccination. (A) Overview of general immune cell populations (leukocytes (n=10), lymphocytes (n=8), neutrophils (n=8), neutrophils/lymphocytes ratio (n=8), and thrombocytes (n=8)), and the concentration of C-reactive protein (CRP, n=9). (B) Overview of flow cytometry analyses to determine subpopulations (CD3+T cells, CD4+T cells, CD8+T cells, B cells (CD19+), and natural killer cells (CD56+), all n=10). Concentration of immune cells and CRP depicted as boxplots showing median, upper and lower quartiles. Whiskers extend into a max of 1.5 times the IQR.

tumor when investigated within a median of 9 days after the intervention. A downregulation of FOXP3, which is mainly expressed by Tregs in the TME, and of innate immune pathways involving neutrophils was evident along with a local upregulation of PD-L1 protein expression after vaccination.

Neoadjuvant immunotherapy is a mainstay in several cancer types, with recent studies showing 100% pathological response in dMMR CRC.^{5,6} However, response rates in pMMR CRC are limited.⁵ A major difference between the pMMR and dMMR phenotypes is the level of immune cell infiltration of especially CD8+T cells, with pMMR tumors commonly reported with a low level of infiltration. Several studies have shown that pretreatment levels of CD8+T-cell infiltration are central for a robust response to ICI treatment.^{29,30} However, interventions that lead to increased infiltration of CD8+T cells are lacking.

Our results show that repurposing the seasonal influenza vaccine increases the density of CD8+T cells and PD-L1 protein expression in pMMR tumors, which may be translated to a possible benefit of ICI treatment that targets either PD-L1 on tumor cells or its ligand programmed cell death protein-1 on T cells to downregulate their immunosuppressive effects and improve the tumor-killing effect of CD8+T cells. Importantly, this analysis was done in comparison of only tumor regions before and after vaccination, without including the invasive

margin. The increase in the proportion of CD8+T cells in tumor areas is in line with preclinical data on IT influenza vaccine treatment.¹⁰ The TCR repertoire diversity score for alpha chain variants also increased within the tumor suggesting that the infiltrating CD8+T cells are more diverse and may have been presented for neoantigens. However, we cannot determine based on the current investigations if the neoantigens stem from the vaccine or the cancer cells, but due to upregulation of PD-L1 on post-vaccination tumor cells there is an indication that at least some of the CD8+T cells are able to interact with the tumor.³¹ Regardless of their specificity, the increased density of CD8+T cells indicate that the intervention has induced a change to a more 'hot' phenotype of the TME.

Along with the influx of CD8+T cells, we see a distinct change in messenger RNA expression in the TME with a change in key cytotoxic genes. The functional enrichment analysis revealed a significant upregulation of the NS validated TH1 cell and TIL pathways.²⁸ Several innate immunity genes were significantly downregulated, along with a significant downregulation of several neutrophil-associated pathways. Indeed, a significant increase in the CD8+T cell to neutrophil ratio suggests a shift towards a more antitumor immune phenotype.

Finally, we were interested in treatment-induced changes in specific regions of the tumors before and after vaccination. The above-mentioned results were found

using common strategies where bulk tissues are investigated, including both normal stroma, invasive margin, and tumor regions. In order to investigate isolated changes within immune-infiltrated tumor regions we applied spatial protein expression analysis and found a striking significant difference in upregulation of PD-L1 and downregulation of FOXP3 after vaccination. The upregulation of PD-L1 is crucial as it is a biomarker of ICI efficacy demonstrated in several studies.^{29,32} Indeed, the preclinical data from mice suggested a synergistic effect of combining IT influenza vaccine treatment with ICI.¹⁰ Together with the increased CD8+T-cell infiltration, the upregulation of PD-L1 suggests that the pMMR tumors have been primed by the IT influenza vaccine treatment to respond to ICI treatment. Baseline levels of FOXP3, a marker of the immunosuppressive Treg type, have also been shown to influence the efficacy of ICI treatment, with lower levels of FOXP3 seen in responding patients.³³ This further adds to the notion that IT influenza vaccine treatment may prime the pMMR tumors for ICI treatment. The spatial protein analysis showed an increased expression of the macrophage markers CD14, CD68 and CD163 in immune-infiltrated tumor areas after IT influenza vaccine treatment. It has earlier been suggested based on in vitro studies that CD163 may represent an M2-like macrophage marker, but newer data using in situ IHC indicates that CD163 may not be a specific marker for M2-like macrophages since the numbers of CD163+macrophages were found to be higher in TME of cases with a cytotoxic/Th1 signature.³⁴ These macrophage markers were increased along with HLA-DRA for presentation of antigenic peptides for CD4+T cells. Among these three macrophage markers, we identified that CD14 and CD163 were selectively enriched within high immune-infiltrated versus low immune-infiltrated tumor regions on vaccination, thus pointing to selective macrophage markers being expressed by macrophages within immune-infiltrating regions. The spatial GeoMx method does not allow for identification of double-positive or triple-positive cells that may otherwise have resulted in a more specific identification of, for example, M1-like polarization over M2-like's within the tumor tissue. Moreover, a concomitant downregulation of three DNA repair proteins and tumor suppressors TP53, PTEN, and PARP1 along with the proliferation MKI67 and the pro-apoptotic proteins FAS and BCL2L11 was observed. This suggests an interesting switch in the tumor cells with less proliferation along with less apoptosis and DNA repair. Finally, we noted a significant downregulation of CD27 at protein level in tumor-infiltrated regions, but not at transcript level within the general tumor tissue, which may also contain normal tissue areas. CD27 is a protein involved at different time points in the differentiation of T cells.³⁵ The expression is downregulated during differentiation and upregulated in memory T cells, while a persistent upregulation can be seen in FOXP3+Tregs. A reduced CD27 protein expression may thus align with our findings of reduced FOXP3 levels in immune-infiltrated

tumor areas. Altogether, the non-proliferative, less apoptotic tumor phenotype combined with increased cytotoxic potential, decreased FOXP3 levels, and increased PD-L1 in immune-infiltrated tumor regions point to induction of an antitumor signature by IT influenza vaccine treatment. Finally, in the preclinical IT influenza vaccine study, a significant role of regulatory B cells were evident in abrogating the antitumor response if the influenza vaccine included a squalene-based adjuvant when compared with the non-adjuvanted version.¹⁰ In our data, we saw no significant changes on the transcript or protein level of regulatory B cells, which was expected as we used a non-adjuvanted influenza vaccine in the study.

A limitation of our study is the inclusion of a low number of patients which was due to expiration of the used influenza vaccine and restricted patient inclusion during COVID-19. The study coordinators deemed that using another influenza vaccine in the same study would convolute the results, and patient inclusion was therefore halted. Further, the 9 days between treatment and surgery may hamper appearance of activated tumor-specific T cells in the tumor tissue, as well as in the circulation, as the expansion of activated clones, and their appearance in the tumor may require at least 14–21 days due to the division rates of T cells on activation (ca. one cell division per day). The short period was due to a requirement from the ethics committee to adhere to the Danish cancer care regulations dictating that surgery needs to be performed within 14 days after diagnosis. The short period could also explain the limited pathological response. As the potential safety and effects of the IT influenza vaccine treatment have been illuminated in the present study, future studies should extend the period from IT influenza vaccine treatment to surgery to allow for increased time for activation and proliferation of tumor-specific CD8+T cells, and consider to investigate the intervention in a randomized setting.

Compared with the recent discovery of neoadjuvant chemoradiation as a means to induce a cytotoxic TME,³⁶ the influenza vaccine is unparalleled in terms of a favorable safety profile, and its wide usage in patients with cancer across age and frailty.³⁷ Furthermore, the influence of previous influenza vaccination was minimal across our analyses, encouraging its prospects. As indicated in the preclinical data and further substantiated by the present study, future studies should combine IT influenza vaccine treatment with ICI in a study design that allows a prolonged time from intervention to surgery. The prolonged time would ensure sufficient recruitment and differentiation of T cells and the potential for a pathological response that reduces the tumor size.

Based on our present study, we can conclude that neoadjuvant IT influenza vaccine treatment is a safe intervention that induces an increased infiltration of CD8+T cells in the pMMR TME, a shift in gene signatures related to CD8+T cells versus neutrophils, with downregulated FOXP3 levels, and enhanced PD-L1 protein expression that may prime pMMR tumors to be susceptible to

ICI treatment. Further studies should investigate a more extended period from the IT influenza vaccine to surgery and combine it with ICI treatment.

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1. Title page

Intratumoral Influenza Vaccine for Early Colorectal Cancer**Sponsor and primary investigator**

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2. Introduction

The remarkable results achieved in the past years with cancer immunotherapies and checkpoint inhibitors have revolutionized the field of oncology. However, recent results also show that it is only a subgroup of all patients benefitting from these treatments¹. Tumor-infiltrating T-cells (TILs) have been highlighted as a paramount factor in the effectiveness of immunotherapies. Patients with tumors with a high amount of TILs, termed “hot” tumors, benefit the most from immunotherapies while patients with a low amount of TILs, termed “cold” tumors, benefit the least²⁻⁴.

The T-cells ability to infiltrate and be effective in the tumor microenvironment is regulated and effected by numerous cells and cytokines⁵. Thus, the research into modulating the tumor microenvironment has started so more patients can be treated with effective immunotherapies. A recent discovery has been the usage of intratumoral interventions as a way to directly affect the tumor and possibly sparing the patients of side effects of systemic treatment^{6,7}. An approach have been to use off-the-shelf vaccinations and administer them intratumorally where rotavirus and influenza virus vaccinations have been the most prominent^{6,7}. The effects of either vaccine was shown to be substantial when administered intratumorally but not intramuscularly. In vivo models of mice with injected tumors in both flanks where only one flank was treated also showed a systemic response in the form of regression of both the treated and non-treated flank⁷.

Both author groups focused on TILs in subsequent analyses of the tumors and both found that T-cell infiltration rose but interestingly also found that tumor cells upregulated PD-1 and CTLA-4 expression. A combination of rotavirus vaccination and anti-CTLA-4 therapy was conducted and showed a more prominent response, thus proving that a combination of the treatments complement each other⁷.

Studies investigating the effects of systemic influenza vaccine in patients with colorectal cancer have shown an increase in unspecific NK cell activity⁸. The authors investigating intratumoral influenza vaccine also analyzed the tumors with Nanostring, a multiplex mRNA gene assay providing information on known

inflammatory and immune pathways, where they could show an increase in IFN-gamma, the key stimulator of NK cells⁶.

Aim of the study

The aim of this explorative phase II clinical trial is to establish the safety and efficacy of intratumoral influenza vaccine in patients with colorectal cancer, as an additive treatment prior to intended curative surgery.

Previous experience at Zealand University Hospital

Professor Ismail Gögenur is the founder of Center for Surgical Science and Zealand Surgical Forum. He is one of the leading colorectal surgeons in Denmark. His primary investigational field covers optimization of colorectal cancer treatment and prognosis for the patients. Department of Surgery, Zealand University Department of endoscopy has all the necessary resources to perform this study. Only senior surgeons with high expertise in endoscopy will perform the procedures.

3. Methods

Objectives

Primary:

To investigate if intratumoral influenza vaccine is a safe treatment modality for tumor down staging prior to intended curative surgery in patients undergoing treatment for colorectal cancer.

Secondary:

To investigate if intratumoral influenza vaccine will induce immunologic invasion of the primary tumor

Tertiary:

To investigate if the treatment will induce a systemic immunologic response.

Quality of recovery

To assess quality of recovery for patients recruited into this trial. A quality of recovery questionnaire (QoR-15) will be given to patients pre- and post-treatment.

Study design

This is an explorative phase 2 clinical trial which will be conducted in two phases. The aim of this study is to establish the safety and efficacy of treating patients with early colorectal cancer with intratumoral influenza vaccine as a down staging and immune response enhancing treatment prior to intended curative surgery.

The first part of the study will be conducted as a pilot study. Six patients with histologically verified or clinically suspicious sigmoid colon cancer who are planned to undergo curative surgery will be included. Patients will be recruited from the Department of Surgery, Zealand University Hospital, Department of Surgery, Slagelse Sygehus and Department of Surgery, Sydvestjysk Sygehus after their case has been reviewed by the multidisciplinary team (MDT). Standard treatment involves intended curative surgery within two weeks after the diagnosis. The treatment will be performed within a few days and it will be ensured that the experimental treatment will not lead to a significant delay of intended curative surgery.

If the pilot study finishes without violating any stop rules and without any serious adverse events the second part of the study will be initiated. This will be conducted as a phase 2 study where 24 patients with histologically verified or clinically suspicious sigmoid colon cancer and rectal cancer will be included. Patients will be recruited from the Department of Surgery, Zealand University Hospital, Department of Surgery, Slagelse Sygehus and Department of Surgery, Sydvestjysk Sygehus after their case has been reviewed by the multidisciplinary team (MDT). Standard treatment involves intended curative surgery within two weeks after the diagnosis. The treatment will be performed within a few days and it will be ensured that the experimental treatment will not lead to a significant delay of intended curative surgery.

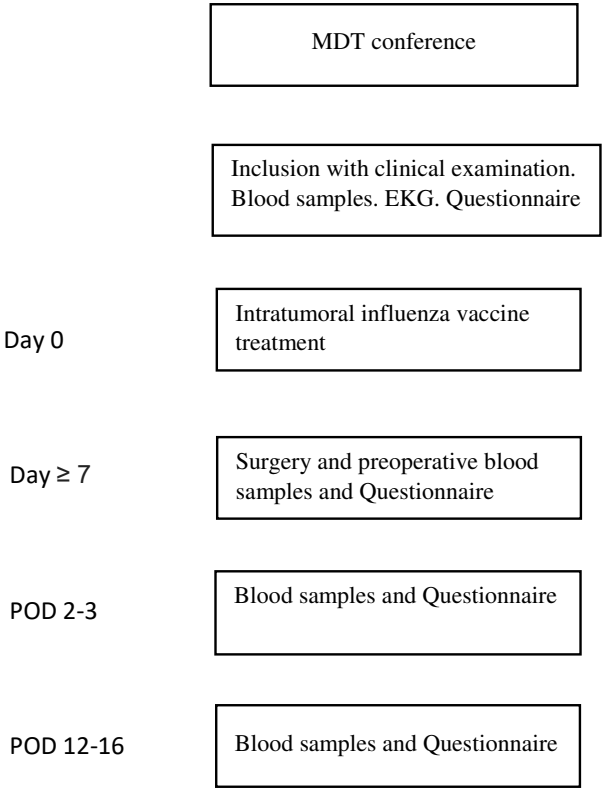
In relation to both parts of the study following samples will be collected:

Blood samples will be collected prior to the treatment with intratumoral influenza vaccine. Furthermore, blood samples will be collected on admission prior to elective surgery, within 3 days after surgery, and when the patients return to the outpatient clinic (approximately 14 days after surgery). The systemic response to intratumoral influenza vaccine will be evaluated through a multiplex gene assay, multiplex cytokine analysis, flow cytometry and NK cell activity analysis. Furthermore, ct/cfDNA will be analyzed and cell adhesion assays will be performed.

In relation to the intervention, eight intraluminal biopsies (8 x 0.5-1.5 cm) from the tumor will be collected and stored in a biobank for later analysis. The samples will be collected just before influenza vaccine is injected. In order to ensure uniformity, all tumor biopsy analyses will be performed when samples have been obtained from all participants. Tumor tissue will be registered and stored in "Dansk Cancer Biobank" according to Danish law. The Biobank will be approved by the Danish Data Protection Agency and subjected to "Persondataloven". An additional approval will be required for future research with remaining tumor

tissue. Further, after the elective surgery, three samples of 0.5 cm³ will be collected from the tumor and adjacent normal tissue and stored for later analysis. Additionally, if pathological examination reveals positive lymph nodes (eg. lymph nodes with live cancer cells) three formalin fixated specimens of the lymph nodes will be stored in the biobank. In order to ensure uniformity, all tumor biopsy analyses will be performed when samples have been obtained from all participants. Any tumor tissue left from the analyses will be stored in “Dansk Cancer Biobank” for future research according to Danish law. The Biobank will be approved by the Danish Data Protection Agency. Tissue collected before the experimental treatment will be characterized and compared with tumor tissue obtained from the final surgical specimen. Additionally, histopathologic characterization will be performed according to current standards (pTNM staging and tumor regression grade). Specific immunohistochemical staining for PD-1/PD-L1 and conventional prognostic marker analysis will be performed on biopsies and the final surgical specimen. Tumor infiltration of T-cells and subtypes will be characterized according to the immunoscore classification system. Quality of Recovery (QOR15) will be evaluated at baseline, prior to surgery on admission to the Department of Surgery, post-operative day 2-3, and at follow-up day 12-16.

Study design:



Dosage

A single vial containing 0.5 ml of Influvactetra® influenza vaccine will be suspended in 2 ml of NaCl in one vial. The treatment will be administered in four quadrants where 0.5 ml will be deposited in each location.

Surgical procedure

Standard surgical procedure for rectal cancer and sigmoid colon cancer, respectively. Surgical treatment outlined by MDT.

Study endpoints**Safety endpoints**

Safety evaluation will be performed via continuous assessment of safety parameters by reviewing events as they arise. We will conduct the pilot study in order to measure potential safety issues in a small cohort of patients before including patients in the phase 2 study.

The investigation will be put on hold if unacceptable safety issues are detected.

Primary safety endpoints:

1. Evaluation of serious adverse events
2. Evaluation of any adverse events reported.

Efficacy endpoints

The efficacy of the treatment will be evaluated according to the pathological examination of the surgical specimen. As such, final tumor staging, tumor regression grade (Mandard classification) T-cell subtype infiltration of the primary tumor and PD1/PDL-1 status before respective to after treatment are considered endpoints parameters. Furthermore, systemic immune responses will be evaluated through a multiplex gene assay, flow cytometry and NK cell activity analysis. A detailed evaluation of patient reported quality of recovery will be performed through repetitive administration of standardized questionnaires before the treatment with influenza vaccine (baseline), before surgery, postoperative day 2-3, and at follow-up (postoperative day 12-16).

4. Statistical considerations

Statistical considerations

The aim of this explorative study is to establish the safety and efficacy of treating patients with primary, rectal cancer and sigmoid colon cancer with intratumoral influenza vaccine prior to intended curative surgery.

Sample Size

This study will be the first to investigate intratumoral influenza vaccine in patients with colorectal cancer. Therefore, it is not possible to conduct formal sample size calculations. It was agreed at the coordinating investigator's pre sub-mission meeting that six patients were an appropriate initial sample size for the pilot study.

For the phase 2 study it was agreed that 24 patients were an appropriate initial sample size for this explorative study corresponding to 24 patients with either sigmoid colon cancer or rectal cancer.

Endpoints

1. **Safety endpoint:** Safety endpoints will be assessed continuously for serious adverse events as they may arise. Review of the adverse event reports for events that may have occurred during the investigation will serve as overall assessment of safety endpoints. Conclusions will be drawn through full analysis of all reports rather than through statistical analysis.

2. **Efficacy endpoint:**

The Department of Pathology at Roskilde Hospital will objectively assess the surgical specimen after resection in accordance to standard guidelines (Mandard classification).

Furthermore, our aim is to investigate changes in immunological invasion in tumor tissue before and after intratumoral influenza vaccine. Finally, we intend to investigate the systemic response to intratumoral influenza vaccine.

Reporting of data and statistical analysis plan

After all participants have undergone treatment, the coordinating investigator will perform the statistical analyses. The analyses will begin with an exploration of the data to check for anomalies that might require data queries to be raised. Data will be presented as mean (95% confidence interval), median (interquartile range [IQR]), or number (%) as appropriate. The level of significance will be set at $p < 0.05$. Data will be analyzed via parametric or non-parametric statistic depending on their distribution. Missing values, selection or exclusion of observations and variables and handling of missing values in the statistical analysis will be described carefully. Missing values will be filled in with last value available (Last Observation Carried

Forward – LOCF). A principal analysis will be performed according to the intention-to-treat principle (ITT). A per-protocol analysis will also be performed. For statistical analysis of data derived from the multiplex gene assay normalization and gene expression index calculation of probe intensities will be done, using the robust multi-array average (rma) method. Only perfect match probes will be used for data analysis. Regularized t-test will be used to calculate differences in gene expression between samples taken at different time sets. The Benjamini Hochberg method using the false discovery rate (FDR) will be used to correct for multiple hypothesis testing.

The results of this trial will be published in international or national peer-review journals regardless of negative, positive or inconclusive research results. In addition, the trial will be registered at www.clinicaltrials.gov and will be updated according to the study progress. In the event that part of the analysis is changed from the statistical analysis plan, these changes will be described and justified. Additional analyses that are not specified in the statistical analysis plan will be described at this stage and will be labeled as 'post-hoc' and the reason for their inclusion will be provided.

5. Participants

The participants in this study are individuals over 18 years with histologically verified or clinically suspicious rectal cancer and sigmoid colon cancer, with no indication for neoadjuvant chemoradiotherapy prior to intended curative surgery. The study involves recruitment of 30 patients in total, 6 in the pilot phase of the study and 24 patients with either sigmoid colon cancer or rectal cancer in the second phase of the study.

Estimated time needed to recruit participants: 24 months

Patients will be recruited from Zealand University Hospital in the Region of Zealand, Denmark, Slagelse Sygehus in the region of Zealand, and Sydvestjysk Sygehus in the Region of Southern Denmark

Inclusion criteria

- Patients must be mentally capable of understanding the information given.
- Patients must give written informed consent.
- Clinically suspected or histologically verified malignant tumor of the rectum or sigmoid colon.
- Tumor described as passable at index endoscopy.
- Men or women aged at least 18 years.
- Case reviewed by MDT (surgery, radiology, oncology). Case considered curable with standard surgical resection.

Exclusion criteria

- Highly inflamed gastrointestinal tissue which is ulcerated and bleeding
- Ongoing immunosuppressive treatment.
- Concurrent treatment with an investigational medicinal product.
- Patients with any other clinical condition or prior therapy that, in the opinion of the investigator, would make the patient unsuitable for the study or unable to comply with the study recruitments.
- Advanced tumor stages, clinical UICC stage IV.
- Indication for neoadjuvant chemoradiation or chemotherapy prior to surgery
- Acute surgical resection.
- Pregnancy
- Any previous allergic reaction to influenza vaccine or constituents, egg and chicken proteins, neomycin, formaldehyde or octoxinol-9
- Acute febrile illness
- Acute infectious disease
- Influenza vaccine administered within 30 days before study inclusion

Discontinuation criteria

Trial subjects are withdrawn from the study if:

- The patient withdraws his or her consent
- The disease progresses so that the patient is in need of another treatment
- Investigator deems that withdrawal is in the best interest of the patient

6. Risks and side effects**Registration of adverse events**

Adverse events / reactions are recorded from day of treatment (Day 0) until the surgery, as it will be difficult to differ between adverse events/reactions to the experimental treatment or surgery. All adverse events / reactions should be described in medical terminology in the patient's file and recorded in case report forms (CRF). The following information must be recorded: start date/date when observed, severity, any initiated treatment, assessment of the AE if it meets the criteria for SAE, end date, and relationship to study drug. For AEs that meet the criteria for SAE, the outcome must be recorded.

The adverse reactions are classified according to CTCAE version 4.0 (Common Terminology Criteria for Adverse Events)

Reporting adverse events

Unexpected and serious suspected adverse reactions that are fatal or life-threatening shall be reported to the Medical Products Agency and Science Ethics Committee as soon as possible, and no later than 7 days after the sponsor has knowledge of such suspected side effect. Within 8 days after reporting, the sponsor must notify the National Board of Health and Ethics Committee with all relevant information on the sponsor and the investigator's follow-up report.

All other unexpected serious suspected adverse reactions should be reported to the same authorities within 15 days after the sponsor has been informed of this. Any report must be accompanied by comments on any consequences for the trial. After completion of the trial, all adverse reactions will be reported in the final report to the Board of Health and Ethics Committee.

Sponsor must report all events related to the medical device to the manufacturer.

Definitions

- **Adverse Event, AE:** Any adverse events in a patient that occur or worsen during the trial and does not necessarily have a causal relationship to study treatments.
- **Adverse Reaction, AR:** All noxious and unintended reactions to a trial drug at any dose. The term “reaction to a trial drug” means that a possible relation between the study drug and the adverse reaction cannot be excluded.
- **Unexpected Adverse Reaction, UAR:** An adverse reaction with a nature or severity that is not in accordance with the current product information.
- **Serious Adverse Event, SAE or Serious Adverse Reaction, SAR:** an event or side effect that at any dose:
 - Results in death.
 - Is life threatening.
 - Leads to hospitalization or prolongation of hospital stay.
 - Results in persistent or significant disability or incapacity.
 - Leads to a congenital anomaly or birth defect.
 - Is a major medical event.
- **Suspected Unexpected Serious Adverse Reactions, SUSARs:** Unexpected and serious suspected adverse reactions that are not described in the product information for the experimental drug.

The investigator must, based on medical and scientific experience, assess whether it is relevant to report the medical event /reaction that is not immediately life-threatening, but which may bring the patient's health at risk or require medical treatment, to prevent one of the above mentioned events/reactions. Such incidents should usually be classified as serious.

Other events to be treated as serious adverse events

If the patient becomes pregnant around the time of treatment or 6 months after treatment, she must immediately be removed from the trial. The patient should be followed throughout the pregnancy. The results of pregnancy and childbirth must be reported, even if the process is normal and without AEs.

Stop rules

If a patient previously treated with influenza vaccine experiences anaphylactic shock to the intratumoral application of influenza vaccine the study will be stopped immediately. If a perforation of the bowel happens at the intervention site eg. at tumor level but not in the remaining bowel, as this will be seen as a known side effect to colonoscopies. Additionally, the trial may be interrupted prematurely and before inclusion of all patients if:

- Unexpected, significant or unacceptable risks to patients.
- Failure to enter patients at an acceptable rate.
- Insufficient adherence to protocol requirements
- Unacceptable compliance.
- Decision by sponsor or regulatory authority based on safety evidence.

Side effects to the influenza vaccine

Very common (> 10%)	Decreased appetite, reactions and discomfort at the insertion site, malaise. Myalgia. Headache, Irritability.
Common (1-10%)	Fever, chills.
Uncommon (0.1-1%)	Abdominal pain. Lymphadenopathy, Thrombocytopenia. Dizziness.
Rare (0.01-0.1%)	Dyspnea. Arthralgia.

	Paresthesia. Allergic reactions, Hypersensitivity.
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Side effects to applying the influenza vaccine intratumorally using a needle in the colon.

Injecting a needle into a tumor or normal tissue in the colon is part of many routine clinical procedures. Every patient with an endoscopically identified malignant polyp or tumor will be marked by injecting ink by a needle in the adjacent tissue⁹, so that part can be identified during the subsequent surgery. This techniques has been used since the 1980’s and has virtually no both short term and long term side effects⁹. Injecting a needle and administering up to 10 ml of saline is also a routine part of endoscopic mucosal resection¹⁰.

Side effects to the additional endoscopy

The study necessitates and additional endoscopy of the sigmoid colon. This is associated with the same risk as in a standard endoscopy of the sigmoid colon, ie. a risk of bleeding, infection and perforation of the colon, up to 0.5% overall. These complications are severe and will require hospitalization and presumably surgery. Prior to the endoscopy, the included patient will have to take a laxative to cleanse the bowel. This may be associated with discomfort.

7. Biological material from participants

Blood samples

Blood samples will be collected four times during the study period. Before intratumoral influenza vaccine treatment (baseline), on admission to surgery, within 72 hours after the surgical procedure, and when patients return to the outpatient clinic for final evaluation (approximately 14 days surgery). Additionally, one blood sample will be collected at baseline and prior to surgery for flow analysis.

Following blood samples will be collected at four specific time points:

- 1 x 6 ml in serum separator tubes
- 5 x 10 ml in EDTA tubes for full blood, buffy coat, flow cytometry, NK cell activity and ct/cfDNA
- 2 x 2.5 ml in PAXgene RNA tubes

A total of 244 ml will be collected during the entire study period.

Systemic immunological responses will be analyzed when all samples have been collected from all patients. Research shows that immune cells and their invasion of the primary tumor correlate to the patient's prognosis, again suggesting that the immune response is important for cancer growth^{11–13}, thus we will perform an evaluation of the systemic immunologic response.

Furthermore we plan to analyze circulating tumor DNA (ctDNA) and cell free DNA (cfDNA). Studies have shown elevated levels of cfDNA in cancer patients compared with healthy individuals^{14,15}. Studies have considered cfDNA as a prognostic marker for outcome, and high levels of cfDNA have been related to poor survival¹⁶. Furthermore, evidence suggest that the level of ctDNA is correlated to the tumor burden¹⁷. Further, we will analyze NK cell activity as this has been shown to increase after systemic influenza vaccine.¹⁸

Finally we plan to perform cell adhesion assays to analyze the metastatic ability of the cancer cells.

All samples will be stored in a biobank created for this study according to Danish legislation and approval from the Danish Data Protection Agency will be obtained prior to initiation of the study. Study blood samples and multiplex gene assays will be analyzed at Zealand University Hospital. Cell adhesion assays will be performed at Roskilde University. Samples will be shipped in anonymized form by applying ID numbers for all samples and blinding for intervention prior to safe transport. All bio-banked blood samples will be kept (without CPR-number, but instead with the patient code), and will be stored until analysis during the course of the trial. The project will be approved by the Danish Data Protection Agency and all formal requirements and maintenance of the biobank will be performed accordingly.

Upon termination of the current study, the samples will be kept in a research biobank for future studies for 10 years. Any remaining samples after this time point will be destroyed. The research biobank for future studies will be approved by the Danish Data Protection Agency, are subjected to Data Protection Act and will follow General Data Protection Regulation (GDPR) guidelines. Any additional analyses require renewed approval from the Ethics Committee and the Danish Data Protection Agency.

Biopsies and tumor samples

Final tumor samples, pre-treatment biopsies (8x0.5-1.5 cm) and a sub-specimen (3x0,5 cm³) of the original surgical specimen will be stored in a biobank according to the instructions issued by the Danish Cancer Biobank. Additionally, if pathological examination reveals positive lymph nodes (eg. lymph nodes with live cancer cells) three formalin fixated specimens of the lymph nodes will be stored in the biobank. The biobank will be approved by the Danish Data Protection Agency prior to initiation, and all formal requirements and maintenance of the biobank will be upheld during the study. Standard histopathological

evaluation will be performed at Zealand University Hospital. Immunohistochemistry and the Immunoscore will be performed in department of Pathology, Zealand University Hospital Roskilde. The Immunoscore is measured by immunohistochemistry by applying a novel clinical available and approved assay. Samples from the core of the tumor and from the invasive margin are stained for CD3 and CD8 positive lymphocytes. Tumor samples, including biopsies, and sub specimens will be sent for analysis. The Data Protection Act and GDPR will still be valid when samples are sent and handled. Samples will be anonymized through specific ID numbers and blinded for intervention prior to safe transport. The sponsor will retain a patient identification list. Upon completion of the current study any excess material will be stored in a biobank. From this time point additional analyses require renewed approval from the Ethics Committee and the Danish Data Protection Agency.

Quality of recovery

Patient perceived quality of recovery should be in focus when implementing new treatments and therefore, we include the widely used and validated “Quality of Recovery” questionnaire in the short 15-item form as a main outcome in our study. The 15-item QOR questionnaire (QoR-15 score) results in a score of 0–150 with a high score indicating a good quality of recovery. The questionnaire will be applied at baseline, prior to surgery, within 72 hours post-operatively and at 14 days after surgery, four times in total.

Photografic documentation

Photografic documentation of tumor and clinical response will be performed.

8. Information from electronic health records

We intend to use patient records of the included participants to register the following items during the admission for surgery.

- Clinical information including birthday, age, ASA score, height and weight
- Procedural details and possible adverse events in relation to the intratumoral influenza vaccine treatment and surgical procedure
- Clinical symptoms and vital signs
- Blood test results
- Fotografic documentation

This information is needed in order to provide safety of the participants during the study. We will first obtain permission from the physician that has the responsible to participant's treatment, before asking the participant for consent for assessing this information.

9. Storage of information from patients

All information about participants is subjected to 'lov om behandling af personoplysninger' and 'Sundhedsloven', and these will be complied. The approval from Danish Data Protection Agency (Datatilsynet) will be applied. Authorized representatives from the Local Ethical Committee are granted inspection of documents related to the study if needed. Each participant will be informed that representatives from the Danish Data Protection Agency and the Local Ethics Committee may inspect their medical journals and trial records, in all confidentiality. The requirements to ensure anonymity of data, data security and confidentiality of data will be explained to the participants and complied. All information will be treated with strict confidentiality and stored as confidential material according to the Data Protection Act and GDPR. TeamSite (a secure website at Zealand University Hospital) will be used for storage of such information. Only the principal- and coordinating investigators will have access to the information. Study results will be reported in anonymous form. The investigator will keep identification lists of all participants including a sample log. This list will include full name and CPR-number (social security number). The recorded data will be kept in an electronic Case Report Form system, EasyTrial, that complies with all data safety requirements in Region Zealand. An individual journal will be created for the study. The information will only be available for inspection for authorized representatives from the relevant authorities upon request (GCP Unit, local ethical committee, Danish Data Protecting Agency, Danish Medicines Authority). The investigators will retain investigational records, copies of CRF and source documentation for the maximum period required by the regulatory authorities.

The informed consent gives the primary investigator, sponsor and sponsor's representatives as well as any control authority (GCP Unit, local ethical committee, Danish Data Protecting Agency, Danish Medicines Authority) direct access to obtain information in the patient's record, including electronic health record, in order to see information on the patient's health conditions which are necessary as part of the implementation of the research project, and for control purposes, including self-control, quality control, and monitoring, which they are required to perform. Changes to the protocol will require written approval from the competent authority prior to implementation except when modifications are needed to eliminate immediate hazard(s) to the patients.

10. Economy

Economy: Financial support from private and public grants to cover the expenses will be applied on ongoing basis. The responsible investigators are employees of Zealand University Hospital.

Time plan: Enrolment of the first participant has been set for August 1st 2020. We are expecting the clinical study with patient enrolment to last for approximately 12 months. Data analyses are expected to last for three months. Lastly we are expecting a three-month period for article writing.

11. Payment to patients

The patients will not receive any payment to be part of the study.

12. Inclusion of patients and informed consent

All required regulatory and ethics approval must have been obtained before enrolment in this trial. Each potential participant must be given a patient information sheet and full written informed consent must be obtained from the patient before registration on to the trial. Potential participants will be screened and enrolled on the clinical trial on the basis of the inclusion/exclusion criteria specified in the protocol. Only patients fulfilling all inclusion criteria, and without any exclusion criteria can be registered. Any queries about eligibility should be addressed directly to the coordinating investigator.

The investigators will screen potential candidates for the study at the biweekly MDT colorectal conference at Zealand University Hospital. The investigator will contact the potential participant at the preoperative examination 2-3 weeks before scheduled surgery. Only if the patient has time and interest in participating in the study, the investigator will continue with an information meeting. In addition to an oral and written presentation of the study, the patient will be given a written copy of information concerning the study and copies of the pamphlets "Forsøgspersoners rettigheder i et sundhedsvidenskabeligt forskningsprojekt" and "Før du beslutter dig" issued by the Central Committee for Health Research. The candidate will be informed in advance that he/she is allowed to bring an assessor for the meeting.

At the information meeting the candidate will receive information concerning the study both orally and in writing. The meeting will be held in a room that will only be used for this purpose to avoid unnecessary disturbances. The investigator will explain content, extent, purpose, expected risks and possible advantages

of the study in plain Danish. The candidate will be given time to ask questions and read the information one more time. The following points will also be covered during the interview:

- A description of the purpose with the study and how it will be organized.
- The permission to access his / her patient records to register any adverse event, medication use, blood tests and other basic clinical parameters during his / her admissions.
- The methods used in the study.
- The permission to use his / her clinical data as well as clinical images, scanning images, pathological images for publication in strictly anonymized form, e.g. case reports, posters, lectures, and future publications.
- The right to ask for additional information at any time.
- The patient's right to withdraw from the trial at any time without having to explain why.
- The insurance.
- The contact person.

The informed consent will include all the elements that are required according to Danish legislation. The participant will be given the necessary and needed time for consideration, to determine the participation in the study. The participant will be asked for permission to be contacted by telephone for final decision regarding study participation within 24 hours. A formal inclusion interview will be arranged if the patient accepts inclusion in the trial. All questions will be answered to the candidate's satisfaction. Once the candidate accepts to take part, the participant will be asked to take part in an inclusion interview and sign the informed consent. After the information meeting, all active participants will be updated with any new and important information or change in the study that can have influence on the participants' willingness to participate or safety. The approved documents will be updated accordingly.

13. Publication

All results, including positive, negative and inconclusive will be published in international peer-reviewed journals.

14. Ethical considerations

The only curative treatment for rectal cancer and sigmoid colon cancer is surgical excision. In this study we aim to examine the effect of intratumoral influenza vaccine on tumor response. The study will provide further knowledge to the understanding of the tumor response in colorectal cancers. The effects may possibly lead to better oncological outcome and alterations in the current management towards less invasive treatment. To our knowledge, no one has completed a study of this kind on patients undergoing treatment for colorectal cancer. The participants of the current study may not directly benefit from the participation. However, the knowledge gained from the study will provide knowledge that has vast clinical implications moving forward. A protective effect of the influenza vaccine will mean that the immune system can be strengthened by simple means to increase its role in the killing of circulating tumor cells. Mostly, patients with solid tumors are offered curative surgery, whereby the influenza vaccine will play a crucial role in the course of these patients.

If the influenza vaccine protects against recurrence of cancer, it will be revolutionary in this area. The influenza vaccine causes few side effects and will thus be a patient-friendly alternative or supplement to chemotherapy.

Further, the multiplex gene assay analysis, is focused on the synthesis of proteins and will analyze RNA sequences, thus not having the risk to discover possible unknown genetic diseases in the patients. When analyzing cfDNA, we do not analyze for known genetic variants related to inherited diseases. Analyzing cfDNA is based on a specific sequence that is found in all human DNA and will thus not reveal any genomic variants related to inherited diseases. Nevertheless, if such a variant should be found, its clinical importance will be evaluated by an expert committee, appointed by Department of Surgery.

The committee is appointed when needed and the members will be chosen according to the potential disease. The committee will include a molecular biologist, specialized in genetic sequencing, and a medical doctor specialized in personalized medicine, a clinical geneticist specialized in inherited diseases, and a medical doctor specialized in the disease in question. If deemed relevant other specialists may be included.

This committee will assess if

- 1) The technological quality of the analysis is sufficient for a reliable result.
- 2) There is sufficient evidence in the literature for a clinical relevance (e.g. expected penetrance)
- 3) The sum of information justifies a relevant risk for a genetic disposition
- 4) The disease, according to current standards, can be treated or prevented

Based on the assessments the committee decides, whether or not the patient (and/or his family) should be informed (by written letter) that the research accidentally has resulted in a finding, with potential influence

on his or hers health, and that further information and advice on the matter is offered to him/her and/or potentially affected family members. If accepted, this will be initiated.

Whether or not to provide feedback to relatives of deceased study participants or to study participants who, themselves, deny information about genetic issues, will be decided based on a medical perspective according to “DNVKs retningslinjer af 29. april 2013, sundhedslovens § 43, stk. 2, nr.2” and in “autorisationsreglerne om lægers omhu og samvittighedsfuldhed”.

This study will be conducted according to the principles of the Helsinki Declaration. This protocol will be submitted to the Regional Committee for Health Research and Ethics, the Danish Data Protection Agency, and the Danish Medicines Agency for approval. Cooperation with the regional GCP unit will be initiated before the study is launched to ensure good clinical practice before during and after the study. The head of department for the experiment site has given the approval. The protocol will be registered at clinicaltrials.gov. All participants in this study have to give their oral and written consent before they can be included in the study. It is the investigators duty to inform the participant orally and in writing, so they are thoroughly informed about all aspects concerning participation in the study. The participant can at any point withdraw the consent concerning participation in the study. If a participant decides to do so it will not impair the relationship to the investigator or the doctors involved.

The clinical investigation shall not begin until the required approvals from the Local Ethics Committee, the Danish Data Protection Agency, and the Danish Medicines Agency have been obtained. Any additional requirements imposed by the Local Ethics Committee or regulatory authority will be followed.

15. Compensation to patients

The Department of Surgery, where participants are admitted, assumes the legal responsibility on behalf of the investigators and the investigators’ assistants for all participants in the trial concerning any injury that is caused by the study procedures either directly or indirectly, assuming that the investigators and assistants have followed the guidelines given in this protocol and in any eventual additions to this protocol. In addition, it is also assumed that the study is performed scientifically and in coherence with existing rules and accepted techniques. The participants are in the case of injury or death without connection to the completion of the trial insured by the hospitals insurance. The participants will be informed that they are

covered by the public participant insurance, and if the participant wishes to complain, the participant will be informed on how to obtain help for this matter.

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Supplementary materials

Title:

Neoadjuvant intratumoral flu vaccine treatment in patients with proficient mismatch repair colorectal cancer leads to increased tumor infiltration of CD8+ T-cells and upregulation of PD-L1: A phase 1/2 clinical trial

Running title:

Intratumoral flu vaccine in pMMR colorectal cancer

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Supplementary methods

Staining of slides for immunohistochemistry analyses

Staining was performed using anti-Cytokeratin clone BS5 (Nordic Biosite Aps, Denmark, # BSH-7124-1), anti-CD3 clone LN10 (Leica/Triolab AS, Denmark, # NCL-L-CD3-565), and anti-CD8 clone C8/144B (Agilent/Dako, Denmark, # GA623). Double-labelling staining was performed on the automated instrument Omnis (Agilent/Dako, Denmark). Briefly, antigen retrieval was accomplished using EnVision™ FLEX Target Retrieval Solution, High pH (Agilent/Dako, Denmark, # GV804) and slides were subsequently incubated with primary antibodies, CD3 (1:50) or CD8 (Ready-to-Use) for 30 minutes at 32°C. Reactions were detected using the standard polymer technique EnVision™ FLEX /HRP Detection Reagent (Agilent/Dako, Denmark, # GV800/GV821) and visualized using EnVision™ Flex DAB+ Chromogen system (Agilent/Dako, Denmark, # GV825) following instructions given by the manufacturer. In the second sequence, slides were incubated with anti-Cytokeratin (1:800) applying the same protocol settings as described above except for visualizing the reactions with EnVision™ Flex Magenta Chromogen system (Agilent/Dako, Denmark, # GV900). Finally, sections were counterstained with hematoxylin and mounted with pertex mounting media (Pertex™/Histolab, Sweden, #00801-EX).

RNA isolation and panel preparation for nCounter analysis of mRNA expression

RNA isolation and panel preparation: Total RNA was extracted from FFPE slides (10 µm sections) using the High Pure FFPET RNA Isolation Kit (Roche Life Science, Germany) according to the manufacturer's instructions. Total RNA was quantified using spectrophotometry (NanoDrop, Thermo Scientific, USA), and RNA quality assessment was done with the Bioanalyzer (Agilent, Denmark). Approximately 300 ng total RNA was used to determine gene expression levels for each sample adequately. We performed RNA hybridization overnight using two distinct panels: for gene expression analysis, we used the nCounter® IO360 panel of 750 endogenous human transcripts; for T-cell receptor (TCR) expression analysis – the nCounter® TCR Diversity panel of 119 T-cell receptor variable and constant regions and lymphocyte

transcripts (NanoString, USA). Sample acquisition was done by using the nCounter® system and following the manufacturer's instructions.

Preparation of plots related to nCounter analysis of mRNA expression:

PCA was performed using the top 400 most variable genes and results were plotted using the functions “pca” and “biplot” of the PCATools package (v.2.5.3) in R, respectively¹. Hierarchical clustering was performed based on sample Euclidean distances and farthest neighbor linkage method. A heatmap of the full gene panel (n = 750) was generated using “Heatmap” function from ComplexHeatmap package (v.2.8.0) in R². DE results were visualized with “EnhancedVolcano” package (v.1.11.1) in R³. Enrichment analysis was performed to test for functional enrichment in samples before and after vaccination based on pair-wise down-regulated (higher expression in samples before) and up-regulated (higher expression in samples after) DE genes. Gene set annotations were downloaded from the Molecular Signatures Database v7.4 and comprised gene sets from the Hallmark gene sets⁴, and “biological processes” from Gene Ontology (GO) database⁵. Additionally, NanoString specific gene sets were retrieved from Danaher *et al*⁶. We used enrichment functions from “clusterProfiler” (v.4.0.2)⁷ specific for each gene set annotation, e.g. “enrichGO” for GO database. Genes were included in enrichment analysis if they met threshold requirements of adjusted p value < 0.1 and log2 fold change (logFC) ≥ 0.5.

Gene expression analysis:

Shortly, quality control was performed by evaluating technical sample quality, generating principal component analysis (PCA) plot, Euclidean distance heat map, and gene count histogram. Raw gene counts were normalized by first running upper quartile normalization, followed by variance stabilizing transformation. Estimated unwanted variation was removed using the “removeBatchEffects” function from limma package (v.3.48.1). After iterative QC and normalization, we removed n = 5 vectors of unwanted variation from the data set.

TCR expression analysis

The same iterative QC process was performed for raw TCR data as described for gene expression data.

Normalization was performed as described in technical note provided by Nanostring⁸. Shortly, we used “procedure 2”, which included three steps: (1) panel standard normalization, (2) creation of housekeeping normalization factors for each sample, and (3) housekeeping normalization. From further analysis we removed n = 1 sample, as normalization could not overcome technical sample artifacts. TCR scores were calculated to quantify the diversity of TCR alpha, beta, delta and gamma variable regions within a sample. The score is based on the Shannon diversity index calculations, which was calculated using base R.

GeoMX data collection

For QC, a FOV detection of >75, binding density of 0.1–2.25, minimum nuclei count of 20, minimum surface area of 0.016 mm, and a positive control normalization factor not between 0.3–3.0 was chosen.

References

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4. Liberzon, A. *et al.* The Molecular Signatures Database (MSigDB) hallmark gene set collection. *Cell Syst.* **1**, 417–425 (2015).
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6. Danaher, P. *et al.* Gene expression markers of Tumor Infiltrating Leukocytes. *J. Immunother. Cancer* **5**, 18 (2017).
7. Yu, G., Wang, L.-G., Han, Y. & He, Q.-Y. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS* **16**, 284–287 (2012).
8. TECH NOTE: Panel Standard and Calibration Sample Usage. 8 https://nanosttring.com/wp-content/uploads/TN_MK3415_Panel-Standard_r9.pdf (2021).

Figure S1 – Flow chart of patient inclusion

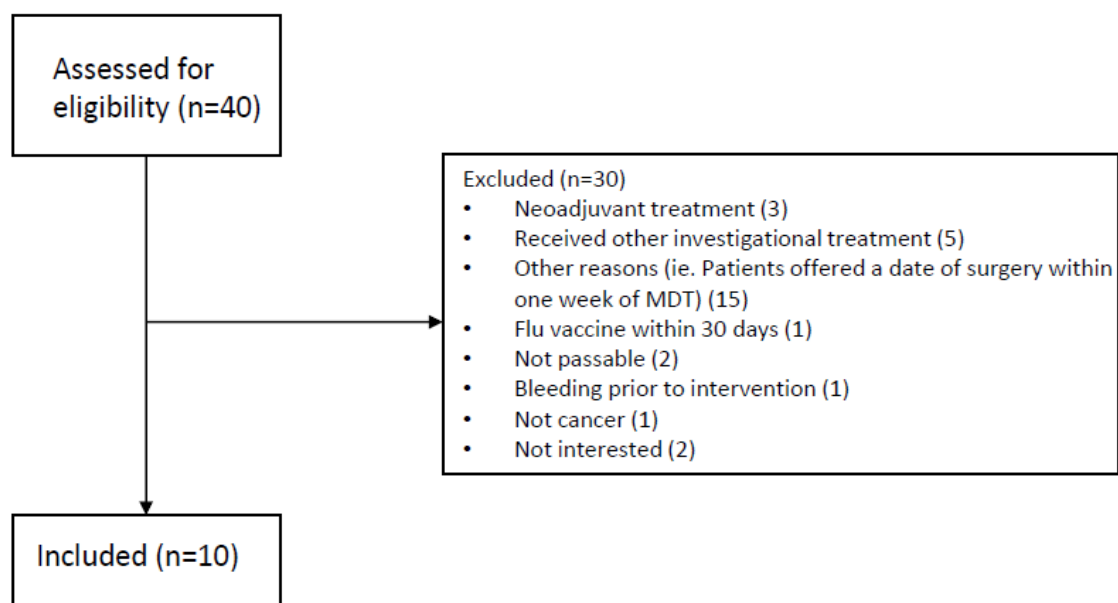


Figure S2 – Overview of distribution and correlation plots after K=5 normalization

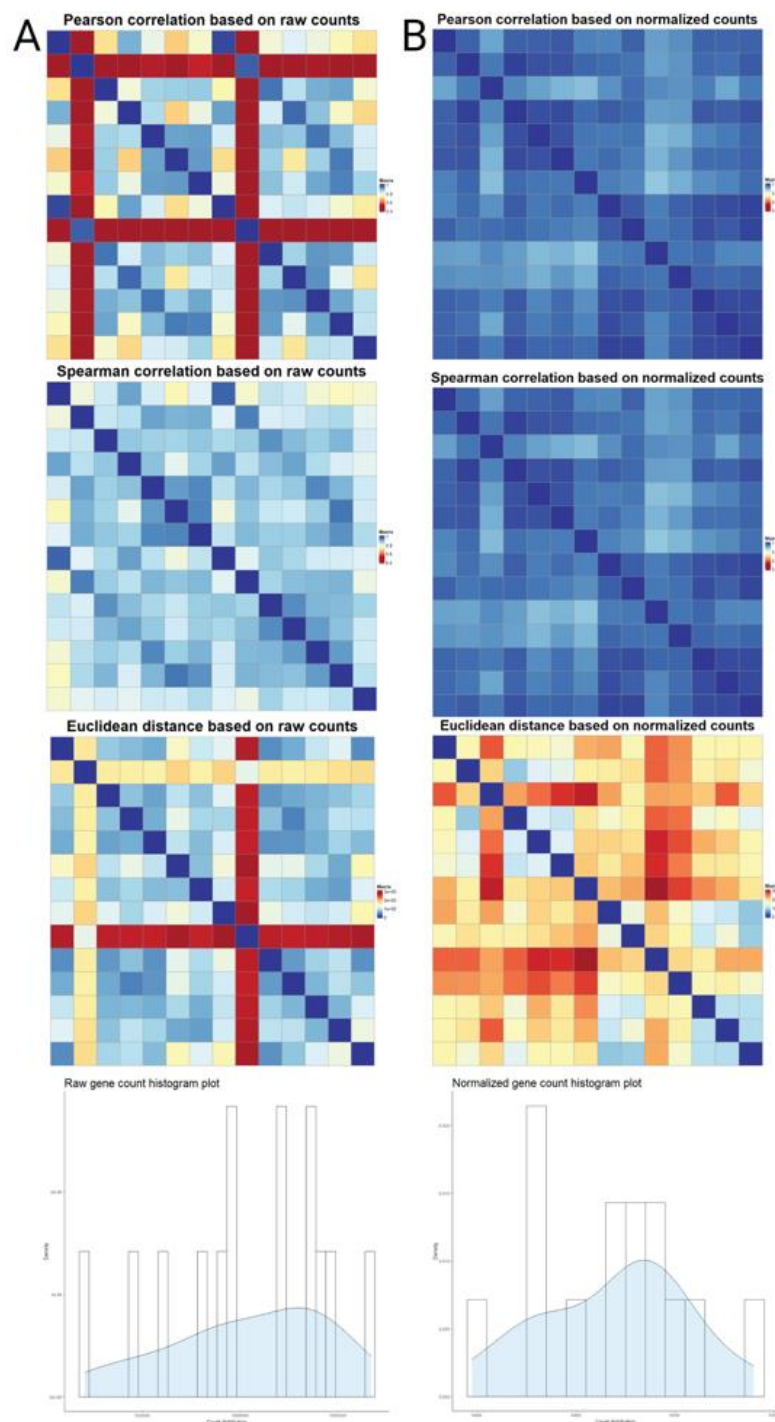


Figure S2 - Overview of distribution and correlation plots before vs. after K=5 normalization. A: Pearson, Spearman, Euclidean distance correlation plots, and data distribution plot before normalization of tumor mRNA gene expression data. B: Pearson, Spearman, Euclidean distance correlation plots, and data distribution plot after RUVseq K=5 normalization of tumor mRNA gene expression data.

Figure S3 – Overview of mRNA gene expression analysis after vs. before IT-flu vaccination

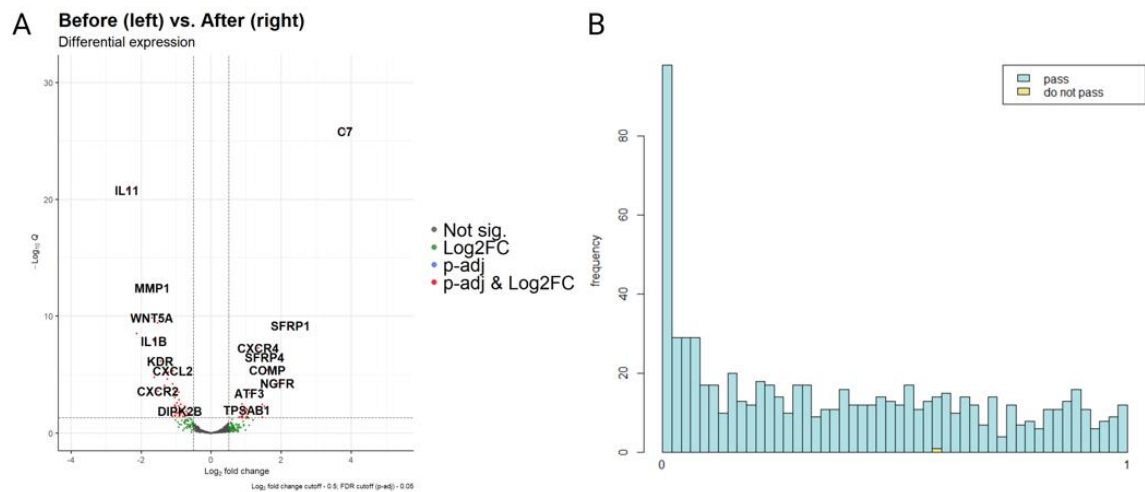


Figure S3 – Overview of mRNA gene expression comparison in tumor samples before vs. after vaccination.
A: Volcano plot, B: P-value distribution plot.

Figure S4 – Overview of upregulated genes in mRNA gene expression analysis

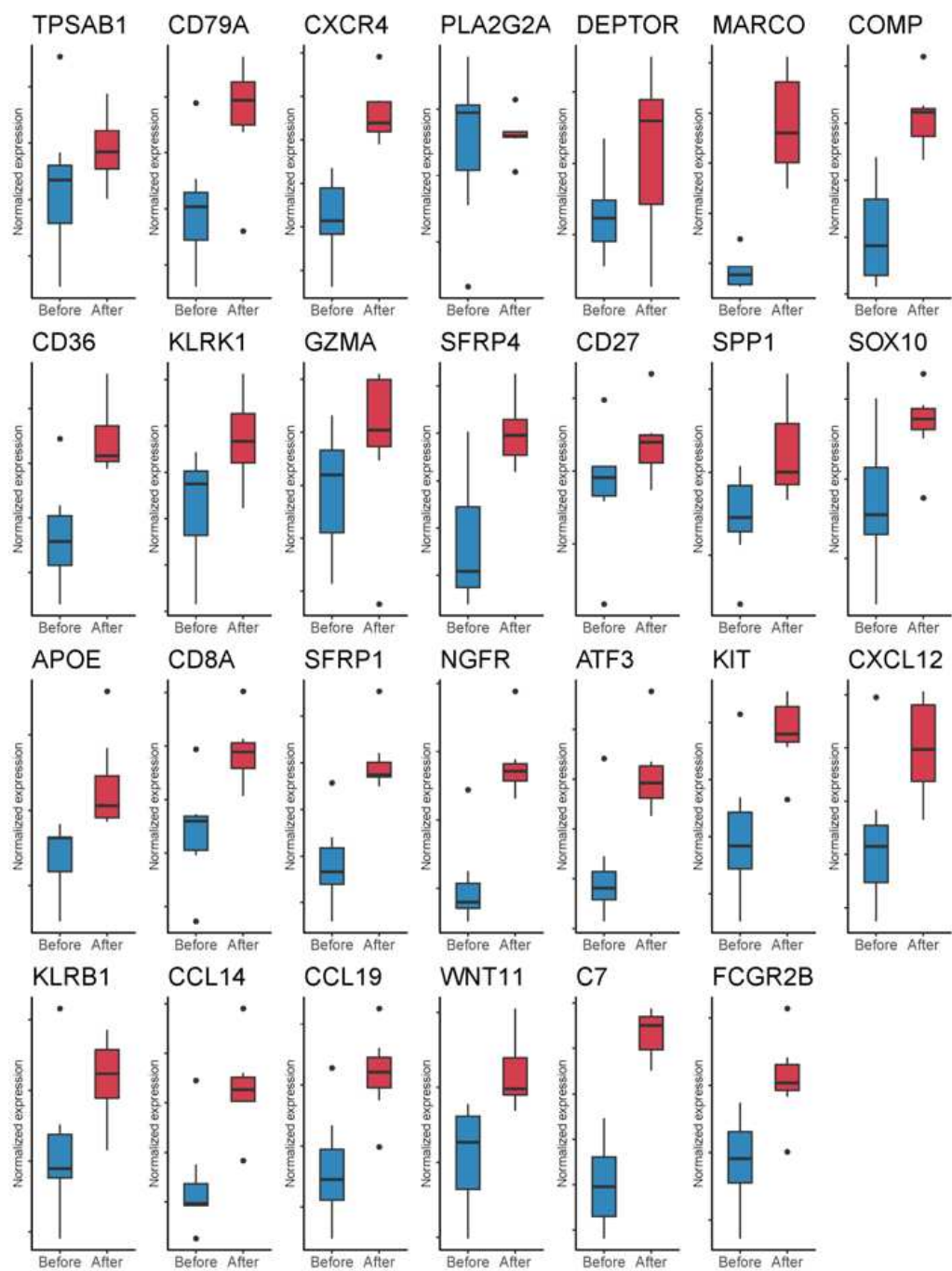


Figure S4 - All upregulated genes after vaccination

Figure S5 – Overview of downregulated genes in mRNA gene expression analysis

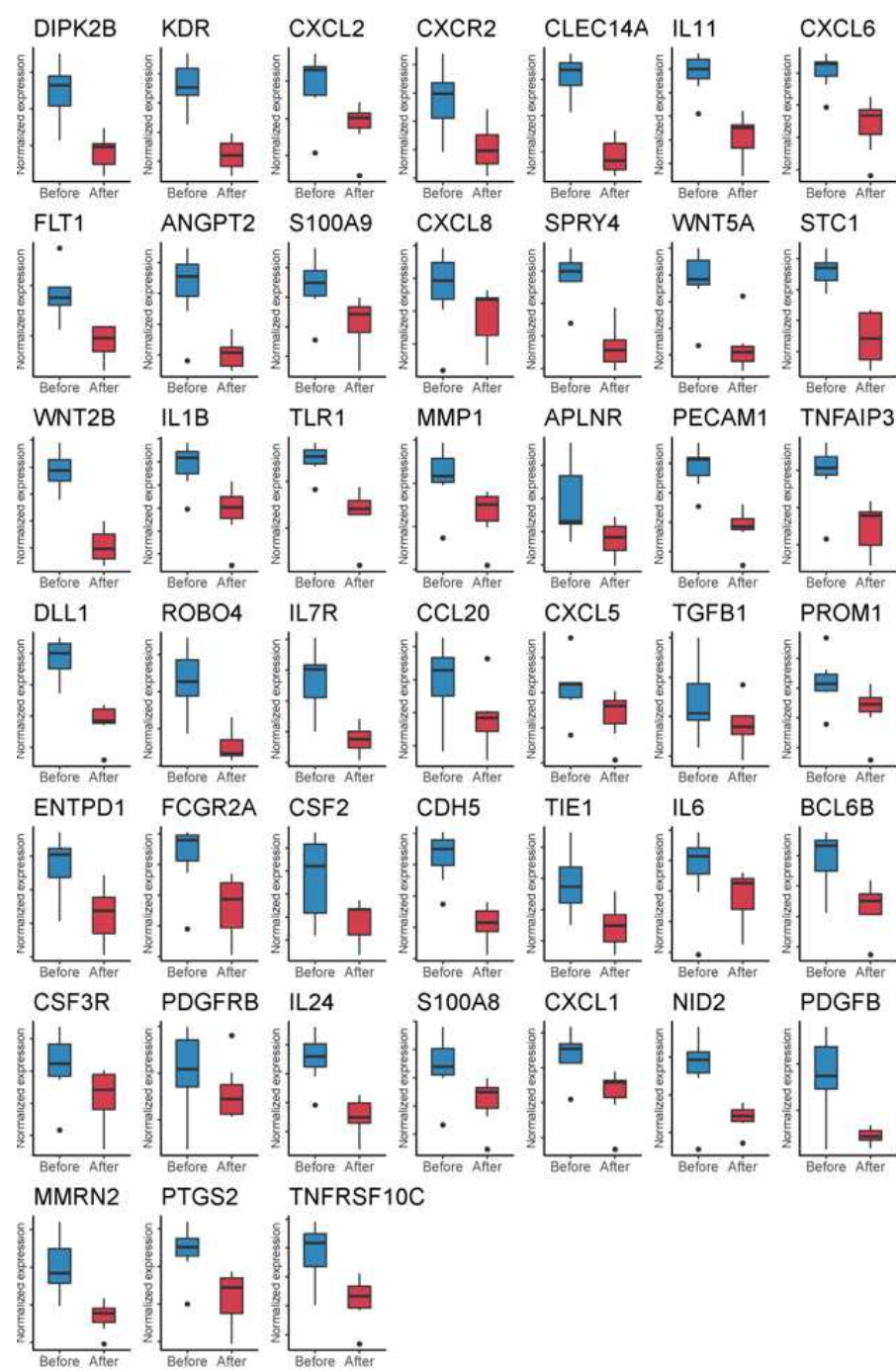


Figure S5 - All downregulated genes at after vaccination.

Figure S6 – Representative ROI’s of normal tissues regions before vs. after IT-flu vaccination

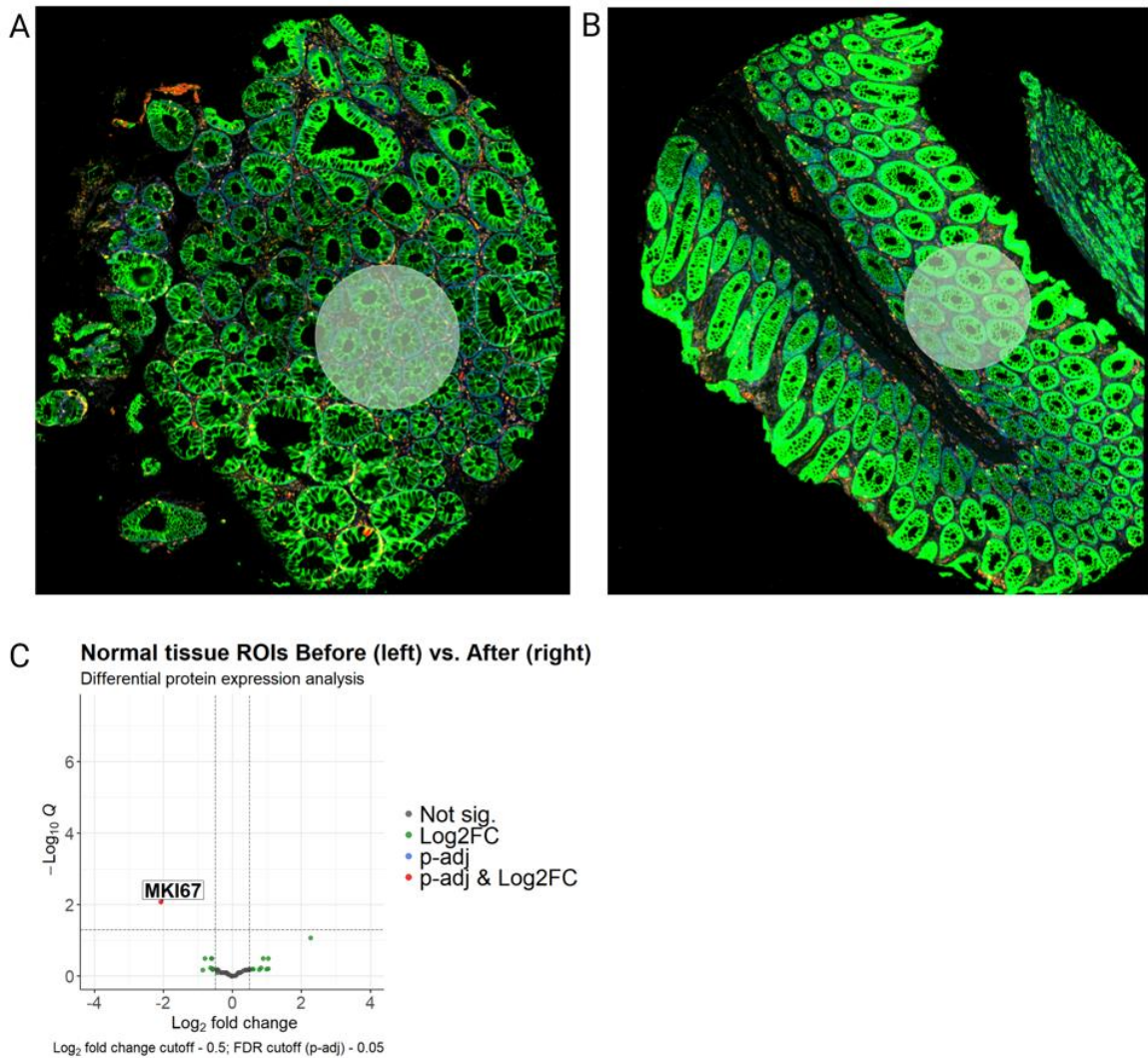


Figure S6 - Representative ROI’s of normal tissue regions before vs. after IT-flu vaccination. A: Normal tissue region before vaccination (baseline). B: Normal tissue region after vaccination. C: Volcano plot of differentially expressed proteins at different time points.