## SUPPLEMENTARY NOTE

### Supplementary Methods

**Patients.** The Phase I escalation trial received approval from the Cancer Therapeutics Evaluation Program of the National Cancer Institute as well as the Internal Review Board of Dana-Farber Partners Cancer Care. Informed written consent was obtained from all six patients enrolled at the first dose level (5 mg/kg bevacizumab, three female, three male). Mean age at treatment was 49 years (range: 34 to 58 years). Mean tumor size at pre-treatment endoscopy was 4 cm (range: 3.5 to 6 cm). Transmural extension of tumors into the mesorectum (T3) was documented by endoscopic ultrasound (two patients) or surface coil MRI (four patients). The six patients have completed the neoadjuvant therapy of the first bevacizumab infusion followed by three two-week cycles of bevacizumab, external beam radiation therapy (EBRT), and concurrent 5-FU without dose-limiting toxicity; all six patients have undergone surgery uneventfully 7 to 9 weeks after completion of all preoperative therapy (two patients underwent abdominoperineal resection and four patients low anterior resection). Molecular, cellular and physiological measurements were made before, during, and after treatment with bevacizumab, EBRT, and 5-FU chemotherapy. Assays on day 11 or 12 following the first bevacizumab infusion – equivalent to approximately the half-life of bevacizumab – permitted evaluation of the specific effect of bevacizumab alone on the tumors, as EBRT and 5-FU treatment was not initiated until the third week of the protocol. The trial is ongoing and dose escalation and treatment continue in additional patient cohorts.

**Tumor imaging analyses**. Functional imaging [perfusion CT scans and 18-fluorodeoxyglucose positron emission tomography (PET) scans] was conducted before and 12 days following bevacizumab infusion, as well as approximately 6 weeks after completion of all therapy (one week prior to surgery). These imaging techniques assess tumor metabolism (PET scan) and perfusion (CT scan). Functional CT was performed on a four-slice multi-detector row CT scanner (GE, light Speed QX/i). A 2 cm region of tumor was selected on non-contrast CT of pelvis. Subsequently, dynamic CT of this region was performed for 45 seconds at the same table position, immediately after initiation of a bolus injection of 125 ml of iodinated non-ionic contrast media at the rate of 7 ml/s through a peripheral 18G intravenous cannula. Data were analyzed on a workstation (Advantage Windows, GE) using commercially available CT perfusion 3.0 software, which implements a deconvolution approach to calculate regional blood flow, blood volume and permeability-surface area (PS) product (**Supplementary Fig**. **1a**). The external iliac artery and vein served as arterial and venous input respectively. Multiple, non-overlapping regions of interest (ROI) were drawn over the tumor for each of four slices and blood perfusion values were obtained. For each patient, a mean of the values from the individual ROIs was used to calculate mean blood perfusion. In previous studies, similar values of blood perfusion were obtained with functional CT and radioactive microspheres[1,2]. Five out of six patients had analyzable data.

**Immunohistochemistry**. Tumor biopsies obtained before and 12 days after bevacizumab infusion were assessed for microvessel density (MVD). Five out of six patients had analyzable biopsies that permitted accurate determination of the number of vessels per micrometer square in areas of invasive adenocarcinoma with desmoplasia using an antibody against human PECAM (Dako, Carpentria, CA). Pericyte coverage of the vessels was assessed by double staining the biopsies for PECAM and α–SMA (monoclonal antibody, Dako, Carpentria, CA, clone 1A4) allowing concomitant analysis of both markers (**Supplementary Fig. 1b**). Concentration of the α–SMA antibody (1:5000 dilution) was optimized to differentiate between the myofibroblasts present in the desmoplastic areas and the perivascular cells.

**Endoscopy and IFP measurements**. Prior to, and then 12 days after the first bevacizumab infusion, flexible sigmoidoscopies were performed on all six patients, which permitted tumor visualization, and assessment of gross response, measurement of tumor IFP and excision of tumor biopsies. To measure IFP a 23-gauge needle, with a 2-3 mm side-hole at 4-5 mm from the tip, was connected to PE 90 tubing that was inserted through a trochar sleeve and the working channel of the endoscope (**Supplementary Fig. 2a**). The IFP was measured in two to five tumor locations. Stable pressure measurements with a good fluid communication between the tumor interstitial space and needle were considered valid[3]. Reliable measurements were obtained for patients three, four, five and six.

**Circulating cells and VEGF measurements**. Peripheral blood was collected for measurements of circulating cells by four-color flow cytometry as described previously[4] (**Supplemental Fig. 2b**). Cell suspensions were evaluated by FACSCalibur (Becton Dickinson, San Jose, CA). The antibodies used were: CD31 (EC and monocytes), CD45 (pan-hematopoietic marker), CD133 (AC133, progenitor/stem cell marker), and CD34 (progenitor/stem cells, EC), and the gate was set on the lymphocyte/mononuclear populations, to avoid RBCs, cell debris and neutrophil contamination. Circulating cells data were obtained as an average of three separate measurements, after scanning 50000 events. For total number of circulating endothelial cells (CECs) and progenitor/stem cells, results are shown as percent of the total number of nucleated cells and were normalized with respect to the baseline value. Fluorescently labeled isotype matched $IgG_1$ antibodies were used as control for analysis. The time points analyzed were: pre-treatment, day three after first bevacizumab administration, day 12 after first bevacizumab administration, and pre-surgery. Soluble VEGF levels in plasma samples were evaluated from samples obtained throughout the course of the protocol using an ELISA kit (R&D System, Minneapolis).

**Statistical analysis.** For the blood flow, blood volume, permeability surface area product and vascular density we used the t-test to determine differences for each individual patient (**Fig. 2**) or the grouped data (**Supplemental Table 1**). For interstitial fluid pressure, circulating endothelial cells and the fraction of α–SMA-positive vessels, with a unique or small number of discrete measurements, the overall mean values before and after bevacizumab were compared with a paired t-test or a Wilcoxon signed-rank test (see **Supplementary Table 1**).

**Pathological description of surgical specimens.** Macroscopic evaluation of five of the six surgical specimens revealed well-circumscribed shallow ulcerations measuring 1.5 to 2.5 cm in diameter (**Fig**. **1a**); tumor from patient three demonstrated a 2.5 cm plaque. Five of the six patients demonstrated rare residual tumor glands overgrown by dense fibrosis (Tumor Regression Grade II by Mandard criteria[5]). In two cases the fibrosis was densely hyalinized, with residual dystrophic calcifications marking tumor necrosis in one patient. In the other three patients, the residual malignant glands were embedded in an edematous and inflamed fibrosis. One case (patient six) showed more abundant malignant glands and was classified as Tumor Regression Grade III by Mandard criteria[5].

Residual irradiated cytologically intact tumor cells were present in the submucosa (pT1) in two cases, in the muscularis propria (pT2) in two other cases and in the perirectal adipose tissue (pT3) in the third and fourth patients. All lymph nodes were negative for malignancy in four cases. In the third and fourth case, nine lymph nodes out of the twenty-one examined were extensively necrotic with only microscopic residual tumor. These preliminary tumor regression rates after bevacizumab, 5-FU and EBRT are encouraging[6].

**References**


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