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Review Article

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A Discussion on Tumor Associated Neutrophils and Arginase-1 In Rectal Cancer. New Insights on the OR1 Scientific Experiment

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ABSTRACT

Introduction: In this paper, a second set of results and graphs of a previously published scientific experiment will be revealed and discussed. Tumor Associated Neutrophils have been revealed to play an important role in regards of tumor progression and etiopathogenesis and several research teams around the world have been focusing on how to gather new intel to transfer this knowledge in a clinical setting in order to aid clinicians and patient management.

Methods: A total of 65 patients with histologically confirmed rectal adenocarcinoma were retrospectively included and stratified into four groups according to neoadjuvant treatment.

Conclusion: Patients with perineural invasion, patients who didn't undergo any neoadj therapy with chemotherapy (so patients who underwent either CTRT or CT) have a statistically significant higher concentration of Arginase-1. Statistically significantly better DFS in patients whose tumor had no perineural invasion who underwent therapy (either CT or CTRT) vs those patients who had perineural invasion.

Introduction

In this paper, a second set of results and graphs of a previously published scientific experiment will be revealed and discussed.

Tumor Associated Neutrophils have been revealed to play an important role in regards of tumor progression and etiopathogenesis and several research teams around the world have been focusing on how to gather new intel to transfer this knowledge in a clinical setting in order to aid clinicians and patient management.

The innate immune system has been an increasingly studied system in terms of its role in tumor genesis, progression and clinical aspect and new factors are being studied in terms of their role in cancer development and progression. Namely Arginase-1 (produced also by neutrophils) that has been found to be a potent immuno-depressor (eg. T cell immunodepression), neural-tumor relation. The importance of developing new tools for the management, understanding and treatment of rectal cancer. This paper aims to shed new light on this complex set of interactions by showing new data and reviewing previously published data of the 2025 OR1 study conducted and led by dr Nicola Sarandria and an open discussion on rectal cancer.

Arginase-1 has been shown by literature to be a potent immunosuppressor in terms of neoplastic course of disease. It is known that tumor stroma rich in macrophages are linked to a poor response to adjuvant and neoadjuvant therapies. Macrophages M2 are also linked to Arginase 1 [1].

Recently, neutralization of NET (neutrophil extracellular trap) linked neutrophils Arg-1 is linked to an increased potency and effect of immunotherapy effect [2].

Neutrophils have a complex role in terms of the tumor microenvironment and rectal cancer. Neutrophils are divided in terms of both anti and pro tumoral action linked to a polarization of the tumor, namely N1 and N2 neutrophils.

Other than functioning as first responders to infection, inflammation and innate-immunity tasks, neutrophils are involved in the tumor microenvironment by for instance acting on tumor growth and invasion/metastasis through systems involving of antibody related cytotoxicity (involving antibody-targeting cells which are destroyed by immune cells that have Fc receptors (FcR) that is present on neutrophils, which express the

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family members of FcγR. The interactions between neutrophils and mAb through FcR induce the release of tumoricidal mediators) and direct mechanisms (direct mechanisms involving reactive oxygen species (ROS), myeloperoxidase (MPO), hydrogen peroxide (H2O2), and proteases). Neutrophils can have this anti-tumoral effect also by activating an anti-tumoral cascade of reactions which involve activation and recalling of natural killer cells, b cells, t cells and dendritic cells [3].

N2 neutrophils, the population of neutrophils causing a pro-tumoral environment, could be linked with Arginase-1, a known compound produced by neutrophils, which has been shown to be associated with pro-tumoral and tumor progression. Furthermore, Arg-1 inhibitors have been shown to be a promising new frontier in therapy. The hypothesis of this study is that the population of N2 neutrophils would be directly linked also in terms of concentration with Arg-1 and this latter would decrease as would decrease the N2 population. It could be worth noting that possibly different stages of rectal cancer, due to tumoral microenvironment milieu and cross-reactions, could contain N1 or N2 populations.

Several co-factors have been shown in the literature to be of impact in onco-progression. Perineural invasion has been shown to have significantly worse prognosis impact, partly possibly due to the immuno-suppressive capabilities of the neuro-tumor relationship leading to a build up in TGF-beta. This is due to Schwann cells and SC-tumor interaction recalling myeloid derived suppressor cells [4].

In this paper, a review of recently published data of the 2025 OR1 study will be discussed with the addition of new graphs and discussion.

Methods

In the OR1 analytical experiment the following criteria was used [5]:

Patient Selection and Clinical Data Collection

Samples were provided by the regional tumor bank of Franche-Comté (University Hospital of Besançon, France; registration number BB-0033-00024). The project was approved by the scientific board of the biobank (#2508).

A total of 65 patients with histologically confirmed rectal adenocarcinoma were retrospectively included and stratified into four groups according to neoadjuvant treatment modality: 20 patients received no neoadjuvant therapy, 20 were treated with combined radiochemotherapy (RTCT), 20 with radiotherapy (RT) alone, and 5 with chemotherapy (CT) alone. The latter group was included despite its limited size, as neoadjuvant chemotherapy without radiotherapy is not a current standard of care and is typically restricted to clinical trial settings (e.g., NORAD), making such cases relatively rare.

Clinical and pathological data were collected from medical records. When available, the following variables were recorded : date of biopsy or surgical sampling (date_prlv), age, sex, histological subtype and grade, Dworak tumor regression grade, pathological TNM stage, vascular and perineural invasion, resection margin status (R), neoadjuvant treatment received (neoadj), date of progression (date_prog), progression-free survival (DFS), date of death (date_death), overall survival (OS), and molecular profiling data including KRAS, NRAS, BRAF mutation status, and microsatellite instability (MSI). Death was considered as the first progression event in the absence of documented disease progression.

Samples from Stage III rectal cancer patients.

Sample Selection

For each surgical specimen, the most representative FFPE tissue block was selected by a pathologist, based on morphological assessment. This approach ensured optimal preservation of tumor architecture and included the relevant tumor regions (invasive margin, tumor center, and luminal surface) required for downstream analysis [6].

Immunohistochemistry

Serial 3–4 μm sections were cut from each FFPE block and mounted on positively charged slides. Immunohistochemical staining was performed on a BenchMark ULTRA automated stainer (Roche Diagnostics) following the manufacturer's protocol. Pre-treatment involved heat-induced epitope retrieval (HIER) under optimized buffer conditions, followed by incubation with primary antibodies and detection using the OptiView DAB IHC Detection Kit.

Tonsil tissue (for CD66b) and liver tissue (for ARG1) served as positive controls. Negative controls were processed identically but omitting the primary antibody.

Procedure Summary

- Deparaffinization and Rehydration: Slides were baked at 72°C for 12 minutes, followed by solvent-based deparaffinization and rehydration.
- Epitope Retrieval: HIER was carried out using Ventana CC1 buffer (pH 8.5) at 95–100°C for 64 minutes (CD66b) or 32 minutes (ARG1).
- Primary Antibody Incubation: Antibodies were applied automatically at optimized dilutions and incubation times.
- Detection: Staining was visualized using a multimer-based DAB chromogen system, with hematoxylin counterstaining.

Antibody	CD66b	Arginase-1 (ARG1)
Clone/Reference	G10F5 (BD Pharmingen)	AB96183 (Abcam)
Type	Mouse monoclonal	Rabbit polyclonal
Dilution	1:50	1:2000
Pre-treatment	CC1 standard (pH 8.5, 64 min)	CC1 short (pH 8.5, 32 min)
Incubation	32 min at 37°C	32 min at 37°C
Detection	OptiView DAB	OptiView DAB

Detection of CD66b and Arginase-1 Staining

CD66b and Arginase-1 immunostainings were quantified on whole slides using QuPath v0.5.

For each slide, representative regions were manually selected to reflect spatial heterogeneity of the immune infiltrate. These included the invasive front, the deep core of the tumor, and the

luminal interface (figure 1). Region selection was performed by one observer and independently reviewed by a board-certified pathologist [7].

Due to the predominantly membranous and/or cytoplasmic localization of these markers in small cells, nuclear detection was sometimes hindered, particularly within dense immune infiltrates. To overcome this limitation, two nucleus detection strategies were applied: optical density (OD) sum (figure 2) and hematoxylin (H) based detection. The former offered higher sensitivity, capturing nuclei partially obscured by cytoplasmic staining, while the latter showed improved specificity by reducing false positives from stromal background or artifacts. Both approaches yielded comparable results overall, with minor variations depending on tissue context and marker expression. Therefore, both datasets were retained to enable downstream statistical comparison and determine the most robust quantification method. Positive cell detection was performed using optimized parameters, with thresholds validated by a pathologist. Results were expressed as the density of positive cells per μm^2 [8,9].

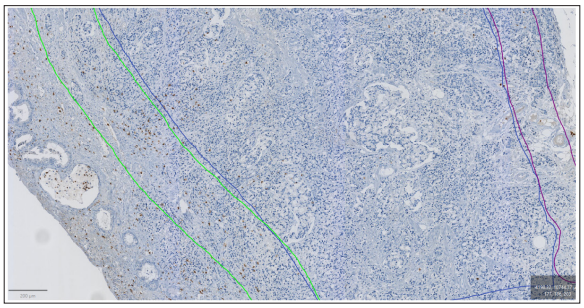

Figure 1: Representative annotation of selected tumor regions used for immunohistochemical quantification. The luminal interface is shown in green, the deep tumor core in blue, and the invasive front in purple. These regions were manually delineated on each slide

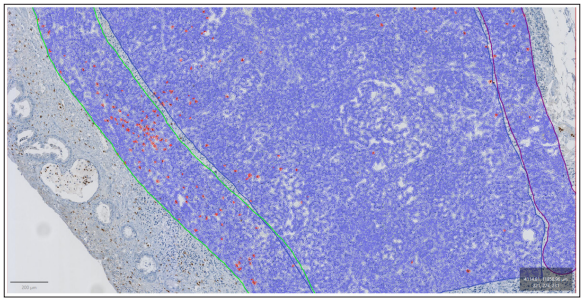

Figure 2: Representative annotation of selected tumor regions after detection with the optical density sum algorithm

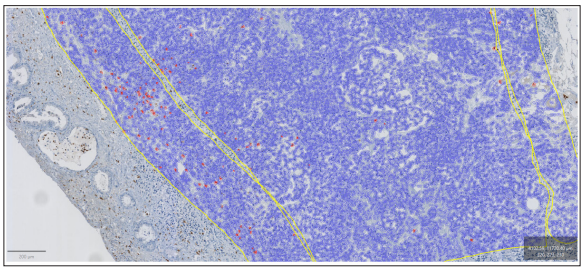

Figure 3: Representative annotation of selected tumor regions after detection with the hematoxylin algorithm

Statistical Analysis software

For the statistical analysis of the raw data, the following software were used:

- Microsoft Excel: For the division in groups and basic statistics such as median, mean.
- Graphpad Prism: For Survival Curves and their statistical analysis, Wilcoxon Test.

Measuring unit « Number of positive cells per square micrometer (cells/μm^2) ».

Results

The results of this study revealed new information regarding the role of TANs, Arginase 1 and perineural invasion in terms of predictivity, prognosis and histopathology.

The main focus of the experiment was to analyze the concentrations of Arginase-1 and TANs (Through CD 66b staining).

In the following graphs, it can be seen how in patients with perineural invasion, patients who didn't undergo any neoadj therapy with chemotherapy (so patients who underwent either CTRT or CT) have a statistically significant higher concentration of Arginase-1 (this trend seen also in the patients whose tumor do not have perineural invasion). This possibly linking the anti-tumoral and Arginase decreasing effect of chemotherapy (Figure 4 and Figure 5). This trend of higher Arginase-1 in patients whose tumor has no perineural invasion is mirrored by a statistically significant higher concentration of tumor associated neutrophils (TANs) in those patients who didn't undergo the therapy (CT or CTRT) (Figure 6) . In figure 5 the graph shows a statistically significantly better DFS in patients whose tumor had no perineural invasion who underwent therapy (either CT or CTRT) vs those patients who had perineural invasion.

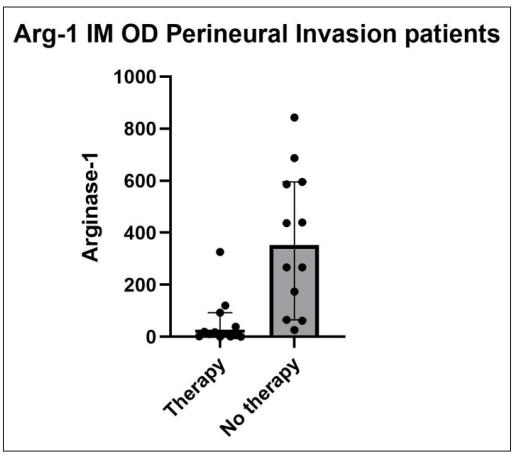

Figure 4: Arginase 1 Im Margin Wilcoxon test, plot graph showing Median and 95 % CI. OD= optical density measurement.

One sample Wilcoxon test	A Therapy	B No therapy
Theoretical median	0.000	0.000
Actual median	12.48	352.2
Number of values	13	12
Wilcoxon Signed Rank Test		
Sum of signed ranks (W)	66.00	78.00
Sum of positive ranks	66.00	78.00
Sum of negative ranks	0.000	0.000
P value (two tailed)	0.0010	0.0005
Exact or estimate?	Exact	Exact
P value summary	***	***
Significant (alpha=0.05)?	Yes	Yes
How big is the discrepancy?		
Discrepancy	12.48	352.2
95% confidence interval	1.377 to 92.88	65.59 to 595.6
Actual confidence level	97.75	96.14

Statistical analysis **Figure 4**

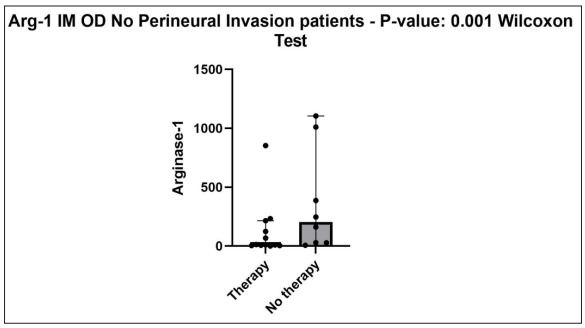

Figure 5: Arginase 1 Im Margin Wilcoxon test, plot graph showing Median and 95 % CI. OD= optical density measurement. Beneath, DFS

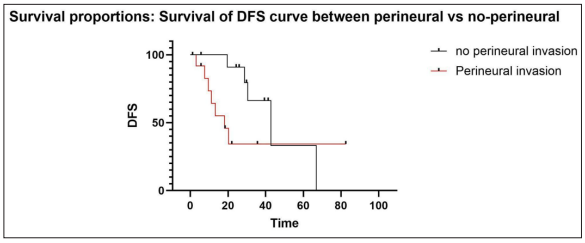

Figure 6: DFS curve showing pathology DFS of patients who underwent therapy (either CT or CTRT) having in the tumor perineural invasion vs no perineural invasion.

Survival Curve comparison	A	B
Table Analyzed	DFS curve between perineural vs no-perineural invasion	
Logrank (Mantel-Cox) test		
Chi square	2.478	
df	1	
P value	0.1154	
P value summary	ns	
Are the survival curves sig different?	No	
Gehan-Breslow-Wilcoxon test		
Chi square	6.422	
df	1	
P value	0.0113	
P value summary	*	
Are the survival curves sig different?	Yes	
Median survival		
no perineural invasion	42.8000	
Perineural invasion	18.1000	
Ratio (and its reciprocal)	2.365	0.4229
95% CI of ratio	0.7505 to 7.451	0.1342 to 1.332
Hazard Ratio (Mantel-Haenszel)	**A/B**	**B/A**
Ratio (and its reciprocal)	0.3820	2.618
95% CI of ratio	0.1153 to 1.266	0.7899 to 8.675
Hazard Ratio (logrank)	**A/B**	**B/A**
Ratio (and its reciprocal)	0.4172	2.397
95% CI of ratio	0.1291 to 1.348	0.7418 to 7.744

Analysis of **Figure 6**

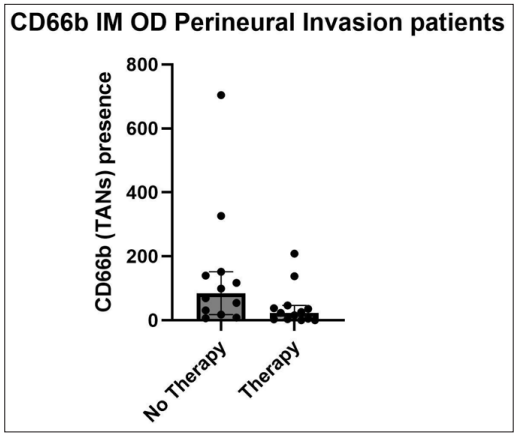

Figure 7: CD66b Im Margin Wilcoxon test, plot graph showing Median and 95 % CI. OD= optical density measurement.

One sample Wilcoxon test	A No Therapy	B Therapy
Theoretical median	0.000	0.000
Actual median	84.27	22.80
Number of values	12	13
Wilcoxon Signed Rank Test		
Sum of signed ranks (W)	78.00	66.00
Sum of positive ranks	78.00	66.00
Sum of negative ranks	0.000	0.000
P value (two tailed)	0.0005	0.0010
Exact or estimate?	Exact	Exact
P value summary	***	***
Significant (alpha=0.05)?	Yes	Yes
How big is the discrepancy?		
Discrepancy	84.27	22.80
95% confidence interval	17.54 to 151.6	2.850 to 46.54
Actual confidence level	96.14	97.75

analysis of **Figure 7**

Further analysis showed that perineural invasion is associated in a statistically significant way to a higher concentration of TANs (CD 66b) and Arg-1. Supporting the neuro-immuno-onocological theory that perineural invasion leads to an immuno-depressive tumor microenvironment associated with TANs, which could possibly be of N2 polarization and associated with an increase in Arginase-1. Also, analysis revealed how CD6b and Arg-1 are statistically significantly higher in the tumors of patients who didn't undergo any therapy. Supporting the concept of neutrophils polarization into N1 caused by therapy (eg 5-FU therapy).

Conclusion and Discussion

In this paper a review on rectal cancer, its features and terminology and previously published data on the OR1 analytical experiment are discussed.

The results of the OR1 study reveal novel knowledge on Arginase-1 and neutrophil in rectal cancer.

No-neoadjuvant therapy patients have a statistically significant higher concentration of Arginase-1 in respect to patients treated with neoadjuvant therapy.

The feature of perineural invasion in rectal cancer especially seems to be a very strong factor correlated with this difference, which is directly proportional with the concentration of neutrophils in the tumor (both in the marginal zone and in the deep core of the tumor tissue sample). Perineural invasion was taken as an independent factor of negative prognosis.

In fact, Arginase-1 seems to be a worsening factor to prognosis. This molecule has been found in literature to be a potent immunodepressor (eg. T cell immunodepression), and Arginase inhibitors are currently being thought of as a potential new category of drugs potent against cancer and in cancer immunotherapies. In fact inhibiting Arginase shows a strong boosting of immune reactions against cancer. When comparing patients whose tumors had no perineural invasion with those with perineural invasion, those without had a much higher Arginase-1 concentration.

Presence of neoadjuvant therapy changes also the impact of perineural invasion presence on the concentration of Arg-1 within the tumour, inverting the relationship seen in the group as a whole (perineural invasion leading to higher Arg-1).

Furthermore, DFS was statistically significantly worse in the group of patients who were not treated with neoadjuvant therapy (when all these patients' tumors had no perineural invasion as a common denominator). When dividing these same patients according to treatment modality, only patients who received only radiotherapy had a statistically significant better prognosis in terms of DFS compared to patients who had no neoadj treatment (patients who also or only receive chemotherapy had a similar prognosis to those without treatment neoadj).

In patients with perineural invasion, patients who didn't undergo any neoadj therapy with chemotherapy (so patients who underwent either CTRT or CT) have a statistically significant higher concentration of Arginase-1 (this trend seen also in the patients whose tumor do not have perineural invasion). This possibly links the anti-tumoral and Arginase decreasing effect of chemotherapy. This trend of higher Arginase-1 in patients whose tumor has no perineural invasion is mirrored by a statistically significant higher concentration of tumor associated neutrophils (TANs) in those patients who didn't undergo the therapy (CT or CTRT). A statistically significantly better DFS in patients whose tumor had no perineural invasion who underwent therapy (either CT or CTRT) vs those patients who had perineural invasion.

To keep in mind is the impact of RT and CT (such as 5-FU in the protocol of FOLFIRI or FOLFOX) in the neutrophil polarisation and tumour microenvironment.

Without taking in consideration the presence of neoadj therapy, the fact that perineural invasion seems to be correlated with this high level of Arginase could be that the schwann cells tumor interaction (including the recalling of MDSC) act as immunosuppressors for instance through the increase in production of TGF beta.

Neoadjuvant therapies, such as chemotherapy, can activate an antitumoral N1 polarization in neutrophils by causing DNA damage (activating cGAS-STING pathway for IFN-β) or inducing ROS and chemokines (like IL-8/CXCL5), therefore leading to a N1 phenotype that kills tumor cells via cytotoxic substances, releasing pro-inflammatory cytokines, and activating other immune cells [6]. The seen inversion of TANs and Arginase-1 concentration in the tumor in patients who underwent neoadj therapy (including chemotherapy) possibly shows an activation of an antitumoral reaction leading to a decrease of N2 neutrophils (the presumed population assessed which is associated with the production of Arginase-1, which also follows the concentration trends of TANs). In fact, Arg-1 has been shown to be associated with pro-tumoral and tumor progression and Arg-1 inhibitors have been shown to be promising new frontiers in therapy.

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