***Fusobacterium nucleatum*, rectal cancer and radiotherapy**

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The interplay between the microbiome and both the evolution and treatment of colorectal cancer (CRC) is a hot topic. The colonic microbiota is a diverse ecosystem that interacts symbiotically with the host to sculpt a robust immune response [1]. However, in CRC the relative abundance of the species and metabolites that together form the microbiota, differ significantly from the healthy bowel (reviewed extensively elsewhere [2-6]). One of the most consistently enriched bacteria in faecal samples from patients with CRC is *Fusobacterium nucleatum* (FN) [7, 8], levels of which have been positively corrected with microsatellite instability and poor prognosis [9]. *In situ* imaging has shown that FN can be localised in biofilms, which are dense microbe aggregates enclosed in an exo-polymeric matrix [10, 11], and has been cultured both from primary colon tumours and matched liver metastases [12].

In the triad between altered microbiome, CRC and dysregulated immune responses, critical questions include what is cause and what is consequence? Using the $APC^{Min/+}$ mouse model of colon cancer, Kostic et al., showed that gavaging mice with FN lead to a heightened tumour burden [13]. This was accompanied by an increased proportion of putatively pro-tumourigenic myeloid cells that were able to suppress the proliferation of CD4+ T cells in an *ex vivo* co-culture system. *In vitro*, treating CRC cell lines with FN suppressed the activity of NK cells which are thought to be protective [14]. Thus there is some evidence of a causative role of FN in driving CRC and promoting the pro-tumourigenic immune milieu.

FN therefore possess interesting promise as an element of the tumour microenvironment which may be amenable to therapeutic manipulation and as a biomarker. In this study by Garazi et al., the authors probed whether intra-tumoural levels of FN can predict response or relapse after neoadjuvant chemoradiotherapy (CRT) in locally-advanced rectal cancer [14]. Utilising a biobank of FFPE tissue from patients with rectal adenocarcinoma pre- and post-CRT, they adapted an RNA *in situ* hybridisation assay for FN which was first published in 2017 [12] to make it automated. Slides were scanned prior to annotating tumour areas and analysing the number of bacterial cells per mm^2 tissue using Visipharm image analysis. The accuracy of this protocol was determined by correlating the data with FN qPCR results in both infected HCT116 colon cancer cells and FFPE tissue sections (n=71 patients; qPCR data from [12]). Although not perfect, qPCR and imaging results did positively correlate and FN automated quantification was successful in 251/254 tissue samples.

FN was primarily localised at the luminal surface of the tumour and in dense aggregates which may well have been part of a biofilm, a finding that could be probed by incorporating a pan-bacterial stain. Baseline FN positivity did not differ according to sex, age or disease stage. Overall 57% of untreated rectal cancers (n=127) were FN positive in this study. Prior qPCR data suggested low rates in rectal cancer (e.g. 2.5% of rectal cancer tumours (n=157) classified FN-"high" in one study [15]) compared to much higher rates in the right colon where FN-positivity varies from 12.5% (n=1102) [15] to 74% (n=149) [16, 17] and 94% (n=544) [17], the variability of which has been discussed elsewhere [18].

Levels of FN in untreated samples did not predict pathological response and neither pre- nor post-treatment levels of FN were associated with CRT responsiveness. The utility of FN as a biomarker to predict treatment responsiveness in rectal cancer is therefore lacking. However, persistent FN-positivity in resection specimens after radiotherapy could predict relapse. Indeed, 15/68 of the samples probed remained FN positive post-CRT, a finding that was associated with a significantly increased risk of relapse at follow-up as compared to all post-CRT FN-negative tissue samples (HR=9; 95% confidence interval:3.0-27.2; $p<0.001$). In contrast all tumours that were FN-negative at baseline (n=23) remained negative whilst CRT reduced FN to undetectable levels in a further 30 patients.

The authors hypothesise that this is due to FN reducing levels of CD8+ T cells thereby allowing tumours to evade immune destruction and therefore increasing risk of disease recurrence. In support of this, the number of CD8+ T cells measured in samples from 45 patients by immunohistochemistry was significantly increased in tumours that were FN-negative post-CRT, but not in those that remained FN-positive. Alongside CD8, expression CD3 and PDL1 was probed by was not significantly affected by CRT. Further work is needed to expand upon this correlative observation to investigate the activation status of the cells, especially since an exhausted CD8+ T cell phenotype may have a detrimental impact [19]. It would also be interesting to probe the impact of CRT on immunosuppressive myeloid populations [13] and NK cells [14] which have been shown to be impacted by FN, and whether they correlate with levels of FN.

The key novelties of this retrospective analysis in our opinion are the automated assay for imaging and quantifying FN *in situ*, a focus specifically on rectal rather than CRC, and the potential to delineate patients with a heightened risk of relapse following CRT. This study raises several intriguing questions but does have some limitations that should be considered. Most notably and acknowledged by the authors themselves, sample numbers are low, particularly in the group for whom FN persists after CRT [n=15], and no validation cohort is included. One key advantage to an automated platform is the ability to investigate larger tissue areas in the future and probe the extent of heterogeneity in FN expression within any one tumour sample which may cause sampling error if used clinically. Towards this end, the authors report that there is a trend towards higher FN in untreated endoscopic versus surgical samples ($p=0.13$). Although this study focusses on FN, other Fusobaterium species may contribute to CRC pathogenesis and must not be overlooked [7, 8, 10, 12]. To fully realise the clinical utility of FN, it may be worth looking at species that co-aggregate with FN and/or other bacterial candidates that are enriched in the faecal CRC microbiota such as *Escherichia coli* or *Bacteroides fragilis* [2-6].

Although this study does not aid our ability to predict who will respond to CRT, it may help to stratify patients prone to relapse post-CRT who might benefit from post-resection microbiome-directed adjuvant therapy, such as faecal microbiota transplant [20]. Careful consideration is needed to decipher whether any microbiome modulation would impact the efficacy of routine treatment regimes. Despite being correlative in nature, this study adds further fuel to the hypothesis that FN promotes an immunosuppressive tumour microenvironment that enables metastatic spread. It further prioritises more holistic analysis

of clinical samples which need to include evaluation of the tumour, stroma, immune response and microbiota.

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