



Original research

Integration of lipidomics with targeted, single cell, and spatial transcriptomics defines an unresolved pro-inflammatory state in colon cancer

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► Additional supplemental material is published online only. To view, please visit the journal online (<https://doi.org/10.1136/gutjnl-2024-332535>).

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Received 28 March 2024
Accepted 9 October 2024

ABSTRACT

Background Over a century ago, Virchow proposed that cancer represents a chronically inflamed, poorly healing wound. Normal wound healing is represented by a transitory phase of inflammation, followed by a pro-resolution phase, with prostaglandin (PGE2/PGD2)-induced 'lipid class switching' producing inflammation-quenching lipoxins (LXA4, LXB4).

Objective We explored if lipid dysregulation in colorectal cancers (CRCs) is driven by a failure to resolve inflammation.

Design We performed liquid chromatography and tandem mass spectrometry (LC–MS/MS) untargeted analysis of 40 human CRC and normal paired samples and targeted, quantitative analysis of 81 human CRC and normal paired samples. We integrated analysis of lipidomics, quantitative reverse transcription-PCR, large scale gene expression, and spatial transcriptomics with public scRNAseq data to characterize pattern, expression and cellular localisation of genes that produce and modify lipid mediators.

Results Targeted, quantitative LC–MS/MS demonstrated a marked imbalance of pro-inflammatory mediators, with a dearth of resolving lipid mediators. In tumours, we observed prominent over-expression of arachidonic acid derivatives, the genes encoding their synthetic enzymes and receptors, but poor expression of genes producing pro-resolving synthetic enzymes and resultant lipoxins (LXA4, LXB4) and associated receptors. These results indicate that CRC is the product of defective lipid class switching likely related to inadequate or ineffective levels of PGE2/PGD2.

Conclusion We show that the lipidomic profile of CRC tumours exhibits a distinct pro-inflammatory bias with a deficiency of endogenous resolving mediators secondary to defective lipid class switching. These observations pave the way for 'resolution medicine', a novel therapeutic approach for inducing or providing

WHAT IS ALREADY KNOWN ON THIS TOPIC

- ⇒ Colorectal cancer (CRC) is a complex disease characterised by the imbalance between proinflammatory and pro-resolving processes resulting in chronic inflammation akin to a poorly healing wound, with resultant immunosuppression and excessive cellular proliferation.
- ⇒ Lipid mediator class switching from arachidonic acid-based, pro-inflammatory mediators (leucotrienes) to specialised pro-resolving mediators has been identified as an essential mechanism for resolving inflammation in normal wound healing.
- ⇒ To date, studies that interrogate the cellular and molecular mechanisms that govern dysregulated CRC lipid metabolism and its impact on the immune tumour microenvironment (TME) are very limited.

resolvins to mitigate the chronic inflammation driving cancer growth and progression.

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in the world and the second leading cause of cancer-related deaths in the USA.^{1–3} Despite the continued poor survival rate for metastatic CRC, the most exciting treatment advances have been related to modification of the immune tumour microenvironment (TME). Notably, microsatellite unstable (MSI-H) rectal cancer has been recently treated with checkpoint inhibitors alone—without surgery, chemotherapy or radiotherapy—producing



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To cite: Soundararajan R, Maurin MM, Rodriguez-Silva J, et al. Gut Epub ahead of print: [please include Day Month Year]. doi:10.1136/gutjnl-2024-332535

WHAT THIS STUDY ADDS

- ⇒ Quantitative LC–MS/MS analysis of 81 human CRC and normal paired samples demonstrated a significant pro-inflammatory bias with little if any evidence of active, endogenous resolution of inflammation.
- ⇒ We demonstrated a marked defect in lipid class switching in CRC tumours with increased expression of genes encoding pro-inflammatory lipid mediators and their receptors, but absence or low expression of key resolving genes (ALOX15, EPHX1), as measured by quantitative reverse transcription-PCR, as a mechanism for the observed inflammatory bias. Moreover, we found that ineffective levels of PGE2/PGD2 (~70% of cases with T<N levels) and poor correlation with LXB4, as well as inefficient PGE2/PGD2 signalling (secondary to low PTGER2, 3, 4 levels in T vs N) may be responsible for defective lipid class switching.
- ⇒ scRNA-seq analysis identified tumour-associated macrophages in the TME as the source for many pro-inflammatory, lipid mediator producing genes. Spatial transcriptomics analysis of colon tumours showed a strong coexpression of ALOX5/ALOX5AP known to encode pro-inflammatory leucotrienes in high versus low LTB4 producing CRC tumour samples and decreased expression of some genes involved in the synthesis of PGE2, lipoxins, resolvins and other resolution mediators.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- ⇒ In this study, we demonstrate that one of the most promising and often overlooked treatment options for CRC is related to harnessing the immune potential of TME.
- ⇒ CRCs have a clear pro-inflammatory bias leading to dysregulated eicosanoid pathway. This study suggests new therapeutic approaches for CRC could induce lipid class switching via production of *endogenous* specialised pro-resolving mediators (SPMs) (eg, CBD oil, AKBA, celastrol) or via provision of *exogenous* SPMs (eg, SPMs containing PDX and resolvins) that bypass deficiencies in ALOX12/15 to promote resolution of inflammation *without* immunosuppression. We believe that our findings are not limited to CRC, but are likely generalisable to many other tumour types.

remarkable, and complete pathological responses, suggesting there is substantial advantage in harnessing the immune potential of the TME.⁴

An emerging area of cancer research focuses on understanding the mechanisms involved in dysregulated lipid metabolism in CRC and its impact on the immune TME. scRNA-seq studies have recently suggested that much of lipid metabolism and its regulation occurs in the TME rather than the epithelial tumour and normal cell compartments.⁵ We now know that lipids play crucial roles in cell signalling, energy storage and membrane structure, and that their dysregulation can contribute to cancer development and progression.⁶ Lipidomic analysis of CRC tissues and plasma suggest that distinct lipid profiles are associated with disease stage and prognosis. There is an unmet need to understand the cellular and molecular mechanisms that govern dysregulated lipid metabolism in the CRC TME.

The western diet, low in fibre and rich in omega-6 fatty acids such linolenic acid (LA), a metabolic precursor of arachidonic acid (AA), has been strongly linked to chronic inflammation

and CRC development and progression, whereas the omega-3-rich diet has been proposed to have the opposite effect.^{7–9} We hypothesise that CRC may be driven by the imbalance of pro-inflammatory>pro-resolving lipid mediators, resulting in chronic inflammation, tumour growth and progression. This imbalance may be governed by an excess of AA and over-expression of key enzymes in the AA pathway (ALOX5, ALOX5AP, LTA4H, online supplemental figure 4, AA metabolic pathway). Chronic inflammation is attributed to the recruitment and maintenance of resident tumour-associated macrophages (TAMs) that are thought to be one of the principal cell types responsible for contributing to dysregulated lipid metabolism in the TME. Cancer has been characterised as a poorly healing chronic wound. During the initial phase of normal wound healing, prostaglandins and leukotrienes (LTs) are synthesised and orchestrate an anti-inflammatory response wherein PGE2/PGD2 induce ALOX15 expression causing a switch from pro-inflammatory mediators (LTs) to pro-resolving lipid mediators (lipoxins, resolvins, maresins, protectins and maresins). This process is called lipid mediator class switching (LCS) and has been identified as an essential mechanism for resolving inflammation.¹⁰ This LCS process appears to be deficient in CRC, resulting in the majority of tumours sustaining a chronic inflammatory state. In this study, we have profiled the untargeted and targeted lipid landscape and associated gene expression of CRC tumours paired with normal tissue counterparts, with a particular focus on the AA pathway. We have proposed novel therapeutic strategies to overcome the observed dysregulated lipid metabolism in the TME.

MATERIALS AND METHODS**Study design**

The demographic data for the patient samples are described in table 1. The inclusion criteria included subjects with biopsy-proven primary and metastatic, majority were microsatellite stable (MSS) CRC. The exclusion criteria included patients less than 18 years of age, with active infections of one or more of the diseases such as tuberculosis, HIV, hepatitis B and hepatitis C or unable to provide written informed consent.

Patient and public involvement

Patients or the public were not involved in the design, or conduct, or reporting, or dissemination plans of our research, however, our knowledge of the relationship between their likely diets and diet preferences and their disease was taken into consideration when we planned to examine the omega-6 and omega-3 lipids and lipid mediators. Individual results will not be disseminated to patients as the data were deidentified.

Sample collection and processing

Tumours were resected per standard of care from two different performance sites (TGH, CHTN). Normal mucosa was harvested ~10 cm away from the tumour that was histologically without evidence of inflammation (documented by Ki67 and TP53 staining, online supplemental figure 11). Normal liver was used as the control for liver metastases (n=13). The freshly obtained samples were flash frozen within 30 min in liquid nitrogen for lipidomics analysis, homogenised in 1 mL of Trizol (Cat. No. 15596026, Thermo Fisher) or formalin-fixed for downstream analyses.

Histochemistry analysis

Tumour and normal samples were fixed in 10% neutral buffered paraffin for 48 hours, processed and paraffin-embedded. A

Table 1 Patient demographics

(n=81, 2 patient samples without any demographic data)		
Patient characteristics	Age, mean±SD	64.025 (\pm 13.08)
	Age, range	30–85
Sex		
Male	44 (55.70%)	
Female	33 (41.77%)	
Not reported	2 (2.53%)	
BMI (kg/m ²)	29.94 (\pm 7.25)	
Not reported	28 (35.44%)	
BMI range (min–max)	18.90–52.90	
Cancer characteristics	Diagnosis	
	Colon cancer	63 (79.75%)
	Rectal cancer	14 (17.72%)
	Not available	2 (2.53%)
	Primary tumour versus metastasis	
	Primary	67 (84.81%)
	Metastasis	13 (12.66%)
	Not available	2 (2.53%)
	Tumour location	
	Right-sided	32 (40.51%)
	Ileocecal/cecal	10 (31.25%)
	Ascending	20 (62.50%)
	Transverse	2 (6.25%)
	Left-sided	39 (49.37%)
	Descending	4 (10.26%)
	Sigmoid/rectosigmoid	21 (53.85%)
	Rectum	14 (35.90%)
	Liver	2 (2.53%)
	Not available	2 (2.53%)
Tumour characteristics	Stage*	
	0	1 (1.27%)
	I	6 (7.59%)
	II	26 (32.91%)
	III	22 (27.85%)
	IV	22 (27.85%)
	Not available	2 (2.53%)
	Microsatellite status†	
	MSI-L/MSS	55 (69.62%)
	MSI-H	12 (15.19%)
	Not available	12 (15.19%)
	T stage‡	
	TX	2 (2.53%)
	T0	1 (1.27%)
	T1	5 (6.33%)
	T2	6 (7.59%)
	T3	45 (56.96%)
	T4	17 (21.52%)
	Not available	3 (3.80%)
	N stage	
	N0	37 (46.84%)
	NX	2 (2.53%)
	N1	25 (31.65%)
	N2	12 (15.19%)
	Not available	3 (3.80%)
	M stage	
	M0	54 (68.35%)
	M1	22 (27.85%)
	Not available	3 (3.80%)
	Recurrence	
	Yes§	6 (7.60%)

Continued

Table 1 Continued

No	70 (88.61%)
Not available	3 (3.80%)
Grade¶	
1	8 (10.13%)
1–2	2 (2.53%)
2	50 (63.29%)
2–3	4 (5.06%)
3	9 (11.39%)
Not available	6 (7.60%)

n=81, two patient samples without any demographic data.

Values are reported as mean±SD or absolute values (%), unless otherwise indicated.

*Stage is the extent of cancer at presentation, and it has clinical, prognostic, pathological and treatment implications. Every cancer has a unique staging system based on its individual tumorigenesis. In our experiment, pathological stage was used for colon cancer and clinical stage prior to neoadjuvant therapy for rectal cancer, to ensure the most accurate and complete baseline stage. Stage is assigned using the TNM staging system as outlined in the universally accepted American Joint Committee on Cancer (AJCC) manual for colorectal cancers.⁵⁴

†Microsatellite status is the pathological presence or absence of mutations in a tissue specimen resulting in the deficiency of DNA mismatch repair functionality. MSI-L/MSS is the absence of these mutations, or intact DNA mismatch repair function, and MSI-H is the presence of mutations that have an increased correlation with inherited predispositions to development of colorectal cancers. The microsatellite status of a tumour has been proven to have an impact on overall clinical prognosis and responsiveness to chemotherapeutic agents.

‡TNM corresponds to tumour depth of invasion, absence or extent of lymph node involvement, and metastasis status. An alphanumerical value is assigned to each letter based on the AJCC manual for colorectal cancer in each category.

§Patient specimen used was a recurrent cancer, despite being previously treated according to the gold standard of care.

¶Tumour grade is a pathological designation of the degree of differentiation of the observed cancer cells, ranging from well-differentiated (1) to poorly differentiated (3). The more differentiated a colorectal cancer cell, the more it resembles its original tissue architecture (colon vs rectum). Degree of differentiation contributes to the heterogeneity of colorectal cancer is considered when determining a patient's overall pathological and clinical prognosis.

BMI, body mass index; MSI-H, microsatellite instability-high; MSI-L, microsatellite instability-low; MSS, microsatellite stable.

5 µm paraffin section was cut and stained with a standard H&E staining protocol at Tissue Core Histology lab at Moffitt Cancer Center (MCC). The images were acquired at Analytical core at MCC using Aperio Imager. The images were processed using Adobe Photoshop V.CS7.

Immunohistochemistry analysis

Immunohistochemistry (IHC) analysis for p53 and Ki67 markers was performed for normal mucosa samples (n=20) by a commercial pathology lab using formalin-fixed, paraffin-embedded sections using tissue-Tek genie anti-p53 mouse monoclonal (BP53.12) and anti-Ki67 mouse monoclonal (GM010) antibodies (Sakura Finetek, USA) as per manufacturer's instructions. The images were acquired by Agilent's C10 imager and images were processed using Adobe Photoshop V.CS7.

Quantitative LC-MS/MS analysis of targeted lipids

Lipids from colon tumours (n=81) and matched controls (n=81) were extracted and supernatant injected into the Sciex QTRAP 6500+Triple quadrupole mass spectrometer. Samples were run using Analyst software V.1.7 and quantified by Analyst software V.1.7 as described.^{11 12} Peak identification, peak annotation and peak area integration were performed keeping Minimum peak width 3-point, minimum peak height 100 points, Gaussian smooth width 0 points, baseline subtract window 2.0 min and peak splitting 2 points. The relative retention time was used as a reference from the standard calibration curve for $\pm 2.5\%$. Precursor mass tolerance and fragment mass tolerance were at 0.4 Da, intensity threshold was at 0.05. The fragment ions from

the m/z values were identified for each compound using the Sciex OS library.

Quantitative LC-MS/MS analysis of non-targeted lipids

The lipid extraction from colon tissues (n=40) and matched normal mucosa controls (n=40), a subset of the 81 paired tumour/normal samples used in targeted lipid analyses, were analysed using a prominence UFC system (Shimadzu, Kyoto, Japan) coupled with a Linear Quadrupole Orbitrap mass spectrometer by the method established in earlier studies.^{13 14} The raw data obtained from the liquid chromatography and tandem mass spectrometry (LC-MS/MS) was processed for lipid annotation, peak extraction and peak alignments using MS-DIAL software (V.4.9). The following parameters were set in the MS-DIAL for data processing: peak height cut-off (1000 amplitude), smoothing level (3 scans), minimum peak width (5 scans), mass slice width (0.1 Da), sigma window (0.5) and signal intensity fivefold (>blank). The MS tolerance and retention times were 0.015 Da and 0.1 min, respectively. All the identified lipids were confirmed by their MS/MS spectral fragmentations which are closely matching with the in-built library of MS DIAL. Further, the peak area of each lipid metabolite was integrated using Xcalibur.

Generation of heatmaps for lipid metabolites

Heatmaps for targeted lipids were generated from geometric normalised data for colon tumours and normal matched controls. For the untargeted lipids, heatmap was generated using the z-score normalised for visualisation purposes. All heatmaps were generated using R (V.4.2.3).¹⁵

Secondary analyses of human CRC tumour databases

Gene expression and Spearman correlation analyses of human CRC tumours were performed using the Affymetrix gene expression dataset of Merck-Moffitt CRC (n=2373)¹⁶ or using the Affymetrix gene expression dataset of the Marisa CRC dataset (n=585).¹⁷ CMS1*, CMS2*, CMS3* and CMS4* scores, that are designated to measure a propensity of a tumour to fall into CMS1, CMS2, CMS2 and CMS4 classes, respectively, were generated for each of Merck-Moffitt 2373 CRC tumours as we previously reported.¹⁸ The analyses were performed using GraphPad Prism V.10 (La Jolla, CA). A public database (Broad) was used to access scRNAseq data from 28 MMRp and 34 MMRd CRC patients. We interrogated The Cancer Genome Atlas Colon Adenocarcinoma Collection (TCGA-COAD) for samples with matched normal controls using the TCGA Application Programming Interface. Duplicate samples were removed.

Spatial transcriptomics (10x Genomics, Visium) analysis of colon tumours

Formalin-fixed paraffin-embedded tumour samples (n=8) were subjected to spatial transcriptomics analysis using the 10x Genomics 10x Visium platform and the libraries were sequenced using Nova Seq X plus. Data was analysed using Space Ranger and data visualised using 10x Genomics Loupe browser.

Statistical analysis

For untargeted lipid analysis, paired Student's t-test was used. Data was visualised as box and whisker plots. For targeted lipidomics data, the raw values (pg/50mg) initially obtained from the quantitated LC-MS/MS were logarithmically transformed to the base 10 and graphed as tumour values subtracted from their matched normal controls for each lipid mediator studied. The

two-tailed non-parametric Wilcoxon signed-rank test was used to assess the statistical significance of the difference between the paired tumour and normal samples.

For quantitative reverse transcription (qRT)-PCR analyses, we used a paired, two-tailed Student's t-test. The mean with SEM is reported for the bar graphs and individual values for the patient samples are represented as a vertical scatter. Spearman's correlation was performed for the secondary analysis of published databases (Merck-Moffitt and Marissa) using GraphPad Prism V.10.0.2. A detailed description of the statistical test used, and the statistically significant values are given in the figure legends. P<0.05 was considered as statistically significant.

A detailed description of all reagents and methods are provided in online supplemental file.

RESULTS

CRC patient samples acquired for lipidomics and qRT-PCR analyses

162 human samples from two different institutions were analysed representing 81 paired tumours and matched normal mucosa, representing a diverse set of CRCs (80% colon and 18% rectal tumours), 85% primary tumours and 13% metastatic tumours comprising both right and left colon. The majority of tumours collected were stage 2–4 and MSS. The median age of the CRC patients ranged from 30 to 85 years, 55.7% were males and 33% were females with body mass index (18.90–52.90) (table 1).

Non-targeted lipid analysis identified enrichment of LA and AA containing structural phospholipids in CRC tumours

We applied an unbiased and untargeted approach to profile the comprehensive lipidome of CRC tumours (figure 1). The orthogonal partial least squares discriminant analysis showed distinct lipid compositions between the tumour (n=40) and matched normal mucosa (n=40) (figure 1A). The volcano plot analysis of the colon tumours depicts the number of significantly altered lipid species in tumour versus the matched normal controls (figure 1B). About 211 lipid molecular species from five major lipid classes (fatty acyls, glycerolipids, glycerophospholipids, sphingolipids and sterols) were identified. Out of these 42 are downregulated, comprising triacylglycerols, and 11 are upregulated, particularly sphingomyelins (SMs) (SM (d18:1/14:0)) and phospholipids (PLs) enriched with 16:1 fatty acid. Further changes in LA/AA-enriched PLs (mainly phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphoglyceride (PG) and phosphatidylserine (PS)) are depicted in the heatmap (figure 1C) and online supplemental figure 1). The structural lipid species such as (PE (16:0/16:1)) and (PE (O-16:1/16:1)) were increased in colon tumours versus matched controls (figure 1B). Phosphoglycerides (PGs) species such as (PG (16:1/18:2)), (PG (18:0/18:1)) and (PG (22:6/22:6)) showed increased fold change in colon tumours relative to matched normal controls (figure 1B). However, the analysis of *all* the PE and PG subspecies combined, respectively, did not show any significant changes in colon tumour relative to matched normal controls (online supplemental figure 1A). The structural lipid species such as PS and PI, were unchanged except for a slight increase in phosphatidylcholine in colon tumours versus matched normal controls (online supplemental figure 1A). Among LA/AA-derived PLs, AA derived PLs showed increased levels in tumours (figure 1C). Significantly upregulated SMs (figure 1D, online supplemental figure 1C) and downregulated triglycerides (TGs) (online

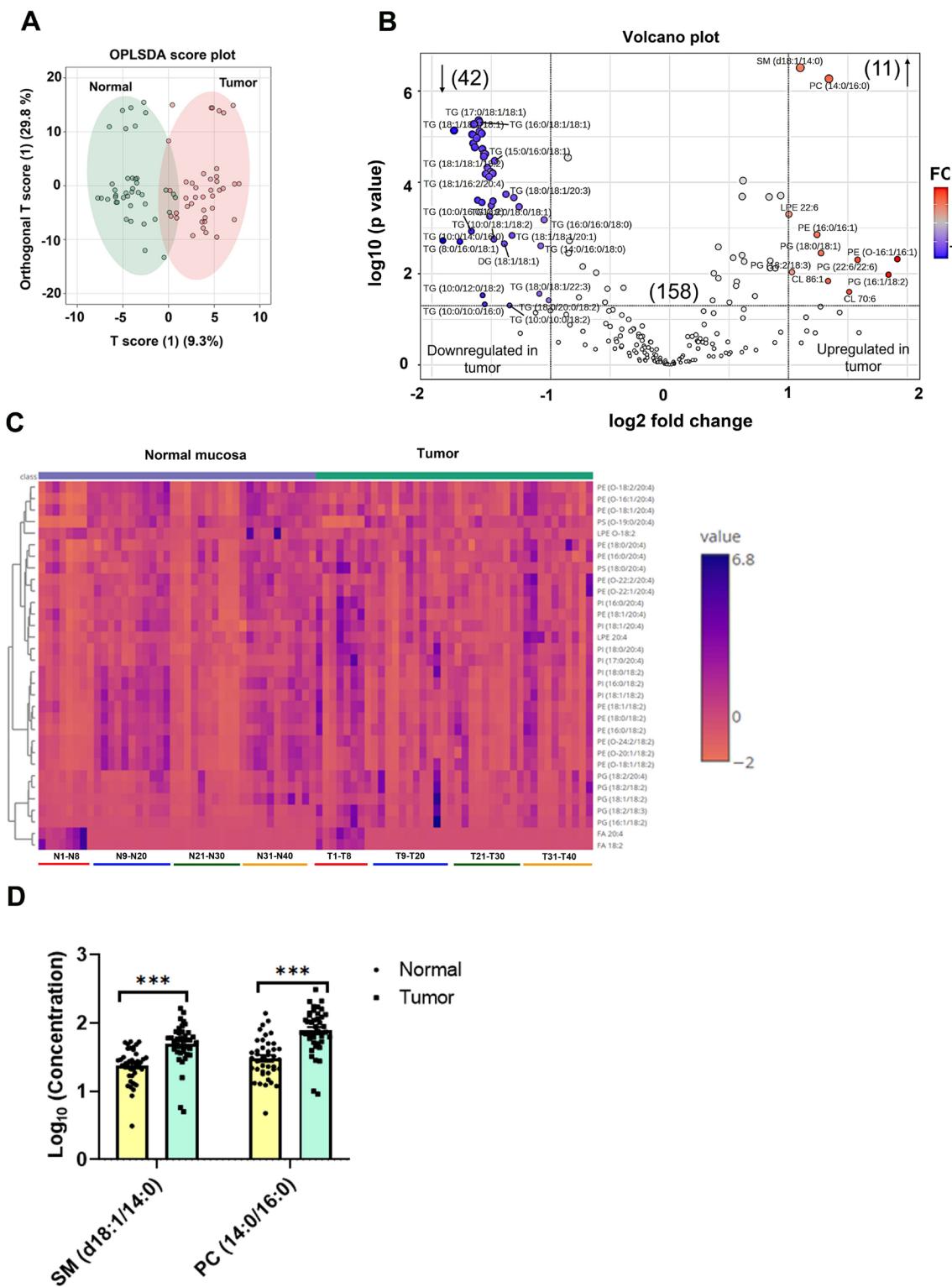


Figure 1 Non-targeted shotgun lipidomic analysis identifies upregulation of linolenic acid and arachidonic acid enriched structural and bioactive lipids in colon tumours. (A) Orthogonal partial least square analysis of colon tumours ($n=40$) and matched normal mucosa ($n=40$) reveals two distinct and tight clusters of tumours and normal controls cleanly separated from each other. The calculated Q2 and R1 values represent the predictability and goodness of fit, R^2X ($p=0.092$, $Q=0.298$), R^2Y ($p=0.407$, $Q=0.131$), Q ($p=0.321$, $Q=0.123$). (B) Volcano plot depicts the upregulated and the downregulated lipid mediators in normal and tumour samples. The p value is shown on the y-axis and the log2 fold change is shown on the x-axis. $P<0.05$ versus normal controls. (C) Heatmap of linoleic acid/arachidonic acid enriched phospholipids (Phosphatidylethanolamine, phosphatidylinositol, phosphoglyceride and phosphatidylserine) in colon tumours (T , $n=40$) and matched normal mucosa (N , $n=40$). The Student's t-test was performed using GraphPad V.10 software. $P<0.05$ versus normal controls. (D) A significant increase in myristic acid bearing sphingomyelin/phosphatidylcholines (SM (d18:1/14:0), PC (14:0/16:0)) in tumours relative to matched normal controls. The relative amount of the lipid mediator is expressed as \log_{10} (concentration) in ng/mg of tissue. Data were expressed as a scatter dot plot. The Student's t-test was performed using GraphPad Prism V.10. $P<0.05$ versus normal controls.

supplemental figure 1D,E) enriched with monounsaturated and polyunsaturated fatty acids were seen. Detailed concentrations of all the annotated lipids, structural formula and retention time are provided in online supplemental table 1. The extracted ion chromatograms of lipid analytes versus standards are shown (online supplemental figure 2A–D). Receiver-operating characteristics curves of lipid subclasses in colon tumour and normal mucosa show percentage specificity versus percentage sensitivity for each untargeted lipids (online supplemental figure 3A–M). The extracted ion chromatogram of TG and SM of a representative tumour normal pair shows specificity of the lipid species identified by LC–MS/MS (online supplemental figure 3N,O).

Quantitative targeted LC–MS/MS lipidomics analysis demonstrated a pro-inflammatory bias in CRC tumours versus matched normal mucosal controls

Our quantitative LC–MS/MS analysis targeted 25 lipid mediators that are byproducts of AA metabolism (online supplemental

figure 4). The heatmap of human CRCs and the matched normal control lipidome ($n=81$ per group) illustrates a distinct shift towards pro-inflammatory versus the pro-resolving lipid mediators in the AA pathway (figure 2A). Specifically, a significant increase in 5-HETE, a byproduct of the ALOX5 pathway, was observed in tumours versus matched controls ($p<0.0001$) (figure 2A,B). The pro-inflammatory lipid products of 5-HETE, called LTs (LTB4, LTC4, LTD4 and LTE4) were all significantly upregulated in colon tumours relative to the normal controls ($p<0.0001$, $p=0.045$) (figure 2C–F). Conversely, the prostaglandins PGD2 and PGE2 show a significant decrease in tumours versus matched normal controls (figure 2A). The charge to mass ratio (m/z) of the various ionic species and the structure of LTB4 is shown (figure 2G,H). The spectra of all the lipid mediators tested in this are provided in online supplemental figures 5A–AD and 6A–AD. The chromatogram and structure of 5-HETE and LTB4 is shown (online supplemental figure 7A–D). The anti-inflammatory lipid mediator, 11-hydroxyeicosapentaenoic acid (11-HEPE) is decreased significantly in colon tumours

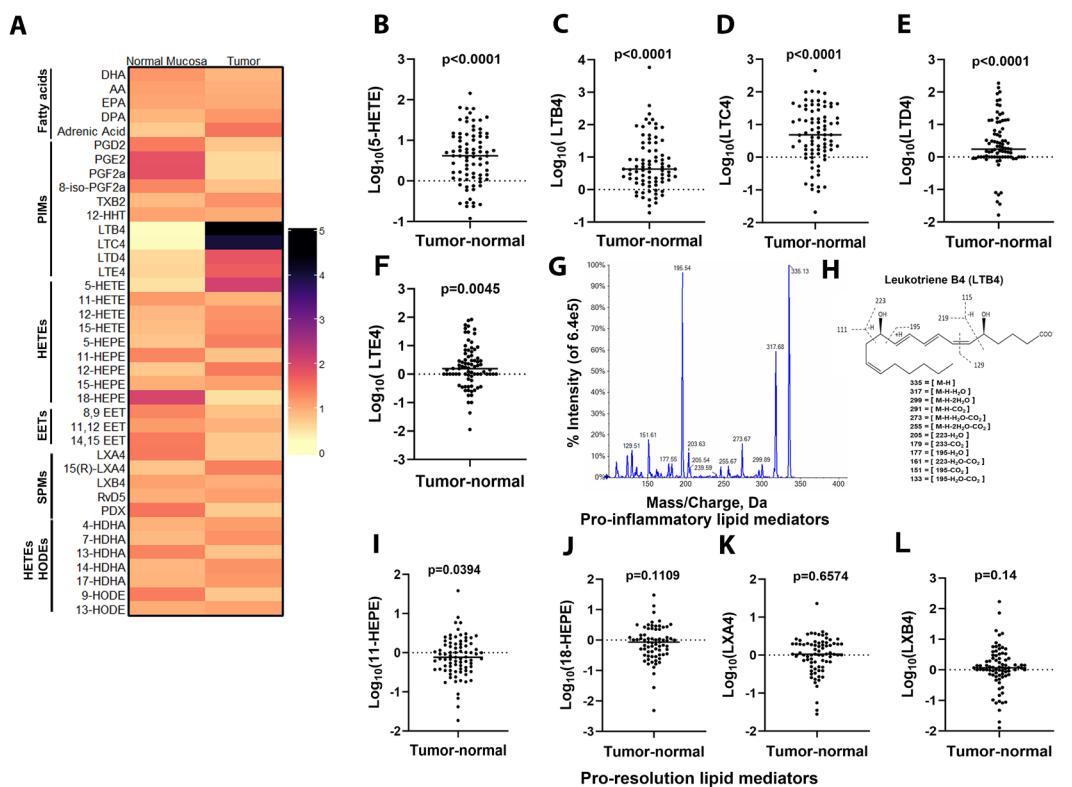


Figure 2 Quantitative targeted lipid analysis shows a significant bias towards pro-inflammatory lipid mediators in colon tumours versus normal matched mucosa. (A) Quantitative LC–MS/MS analysis of targeted lipids in colon tumours and matched normal mucosa controls ($n=81$ per group) is visualised as a heat map generated using the R program. The fatty acid substrates, arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and adrenic acid; pro-inflammatory lipid mediators of ALOX5 pathway (HETEs and leukotrienes) and COX pathway (prostaglandins and thromboxanes) are shown in the heatmap. The pro-resolving lipoxins (LXA4) and (LXB4) are also listed. Lipid mediators that were also tested included the EETs, HEPEs and the HODEs. There is a significant increase in pro-inflammatory lipid mediators including 5-HETE and leukotrienes (LTB4, LTC4, LTD4 and LTE4) in tumours versus controls. Conversely, prostaglandins (PGD2 and PGE2) show a significant decrease in tumours relative to normal controls. (B–F, I–L) Quantitative LC–MS/MS analysis of targeted lipid mediators (pro-inflammatory and pro-resolution) was performed using colon tumours and matched normal controls ($n=81$ per group). (B) 5-HETE, (C) LTB4, (D) LTC4, (E) LTD4 and (F) LTE4, known pro-inflammatory mediators, are significantly increased in tumours relative to matched normal controls. $P<0.0001$ and $p<0.01$ relative to matched normal controls. (G) LC–MS/MS analysis of LTB4 reveals distinct ionic species. (H) The LTB4 structure is shown and the charge to mass ratio (m/z) of the ionic species is listed. (I) 11-HEPE is decreased in tumours versus normal controls. $P=0.0045$ relative to matched normal controls. (J–L) The other pro-resolution mediators 18-HEPE ($p=0.1109$), LXA4 ($p=0.6574$) and LXB4 (0.14) levels remain unchanged in tumours versus matched controls. The data was logarithmically transformed to the base 10. The y-axis represents the tumour values subtracted from their matched normal controls for each of the lipid mediators in our study. The individual values are shown above and below the median. The quantity of lipid mediators was expressed as log₁₀ (pg/50 mg of tissue). The data were analysed statistically using paired two-tailed t-test, Wilcoxon rank sum analysis (GraphPad Prism V.10).

versus matched normal controls ($p=0.0394$), whereas 18-HEPE is unchanged (figure 2I,J). Key pro-resolving lipid mediators such as the lipoxins (LXA4 and LXB4) were low and/or did not show any change in colon tumours versus the normal controls (figure 2K,L). The supporting data for the targeted lipidomics analysis are provided in online supplemental table 2.

Significant upregulation of AA processing pro-inflammatory genes and myeloid immune cell markers in colon tumours suggest an inflammatory milieu in the TME

The genes encoding ALOX5, its binding partner ALOX5AP, the LTA4H enzyme synthesising LTB4, and its receptor LTB4R were all analysed by qRT-PCR. ALOX5 mRNA was significantly upregulated in colon tumours versus matched normal controls ($n=80$ per group, $p<0.0001$) (figure 3A). Similarly, ALOX5AP ($n=77$ per group, $p<0.01$) (figure 3B), LTA4H ($n=60$ per group, $p<0.05$) (figure 3C) and LTB4R ($n=78$ per group, $p<0.001$) (figure 3D) were all increased in colon tumours versus matched normal controls. The genes encoding the leukotriene cysteinyl receptors, CYSLTR1 and CYSLTR2 were unchanged in both groups ($n=15$ per group, respectively) (online supplemental figure 8A,B). LTC4S which encodes leucotriene cysteinyl 4 synthases, was significantly downregulated in the colon tumours versus matched normal controls ($n=15$ per group, $p<0.05$) (figure 3I). Leukotrienes are synthesised by ALOX5 predominantly in immune cells such as macrophages, monocytes, mast cells and neutrophils.¹⁹ ALOX5 has a dual role in initiating pro-inflammatory/pro-resolution signalling depending on the availability and usage of fatty acids substrates (omega-6 vs omega-3 fatty acids).²⁰ We observed a significant increase in CCR2 mRNA that encodes a recruitment receptor for macrophages in colon tumours ($n=78$ per group, $p<0.01$) (figure 3J). In addition, the CCL2 gene encoding a chemokine ligand for CCR2²¹ is also trending as upregulated in colon tumours relative to adjacent normal matched tissues (figure 3K). Dysregulation of TGF β pathway has been implicated in solid tumours.^{22,23} Because TGF β is one of the critical regulators of the immune cells in TME and its pro-inflammatory/pro-metastatic effects are regulated by NF- κ b,²⁴ we analysed TGF β expression in colon tumours. There was a significant increase in TGF β mRNA levels in all colon tumours tested relative to normal controls (figure 3L). Interestingly, LTC4S, the gene responsible for LTC4 synthesis, is highly expressed in mast cells as well as macrophages (figure 3M).

The expression of ALOX5, ALOX5AP, LTA4H, LTB4R, CYSLTR1, CYSLTR2, TGF β , CCLR2 and CCL2 genes was evaluated at single cell resolution using public colon cancer scRNA-seq data ($n=62$).²⁵ ALOX5 is expressed in macrophages, mast cells, B cells, epithelial tumour cells, stroma and plasma cells (figure 3E). Interestingly, ALOX5AP showed a similar pattern of cellular expression as ALOX5 consistent with its collaborative role, except for notably strong expression in immune cells (figure 3F). See spatial transcriptomics data figure 8 for more clarification. LTA4H was present in many cell types including epithelial tumour cells (figure 3G). LTB4R was primarily expressed in macrophages, tumour cells and immune cells (figure 3H). CYSLTR1 was ubiquitously expressed within the different cellular compartments of the TME (online supplemental figure 8C), whereas CYSLTR2 was only present in the myeloid compartment (online supplemental figure 8D). CCR2 was expressed primarily in myeloid cells, plasma cells and T cells (figure 3N) and CCL2 was expressed in myeloid cells and stromal cells (figure 3O). scRNA-seq analysis suggested that TGF β gene expression is ubiquitous in most cell types other than

normal epithelial cells (figure 3P). Top Cluster scRNA-Seq view of different cell types (macrophages, stroma, epithelial tumour cell, immune B and T cells, plasma cells and mast cells) is shown (figure 3Q). We further interrogated the genes using the MMRd and MMRp classification (scRNA-seq data) and did not find any difference (data not shown).

Enrichment of enzymes linked to pro-inflammatory lipids and signalling, CMS classes and TAMs in large Affymetrix microarray datasets of CRC tumours

To validate our experimental clinical findings in a large cohort of CRC tumours, we performed a secondary analysis on a proprietary Merck-Moffitt ($n=2373$ CRC tumours)¹⁶ and Marissa ($n=585$ CRC tumours)¹⁷ Affymetrix microarray datasets. Notably, in both datasets and consistent with our qRT-PCR data, there is strong expression of genes (ALOX5, ALOX5AP, LTA4H, LTC4A and LTB4R) encoding pro-inflammatory lipids/receptors, but weak expression of pro-resolution lipids/receptors (ALOX12, ALOX15 and GPR32) (figure 4A,B). To test if gene expression of lipid metabolising enzymes/receptors was linked to genes measuring inflammatory signalling and EMT, and the consensus molecular subtyping (CMS1-4) scores, we carried out a larger Spearman correlation analysis (heatmap, figure 4). Our analysis revealed that genes linked to AA lipid production and membrane release (PLA2G4A and TNFa), genes related to pro-inflammatory mediators (ALOX5, ALOX5AP, LTA4H, LTC4S) and the receptors (LTB4R, CYSLTR1, CYSLTR2) all appear to be coordinately expressed along with inflammatory biomarkers (TGFB1, NFkB1, NFkB2) and associated macrophage markers (CCL2, CCR2) in colon tumours. TGFB1 gene expression was strongly correlated to EMT, with a strong positive correlation to vimentin and negative expression of CDH1. Notably, genes linked to lipid class switching (ALOX12, ALOX15, IL4, IL13, EPHX1) were weakly correlated with the inflammatory gene programme in colon tumours (figure 4C). Finally, the pro-inflammatory milieu was over-represented in CMS1 (immune activated) and CMS4 (immune inflamed) subtypes (figure 4C) relative to CMS2 (immune desert) and CMS3 (immune excluded) subtypes.¹⁸ The strong correlation between ALOX5 and ALOX5AP in colon tumours confirmed their close functional association (figure 4C). In addition, ALOX5/ALOX5AP showed a strong relationship with NF- κ b and TGF- β signalling. These gene expression data are consistent with our lipidomics data from human CRC tumours and indicate a strong prevalence of pro-inflammatory mediators (5-HETE, LTB4) (figure 2B,C) with extremely low levels of LXA4 and limited LXB4 (figure 2K,L).

Key genes linked to pro-resolving lipid class switching showed extremely low expression in CRC tumours

scRNA-seq analysis of the public colon scRNA-seq data showed that the genes associated with lipid class switching (ALOX12, ALOX15, IL4, IL13) are poorly expressed (figure 5A-D). These data were consistent with spatial transcriptomics data. scRNA-seq analysis shows EPHX1 expression in the myeloid compartment (figure 5E). The genes in the COX pathway, PTGS1 and PTGS2, that synthesise PGH2/PGG2—precursors for the synthesis of PGE2/PGD2—were expressed in macrophages and mast cells (figure 5H,I). Further, prostaglandin E synthase (PTGES) was weakly expressed in tumour cells while PTGES2 was ubiquitously expressed in all sampled cells (figure 5J,K).

Colon

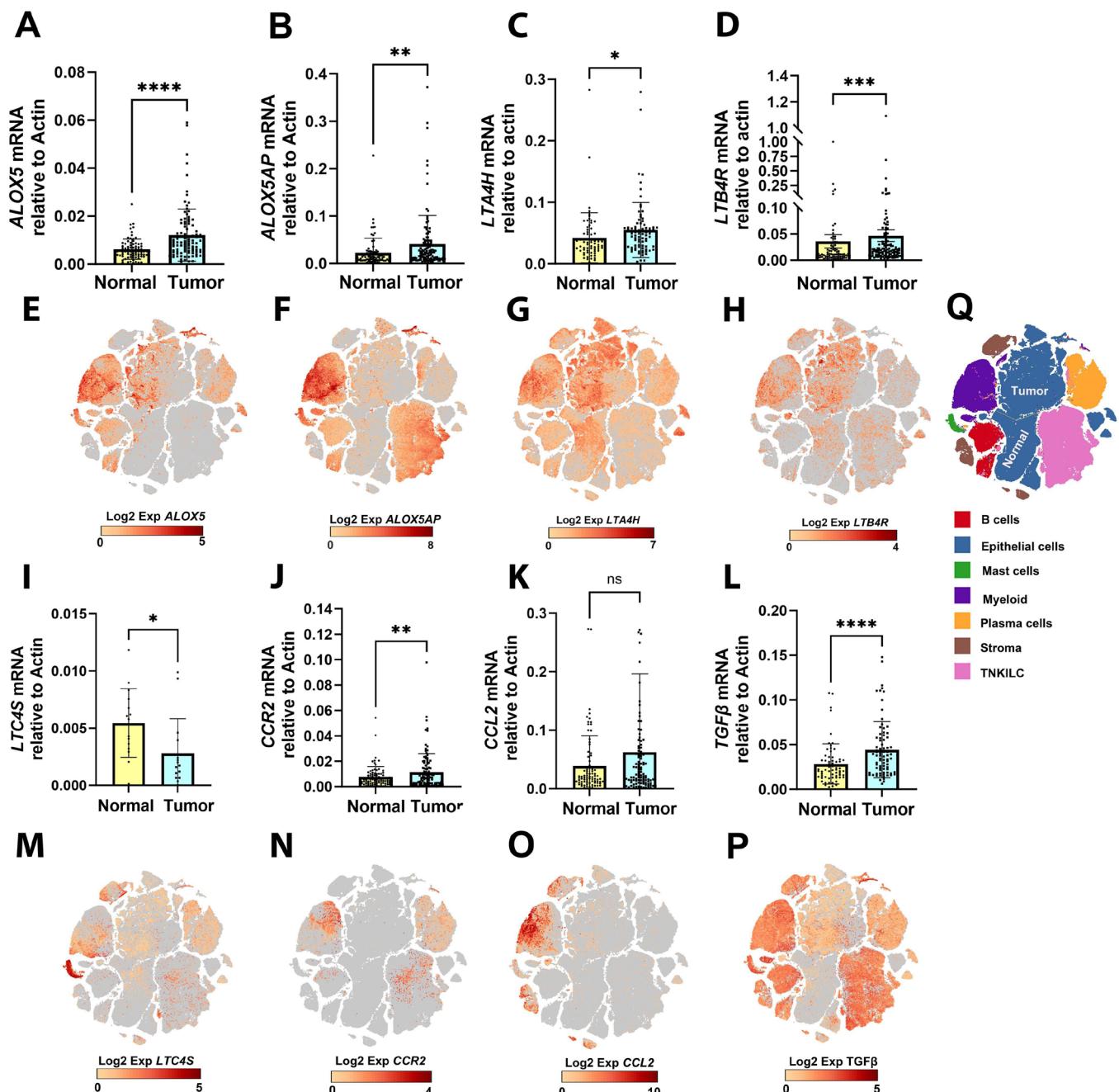


Figure 3 Uptregulation of genes producing LTB4 and its receptor LTB4R and immune cell markers in the myeloid compartment (*CCR2* and *CCL2*) supports the presence of an inflammatory milieau in the colorectal cancer tumour microenvironment. (A) qRT-PCR analysis revealed a significant increase in *ALOX5* mRNA in tumour versus matched normal controls (n=80 per group, ****p<0.00001). (B) *ALOX5AP* mRNA levels were significantly upregulated in tumours relative to the matched normal controls (n=77 per group, **p<0.01). (C) There was an upregulation of *LTA4H* transcript in tumours when compared with matched normal controls ((n=60, *p<0.05). (D) *LTB4R* mRNA was significantly increased in tumours relative to matched normal control (n=78 per group, ***p<0.001). (A–D) For these comparisons, the data is expressed as mean±SEM and graphed as a vertical scatter plot, GraphPad Prism V.10, two-tailed paired Student's t-test. (E–H, M–P) Top cluster view of transcripts expressed as log2 in various cell types based on analysis of colon cancer single cell RNA-seq data²⁵ (n=62). (E) *ALOX5* was expressed mainly in macrophages, mast cells, immune B cells, epithelial tumour cells, stroma and plasma cells. (F) *ALOX5AP* expression mirrored *ALOX5* and in addition it is also remarkably expressed in immune T cells. (G) *LTA4H* was present in many cell types including epithelial tumour cells. (H) *LTB4R* was most restricted in expression and primarily expressed in myeloid cells, tumour cells and immune T cells. (I) *LTC4S* was downregulated in tumour relative to matched normal controls (n=15 per group, *p<0.05). (J) *CCR2*, a recruitment receptor for macrophages is upregulated in tumour versus matched normal controls (n=78, **p<0.01). (K) *CCL2*, the chemokine ligand for *CCR2* was unchanged in tumours versus matched controls (n=78 per group, p=0.0731). (L) *TGFβ* is markedly upregulated in tumours (n=61 per group, p<0.0001). (I–L) For these comparisons, the data was expressed as mean±SEM and graphed as a vertical scatter plot. GraphPad Prism V.10, two-tailed paired t-test. (M) *LTC4S* was mainly expressed in mast cells and at an average level in all other cellular compartments. (N) *CCR2* was expressed primarily in myeloid cells, plasma cells and T cells. (O) *CCL2* was expressed in myeloid cells and stromal cells. (P) *TGFβ* was ubiquitously expressed in most cell types other than normal epithelial cells. (Q) A schematic of top cluster view of different cell types from single-cell colon RNA-seq portal.

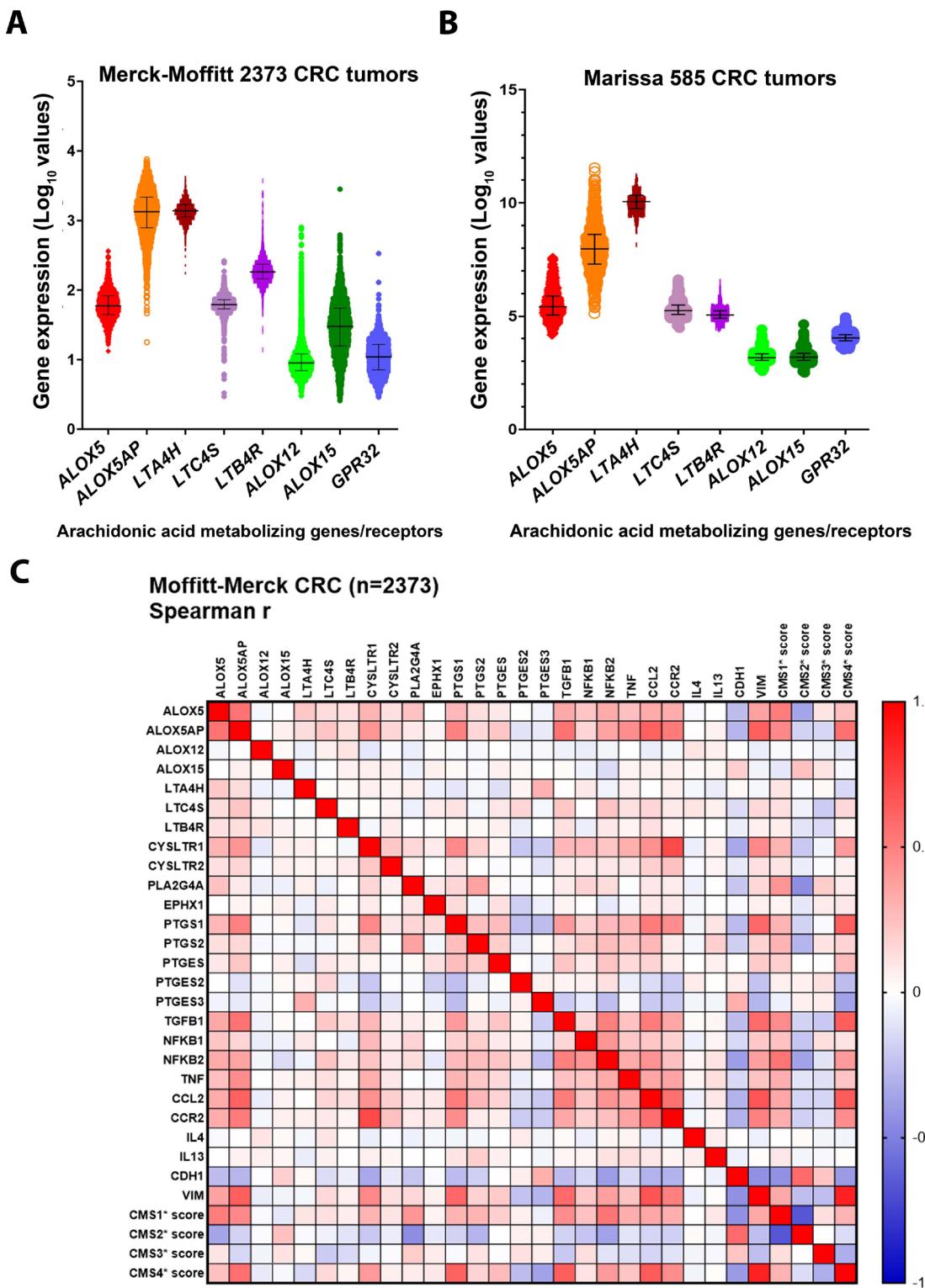


Figure 4 Increased expression of enzymes synthesising pro-inflammatory lipid mediators ALOX5 in the microarray datasets of human colorectal cancer (CRC) tumours. The Affymetrix gene expression of representative arachidonic acid metabolising enzymes/receptors in (A) Merck-Moffitt 2373 CRC tumours and (B) Marissa 585 CRC tumours is shown. (C) Spearman correlation of the gene expression of lipid metabolising enzymes/receptors with genes measuring inflammatory signalling and EMT as well as the CMS1-4* scores Merck-Moffitt 2373 CRC tumours. Of note, genes linked to arachidonic acid lipid production (PLA2G4A and TNF), genes related to pro-inflammatory mediators (ALOX5, ALOX5AP, LTA4H, LTC4S) and their receptors (LTB4R, CYSLTR1, CYSLTR2) all appear to be coordinately expressed along with the inflammatory biomarkers (TGFB1, NFKB1, NFKB2) and associated macrophage markers (CCL2, CCR2) in colon tumours. TGFB gene expression is strongly linked to EMT, with relative overexpression of vimentin and under expression of CDH1. Notably, genes linked to lipid class switching (ALOX12, ALOX15, IL4, IL13, EPHX1) were poorly expressed in colon tumours. Finally, the pro-inflammatory milieu is present in CMS1 and CMS4. CMS1*, CMS2*, CMS3* and CMS4* scores are designated to measure the propensity of a tumour to fall into CMS1 (immune activated), CMS2 (immune desert), CMS3 (immune excluded) and CMS4 (immune inflamed) classes, respectively. These analyses were performed using GraphPad Prism V.10.

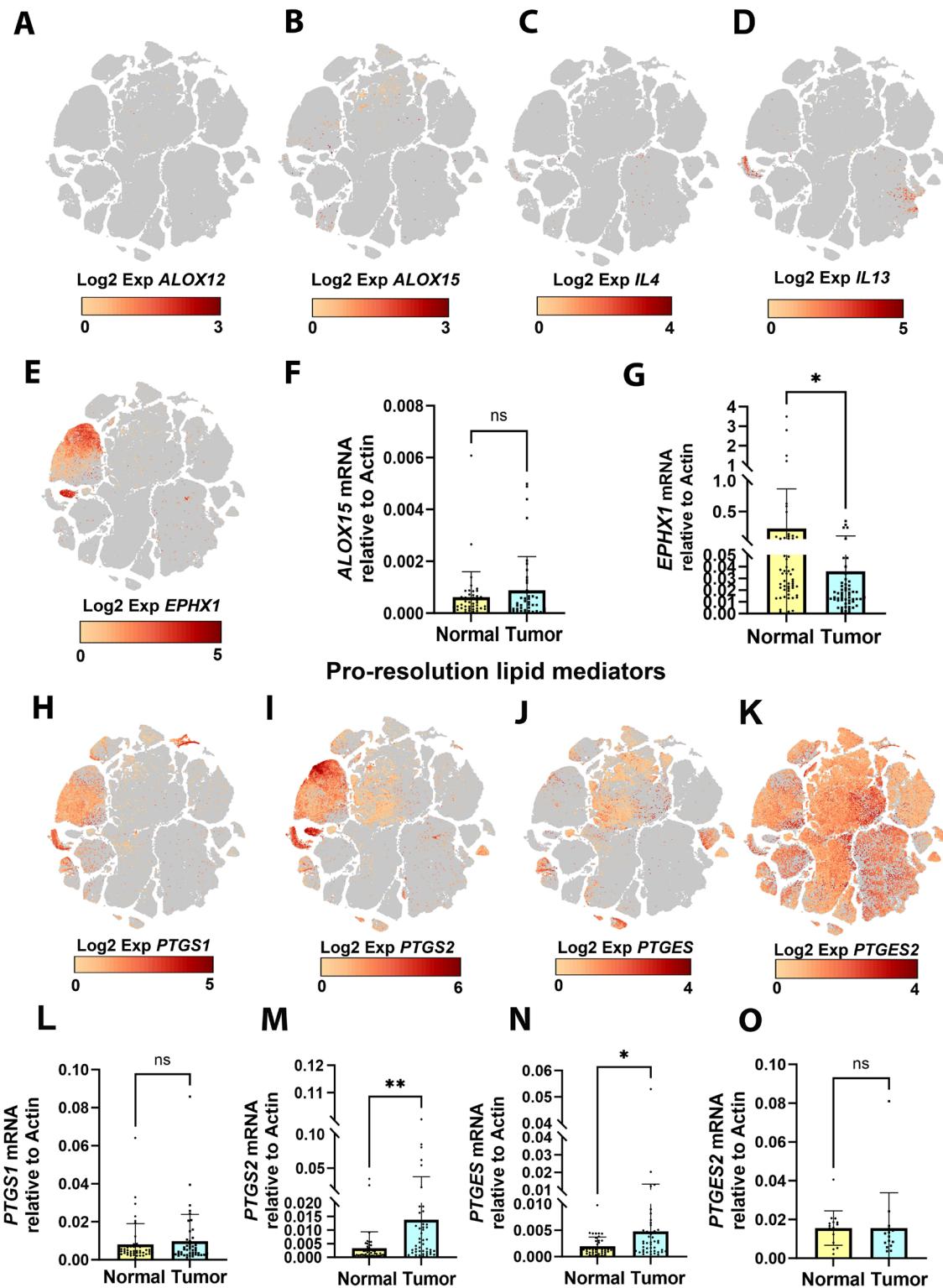


Figure 5 The low levels of prostaglandins (PGD2 and PGE2) and decreased expression of pro-resolution lipid mediators suggest defective class switching in colon tumours. (A–E) scRNA-seq analysis reveals that genes associated with lipid class switching (*ALOX12*, *ALOX15*, *IL4*, *IL13*) except *EPHX1* are poorly expressed in colon tumours. *EPHX1* was present exclusively in the macrophages (myeloid compartment). (F) The *ALOX15* transcript was weakly expressed by quantitative reverse transcription-PCR and did not show any quantitative difference among colon tumour and matched normal controls (n=42 per group). (G) Interestingly, *EPHX1* mRNA was significantly downregulated in colon tumours versus matched normal controls (n=55 per group, *p<0.05). (H–K) COX pathway genes, *PTGS1* and *PTGS2*, that are linked to the synthesis of PGG2/PGH2, precursors for PGE2/PGD2 synthesis, are expressed predominantly in myeloid cells and mast cells, whereas prostaglandin E synthase (*PTGES*) was weakly expressed in epithelial cells while *PTGES2* was ubiquitously expressed in all the sampled cells. (L, M) *PTGS1* was poorly expressed in tumour and normal samples (n=46 per group), but *PTGS2* was, in fact, moderately overexpressed in tumour versus normal samples (n=46 per group, **p<0.01). (N, O) PGE2 synthesising genes, *PTGES* (n=43 per group) and *PTGES2* (n=16 per group), were weakly expressed in both normal and tumour samples with *PTGES* showing some modest increased expression in tumour samples versus matched normal controls (*p<0.05).

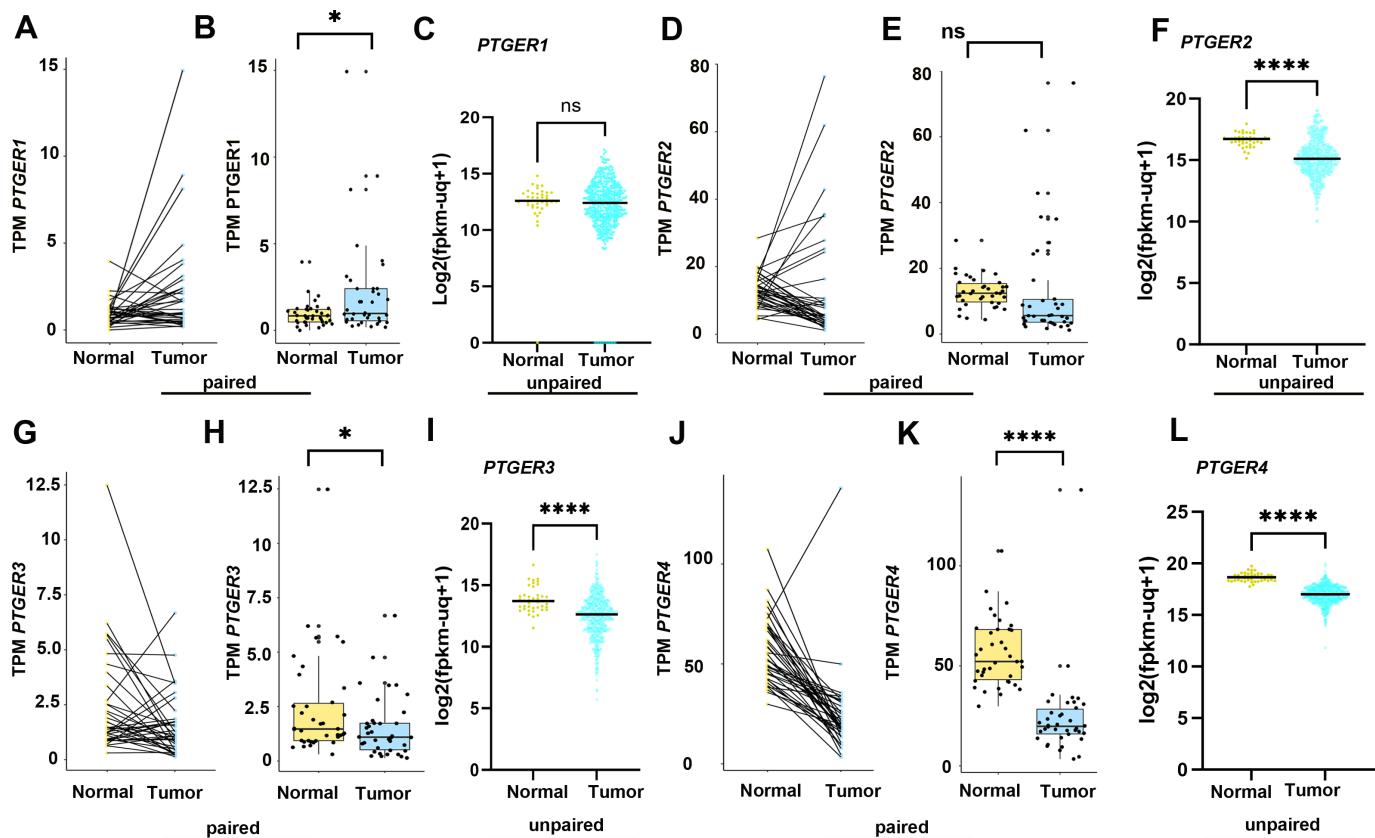


Figure 6 Analysis of the Cancer Genome Atlas database (TCGA) reveals a significant decrease in transcripts encoding PGE2 receptors in colon tumours. Paired and unpaired analysis of (A–C) *PTGER1*, (D–F) *PTGER2*, (G–I) *PTGER3* and (J–L) *PTGER4* transcripts in tumour and matched normal controls. For paired samples ($n=39$ per group), the y-axis represents transcript per million (TPM) and is expressed as median. Unpaired data ($n=39$ normal and $n=144$ tumour) is expressed as mean \pm SEM. Statistical analysis was performed using GraphPad Prism V.10. Paired or unpaired two-tailed t-test and Wilcoxon rank sum analysis were performed. * $p<0.05$ and *** $p<0.00001$ versus normal mucosa controls.

qRT-PCR

While *ALOX15* gene expression was detected by qRT-PCR, it was equally low in both tumour and normal samples ($n=42$ per group, [figure 5F](#)). Further, qRT-PCR analysis of tumour versus matched normal controls showed reduced tumour expression of *EPHX1* ($n=55$ per group, $p<0.05$, [figure 5G](#)). By qRT-PCR, however, *PTGS1* was expressed at low and equivalent levels in tumour and normal samples, while *PTGS2* was, in fact, overexpressed in tumour versus normal samples ($n=46$ per group, [figure 5L,M](#)). On the other hand, *PTGES* showed weak overall expression ($RFC<0.005$) in both normal and tumour samples and *PTGES2* showed no increased expression in tumour samples ($n=16$ – 43 per group) ([figure 5N,O](#)).

TCGA public data analysis reveals reduced expression of *PTGER2, 3, 4* transcripts in CRC tumours versus normal mucosa

We further analysed PGE2/PGD2 receptors (*PTGER1*-4) using paired and unpaired tumour and normal mucosa samples from the TCGA database. Only paired analysis showed that there was a significant increase in *PTGER1* in tumour versus normal controls ([figure 6A,B vs 6C](#)). *PTGER2* transcript was significantly decreased in tumour versus normal controls by unpaired analysis ([figure 6F vs 6D,E](#)). Both *PTGER3* and *PTGER4* transcripts were significantly downregulated in tumour versus normal controls irrespective of paired or unpaired analysis ([figure 6G–L](#)).

Low levels of PGD2/PGE2 in the majority of cases suggested defective lipid class switching in CRC tumours likely related to poor PG synthesis

LC-MS/MS analysis showed a significant decrease in PGD2 ($n=76$ per group, $p<0.01$) ([figure 7A](#)) and PGE2 levels ($n=81$ per group, $p<0.05$) ([figure 7B](#)) in ~70% of tumour cases relative to matched normal controls. In online supplemental figure 9A,B, we show that in TGH samples ($n=51$ pairs) as well as the CHTN samples ($n=30$ pairs) obtained from two different performance sites, we observe PGE2 levels are consistent with either significantly lower or higher trends in T versus N samples. In the 30% of cases showing increased PGE2 levels, these levels do not correlate well with LXA4 or LXb4 levels in paired T:N samples (online supplemental figure 9C–D). PGF2a levels remained unchanged in colon tumours relative to matched normal mucosa ($n=81$ per group) ([figure 7C](#)). Low PGD2 levels could be related to poor prostaglandin D synthase (PTGDS) gene expression noted in scRNA-seq data although this is difficult to quantify ([figure 7D](#)). HPGD, a PGE2 degrading enzyme, was ubiquitously expressed in scRNA-seq data ([figure 7E](#)) but its relative expression by qRT-PCR was decreased in tumour versus matched controls ($n=14$ per group, $p<0.05$) ([figure 7F](#)) thus does not explain the low PGE2 levels in tumours. A top cluster view of various cell types is shown ([figure 7G](#)). While it appears that *PTGS2* gene encoding the enzyme that produces PGH2 and PGG2 (precursors of PGE2) is overexpressed in tumour versus normal sample, it cannot explain reduced PGE2 levels. Low

Colon

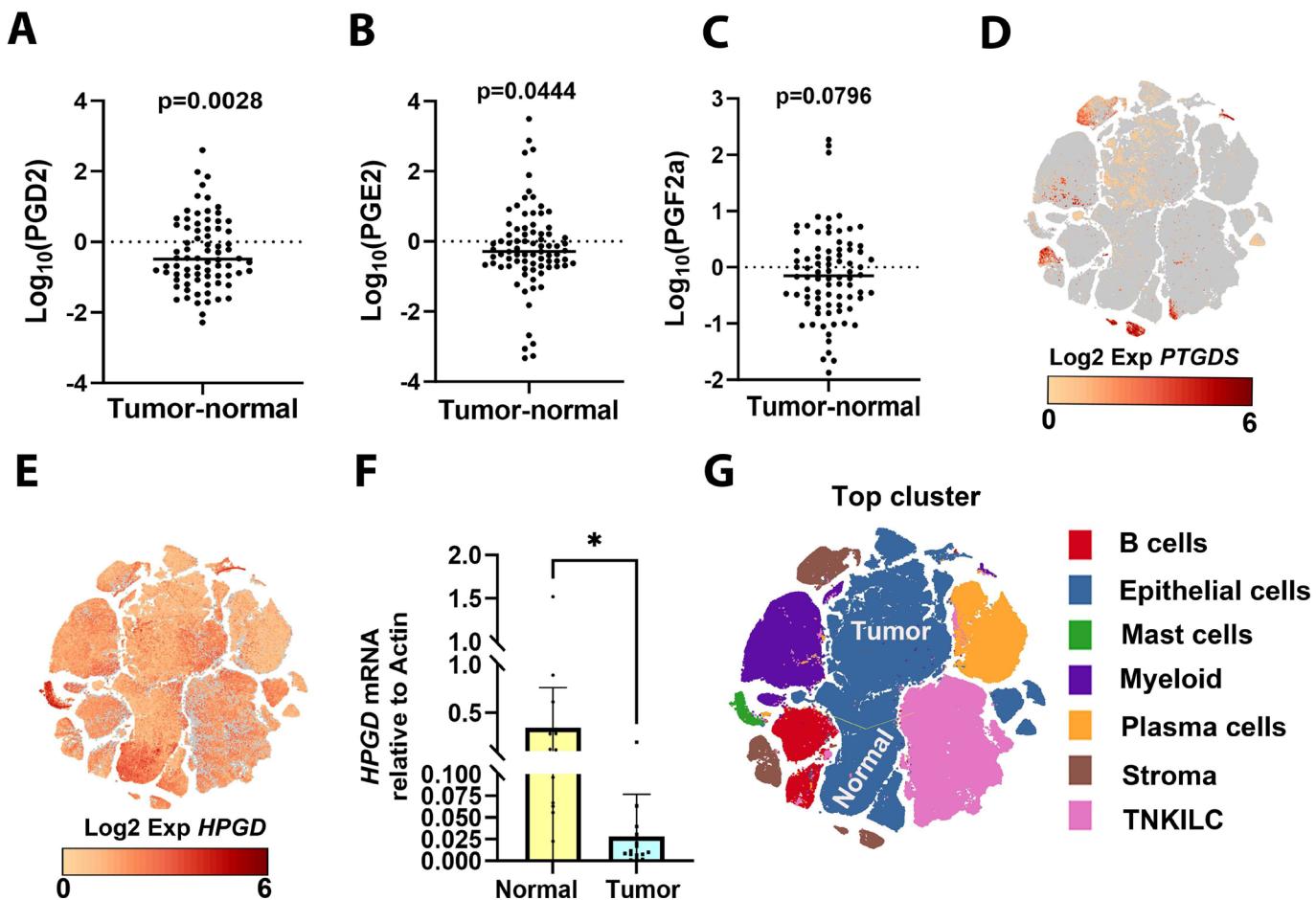


Figure 7 Quantitative LC-MS/MS analysis reveals low levels of PGD2/PGE2 indicative of a defective lipid class switching in colorectal cancer tumours. (A–C) LC-MS/MS analysis of prostaglandins in tumour and matched normal controls. (A) PGD2 levels in colon tumours showed a significant decrease relative to the matched normal controls ($n=76$ per group, $p=0.028$). (B) PGE2 levels also showed a significant decrease in colon tumours relative to matched normal controls ($n=81$ per group, $p=0.0444$). (C) PGF2a levels remained unchanged in colon tumours versus matched normal mucosa controls ($n=82$ per group, $p=0.0796$). The quantity of lipid mediators was expressed as \log_{10} (pg/50 mg of tissue). The y-axis represents the tumour values subtracted from their matched normal controls for each of the lipid mediators in our study. The individual values are shown above and below the median. The data was analysed statistically using GraphPad Prism V.10 and expressed as mean \pm SEM. Paired two-tailed t-test, Wilcoxon rank sum analysis. (D) Prostaglandin D synthase (PTGDS) expression was extremely low in all the cellular compartments as determined by scRNA seq data. (E) The HPGD encoding the key enzyme responsible for PGE2 degradation is ubiquitously expressed. (F) HPGD mRNA, as measured by quantitative reverse transcription-PCR, was significantly downregulated in colon tumours relative to matched normal controls ($n=14$ per group, $*p<0.05$). (G) Top cluster view of transcripts expressed as log2 in various cell types based on single cell RNA-seq data from colon tumours²⁵ ($n=62$).

PGE2 levels in 70% of our cases may be attributed to relatively low levels of PTGES that act on the PGE2 precursors (PTGES, PTGES2) seen in tumour versus normal samples measured by qRT-PCR (figure 5N,O).

Histological and IHC analysis of normal colon mucosa confirms no evidence of malignancy in normal samples that could bias results

We performed H&E and IHC analysis of normal mucosa samples ($n=20$). The representative H&E-stained images clearly shows the presence of normal epithelial crypts and goblet cells (online supplemental figure 10A–F, $n=6$). Further, we analysed the expression of Ki67 and p53 (markers of tumour epithelium) in normal mucosa samples. From online supplemental figure 11A–F ($n=6$), it is very evident that the Ki67 immunostaining is restricted to the proliferative basal normal epithelial cells in the normal crypt. Further, cytoplasmic p53 is minimally expressed, whereas nuclear p53 staining (marker of tumour epithelium) is completely

absent in normal mucosa samples (online supplemental figure 11G–L, $n=6$). Together, these data strongly indicate that these are indeed normal colon mucosa samples and were unlikely contaminated with tumour or associated tumour TME with PGE2/PGD2 producing myeloid cells.

Spatial transcriptomics provides new insight into increased coexpression of ALOX5/ALOX5AP genes linked to 5-HETE/LTB4 production in high versus low LTB4 producing tumours

The spatial distribution and degree of expression of genes linked to LTB4 and PGE synthesis and pro-resolution lipid mediator genes were analysed in representative low LTB4 ($n=4$) versus high LTB4 ($n=4$) colon tumour sections. The H&E stained sections of low LTB4 tumour samples (figure 8A,F,K,P) and high LTB4 (figure 8U,Z,AE,AM) are shown. The gene clusters identified based on unbiased, unsupervised k-nearest neighbour analysis of differential gene expression are shown for both the low and high LTB4 samples (figure 8B,G,L,Q,V,AA,AF,AN, respectively). Here,

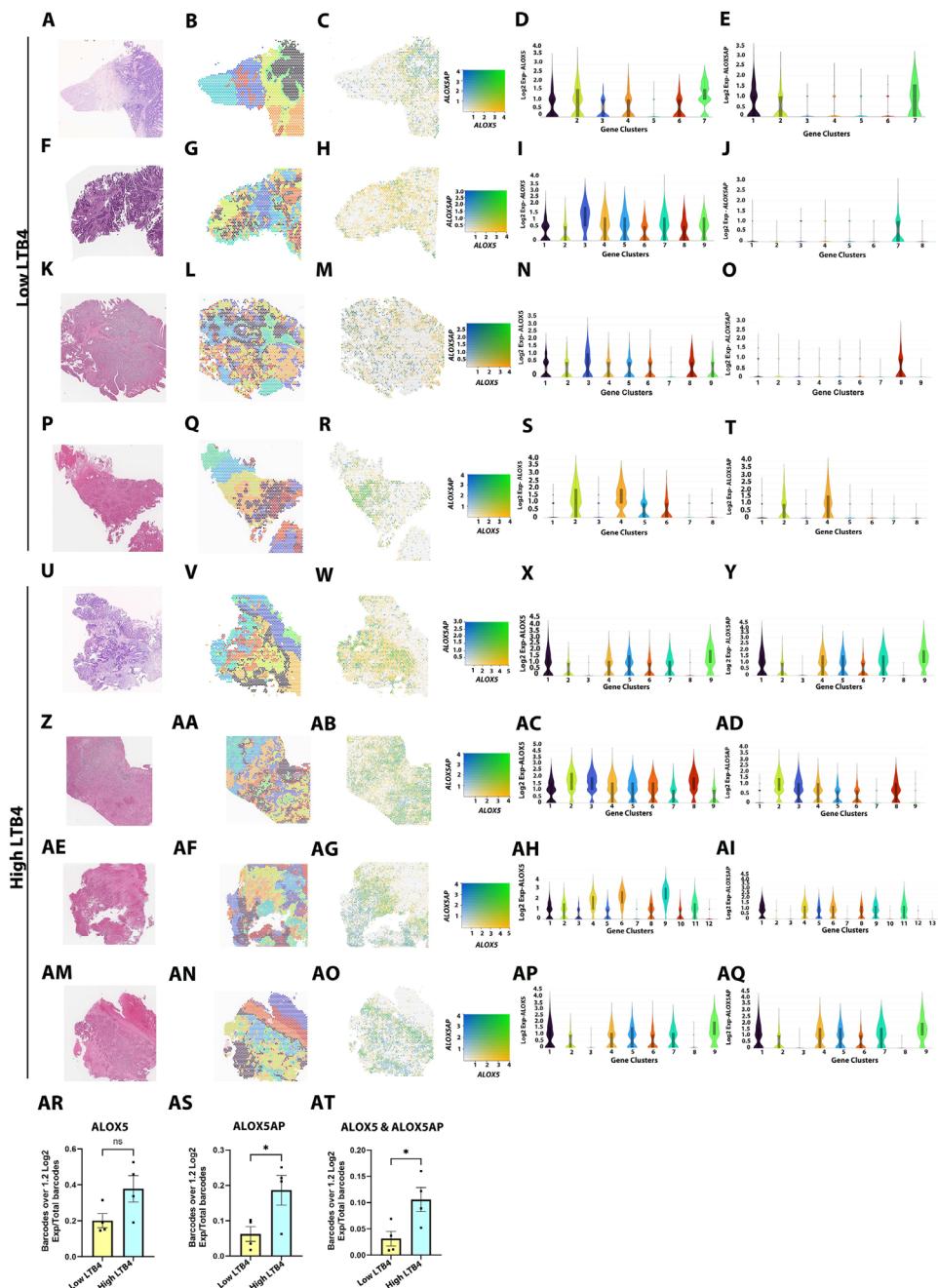


Figure 8 Spatial transcriptomics analysis reveals a relative increase in expression of pro-inflammatory genes linked to LTB4 synthesis in tumours with higher LTB4 levels. H&E-stained images, mapped gene clusters, coexpression of *ALOX5/ALOX5AP* genes in various clusters of low LTB4 (A–T) and high LTB4 colon tumours (P–AQ). (AR) Quantitative analysis of *ALOX5*, (AS) *ALOX5AP* and (AT) *ALOX5+ALOX5AP* show a significant increase in *ALOX5AP* and *ALOX5+ALOX5AP* expression in high LTB4 versus low LTB4 producing colon tumours. Paired two-tailed t-test with Wilcoxon rank sum analysis was performed using GraphPad Prism V.10. N=4 low LTB4 and n=4 high LTB4 tumours were used for the spatial transcriptomics analysis.

we have established the considerable heterogeneity of CRC gene expression as represented by up to 9–12 different subclasses for each tumour. The data analysis from n=4 low LTB4 and n=4 high LTB4 showed a marked difference in the spatial distribution of *ALOX5* and *ALOX5AP* transcripts (figure 8C,H,M,R vs figure 8W,AB,AG,AO, respectively). The coexpression analysis of *ALOX5* and *ALOX5AP* genes suggested that there is low coexpression in low LTB4 tumours relative to high LTB4 tumours (gene clusters) (figure 8D–E,I–J,N–O,S–T vs X–Y,AC–AD,AH–AI,AP–AQ, respectively) that might lead to differential LTB4 levels. Further, quantitative analysis by cluster showed a significant

increase in *ALOX5/ALOX5AP* coexpression with *ALOX5AP* as a main determinant for LTB4 production in high LTB4 tumours versus low LTB4 tumours, indicating the importance of this colocalisation as a requirement to produce pro-inflammatory lipid mediators (figure 8AS,AT vs figure 8AR).

Spatial transcriptomics analysis supported the lack of lipid class switching in colon tumours

We interrogated the spatial location and degree of expression of genes (*ALOX12/15*) linked to the synthesis of pro-resolution lipid mediators in both low LTB4 (n=4) and high

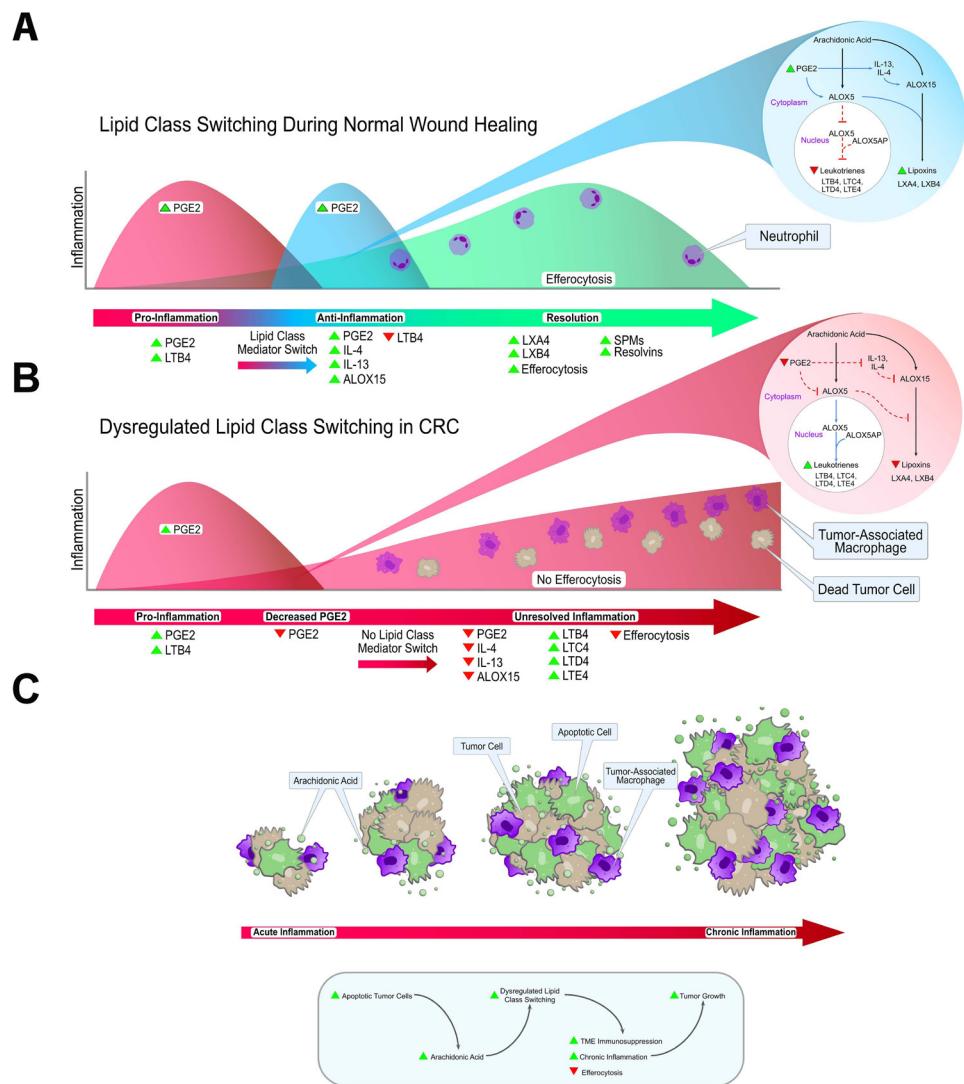


Figure 9 Proposed new colorectal cancer (CRC) harmonised model linking tumour-derived arachidonic acid (AA) to dysregulated lipid class switching, diminished efferocytosis, chronic inflammation, immunosuppression and tumour growth. (A) Normal wound healing is characterised by initial phase of inflammation with a subsequent resolution that is secondary to lipid class switching and efferocytosis of dying neutrophils. (B) Dysregulated lipid class switching in CRC is linked to low PGE2 levels resulting in a failure to produce the pro-resolution lipoxins. (C) Apoptotic tumour cells release excessive AA that fuels chronic inflammation, diminished efferocytosis, immunosuppression and tumour growth.

LTB4 producing tumours ($n=4$) (online supplemental figure 12). We showed that in all the CRC tumour samples, the genes encoding the *pro-resolving* lipid mediators (*ALOX12* and *ALOX15*) were poorly expressed (online supplemental figure 12A–P2, respectively). Normalised expression is shown as a heatmap for *ALOX12* and *ALOX15* (online supplemental figure 12Q,R).

DISCUSSION

Our unsupervised lipidomics landscape analysis of CRC demonstrated dysregulated lipid metabolism in colon tumours versus matched normal controls in matched sets. We then focused on targeted, quantitative analysis of ~25 AA pathway lipid mediators and related gene expression in a larger and more diverse set of CRCs and their paired normal mucosal samples. We found that AA-derived lipid mediators in CRC were principally pro-inflammatory, with little evidence of pro-resolving mediators derived from lipid class switching. 5-HETE was one of the most overexpressed AA pathway lipid mediators. 5-HETE plays an

important role in promoting the growth and spread of certain types of cancers, including prostate, lung, colorectal, pancreatic and gastric cancers.^{20 26–30} 5-HETE activates signalling pathways like MEK/ERK that drive cancer cell proliferation and survival.²⁸ Importantly, the leukotriene (LT) derivatives of 5-HETE (LTB4, LTC4, LTD4 and LTE4), were also all significantly overexpressed in tumour versus paired normal samples. These lipid mediators have been linked to inflammation and stimulation of proliferation, migration and survival of various cancers through signalling pathways involving ERK, PI3K/AKT and NF- κ B.^{20 31 32}

5-HETE is produced by the enzyme 5-lipoxygenase (ALOX5), which is overexpressed in various cancer cells as a consequence of their malignant transformation.³³ The ALOX5 enzyme, through its actions converting AA to pro-inflammatory LTs is known to play an important role in tumour initiation and progression, and is overexpressed in numerous epithelial tumours.^{20 33} Importantly, ALOX5 is known to have a *dual* function, as it can produce either pro-inflammatory LTs or pro-resolution lipid mediators based on the availability of omega-6 versus omega-3 fatty acids as substrates.³⁴ On stimulation, the migration of leucocytes to

inflammatory sites triggers the re-localisation of ALOX5 from cytoplasmic to perinuclear and nuclear locations where it associates with ALOX5AP and its substrates to synthesise LTs.³⁵ Further, mutation of nuclear localisation signals, phosphorylation of ALOX5 or both decreases LTB4 synthesis significantly reinforcing its role.³⁶ Our spatial transcriptomics data supported this important association. In addition to ALOX5, we found that ALOX5AP and LTA4H, that are collectively responsible for LTB4 production, were both overexpressed in tumour versus normal matched samples. LTB4 targets the LTB4 receptor (*LTB4R*) which was also overexpressed in CRC. LTB4 is considered as one of the most reactive pro-inflammatory lipid mediators. LTB4 homeostasis is important as it is required for mounting host defence responses to infection. However, excess production of LTB4 can also lead to chronic inflammatory disease²⁰ and cancer.^{31 32} While we found LTB4 and other pro-inflammatory LTs were elevated in CRC tumours, pro-resolving lipoxins (LXA4, LXB4) linked to the AA pathway, as well as other resolvins and the genes encoding their principal synthetic enzymes (ALOX15) were difficult to detect or were poorly expressed in both tumour and normal samples. 11-HEPE, just like lipoxins, was also downregulated in tumours versus normal samples. It has been suggested that 11-HEPE, an EPA-derived eicosanoid, may provide an as yet, undefined, pro-resolving mechanism to balance the inflammation in pre-malignant lesions or early-stage tumours.³⁷

The pro-resolving, lipoxin-producing, 'lipid class switching'¹⁰ process present in *normal* wound healing appears to be driven primarily by PGE2 and possibly PGD2. PGE2 has been proposed to have both pro-inflammatory as well as pro-resolving functions that may be context-dependent and temporally controlled.³⁸ While PGE2 initiates and amplifies inflammation early on, it also plays a crucial role in actively resolving inflammation and restoring homeostasis at later stages through immunomodulatory mechanisms.^{38–41} Moreover, increasing concentrations of PGE2/PGD2 are thought to inhibit ALOX5 protein translocation from the cytoplasm to the nucleus, mitigating leukotriene synthesis,⁴² thereby skewing the balance towards increased lipoxin synthesis. The function of lipoxins is to mitigate the effects of pro-inflammatory mediators, neutrophil-induced damage⁴³ while promoting macrophage efferocytosis.⁴⁴ This normal wound healing process,¹⁰ appeared to be dysregulated in our CRC samples.

The switch from LTB4 to lipoxin (LXA4/LXB4) production has been definitively linked to elevated PGE2/PGD2 levels. We have shown decreased PGE2/PGD2 levels in the majority (~70%) of T versus N paired samples, which by itself can explain the diminished lipid class switching we observe. This is not entirely inconsistent with previous literature where only 50%–60% of tumours versus matched normal may have shown elevated PGE2.⁴⁵ Although overexpressed PTGS2 is responsible for PGG2/PGH2 production (precursors of PGE2), three penultimate synthesis enzymes produce PGE2 (PTGES, PTGES2, PTGES3). And while PTGES is modestly overexpressed in T versus N, the PTGES2 is not, and may be partly responsible for the reduced PGE2 levels seen in our data. More importantly, using unpaired and paired TCGA data (figure 7) we found that the genes encoding key receptors for PGE2 signalling (PTGER2, 3, 4) were remarkably under-expressed in T versus N suggesting that, even when PGE2 is produced in excess, its downstream signalling may be muted. In summary, we propose that PGE2 levels may be inadequate/ineffective in the majority (70%) of cases to induce lipid class switching either due to *reduced synthesis* or to *reduced signalling*.

To gain deeper biological insights regarding dysregulated lipid metabolism in CRC, we evaluated the coordinated expression

of lipid-metabolising genes seen with qRT-PCR in our tumour/normal samples by analysing a proprietary database of >2300 highly curated CRC tumours.¹⁶ We confirmed that pro-inflammatory gene expression programmes (ALOX5, ALOX5AP, LTA4H, LTC4s, LTB4R) were strongly correlated with TNFα/TGFβ/NFKB, the gene that releases AA from the membrane (PLA2G4A) and myeloid marker genes (CCR2/CCL2). This was further confirmed by spatial transcriptomics data showing increased expression of genes encoding LTB4 synthesising enzymes in the invasive portions of cancer. On the other hand, we found poor expression and correlation with genes linked to pro-resolving functions (IL4, IL13, ALOX12, ALOX15 and EPHX1).

Using a public domain scRNA-seq CRC dataset of 62 individuals,²⁵ we were able to identify the cellular origin of expression of the pro-inflammatory and pro-resolving genes linked to the AA pathway, but did not see a significant influence of MMRp versus MMRd status. Surprisingly, we found that most of the lipid-regulating genes were expressed more so by the cells in the TME rather than by tumour or normal cells. Our data support the hypothesis that TAMs, neutrophils and other immune cells, initially attracted in the inflammatory process, are hijacked to support tumour growth (figure 9). TAMs appear to be incapable of pro-resolving activities associated with normal wound healing such as phagocytosis of dead infiltrating neutrophils and dead tumour cells, also known as 'efferocytosis'.⁴⁴

Strengths and weaknesses of the study relative to other studies

Our finding that the PGE2/PGD2 levels were relatively low in tumours when compared with paired normal samples was a surprise given older literature suggesting PGE2/PGD2 levels may be elevated in *early* CRC disease.^{45 46} However, numerous older studies were performed using less robust and sensitive radioimmunoassays, smaller sample sizes, unpaired tumour versus normal samples and pre-cancerous polyps versus invasive cancers. PGE2 is one of the most important prostanoids that shows a biphasic response in normal wound healing.³⁸ We have suggested there may be two mechanisms leading to defective lipid class switching: (1) *ineffective PGE2 synthesis*, as not all three genes encoding PGE2 synthetic enzymes (PTGES, PTGES2, PTGES3) are overexpressed in T>N; (2) *inefficient PGE2 downstream signalling* due to under-expression of PTGER2-4 in T versus N, unpaired and paired samples (figure 7).

While it is possible that some tumours (~30% had elevated PGD2/PGE2 levels) may undergo an 'attempt' at lipid class switching that may be present in ulcerative colitis known for 'relapsing' and 'remitting' disease,^{47 48} there was only minimal correlation of elevated PGE2/PGD2 levels with LXB4/LXA4 levels (0.2 Spearman's, online supplemental figure 9). Finally, some of the ~30% of cases where PGE2 was found to be higher in T versus N may be related to the use of normal liver as the control for CRC liver metastases; removing these samples from the analysis would strengthen our argument. We were unable to find differences in PGE2 levels in T versus N by performance site (TGH vs CHTN) suggesting our results are robust (online supplemental figure 9).

In summary, we propose a new, harmonised preclinical and clinical model whereby AA excess may contribute to the observed lipid class imbalance (figure 9). Our data are supportive of the compelling murine model whereby non-viable tumour cells (n=900,000) support the growth of otherwise non-tumorigenic, viable tumour cells (n=10,000), likely by providing excess

AA⁴⁹ and ultimately suppressing the immune function of the TME.⁵⁰ Thus, dead tumour cells in concert with deficient lipid class switching and inadequate efferocytosis may contribute to chronic inflammation promoting CRC growth and progression.

Meaning of the study

Because it has been suggested that PGE2 can have both pro-tumorigenic and anti-tumorigenic effects,^{51 52} there would be some risk in administering exogenous PGE2 to subvert its temporally related observed deficiency. Our observations suggest that there may be novel approaches to treat CRC that subvert defective lipid class switching.

Unanswered questions and future research

There is the possibility that providing *exogenous* specialised pro-resolving mediators (SPMs) such as PDX and specific resolvins could essentially *bypass* the need for PGE2/PGD2 and endogenously deficient enzymes (ALOX15/EPHX1). Other approaches, using agents such as CBD oil, AKBA and celastrol recently shown to induce lipid class switching, could induce the *endogenous* production of SPMs.⁵³ Unlike steroids, these anti-inflammatory mediators are thought to promote resolution of inflammation *without* immunosuppression. This opens the possibility for a novel and holistic therapeutic approach to CRC using ‘resolution’ medicine.

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Correction notice This article has been corrected since it published Online First. Minor amendments have been made including a change to an author name and the legend for figure 3.

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Acknowledgements We acknowledge Sarah E Glass for editorial assistance. The authors acknowledge the valuable support from Avennette Pinto, McKayla Carr and Ricardo Perez for helping with patient sample processing.

Contributors TJY is the guarantor of the article. All authors were involved in conceptualisation and study design. TJY was responsible for funding acquisition and resources. RS, MM, JR, AA and HW processed patient samples, performed qRT-PCR, analysed, graphed and interpreted the data. JR-S and AA consented patients, procured patient tissue samples, wrote IRB protocol and generated patient database. AA compiled and prepared the patient demography table. GU prepared patient samples for targeted lipidomics, carried out LC-MS/MS, analysed and interpreted the data, prepared figures and wrote targeted lipidomics methodology under the guidance of GH. DG, PS and SH prepared samples, performed non-targeted lipidomics, analysed and interpreted the data and prepared the figures under supervision of SG. RS and MM processed and stained tumour sections for spatial transcriptomics analysis, analysed and interpreted data using Loupe browser and prepared the figure for spatial data. RS imaged and analysed H&E and IHC data

under the supervision of DC, who additionally provided pathological report of the tumours for spatial transcriptomics. MM, MY and LP analysed and interpreted the secondary analysis of databases and prepared figures. NJL designed and prepared the schematic figure and graphical abstract. MJS provided expert guidance and help analyse and interpret the statistical data. MVN and AL contributed Merck data and interpreted the associated analysis. RC provided human colon tumours and normal controls from CHTN database and discussed experimental plans as well as data interpretation. JM, CM, RDB, AC, AK, TMN, PC and MLA performed surgeries on patients to provide tissue samples/data for this study and discussed experimental plans and interpreted the findings. RS prepared all the figures for the manuscript, wrote the first draft, revised the manuscript with edits and contributions from TJY in addition to input from all the authors. TJY, GH, JP and MY provided critical evaluation of the manuscript. All authors read the manuscript and approved the final version of the manuscript.

Funding This study received support from the Tampa General Hospital Foundation and NCI UH3CA227955 (TJY), R21CA256372 (TJY), R21CA255312 (TJY), NIHRO1HL144788 (GH) and NIH50CA236733 (RC).

Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and all experimental protocols were approved by the Institutional Review Board (IRB) of the University of South Florida. Informed consent was obtained for patients undergoing colorectal surgery at Tampa General Hospital (TGH) as per the approved IRB protocol for University of South Florida STUDY000356 prior to collection of deidentified matched tumour and normal mucosa samples.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. All data relevant to the study are included in the article or uploaded as supplementary information. Not applicable as data is uploaded as supplementary information.

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